Engineering aspects, pathways and mechanisms of nitrogen removal in engineered wetland systems

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This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy (PhD)

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DECLARATION AND STATEMENTS

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This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

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ABSTRACT

While constructed wetlands (CWs) have been applied successfully to achieve contaminant removal from several types of wastewater, their efficiency and capacity is still low with respect to nutrients (nitrogen, N and phosphorus, P) removal. Through typical configurations, the removal efficiency for total nitrogen (TN) varied between 40 and 50% and total phosphorus (TP) between 40 and 60% depending on CWs type and inflow loading. This limits their implementation in delivering advanced wastewater treatment. Subsequently, the main aim of this study was to develop innovative CW processes to significantly enhance the nitrogen removal efficiency by investigating a new approach to the configuration of CWs and exploring substitutional operational strategies. If this is accomplished, it could make CWs a main wastewater treatment unit instead of conventional wastewater treatment systems. To accomplish this aim, two systems (multistage and single stage CW) were developed and comprehensively investigated and studied by applying a tidal flow operational strategy with gravel used as the main media for both systems. Tidal flow enhances and promotes the oxygen transfer to the CW system by batch pulse feeding and producing alternant wet/dry conditions of the CW media with wastewater. The multistage configuration is connected by four identical units in series and is divided into two phases depending on the seasonal temperature. The results of the multistage configuration showed average values for removal of 97.3%, 98.1%, 77.3% and 16.6% for chemical oxygen demand (COD), ammonium (NH_4^+-N), TN and TP respectively for phase 1, and 87.5%, 78.5%, 60.3% and 10.2% for COD, NH_4^+-N, TN and TP respectively for phase 2. However, TN removal was still not desirable with a mean removal efficiency in both phases 1 and 2, whereas the satisfactory removal for TN is >80%. This is due to the seasonal temperature and because all the stages were under aerobic conditions to a certain extent, which is unfavourable for denitrifying bacteria. Subsequently, to obtain a satisfactory efficiency of TN removal performance, the original multistage configuration system has been modified. To obtain a high and efficient TN removal performance for the multistage system, further investigations and developments have been made regarding system modification and alternative operational options. These have resulted in the development of improved nitrogen removal processes by applying a step-feeding strategy to the system with different step-feeding schemes. As a result, 81.1% of TN removal was achieved. In addition, TP removal was poor because the system was not designed to removal phosphorus. The results of the single stage CW showed average values for removal of 86.7-99.3% and 64.1-74.8% for NH_4^+-N and TN respectively, depending on the recirculation number (Rn) and inorganic carbon concentrations (IC); a single stage CW was used to follow the new route of nitrogen removal, called the Complete Autotrophic Nitrogen removal Over Nitrite (CANON) process. Microbial community analysis revealed that the dominant phyla for the multistage system were Proteobacteria, Planctomycetes, Firmicutes, and Bacteroidetes, whereas the dominant phyla for the single stage were Proteobacteria, Planctomycetes, Acidobacteria, and Chloroflexi. All these phyla were responsible for nitrogen and organic matter removal in both systems. The difference in microbial community and structure between both systems might attributed to the differences in the operational conditions. Overall, the results of this research study enhance the treatment capacity, which enables CWs to have the potential to work as the fundamental technology in advanced wastewater treatment and to increase our understanding of nitrogen removal in CW systems.

DEDICATION

Dedicated to my lovely family

For their unconditional love and support

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List of Publications

Papers

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Conferences

1. Khajah, M. and Babatunde A.O. (2016). Operational variables influence on nitrogen removal in redox-stratified multistage vertical constructed wetland system. Presented at the 10th IWA World Water Congress and Exhibition; 9-13 October 2016, Brisbane, Queensland, Australia.

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LIST OF ABBREVIATIONS

Activated sludge (AS)

Ammonia (NH_3)

Ammonia-oxidizing bacteria (AOB)

Ammonium (NH_4^+)

Ammonium chloride (NH_4Cl)

Anaerobic ammonium oxidation (Anammox)

Analysis of variance (ANOVA)

Biochemical oxygen demand (BOD)

Carbon dioxide (CO_2)

Chemical oxygen demand (COD)

Completely autotrophic nitrogen removal over nitrite (CANON)

Constructed wetlands (CWs)

Continuous aeration (CA)

Continuous flood (CF)

Dinitrogen (N_2)

Dissolved oxygen (DO)

Fluorescent in-situ hybridisation (FISH)

Free ammonia (FA)

Free water surface flow (FWSF)

Horizontal flow (HF)

Horizontal flow constructed wetlands (HFCWs)

Horizontal subsurface flow constructed wetland (HSSFCW)

Hydraulic loading rate (HLR)

Hydraulic retention time (HRT)

Hydrochloric acid (HCl)

Inorganic carbon (IC)

Intermittent aeration (IA)

Intermittent flood (IF)

Magnesium-ammonium-phosphate (MAP)

Nitrate (NO_3^-)

Nitrate accumulating ratio (NAR)

Nitric oxide (NO)

Nitrite (NO_2^-)

Nitrite-oxidizing bacteria (NOB)

Nitrogen (N)

Nitrogen loading rate (NLR)

Nitrous oxide (N_2O)

Operational taxonomic units (OTUs)

Organic matter (OM)

Orthophosphate (PO_4^{3-}-P)

Oxidation-reduction potential (ORP)

Partial nitrification (PN)

Person equivalent (pe)

Phosphorus (P)

Polymerase chain reactions (PCR)

Principal coordinates analysis (PCoA)

Recirculating vertical flow constructed wetland (RVFCW)

Root Zone Method (RZM)

Single reactor system for high rate ammonium removal over nitrite (SHARON)

Sodium hydroxide (NaOH)

Subsurface flow (SSF)

Surface flow (SF)

Suspended solids (SS)

Tennessee Valley Authority (TVA)

Tidal flow (TF)

Tidal flow constructed wetland (TFCW)

Tidal flow vertical subsurface flow constructed wetlands (TFVSSFCWs)

Total nitrogen (TN)

Total phosphorus (TP)

United Kingdom (UK)

United State of America (USA)

US Department of Agriculture (USDA)

Vertical flow (VF)

Vertical flow constructed wetlands (VFCWs)

Vertical subsurface flow constructed wetland (VSSFCW)

1 Introduction

1.1 Background of the study

The rapid growth of population across the world, climate change, and changing lifestyles in municipalities (i.e. municipalities in the arid and semi-arid regions) and industrialized locations have led to a reduction in limited freshwater resources and supplies (Campa, 2014; Almuktar and Scholz, 2015). This means the consumption of freshwater has increased with the increase in human activities leading to a corresponding increase in wastewater discharges. Consequently, concern over water problems is not limited to the amount of water available to meet demand, but also includes concerns regarding the quality of freshwater (such as ground water and river). Wastewater treatment seems to be one of the sources of increasing the availability of freshwater as other sources are diminishing. Therefore, new management strategies should be developed to provide a supply of freshwater. This means wastewater treatment and recirculation will play a significant role in the issues of freshwater shortage and environmental protection. Wastewater treatment can be considered as a tool generated by engineers and scientists to counter the various threats caused by wastewater and to enhance the water quality. However, the main concern with wastewater treatment reuse or recirculation is its composition (i.e. nutrients (nitrogen; N, phosphorus; P), chemicals and pathogens) which can be detrimental to human health and aquatic life and can present environmental hazards (Xinshan et al., 2010; Rasool et al., 2014). To be more specific, inappropriate discharge of the treated wastewater would release nutrients and waterborne pathogenic microbes into the receiving water bodies or soils. Therefore, environmental issues would arise.

Nitrogen is considered as a key element for the environment. Forms of nitrogen include ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) as compounds that commonly exist in water and wastewater. Excessive nitrogen would contaminate the water, causing serious problems for human health and the environment, and affecting the economy, for example, the eutrophication phenomenon, deterioration water quality, contamination of soils and plants, and transmission of diseases (Sun et al., 2012; Chen et al., 2014), as shown in Figure 1.1. Eutrophication can be defined as an ecosystem that is rich in nutrients in the water body, which causes a dense growth of plant life (e.g. algae) on the surface of the water body. This reduces the light reaching the bottom of the water body resulting in a decreased amount of sunlight for infiltration (Selman and Greenhalgh, 2010). As a result, death and decomposition of blooming algae affects the dissolved oxygen (DO) levels by depleting and minimizing the DO in the water. This, reduces the DO that fish and other aquatic life need to survive, which leads to the sickness and subsequent death of large numbers of fish (Håkansson, 2002). Moreover, anoxic and anaerobic reactions take place in the water system. In addition, human health is affected by blooming algae, due to high toxins produced from the algae and from bacterial growth, which can make people sick if they come into contact with or drink contaminated water, consume tainted fish or shellfish (USEPA, 2000). Generally, in drinking water, nitrate is not toxic to human health, unless high concentrations in the body may be transformed from nitrate to nitrite. Nitrite is a toxic salt which disrupts the transport of oxygen in the blood by disrupting the haemoglobin to methaemoglobin transformation. This causes stomach pains and nausea for adults, and causes blue baby syndrome and blood oxygen deficiency for infants (Coffman, 2007). Therefore, (USEPA, 2000) set 10 mg/l and 1 mg/l as the maximum concentrations for nitrate and nitrite respectively in drinking water.

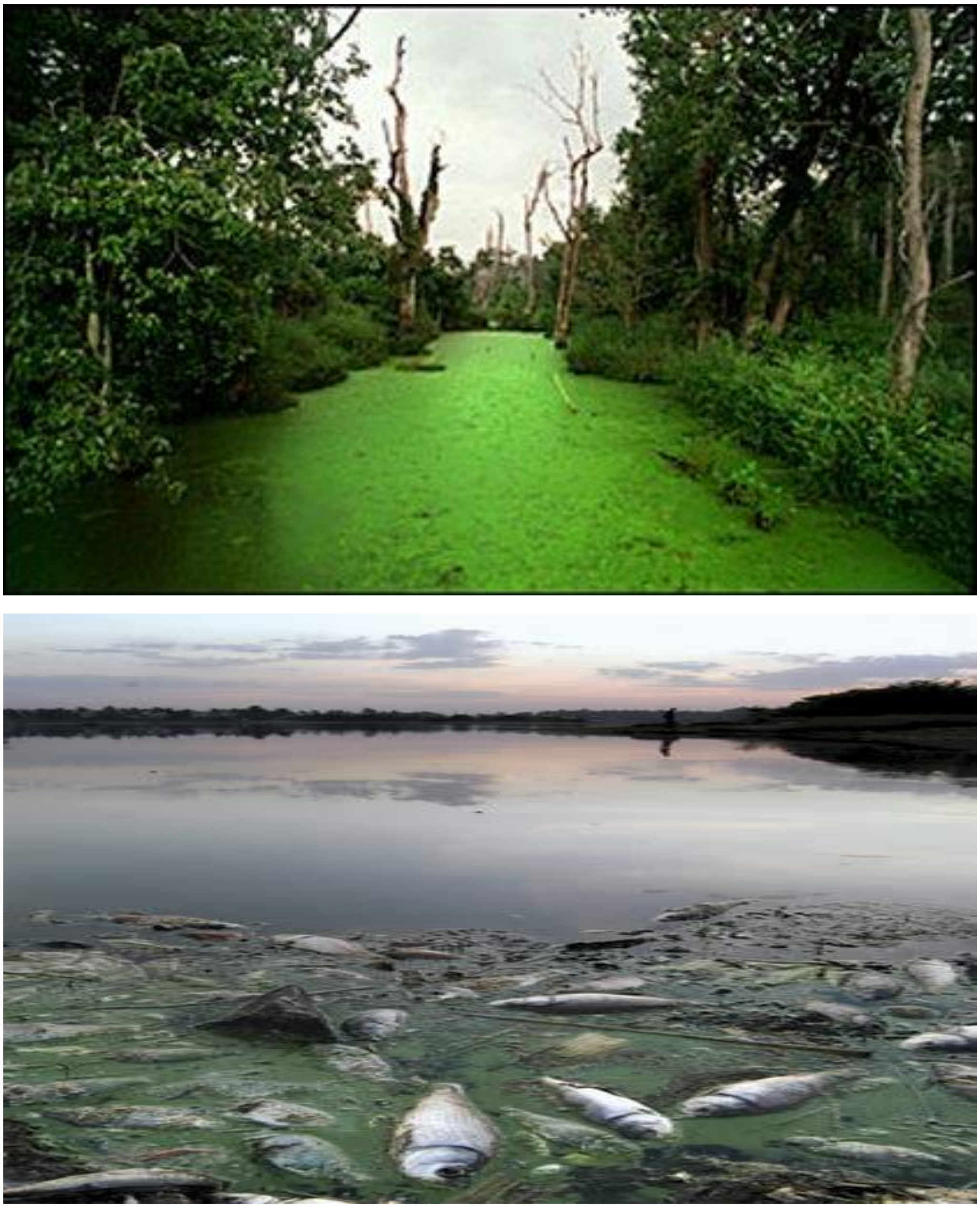


Figure 1.1 Eutrophication and its impact on aquatic life

People’s understanding and realization of the serious influence and impact of these contaminants on ecosystems and on human health have forced governments around the world to impose legislation guidelines and regulations regarding wastewater treatment before disposal to the receiving water bodies (sea, rivers, lakes, etc.) to protect water resources through providing a convenient treatment for reuse and recirculation (Tchobanoglous et al., 2004; Doble and Kumar, 2005; Vo et al., 2014). Consequently, the human usage of water needs to be adjusted to become more sustainable. However, regarding the above mentioned problems, the requirements for sustainable wastewater treatment technologies are that they are easy to operate and cost effective, consume less energy, and are environmentally friendly (green systems). There are several technologies for wastewater treatment, such as activated sludge, constructed wetland, septic tank, trickling filter, stabilization pond, etc. This thesis focuses on applying a constructed wetland (CW) system for domestic wastewater treatment, particularly for nitrogen removal.

1.2 Constructed wetland (CW) system

Constructed wetlands (CWs), as a system for wastewater treatment, have been developed rapidly in recent years. The concept of CWs was not only to solve environmental contamination issues, but also to promote eco-friendly values (Stefanakis et al., 2014). CWs are engineered systems that are used globally for their low operation and maintenance costs, low energy requirements and ease of operation; they are aesthetically pleasing treatment systems and are environmentally friendly compared with conventional wastewater treatment processes (activated sludge, AS), especially for small rural communities, areas, and villages (Wu et al., 2014). Moreover, CWs also provide wildlife habitats, landscape beautification, and research laboratories (Kadlec and Knight, 1996). With respect to the development of CW systems, CWs have become a sensible choice for treating groundwater and several types of wastewater, such as domestic wastewater, industrial wastewater, agricultural wastewater, landfill leachate, mine water discharge, stormwater, livestock wastewater, etc. (Kadlec and Wallace, 2008). CWs are designed to simulate the same principle as natural wetlands with physical, chemical, and biological treatment processes involving wetland plants (vegetation), soils, and associated microbial assemblages to improve the wastewater treatment capacity (Vymazal, 2014). However, vegetation and microbial communities in CWs can adapt to the influent of the wastewater and use different organic and inorganic contaminants through their metabolic and other life processes (Brix, 1994b). There are two typical types of CWs as shown in Figure 1.2, namely, surface flow (SF) and subsurface flow (SSF), which can be further divided into horizontal subsurface flow (HSSF) and vertical subsurface flow (VSSF) (Kadlec and Knight, 1996). These types are classified into three categories depending on the water level on the bed or hydrology (SF or SSF), wetland plants (floating, emergent, and submerged), and water flow path or direction (HSSF or VSSF). In addition, combining these two types of CWs results in another type of CW (HSSF and VSSF), called a hybrid system to obtain the maximum removal efficiency of contaminants (Vymazal, 2014).

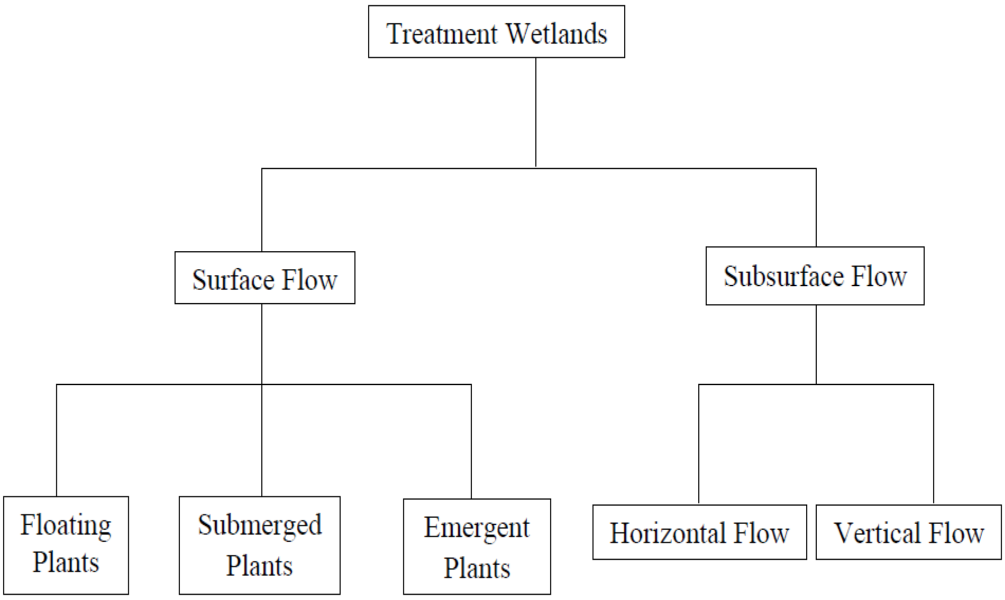


Figure 1.2 Constructed wetlands classification (Kadlec and Wallace, 2008)

Various contaminants can be removed by CWs, such as biochemical oxygen demand (BOD), chemical oxygen demand (COD), heavy metals, nitrogen (N), phosphorus (P), and suspended solids (SS). Among these contaminants, N and P commonly occur in several wastewater types and their removal is one of the main objectives of CW treatment; thus, this study focuses on N removal in CW systems. N can be transform by ammonification, and nitrification and can be removed by several conventional processes, such as, denitrification, plant uptake, microbial assimilation, ammonia volatilization, and adsorption (Kadlec and Wallace, 2008) and novel pathways of nitrogen removal processes include a single reactor system for high rate ammonium removal over nitrite (SHARON), anaerobic ammonium oxidation (Anammox), and completely autotrophic nitrogen-removal over nitrite (CANON) (Khin and Annachhatre, 2004).

Given the advantages of VSSFCWs, in that they require less surface area than HSSFCWs and enhancing the oxygen transfer and supply to the system for the nitrification process, VSSFCWs have been used in this study to investigate the mechanisms and pathways of N removal. These are successfully applied to treat domestic wastewater from organic matters, but have limited N removal because of their incapability of denitrifying water. Therefore, this study applies different techniques and strategies to enhance and improve N removal through VSSFCWs by using conventional and novel pathways of N removal.

1.3 Aim and objectives

The overall aim of the study is to further our mechanistic understanding of nitrogen removal in such systems (i.e. CW system), to examine the engineering aspects (hydraulic loading rate (HRL), hydraulic retention time (HRT) and pollutant loading) and determine the pathways and mechanisms involved in nitrogen removal in an engineered wetland system. To achieve this aim, the following key objectives are addressed:

- to construct a VSSFCW systems configuration design to apply different strategies to investigate and understand nitrogen removal
- to determine the effect of operational variables on nitrogen removal and the performance of the system
- to investigate and determine the mechanisms and pathways of nitrogen removal in engineered wetland systems
- to investigate these different strategies to enhance and improve nitrogen removal in engineered wetland systems
- to investigate and profile the structure of the microbial communities (i.e. characteristics and composition) that develop in the engineered wetland system.

1.4 Thesis outline

The thesis is organised into seven chapters, including one chapter that provides literature review and other chapters that focus on experimental investigation and data analysis. This chapter, Chapter One, has given a background introduction to the thesis, setting it in the overall context of CWs in wastewater treatment and highlighting the specific aim and objectives of the study. Chapter Two presents a comprehensive up to date literature review on nitrogen removal, focusing on the problems and removal mechanisms, and discusses the emerging novel pathways for nitrogen removal processes, such as the Anammox and CANON processes. Chapter Three presents the materials and methods for both CW systems (multistage and single stage CW) and their operational strategy. Chapter Four evaluates the performance of a four-stage tidal flow CW (TFCW). The main purpose is to identify the nitrogen removal in the TFCW configuration by examining the engineering aspects of nitrogen removal in CWs, which will direct the process modifications and amendments in the following chapter. Chapter Five suggests a step-feeding strategy to enhance total nitrogen (TN) removal for a four-stage TFCW. The effects of various step-feeding schemes and operational conditions on the performance of the treatment are widely investigated and evaluated. Chapter Six reports on a new route for nitrogen removal in a single CW system, called the CANON process. The system has followed different strategies to achieve high nitrogen removal in a single CW system with respect to the CANON process. Chapter Seven investigates the structure (characteristics and composition) of the microbial communities associated with the multistage and single stage CW systems, while Chapter Eight presents a general discussion of the issues followed by the conclusion and the recommendations from the study.

2 Literature Review

2.1 Constructed wetlands history

Constructed wetlands (CWs) are engineered (man-made) wastewater treatment and purification systems that include chemical (adsorption, precipitation), physical (sedimentation, filtration), and biological (plant uptake, microbial processes) processes. These processes are similar to the processes that happen in natural wetland systems (Vymazal, 2014; Song et al., 2015). They also comprise the interaction and combination of wetland vegetation, substrate, and microbial communities. Kadlec and Knight (1996) found that natural wetlands were possibly used for the removal and disposal of collected wastewater in the beginning of last century. In the 1950s, Kathe Seidel a German scientist researcher, invented the first experimental research with wastewater treatment wetland systems planted with macrophytes at the Max Planck Institute, Germany (Seidel, 1955). Seidel carried out several experiments using macrophytes on dairy, livestock, and phenol wastewaters (Seidel, 1955; Seidel, 1961; Seidel, 1965b). In addition, in the 1960s, Seidel increased her efforts in researching and discovering the diverse types of sludge and the use of wetland plants, and focused on the improvement of rural wastewater systems that were suffering from low performance effectiveness (Vymazal, 2005). Since septic tank systems are anaerobic, and need to be enhanced and developed, Seidel inserted and introduced a mud separation system across the infiltrated substrate bed and an enucleation set-up in the horizontal trend to enhance the treatment performance (Seidel, 1965a). This led to the initiation of new types of wetlands, called hybrid wetlands; these were regenerated at the end of year 2000 as outlined by (Vymazal, 2005). Vertical flow (VF) wetlands were started in Germany by Seidel as reported in several studies (Cooper et al., 1996; Vymazal, 2005, 2014). Furthermore, there was decreasing interest in the usage of VF wetlands in their original design; however, years later, these were recovered because their capability in the nitrification process was better than that of horizontal flow (HF) wetlands. Additionally, HF wetlands were not favoured by the designers and operators due to their ability and capability of oxidizing ammonia to nitrate. Nonetheless, currently, vertical flow constructed wetlands (VFCWs) are being used much more than horizontal flow constructed wetlands (HFCWs) in Europe (Vymazal, 2014).

A new HF wetlands system, called the Root Zone Method (RZM), was invented by Seidel and Kickuth in the 1960s. In 1974, at Othfresen in Germany, the municipal sewage was treated by the RZM system, which is completely different from the 1950s Seidel design, as it uses the clay soil as a substrate in the wetlands (Kickuth, 1977; Kickuth, 1981; Brix, 1987). Moreover, Kickuth et al. established around 200 municipal and industrial wastewater treatment systems by spreading their ideas and concepts and their experimental research (Bastian and Hammer, 1993). This led to increased interest in using RZM systems in Europe in the mid-1980s. Moreover, in the 1950s and 1960s, CWs research experimental studies for wastewater treatment were carried out and were implemented in Europe and the United States of America (USA). In the USA, the research was developed and expanded highly between 1970 and 1990 (Davis, 1995). Federal agencies, such as the Tennessee Valley Authority (TVA) at the end of 1980s and the US Department of Agriculture (USDA) at the beginning of 1990s also played a role in the development of the research in the USA. However, in the mid-1980s, particularly in the United Kingdom (UK), CW systems became popular and acceptable when the UK Water Industry popularised use of the RZM. Subsequently, Denmark began to operate CWs in its wastewater treatment systems (Cooper et al., 1996). Furthermore, because of the capability of CW systems to treat wastewater in small communities with a population of 50-1000 person equivalent (pe), water authorities accepted and approved CWs as wastewater treatment systems. However, water authorities discovered that there were several problems in the CW systems that needed to be solved; as a result, researchers and designers developed the CWs, which led to the selection of the subsurface VFCW system (Cooper et al., 1996).

Nowadays, CW systems are applied all over the world to treat several kinds of wastewater, such as storm run-off, domestic wastewater, industrial wastewater, agricultural drainage, acid mine waste, and landfill leachate (Wu et al., 2014; Vymazal et al., 2015; Vymazal and Březinová, 2016). Since the 1990s, the applications and studies of CW systems in wastewater treatment have notably increased around the world, as the systems have many advantages (Zhao et al., 2010; Abou-Elela et al., 2013; Chang et al., 2014); for example, they can be built in the same place as the generated wastewater and can be operated by un-trained personnel; they provide a low energy, low cost system in terms of construction, operation and maintenance; and they produce less sludge and have the potential for creating biodiversity.

2.2 Constructed wetlands classification

2.2.1 Surface flow constructed wetlands (SFCWs)

SFCWs are also known as free water surface flow constructed wetlands (FWSFCWs) and are considered as one of the oldest and most prevalent types of artificial wetland. Moreover, such wetlands are extremely similar to natural wetlands compared to other types (Vymazal et al., 1998; Wu et al., 2014). The typical configuration of SFCWs is a closed shallow basin including 20-30 cm of submerged bed media (gravel, soil and sand, etc.), allowing the wetland plants to become established with a depth of water of about 20-40 cm (Kadlec, 1995; Kadlec and Wallace, 2008) as shown in Figure 2.1. SFCWs are flooded from the top, and the water flows on the top of the wetland substrate media in a horizontal direction.

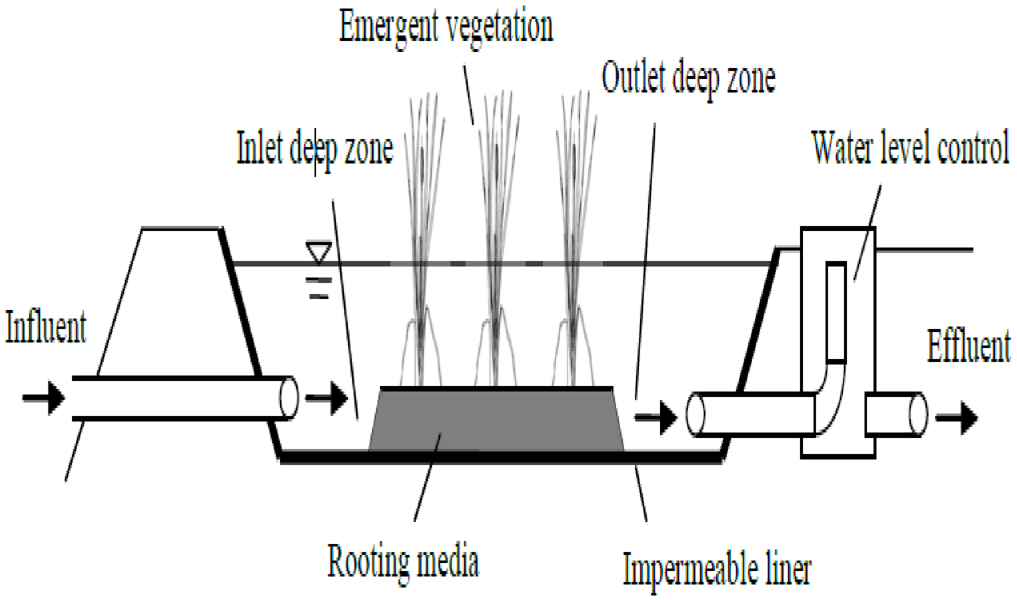


Figure 2.1 Typical schematic of surface flow constructed wetland (Kadlec and Wallace, 2008)

SFCWs are widely used in the USA, mostly for low wastewater concentrations, such as effluent from secondary or tertiary treatment (Kadlec and Wallace, 2008).

Moreover, SFCWs are rarely used for secondary treatment because of the pathogens that would affect human health and expose them to danger (USEPA, 2000). A range of plants can be planted in SFCW systems, e.g., floating plants, submerged plants, and emergent plants. Nevertheless, despite the simple technology and the low operating costs, SFCWs require a large land area, and human contact with the water is possibly risky. In addition, populations of insects, particularly mosquitos, can be intensified in the nearly standing water (Stefanakis et al., 2014). Nonetheless, SFCWs are effective in removing suspended solids (SS), organic matter (OM), and heavy metals (Kadlec and Knight, 1996; Kadlec and Wallace, 2008; Stefanakis et al., 2014). Agricultural runoff, mine water, treatment of animal waste, storm water treatment, groundwater, industrial wastewater, and leachate treatment are considered the most common application of SFCWs (Kadlec, 1995; Kadlec and Wallace, 2008).

2.2.2 Subsurface flow constructed wetlands (SSFCWs)

SSFCWs are very different from SFCWs, as the wastewater flows beneath the surface, either horizontally or vertically through the porous substrate media. In the following sections, types of SSFCWs will be discussed in detail.

2.2.2.1 Horizontal subsurface flow constructed wetlands (HSSFCWs)

In HSSFCWs, the wastewater from the inlet movement in a horizontal direction and gradually passes through the substrate media (filter media), macrophyte (plant) roots, and rhizomes below the surface until it reaches the outlet place where samples are collected for analysis (Cooper et al., 1996). A typical HSSFCW is shown in Figure 2.2.

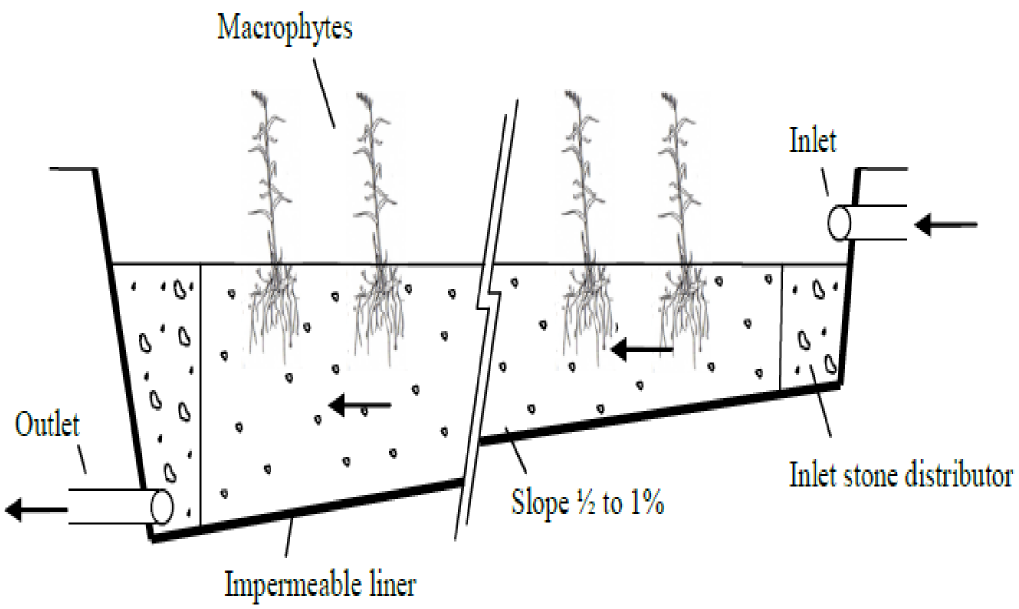


Figure 2.2 Typical schematic of horizontal subsurface flow constructed wetland (Cooper et al., 1996)

In the 1970s, HSSFCWs were developed in Germany. Currently, HSSFCWs are widely used in Europe and the USA (Vymazal, 2014); they require a small land area compared to SFCWs, but it has high investment cost (Kadlec and Wallace, 2008). The components of HSSFCWs are gravel and/or soil as the substrate media planted with macrophytes (Kadlec and Wallace, 2008; Vymazal, 2014). The depth of the substrate media varies between 0.3 and 0.8 m, depending on the type of plants and their root depth, and acts as support for the wetland plants to grow (Vymazal et al., 2006; Akratos and Tsihrintzis, 2007). The HSSFCW beds are slightly inclined (gradient slope ≥ 1%), as shown in Figure 2.2, to facilitate the movement of the water flow from inlet to outlet (Kadlec and Wallace, 2008). Since the wastewater flow is beneath the surface, the risk to humans and wildlife habitats will be reduced and the risk of mosquitoes breeding is reduced compared to SFCWs (Vymazal et al., 2006; Kadlec and Wallace, 2008). Also, HSSFCWs are appropriate for treating primary effluent and for use in cold temperatures because of the natural insulation provided by the media, thus preventing freezing and hydraulic failure (Kadlec and Wallace, 2008). The treatment of contaminants takes place through various interactions between physical, chemical, and biological processes (Kadlec and Knight, 1996). As HSSFCWs are continuously in a saturated condition, aerobic zones will be limited in the system. Therefore, aerobic treatment processes, such as the nitrification process, will be limited in HSSFCWs. Several research studies have revealed that the porous media and the presence of the plant roots in HSSFCWs favour the growth of the biofilm, which enhances OM and SS removal, consequently making HSSFCWs efficient in domestic wastewater treatment, though with low treatment of the nutrients (nitrogen (N) and phosphorus (P)) (Vymazal et al., 2006; Kadlec and Wallace, 2008; Vymazal, 2014).

2.2.2.2 Vertical subsurface flow constructed wetlands (VSSFCWs)

Seidel was the first to initiate the VSSFCW system in Europe, particularly in Germany in 1965 (Vymazal et al., 1998), when she introduced VSSFCWs between septic tanks and HSFCWs (Vymazal et al., 2006; Vymazal and Kröpfelová, 2011). The reason behind the development of VSSFCWs was to achieve fully nitrified effluents (Cooper, 1999). To date, VSSFCW systems have become widely used all over the world because of their high capacity treatment compared to SFCWs and HSSFCWs (Kadlec and Knight, 1996). There are many varieties of VSSFCWs regarding the water flow direction or the scheme of wastewater feeding, such as the upflow or downflow system (Vymazal, 2007). From the 1990s, upflow VSSFCWs have been established and used as household treatment systems, particularly for phosphorus removal (Bavor et al., 1995). In most VSSFCWs, the wastewater flows downward, travels by gravity (Cooper et al., 1996) through the wetland substrate media, and is then collected from a perforated pipe located at the bottom (drainage) layer of the CW, as shown in Figure 2.3.

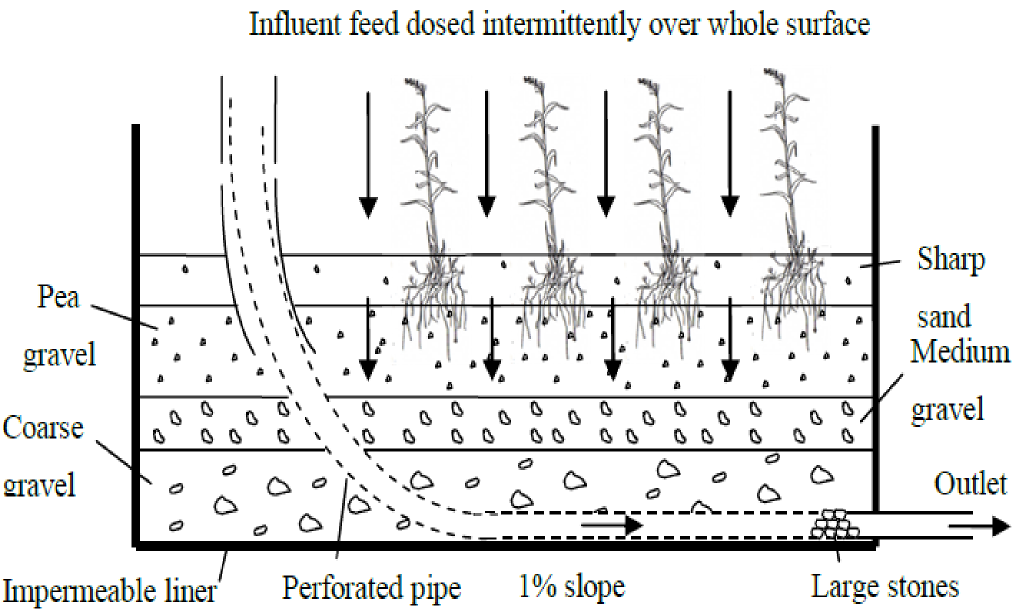


Figure 2.3 Typical schematic of vertical subsurface flow constructed wetland (Cooper et al., 1996)

The components of typical VSSFCWs are layers of sand and gravel; the size gradation increases with depth, and macrophytes are planted in the top layer of the system (Vymazal et al., 2006). The depth of the filtration layer (media layer) is between 0.45 m and 1.2 m and the slope is 1-2%, which simplifies and facilitates the movement of the effluent treated wastewater out of the system. Unlike HSSFCWs, the wetland substrate media in VSSFCWs is not always or permanently saturated because of the intermittent batches loading on the system (Wallace, 2013; Li et al., 2015a). As a result, the oxygen transfer in the system is significantly enhanced (Knowles et al., 2011; Stefanakis et al., 2014). When the wastewater enters the VSSFCW, air enters the substrate media pores, which improves and increases the aeration and the microbial activities in the system (Vymazal et al., 2006; Song et al., 2015).

Several research studies (Chang et al., 2012; Paing et al., 2015; Fan et al., 2016) were carried out to evaluate the performance efficiency of VSSFCW systems, and these revealed that the system was good treatment technology as regards the water quality parameters. These studies revealed that VSSFCWs performance was satisfactory in SS and OM as represented by chemical oxygen demand (COD) and biochemical oxygen demand (BOD) and was limited in P removal due to the insufficient interaction between the wastewater and the substrate media. In addition, other studies mentioned that VSSFCWs can also achieve a satisfactory level of nitrification (Vymazal et al., 2006; Zhi et al., 2015; Fu et al., 2016). However, some research studies pointed out that the performance of VSSFCWs was poor regarding denitrification (Vymazal, 2005; Vymazal and Kröpfelová, 2011). Furthermore, many recent research studies have demonstrated that VSSFCWs with intermittent loading regimes can denitrify effectively with some amendments and modifications to the system (Foladori et al., 2013; Song et al., 2015; Fan et al., 2016). The intermittent loading application in VSSFCWs improves and enhances the oxygen transfer (aeration) in the bed. Between doses and batches, wastewater is drained from the CW, and oxygen can then enter into the substrate media, consequently providing aerobic treatment for the next cycle. As a result, VSSFCWs are preferred for the nitrification step. The rhizomes and roots of plants are considered as oxygen suppliers in VSSFCWs, which can be negligible compared to the oxygen retained in the system between doses and batches. The high oxygenation in the VSSFCWs enhances the conditions for OM decomposition and nitrification step in comparison to HSSFCWs (Kadlec and Wallace, 2008; Stefanakis et al., 2014). It is known that VSSFCWs have a good performance regarding treatment of SS, OM, N and P. While many research studies have revealed that clogging could be an operational issue (Fu et al., 2013; Song et al., 2015; Du et al., 2016), numerous research studies have stated that bioclogging can be minimized when the intermittent loading application is used in VSSFCWs, as the resting period could efficiently improve and increase the porosity and the hydraulic conductivity after a certain resting period (Fan et al., 2013; Paing et al., 2015). VSSFCWs are largely used in Europe, predominantly in Austria, Denmark, Germany, the UK, and France and in the USA as well (Kadlec and Wallace, 2008). Currently, the application of VSSFCWs is gradually developing in Asia and Africa (Abou-Elela et al., 2013; Song et al., 2015). However, trickling filter system has the same concept as VFCW system, being a biological treatment system that operates under mostly aerobic conditions which can be used to remove organic matter from wastewater, and also other aerobic microbial processes such as nitrification (Kim et al., 2014; Kim et al., 2015; Godoy-Olmos et al., 2016). In addition, it requires low additional carbon source for the denitrification step, which can remove up to 50% and 67% of nitrate with 100% and 200% recycling of the effluent respectively (Biesterfeld et al., 2003). It has similar features to VFCW systems such as low cost; simplicity of design, construction, operation and management; good oxygen transfer and supply for nitrification process; low energy requirement and efficient organic matter removal (Guillén et al., 2015; Godoy-Olmos et al., 2016; Zhang et al., 2016).

Table 2.1 shows the comparison between SF and SSF (HSSF and VSSF) regarding their advantages and disadvantages in CW systems:

Table 2.1 Advantages and disadvantages of surface flow and subsurface flow constructed wetlands (Vymazal et al., 2006; Vymazal, 2007)

Constructed wetland types	**Advantages**	**Disadvantages**
Surface flow constructed wetlands	• Inexpensive and simple to construct and operate, and easy to maintain • Greater aesthetic appeal and provides wildlife habitats • Less energy required	• Larger land area required • Long start-up time to reach full working capacity • Pest and odour problems
Horizontal flow constructed wetlands	• Long flowing distance, and contaminant gradients can be established • Efficient in the removal of SS and OM • Denitrification of nitrogenous compounds • Reduced risk of exposing humans or wildlife to toxics • Greater cold tolerance	• Larger land area requirement • Clogging • Limited aerobic zones for ammonium oxidation and other oxygen-dependent processes • Uniform passage of wastewater through the packed media is complicated (due to possible presence of dead zones)
Vertical flow constructed wetlands	• Smaller land area required • Good oxygen transfer and supply • Enhanced nitrification process • Simple hydraulics • Efficient in SS and OM removal • Greater cold tolerance	• Shorter retention time • Poor denitrification process • Less effective in phosphorus and nitrate reduction

2.2.2.2.1 Tidal flow vertical subsurface flow constructed wetlands (TFVSSFCWs)

Tidal flow vertical subsurface flow constructed wetlands (TFVSSFCWs) are considered one of the most substantial developments in wetland treatment systems in the last two decades. The tidal flow (TF) strategy is a process that repeatedly allows CWs to be filled with wastewater so the main media layer is submerged and then fully drained by pumps, which means that the process is operated as a filling, contact, drain, and rest sequence. In the filling time, the CW is gradually submerged, the air in the CW is continuously consumed, the influent of ammonium is absorbed by wetland media, and OM is degraded by the microbial attached to the wetland media. After the filling period, the CW is preserved for a particular time, which allows contact between the wastewater and the wetland media. Moreover, denitrification will be enhanced due to the anoxic or anaerobic conditions that can be established during this period. In the draining time, the treated wastewater is rapidly drained out of the CW, fresh air is dragged into the CW, and the CW is replenished with oxygen. The atmospheric prevalence in the CW will oxidize the biofilm, causing the nitrification process to occur in the system. After that, the CW is refilled again, and the oxidized nitrogen is thereafter released from the wetland media, because the positive ammonium ion (NH_4^+) is oxidized and converted to negative ions of nitrite (NO_2^-) and nitrate (NO_3^-), which are repulsed by the negative charges of the wetland media. On the other hand, the influent of OM supplies a source of carbon for the denitrification process. During the consistent cycle of wet (saturated) and dry (unsaturated) time, the wastewater acts as a passive pump to drag and expel air into the CW. Therefore, oxygen supply and consumption are basically enhanced, and this improves the treatment ability of the system. TF is very efficient for promoting the wastewater treatment in CWs and in the performance for high contamination loads (Green et al., 1997; Li et al., 2015a; Wang et al., 2016d). Figure 2.4 illustrates the procedure of the tidal flow process in CWs.

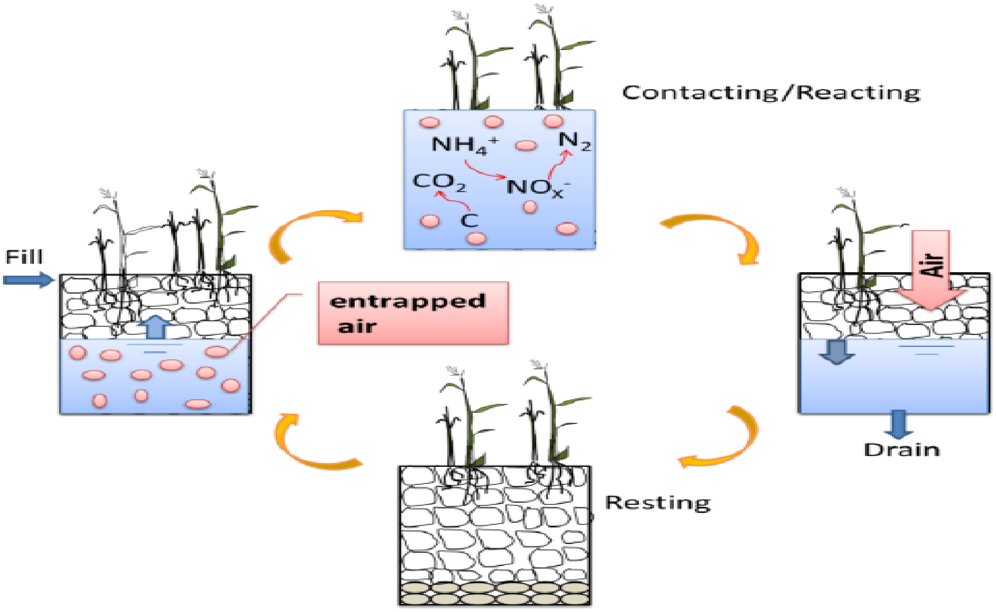


Figure 2.4 Tidal flow process in constructed wetland (Green et al., 1997)

Table 2.2 shows that VFCWs system can reach nearly complete nitrification (above 90%). The general NLR implemented is still below 10 g/m³.d. However, a number of factors affected the nitrification performance (above 80%) when high loading rate above 10 g/m³.d is used. Weedon (2010) achieved 90% NH_4^+-N removal with high NLR (32 g/m³.d). These results show that VFCWs system have no limiting step for nitrification for TN removal. By contrast, denitrification is limited in VFCWs system because of the aerobic condition in the system. As presented in Table 2.2, in most cases it is clear that NOx^--N is significantly accumulated beside the NH_4^+-N reduction, and as a result, a highly nitrified effluent. Therefore, TN reduction was less than 50%. Overall, these results indicate that a single stage VFCW does not have the ability to deliver high TN removal. To achieve effective TN removal, some strategies were applied to enhance the denitrification process in the VFCWs system. One of the most common strategy that has been used is recirculating the nitrified effluent back to the influent source, where the nitrified effluents mixes with the raw wastewater, and then the microorganisms in the system can increase the treatment efficiency for denitrification step, however it has a limited effect (Bahlo, 2000; Zhao et al., 2004c; Arias et al., 2005; Lavrova and Koumanova, 2010). For instance, limited reduction of the effluent NO_3^--N with 100% recirculation effluent ratio from 60 mg/l to 46 mg/l was reported by Bahlo (2000). Similarity, Arias et al. (2005) obtained significant improvement of TN removal from 1% without recirculation to 66% and 68% with

200% and 300% effluent recirculation respectively. Nonetheless, there are some research studies that have observed contrary effect of the effluent recirculation on TN removal efficiency (Brix and Arias, 2005; Lavrova and Koumanova, 2010). Overall, VFCWs system are in aerobic condition which is favourable for the nitrification process, but hinders denitrification even if sufficient carbon source is provided. Therefore, anoxic/anaerobic zone must be created to enhance the denitrification process by increasing the contact time of the wastewater with the microorganisms (Langergraber et al., 2007). Moreover, Table 2.2 shows the general treatment performance of TFCWs with various configurations. Tidal flow strategy could enhance the oxygen transfer and treatment efficiency of the VFCWs system for nitrification and the limited denitrification. This has been confirmed with the highly nitrified effluents and limited TN reduction (Sun et al., 2005; Jia et al., 2010; Wu et al., 2011). In addition, NH_4^+-N removal was incomplete in some research studies due to the high strength of the wastewater (Sun et al., 2005; Wu et al., 2011).

Table 2.2 Treatment performance of different VFCW configurations in nitrogen removal

Wastewater	NH_4^+-N reduction			NOx^--N reduction		TN reduction			NLR* g/m^3.d	Configuration	Reference
	Influent (mg/l)	Effluent (mg/l)	Removal (%)	Influent (mg/l)	Effluent (mg/l)	Influent (mg/l)	Effluent (mg/l)	Removal (%)			
Potato processing	116	26	78	1	43	120	82	32	13.4	VFCW + pond	(Kadlec et al., 1997)
Domestic	80	2	98	1	60	81	62	23	2	VFCW with recirculation	(Bahlo, 2000)
Domestic	25	3	88	1.2	14	32	19	41	5.3	Two pilot VFCW	(Korkusuz et al., 2004)
Domestic	68	0.2	99.7	0.4	54	85	>55	<35	6.2	2 stages of VFCW	(Langergraber et al., 2007)
Diluted leachate	76	16	79	10	13	90	29	68	13	Six VFCW different wastewater	(De Feo, 2007)
Domestic	66	0.3	99.5	0.4	39	78	41	47	11	Single + two stages of VFCW	(Langergraber et al., 2010)
Combined settled sewage and abattoir effluent	94	9	90	0.4	78	132	89	33	32	VFCW	(Weedon, 2010)
Piggery	330	33	93	5.3	184	335	207	38	17	Combined TFCWs	(Sun et al., 1999)
Piggery	121	46	62						80	Four stages with recirculation	(Zhao et al., 2004c)

Piggery	28-159		33						60	Four stages TFCWs	(Zhao et al., 2004a)
Piggery	104	76	30	5.8	195				67	Four stages TFCWs	(Sun et al., 2005)
Piggery	121	63	48	8.8	14				78	Four stages TFCWs with recirculation	(Sun et al., 2005)
Piggery	201	84	58	13	2.5	214	87	59	26	Five stages TFCWs	(Sun et al., 2006)
Synthetic domestic	43	3	92	6	24	50	27	47	2.5	Single stage TFCW	(Jia et al., 2010)
Synthetic domestic	37	7	81	1	15	39	22	44	38	Single stage TFCW with recirculation	(Wu et al., 2011)

*NLR – nitrogen loading rate

With tidal flow strategy, high organic matter removal can be achieved (up to >90%). In addition, the two operation modes: intermittent flood (IF) and continuous flood (CF) had no major effect on enhancing or deteriorating the removal of COD in HFCW (Zhang et al., 2012b) and VFCW (Jia et al., 2010; Jia et al., 2011) (see Table 2.3). On the other hand, the contribution for the treatment of low, medium and high strength wastewater for both VFCW and HFCW was different in hybrid CW systems. For example, for the medium strength wastewater, the overall removal efficiency of COD was 93% by hybrid CW system in which VFCW and HFCW contribution 65% and 28% respectively (Zapater-Pereyra et al., 2015).

Moreover, numerous studies have revealed that IF in VFCW is very efficient for the removal of NH_4^+-N (above 90%) and TN (above 60%) (Wu et al., 2011; Zhao et al., 2011; Hu et al., 2014b; Wu et al., 2015b). Jia et al. (2010); Jia et al. (2011) showed that IF provided more oxidized condition for NH_4^+-N removal, while the CF system had the highest TN removal. In the CF system, NH_4^+-N removal is incomplete because of the consumption of dissolved oxygen. Therefore, denitrification became favourable due to the anoxic/anaerobic condition. Consequently, CF systems have lower NH_4^+-N removal and higher TN removal. In addition, in hybrid CW system with IF mode, Zapater-Pereyra et al. (2015) achieved >80% NH_4^+-N and TN removal efficiencies. The contribution of both VFCW and HFCW in hybrid CW was varied in terms of the removal efficiency for NH_4^+-N and TN. For example, the removal efficiency for NH_4^+-N in hybrid CW with low, medium and high strength wastewater was 85, 73 and 55% respectively, whereas VFCW contributed 82%, 51% and 46% removals respectively. Also, the removal efficiency of TN in hybrid CW with low, medium and high strength wastewater was 72%, 82% and 78% respectively, whereas HFCW contributed 38%, 49% and 48% removals respectively (see Table 2.3).

Table 2.3 Comparison of studies using tidal flow strategy

Wetland type/scale	Area (m²)	Wastewater type	HLR (m³/m².d)	Operation mode	Fill and drain time ratio (hr:hr)	COD removal (%)	NH_4^+-N removal (%)	TN removal (%)	TP removal (%)	Reference
VFCW										
Pilot	0.25	Piggery	0.12	Intermittent flood	NA	80	58	NA	NA	(Sun et al., 2006)
Lab	3.3	Synthetic	0.10	Intermittent flood	1:2	96	94	47	92	(Jia et al., 2010)
Lab	3.3	Synthetic	0.10	Intermittent flood	2:1	97	90	56	92	(Jia et al., 2010)
Lab	3.3	Synthetic	0.10	Continuous flood	3:0	92	63	67	87	(Jia et al., 2010)
Lab	4	Synthetic	0.08	Intermittent flood	1:2	96	94	47	NA	(Jia et al., 2011)
Lab	4	Synthetic	0.08	Continuous flood	3:0	92	63	67	NA	(Jia et al., 2011)
Lab	4	Synthetic	0.08	Intermittent flood	4:3	93	76	67	NA	(Jia et al., 2011)
Lab	4	Synthetic	0.08	Continuous flood	7:0	94	78	69	NA	(Jia et al., 2011)
Pilot	5	Animal farm	0.29	Intermittent flood	4:4	84	93	78	94	(Zhao et al., 2011)
Pilot	0.3	Animal farm	0.29	Intermittent flood	4:4	36	49	11	75	(Zhao et al., 2011)
Lab	0.3	Synthetic	0.90	Intermittent flood	3:3	NA	82	43	NA	(Wu et al., 2011)
Lab	0.2	Synthetic	0.90	Intermittent flood	3:3	NA	33	21	NA	(Wu et al., 2011)
Lab	2.2	Piggery	0.44	Intermittent flood	1:1	70	96	60	88	(Hu et al., 2014b)
Lab	0.6	Piggery	0.44	Intermittent flood	1:1	62	94	60	88	(Hu et al., 2014b)
Pilot	1	Synthetic	0.39	Intermittent flood	3:3	93	93	74	NA	(Wu et al., 2015b)
Hybrid CW										
Lab (VF + HF)	7.9	Domestic	0.046	Intermittent flood	1:2	87	85	72	80	(Zapater-Pereyra et al., 2015)
Lab (VF + HF)	3.4	Domestic	0.046	Intermittent flood	1:2	93	73	82	61	(Zapater-Pereyra et al., 2015)
Lab (VF + HF)	2.6	Domestic	0.046	Intermittent flood	1:2	91	55	78	44	(Zapater-Pereyra et al., 2015)

2.2.2.3 Hybrid constructed wetlands

Previous sections have discussed in detail how SFCWs and HSSFCWs are limited in the nitrification process and VSSFCWs are limited in the denitrification process. Thus, none of these CW systems alone can achieve a high and effective removal of total nitrogen (TN) (>80%). However, if SFCWs or HSSFCWs are combined with VSSFCWs to form hybrid CWs, high and effective TN removal can be achieved. Hybrid CWs are treatment systems that have been generally used to obtain high N removal in wastewater treatment by combining VSSFCWs (nitrification process, the capability to oxidize ammonia due to the high oxygenation in the bed) and HSSFCWs (denitrification process, effectively treating nitrate) (Vymazal, 2013a; Vymazal and Kröpfelová, 2015). Hybrid CWs or VFCW multistage systems are used to denitrify the nitrifying effluents from the CW system and to achieve higher removal of TN. As stated earlier, a hybrid CW system, known as the Seidel system, was initially used by Seidel in the 1960s at the Max Planck Institute in Krefeld, Germany (Vymazal, 2005). The system was modified at Saint Bohaire, France in the 1980s and at Oaklands Park in the UK in the 1990s (Cooper, 1999; Vymazal, 2005). By the end of the 1990s and beginning of the 2000s, the hybrid systems were getting more attention, and their use was expanded in several countries in Europe and all around the world because of their capability of removing ammonia, nitrate, and total nitrogen from different types of wastewaters (Vymazal, 2007; Vymazal, 2013a; Vymazal and Kröpfelová, 2015).

2.3 Composition of wetlands

2.3.1 Wetland plants

Wetland plants are also known as macrophytes, and these are a substantial and prime component of the wetland system (Vymazal, 2013b; Chen et al., 2015; Jesus et al., 2016). CW systems have been called a green technology most probably because of the presence of these plants (Stefanakis et al., 2014). The roots of the plants in the rhizosphere are considered an important area for microbial growth and for the aerobic zone in CWs. The macrophytes absorb the pollutants in their tissues and provide a suitable growing environment for the microorganisms (Vymazal, 2011). Thus, the rhizosphere is considered the most active area for reaction because of these microorganisms. Macrophytes have advantages such as providing a supply of oxygen, absorbing N and P, and allowing bacterial growth in the rhizosphere (Stefanakis et al., 2014). Several research studies have shown that planted CW systems are more efficient in contaminant removal than are unplanted CW systems (Akratos and Tsihrintzis, 2007; Carranza-Diaz et al., 2014). The macrophytes that are used in CWs can be divided into four categories, as shown in Figure 2.5 (Kadlec and Wallace, 2008; Saeed and Sun, 2012), and these are briefly explained in the following subsections.

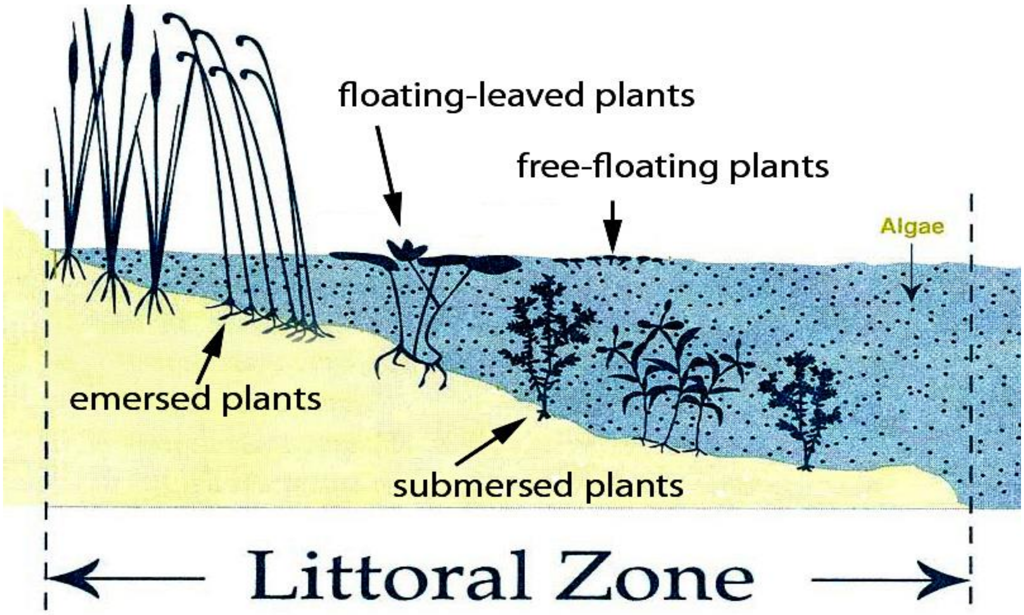


Figure 2.5 Types of macrophytes in constructed wetland (Kadlec and Wallace, 2008)

2.3.1.1 Freely floating plants

These types of plants are able to remove N, P, and SS. Examples of these plants are *Eichhornia crassipes*, *Lemna minor* and *Spirodela polyrhiza*.

2.3.1.2 Floating leaved plants

These types of plants have floating or slightly aerial leaves and are rooted in submersed sediments with a water depth of 0.5-3.0 m. Examples of these plants are *Nuphar lutea* and *Nymphaea odorata*.

2.3.1.3 Emergent plants

These type of plants are observed on submersed or water saturated soil and grow in a water depth of 0.5 m or more above the substrate surface. The oxygen is transferred from their roots to the surrounding rhizosphere to enhance the degradation of the pollutants. Examples of these plants are *Typha latifolia, Carex rostrata, Scirpus lacustris, Acorus calamus,* and *Phragmites australis*.

2.3.1.4 Submerged plants

These types of plants grow well in oxygenated water with their photosynthetic tissue submersed in the water. Examples of these plants are *Ceratophyllum demersum, Myriophyllum spicatum* and *Rhodophyceae*.

2.3.2 Substrate

Substrate refers to the media used in the layers (distribution, filter, drainage) of a CW system. Many different types of wetland media have been used to develop, enhance, and optimize the performance removal of nutrients and OM from wastewater through a CW system, such as organic wood-mulch, rice husk, zeolite, sand, light weight aggregates (gravel), alum sludge, maerl, compost, slag, and peat (Saeed and Sun, 2012). In addition, the hydraulic mechanism of the system is significantly influenced by the structure and the composition of the media. Besides the ability of the substrate media in CWs to adsorb the pollutants, the substrate media also plays a key role in providing a suitable environment for microorganisms and wetland plants to biodegrade the contaminants in the wastewater (Ge et al., 2015b). Nevertheless, the substrate media should not be extremely large particles, because this would provide insufficient surface area for microbial establishment, whereas small particles provide a surface area for microbial establishment; narrow pore diameter media leads to blockage (Meng et al., 2014; Song et al., 2015). Sand and gravel combined together provide a recommended media to enhance the efficient removal of pollutants and hydraulic conditions as well (Kadlec et al., 2000).

2.3.3 Microorganisms

Many research studies have revealed that both aerobic and anaerobic zones of a CW system have numerous microbial communities existing in different forms, such as bacteria, algae, fungi, and protozoa (Faulwetter et al., 2009; Zhao et al., 2015; Cao et al., 2017). The interaction of physical, chemical, and biological processes in a CW system results in wastewater conversion of nutrient and organic contaminants. Therefore, the microbial communities in CWs are the reason behind the reduction of the contaminants in wastewater. In addition, the microbial communities in a CW system play a significant role in the wastewater treatment by feeding the microorganisms of the contaminants (Truu et al., 2009; Yuan et al., 2016). However, the removal of contaminants in a CW system relies on the interaction between microorganisms, plants, and substrate media. Furthermore, the aerobic and anaerobic microorganisms can achieve the decomposition of OM in a CW system. OM biodegradation is commonly correlated to autotrophic and heterotrophic bacteria, fungi, yeast, and protozoa (Meng et al., 2014; Park et al., 2015). The microorganisms can grow rapidly when a suitable environment and adequate nutrients are available. Microorganisms usually live in water, the roots of plants, or substrate media, and they consume nutrients and OM, therefore removing or reducing the level of different contaminants from wastewater.

Microorganisms carry out significant activities in a CW system, for example, changing oxidation and reducing the reactions of the substrate media, and converting several organic and inorganic constituents from harmful to harmless constituents. Several types of microorganisms are involved in nitrogen removal, such as bacterial groups, like β-*Proteobacteria* and γ-*Proteobacteria* for the nitrification process (Purkhold et al., 2000; Faulwetter et al., 2009); bacterial groups, like *Enterobacter* and *Micrococcus,* for the denitrification process (Gómez-Villalba et al., 2006; Meng et al., 2014); and planctomycete-like bacteria *Candidatus Brocadia anammoxidans* for the anaerobic ammonium oxidation (Anammox) process (Schmid et al., 2000).

2.3.4 Hydrology

Hydrology is a constant or intermittent saturation of the CW substrate media, so it can be said that hydrology contributes to the anaerobic condition and serves as a path to where the common biogeochemical operations are taking place (Postila et al., 2015). In CWs, hydrology can be defined by two parameters: hydro period and depth of flooding (Ronkanen and Kløve, 2008). The hydro period can be defined as the time during which the soil is saturated or flooded, and it is affected by several natural aspects, such as geology, subsurface soil characteristics, groundwater, and weather conditions. The depth of flooding in a natural wetland varies between +2 m and –1 m; it is related to the ground surface, with an average of around +1 m. These two parameters strongly influence the stability and the characteristics of the wetlands (i.e. pH, oxygen concentration, redox, plants, nutrients, etc.) (Scholz and Lee, 2005).

Hydraulic retention time (HRT) is the average time that water stays in the wetland and is an extremely important variable in designing, operating, and evaluating the performance treatment of wetland systems (Zhang et al., 2012a; Lee et al., 2014) and in determining the efficiency performance, plant uptake and biochemical processes (Kadlec and Knight, 1996).

2.4 Mechanisms and pathways for nitrogen removal in constructed wetlands

Various compounds of N are amongst the most significant components of wastewater and so are of concern to scientists and engineers due to their role in and contribution to eutrophication along with other nutrients like P. However, eutrophication can lead to blooming algae, thus depleting the DO in bodies of water. Nitrogen, a fundamental nutrient for all living organisms, is a key element in wetland biogeochemical cycles. It exists in organic and inorganic N compound forms. Organic N compounds are nucleic acids, amino acids, purines, proteins, uric acid, urea, and pyrimidines. Inorganic N compounds are ammonia (NH_3), ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), dinitrogen (N_2), nitric oxide (NO) and nitrous oxide (N_2O) (Kadlec and Knight, 1996). There are numerous processes for the removal of N from wastewater in a wetland environment, by either physicochemical or biological processes. Physicochemical processes include ammonia volatilization, adsorption, and sedimentation, whereas biological processes include ammonification, nitrification, denitrification, plant uptake, biomass assimilation, and anaerobic ammonium oxidation (Anammox). Figure 2.6 shows the numerous forms of N and major N conversion processes in wetlands. This thesis focuses on the biological process for nitrogen removal in an SSFCW system. Further details are given in the following sections.

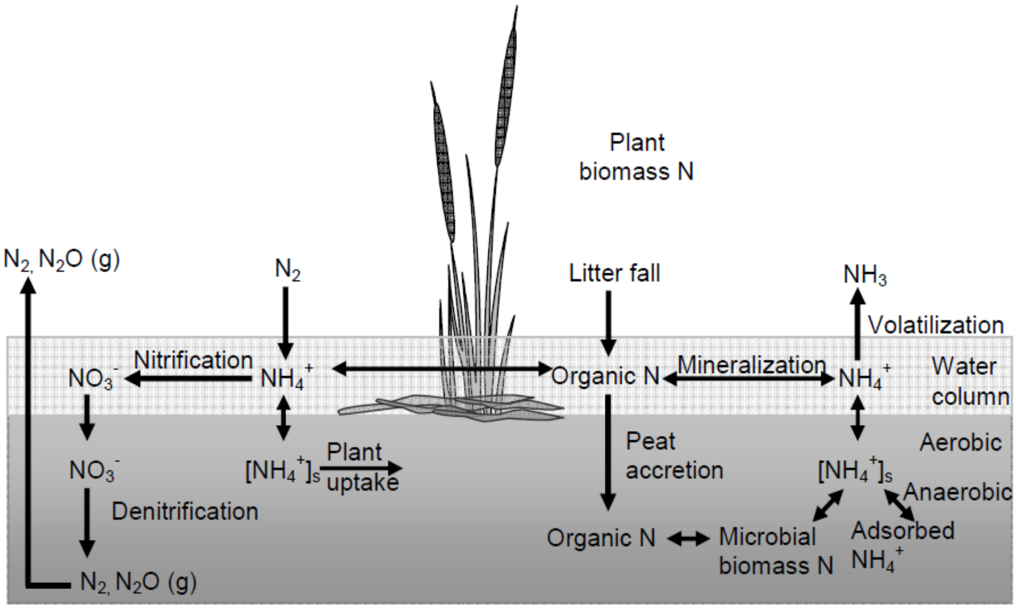


Figure 2.6 Schematic diagram of major nitrogen conversion in wetland (Reddy and DeLaune, 2008)

2.4.1 Classical nitrogen removal pathways

2.4.1.1 Biological process

Figure 2.7 shows the conventional biological nitrogen removal pathways in SSF wetland systems; this will be discussed in more detail in the following sections.

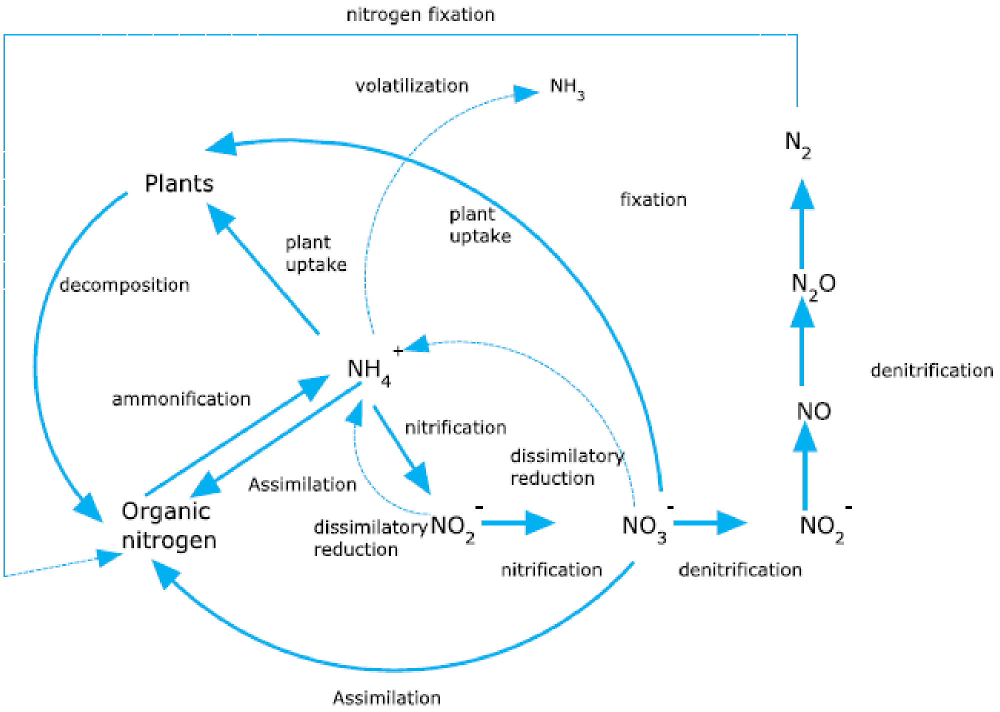


Figure 2.7 Biological nitrogen removal pathways in subsurface wetland (Saeed and Sun, 2012)

2.4.1.1.1 Ammonification

Ammonification, also called mineralization, is a biological process that converts organic nitrogen into ammonia, which can then be presented for other processes, such as volatilization, adsorption, plant uptake, and nitrification. This process can be affected by C/N ratio, temperature, pH, soil structure and available nutrients (Reddy et al., 1984). The process decreases with depth, as due to the aerobic conditions, ammonification in the upper zone will be faster than in the lower zone, where anaerobic conditions occur (Reddy et al., 1984; Kadlec et al., 2000). The optimal pH range is 6.5-8.5, and the high temperatures (40 - 60°C) will speed up the process (Hammer and Knight, 1994; Vymazal, 2007). The ammonification process can go through many kinds of deamination reaction; these are expressed by (Saeed and Sun, 2012) as the following equation 2.1:

$$\text{Amino acids} \rightarrow \text{Imino acids} \rightarrow \text{Keto acids} \rightarrow NH_3 \qquad \text{eq. 2.1}$$

2.4.1.1.2 Nitrification

Nitrification is a biological aerobic oxidation that converts ammonia to nitrate using nitrifying bacteria (Kadlec and Knight, 1996). The nitrifying bacteria, such as *Nitropira*, *Nitrosococcus*, *Nitrosomonas* and *Nitrobacter*, are aerobic, autotrophic and chemoautotrophic (Purkhold et al., 2000). They use carbon dioxide (CO_2) and its aqueous forms as a carbon supply source, while they use the oxidation of ammonia or nitrate as the energy source for growth (Vymazal, 2007). The nitrification process is one of the most important of the mechanisms that reduce the concentration of ammonia nitrogen. It is a two-step process under aerobic conditions using autotrophic bacteria (Kadlec and Knight, 1996; Vymazal, 2007). The two nitrification processes are nitritation and nitratation (Metcalf and Eddy, 2003). The most commonly known genus of bacteria that convert ammonia to nitrite is *Nitrosomonas* (ammonium oxidizing bacteria (AOB), nitritation), and the conversion of nitrite to nitrate in the presence of oxygen is by *Nitrobacter* (nitrite oxidizing bacteria (NOB), nitritation), where the oxygen is the electron acceptor (Lee et al., 2009). Nitrifying bacteria occur in the surface layers and decrease with depth as the upper zones have aerobic conditions whereas in the lower zones, the conditions are anaerobic (Kadlec and Wallace, 2008). The process can be written by the following two reaction equations 2.2 and 2.3 (Reddy et al., 1984):

$$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + H_2O + 2\ H^+ \quad \text{(Ammonia oxidizing bacteria, AOB)} \qquad \text{eq. 2.2}$$

$$NO_2^- + 0.5\ O_2 \rightarrow NO_3^- \quad \text{(Nitrite oxidizing bacteria, NOB)} \qquad \text{eq. 2.3}$$

Therefore, the nitrification process reaction can be written as the following reaction equation 2.4:

$$NH_4^+ + 2\ O_2 \rightarrow NO_3^- + 2\ H^+ + H_2O \qquad \text{eq. 2.4}$$

Several environmental factors can affect the growth of nitrifying bacteria, and therefore, affect the efficiency of the nitrification process. Examples of environmental factors include ammonium concentrations, temperature, pH value, alkalinity of the water, microbial population, inorganic carbon source, DO concentration, and soil moisture (Reddy et al., 1984; Vymazal, 1995). The optimum temperature for the nitrification process is between 16.5 to 32 °C (Demin and Dudeney, 2003; Katayon et al., 2008) while the minimum temperatures required for *Nitrosomonas* and *Nitrobacter* growth are 5 and 4 °C, respectively (Cooper et al., 1996). The optimum pH for the nitrification process is between 7.2 and 9 (Kadlec and Wallace, 2008). Moreover, heterotrophic bacteria (that is, those that use the organic carbon source as a carbon source for growth) will be the predominant bacteria when a high concentration of biodegradable organics exists in the wastewater. For this reason, a significant nitrification process does not occur before significant OM removal has occurred (Kadlec and Knight, 1996).

2.4.1.1.3 Denitrification

The denitrification process is the main mechanism for N removal from a wetland system (Hammer and Knight, 1994). This is a biochemical process that converts NO_3^- to N_2 by intermediates NO_2^-, NO, and N_2O (Vymazal, 2007), as shown in equation reaction 2.5:

$$2\ NO_3^- \rightarrow 2\ NO_2^- \rightarrow 2\ NO \rightarrow N_2O \rightarrow N_2 \qquad \text{eq. 2.5}$$

It is carried out by autotrophic and heterotrophic anaerobic bacteria, for example, *Pseudomonas, Achromobacter, Alcaligenes, Thiobacillus, Paracoccus, Bacillus,* and *Micrococcus* (Kadlec and Knight, 1996). Denitrification uses either nitrate as the electron acceptor instead of oxygen to oxidize organic matter (heterotrophic denitrification) or uses inorganic matter such as sulphur or hydrogen (autotrophic denitrification) for the growth of the microorganisms and to gain energy under anoxic/anaerobic conditions (Kadlec and Wallace, 2008). Denitrification occurs when oxygen levels are depleted and nitrate becomes the primary oxygen source for microorganisms. The denitrification rate can be increased by adding an external carbon source (Wu et al., 2014), as the denitrification process demands an external carbon source which is used as an electron donor for the process, such as acetate, acetic acid, lactate, methanol, ethanol and glucose (Khin and Annachhatre, 2004). The denitrification process can be written as the following reaction equations 2.6 and 2.7:

$$4\ (CH_2O) + 4\ NO_3^- \rightarrow 4\ HCO_3 + 2\ N_2O + 2\ H_2O \qquad \text{eq. 2.6}$$

$$5\ (CH_2O) + 4\ NO_3^- \rightarrow H_2CO_3 + 4\ HCO_3 + 2\ N_2 + 2\ H_2O \qquad \text{eq. 2.7}$$

where (CH_2O) represents the organic matter.

Several factors can influence the denitrification rate, such as temperature, pH value, quantity and quality of organic carbon source, NO_3^- concentrations, absence of oxygen, soil moisture, soil type, redox potential, presence of denitrifying bacteria, different plant species residues, hydroperiods, and water level (Vymazal, 1995). The denitrification rate is slower in acidic conditions compared to neutral or alkaline conditions. The optimum pH for the denitrification process is between 6 and 8. When the pH is less than 6, the transformation of N_2O to N_2 will be inhibited. However, at a pH below 4, denitrification will be negligible or no denitrification will take place (Paul and Clark, 1996). The denitrification process can occur in temperatures between 25 and 75 °C (Hammer and Knight, 1994). The rate of denitrification doubles between 11-35 °C and decreases rapidly below 5 °C, even though it is still detectable at 2 °C (Paul and Clark, 1996). However, about 60-95% of TN and 75-95% of NO_3^- removal results from the denitrification process (Hammer and Knight, 1994).

The differences between the nitrification and denitrification processes are shown in Table 2.4:

Table 2.4 Differences between nitrification and denitrification processes (Hernando and Martínez, 2010)

Characteristics	Nitrification process	Denitrification process
Type of microorganism	Autotrophic	Generally Heterotrophic
Grow rate	$Days^{-1}$	$Hours^{-1}$
Type of respiration	Aerobic	Anoxic/Anaerobic
Limiting factors during the process	Low oxygen concentration Presence of OM	Presence of oxygen Low OM concentration

It can be concluded that the biological nitrification-denitrification process is achieved by passing wastewater through the aerobic and anaerobic steps, as shown in Figure 2.8.

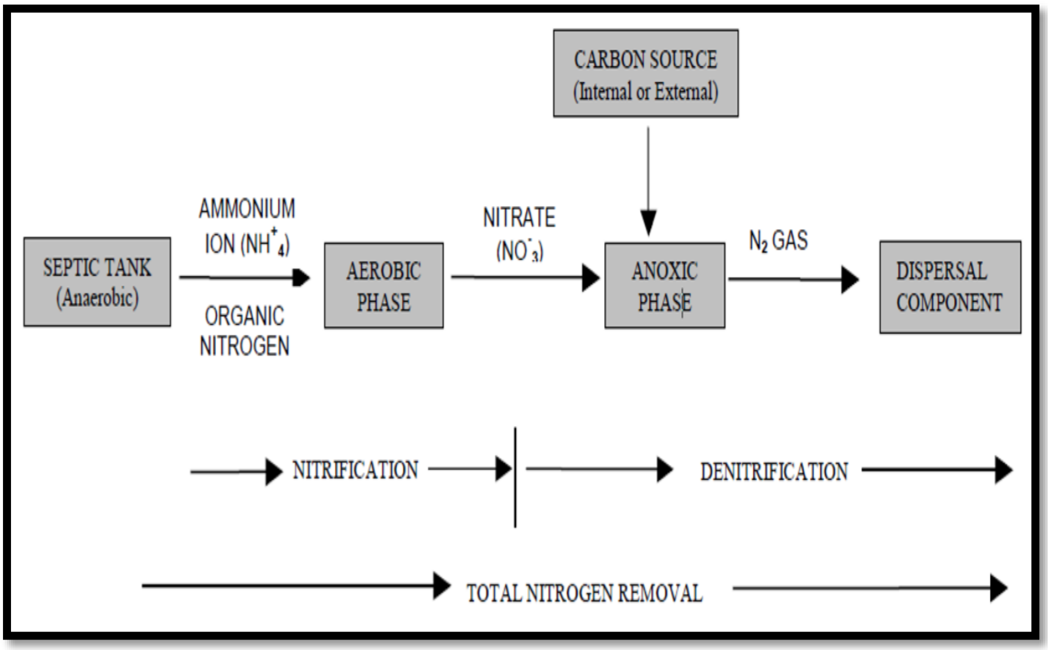


Figure 2.8 Biological nitrification-denitrification process (Serra and Vazaiou, 2012)

2.4.1.1.4 Nitrogen assimilation

The nitrogen assimilation process is a biological process that converts inorganic nitrogen into the form of organic components to build up blocks for cells and tissues for the growth of microorganisms and macrophytes (Vymazal, 1995). Both ammonia and nitrate are considered forms of nitrogen that are used for assimilation. Emergent and rooted floating-leaved macrophytes uptake nutrients from sediments, whereas free floating macrophytes uptake nutrients from the water (Wetzel, 2001). However, plants act only as a temporary storage for nutrients. When wetland plants die, biomass is returned to the wetland for nutrient burial and recycling. A small portion of the biomass is buried and stored as new soil and sediment for the wetland, whereas the majority of the plant biomass decomposes, and then the nutrients are recycled (Kadlec and Knight, 1996). Plants in a CW system improve the N removal efficiency, because they enhance the nitrification process by providing surface area and oxygen, and they enhance the denitrification process by providing about 5-25% of the carbon source from their roots (Wang et al., 2012a).

2.4.1.1.5 Nitrogen fixation

Nitrogen fixation is a process that converts atmospheric nitrogen (N_2) to organic nitrogen. In wetland systems, mostly nitrogen fixation is carried out by autotrophic and heterotrophic bacteria, such as cyanobacteria (blue-green algae), free-living bacteria (*Bacillus*, *Klebsiella*, *and Clostridium*), Rhizobium bacteria and actinomycetes (*Frankia*) (Kadlec and Wallace, 2008). Nitrogen fixation has a basically negligible loading impact on CWs that receive high nitrogen loading in the wastewater, because high ammonium concentrations would inhibit nitrogen fixation (Kadlec and Wallace, 2008). However, this process can occur in several locations, such as in aerobic and anaerobic soil layers, in the plant rhizosphere, and on the leaf and stem of the plants (Reddy and DeLaune, 2008).

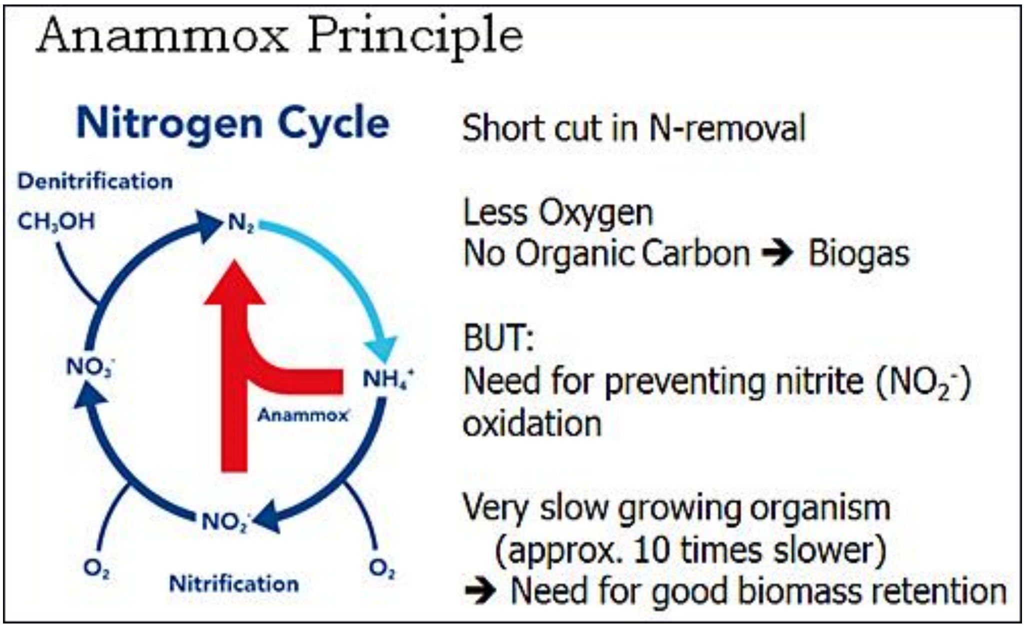
2.4.2 New and emerging nitrogen removal routes

2.4.2.1 Anaerobic ammonium oxidation (Anammox)

The anaerobic ammonium oxidation (Anammox) process was originally discovered in 1995 in a denitrifying fluidized bed reactor (Mulder et al., 1995). Many research studies for nitrogen removal using the Anammox process have been conducted at relatively high water temperatures, since the optimal temperature for Anammox bacteria is approximately 30-40°C and the optimal pH is between 6.7 and 8.3 (He et al., 2012). If the temperature is below 25°C, the Anammox process would be expected to be applicable to many kinds of industrial wastewater treatments, where there is a high ammonium content and low organic carbon content (Mulder et al., 1995). In 2002, the first full-scale Anammox reactor in the world was put into operation at Sluisjesdijk, a sludge treatment plant in Rotterdam, the Netherlands. The Anammox process is being used as an alternative way to remove nitrogen from wastewater instead of the conventional way of using the nitrification-denitrification process, since it has low operating costs. The Anammox process is a biological autotrophic process where ammonium and nitrite are converted to nitrogen gas (N_2) under anaerobic conditions, using organic intermediates, such as hydrazine (N_2H_2) and hydroxylamine (NH_2OH). It can also be conducted without any additional or external carbon source, since the carbon dioxide (CO_2) will be the main source of carbon for bacterial growth (Mulder et al., 1995). Nitrite will be used as the electron acceptor instead of using organic compounds. The main product of Anammox is nitrogen N_2, but around 10% of ammonium and nitrite is converted to nitrate. Therefore, the Anammox reaction equation can be written as the following equation 2.8:

$$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15}\ (\text{Anammox bacteria}) + 2.03\ H_2O \qquad \text{eq. 2.8}$$

That means Anammox bacteria need to be provided by nitrite; however, the substrate of nitrite is toxic to Anammox organisms at a level above 70 mg/l (Van der Star et al., 2007) and can stop the process completely. The disadvantage of the Anammox process is mainly the very slow growth of Anammox bacteria (Jetten et al., 2001). The Anammox bacteria consume ammonium to live under anaerobic conditions, which makes the process a good alternative for the conventional nitrogen removal process (nitrification-denitrification process). Anammox bacteria growth can be activated and enriched by adding ammonium chloride (NH_4Cl) to the inlet of wastewater before the wastewater enters the treatment process, e.g., CW systems (He et al., 2012). Anammox bacteria are found in wastewater treatment systems and in sea water according to their genus: *Brocadia*, *Kuenenia* and *Scalindua*. The following Figure 2.9 illustrates the possible nitrogen cycle using the Anammox process.



Anammox Principle

Short cut in N-removal

Less Oxygen
No Organic Carbon ➔ Biogas

BUT:
Need for preventing nitrite (NO_2^-) oxidation

Very slow growing organism
(approx. 10 times slower)
➔ Need for good biomass retention

Figure 2.9 Short-cut in nitrogen cycle using the Anammox process (Jetten et al., 1997)

Ammonium, nitrite, nitrate, the availability of OM, pH, temperature, and presence of other oxidants (manganese, iron oxides, etc.) control the Anammox process (Reddy and DeLaune, 2008). The Anammox process is also appropriate for wastewater flows with high nitrogen concentrations and a low organic carbon source (Hernando and Martínez, 2010; Jin et al., 2012) such as reject water from digested sludge, landfill leachate, fish canning effluent, nitrogen fertilizer production, cocking wastewater, and industrial wastewater. There are several advantages and benefits of using the Anammox treatment process (Khin and Annachhatre, 2004; Hernando and Martínez, 2010) such as the following:

- high efficiency of nitrogen removal
- no need for additional external OM source because it is an autotrophic process
- reduced oxygen demand for the nitrification reactor, therefore reducing the energy supply for aeration by around 60% and so reducing the operating costs.
- lower environmental impact by reducing 90-95% of CO_2 emissions to the air because the Anammox process consumes CO_2 and also produces no N_2O emissions
- low biomass, meaning little sludge is produced, resulting in a low operating costs
- up to 90% lower operating costs compared with the conventional process (nitrification-denitrification process)
- no chemicals addition required
- needs up to 50% less space.

In addition, there are many substances that prevent improvement of the Anammox process, such as substrates (e.g. ammonia and nitrite), OM (e.g. toxic OM and non-toxic OM), sulphide, heavy metals, phosphate, and salts (Jin et al., 2012).

2.4.2.2 Completely autotrophic nitrogen removal over nitrite (CANON)

CANON stands for a Completely Autotrophic Nitrogen Removal over Nitrite, where ammonium is the electron donor, and oxygen and nitrite are the electron acceptors. The CANON process is suitable for wastewater with a high ammonium concentration and low organic carbon source. Also, this process can function in a single reactor (Hu et al., 2014a). The CANON process relies on the interaction between two bacteria cooperating with each other in a single reactor, namely, *Nitrosomonas* and *Nitrospira* aerobic bacteria, and *Planctomycete* anaerobic ammonium oxidizing (Anammox) bacteria due to the oxygen limitation (Third et al., 2001; Khin and Annachhatre, 2004). Regarding the aerobic ammonium oxidizing bacteria, these bacteria will oxidize ammonium to nitrite in conditions of limited oxygen (Third et al., 2001) as in the following reaction equation 2.9:

$$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + 2\ H^+ + H_2O \qquad \text{eq. 2.9}$$

while the Anammox bacteria will use the nitrite that is produced from equation 2.9 as an electron acceptor to oxidize ammonium to convert it to dinitrogen gas and trace amounts of nitrate, as in the following reaction equation 2.10:

$$NH_4^+ + 1.3\ NO_2^- \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 2\ H_2O \qquad \text{eq. 2.10}$$

In the Anammox process, Anammox bacteria convert ammonium together with nitrite, which is the electron acceptor, directly to dinitrogen gas in the absence of oxygen. It is a precondition for the Anammox process that the antecedent nitrification process stops at nitrite due to ammonia-oxidizing bacteria (AOB) (partial nitrification, PN), i.e., the oxidation of nitrite to nitrate by nitrite-oxidizing bacteria (NOB) has to be avoided (without affecting the activities of AOB and Anammox bacteria) due to the rapid conversion of nitrite to nitrate (Hu et al., 2014a). NOB competes with AOB and Anammox bacteria for DO and nitrite respectively (Bagchi et al., 2010). This can be achieved by making selective conditions where AOB grows faster than NOB. Numerous operational parameters have been manipulated for this purpose, including DO, free nitrous acid, free ammonia (FA), temperature, pH, inorganic carbon, etc. Amongst them, low DO (≃1 mg/l), a pH value between 7.8 and 8.5, and especially elevated temperatures (above 25°C) have been demonstrated to be the key controlling factors for partial nitrification (He et al., 2012; Hu et al., 2014a). In addition, it is worth mentioning that adding ammonium chloride (NH_4Cl) to the influent before feeding the wetlands enhances and improves the growth of AOB and Anammox bacteria (He et al., 2012).

Since different conditions are required for PN (aerobic) and Anammox (anoxic), a two-reactor (aerobic/anaerobic) system was primarily adopted to support and facilitate these autotrophic nitrogen conversion processes. After that, it was found possible to combine and integrate these two processes within a single reactor under limited oxygen conditions, referred to as the CANON process as described in reaction equation 2.11 (Third et al., 2001):

$NH_4^+ + 0.79\ O_2 + 1.11\ HCO_3^- \rightarrow 0.0103\ C_5H_7O_2N$ (AOB) $+ 0.028\ CH_2O_{0.5}N_{0.15}$ (Anammox bacteria) $+ 0.11\ NO_3^- + 0.44\ N_2 + 1.06\ CO_2 + 2.49\ H_2O$ eq. 2.11

CANON is an autotrophic (self-feeding) process, which avoids the need for an additional carbon source. The entire nitrogen removal process can be achieved in a single reactor with very low aeration (Bagchi et al., 2010; Hu et al., 2014a) leading to a reduction in space, energy and operating costs (Third et al., 2001; Khin and Annachhatre, 2004). The CANON process requires limited oxygen for the process to take place; therefore, it requires a shorter unsaturation time and a longer saturation time in the bed. Moreover, the CANON process consumes 63% less oxygen and 100% less reducing chemicals than the conventional nitrification-denitrification process for nitrogen removal (Khin and Annachhatre, 2004).

Table 2.5 shows a comparison between conventional, Anammox, and CANON processes for nitrogen removal.

Table 2.5 Comparison of the conventional and new processes for nitrogen removal (Khin and Annachhatre, 2004)

System	**Conventional nitrification-denitrification**	**Anammox**	**CANON**
Number of reactors	2	1	1
Discharge	NO_2^-, NO_3^-; N_2	NO_3^-, N_2	NO_3^-, N_2
Conditions	Oxic; anoxic	Anoxic	Oxygen limited
Oxygen requirement	High	None	Low
carbon requirement	Yes	None	None

Bacteria	Nitrifiers + various heterotrophs	Planctomycete	Aerobic NH_4^+ oxidizers + Planctomycete
Advantages	High efficiency in removal. High process reliability, therefore more stability. Easy process control.	High efficiency of nitrogen removal. No need for additional external OM source. Biomass is very low. Less energy, operational costs and area requirements.	Almost complete transformation of ammonium to dinitrogen gas. High loss of nitrogen. Low organic carbon.
Disadvantages	High energy for aeration, therefore high cost	Slow growth of Anammox bacteria	Trace amounts of nitrate

Also, Table 2.6 summarizes the classical and emerging N removal routes from wastewater.

Table 2.6 Summary of classical and emerging nitrogen removal routes from wastewater (Saeed and Sun, 2012)

Route	Mode	Significance
Classical N removal routes		
Ammonification	Microbiological	Changes the form of nitrogen in wastewater, e.g. from organic nitrogen to NH_4^+-N.
Nitrification	Microbiological	Transforms NH_4^+-N to NO_2^--N and NO_3^--N. Net quantity of TN remains constant.
Denitrification	Microbiological	Reduces NO_3^--N to N_2 gas. The process reduces TN quantity in wastewater, when combined with nitrification.
Nitrogen assimilation	Microbiological	Adsorbs NH_4^+-N, thereby reducing nitrogen content of wastewater.

emerging N removal routes		
Partial nitrification, Anammox and CANON	Microbiological	Allows nitrite removal over nitrate, reducing net TN content of wastewater.

2.5 Effects of environmental factors and operational conditions on nitrogen removal in subsurface flow constructed wetlands

As has been discussed in previous section 2.4, nitrogen removal in a CWs system depends on several environmental factors. A brief discussion on the interactions between environmental and operational factors with wetland performance in nitrogen removal is provided in the following sections.

2.5.1 pH

The nitrification process consumes alkalinity (Kadlec and Knight, 1996), as is shown in reaction equation 2.12:

$$NH_4^+ + 1.83\ O_2 + 1.98\ HCO_3^- \rightarrow 0.021\ C_5H_7O_2N + 0.98\ NO_3^- + 1.041\ H_2O + 1.88\ H_2CO_3 \quad \text{eq. 2.12}$$

The nitrification process can result in a significant drop in the pH of the wastewater, consequently obstructing the denitrification process. The denitrification process can be inhibited at a pH less than 6 and above 8, and the highest rate of the denitrification process was detected at a pH between 7 and 7.5. In addition, the denitrification process occurs at a slower rate at pH 5 in wetland systems (Vymazal, 2007). However, in a wetland system with intensive algae vegetation in the day time, the pH increases because of the photosynthesis, and decreases in the night time because algae respiration results in a weak carbonic acid that causes a reduction in the pH of the wetland (Kadlec and Knight, 1996). Furthermore, the pH in wastewater may increase or decrease due to the biological reaction by microbial degradation, plant uptake, and type of soils (Campbell et al., 2000). In addition, both chemical reagents sodium hydroxide (NaOH) and hydrochloric acid (HCl) can be used to increase and decrease the pH in the wastewater respectively.

2.5.2 Temperature

Temperature affects all of the biological nitrogen removal processes. Temperature is considered one of the important environmental factors that affect microbial growth rates and on which their activities depend (Gikas et al., 2007). Several research studies have shown that the efficiency of nitrogen removal at high temperatures is more efficient than at low temperatures (Akratos and Tsihrintzis, 2007; Sani et al., 2013; Mietto et al., 2015). In addition, nitrogen removal by plant uptake is greater at higher temperatures. However, the optimum temperature range for ammonification is between 40 and 60 ˚C (Vymazal, 2007) while the preferred temperature for the nitrification process in CWs is between 16.5 and 32 ˚C (Demin and Dudeney, 2003; Katayon et al., 2008). Little of the nitrification process happens below 5 ˚C and above 40 ˚C (Vymazal, 2007), whereas a slow denitrification process can take place at low temperatures, e.g., 5 ˚C with its reaction rate increasing with the increasing temperature (optimal temperature between 11 and 35 ˚C) (Paul and Clark, 1996).

2.5.3 Availability of oxygen

There are three ways for oxygen to diffuse from the atmosphere into a wetland system: direct dissolution to the wetland surface water, convective translocation through live leaves and stems, and convective transport through dead roots and stems (Kadlec and Knight, 1996). DO concentration in wetlands depends on dissolved salt, temperature, and biological activities (Kadlec and Knight, 1996). DO concentrations increase with decreasing temperatures in wetlands (Kaseva, 2004), so DO concentrations are lower in summer than in winter. The oxygen level in wetlands can affect nitrification, denitrification, and OM decay. The oxygen level increases, nitrification and decomposition activities increase, and denitrification activity decreases. The presence of oxygen is very important for the nitrification process in CW systems as is shown in equations 2.13 and 2.14 (Kadlec and Knight, 1996), where the oxygen level controls the nitrification process as well as organic biomass degradation in the CW system.

$$NH_4^+ + 1.5\ O_2 \rightarrow H^+ + H_2O + NO_2^- \qquad \text{eq. 2.13}$$

$NH_4^+ + 1.83\ O_2 + 1.98\ HCO_3^- \rightarrow 0.021\ C_5H_7O_2N + 0.98\ NO_3^- + 1.041\ H_2O + 1.88\ H_2CO_3$ eq. 2.14

Numerous research studies show the important role of oxygen in N and OM removals in aerated and non-aerated SSFCWs (Liu et al., 2013b; Chyan et al., 2016; Wu et al., 2016). Artificial aeration is adopted to increase the DO concentration in the CW, which requires more energy consumption. Therefore, operating costs will be increased. It is noticed that artificial aeration has a huge influence on and leads to improvement in removing ammonium-nitrogen. There are two artificial aeration techniques: continuous aeration (CA) and intermittent aeration (IA) (Liu et al., 2013b). Compared with CA, IA could not only minimize the operating costs, but also could create different micro-environments (improve microbial activities) for effective N removal. Therefore, IA is an appropriate choice to improve N removal in a CW system.

2.5.4 Availability of organic carbon source

The organic carbon source is a very significant factor for the removal of biological nitrogen, as it is often restricted by insufficient organic carbon in a CW system, as shown in the following equations 2.15 and 2.16 (Kadlec and Knight, 1996). Organic carbon has an important relationship with denitrification activities, as it provides the electron donors necessary for denitrifying bacteria.

$NO_3^- + 0.833\ CH_3OH \rightarrow 0.5\ N_2 + 0.833\ CO_2 + 1.167\ H_2O + OH^-$ eq. 2.15

$NO_3^- + 1.08\ CH_3OH + 0.24\ H_2CO_3 \rightarrow 0.056\ C_5H_7NO_2 + 0.4\ 7N_2 + 1.68\ H_2O + HCO_3^-$ eq. 2.16

To overcome this issue of a lacking or limited carbon source, the researchers added an external carbon source to the wetlands to assist and support the denitrification process (Kadlec and Knight, 1996). Acetate, acetic acid, lactate, methanol, ethanol, and glucose are examples of an external carbon source (Khin and Annachhatre, 2004). The denitrification performance rate depends on the carbon source, which varies between different systems, i.e., type of wastewater, type of plants, wetland configurations, and nitrogen composition (Zhao et al., 2010). Moreover, it is possible to supply organic carbon (internally) for the denitrification step from the media, such as wood-mulch, rice husk, compost and peat (Saeed and Sun, 2012). However, the biodegradation of

OM in CWs depends on the hydraulic contact time applied and the OM contents during wastewater treatment (Stefanakis et al., 2014). In addition, the biodegradation of OM predominantly takes place in the top 10-20 cm layers because of the high availability of oxygen and the microbial community in the upper wetland media layer (Kadlec and Wallace, 2008; Stefanakis and Tsihrintzis, 2012).

2.5.5 Hydraulic load rate and retention time

The hydraulic loading rate (HLR) is one of the main factors in monitoring the performance of a CW system. HLR is the volume of water that enters the CW divided by the surface area of the CW. HLR is inversely related to hydraulic retention time (HRT). Increasing HLR facilitates the faster passage of wastewater through the wetland media, subsequently decreasing the contact time between the wastewater and the removal components of the wetland, and reducing the system`s N removal performance efficiency (Saeed and Sun, 2012). Increasing HRT usually enhances the efficiency performance of N removal from a CW because of the longer contact time between N pollutants and microorganisms in the bed (Akratos and Tsihrintzis, 2007).

2.5.6 The mode of influent feed

The appropriate choice of the feed mode is very significant to improve the treatment performance efficiency. There are various kinds of influent feed modes for a CW system to promote N removal performance, such as continuous, intermittent, tidal, and step-feeding modes (Saeed and Sun, 2012). An intermittent feeding mode can improve N removal efficiency in VFCWs by the diffusion of atmospheric oxygen into the wetland media through wet and dry periods. This feeding mode has more influence on N removal than has the continuous feeding mode in a CW system because of the increase in the oxidizing conditions, and it can also create aerobic and anoxic/anaerobic zones in the CW bed (Jia et al., 2010). Tidal flow in a VFCW system is effective to enhance the oxygenation in the wetland media (Wang et al., 2016d), as is disscused in detail in section 2.2.2.2.1. The step-feeding mode is an effective alternative option to improve TN removal by the stepwise introduction of the influent to the nitrified liquid, consequently making more efficient use of the influent carbon source for the denitrification step (Hu et al., 2012). In general, the intermittent, tidal, and step-feeding modes are more effective and efficient than the continuous mode in N removal (Saeed and Sun, 2012).

2.5.7 Nitrogen and organics loading

Excessive nitrogen loading in wastewater would have an impact on the nitrogen removal rates in the SSFCW system. The excess of ammonium in the wastewater affects the growth of specific plants and biomass. To illustrate this point, an ammonium-nitrogen concentration of above 100 mg/l can affect and inhibit the growth of some plants, such as *Schoenoplectus tabernaemontani*, *J. effusus* and *T. latifolia L.*, and therefore, can affect and reduce the performance efficiency of N removal in wetland systems (Saeed and Sun, 2012). However, Paredes et al. (2007) indicted that at a high nitrite concentration, plants are not a major factor for N removal because of the toxicity of the nitrite. Subsequently, the microorganisms will play a major function and role in the N removal process. In addition, the existence of organics in the wastewater affects the nitrification rates in wetland systems; this is because the growth rate of heterotrophic bacteria is higher than that of autotrophic bacteria, therefore, increasing and enhancing the available oxygen consumption and limiting nitrification process (Saeed and Sun, 2012).

2.5.8 Recirculation

The aim of this process is to recirculate the effluent wastewater to the wetland again without discharging it to achieve the desired quality of the effluent. For the VFCW, the effluent recirculation improves and enhances the nitrification process when the bed matrices are exposed to oxygen depletion. In addition, it promotes the denitrification process because of the higher contact time between wastewater and microorganisms, as well as providing the OM supply to the system (Saeed and Sun, 2012). Some research studies have demonstrated different recirculation ratios that should be considered for the appropriate design of a CW system by examining the impact of the wastewater recirculation ratio on the system. These studies (Sun et al., 2003; Zhao et al., 2004c; Ayaz et al., 2012) have shown that a ratio of 1:1 is effective for N removal. The recirculating vertical flow constructed wetland (RVFCW) is a modification of the VFCW with these following advantages (Prost-Boucle and Molle, 2012): reduced footprint, mixing of the treated wastewater (effluent) with the untreated wastewater (influent) to increase the treatment value, and improved efficiency of the nitrification and denitrification processes. However, one disadvantage of the recirculating is the high energy consumption involved in using electrical equipment for pumping purposes to recirculate the effluent wastewater, thereby increasing the operating costs for the system (Prost-Boucle and Molle, 2012).

2.5.9 Plant harvesting

Plant harvesting can enhance the N removal performance in CWs, mostly for not highly loaded systems (Vymazal, 2007), as it can remove N at a level of between 0.27 and 0.68 $g/m^2/d$ (Kadlec et al., 2000). Plants grow rapidly in the summer time, which facilitates the N uptake (Saeed and Sun, 2012). The efficiency of frequent harvesting might also assist the preservation of a high quality standing crop of young and actively growing plants. Thereby, N uptake and storage by growing plants in CWs could be almost continuous and could represent a substantial pathway for N removal from wastewater (Kadlec and Wallace, 2008).

2.6 Nitrogen transforming bacteria community in wetlands

2.6.1 Nitrifying bacteria

Nitrification depends on two types of bacteria: AOB, which convert ammonium to nitrite, and NOB, which convert nitrite to nitrate (Vymazal, 2007). AOB and NOB use NH_4^+ or NO_2^- as the energy source and bicarbonate and CO_2 for cell synthesis. AOB belongs to β-*Proteobacteria*, which consists of two genera, namely, *Nitrosospira* and *Nitrosomonas,* which are divided into nine various phylogenetic clusters based on 16S rRNA gene (rDNA) sequences (Purkhold et al., 2000). In general, the optimal temperature for nitrifying bacteria is 16.5‑32 ºC. Nevertheless, nitrification has been observed in wetland systems at temperatures between 0 and 5 ºC (Vymazal, 2007). However, nitrifying bacteria can adapt to the temperature changes and can continue their activities at cold temperatures due to their metabolic adaptation. In addition, some published research studies have concluded that nitrification is inhibited by water temperatures less than 10 ºC and drops off quickly below 6 ºC (Herskowitz et al., 1987;

Xie et al., 2003). On the other hand, the optimum pH for the nitrification process is between 7.2 to 9 (Kadlec and Knight, 1996). In addition, organic loading rates can affect the nitrification step in CWs due to nitrifying and heterotrophic bacteria because of the competition for inorganic nitrogen and oxygen, where intensive competition takes place in the presence of high organic carbon concentrations (Kadlec and Knight, 1996).

There are various environmental factors that can influence and affect the diversity of the nitrifiers, such as pH, temperature, salinity, and inhibitor substances like organic carbon and ammonia (Faulwetter et al., 2009). The AOB communities are very diverse; depth and variances between inflow and outflow wastewater characteristics are the most important factors that affect AOB distributions. Thus, the potential nitrification rates are higher in the rhizosphere area (Sleytr et al., 2009).

2.6.2 Denitrifying bacteria

Most of the denitrifying bacteria consume organic compounds as a source of cellular carbon and as electron donors. However, nitrogen oxides in ion and gaseous forms function as electron acceptors (Vymazal, 2007). The most common bacteria generated in the soil environment are *Micrococcus*, *Bacillus* and *Pseudomonas*, whereas the most common bacteria in the aquatic environment are *Aeromonas*, *Pseudomonas* and *Vibrio* (Kadlec and Knight, 1996). AOB *Nitrosomonas eutropha* is capable of denitrifying under anoxic conditions by using nitrite as an electron acceptor and hydrogen as an electron donor and thus producing nitric oxide (NO) and nitrous oxide (N_2O). Denitrifying bacteria in the environment can be detected by targeting nirK/nirS genes (Angeloni et al., 2006) and nosZ genes (Gómez-Villalba et al., 2006); these are the molecular markers for the conversion of NO_2^- to NO and N_2O to N_2 respectively.

Various environmental factors can affect the denitrification process, such as pH, temperature, availability of organic matter and nitrate, redox potential, the absence of oxygen, soil type, and the degree of moisture saturation (Vymazal, 1995). The activity of denitrifying bacteria increases with any increase in the temperature up to 60–75 ºC (Hammer and Knight, 1994). Denitrification rates decrease at low temperatures in wetlands (e.g. 5 ºC). However, greenhouse gases (i.e. N_2O and NO) are usually produced (Paul and Clark, 1996). On the other hand, the optimal pH for the denitrification process is between 6 and 8 (Paul and Clark, 1996), and low activity is observed at pH 5, but is absent at a pH of below 4 (Vymazal, 2007).

Organic carbon has the ability to increase the denitrifying activities in a CW system, while higher C:N ratios lead to more complete denitrification to dinitrogen gas (Wu et al., 2014). In addition, plants are able to affect the denitrification process by providing organic carbon through their roots. It is clear that the nitrate removal rate in planted CW is higher than in unplanted CW. However, the effect of plants varies from one species to another (Lin et al., 2002).

2.6.3 Anammox bacteria

The Anammox process is performed by planctomycete-like bacteria *Candidatus Kuenenia stuttgartiensis,* which often predominate in bacterial biofilms, and *Candidatus Brocadia anammoxidans,* which predominate in wastewater treatment (Jin et al., 2012). These bacteria were discovered by fluorescent in-situ hybridisation (FISH) in wastewater treatment systems. Anammox bacteria coexist more easily with heterotrophic bacteria compared to nitrifying bacteria, because heterotrophic bacteria consume oxygen, which can create an anoxic environment that is favourable to Anammox bacteria but in competition with nitrifying bacteria without generating excessive greenhouse gases (Faulwetter et al., 2009). In the Anammox reaction, ammonia is transformed with about 90% transformed to N_2, 10% to NO_3^-, and less than 0.1% to N_2O, whereas denitrifiers could transform the created NO_3^- to N_2 (Mulder et al., 1995). However, there are various environmental factors which can affect the Anammox process, such as ammonium, nitrite, nitrate, availability of organic matter, pH, and temperature. The optimal temperature for Anammox bacteria is approximately 30-40°C, and the optimal pH is between 6.7 and 8.3 (He et al., 2012). If the temperature is below 25°C, the Anammox process would be expected to be applicable to many kinds of industrial wastewater treatments where there is a high ammonium content and low organic carbon content (Mulder et al., 1995).

2.7 Choice of vertical flow constructed wetlands over horizontal flow constructed wetlands

The exploration of VF and HF SSFCWs is increasing worldwide as a substitutional wastewater treatment for several kinds of wastewater because of their low operating costs and ease of operation and maintenance (Chang et al., 2012; Abou-Elela et al., 2013; Stefanakis et al., 2014). However, despite these benefits of VFCWs and HFCWs, some research studies have suggested that VFCWs are better in the treatment and efficient removal of SS, OM, nutrients, and heavy metals as they offer several strategies (i.e. tidal flow, recirculation, intermittent loading regimes, etc.) rather than continuously saturated HFCWs (Jia et al., 2010; Chang et al., 2012; Paing et al., 2015; Wu et al., 2016). VFCWs are satisfactory for the nitrification process and poor for the denitrification process because of the high oxygenation in VFCWs and the absence of oxygen that is prevalent in HFCWs (Vymazal, 2014; Song et al., 2015). However, with some modifications to the system, it can be used for denitrification as well.

In a CW system, VFCWs are the state of the art technologies globally applied in wastewater treatment because of the smaller area required compared with HFCWs (Stefanakis et al., 2014). As reported by (Stefanakis et al., 2014), VF requires 1-2 m^2/pe, whereas HF requires 5-10 m^2/pe in the UK, 1.5 m^2/pe in the Czech Republic, 3.2 m^2/pe in Denmark, 1.6-3 m^2/pe in Germany, 2-2.5 m^2/pe in France, 1-1.5 m^2/pe in Greece, 1.3-3.2 m^2/pe in Italy, 1-3.2 m^2/pe in Spain, and 1.5 m^2/pe in the Canary Islands.

2.8 Conclusion

The VFCW design has limited total nitrogen (TN) removal, which is considered as a significant contaminant in the domestic wastewater. This issue was the major motivation behind this thesis: to develop a VFCW system that can efficiently treat domestic wastewater for nitrogen (N) and organic matter (OM), and to understand the process and the mechanisms of N removals and pathways in a CW system.

This chapter has described the historical development of a CW system and covers the classification and types of CWs. Moreover, the composition of the wetlands and the mechanisms and pathways for nitrogen removal in CWs have been discussed.

Furthermore, the effects of environmental factors and operational conditions on N removal in subsurface flow constructed wetlands (SSFCWs) have been covered in detail. Additionally, microbial communities for nitrogen transformation in a wetland system were illustrated. Finally, the chapter closed with the choice of VFCW systems over HFCW systems.

Review of literature showed that VFCWs system have the potential to be used for nitrogen removal by following some strategy and techniques (i.e. tidal flow, step-feeding, artificial aeration, etc.). However, there are still some gaps that need to be addressed to enhance and optimize the nitrogen removal performance. In relation to nitrification process, using multistage VFCW to examine and investigate the impact of long unsaturated time on nitrogen removal. Therefore, using four stages VFCW with tidal flow strategy (2 hrs saturated and 6 hrs unsaturated time) will improve the nitrification process by enhancing the aeration efficiency of the CW system. In relation to denitrification process, it requires external carbon source to enhance the process to convert nitrate to dinitrogen gas. As a result, using four stages VFCW with step-feeding strategy as external carbon source to enhance the denitrification process with different distribution ratios, to examine the effect of carbon source on nitrogen removal. In relation to the new route of nitrogen removal called CANON process, CANON can be achieved via partial nitrification and Anammox processes under limited oxygen in a single unit, and inorganic carbon is a substantial factor to achieve CANON process (i.e. inorganic carbon is considered as a carbon source for all autotrophic microorganisms). Subsequently, using single stage CW with tidal flow to examine the impact of different concentrations of inorganic carbon on nitrogen removal and CANON process. In doing so, this study will contribute to enhancing our understanding of nitrogen removal in CW systems.

3 Materials and methods

This chapter describe the materials and methods for two CW system: multistage (Chapter 4 and 5) and single stage (Chapter 6) CW systems as the following sections.

3.1 Multistage CW system (Chapter 4)

Figure 3.1 shows the multistage CW system. The system consists of four identical stages made from PVC plastic with each stage being 100 cm in height and 10 cm in diameter with a total treatment surface area of 0.032 m² (0.008 m² each). The experiment was constructed outdoors at Cardiff University, where the temperature range between 5 to 25˚C (BBC Weather, 2016). Thus, phase 1 the temperature range between 15 and 25 ˚C, whereas phase 2 between 5 and 15 ˚C.

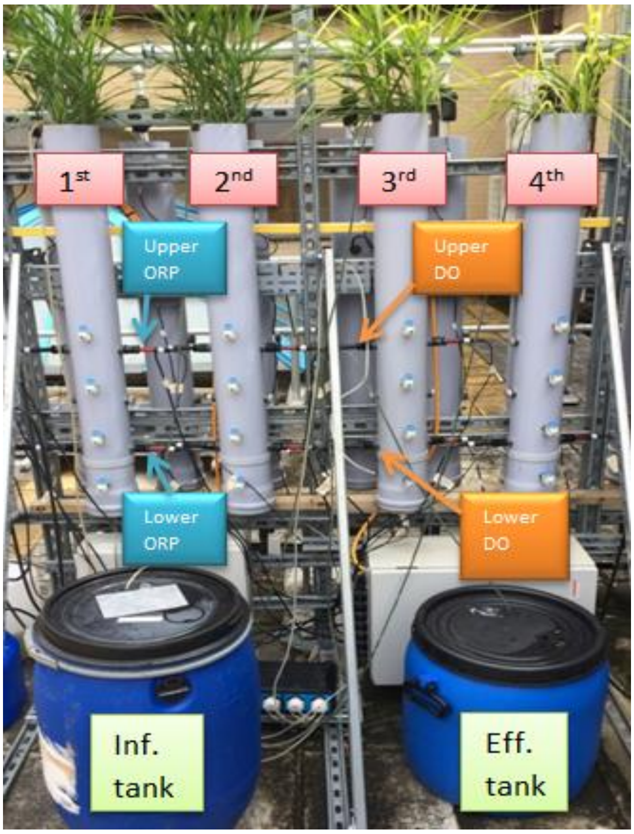


Figure 3.1 Multistage constructed wetland system

3.1.1 Media

Gravel was used as the main substrate in all four stages. Sieve analysis was used to obtain the required gravel size, and the gravel was then washed with tap water at the concrete laboratory to clean the mud from it (UN-HABITAT, 2008). In each stage, a 10 cm bottom layer was filled with coarse gravel (20-25 mm), and this served as the supporting and drainage layer; the following layer was filled with gravel (4-9 mm) as the main substrate layer with a depth of 50 cm; a 10 cm top layer of gravel (10-19 mm) was added to facilitate the dispersion and the distribution of wastewater and the growth of plants, as shown in Figure 3.2. The porosity of the gravel is 40%. Moreover, the gravel was obtained from a local company called Travis Perkins and the composition of the gravel is from a natural aggregates that are produced from naturally occurring rock, sand and gravel deposits, and consists of combinations of various minerals, including silica (no further details available).

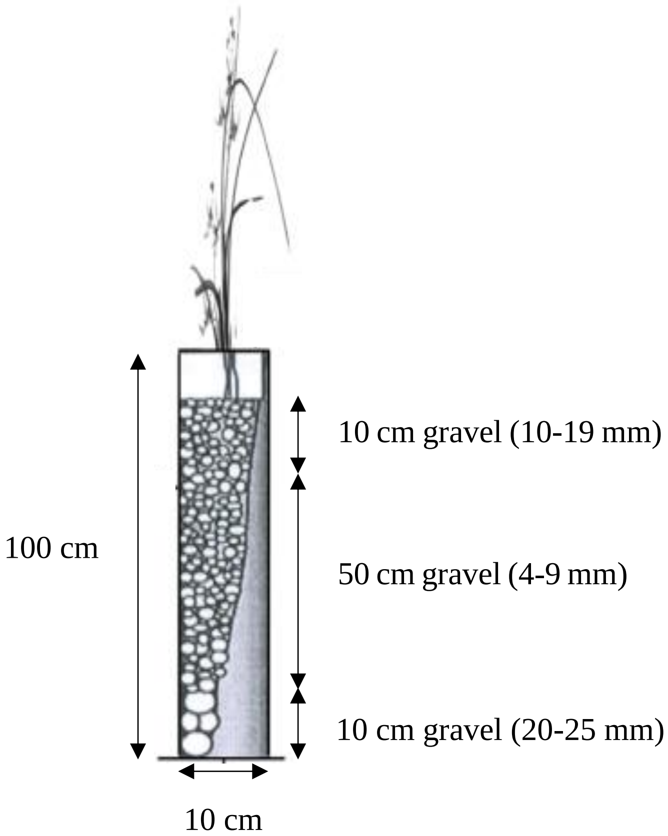


Figure 3.2 Schematic drawn of the media size in the constructed wetland

3.1.2 Plants

Each stage was planted with *Phragmites australis* at the beginning of the experiment, and good growth with lush vegetation was observed after 2 months by feeding the system with synthetic wastewater, as shown in Figure 3.3. The reason behind choosing this type of plant was because they are the most commonly used reference plant for CWs in Europe and are able to survive in most conditions (Brix, 1994a; Sun et al., 2005; Kadlec and Wallace, 2008; Vymazal, 2010a). Moreover, they have a comparatively high oxygen transfer ability from their leaves through the stems and rhizomes, which facilitates the aerobic degradation of pollutants (Barbera et al., 2009; Wang et al., 2012a).

Figure 3.3 Growth of the plant in the system after the start-up period

3.1.3 Wastewater

The system was fed with synthetic wastewater to simulate domestic wastewater. It was prepared at the beginning of every week at Cardiff University laboratory at the Characterization Laboratories for Environmental Engineering Research (CLEER) laboratory. About 60 litres of synthetic wastewater was used to fill the influent tank, which it was enough to feed the system for one week. The composition of the synthetic wastewater was 0.95 g/l (CH_3COONa),0.025 g/l (KH_2PO_4), 0.025 g/l (K_2HPO_4), 0.74 g/l (KCl), 0.58 g/l (NaCl), 0.2181 g/l (NH_4Cl), 0.1 g/l ($MgSO_4 \cdot 7H_2O$) and 0.1 g/l

($CaCl_2 \cdot 2H_2O$) with 1 ml/l of the trace elements mixture consisting of 0.5 g/l ($FeSO_4 \cdot 7H_2O$), 0.035 g/l ($ZnCl_2$), 0.05 g/l ($MnCl_2 \cdot 4H_2O$), 0.003 g/l (H_3BO_3), 0.065 g/l ($CaCl_2 \cdot 6H_2O$), 0.001 g/l ($CuCl_2 \cdot 2H_2O$), 0.012 g/l ($NiCl_2 \cdot 6H_2O$), 0.018 g/l ($Na_2MoO_4 \cdot 2H_2O$) and 0.119 g/l ($CoCl_2 \cdot 6H_2O$) (Sleutels et al., 2011). The purities of chemicals used were analytical grade and supplied from two local companies: Sigma-Aldrich and Fisher Scientific. The synthetic wastewater simulates typical domestic wastewater with a high concentration of nitrogen and organic carbon source to obtain approximately 700 mg/l of COD and 60 mg/l of NH_4^+-N. Tidal flow was generated in each stage with the five peristaltic pumps, which were controlled by specific programmable timers. The tidal flow strategy is a process that repeatedly allows CWs to be filled with wastewater until the main media layer is submerged and then fully drained. In the filling time, the CW is gradually submerged and the air in the CW is continuously consumed. In the draining time, fresh air is dragged into the CW, and the CW is replenished with oxygen. During the consistent cycle of wet (saturated) and dry (unsaturated) time, the wastewater acts as a passive pump to drag and expel air into the CW. Therefore oxygen supply and consumption are basically improved in the system (Li et al., 2015b).

Synthetic wastewater was batch loaded to the first stage and sequentially passed through the other stages, generating alternate wet/dry periods in individual stages as shown in Figure 3.4. In particular, the synthetic wastewater was pumped down flow pattern from the influent tank to the top of the first stage, so the main media layer was submerged and remained so for 2 hours (contact between the wastewater and the attached microbial on the gravel media); the rest of the stages (second, third, and fourth) were unsaturated for 6 hours. After the 2 hours, the synthetic wastewater was pumped to the next stage, so the main media layer was submerged and remained so 2 hours as well; the rest of the stages (first, third, and fourth) were unsaturated for 6 hours. After the 2 hours, the synthetic wastewater was pumped to the next stage, so the main media layer was submerged and remained so for 2 hours as well; the rest of the stages (first, second, and fourth) were unsaturated for 6 hours. After the 2 hours, the synthetic wastewater was pumped to the next stage (final stage), so the main media layer was submerged and remained so for 2 hours; the rest of the stages (first, second, and third) were unsaturated for 6 hours. After the 2 hours, the synthetic wastewater was pumped to the effluent tank and then synthetic wastewater from the influent tank was pumped again to the first stage and the process was repeated three times a day. Therefore, the system was operated with three batch cycles per day, with each cycle entailing 2 hours saturation and 6 hours unsaturation giving a total of 8 hours per cycle. About 2 litres of synthetic wastewater was pumped into the system in each cycle, giving 6 litres per day being pumped into the system with a hydraulic loading rate (HLR) of 0.76 m^3/m^2d (i.e. 0.25 m^3/m^2 cycle).

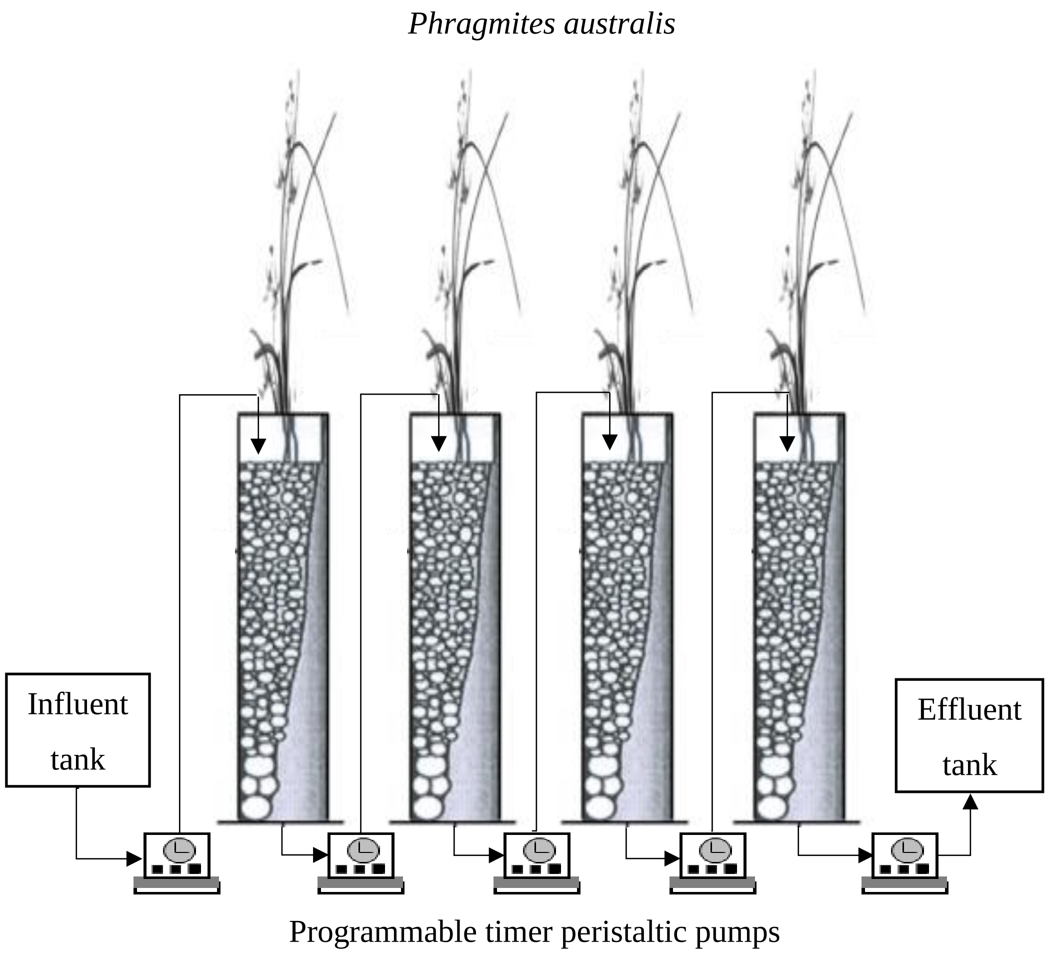


Figure 3.4 Schematic description of the constructed wetland system

Before starting the experiment and loading the synthetic wastewater to the CW, the system was inoculated with activated flocs obtained from the aeration basin of a local domestic wastewater treatment plant for about 2 weeks to provide seed microorganisms for the system.

3.1.4 Chemical analysis

Grab samples were collected on a once weekly basis from the influent tank and the effluent of each stage and analysed directly *in situ* for pH and temperature using a pH/EC/TDS meter (HANNA HI 991301), whereas each stage was instrumented with an MCC-SYSti-18f multiparameter monitoring and control module that provided continuous real-time monitoring and measurement of oxidation-reduction potential (ORP) and dissolved oxygen (DO), as shown in Figure 3.5. The probes of ORP and DO were installed at depths of 20 and 50 cm from the surface of the distribution layer of the first, second and third stages, and only at a depth of 50 cm from the surface of the distribution layer in the fourth stage (see Figure 3.1). The reason behind selecting the upper point is because it is close to the surface where you would expect nitrifying bacteria to be predominant because the aeration is closer to the surface. However, the lower point is deeper, and this is where anoxic or anaerobic conditions can be expected due to the lack of available oxygen.

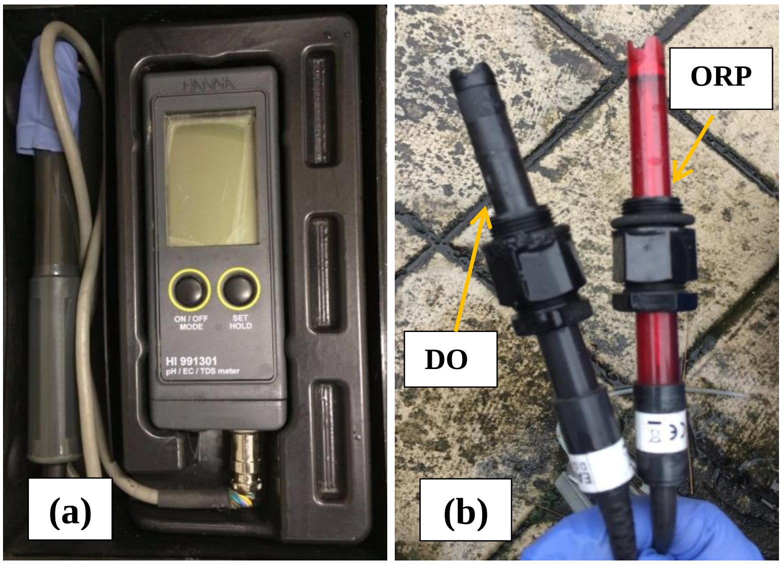


Figure 3.5 (a) pH and temperature probe and (b) ORP and DO probes

The MCC-SYSti module was connected with computer software so that the data could be easily collected, downloaded, and analysed to demonstrate and explain the performance of the CW system, as shown in Figure 3.6.

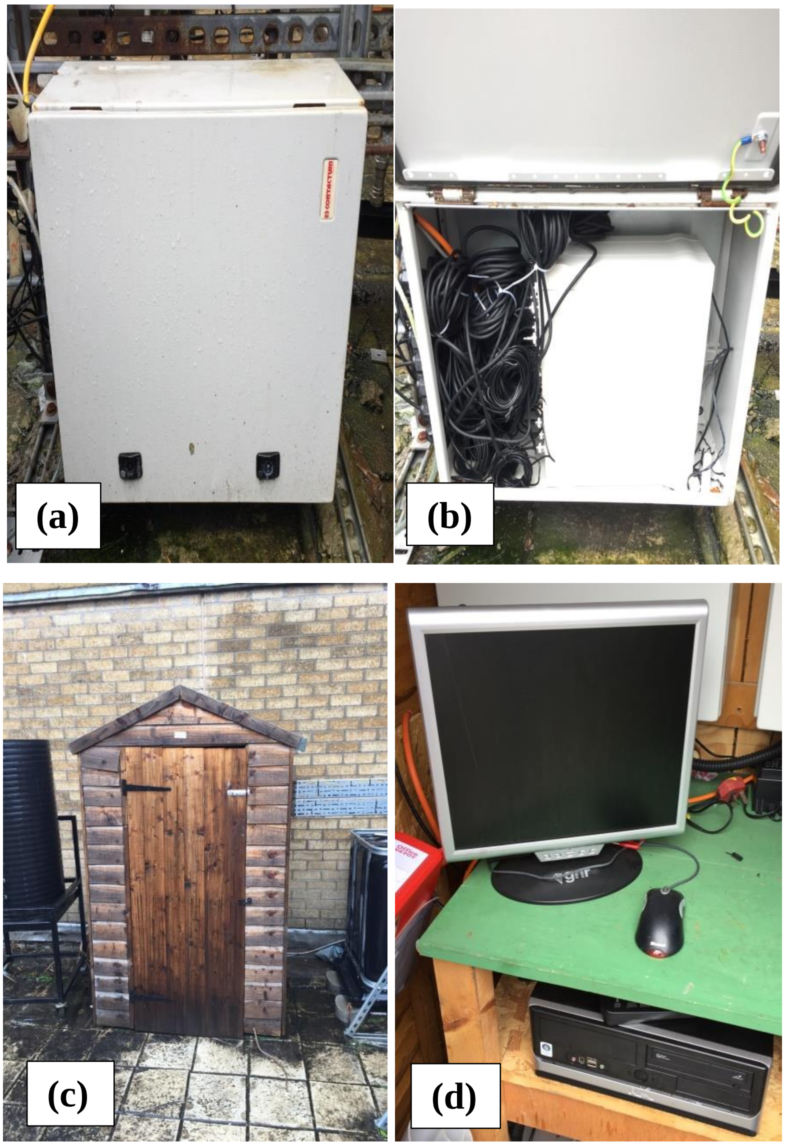


Figure 3.6 (a) Box to protect the multiparameter device, (b) Multiparameter device, (c) Shed to protect the computer from raining weather and (d) Computer software system

Chemical oxygen demand (COD), nitrite (NO_2^--N), nitrate (NO_3^--N), ammonium (NH_4^+-N), total nitrogen (TN), and orthophosphate (PO_4^{3-}-P) were analysed using a Hach DR/3900 spectrophotometer and digester, as shown in Figure 3.7, according to its standard operating procedures, and total phosphorus (TP) was measured by an ICP-OES machine (Optima 2100DV, Singapore).

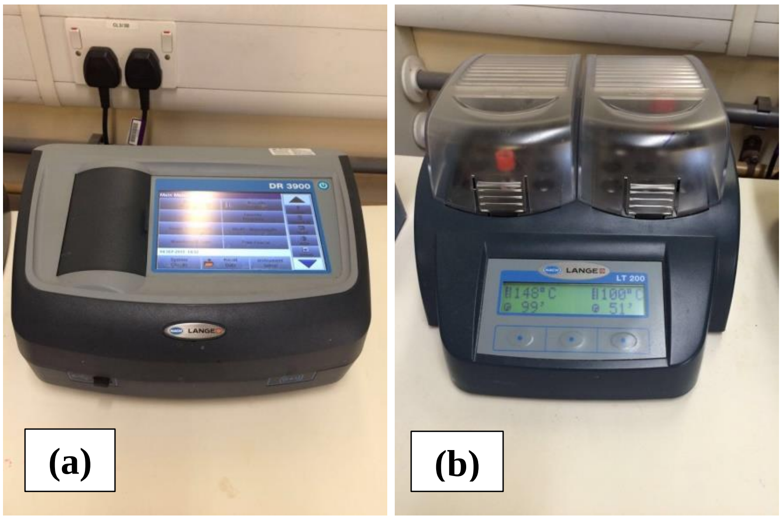


Figure 3.7 (a) Hach DR/3900 spectrophotometer and (b) Digester

3.1.5 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 20 version, and Spearman`s correlation coefficient analysis was used to determine any correlations between the monitor parameters (temperature, ORP, DO, and pH) and water quality parameters (COD, NH_4^+-N, TN, and TP). Therefore, it would lead to improving and enhancing our understanding of the efficiency performance in the system.

3.2 Multistage CW system with step-feeding strategy (Chapter 5)

The VFCWs system is a continuous experiment that had been already installed and constructed outdoors at Cardiff University in a previous study (Chapter 4), with the same four stages, media, and plants. In addition, there was an extra peristaltic pump and its tube for the step-feeding purposing to the third stage. Subsequently, six peristaltic pumps with specific programmable timers were used for this experiment, as shown in Figure 3.8.

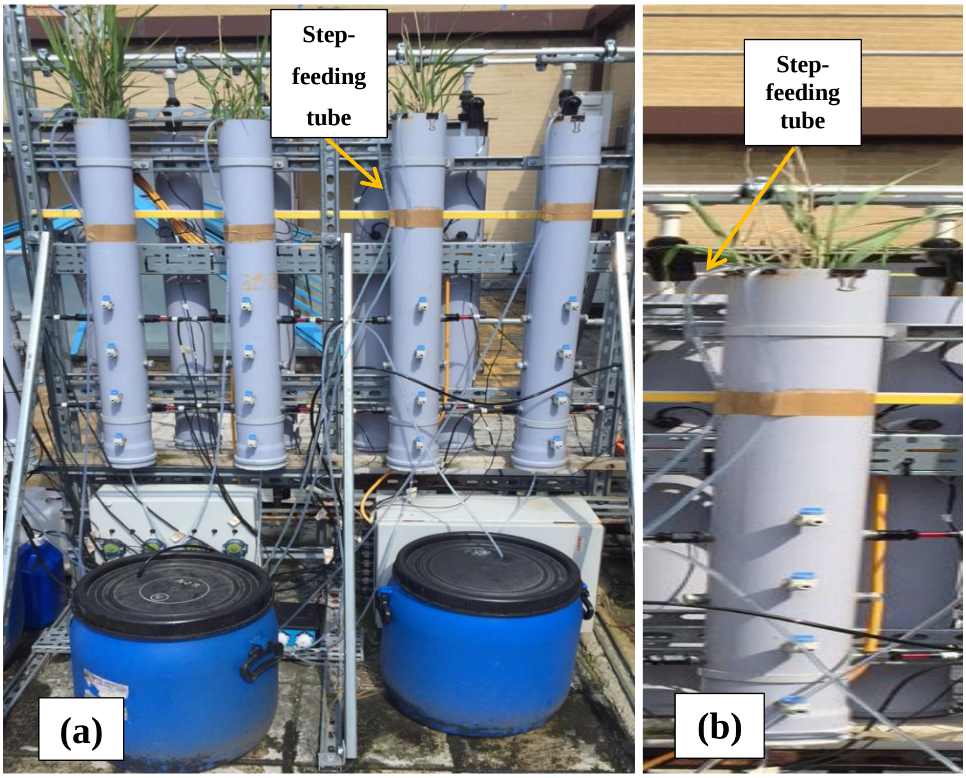


Figure 3.8 (a) Multistage constructed wetland system with step-feeding strategy and (b) Closer picture to the third stage (step-feeding strategy)

3.2.1 Step-feeding strategy

The step-feeding strategy was applied to the system by distributing the influent synthetic wastewater into the first and third stages according to the schemes corresponding to each phase as shown in Figure 3.9. Subsequently, three step-feeding schemes were tested with different distribution ratios as shown in Table 3.1 to investigate and examine different ratios on nitrogen removal, similar research study to (Hu et al., 2012) but with different ratios; 2 litres per cycle and 6 litres per day (3 cycles per day). For the first phase, influent was introduced into Stage 1 and Stage 3 with flow distribution ratios of 80% and 20% respectively. Then, for the second phase, influent was introduced into Stage 1 and Stage 3 with flow distribution ratios of 70% and 30% respectively. Finally, for the third phase: influent was introduced into Stage 1 and Stage 3 with flow distribution ratios of 60% and 40% respectively. The main flow pattern for all experiments was the typical tidal flow down-flow pattern. The hydraulic retention time (HRT) remained the same with 2 hours saturated and 6 hours unsaturated, to enhance the nitrification step and the difference in the distribution ratios was to optimize the carbon source availability for the denitrification step in the third and fourth stages (as they had high NO_3^--N effluent, see Table 4.2 in Chapter 4).

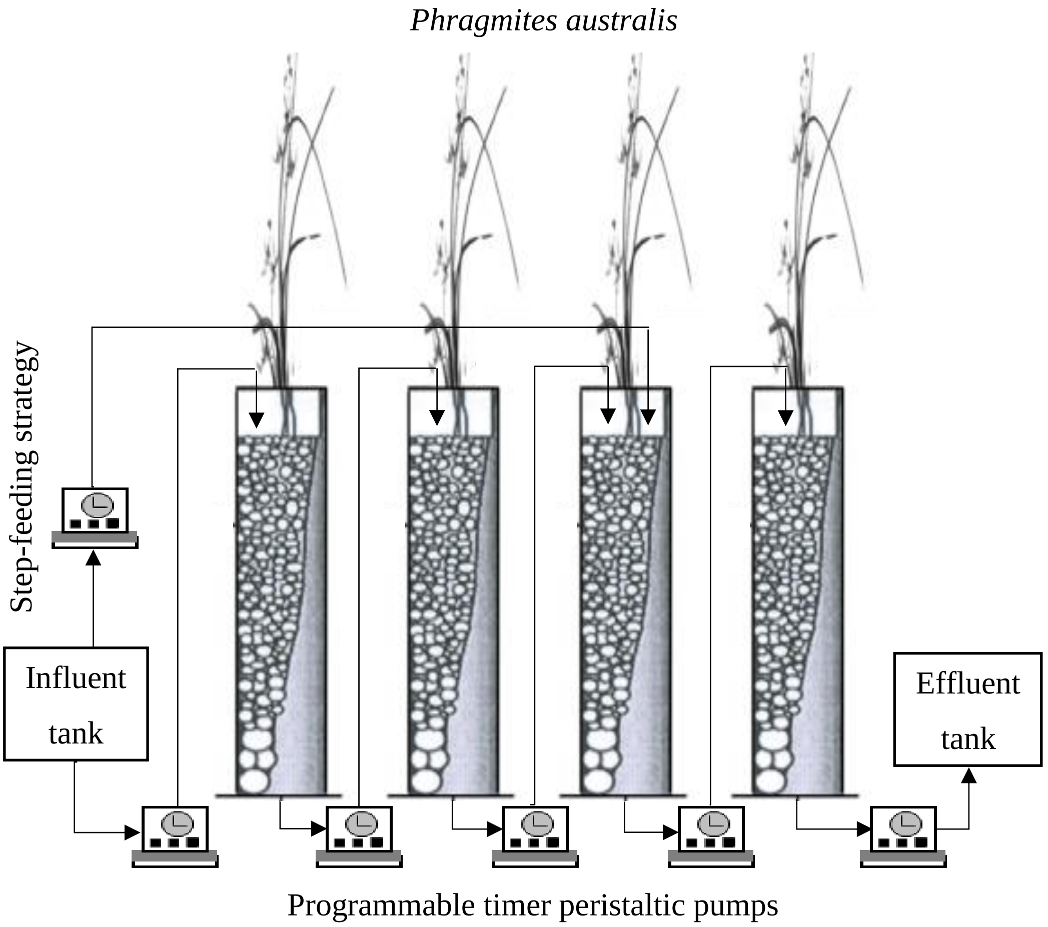


Figure 3.9 Step-feeding strategy schematic diagram

Table 3.1 Summary of the step-feeding strategy

Distribution Ratio (%) (Stage 1:Stage 3)	duration (days)	Volume (ml/cycle) (Stage 1: Stage 3)	Wet: Dry (hrs)	Main flow pattern	HRL* (m^3/m^2d)	HRL** (m^3/m^2d)
80:20	46	1600:400	2:6	Down flow	0.611	0.764
70:30	49	1400:600	2:6	Down flow	0.535	0.764
60:40	42	1200:800	2:6	Down flow	0.458	0.764

HLR*: Hydraulic loading rate on first and second stages

HRL**: Hydraulic loading rate on third and fourth stages

3.2.2 Wastewater

The same wastewater that had been used in the previous experiment, that is, synthetic wastewater, was used to simulate domestic wastewater with the same characteristics as mentioned in section 3.1.3. It was prepared at the beginning of every week at Cardiff University laboratory, specifically, at the Characterization Laboratories for Environmental Engineering Research (CLEER) laboratory and it was used to fill the influent tank for about 60 litres, which was sufficient to feed the system for one week. Tidal flow strategy was also used in this experiment.

3.2.3 Sampling and chemical analysis

Grab samples from the influent tank and the effluent of each stage were collected once a week and analysed *in situ* for pH and temperature and at the CLEER laboratory for chemical oxygen demand (COD), nitrite (NO_2^--N), nitrate (NO_3^--N), ammonium (NH_4^+-N), total nitrogen (TN), total phosphorus (TP), and orthophosphate (PO_4^{3-}-P).

Oxidation-reduction potential (ORP) and dissolved oxygen (DO) were monitored in real time using the multiparameter (the same procedure as in section 3.1.4).

3.2.4 Statistical analysis

In order to investigate the differences in treatment performance between different step-feeding schemes, statistical analysis was carried out using IBM SPSS Statistics 20 version. One way ANOVA at the 95% significance level was used to examine the differences in treatment performance of various step-feeding schemes. Post hoc multiple comparisons for the mean removal efficiency between different step-feeding schemes were also computed with Tamhane's T2 test at the 95% confidence level (α=0.05). Tamhane's T2 method was selected because equal variance between groups was not assumed.

3.3 Single stage (Chapter 6)

It is known that the CANON process requires limited aeration, and this can be achieved by using upflow instead of downflow recirculation (Hu et al., 2014a). Otherwise, this experiment used a downflow stream with a very short unsaturated time to obtain the limited aeration. A long saturation time would limit the oxygen in the bed; this weakens the nitrification step and protects the Anammox bacteria (Dong and Sun, 2007).

The single reactor CW used in this study was constructed from a PVC plastic pipe having a diameter of 10 cm and a length of 100 cm (see Figure 3.10). The bottom layer of the reactor was filled with coarse gravel (up to 10 cm in depth) to act as a support and drainage layer (20-25 mm) followed by 50 cm depth of gravel (4-9 mm) constituting the main media layer, and then a 10 cm depth of distribution layer comprising gravel (11-19 mm).

Figure 3.10 Single constructed wetland (a) side view and (b) front view

The system was controlled by two peristaltic pumps with pre-programmed timers for feeding and draining purposes. The single stage system was planted with *Phragmites australis*. Oxidation-reduction potential (ORP) and dissolved oxygen (DO) probes were inserted into the column at depths of 20 cm (upper point) and 50 cm (lower point) from the surface of the distribution layer and were monitored for 24 hours a day.

3.3.1 Wastewater

The synthetic wastewater was prepared at the Characterization Laboratories for Environmental Engineering Research (CLEER) laboratory at Cardiff University and fed to the CW every two days (Mondays and Wednesdays). The wastewater simulated domestic wastewater after secondary treatment, and the recipe was as follows: 0.1275 g/l (CH_3COONa), 0.0125 g/l (KH_2PO_4), 0.0125 g/l (K_2HPO_4), 0.1 g/l (KCl), 0.1 g/l (NaCl), 0.0575 g/l (NH_4Cl), 0.1 g/l ($MgSO_4.7H_2O$), 0.1 g/l ($CaCl_2.2H_2O$) and 0.1, 0.15, 0.2 and 0.3 g/l ($NaHCO_3$) as IC (depending on the phase period) with 1 ml/l of the trace elements mixture consisting of 0.5 g/l ($FeSO_4.7H_2O$), 0.035 g/l ($ZnCl_2$), 0.05 g/l ($MnCl_2.4H_2O$), 0.003 g/l (H_3BO_3), 0.065 g/l ($CaCl_2.6H_2O$), 0.001 g/l ($CuCl_2.2H_2O$), 0.012 g/l ($NiCl_2.6H_2O$), 0.018 g/l ($Na_2MoO_4.2H_2O$), and 0.119 g/l

($CoCl_2.6H_2O$) (Sleutels et al., 2011). This gave a wastewater concentration of (approximately) ammonium (NH_4^+-N) ≈15 mg/l, chemical oxygen demand (COD) ≈100 mg/l, total phosphorus (TP) ≈6 mg/l and sodium bicarbonate depending on the phase condition (Metcalf et al., 1991). Tidal flow strategy was applied in each cycle with the two peristaltic pumps.

Synthetic wastewater was used in this study to simulate domestic wastewater after the secondary treatment, because the CANON process would be hindered by high pollutant concentrations. In particular, if a high COD was applied, Anammox could be inhibited (Chamchoi et al., 2008; Jin et al., 2012). This is because in such a case, nitrite would be utilized predominantly by the heterotrophic denitrifiers, leaving little or none for the Anammox bacteria. Two litres of the synthetic wastewater were pumped from the influent and holding tank (the tank that holds the synthetic wastewater after being pumped out and then pumped in again to the reactor CW for recirculation purposes) into the bed for 7 minutes to completely submerge the main media layer. Wastewater stayed in the bed for a certain time (depending on the strategy operation) before being pumped out (drained) for 7 minutes into the influent and holding tank with the bed remaining unsaturated for 30 minutes. The same procedure was repeated several times a day depending on the recirculation number strategy with the hydraulic loading rate (HLR) equal to 0.25 m^3/m^2d.

3.3.2 Operational strategy

There were two operating strategies established for nitrogen removal through the CANON process in the single TFCW, as shown in Table 3.2. In the first strategy, the recirculation number (Rn, times/ day) was used as a tool to control the oxygen supply into the system for the PN step. The second strategy was dosing different IC concentrations as sodium bicarbonate ($NaHCO_3$) into the influent wastewater to enhance ammonia-oxidizing bacteria (AOB) and Anammox bacteria activities to achieve the CANON route.

Table 3.2 Operational strategy for the single constructed wetland system

Operational strategy	**Phases**	**Start and finish (date)**	**Duration/cycle (wet : dry) (minutes)**
	Start-up period, Rn = 6 cycles / day, IC = 150 mg/l	3 August 2015 to 4 October 2015	210 : 30
Recirculation number (Rn)	Phase 1 period, Rn = 6 cycles / day, IC = 150 mg/l	5 October 2015 to 29 November 2015	210 : 30
	Phase 2 period, Rn = 4 cycles / day, IC = 150 mg/l	30 November 2015 to 31 January 2016	330 : 30
	Phase 3 period, Rn = 3 cycles / day, IC = 150 mg/l	1 February 2016 to 27 March 2016	450 : 30
Inorganic carbon (IC)	Phase 1 period, Rn = 6 cycles / day, IC = 100 mg/l	4 April 2016 to 1 May 2016	210 : 30
	Phase 2 period, Rn = 6 cycles / day, IC = 200 mg/l	2 May 2016 to 22 May 2016	210 : 30
	Phase 3 period, Rn = 6 cycles / day, IC = 300 mg/l	23 May 2016 to 26 June 2016	210 : 30

Before starting the experiment and loading the synthetic wastewater to the CW, the system was inoculated with activated flocs obtained from the aeration basin of a local domestic wastewater treatment plant for about 2 weeks to provide seed microorganisms for the system.

3.3.3 Water quality monitoring and performance evaluation

Grab samples were taken from the influent and the effluent of the CW twice a week and analysed for nitrite (NO_2^--N), nitrate (NO_3^--N), ammonium (NH_4^+-N), and total nitrogen (TN) (since this study focused on the CANON process and nitrogen removal). Temperature and pH were measured *in situ* by a pH/ EC/ TDS meter (HANNA HI

991301). NO_2^--N, NO_3^--N, NH_4^+-N and TN were determined by using a Hach DR/3900 spectrophotometer according to its standard operating procedures, whereas the CW was instrumented with MCC-SYSti multiparameter monitoring and a control module that provides continuous real-time monitoring and measurement of ORP and DO. The probes of ORP and DO were installed at 20 and 50 cm depths from the surface of the distribution layer of the CW system, as shown in Figure 3.10. The reason behind selecting the upper point is because it is close to the surface where you would expect nitrifying bacteria to be predominant whereas the lower point is deeper, which is where anoxic or anaerobic conditions can take place due to the lack of available oxygen. The MCC-SYSti module was connected with computer software, so that the data could be easily collected, downloaded, and analysed to demonstrate and understand the performance of the CW system.

The efficiency of nitrogen removal was determined from the decrease of total inorganic nitrogen (TN). In particular, the nitrate accumulating ratio (NAR) (Hu et al., 2014a) was used to estimate and evaluate the nitrogen transformation activities, as shown in equation 3.1. If aerobic ammonium oxidization (full nitrification) is the major transformation path of NH_4^+-N, then most of the influent NH_4^+-N will be transformed to NO_3^--N, causing a high NAR close to 1, whilst if the transformation follows the CANON route, a low NAR of 0.11 can be expected according to equation 3.2 (CANON process equation). This method or hypothesis was justified in this study because no significant nitrite build-up was detected throughout the experimental period.

$$\text{NAR} = \text{effluent } NO_3^-\text{-N} / NH_4^+\text{-N removed} \qquad \text{eq. 3.1}$$

$$NH_4^+ + 0.79\ O_2 + 1.11\ HCO_3^- \rightarrow 0.0103\ C_5H_7O_2N\ \text{(AOB)} + 0.028\ CH_2O_{0.5}N_{0.15}$$
$$\text{(Anammox bacteria)} + 0.11\ NO_3^- + 0.44\ N_2 + 1.06\ CO_2 + 2.49\ H_2O \qquad \text{eq. 3.2}$$

3.3.4 Statistical analysis

In order to investigate the difference of the overall effluent quality between different Rn and IC concentrations, statistical analysis were carried out using IBM SPSS Statistics 20 version. One way ANOVA at a 95% significance level was used to examine the difference in treatment performance for NH_4^+-N and TN removal of various recirculation number and inorganic carbon concentrations. Post hoc multiple comparisons for the mean removal efficiency for NH_4^+-N and TN between different recirculation number and inorganic carbon concentrations were also computed with Tamhane's T2 test at a 95% confidence level (α=0.05). Tamhane's T2 method was selected because equal variance between groups was not assumed.

3.4 Microbial community (Chapter 7)

3.4.1 Sampling

Microbial samples (gravels) were collected at the end of both experimental periods at two different locations from each of the five stages of the two CW systems (multistage and single stage), giving a total of 10 samples. The sampling locations were set at two depths, that is, 20 and 50 cm height from the bottom of the CW, as shown in Figure 3.11. After the on-site collection, the microbial samples were stored in an ice incubator, and then immediately sent to the Microbial Laboratory at Bioscience Department at Cardiff University for analysis. The samples were stored in a freezer at below -80 ˚C until the analysis could commence.

Figure 3.11 Microbial samples points

3.4.2 DNA extraction

DNA was extracted from the gravel samples using Metagenomic DNA Isolation Kit according to its standard procedure. Each sample (10 g) was put in a sterile tube (20 mL) including 1 ml of wash buffer (0.2% Tween 20). After that, the tubes were shaken to wash off the trapped microbes on the gravel samples. The cell suspension was transferred to a clean microcentrifuge tube, then the tube was centrifuged at 14,000 x g for 2 minutes to pellet the cells and discard the supernatant. The cell pellet was resuspended in 300 µl of TE Buffer, then 2 µl of Ready-Lyse Lysozyme Solution and 1 µl of RNase A were added to the cell suspension. Next, the tube was mixed by vortexing and then incubated at 37°C for 30 minutes. Following that, 300 µl of Meta-Lysis Solution (2X) and 1 µl of Proteinase K were added to the tube and mixed by vortexing. Then, the tube was briefly pulse-centrifuged to ensure that all of the solution was in the bottom of the tube; it was subsequently incubated at 65°C for 15 minutes and then cooled to room temperature and placed on ice for 3-5 minutes. After that, 350 µl of MPC Protein Precipitation Reagent was added to the tube; it was mixed by vortexing vigorously for 10 seconds, then the debris was pelleted by centrifugation for 10 minutes at 14,000 x g in a microcentrifuge at 4°C. Then, the supernatant was transferred to a clean 1.7 ml microcentrifuge tube and the pellet was discarded. After that, 570 µl of isopropanol was added to the supernatant, and it was mixed by inverting the tube multiple times. Next, the DNA was pelleted by centrifugation for 10 minutes at 14,000 x g in a microcentrifuge at 4°C and a pipet tip was used to remove the isopropanol without dislodging the DNA pellet. Then, the sample was pulse-centrifuged briefly and any residual liquid removed with a pipet tip, without disturbing the pellet. Next, 500 µl of 70% ethanol was added to the pellet, without disturbing the pellet, and it was centrifuged for 10 minutes at 14,000 x g in a microcentrifuge at 4°C. After that, a pipet tip was used to remove the ethanol without dislodging the DNA pellet. In the following step, the sample was briefly pulse-centrifuged, and any residual liquid was removed with a pipet tip, without disturbing the pellet. Next, the pellet was air-dried for 8 minutes at room temperature, and then was resuspended in 50 µl of TE Buffer. Finally, the size and the concentration of the isolated DNA were validated by being compared to the Fosmid Control DNA provided in the kit.

3.4.3 PCR amplification and 16S rRNA sequencing with the Illumina MiSeq

The quantity and size of the extracted DNA fragments were tested by using a Qubit machine and an Agilent Tape Station respectively. After the DNA extraction, bacterial 16S rRNA genes were amplified and sequenced via PCR primers to target the V4 variable (region of 16S rRNA genes) in a 96-well PCR plate. The PCR primers, which are called forward and reverse primers (Kozich et al., 2013), consist of the Illumina adapter sequence (forward primer, F: 5’-AATGATACGGCGACCACCGAGATCTACAC-3’; reverse primer, R: 5’-CAAGCAGAAGACGGCATACGAGAT-3’), an 8-bp i5 and i7 index sequences, a pad sequence to increase the primer sequencing melting temperature (F: 5’-TATGGTAATT-3’; R: 5’-AGTCAGTCAG-3’), and a 2-bp linker sequence that is anti-complementary to the known sequences. (F: 5’-GT-3’; R: 5’-CC-3’), and 16S V4 specific sequences (F: 5’-GTGCCAGCMGCCGCGGTAA-3’; R: 5’-GGACTACHVGGGTWTCTAAT-3’).

The procedure for PCR was as follows: 17 ul of Accuprime Pfx Supermix was dispensed into each well of a new 96 well plate. Then, 1 µl of template DNA per well was transferred to the corresponding well on the PCR plate. After that, 2 µl of each paired set of index primers was transferred from the primer plate to the corresponding well on the PCR plate. Next, 1 µl of PCR grade H_2O was added to the negative control well, and 1 µl of Mock Community at a 1:3 dilution was added to the positive control well. Finally, this was repeated for up to four 96 well plates, after which the plates were sealed and briefly vortexed; the contents were spun down and placed in the thermocycler. The procedure used for the thermocycler was as follows: at 95 °C for 2 minutes, followed by 30 cycles at 95 °C for 20 seconds, at 55 °C for 15 seconds, at 72 °C for 5 minutes, and then a final extension at 72 °C for 10 minutes.

3.4.4 High-throughput amplicon sequencing (Illumina MiSeq)

The DNA samples were examined, and 10 samples were analysed by Illumina sequencing. Illumina sequencing was conducted at the Institute of Medical Genetics at the Cardiff University Heath Campus using 2 bp × 250 bp paired-end flow cells and reagent cartridges. The data resulting from the Illumina sequencing was analysed using the MiSeq standard operating procedure (Mothur v1.38.1). Overall, the process began with the creation of a contigs formation. After that, sequences filtration was used to eliminate any ambiguous contigs readings, that is, those shorter than 245 bp and longer than 275 bp. The resulting sequence readings were clustered into operational taxonomic units (OTUs) (similarity threshold of 95%).

3.4.5 Statistical analysis

Chao1 and the Shannon index were used to estimate the richness and diversity of the microbial communities by using R software. Weighted UniFrac analysis was performed to compute the differences between microbial communities in each column based on phylogenetic information. Principal coordinates analysis (PCoA) was used to visualize the similarities of the microbial community relationships based on the distance matrix (Gao et al., 2016). After clustering, the heat map was generated via R software to reflect similarities and differences of community composition among the bacterial samples at the family level.

3.5 Conclusion

This chapter has described the set-up of the two experimental designs (multistage VFCWs and single stage VFCW) including its design and operational conditions. The detailed information of the two experimental designs have also been presented.

4 General Performance Evaluation of Four Stages Tidal Flow Constructed Wetlands

4.1 Introduction

A constructed wetland (CW) system is considered as a cost effective and environmentally friendly system for wastewater treatment, and in particular, for the removal of nutrients and organic matter from wastewater. Nitrogen and phosphorus are nutrients which if present in high concentrations can lead to eutrophication. Eutrophication is an excessive richness of nutrients in a lake or other body of water, frequently due to run-off from the land, which causes a dense growth of plant life on the water course that consumes oxygen and enhances the growth of algae and therefore depletes the oxygen in the water and affects aquatic life (Chen et al., 2014).

Numerous research studies have been conducted on the effectiveness of CWs in removing nitrogen from wastewater; and it is generally known that CWs are poor in removing nitrogen from ammonium-rich wastewater due to the limiting nitrification process (Hunt and Poach, 2001; Saeed and Sun, 2012; Chang et al., 2014; Vymazal and Kröpfelová, 2015; Guo et al., 2016; Uggetti et al., 2016). In recent years, vertical flow constructed wetlands (VFCWs) have been developed to achieve nitrification and remove organic matter and suspended solids. Via the tidal flow operational strategy, this allows more oxygen to enter the wetland media and promotes nitrification. Hybrid CWs or VFCW multistage systems are used to denitrify the nitrifying effluents from the CW system and to achieve higher removal of TN (Zhao et al., 2004c).

This chapter reports on the performance of a VFCW, which combines four stages to achieve nitrification and obtain good organic matter (COD) removal. It also examines how the VFCW is limited regarding the denitrification process; therefore, a multistage system was used in this research study to achieve high nitrogen removal. This is based on the process design, whereby the first stage is aimed at organic matter removal and initiation of the nitrification step, the second stage is for the nitrification and denitrification steps, and the third stage and fourth stages (depending on the load) are for further treatment.

The aim of this chapter is to examine via laboratory experiments the engineering aspects (i.e. HLR, HRT, and pollutant loading) and determine the pathways and mechanisms involved in nitrogen removal in an engineered wetland system. To achieve this aim, the following key objectives were addressed:

- to determine the effect of seasonal temperature of the operational variables (tidal flow, hydraulic loading rate, hydraulic retention time, pollutant loading) on nitrogen removal and performance

- to investigate and determine the pathway and mechanisms for nitrogen removal in the engineered wetland system.

Findings from the study can further our understanding of nitrogen removal and thus promote the efficient removal of nitrogen.

4.2 Results and discussion

The experiment was carried out over around 9 months (from May 2015 to January 2016) including the start-up period, and it was divided into two phases excluding the start-up period according to the seasonal weather. Table 4.1 shows the phases.

Table 4.1 Experiment phases and their temperatures and periods

Periods	Phases	Temperature range	No. of days
4 May to 26 July 2015	Start-up period	13 – 24 ˚C	83
27 July to 1 November 2015	Phase 1	15 - 25 ˚C	98
2 November to 31 January 2016	Phase 2	5 – 15 ˚C	91

4.2.1 Redox potential (ORP) and dissolved oxygen (DO)

The measurement of redox potential is used to describe the oxidation-reduction conditions of wetland soils (Likens, 2009). ORP is a measurement of the ability or potential of wastewater to permit the occurrence of specific biological (oxidation-reduction) reactions, such as nitrification, denitrification, and phosphorus removal (Szögi et al., 2004; Myers et al., 2006). ORP is measured in millivolts (mV). On the ORP scale, the existence of an oxidizing agent, such as oxygen, increases the ORP value, while the existence of a reducing agent, such as a substrate or carbon BOD, decreases the ORP value. ORP has been shown to be an effective technique for monitoring wastewater processes that can determine which biological reaction is taking place in the system (Seo and DeLaune, 2010; Białowiec et al., 2012; Corbella et al., 2014). Oxygen is a fundamental factor in the operation of the system. In the nitrification process, oxygen plays a role as a co-substrate with nitrogenous compounds, and its concentration significantly affects the metabolism of both ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) (Myers et al., 2006; Dušek et al., 2008).

The relationship between ORP and DO is crucial to this study. As a matter of fact, ORP is heavily driven by DO (Pennington and Walters, 2006; Białowiec et al., 2012); when oxygen is present, DO and ORP increase and vice versa. Real-time data of ORP and DO were collected over a 9-month period for monitoring and measuring purposes at every 10 minutes at two specific depth points, as shown in Figure 3.1. The first point, called the upper point, was at 20 cm beneath the surface of the distribution layer, whereas the second point, named the lower point, was at 50 cm below the surface of the distribution layer. Figure 4.1 illustrates the scale of ORP for different wastewater processes. ORP values above +200 mV indicate aerobic conditions. ORP values between -200 and +200 mV indicate anoxic conditions. ORP values below -200 mV indicate anaerobic conditions. ORP data will be based on Figure 4.1.

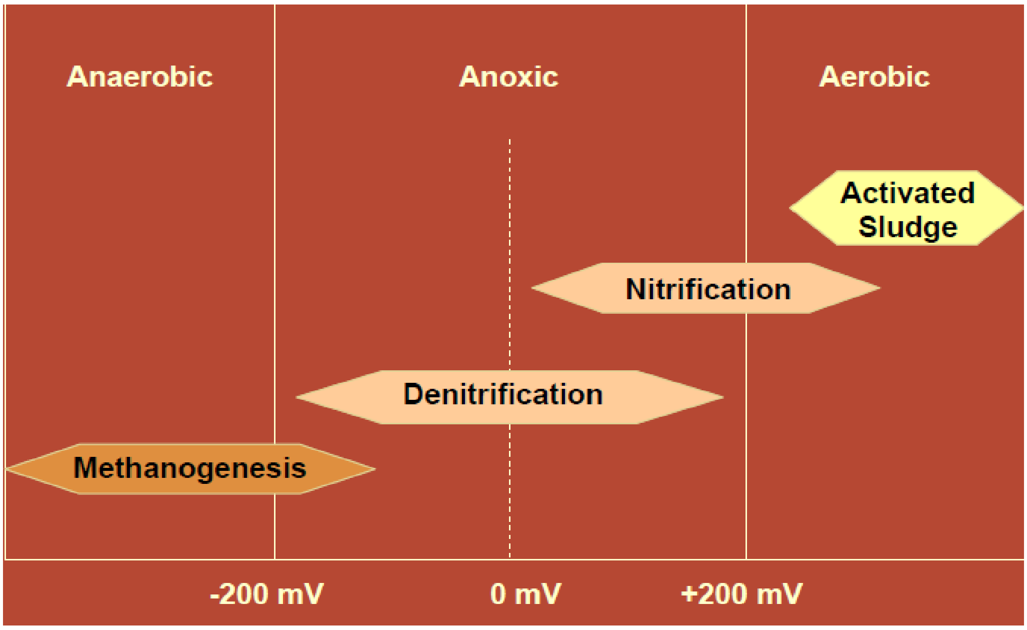


Figure 4.1 ORP scale (mV) for typical wastewater processes (Hernando and Martínez, 2010)

In this study, ORP was monitored in the CW at two depths (20 cm and 50 cm beneath the surface) as per the average data shown in Figure 4.2. After the start-up period (May to July), it was noticed that the first stage (upper point) began with positive ORP values below 200 mV and decreased to negative OPR values at the end of the start-up period. After the start-up period, the OPR values in the first stage were negative due to intense microbial activity. The other stages, ORP values were in the range of -200 and +200 mV, which means anoxic conditions occurred, and so did the activities of nitrifying and denitrifying bacteria (Dušek et al., 2008; Likens, 2009). Regarding the lower point, the first stage began with positive ORP values in the first month of the start-up period (May); it then decreased to negative OPR values because of microbial activities, and the depth played a role in this. The rest of the stages varied between -200 and +200 mV, which means anoxic conditions occurred, and so did the activities of nitrifying and denitrifying bacteria. Regarding DO, this was also monitored at two specific depths (20 cm and 50 cm beneath the surface) as per the average data, which are shown in Figure 4.3. DO decreases with depth and due to low oxygen availability. It was noticed that in the upper and lower points after the start-up period in the first stage, DO varied between 0-0.5 mg/l and had the lowest reading compared to the other stages due to the bacteria consuming the oxygen in the wastewater; therefore, ORP was in negative values.

In general, positive ORP values indicate the existence of oxygen in the system (Rowell, 1981). Such a situation may be attributed to the plants ability to oxygenate reed beds by their roots and from the tidal flow process. Negative ORP values specify that the oxygen is used by microbes for the mineralisation of root exudates (Kankaala et al., 2003; Altor and Mitsch, 2006). In addition, ORP could be affected by many factors, such as temperature, which has a straight effect on the solubility of oxygen in the water and consequently reduces the ORP value. However, temperature also influences ORP indirectly by accelerating the plant growth and the microbial activity processes (Kadlec and Reddy, 2001). In addition, an increase in temperature will accelerate biochemical processes including bacterial activity; as a result, oxygen solubility in the water decreases, and so does ORP. Moreover, the capability of plants to transport oxygen and the release of plant root exudates into the rhizosphere served as a substrate for heterotrophic microorganisms, causing an increase in microbial activity and in the consumption of the oxygen, thus reducing the ORP value. The depth could also have affected the ORP reading, which decreased as the depth increased. The different wastewater concentrations (organic matter and nutrients concentrations) that the system received, pH and DO, could have affected the ORP readings; therefore, the results varied from one month to another. In general, the system is driven by the physicochemical and biological factors (Szögi et al., 2004; Corbella et al., 2014).

One of the unique advantages of the tidal flow in a CW system is the improvement of the oxygen supply (Sun et al., 2005; Sun et al., 2006; Chang et al., 2014), which has been successfully applied during the operational period, as shown in Figure 4.4. The nitrification process requires aeration to take place to remove nitrogen from the wastewater. The relationship between ORP and DO and between DO and nitrogen logically indicates a relationship between ORP and nitrogen; nitrogen decreases with a decrease in ORP and DO because the denitrification process requires anoxic or anaerobic conditions (limited oxygen and the presence of organic carbon to enhance the denitrification process).

Overall, the results (see Table 4.2) highlight the enhanced ammonium removal performance of the CW due to the oxygen that entered into the matrices bed according to the reading from the DO probes. As a result, nitrification took place because the nitrification process is an aerobic process which prefers aeration (2 hours saturated and

6 hours unsaturated). In the first stage, it is clear that most of the ORP values were negative, which means that the microorganisms were active in the contaminant and nutrient removal. In the second stage, most ORP values were below 200 mV with a low DO concentration, which was suitable for the denitrification process (anoxic). In the last two stages (third and fourth), ORP was below 200 mV with a low DO concentration; as a result, a partial nitrification (not full nitrification) step took place. More details will be discussed in the nitrogen removal sections.

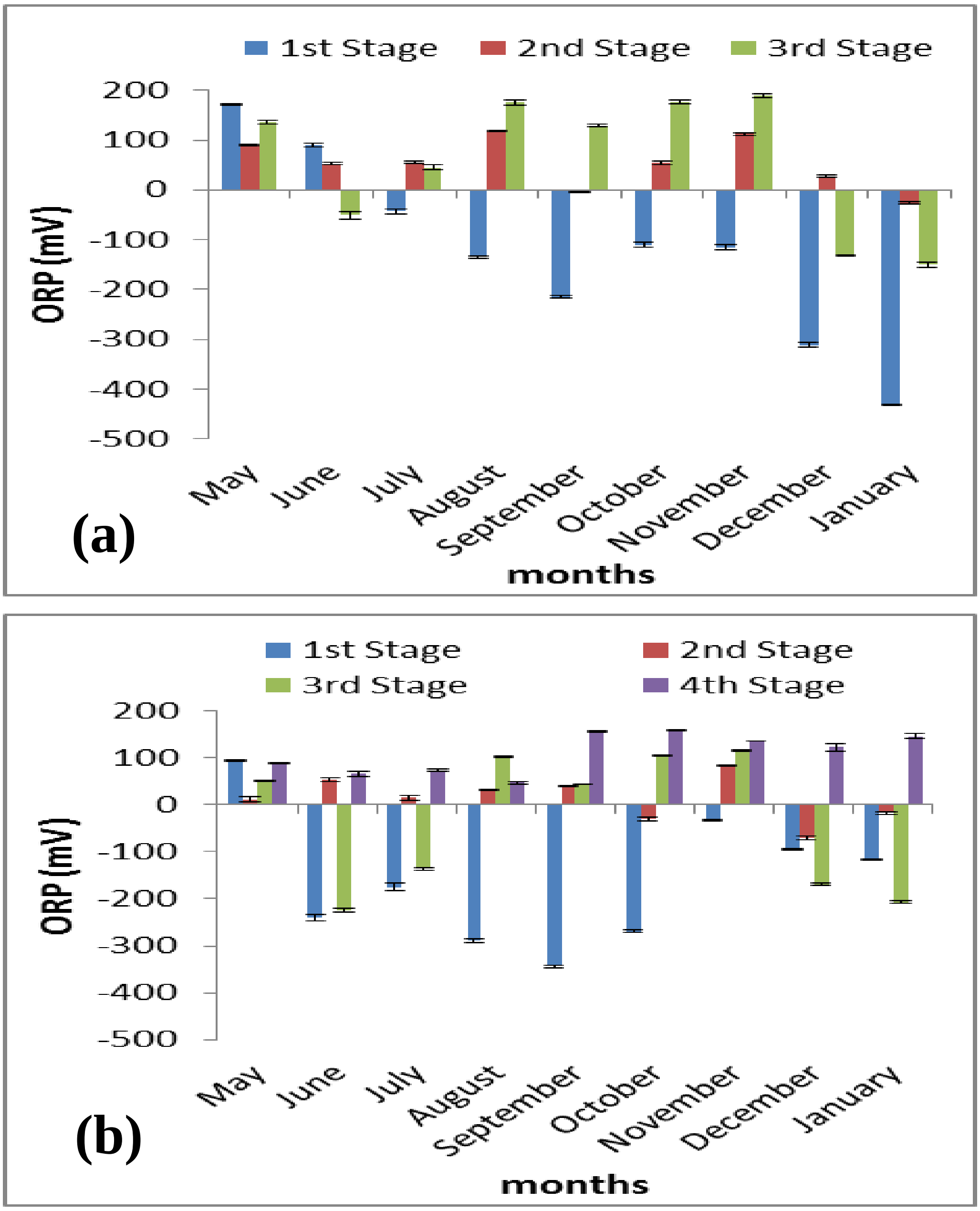


Figure 4.2 Average ORP values at (a) upper point and (b) lower point (error bars represent the standard error)

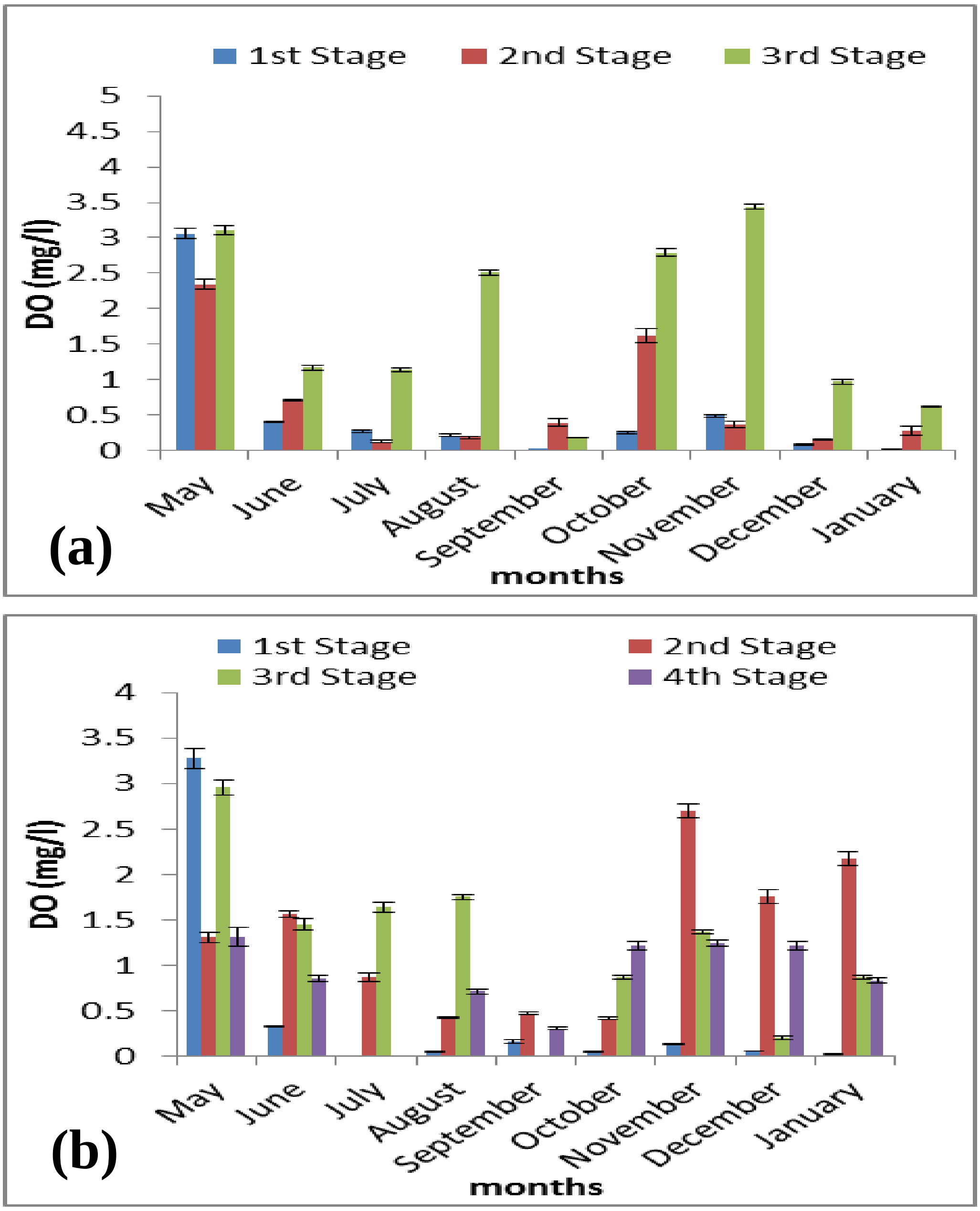
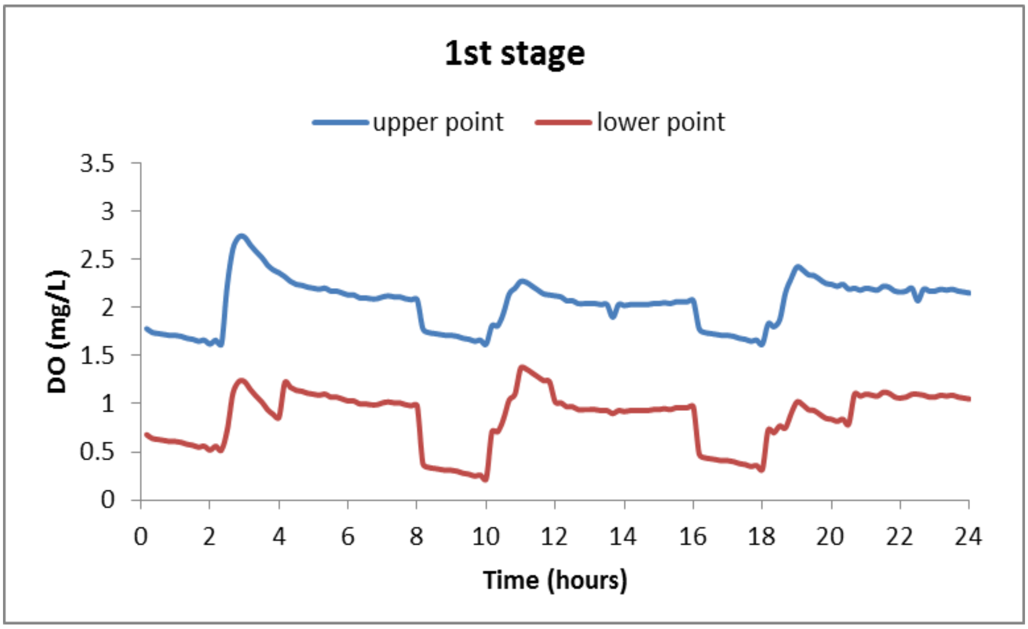
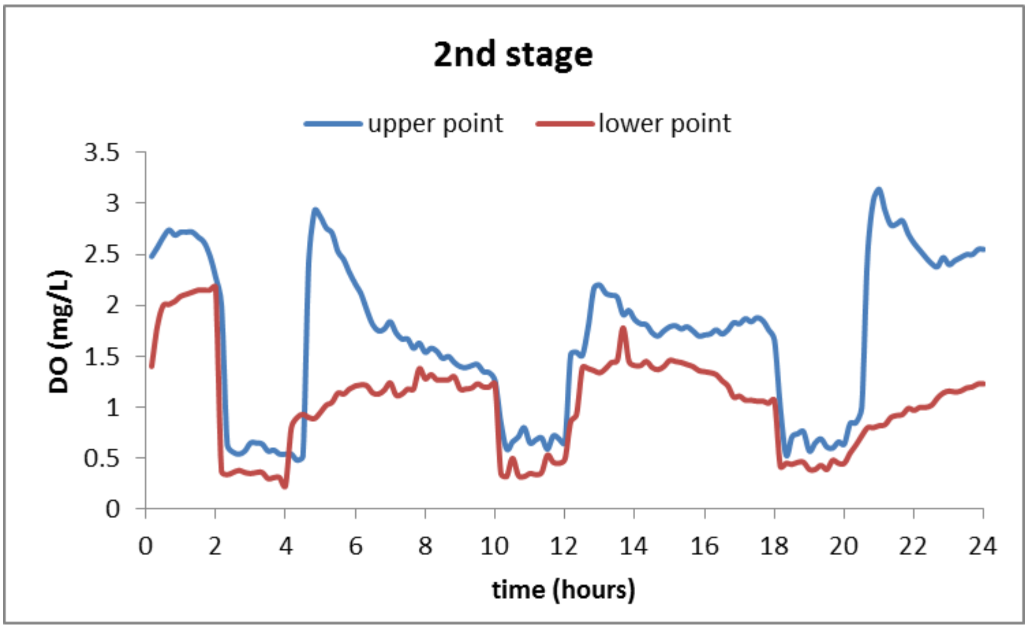


Figure 4.3 Average DO values at (a) upper point and (b) lower point (error bars represent the standard error)


1st stage
upper point
lower point
DO (mg/L)
3.5
3
2.5
2
1.5
1
0.5
0
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (hours)


2nd stage
upper point
lower point
DO (mg/L)
3.5
3
2.5
2
1.5
1
0.5
0
0
2
4
6
8
10
12
14
16
18
20
22
24
time (hours)

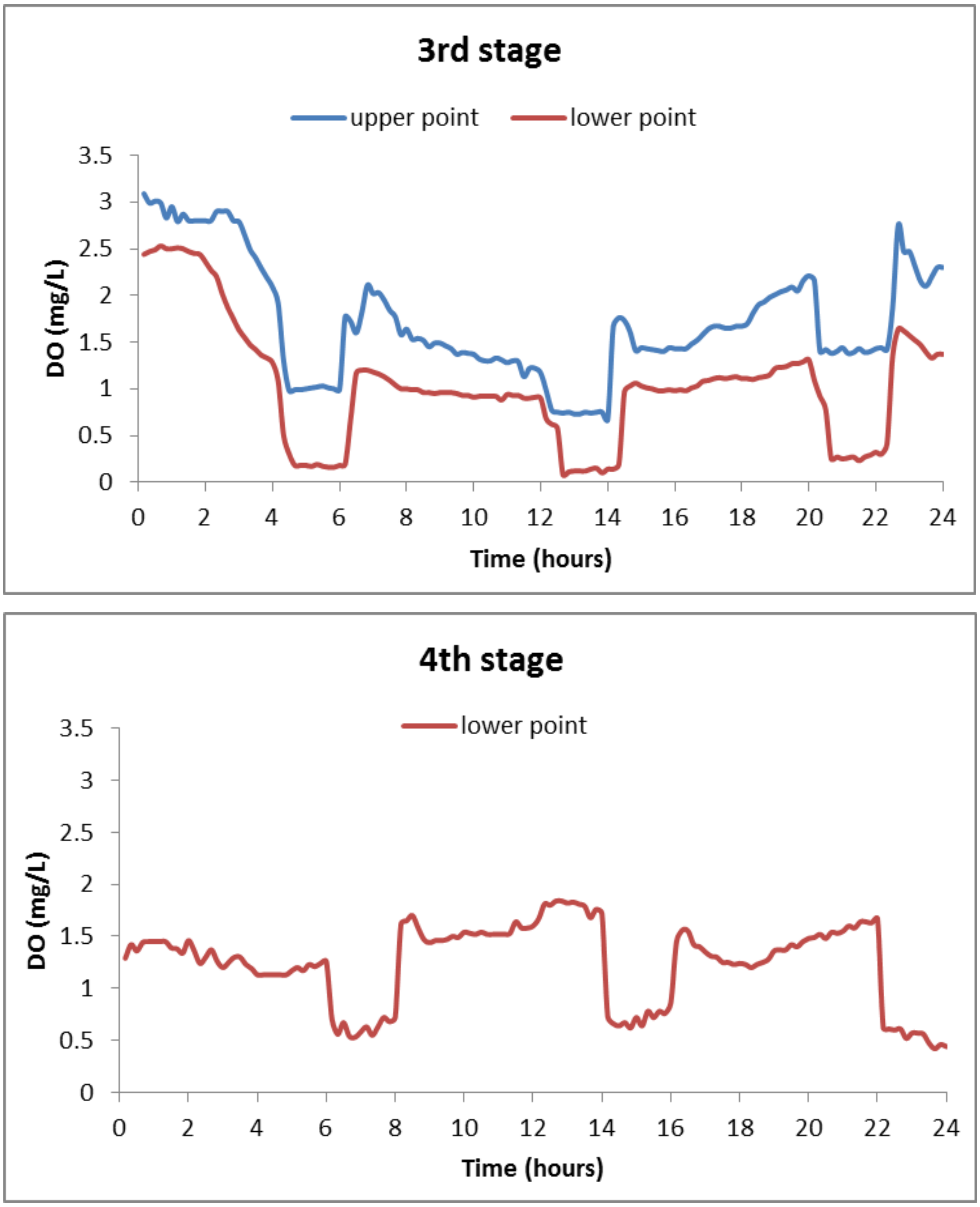


Figure 4.4 Tidal flow strategy during the filling and draining period

4.2.2 Overall treatment performance

The overall treatment efficiency for nutrients and organic matter expressed as COD during the study period for phase 1 and 2 is presented in Table 4.2.

Table 4.2 Overall treatment performance of multistage vertical constructed wetlands excluding the start-up period (between brackets represent standard deviation)

Phase 1	**Influent**	**1st stage**	**2nd stage**	**3rd stage**	**4th stage**	**Re. %***
pH	7.49 (±0.04)	7.48 (±0.15)	7.21 (±0.05)	7.36 (±0.07)	7.67 (±0.13)	
Temperature**	16.2 (±1.6)	16.8 (±1.7)	18.3 (±1.6)	19.4 (±2.1)	20.2 (±2.3)	
NH_4^+-N (mg/l)	57.71 (±0.54)	38.94 (±5.58)	15.36 (±5.74)	4.54 (±1.88)	1.11 (±0.49)	**98.1**
NO_2^--N (mg/l)	0.022 (±0.019)	0.003 (±0.002)	0.015 (±0.008)	0.350 (±0.096)	0.463 (±0.095)	
NO_3^--N (mg/l)	0	4.21 (±2.26)	1.09 (±0.84)	3.06 (±0.54)	3.52 (±0.91)	
TN (mg/l)	61.6 (±0.4)	nm***	nm***	nm***	14.02 (±1.74)	**77.3**
COD (mg/l)	706 (±15.52)	327.21 (±117.90)	61.61 (±13.25)	36.27 (±7.08)	18.89 (±4)	**97.3**
TP (mg/l)	10.12 (±0.57)	9.36 (±1.51)	8.15 (±1.95)	7.85 (±2.45)	8.44 (±1.41)	**16.6**
PO_4^{3-}-P (mg/l)	9 (±0.85)	8.29 (±1.31)	6.95 (±1.83)	6.84 (±2.22)	7.39 (±1.5)	**17.9**
Phase 2	**Influent**	**1st stage**	**2nd stage**	**3rd stage**	**4th stage**	**Re. %***
pH	7.51 (±0.05)	7.50 (±0.15)	7.31 (±0.13)	7.43 (±0.3)	7.58 (±0.22)	
Temperature**	12.9 (±2.8)	12.5 (±3.2)	14.7 (±2.6)	14.8 (±2.5)	14.7 (±2.1)	
NH_4^+-N (mg/l)	58.13 (±0.43)	46.49 (±4.43)	30.14 (±9.34)	19.9 (±12.96)	12.48 (±11.17)	**78.5**
NO_2^--N (mg/l)	0.022 (±0.016)	0.005 (±0.003)	0.012 (±0.012)	0.207 (±0.193)	0.313 (±0.246)	
NO_3^--N (mg/l)	0	5.84 (±3.18)	0.85 (±0.33)	3.49 (±2.14)	3.93 (±2.38)	
TN (mg/l)	61.93 (±0.46)	nm***	nm***	nm***	24.58 (±6.11)	**60.3**
COD (mg/l)	716.63 (±16.88)	470.88 (±98.96)	169.88 (±124.82)	129.79 (±105.46)	89.24 (±75.48)	**87.5**
TP (mg/l)	10.44 (±0.54)	10.01 (±1.24)	7.63 (±1.76)	9.33 (±2.82)	9.37 (±0.66)	**10.2**
PO_4^{3-}-P (mg/l)	9.63 (±0.82)	9.32 (±1.22)	6.96 (±1.6)	8.43 (±2.7)	8.49 (±0.83)	**11.8**

*removal efficiency (%); **sample temperature; ***nm - not measured

4.2.2.1 Nitrification performance

The overall nitrification performance is shown in Figure 4.5. Nitrification was well established after the start-up period, which is indicated by the significant NH_4^+-N reduction (Figure 4.5a) and an increase of NO_3^--N in the effluent (Figure 4.5b).

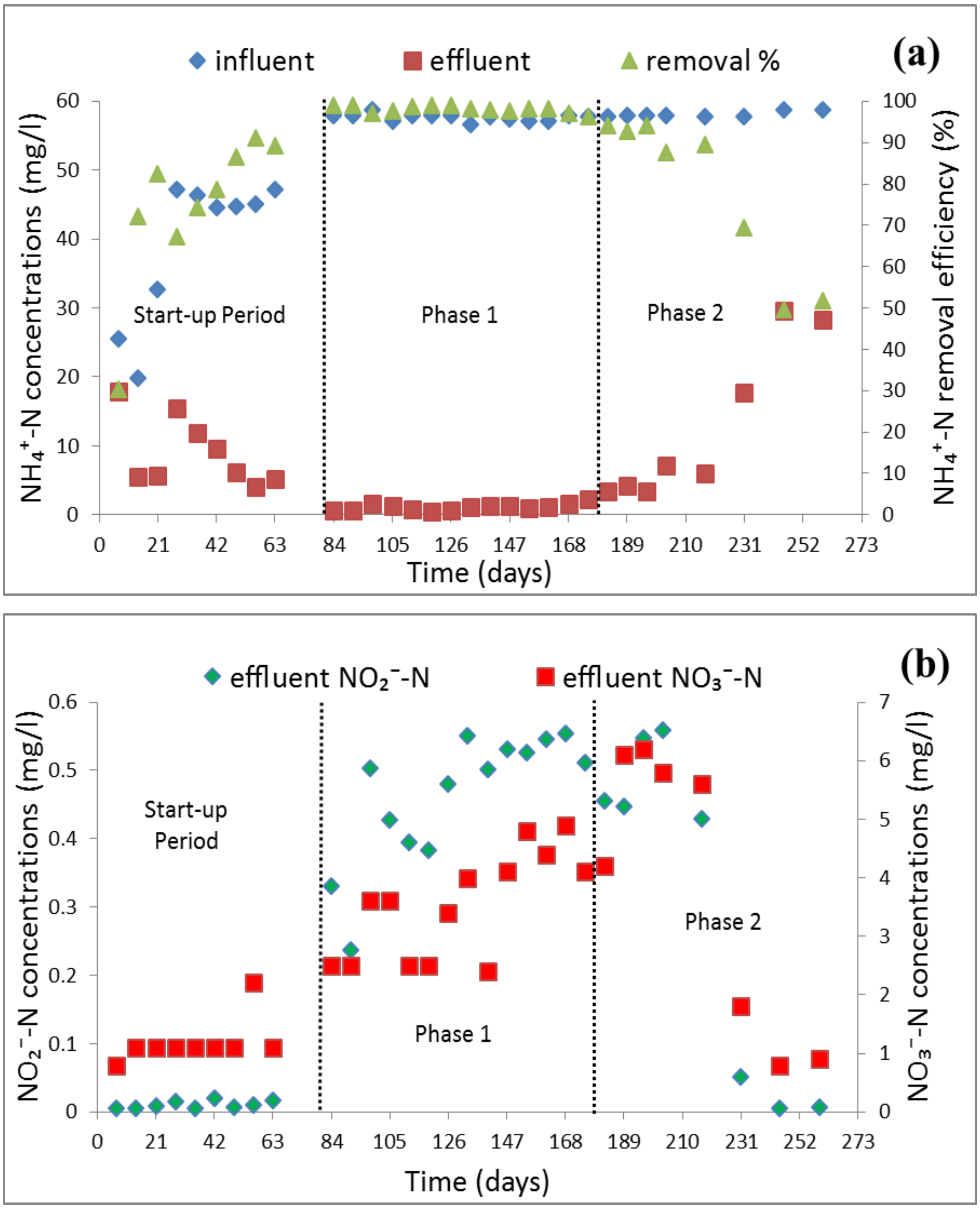


Figure 4.5 Nitrification performance (a) NH_4^+-N reduction; (b) effluent of NO_2^--N and NO_3^--N

The average NH_4^+-N reduction in the start-up period was 30.5-91.1% (average 70.9%). The influent of NH_4^+-N was increased in the phase 1 period to simulate a high strength of nitrogen concentration of domestic wastewater from about 19.8-47.2 mg/l (average 39.3 ± 10.5 mg/l, start-up period) to about 56.6-58.8 mg/l (average 57.7 ± 0.5 mg/l). The average NH_4^+-N reduction maintained 96.2-99.1% (average 98.1%) in phase 1 whereas the NH_4^+-N effluent was 0.54-2.2 mg/l (average 1.1 ± 0.5 mg/l). In the phase 2 period, the effective nitrification performance decreased sharply from 94.1% to 49.7% whereas the NH_4^+-N effluent was 3.4-29.6 mg/l (average 12.5 ± 11.2 mg/l). This was caused by the fall of the ambient temperature as it shown in Figure 4.6. Consequently, there was de-growth of the plants in the system in phase 2 as shown in Figure 4.7, where it is clearly observed the difference of the plants height between Figure 3.3 (phase 1) and 4.7 (phase 2). The removal efficiency of NH_4^+-N in this study reached >90% similar to other studies (Jia et al., 2010; Jia et al., 2011; Zhao et al., 2011; Hu et al., 2014b; Wu et al., 2015b). However, this study obtained higher NH_4^+-N removal than other studies; 58% (Sun et al., 2006), 33-82% (Wu et al., 2011), and 55-85% (Zapater-Pereyra et al., 2015). Overall, the four stages in the tidal flow constructed wetlands (TFCWs) system demonstrated an efficient nitrification in the study period. Moreover, temperature plays a key role in the treatment performance as shown by the results above in Table 3.2.

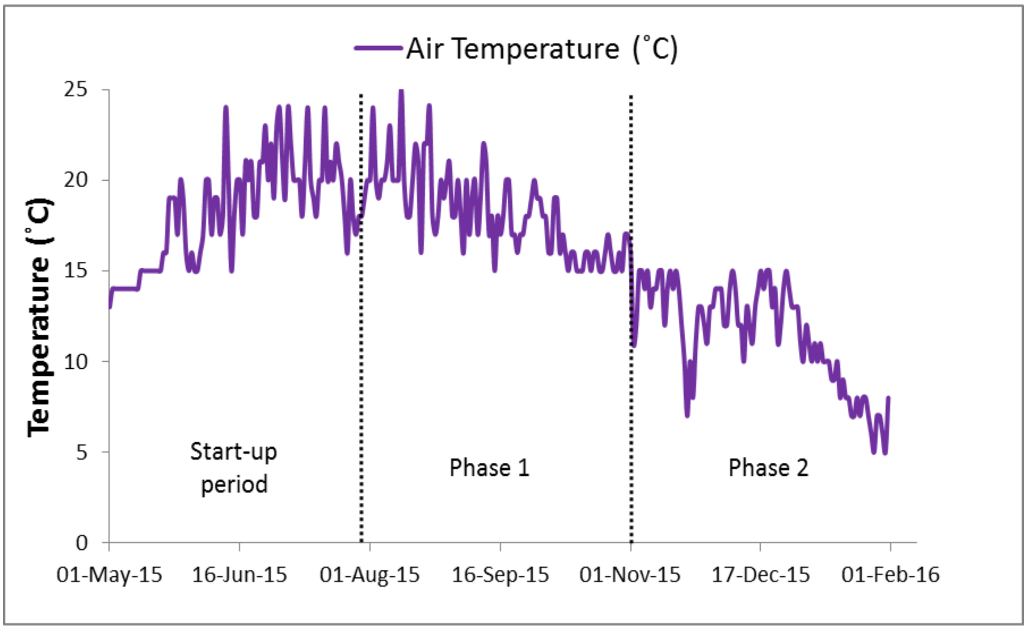


Figure 4.6 Air temperatures during the study period

Figure 4.7 De-growth of the plant in phase 2 period

4.2.2.2 TN reduction

Figure 4.8 illustrates the overall TN removal performance in the study period. The TN elimination showed the same trend as the NH_4^+-N reduction. During the start-up period, the influent TN was 23.2-48.8 mg/l (average 40.9 ± 9.9 mg/l) with 62.8% removal (average 15.2 ± 2.8 mg/l). In the phase 1 period, the TN influent was increased to 61.6 ± 0.4 mg/l on average, with 12-18.1 mg/l effluent (average 14 ± 1.7 mg/l) and with 77.3% removal efficiency, and that means there was a sharp increase in the removal performance. However, in the phase 2 period, the influent maintained almost the same influent as in period 1 with 61.9 ± 0.5 mg/l on average, with 19-34 mg/l effluent (average 24.6 ± 6.1 mg/l) and with 60.3% removal efficiency, which is a significant decrease in performance efficiency compared to the phase 1 period due to the decrease in the nitrification performance (see Figure 4.5) and the drop in the temperature (see Figure 4.6). The nitrogen removal rate for phase 1 and 2 were 72.7 $gN/m^3.d$ and 57.1 $gN/m^3.d$ respectively, where this study was better than other research studies that were using four stages TFCW with 60 $gN/m^3.d$ (Zhao et al., 2004a) and 67 $gN/m^3.d$ (Sun et al., 2005), and with 26 $gN/m^3.d$ for five stages TFCW (Sun et al., 2006). This is because of the different HLR and HRT applied which affect the treatment performance of the system. In addition, there were research studies which gave better removal rates due to recirculation of the effluent in the four stages TFCW to enhance the treatment performance of the system; for instance 78 gN/m³.d was achieved by Sun et al. (2005) and 80 gN/m³.d was achieved by Zhao et al. (2004c). In general, denitrification will easily become the limiting factor in TN reduction unless there is an abundant influent carbon source to facilitate the removal efficiency. Note that TN was measured only in the influent and the effluent of the final stage (fourth stage) due to financial constraints.

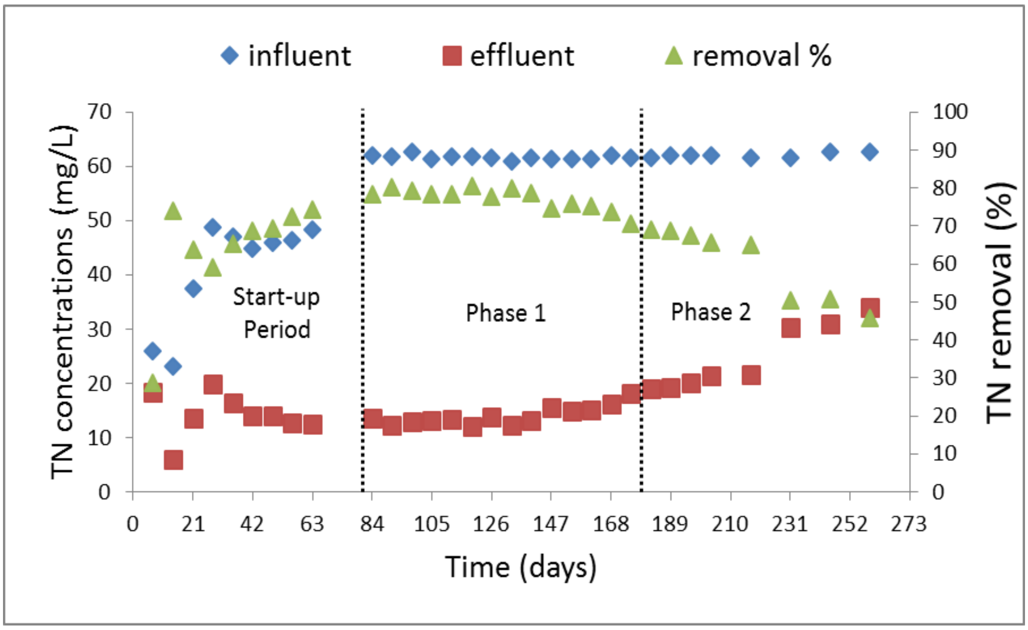


Figure 4.8 TN removals through the study period

4.2.2.3 Organic matter removal

The overall performance for COD is shown in Figure 4.9. The COD influents in the start-up period were 460-793 mg/l (average 685.3 ± 98.1 mg/l) with effluents 43.1-172 mg/l (average 90.5 ±43.8 mg/l) and 85.9% was the removal efficiency performance. In phase 1, the COD influents were 680-731 mg/l (average 706 ± 15.5 mg/l); the effluents decreased to 14.4-27.3 mg/l (average 18.9 ± 4 mg/l) with an increase in the removal efficiency performance to 97.3%. However, in phase 2, the COD influents were 685-734 mg/l (average 716.6 ± 16.9 mg/l); the effluent increased to 31.7-217 mg/l (average 89.2 ± 75.5 mg/l) with a decrease in the removal efficiency performance to 87.7%, due to the decrease of the temperature in this phase, therefore, it impacted on the microbial activities for the removal of organic matter. The significant COD removal obtained during the experiment is predominantly due to the enhanced oxygenation ability of the tidal flow (DO probes reading) (Zhao et al., 2004b; Hu et al., 2012; Chang et al., 2014) and the intensive microbial activities (Dušek et al., 2008).

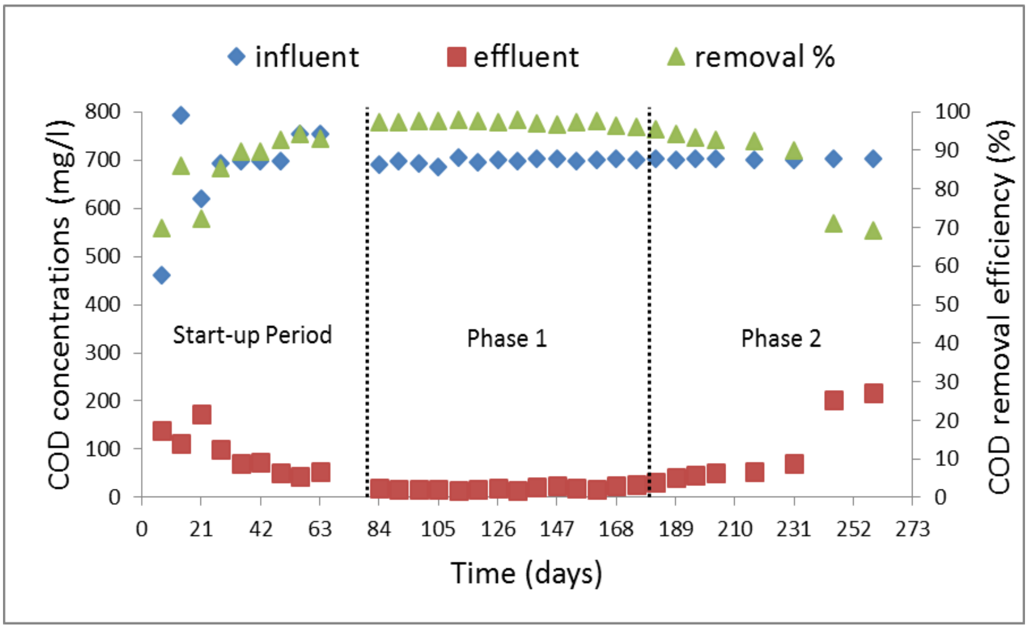


Figure 4.9 COD performance removal during the study period

In most cases, insufficient oxygen supply is the main reason for the poor biological COD removal (Korkusuz et al., 2005; Ayaz et al., 2012; Wu et al., 2015a). In such cases, nitrification will be restrained more seriously than COD reduction because oxygen will be utilized for carbon oxidization prior to nitrification due to the much faster growth rate of heterotrophic organisms than of nitrifiers (Henze et al., 1987; Wu et al., 2011; Bassin et al., 2015; Ge et al., 2015a).

In the phase 1 period, COD removal remained above 97% while at the same time, nitrification remained above 98%. This indicates that the tidal flow operation strategy can provide sufficient oxygen for both COD oxidization and nitrification. Meanwhile, in the phase 2 period, COD removal dropped to 87%, and the same time, nitrification performance dropped to 78% due to the drop in the temperature. The removal efficiency of COD in this study reached >90% similar to other studies (Jia et al., 2010; Jia et al., 2011; Wu et al., 2015b; Zapater-Pereyra et al., 2015), due to the system was under aerobic condition. However, this study obtained better COD removal than other studies: 80% (Sun et al., 2006), 36-84% (Zhao et al., 2011), and 62-70% (Hu et al., 2014b).

4.2.2.4 Phosphorus removal

As the system was not designed for phosphorus removal, the removal efficiency performance was quite low. The overall performances for TP and PO_4^{3-}-P are shown in Figure 4.10. It can be seen that both TP and PO_4^{3-}-P were not removed in the start-up period, but instead, the levels increased in that period. This is because the biological activities in the CW may have a limited impact and influence on phosphorus removal from the system. In addition, because the microorganisms release phosphorus when the anaerobic condition takes place (Likens, 2009). In the start-up period the TP influent was 7.8-11.5 mg/l (average 10.3 ± 1.1 mg/l) and the effluent was 12.4-30.5 mg/l (average 21.7 ± 5.8 mg/l), whereas the PO_4^{3-}-P influent was 8.8-10.6 mg/l (average 10 ± 0.5 mg/l) and its effluent was 11.4-28.5 mg/l (average 20.5 ± 5.2 mg/l). In phase 1, the TP influent was 9.4-11.5 mg/l (average 10.1 ± 0.6 mg/l) and its effluent was 6.7-11.3 mg/l (average 8.4 ± 1.4 mg/l) with 16.6% removal efficiency, whereas the PO_4^{3-}-P influent was 8.1-10.6 mg/l (average 9 ± 0.8 mg/l) and its effluent was 6-10.4 mg/l (average 7.4 ± 1.5 mg/l) with 17.9% removal efficiency. In phase 2, the influent of TP was 9.8-11.6 mg/l (average 10.4 ± 0.5 mg/l) and its effluent was 8.6-10.8 mg/l (average 9.4 ± 0.7 mg/l) with 10.2% removal efficiency, while the PO_4^{3-}-P influent was 8.3-10.6 mg/l (average 9.6 ± 0.8 mg/l) and its effluent was 7.2-9.7 mg/l (average 8.5 ± 0.8 mg/l) with 11.8% removal efficiency.

In conclusion, it was observed that the performance removal efficiency in phase 1 was better than in phase 2 due to the higher temperature in phase 1. In addition, the application of the gravel as the main wetland media provides poor phosphorus removal performance in CWs. Moreover, this study was not targeting phosphorus removal, this is why other studies have higher phosphorus removal than this study; 87-92% (Jia et al., 2010), 75-94% (Zhao et al., 2011), 88% (Hu et al., 2014b), and 44-80% (Zapater-Pereyra et al., 2015).

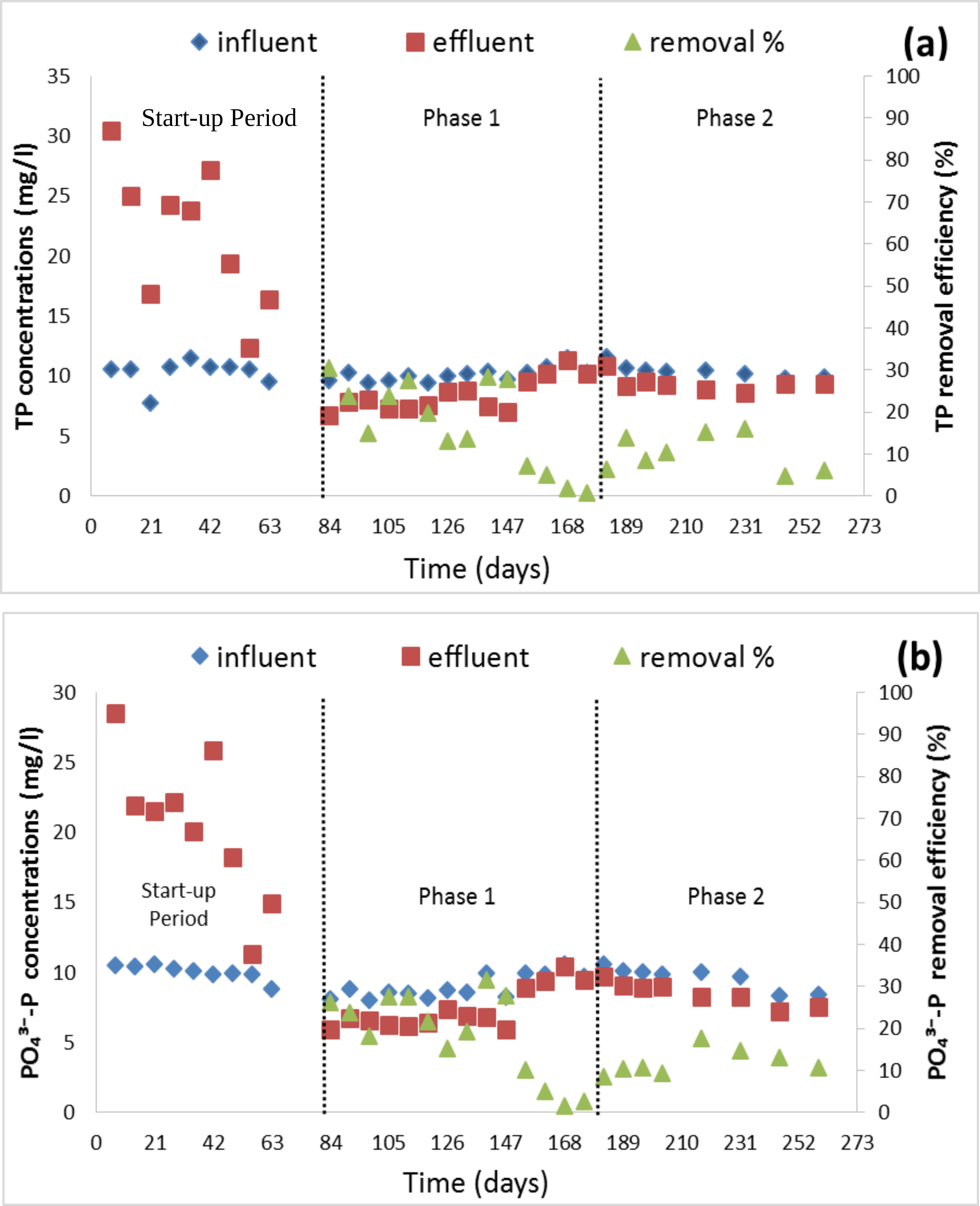


Figure 4.10 (a) TP and (b) PO_4^{3-}-P removal performance through the study period

4.2.3 COD and nutrients profiles in individual stages

4.2.3.1 COD removal performance

COD concentration decreased in all the four stages, with the highest removal efficiency in the second stage just after the start-up period with 81.2% and 63.9%, in phases 1 and 2 respectively. The contribution of each individual stage to the overall removal percentage of COD during all phases (including start-up period) is shown in Figure 4.11. The significant COD removal obtained during the experiment is predominantly due to the enhanced oxygenation ability of the tidal flow (Zhao et al., 2004c; Cui et al., 2012; Chang et al., 2014; Li et al., 2015b; Wu et al., 2015b) (see Figure 4.4) and the intense microbial activity (Dušek et al., 2008). The time of filling and draining of the CWs ensured that the oxygen presented in the beds was frequently replenished (see Figure 4.4).

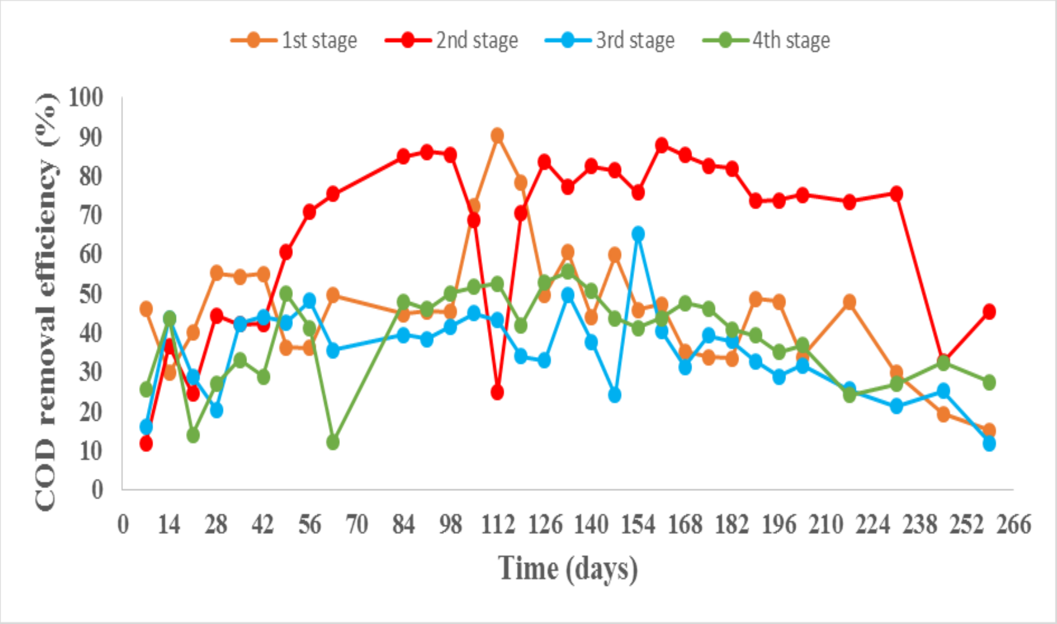


Figure 4.11 Contribution of individual stage to overall removal percentage of COD during the operation period

4.2.3.1.1 Phase 1

COD reduction mainly occurred within the first two stages; as shown in Figure 4.12, 91.3% of influent loading was removed in the first two stages for COD, which contributed to 93.8% (stage 1, 55.1%; stage 2, 38.7%) of the total COD reduction. The contributions in stages 3 and 4 were limited to 3.7% and 2.6% respectively.

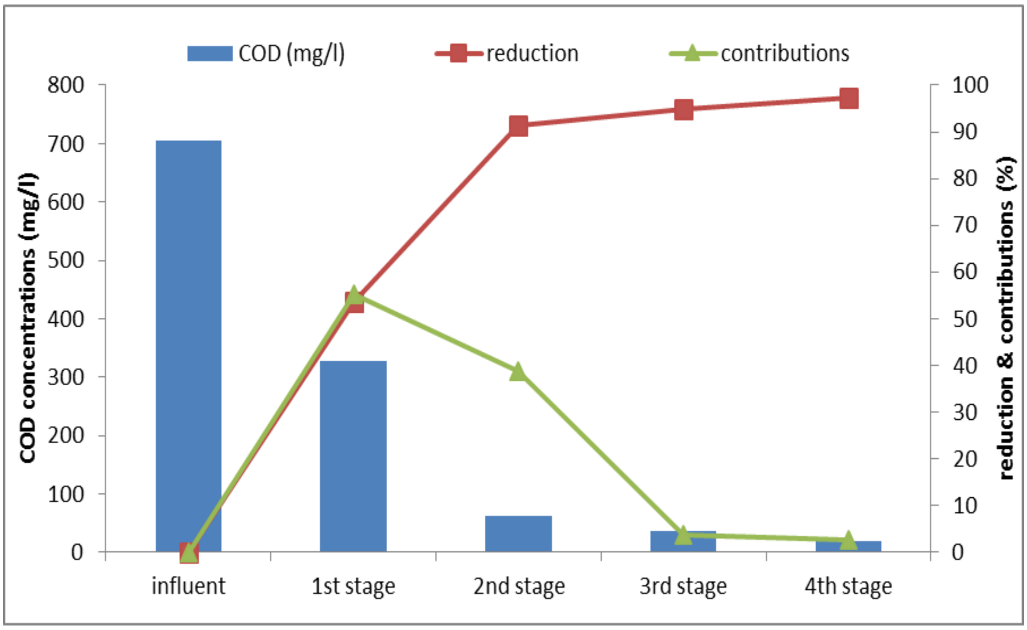


Figure 4.12 Typical COD profile in individual stages: COD concentration; reduction and contribution of individual stages to the total reduction in COD in the phase 1 period

4.2.3.1.2 Phase 2

In addition, COD reduction mainly occurred within the first two stages; as shown in Figure 4.13, 76.3% of influent loading was removed in the first two stages for COD, which contributed to 87.2% (stage 1, 39.2%; stage 2, 48%) of the total COD reduction. The contributions in stages 3 and 4 were limited as well as in phase 1 with 6.4% and 6.5% respectively.

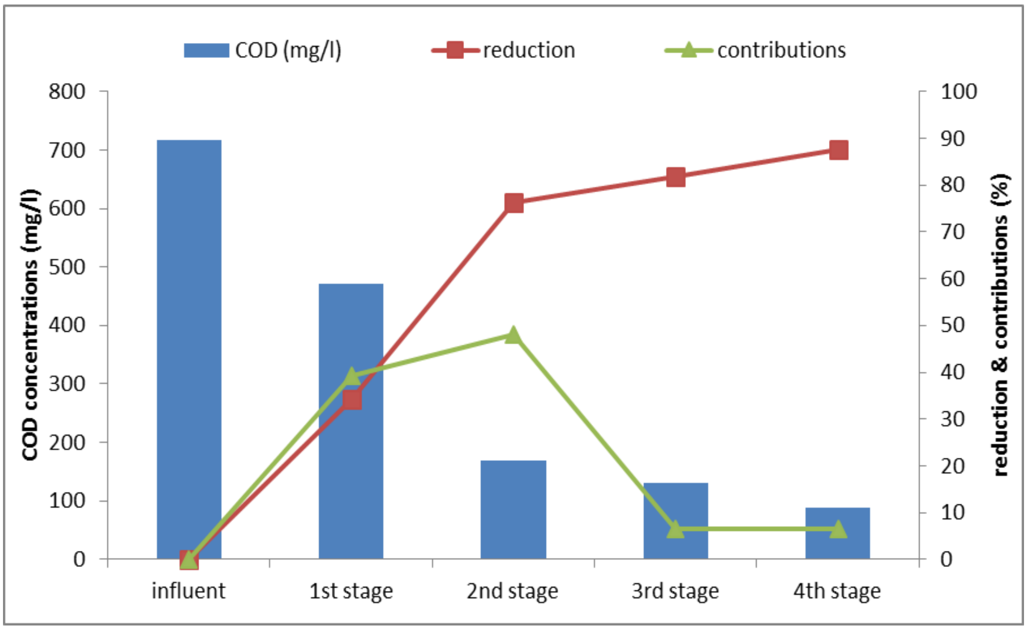


Figure 4.13 Typical COD profile in individual stages: COD concentration; reduction and contribution of individual stages to the total reduction in COD in the phase 2 period

Overall, the limited COD reduction in the last two stages could be because most of the easily biodegradable COD in the influent had been degraded already in the first two stages, and the major COD fraction in the last two stages was slowly biodegradable or non-biodegradable (Orhon and Çokgör, 1997; Namour and Müller, 1998; Orhon et al., 1999).

4.2.3.2 Nitrification and denitrification performance

The NH_4^+-N and TN of the synthetic wastewater were reduced in both phases 1 and 2 through the four stages of the system. Most of the oxygen was consumed by heterotrophic bacteria, not nitrifying bacteria which is a slow process compared to the COD reduction reaction. Consequently, under high organic loading, most of the available oxygen was used to reduce the COD of the influent. Figure 4.14 illustrates the contribution of each individual stage to the overall removal percentage of NH_4^+-N during the whole period of operation, and it shows a continuous reduction of NH_4^+-N through each stage due to microbial activities. In CW systems, the reduction of NH_4^+-N usually results in an increase of NO_2^--N and NO_3^--N levels, as NH_4^+-N can be bio-oxidised by nitrifying bacteria to NO_2^--N and further to NO_3^--N. The removal of NH_4^+-N in the current study may result from a combination of numerous processes, which include nitrification, assimilation associated with decomposition of organics, volatilisation, and plant uptake. From Figure 4.15, the concentration levels of NO_2^--N and NO_3^--N in the effluents were slightly higher than in the influents in most of the cycles, which indicated that the nitrification process was occurring during the experiment due to the aerobic conditions (see section 4.2.4 mass balance). Note that the nitrification and denitrification processes may have occurred simultaneously in the system, and this would have resulted in a decrease in the NO_2^--N and NO_3^--N levels in the effluent, which contributed to the NH_4^+-N reduction.

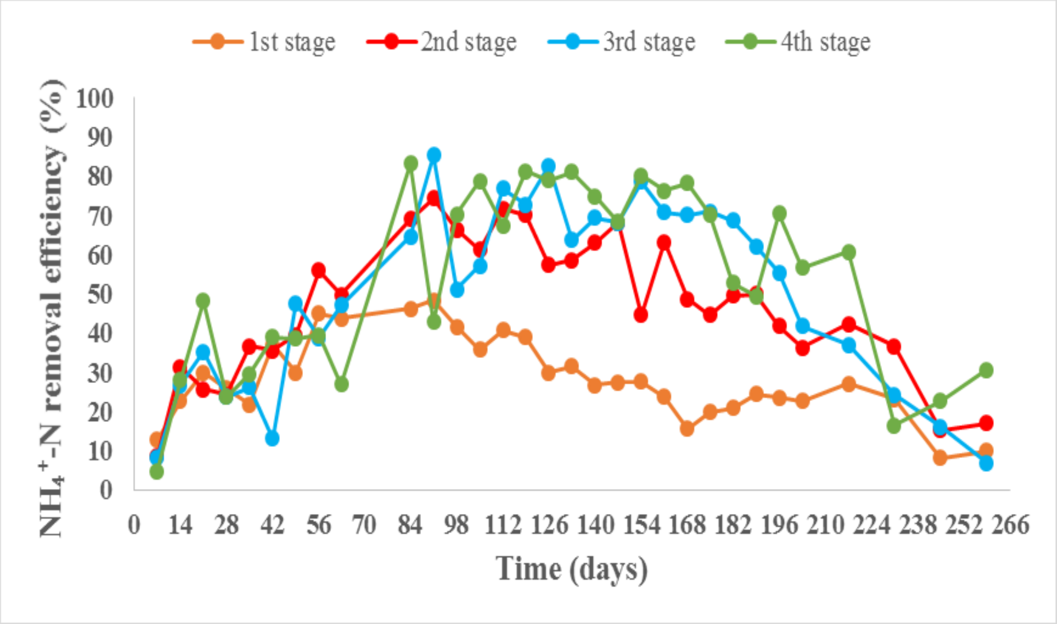


Figure 4.14 Contribution of individual stage to overall removal percentage of $NH4^+$-N during the period of operation

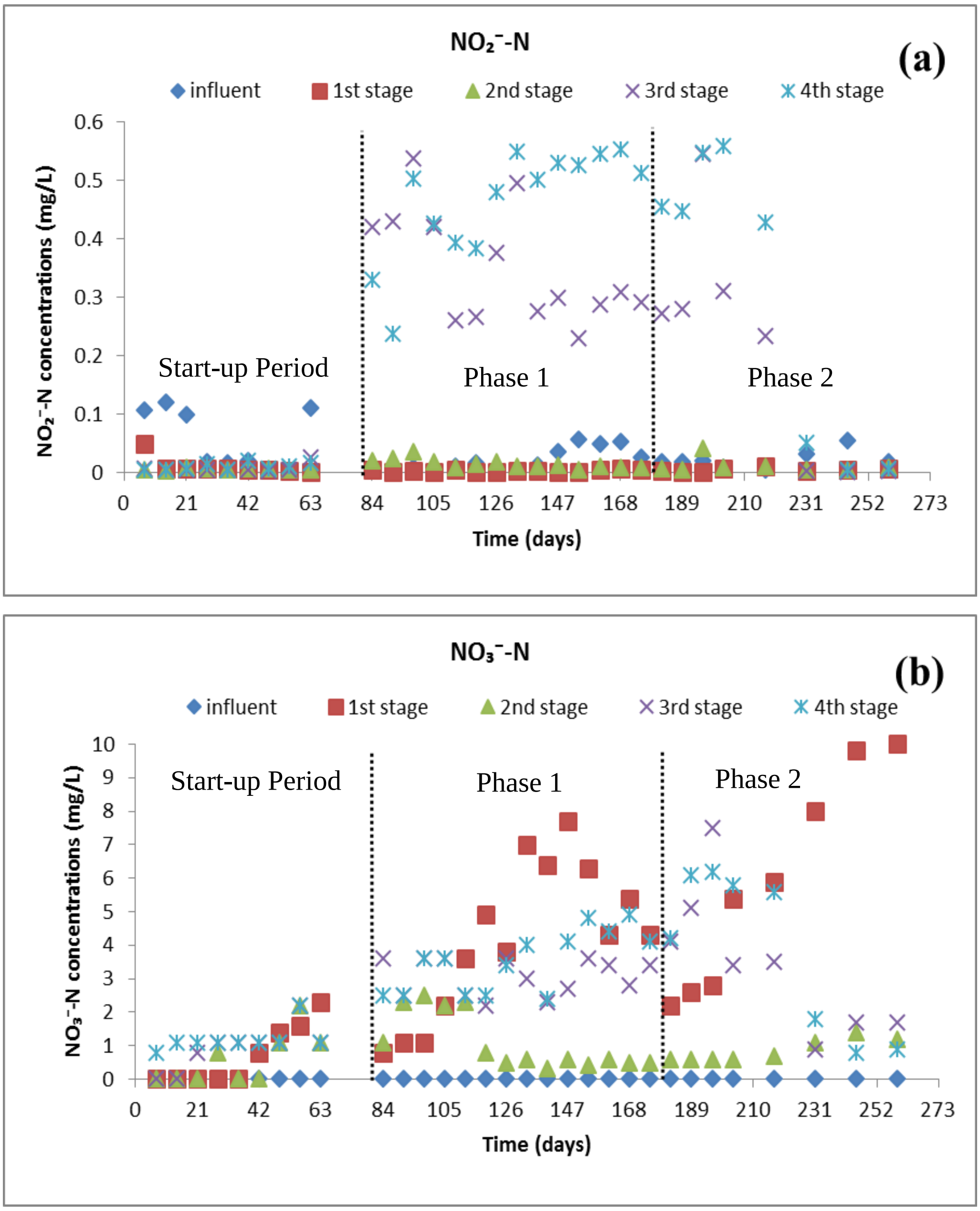


Figure 4.15 (a) NO_2^--N and (b) NO_3^--N conversion through the multistage VFCW system

The trend of NH_4^+-N reduction was similar to that of COD reduction (Figures 4.12 and 4.13).

4.2.3.2.1 Phase 1

Overall, the influent NH_4^+-N was reduced by 98.1% from 57.71 mg/l to 1.11 mg/l. Of that, 73.4% of the influent was reduced in the first two stages, contributing 74.9% (stage 1, 33.2%; stage 2, 41.7%) to the total reduction, as shown in Figure 4.16. The further reduction occurred in stage 3, which contributed 19.1% to the total reduction, and the influent NH_4^+-N was reduced by 92.1% after stage 3. Thereafter, no further significant reduction occurred in stage 4, which contributed only 6.1% to the total reduction. It is interesting to note that only limited nitrate accumulation (NO_3^--N produced/ NH_4^+-N reduced in each stage) happened in stage 2 despite the significant NH_4^+-N reduction, which means almost all of the oxidized ammonia was denitrified. However, from stage 3, the nitrate accumulation increased and 18.2% of the oxidized NH_4^+-N remained as NO_3^--N in stage 3. This suggests that denitrification was limited due to lack of carbon source as well as for the fourth stage (Figure 4.12 COD).

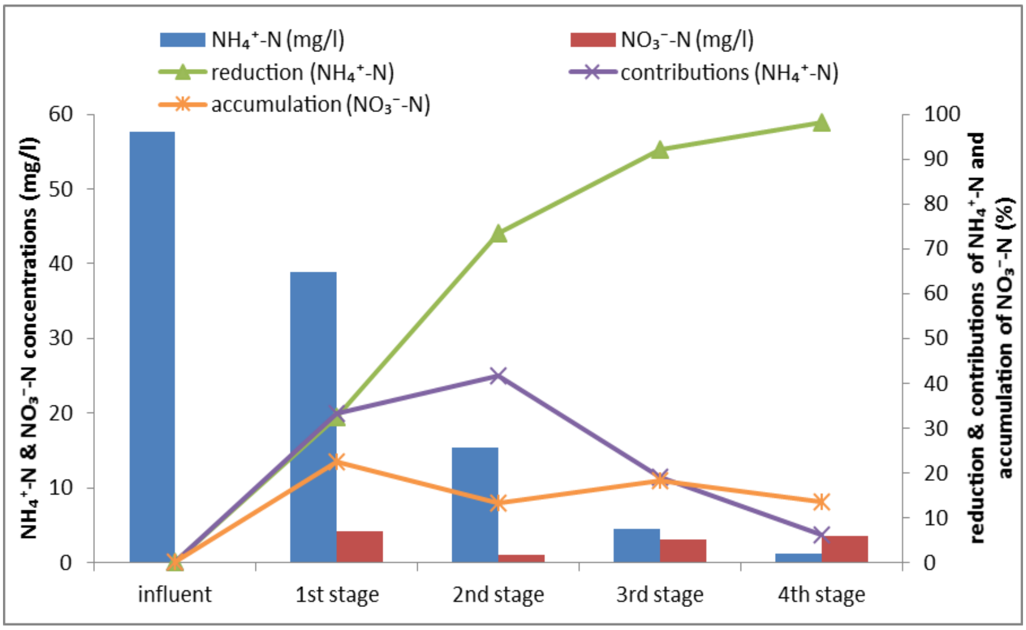


Figure 4.16 Typical nitrogen profile in individual stages: NH_4^+-N and NO_3^--N concentration; reduction of influent NH_4^+-N in each stage, contributions of individual stages to the total NH_4^+-N reduction and NO_3^--N accumulation in each stage in the phase 1 period

4.2.3.2.2 Phase 2

Overall, the influent NH_4^+-N was reduced by 78.5% from 58.13 mg/l to 12.48 mg/l. Of that, 48.2% of the influent was reduced in the first two stages, contributing 61.3% (stage 1, 25.5%; stage 2, 35.8%) to the total reduction, as shown in Figure 4.17. The further reduction occurred in stage 3, which contributed 22.4% to the total reduction, and the influent NH_4^+-N was reduced by 65.8% after stage 3. Thereafter, no further significant reduction occurred in stage 4, which contributed only 16.3% to the total reduction. In addition, it is interesting to note here that only limited nitrate accumulation occurred in stage 2 despite the noteworthy NH_4^+-N reduction, which means the oxidized ammonia was denitrified. However, from stage 3, the nitrate accumulation increased, and 25.8% of the oxidized NH_4^+-N remained as NO_3^--N in stage 3. This suggests that denitrification was limited due to the lack of any carbon source (Figure. 4.13 COD).

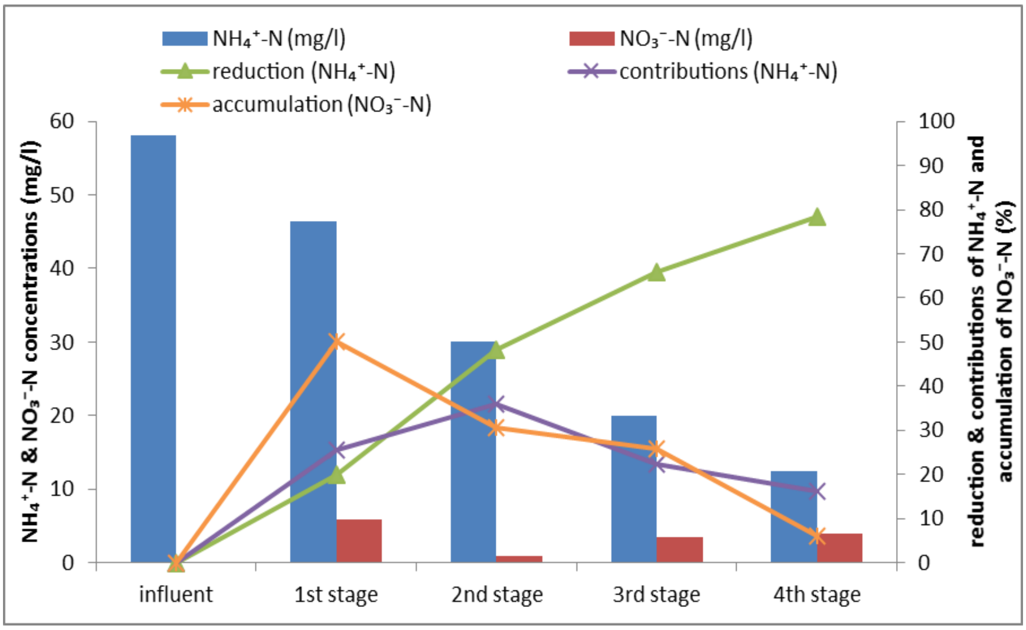


Figure 4.17 Typical nitrogen profile in individual stages: NH_4^+-N and NO_3^--N concentration; reduction of influent NH_4^+-N in each stage, contributions of individual stages to the total NH_4^+-N reduction and NO_3^--N accumulation in each stage in the phase 2 period

These results (phase 1 and 2) actually revealed an inherent restriction of TFCWs in TN reduction. That is why all the stages of TFCWs are aerobic (2 hours saturated and 6 hours unsaturated) to a certain extent due to the enhanced oxygen transfer. In such conditions, organic matter (expressed as COD) will be directly oxidized by oxygen rather than utilized for denitrification (George et al., 2003; Wiesmann et al., 2007). As a result, denitrification can easily become the limiting factor in TN elimination in TFCWs.

4.2.3.3 Phosphorus removal performance

As mentioned in section 4.2.2.4, the system was not designed for phosphorus removal; TP and PO_4^{3-}-P removal efficiencies were observed to be quite low throughout the study period. Figure 4.18 shows the removal of both TP and PO_4^{3-}-P through the stages. After the start-up period, TP and PO_4^{3-}-P began to decrease slightly.

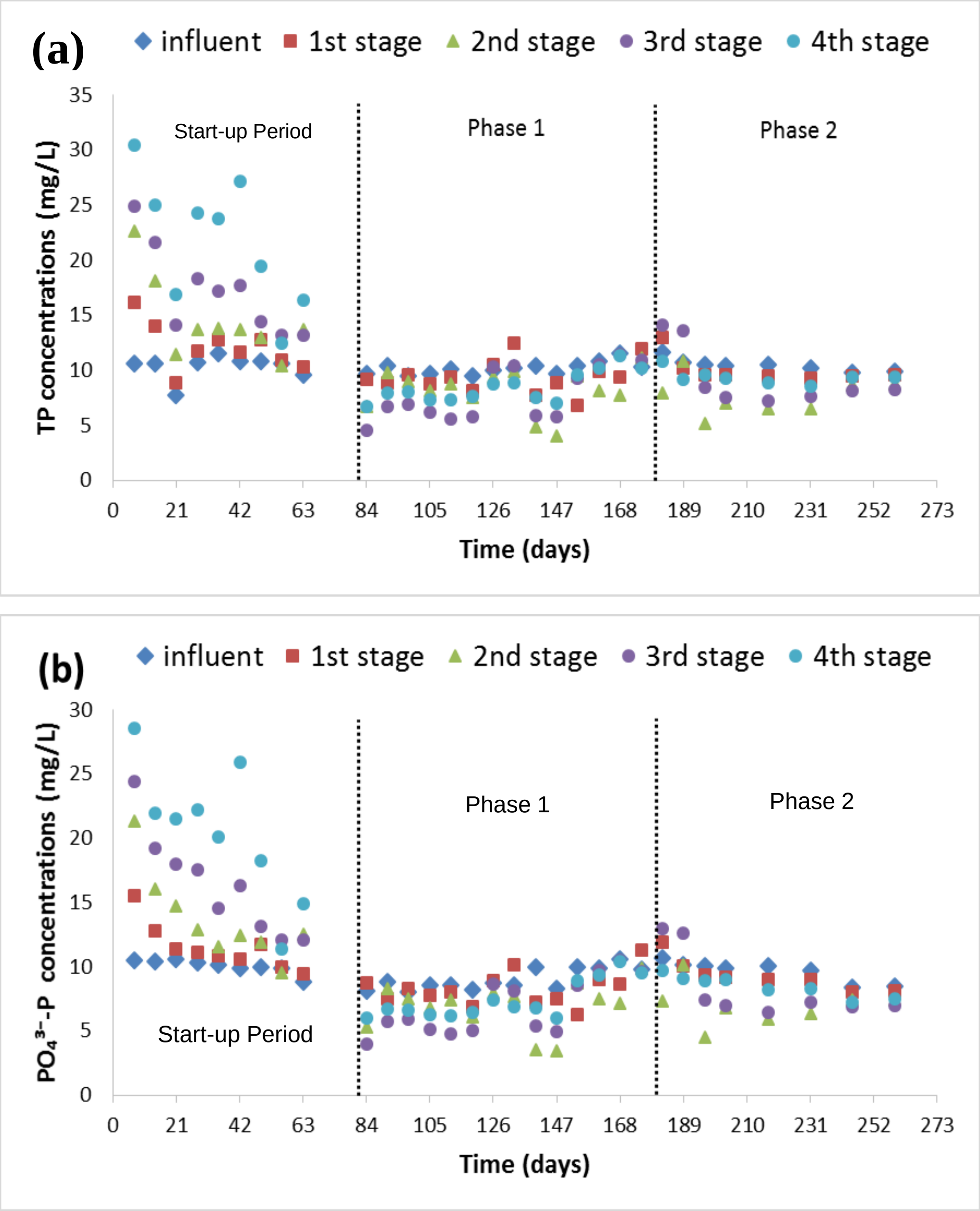


Figure 4.18 (a) TP and (b) PO_4^{3-}-P removal through multistage VFCW

Compared with NH_4^+-N and COD reduction, the reduction of phosphorus in both phases 1 and 2 was significantly less (see Figures 4.19 and 4.20).

4.2.3.3.1 Phase 1

Overall, the influents of TP and PO_4^{3-}-P were reduced by 16.6% and 17.9% from 10.12 mg/l to 8.44 mg/l and from 9 mg/l to 7.39 mg/l respectively. The influents of TP and PO_4^{3-}-P were reduced slightly in the first stage with 7.5% and 7.9% respectively, whereas the main reduction of TP and PO_4^{3-}-P occurred in the rest of the stages: the second stage with 19.5% and 22.8%, the third stage with 22.4% and 24%, and the fourth stage with 16.6% and 17.9% respectively, as shown in Figure 4.19.

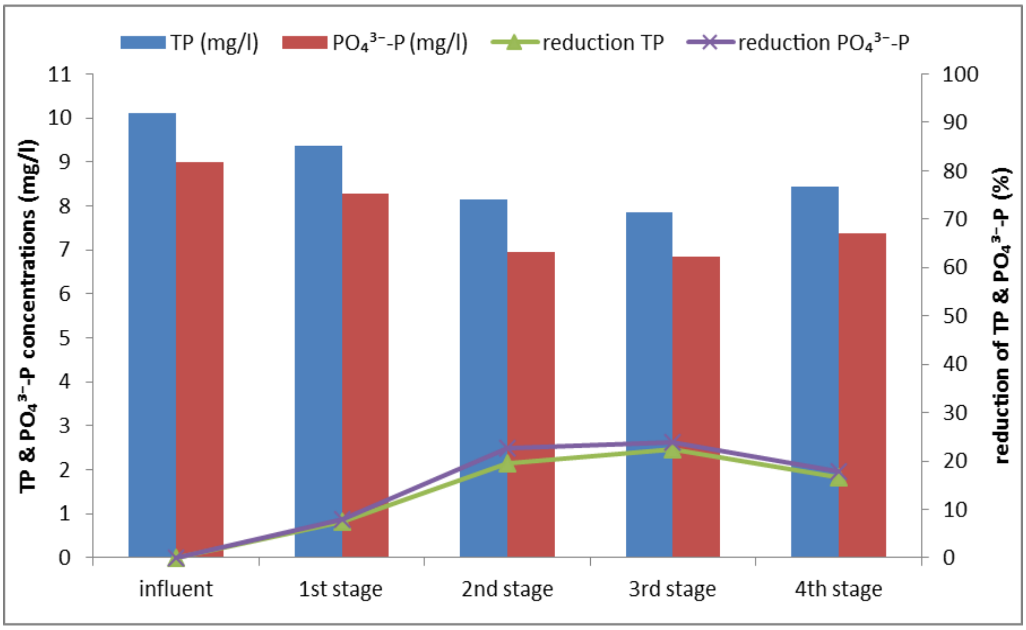


Figure 4.19 Typical phosphorus profile in individual stages: TP and PO_4^{3-}-P concentrations, reduction of the influent for both TP and PO_4^{3-}-P in each stage and the reduction of the individual stages for TP and PO_4^{3-}-P in phase 1

4.2.3.3.2 Phase 2

In this phase, the influents of TP and PO_4^{3-}-P were reduced by 10.2% and 11.8.2% from 10.44 mg/l to 9.37 mg/l and from 9.63 mg/l to 8.49 mg/l respectively. The influents of TP and PO_4^{3-}-P were also slightly reduced in the first stage with 4.1% and 3.2% respectively, whereas the main reduction of TP and PO_4^{3-}-P occurred in the second stage with 26.9% and 27.7% respectively. However, almost same reductions of TP and PO_4^{3-}-P happened in the third and fourth stages with 10.6% and 12.5%, and 10.2% and 11.8% respectively, as shown in Figure 4.20.

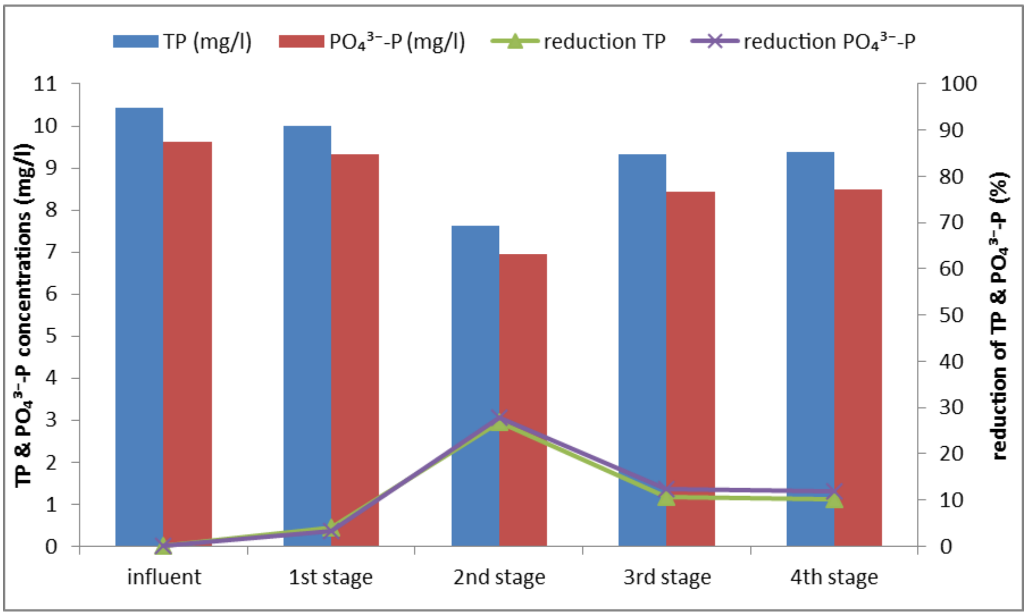


Figure 4.20 Typical phosphorus profile in individual stages: TP and PO_4^{3-}-P concentrations, reduction of the influent for both TP and PO_4^{3-}-P in each stage and the reduction of the individual stages for TP and PO_4^{3-}-P in phase 2

4.2.4 Mass balance

Mass balance was estimated in this study in order to explore the characteristics and features of nitrogen removal in individual stages under different operational conditions and to calculate the contributions of each stage to overall nitrogen removal as shown in Figure 4.21 and Table 4.3. The net mass flux of NH_4^+-N reduction (φNH_4^+-N, Re.) and NOx^--N (NO_2^--N + NO_3^--N) accumulation (φ NOx^--N, Acc.) in each stage are calculated according to Table 4.4. Here, the calculation is based on the simplifying assumption that NH_4^+-N reduction is due to nitrification; thus the nitrogen loss by biomass assimilation and plant uptake is negligible (Stottmeister et al., 2003; Hu et al., 2012). It is worth mentioning that the wastewater used in this study is a synthetic wastewater; therefore, the nitrogen in the influent is in the dissolved form, which is unlikely to be removed via sedimentation. The negative value for φ NH_4^+-N, Re. indicates that the amount of NH_4^+-N produced via ammonification exceeds the amount of NH_4^+-N reduced due to nitrification. Accordingly, the negative value for φ NOx^--N, Acc. suggests that the amount of NOx^--N removed via denitrification is above the amount of NOx^--N produced through nitrification. The removal percentage of the influent NH_4^+-N in each stage is calculated as the φ NH_4^+-N, Re. of each stage divided by the influent NH_4^+-N loading, as shown in Table 4.5.

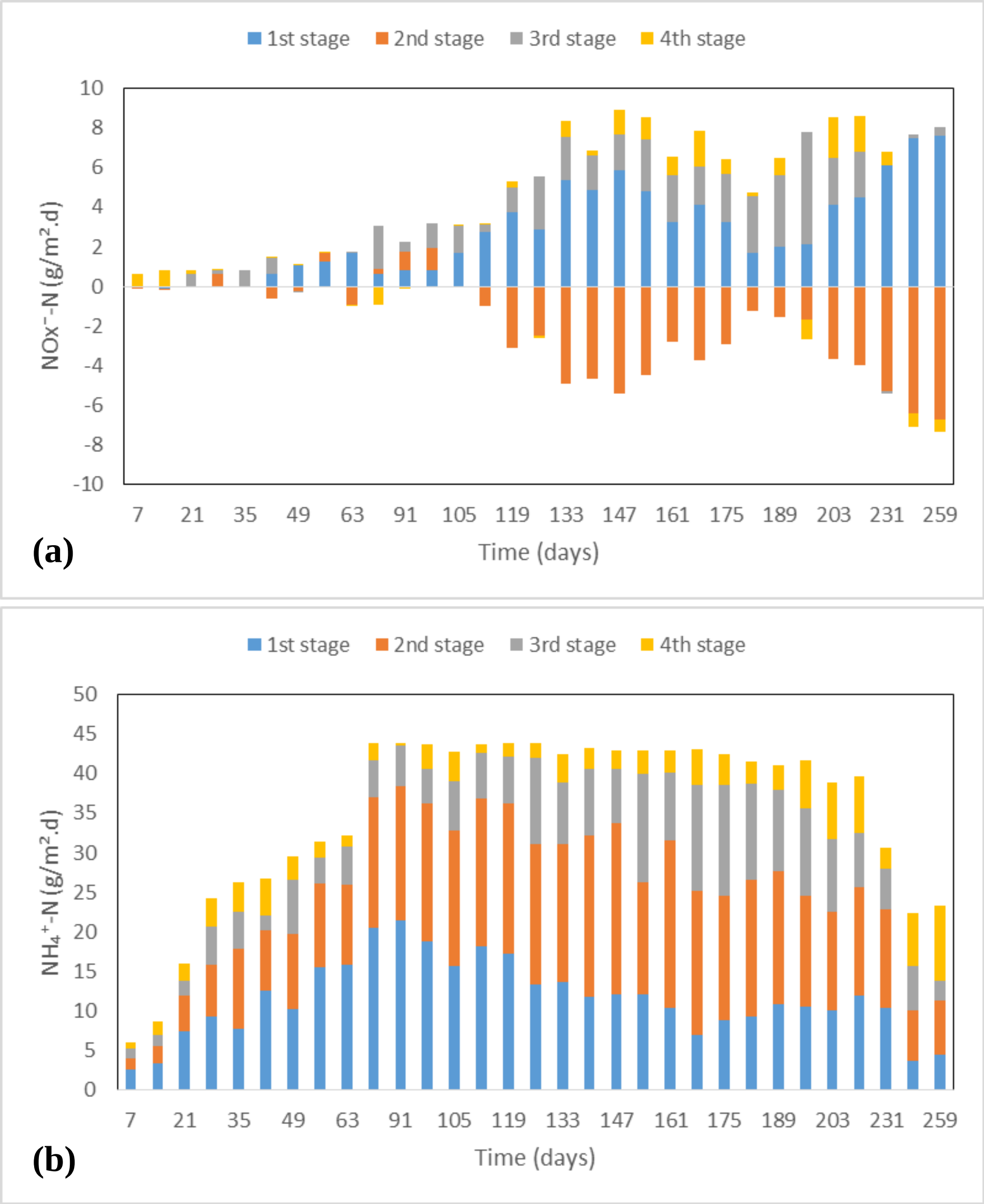


Figure 4.21 Mass balance for (a) NH_4^+-N and (b) NOx^--N accumulation in each stage

Figure 4.21 describes the contribution of individual stages to the overall NH_4^+-N and NOx^--N accumulation in each stage during the whole operation period. Clearly, the NOx^--N accumulation was negative in stage 2 in most of the time, which specified that not only the nitrified N in the current stage is fully denitrified, but also certain amount of NOx^--N from the previous stage was reduced.

Table 4.3 Mass balance for the multistage system (g/ m².d)

	1st Stage		2nd Stage		3rd Stage		4th Stage	
Phase 1	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent
NH_4^+-N	44.09	14.34	14.34	18.01	18.01	8.27	8.27	2.62
NOx^--N	0.017	3.2	3.2	-2.37*	-2.37*	1.76	1.76	0.44
Phase 2	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent
NH_4^+-N	44.41	8.89	8.89	12.49	12.49	7.82	7.82	5.67
NOx^--N	0.017	4.45	4.45	-3.81*	-3.81*	2.17	2.17	0.42

*indicated that denitrification step took place

Table 4.4 Mass flux of NH_4^+-N reduction (φNH4, Re.) and NOx^--N accumulation (φNOx-N, Acc.) in each stage (mg/d)

Stages	**1**	**2**	**3**	**4**
φNH_4^+-N, Re.	Q × (NH_4,in-NH_4,S_1)	Q × (NH_4,S_1-NH_4,S_2)	Q × (NH_4,S_2-NH_4,S_3)	Q × (NH_4,S_3-NH_4,S_4)
φNOx-N, Acc.	Q × (NOx,S_1-NOx,in)	Q × (NOx,S_2-NOx,S_1)	Q × (NOx,S_3-NOx,S_2)	Q × (NOx,S_4-NOx,S_3)

Q: flow rate, l/d; Q = 6, NOx^--N = NO_2^--N + NO_3^--N

NH_4,in, NOx,in: influent NH_4^+-N and NOx^--N, mg/l

NH_4,Si, NOx,Si: effluent NH_4^+-N and NOx^--N of each stage, mg/l; i=1-4

Table 4.5 Removal percentage of the influent NH_4^+-N in each stage

Stages	**1**	**2**	**3**	**4**
% removal of NH_4^+-N inf.	φ NH_4^+-N, Re.$_1$ / (NH_4,in × Q)	φ NH_4^+-N, Re.$_2$ / (NH_4,S_1 × Q)	φ NH_4^+-N, Re.$_3$ / (NH_4,S_2 × Q)	φ NH_4^+-N, Re.$_4$ / (NH_4,S_3 × Q)

Q: flow rate, l/d; Q = 6,

NH_4,in: influent NH_4^+-N

NH_4,Si: effluent NH_4^+-N of each stage, mg/l; i=1-3

4.2.4.1 Phase 1

Table 4.6 shows the mass balance for the phase 1 period according to the simplified calculations in Table 4.4, whereas Table 4.7 shows the removal percentage of the influent NH_4^+-N in each stage according to the calculations in Table 4.5.

Table 4.6 Mass balance for phase 1 (mg/d)

Stages	**1**	**2**	**3**	**4**
φNH_4^+-N, Re.	112.62	141.48	64.92	20.58
φNOx-N, Acc.	25.15	-18.65	13.83	3.44

Table 4.7 Removal percentage of the influent NH_4^+-N in each stage for phase 1

Stages	**1**	**2**	**3**	**4**
% removal of NH_4^+-N inf.	32.5	60.6	70.4	75.6

From Table 4.6, it can be concluded that the net mass flux of NH_4^+-N reduction for the first, second, third, and fourth stages is equal to 112.62, 141.48, 64.92 and 20.58 mg/d respectively, whereas NOx^--N accumulation for the first, second, third and fourth stages is equal to 25.15, -18.65, 13.83 and 3.44 mg/d respectively. On the other hand, from Table 4.7, the removal percentages of the influent NH_4^+-N in each stage were 32.5%, 60.6%, 70.4% and 75.4% for the first, second, third, and fourth stages respectively.

4.2.4.2 Phase 2

Table 4.8 shows the mass balance for the phase 2 period according to the simplified calculations in Table 4.4, whereas Table 4.9 shows the removal percentage of the influent NH_4^+-N in each stage according to the calculations in Table 4.5.

Table 4.8 Mass balance for phase 2 (mg/d)

Stages	**1**	**2**	**3**	**4**
φNH_4^+-N, Re.	69.84	98.1	61.44	44.52
φNOx-N, Acc.	34.94	-29.9	17.01	3.28

Table 4.9 Removal percentage of the influent NH_4^+-N in each stage for phase 2

Stages	**1**	**2**	**3**	**4**
% removal of NH_4^+-N inf.	20	35.2	34	37.3

From Table 4.8, it can be concluded that the net mass flux of NH_4^+-N reduction for the first, second, third and fourth stages is equal to 69.84, 98.1, 61.44 and 44.82 mg/d respectively whereas NOx^--N accumulation for the first, second, third, and fourth stages is equal to 34.94, -29.9, 17.01 and 3.28 mg/d respectively. However, from Table 4.9, the removal percentages of the influent NH_4^+-N in each stage were 20%, 35.2%, 34% and 37.3% for the first, second, third, and fourth stages, respectively.

For both phases 1 and 2, from Tables 4.2, 4.3, 4.6 and 4.8, it can be concluded that in the first stage, the nitrification process took place under an aerobic process. As a result, NH_4^+-N was converted to a small amount of NO_2^--N, and this increased the amount of NO_3^--N in the effluent. The effluent from the second stage, NO_2^--N increased slightly, whereas NO_3^--N decreased sharply, which indicates that nitrification and denitrification occurred simultaneously (the amount of NOx^--N removed via denitrification is above the amount of NOx^--N produced through the nitrification process, -18.65 and -29.9 mg/d for phases 1 and 2 respectively). In both the third and fourth stages, the NO_2^--N and NO_3^--N levels of the effluent were significantly increased indicating the nitrification process did not take place fully due to the increase in nitrite and nitrate in the effluent of each stage and the lack of any denitrification process due to the low amount of any available organic carbon source.

4.2.5 Statistical analysis

The correlations between the monitored parameters and the water quality parameters were investigated and studied to identify the influencing factors on the system performance. Table 4.10 shows that temperature has a significant impact on all parameters. Increases in the temperature will increase the efficiency removal for COD, NH_4^+-N, TN and TP, whereas increases in the temperature will decrease ORP, DO and pH. In addition, it can be concluded that there is a relationship between COD, NH_4^+-N, and TN, i.e., organic matter as a carbon source for nitrogen removal process. Moreover, ORP has a negative significant impact correlation with temperature, COD, NH_4^+-N, and TN and a positive significant impact correlation with DO. Furthermore, DO has a negative significant impact correlation with temperature, COD, NH_4^+-N, and TN, and a positive significant impact correlation with ORP. The correlation analysis suggests that temperature, ORP, and DO are the key influencing factors for the removal performance efficiencies of organic matter and nutrients (nitrogen and phosphorus).

Table 4.10 Correlation analysis for the system performance

		Temp.	**COD**	**NH_4^+**	**TN**	**TP**	**ORP**	**DO**	**pH**
Temp.	Correlation coefficient	-	-	-	-	-	-	-	-
COD	Correlation coefficient	0.77^{**}	-	-	-	-	-	-	-
NH_4^+	Correlation coefficient	0.75^{**}	0.83^{**}	-	-	-	-	-	-
TN	Correlation coefficient	0.79^{**}	0.89^{**}	0.85^{**}	-	-	-	-	-
TP	Correlation coefficient	0.54^{**}	0.365	0.45^{*}	0.50^{*}	-	-	-	-
ORP	Correlation coefficient	-0.71^{**}	-0.65^{**}	-0.58^{**}	-0.68^{**}	-0.35	-	-	-
DO	Correlation coefficient	-0.59^{**}	-0.59^{**}	-0.56^{**}	-0.59^{**}	-0.16	0.72^{**}	-	-
pH	Correlation coefficient	-0.63^{**}	-0.60^{**}	-0.45	-0.45	-0.22	0.35	0.29	-

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

4.3 Conclusion

This chapter has discussed the overall treatment performance and the individual stages of treatment performance. Moreover, this chapter has covered a specific method for treatment called the tidal flow strategy. Furthermore, mass balance has been presented to indicate the nitrification and denitrification process. Finally, statistical analysis has been discussed to illustrate the impact factors on organic matter and nutrients removal. The results outlined within this chapter have improved the current scientific understanding of nitrogen removal using a VFCW system with a tidal flow strategy. The key conclusions derived from the study are as follows:

- The four stages vertical flow constructed wetlands (VFCWs) system has demonstrated efficiently how the capability of organic matter (expressed as COD) and nitrogen removal and the treatment capacity was substantially promoted by the tidal flow operation.
- The system applied a tidal flow with 2 hours saturated and 6 hours unsaturated and proved that the system was able to deal with high concentration levels and remove the contaminants efficiently.
- It was clearly demonstrated that with a shorter saturated time and longer unsaturated time in the tidal flow, the CW system was more efficient in the removal of various pollutants due to the enhanced oxygen supply into the CW.
- The improvement of removal efficiency was attributed to the aeration of the CW during the prolonged unsaturated time, which enhanced the oxygen transfer.
- The removal efficiencies for COD, NH_4^+-N, TN, TP, and PO_4^{3-}-P in the phase 1 period were better than in the phase 2 period.

- The correlation statistical analysis shows that temperature, ORP, and DO are the key influencing factors for the removal performance efficiencies of organic matter and nutrients.

The limited denitrification step in the third and fourth stages due to the greatly enhanced oxygen transfer was the main challenge in this study, which needs appropriate process adjustments and modifications, such as the creation of anoxic stages and the rearrangement of the wastewater flow for more efficient utilization of the influent carbon source. This modification process will be discussed in detail in Chapter Five by using a step-feeding strategy.

5 Applying Step-feeding Strategy to Enhance Biological Nitrogen Removal in Tidal Flow Constructed Wetlands

5.1 Introduction

In the previous study discussed in Chapter 4 regarding a four-stage vertical flow constructed wetlands (VFCWs), the efficiency of NH_4^+-N and TN removal was 98.1% and 78.5% and 77.3% and 60.3% for both phases 1 and 2 respectively. This is because all four stages were under aerobic conditions due to the prolonged unsaturated time, which enhanced the oxygen transfer into the system. However, this is an unfavourable condition for the denitrification step (Vymazal and Kröpfelová, 2011). Analysis of the performance of the individual stages detected that the carbon source that came from the influent tank was almost depleted within the first two stages for both phases. Therefore, the denitrification step was becoming limited as the stages progressed due to the limited carbon source received in the third and fourth stages, causing high NO_3^--N effluent. Consequently, in order to reach and achieve a satisfactory TN removal performance, adjustment and modification of the original VFCWs configuration were needed.

Step-feeding has been generally illustrated in a conventional activated sludge process as an effective alternative and option to improve and enhance TN removal by stepwise introduction of the influent to nitrified liquid and consequently making more efficient use of the influent carbon source for the denitrification step (Miyaji et al., 1980; Fillos et al., 1996; Fujii, 1996; Puig et al., 2004; Tang et al., 2007; Hu et al., 2012).

In this study, step-feeding was adopted as the key strategy and process that aimed to improve and enhance the TN removal performance for the four-stage VFCWs system (TN removals were 77.3% and 60.3% in phase 1 and phase 2 respectively in Chapter 4). To achieve this aim, the following key objectives were addressed:

- to investigate the effectiveness of the different step-feeding ratios (schemes) and the operational conditions on the treatment performance
- to investigate the nitrogen removal pathways and their performance in the individual stages.

5.2 Results and discussion

The experiment was carried out over 5 months (from February to June 2016), and it was divided into three phases according to the distribution ratios of the step-feeding (80:20, 70:30 and 60:40).

5.2.1 Redox potential (ORP) and dissolved oxygen (DO)

This section was discussed in detail in Chapter 4, particularly in section 4.2.1. ORP and DO data were monitored and measured in real-time at every 10 minutes at two depth points over a 5-month period, as shown in Figure 3.8. The first point was named the upper point at 20 cm beneath the surface of the distribution layer, whereas the second point was called the lower point at 50 cm below the surface of the distribution layer.

In this study, ORP was monitored in the VFCWs at two depths (20 cm and 50 cm beneath the surface) as per average data, which are shown in Figure 5.1. However, it was noticed that all the stages at the upper points began with negative values due to the intense microbial activity, and they remained having negative values for the whole period except for the third stage, which began in positive values below 100 mV in February and March, and from April to June, its values were in negative, which means anoxic conditions occurred, and so did the activities of nitrifying and denitrifying bacteria (Dušek et al., 2008; Likens, 2009). Regarding the lower point, the first and second stages remained in negative values due to microbial activity, and the depth also played a role. For both upper and lower points in the third stage, they began in positive values in February and March then went down to negative values in the remaining study period, because a carbon source (step-feeding) was introduced to this stage, which enhanced microbial activities for the denitrification process. The fourth stage was in positive values in the whole study period below 100 mV, which means anoxic conditions had occurred and denitrification process as well. Regarding DO, this was also monitored at two depths (20 cm and 50 cm beneath the surface) as per average data, which are shown in Figure 5.2. DO decreased with depth and due to low oxygen availability. It was noticed that in the upper point values in all stages, the average DO was below 1 mg/l. Therefore, ORP values were almost in negative in all the stages, except for the third stage in February and March, when the values were in positive. In general, positive ORP values indicate the existence of oxygen in the system (Rowell, 1981). Such a situation may be attributed to the plants ability to oxygenate reed beds via their roots and from the tidal flow process. Negative ORP values specify that the oxygen is used by microbes for the mineralisation of root exudates (Kankaala et al., 2003; Altor and Mitsch, 2006). In addition, ORP could be affected by many factors, as was mentioned in Chapter 4, such as temperature, plants, depth, and the different wastewater concentrations that the system had received. Therefore, the results varied from one month to another. In general, the system is driven by the physicochemical and biological factors.

Overall, in the first stage, it is clear that most of the ORP values were negative, which means that the microorganisms were active in the contaminant and nutrient removal in low DO concentrations. In the second stage, ORP values were in negative values with a low DO concentration, which was suitable for the denitrification process (anoxic). In the third stage, February and March were in positive values below 100, which indicates that the nitrification process took place. In the fourth stage, ORP values were all positive below 100 mV, and the denitrification process took place. More details will be discussed in the nitrogen removal section.

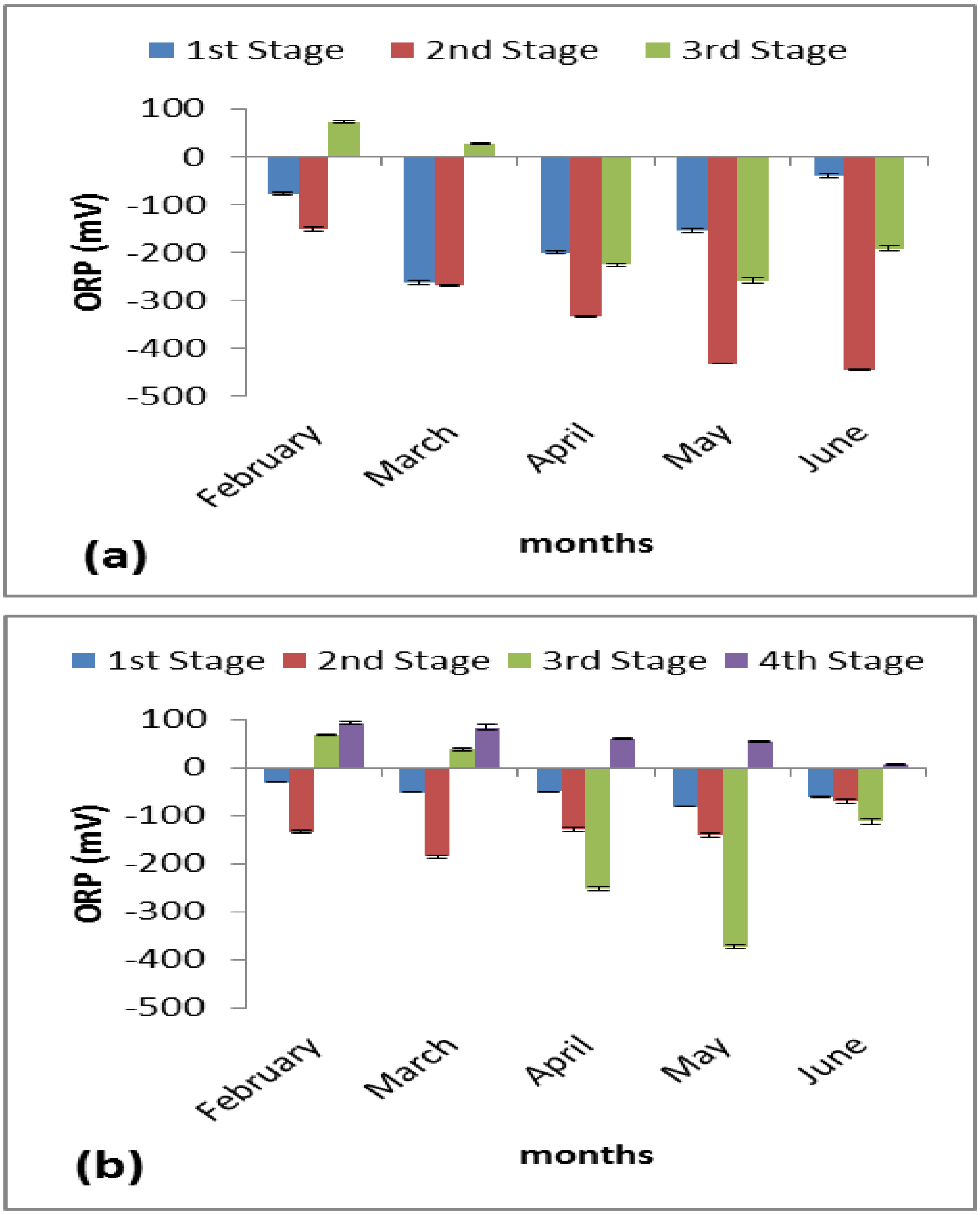


Figure 5.1 Average ORP values during the step-feeding strategy at (a) upper point and (b) lower point (error bars represent the standard error)

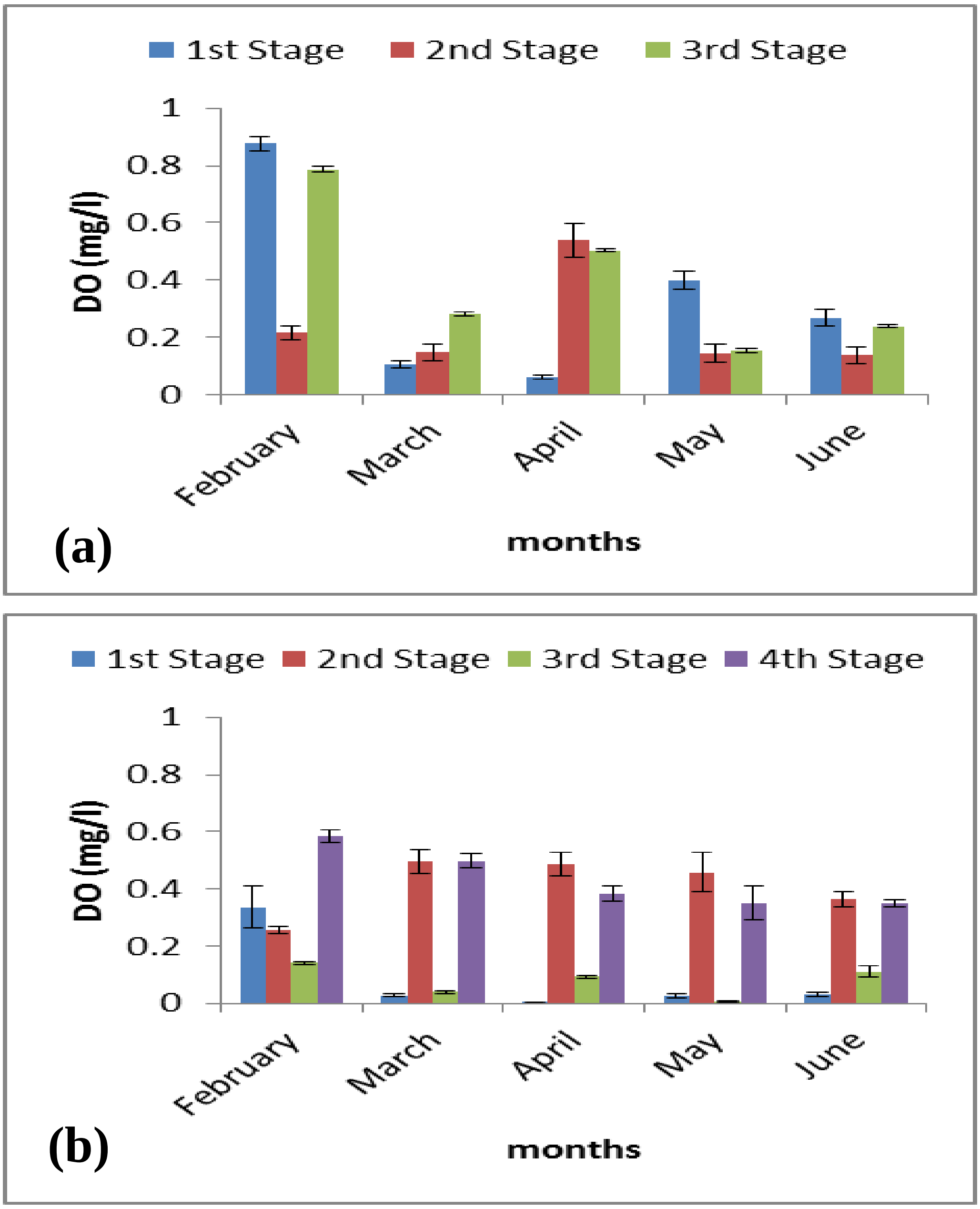


Figure 5.2 Average DO values during the step-feeding strategy at (a) upper point and (b) lower point (error bars represent the standard error)

5.2.2 Overall treatment performance

The overall treatment efficiency for nutrient and organic matter expressed as COD during the study period for phase 1, 2 and 3 is presented in Table 5.1.

Table 5.1 Overall treatment performance of multistage vertical constructed wetlands with different step-feeding distribution ratios (between brackets represent standard deviation)

Phase 1 (80:20)	**Influent**	**1st stage**	**2nd stage**	**3rd stage**	**4th stage**	**Removal %**
pH	7.39 (±0.1)	7.51 (±0.09)	7.44 (±0.08)	7.22 (±0.19)	7.32 (±0.08)	
Temperature*	11.26 (±0.71)	11.04 (±0.99)	13.34 (±1.73)	13.72 (±0.81)	14.2 (±1.42)	
NH_4^+-N (mg/l)	58.28 (±0.74)	46.7 (±3.01)	42.22 (±3.31)	9.44 (±1.89)	6.31 (±0.71)	**89.2**
NO_2^--N (mg/l)	0.039 (±0.02)	0.009 (±0.004)	0.01 (±0.004)	0.019 (±0.014)	0.023 (±0.018)	
NO_3^--N (mg/l)	0	2.94 (±2.22)	1.8 (±1.3)	3.28 (±1.63)	3.12 (±1.61)	
TN (mg/l)	62.12 (±0.66)	nm**	nm**	nm**	11.76 (±1.53)	**81.1**
COD (mg/l)	712.8 (±18.02)	393.2 (±86.37)	303.4 (±97.57)	73.44 (±19.39)	56.14 (±12.78)	**92.1**
TP (mg/l)	10.77 (±0.5)	9.67 (±1.94)	10.21 (±1.58)	4.92 (±2.48)	7.89 (±0.75)	**26.7**
PO_4^{3-}-P (mg/l)	9.81 (±0.15)	8.87 (±1.87)	9.33 (±1.01)	4.06 (±2.35)	6.91 (±0.79)	**29.6**
Phase 2 (70:30)	**Influent**	**1st stage**	**2nd stage**	**3rd stage**	**4th stage**	**Removal %**
pH	7.38 (±0.04)	7.25 (±0.08)	7.48 (±0.11)	7.81 (±0.13)	7.29 (±0.08)	
Temperature*	12.4 (±0.8)	13.14 (±1.06)	16.42 (±1.89)	18.02 (±1.79)	17.86 (±1.67)	
NH_4^+-N (mg/l)	58.16 (±0.63)	49.72 (±1.24)	42.5 (±1.4)	40.42 (±1.8)	15.26 (±3.11)	**73.8**
NO_2^--N (mg/l)	0.025 (±0.009)	0.006 (±0.002)	0.007 (±0.004)	0.009 (±0.007)	0.03 (±0.02)	
NO_3^--N (mg/l)	0	4.1 (±0.86)	2.78 (±1.12)	2.56 (±1.4)	1.48 (±0.71)	
TN (mg/l)	62 (±0.58)	nm**	nm**	nm**	20.98 (±2.11)	**66.2**
COD (mg/l)	713.2 (±16.45)	486 (±30.07)	261.2 (±39.51)	145.78 (±45.14)	41.9 (±6.18)	**94.1**
TP (mg/l)	11.13 (±0.5)	10.69 (±0.34)	10.63 (±1.01)	10.34 (±2.56)	8.95 (±0.67)	**19.6**
PO_4^{3-}-P (mg/l)	10.23 (±0.16)	10 (±0.38)	9.86 (±0.82)	9.42 (±2.56)	7.98 (±0.59)	**22**
Phase 3 (60:40)	**Influent**	**1st stage**	**2nd stage**	**3rd stage**	**4th stage**	**Removal %**
pH	7.35 (±0.03)	7.15 (±0.08)	7.33 (±0.13)	7.45 (±0.22)	7.16 (±0.07)	
Temperature*	17.46 (±2.03)	18.28 (±2.13)	21.04 (±2.41)	23.32 (±3.25)	22.54 (±2.87)	
NH_4^+-N (mg/l)	58.12 (±0.69)	44.78 (±4.46)	31.86 (±5.75)	24.8 (±8.41)	10.55 (±2.96)	**81.9**

NO_2^--N (mg/l)	0.035 (±0.017)	0.003 (±0.001)	0.006 (±0.002)	0.002 (±0.001)	0.116 (±0.043)	
NO_3^--N (mg/l)	0	4 (±1.07)	1.42 (±0.91)	2.94 (±2.03)	2.6 (±0.62)	
TN (mg/l)	61.98 (±0.61)	nm**	nm**	nm**	16.34 (±2.87)	**73.6**
COD (mg/l)	715.6 (±15.88)	355 (±83.01)	97.06 (±34.18)	63.38 (12.80)	32.74 (±4.46)	**95.4**
TP (mg/l)	11.29 (±0.4)	11.15 (±0.76)	10.75 (±1.43)	8.34 (±2.76)	9.99 (±0.8)	**11.5**
PO_4^{3-}-P (mg/l)	10.71 (±0.35)	10.65 (±0.6)	10.15 (±1.24)	7.65 (±2.68)	8.96 (±0.76)	**16.3**

Temperature*: Temperature of the sample; nm**: mot measured

5.2.2.1 Nitrification performance

The overall nitrification performance is shown in Figure 5.3. The nitrification process was already established from the last experiment (Chapter 4), which indicated the NH_4^+-N reduction (Figure 5.3a) and an increase of NO_3^--N in the effluent (Figure 5.3b).

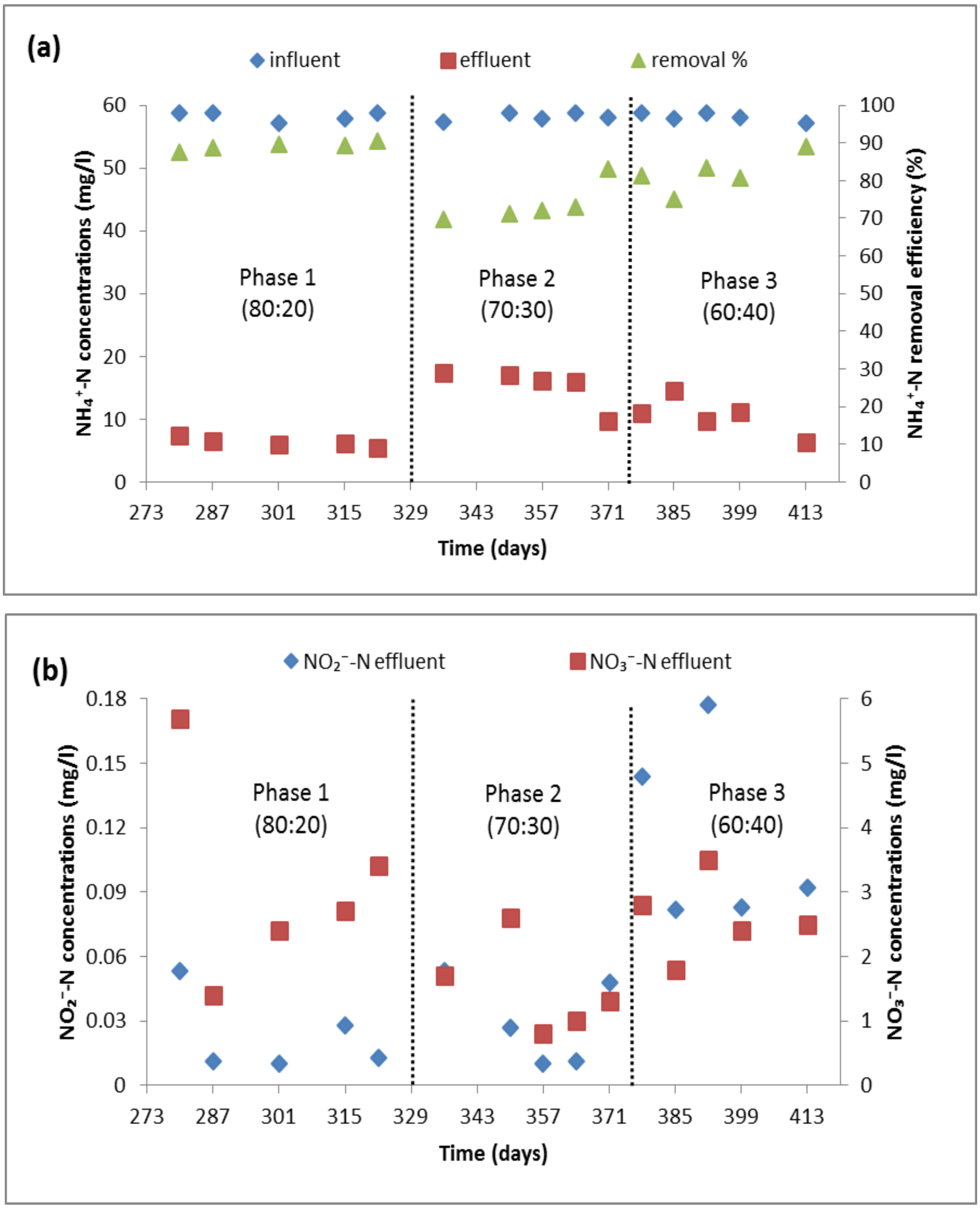


Figure 5.3 Nitrification performance (a) NH_4^+-N reduction; (b) effluent of NO_2^--N and NO_3^--N

The NH_4^+-N reduction in phase 1 period was 87.4-90.6% (average 89.2%) with NH_4^+-N effluent 5.5-7.38 mg/l (average 6.31 ± 0.71 mg/l). However, in phase 2 period, the nitrification performance was decreased from 83.1% to 69.7% (average 73.8%) with NH_4^+-N effluent 9.8-17.4 mg/l (average 15.26 ± 3.11 mg/l). Finally, in phase 3 period; the nitrification performance was backed again to increase from 74.9% to 89% (average 81.9%) with NH_4^+-N effluent 6.27-14.5 mg/l (average 10.55 ± 2.96mg/l). In addition, the removal efficiency for NH_4^+-N in this study was lower compared to what was obtained previously in Chapter 4 due to the operational variables, mainly the HLR which is different in each phase; but achieved 70-90% similar to other studies (Jia et al., 2011; Wu et al., 2011; Zapater-Pereyra et al., 2015), due to different operational variables (i.e. HLR, HRT, contaminants loading, etc.). However, this study obtained higher NH_4^+-N removal than other studies; 58% (Sun et al., 2006), 63% (Jia et al., 2010), and 49% (Zhao et al., 2011). Overall, the four-stage VFCW system demonstrated an efficient nitrification process in the study period.

5.2.2.2 TN reduction

Figure 5.4 illustrates the overall TN removal performance in the study period. The TN elimination showed the same trend as for NH_4^+-N reduction. During the phase 1 period, the average TN influent was 62.12 mg/l with TN effluent 9.7-13.5 mg/l (average 11.76 ± 1.53 mg/l) and 81.1% removal efficiency performance. In phase 2, the TN influent maintained almost the same influent as in the phase 1 period with 62 mg/l on average, and with effluent 17.8-23.3 mg/l (average 20.98 ± 2.11 mg/l). It had 66.2% removal efficiency, which is a significant decrease in performance efficiency compared to phase 1 due to the decrease in nitrification performance, as shown in Figure 5.3, and the availability of the carbon source for the denitrification step. In phase 3, also, the TN influent remained almost the same as in phases 1 and 2 with 61.98 mg/l on average, and with effluent 12.5-20.3 mg/l (average 16.34 ± 2.87 mg/l). It had 73.6% removal efficiency, which is an increase in performance efficiency compared to phase 2 due to the increase in the nitrification step, as shown in Figure 5.3 and the availability of the carbon source for the denitrification step. The best TN removal performance was achieved in phase 1 with 81.1% as the average removal efficiency, which is an improvement compared with the TN removal of the previous study in Chapter 4 in phases 1 and 2 with 77.3% and 60.3% respectively. This shows that the step-feeding is an effective process and strategy for TFCWs to enhance TN removal performance. The nitrogen removal rate for phase 1, 2 and 3 were 76.9 gN/m³.d, 62.7 gN/m³.d and 69.7 gN/m³.d respectively. The removal rates were higher than those achieved in Chapter 4 due to the step-feeding strategy. Similar results were achieved in other studies that used four stages TFCW with effluent recirculation strategy to enhance the TN removal; for instance 78 gN/m³.d was achieved by Sun et al. (2005) whilst Zhao et al. (2004c) achieved 80 gN/m³.d. Moreover, this study achieved higher removal rates than similar studies that used four and/or five stages due to HLR, HRT, and step-feeding strategy. In addition, TN was measured only in the influent and the effluent of the final stage (fourth stage) due to financial constraints.

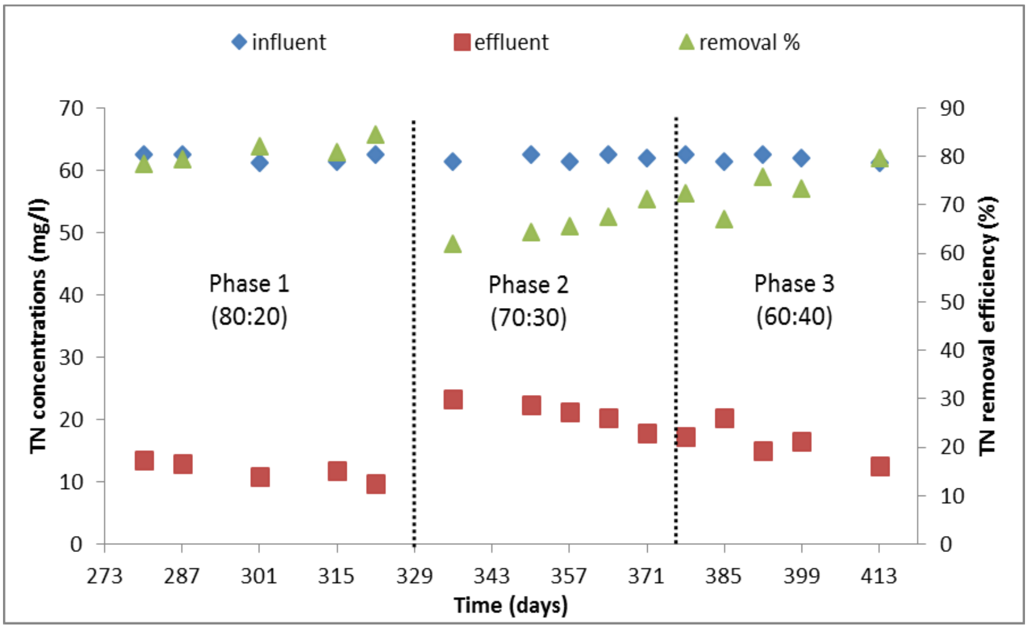


Figure 5.4 TN removals through the study period

5.2.2.3 Organic matter removal

The overall performance for COD is shown in Figure 5.5. The influent COD in the phase 1 period was 689-736 mg/l (average 712.8 ± 18.02 mg/l) with effluent 38.3-73.9 mg/l (average 56.14 ± 12.78 mg/l) and 92.1% removal efficiency performance. In the phase 2 period, the COD influent was 692-734 mg/l (average 713.2 ± 16.45 mg/l); the effluents were reduced compared to phase 1 with 31.7-47.6 mg/l (average 41.9 ± 6.18 mg/l) and the removal efficiency increased slightly to 94.1%. In the phase 3 period, the COD influent was 690-730 mg/l (average 715.6 ± 15.88 mg/l); the effluents were further reduced compared to phases 1 and 2 with 25.7-37.2 mg/l (average 32.74 ± 4.46 mg/l), and the removal efficiency also increased slightly to 95.4%. In addition, the removal efficiency of COD in this study was maintained >90%, similar to other studies (Jia et al., 2010; Jia et al., 2011; Wu et al., 2015b; Zapater-Pereyra et al., 2015). However, this study obtained higher COD removal than other studies; 80% (Sun et al., 2006), 36-84% (Zhao et al., 2011), and 62-70% (Hu et al., 2014b). The significant COD removal obtained during the experiment is predominantly due to the enhanced oxygenation ability of the tidal flow (Zhao et al., 2004b; Hu et al., 2012; Chang et al., 2014), the intense microbial activities (Dušek et al., 2008), and the external carbon source from the step-feeding strategy (Hu et al., 2012). Overall, the system indicates that it can significantly remove the organic carbon from the influent. In general, the main reason for the poor biological COD removal is the insufficient oxygen supply. In such a case, nitrification will be more limited than will COD reduction because oxygen will be utilized for carbon oxidization before the nitrification process due to the much slower growth rate of nitrifiers than heterotrophic organisms (Henze et al., 1987).

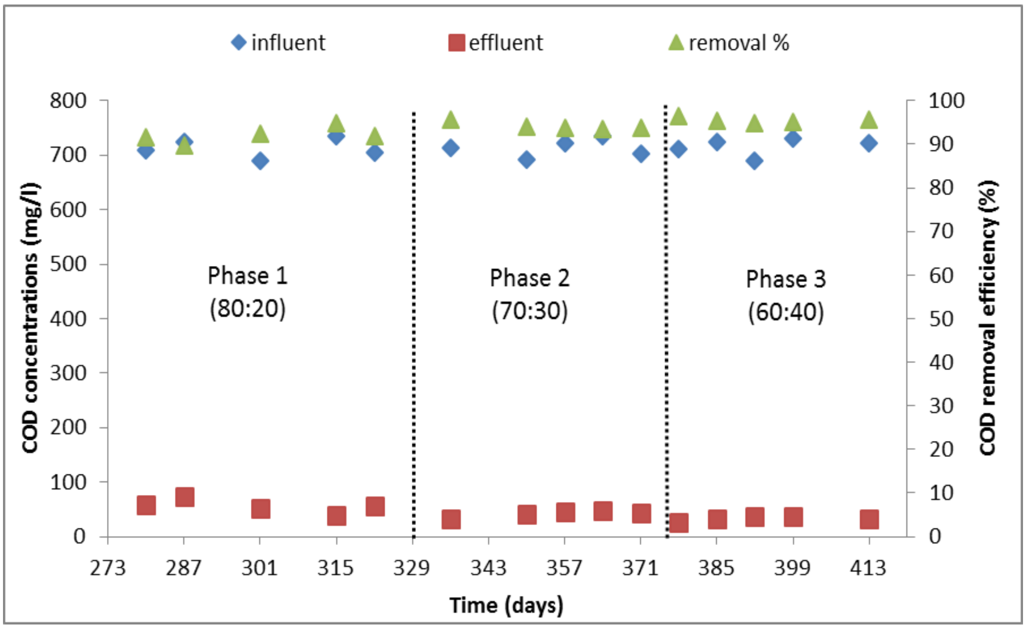


Figure 5.5 COD performance removal during the study period

5.2.2.4 Phosphorus removal

As was mentioned before, the system was not designed for phosphorus removal, and therefore, the removal efficiency performance was quite low. The overall performances for TP and PO_4^{3-}-P are shown in Figure 5.6. In the phase 1 period, the TP influent was 9.97-11.23 mg/l (average 10.77 ± 0.5 mg/l), and its effluents were 7.18-8.8 mg/l (average 7.89 ± 0.75 mg/l) with 26.7% removal efficiency, while the average PO_4^{3-}-P influent was 9.81 ± 0.15 mg/l and its effluents were 6.22-7.97 mg/l (average 6.91 ± 0.79 mg/l) with 29.6% removal efficiency. In the phase 2 period, the TP influent was 10.48-11.79 mg/l (average 11.13 ± 0.5 mg/l) and its effluents were 8.05-9.7 mg/l (average 8.95 ± 0.67 mg/l) with 19.6% removal efficiency, whereas PO_4^{3-}-P influent was 9.97-10.4 mg/l (average 10.23 ± 0.16 mg/l) and its effluents were 7.05-8.53 mg/l (average 7.98 ± 0.59 mg/l) with 22.1% removal efficiency. In the phase 3 period, the TP influent was 10.8-11.84 mg/l (average 11.29 ± 0.4 mg/l) and its effluents were 8.7-10.72 mg/l (average 9.99 ± 0.8 mg/l) with 11.6% removal efficiency, whereas the PO_4^{3-}-P influent was 10.3-11.23 mg/l (average 10.71 ± 0.35

mg/l) and its effluents were 7.74-9.79 mg/l (average 8.96 ± 0.76 mg/l) with 16.4% removal efficiency.

In conclusion, it was observed that the performance of phosphorus removal efficiency decreased when the step-feeding ratio increased in the system (third stage) due to the high phosphorus concentrations introduced into the system. Nevertheless, the gravel media as the main wetland media provides a poor level of phosphorus removal in CWs. In addition, with the step-feeding strategy, the removal efficiency for phosphorus was improved and enhanced compared to the previous study in Chapter 4. However, the removal efficiency of phosphorus was still low comparing with other studies; 78-92% (Jia et al., 2010), 75-94% (Zhao et al., 2011), 88% (Hu et al., 2014b), and 44-80% (Zapater-Pereyra et al., 2015).

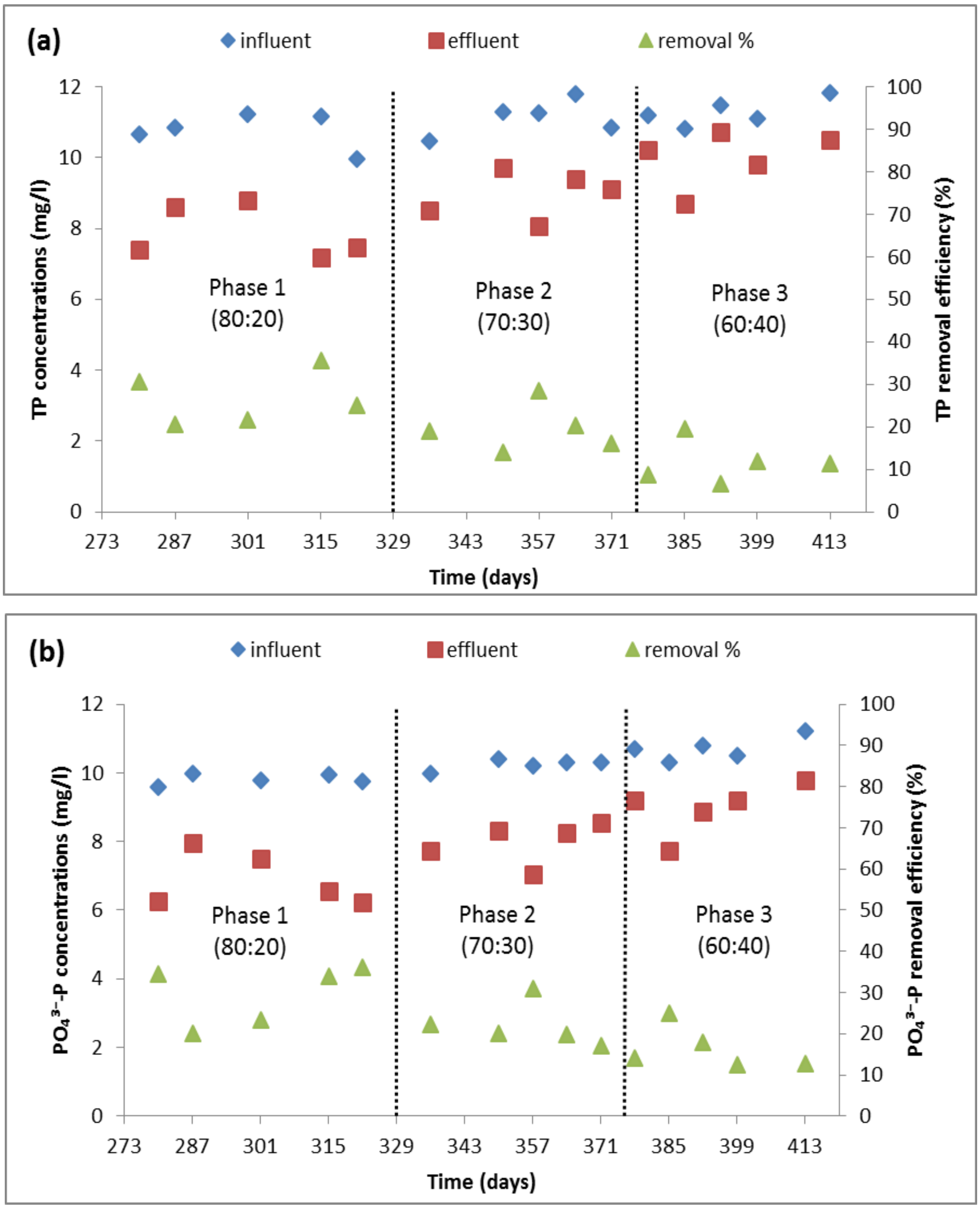


Figure 5.6 (a) TP and (b) PO_4^{3-}-P removal performance through the study period

5.2.3 Effect of step-feeding distribution ratios on COD and nutrients removal performance efficiency on individual stages

5.2.3.1 COD removal performance

The contribution of each individual stage to the overall removal percentage of COD during all phases is shown in Figure 5.7. The COD removal obtained during the experiment is mainly due to the enhanced oxygenation ability of the tidal flow (Zhi and Ji, 2014; Li et al., 2015a; Wu et al., 2015b), the intense microbial activities (Dušek et al., 2008), and the amount of carbon source that had been introduced into the third stage (step-feeding) (Hu et al., 2012).

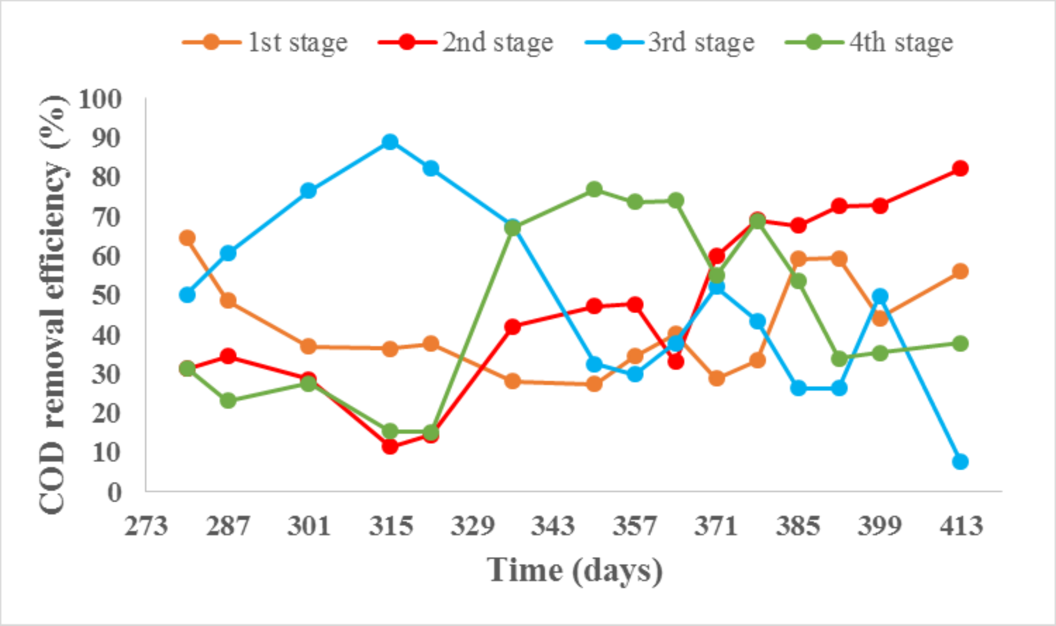


Figure 5.7 Contribution of individual stage to overall removal percentage of COD during the operation period

5.2.3.1.1 Phase 1

COD reduction mainly occurred within the first three stages, as shown in Figure 5.8; 89.7% of influent loading was removed in the first three stages for COD, which contributed to 97.4% (stage 1, 48.7%; stage 2, 13.7% and stage 3, 35%) of the total COD reduction. The contribution in the final stage (fourth stage) was very limited, with 2.6% compared to the other stages.

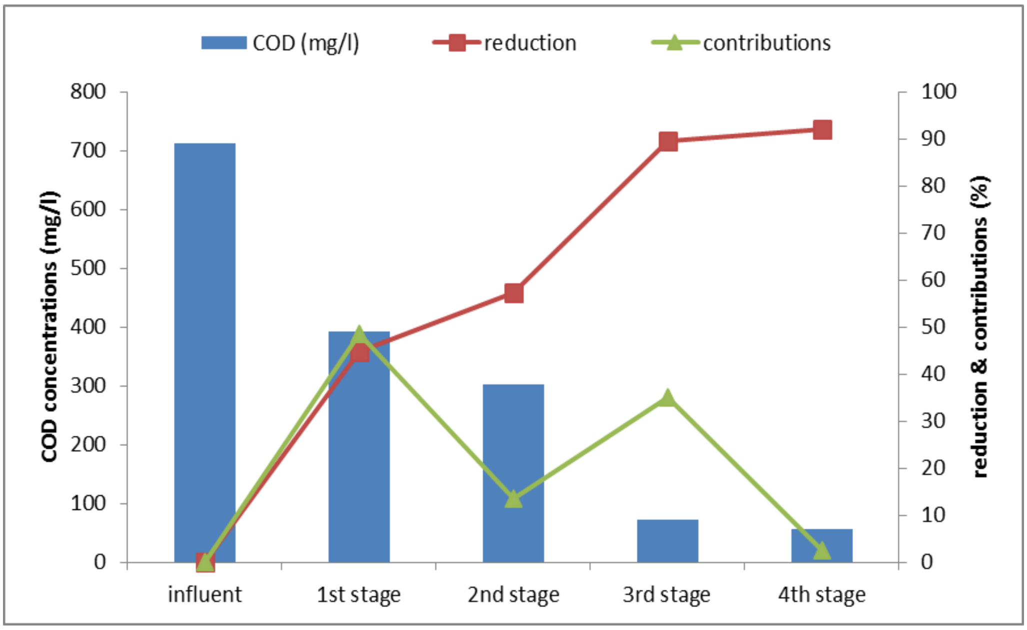


Figure 5.8 Typical COD profile in individual stages: COD concentration; reduction and contribution of individual stages to the total reduction in COD in the phase 1 period

5.2.3.1.2 Phase 2

In addition, the COD reduction mainly happened within the first three stages, as shown in Figure 5.9; 79.6% of influent loading was removed in the first three stages for COD, which contributed to 84.5% (stage 1, 33.8.2%; stage 2, 33.5% and stage 3, 17.2%) of the total COD reduction. The contribution in the fourth stage was 15.5%, which increased the percentage of the contribution compared to the phase 1 period.

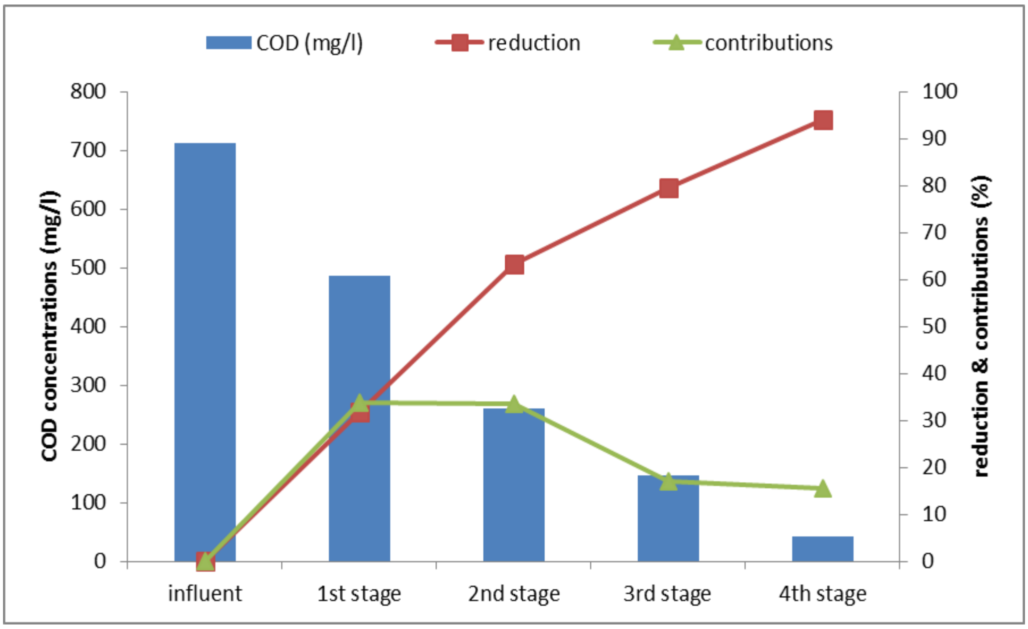


Figure 5.9 Typical COD profile in individual stages: COD concentration; reduction and contribution of individual stages to the total reduction in COD in the phase 2 period

5.2.3.1.3 Phase 3

In this period, the COD reduction mainly happened within the first two stages, as shown in Figure 5.10; 86.4% of influent loading was removed in the first two stages for COD, which contributed to 90.6% (stage 1, 52.8%; stage 2, 37.8%) of the total COD reduction. The contributions in the final two stages (third and fourth) were limited, with 4.9% and 4.5% respectively.

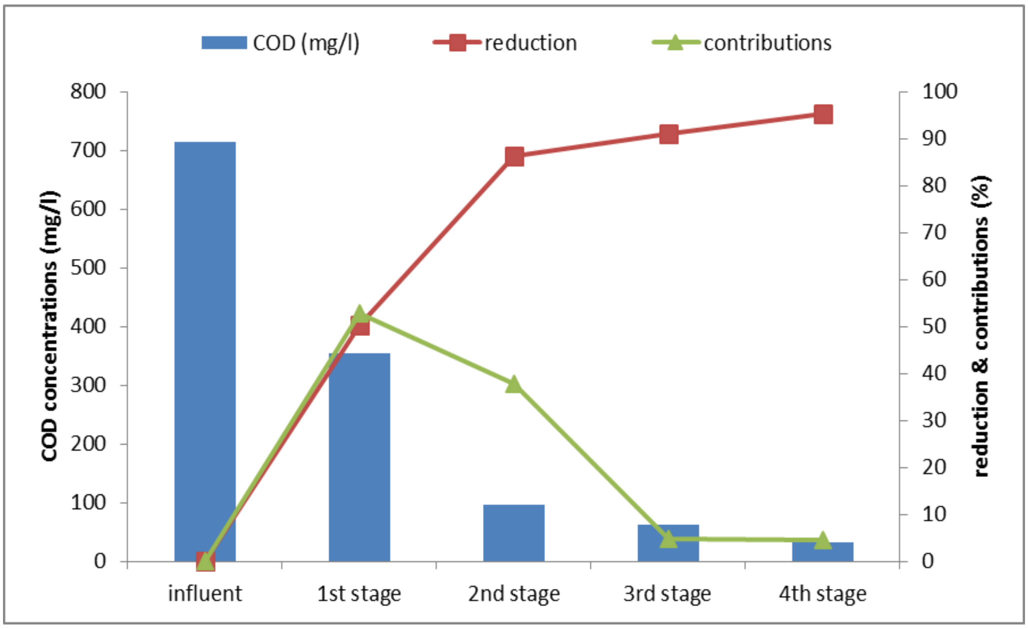


Figure 5.10 Typical COD profile in individual stages: COD concentration; reduction and contribution of individual stages to the total reduction in COD in the phase 3 period

Overall, in this study, as long as the step-feeding distribution ratios were increased, more COD removal could take place in the system effluents. The greater the amount of organic matter that was introduced to the system, the more COD removal occurred due to the bacterial activities in the CW system.

5.2.3.2 Nitrification and denitrification performance

The NH_4^+-N and TN of the synthetic wastewater were reduced in phases 1, 2 and 3 through the four stages of the system. Figure 5.11 illustrates the contribution of each individual stage to the overall removal percentage of NH_4^+-N during the whole operation period, and it shows a continuous reduction of NH_4^+-N through each stage due to the microbial activities. The removal of NH_4^+-N in the current study may result from a combination of numerous processes, which include nitrification, assimilation associated with the decomposition of organics, volatilisation, and plant uptake, and in addition, the amount of carbon source that is introduced to the system (third stage).

From Figure 5.12, the concentration levels of NO_2^--N and NO_3^--N in the effluents were slightly higher than in the influents in most of the cycles, which indicated that the nitrification process was taking place during the experiment due to the aerobic conditions (see section 5.2.4 mass balance). Note that the nitrification and denitrification processes may have occurred simultaneously in the system, and this resulted in a decrease in the NO_2^--N and NO_3^--N levels in the effluent, which contributed to the NH_4^+-N reduction.

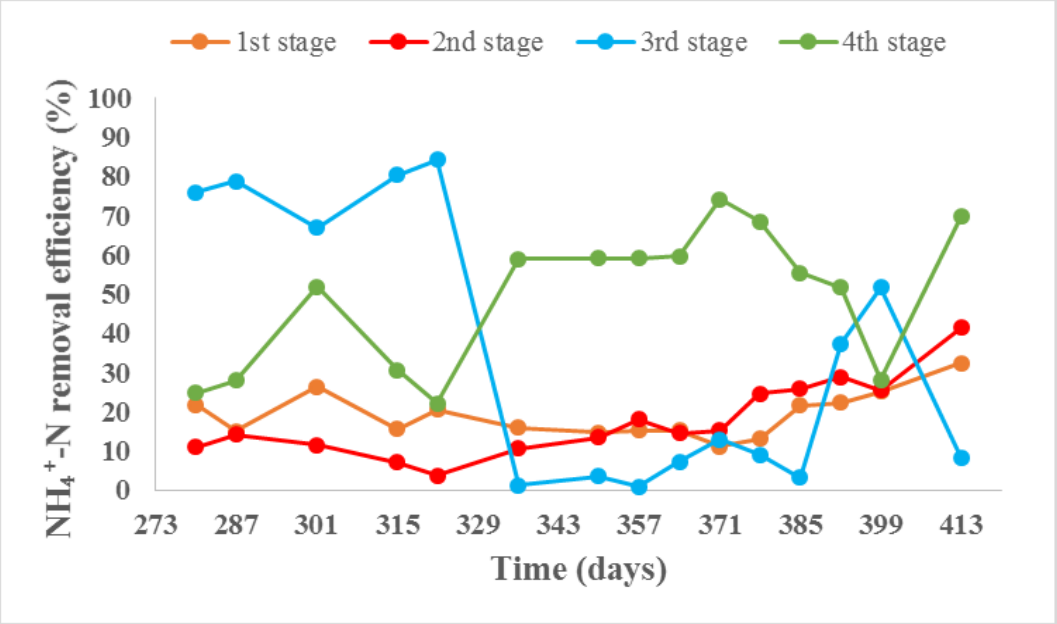


Figure 5.11 Contribution of individual stage to overall removal percentage of NH_4^+-N during the period of operation

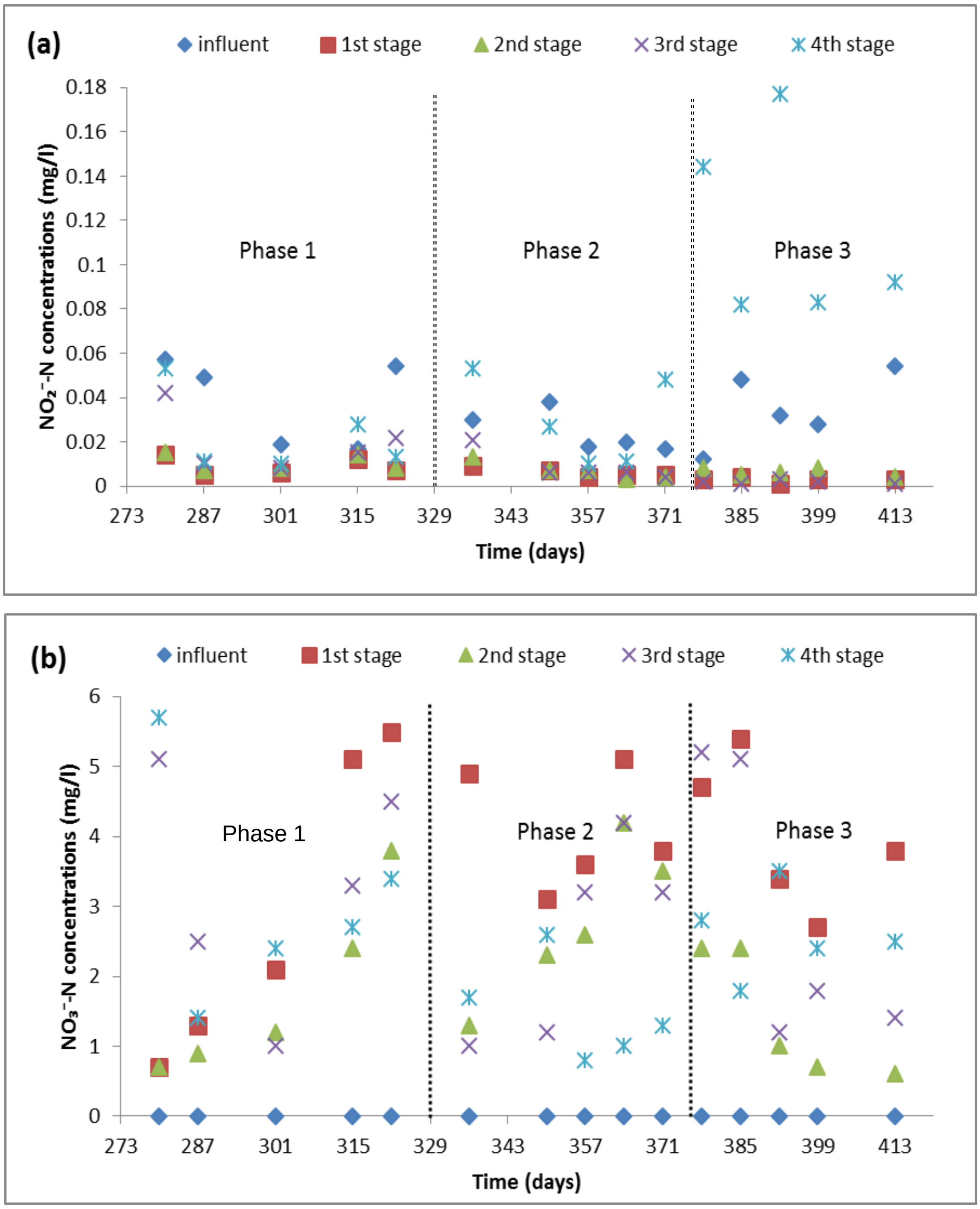


Figure 5.12 (a) NO_2^--N and (b) NO_3^--N conversion through the multistage VFCW system

The trend of NH_4^+-N reduction was similar to that of COD reduction (Figure 5.8, 5.9 and 5.10).

5.2.3.2.1 Phase 1

Overall, the influent NH_4^+-N was reduced by 89.2% from 58.28 mg/l to 6.31 mg/l. Of that, 83.8% of the influent was reduced in the first three stages, contributing 94%

(stage 1, 22.3%; stage 2, 8.6% and stage 3, 63.1%) to the total reduction, as shown in Figure 5.13. No further significant reduction happened in the final stage (fourth stage), which only contributed 6% to the total reduction. Nitrate accumulation started from stage 1, which indicates that the denitrification step became the limiting process for TN reduction due to the carbon deficiency. It is interesting to note that only limited nitrate accumulation happened in stages 3 and 4 despite the significant NH_4^+-N reduction, which means almost all the oxidized ammonia (the nitrified nitrogen) was denitrified within these stages, i.e., simultaneous nitrification and denitrification (SND). This is because the external carbon source was sufficient in stages 3 and 4 to support the denitrification step of the nitrified nitrogen.

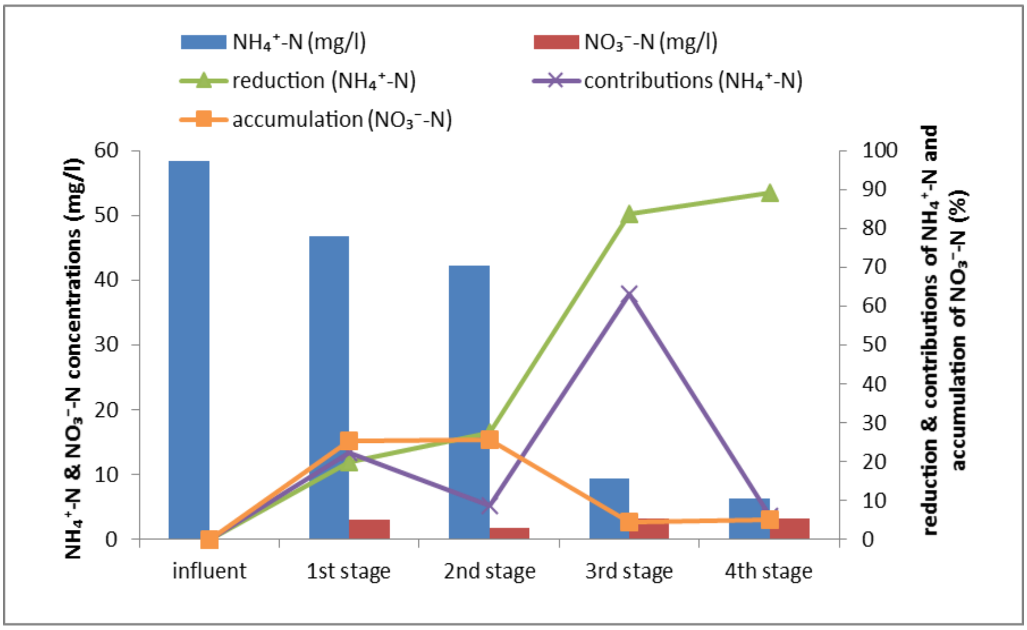


Figure 5.13 Typical nitrogen profile in individual stages: NH_4^+-N and NO_3^--N concentration; reduction of influent NH_4^+-N in each stage, contributions of individual stages to the total NH_4^+-N reduction and NO_3^--N accumulation in each stage in the phase 1 period

5.2.3.2.2 Phase 2

Overall, the influent NH_4^+-N was reduced by 73.8% from 58.16 mg/l to 15.26 mg/l. Of that, 30.8% of the influent was reduced in the first three stages, contributing 41.8%

(stage 1, 19.7%; stage 2, 16.8%; and stage 3, 5.3%) to the total reduction, as shown in Figure 5.14. Subsequently, in the fourth stage, there was a significant reduction in NH_4^+-N, which contributed 58.2% to the total reduction. In addition, nitrate accumulation started from stage 1, which indicates that the denitrification step became the limiting process for TN reduction due to the carbon deficiency. It is interesting to note that the percentage of nitrate accumulation decreased through the stages, which means the nitrified nitrogen (oxidized ammonia) was denitrified within these stages, i.e., there was SND. This is because the carbon source was sufficient in these stages to support the denitrification step of the nitrified nitrogen (see NO_2^--N and NO_3^--N concentrations in third and fourth stages in Table 5.1 compared to Table 4.2 in Chapter 4).

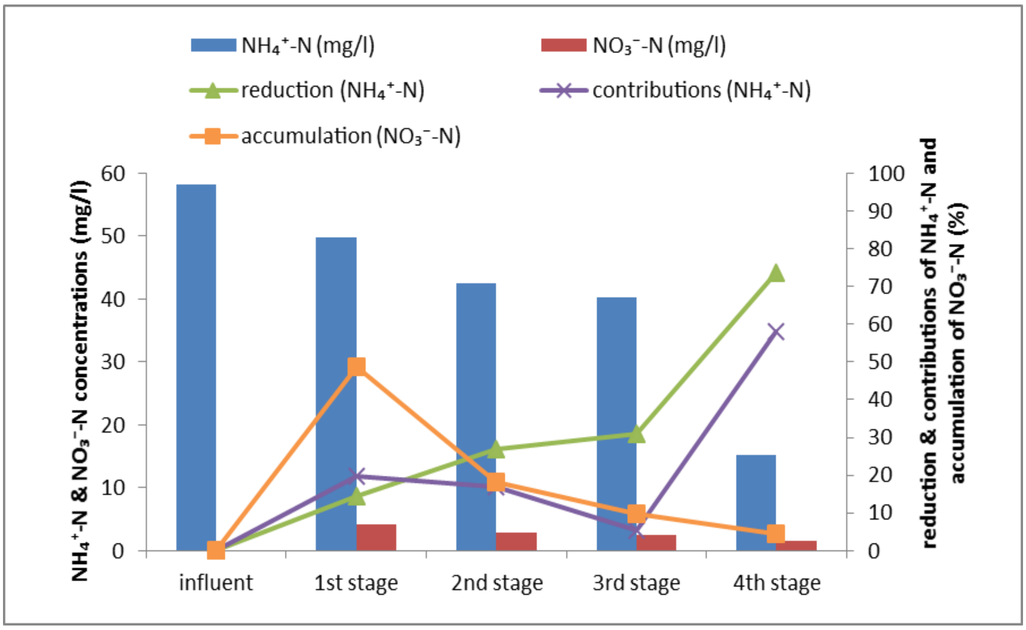


Figure 5.14 Typical nitrogen profile in individual stages: NH_4^+-N and NO_3^--N concentration; reduction of influent NH_4^+-N in each stage, contributions of individual stages to the total NH_4^+-N reduction and NO_3^--N accumulation in each stage in the phase 2 period

5.2.3.2.3 Phase 3

Overall, the influent NH_4^+-N was reduced by 81.9% from 58.12 mg/l to 10.55 mg/l. Of that, 57.3% of the influent was reduced in the first three stages, contributing 70% (stage 1, 28%; stage 2, 27.2%; and stage 3, 14.8%) to the total reduction, as shown in Figure 5.15. Subsequently, in the fourth stage, there was a significant reduction in NH_4^+-N, which contributed 30% to the total reduction. It is interesting to note that only limited nitrate accumulation happened in stage 4 despite the significant NH_4^+-N reduction, which means almost all the oxidized ammonia (the nitrified nitrogen) was denitrified within this stage, i.e., SND. This is because the external carbon source was sufficient in stage 4 to support the denitrification step of the nitrified nitrogen.

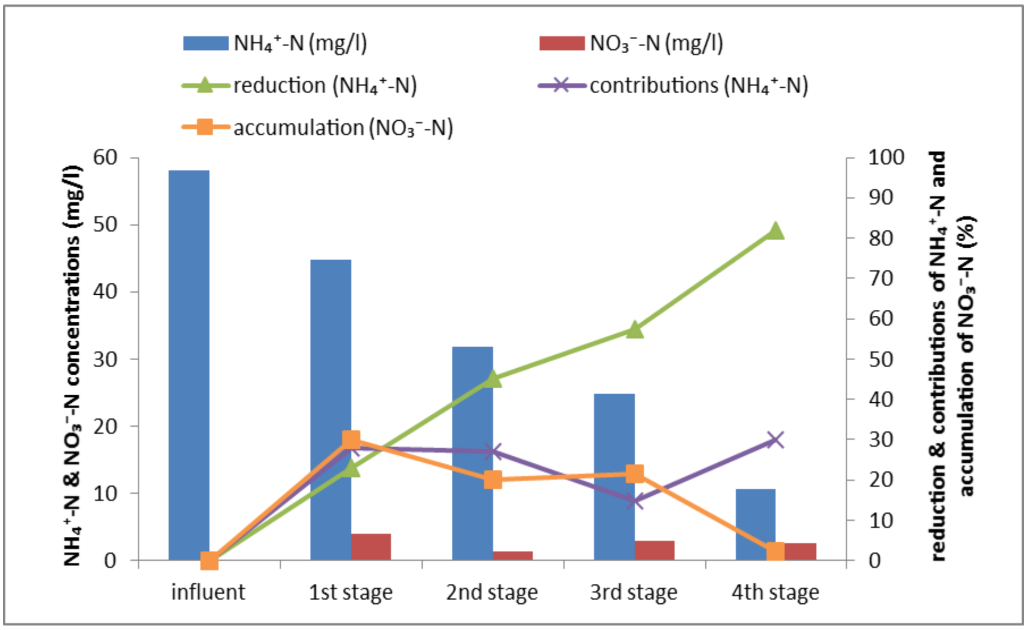


Figure 5.15 Typical nitrogen profile in individual stages: NH_4^+-N and NO_3^--N concentration; reduction of influent NH_4^+-N in each stage, contributions of individual stages to the total NH_4^+-N reduction and NO_3^--N accumulation in each stage in the phase 3 period

These results (phases 1, 2 and 3) actually revealed an inherent restriction of TFCWs in TN reduction. That is why all the stages of TFCWs are aerobic (2 hours saturated and 6 hours unsaturated) to a certain extent due to the enhanced oxygen transfer. In such conditions, organic matter (expressed as COD) will be directly oxidized by oxygen rather than utilized for denitrification. As a result, denitrification can easily become the limiting factor in TN elimination in TFCWs. The step-feeding played a role in the results of each phase for supporting the denitrification step by adding a percentage of a carbon source to the system.

In conclusion, the best NH_4^+-N removal was 89.2% in phase 1 due to sufficient carbon source having been introduced in the third stage. However, denitrification can still proceed by introducing a carbon source to the nitrified liquid (step-feeding strategy). This effect can be observed with the reduction of the nitrate accumulation, such as in the third and fourth stages for phases 1 and 2, and in the fourth stage for phase 3.

5.2.3.3 Phosphorus removal performance

As mentioned in section 5.2.2.4, the system was not designed for phosphorus removal; thus, TP and PO_4^{3-}-P removal efficiencies were observed to be quite low throughout the study period. Figure 5.16 shows the removal of both TP and PO_4^{3-}-P through the stages.

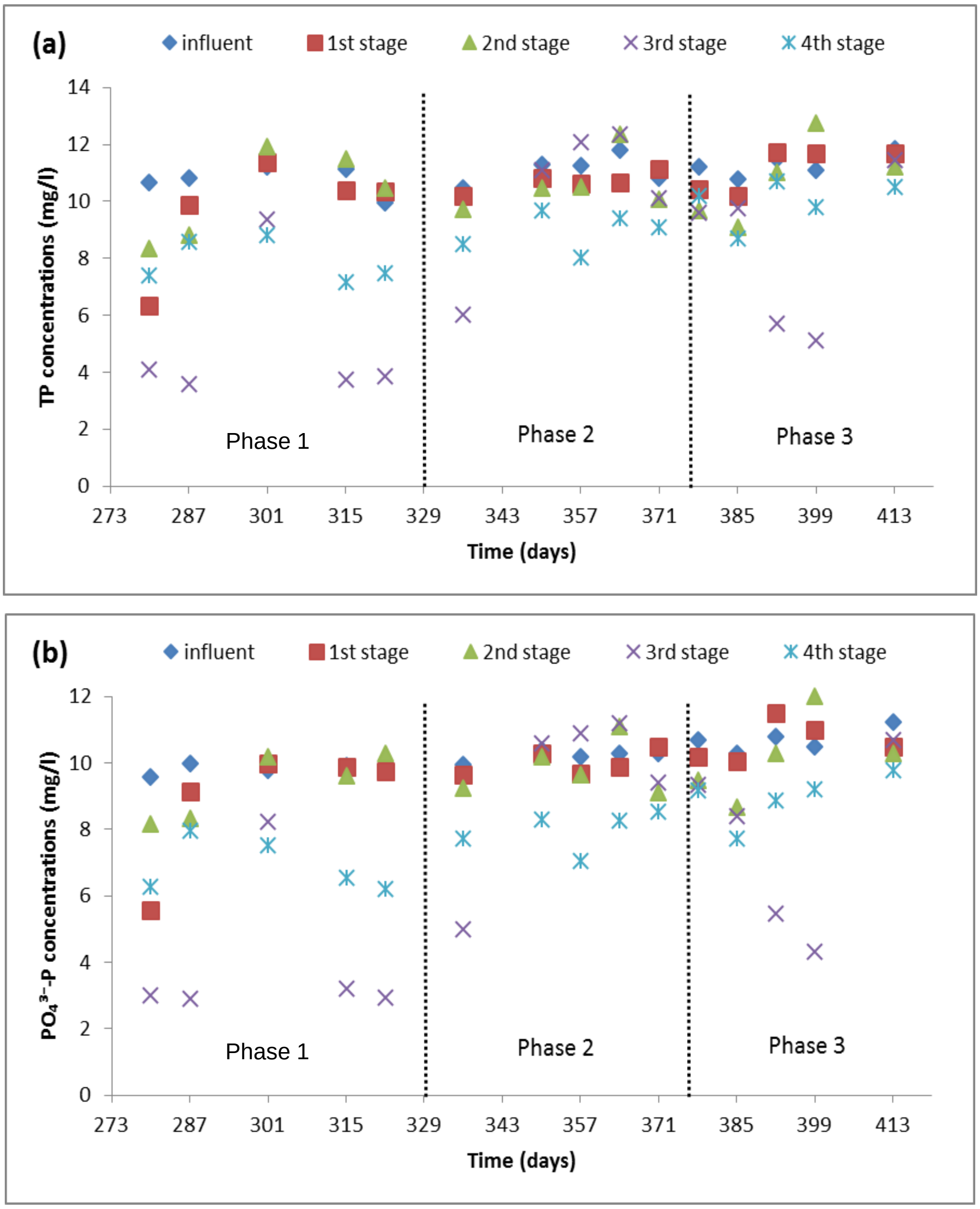


Figure 5.16 (a) TP and (b) PO_4^{3-}-P removal through multistage VFCW

Compared with NH_4^+-N and COD reduction, reduction of phosphorus in all phases 1, 2 and 3 was significantly less (see Figures 5.17, 5.18 and 5.19).

5.2.3.3.1 Phase 1

Overall, the influents of TP and PO_4^{3-}-P were reduced by 26.7% and 29.7% from 10.77 mg/l to 7.89 mg/l and from 9.81 mg/l to 6.91 mg/l respectively. TP and PO_4^{3-}-P reductions of the influent were slight in the first and second stages with 10.2% and

9.6%, and 5.2% and 4.9% respectively. However, the main reduction occurred in the third stage with 54.3% and 58.6% for TP and PO_4^{3-}-P respectively. In the fourth stage, the reduction efficiency was reduced to 26.7% and 29.6% for TP and PO_4^{3-}-P respectively, as shown in Figure 5.17.

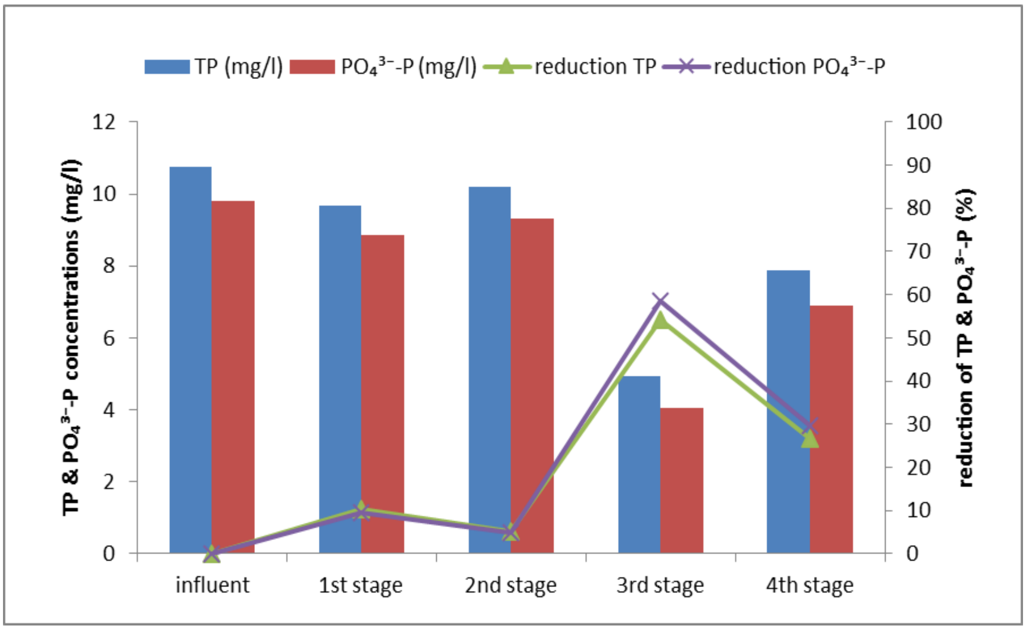


Figure 5.17 Typical phosphorus profile in individual stages: TP and PO_4^{3-}-P concentrations, reduction of the influents for both TP and PO_4^{3-}-P in each stage and the reduction of the individual stages for TP and PO_4^{3-}-P in phase 1

5.2.3.3.2 Phase 2

In this phase, the influents of TP and PO_4^{3-}-P were reduced by 19.6% and 22% from 11.13 mg/l to 8.95 mg/l and from 10.23 mg/l to 7.98 mg/, respectively. No significant reductions in the influent happened in the first three stages for TP and PO_4^{3-}-P, with 4% and 2.2%, 4.5% and 3.6%, and 7.1% and 7.9% for TP and PO_4^{3-}-P in the first, second and third stages respectively. The main reduction occurred in the fourth stage with 19.6% and 22% for TP and PO_4^{3-}-P respectively, as shown in Figure 5.18.

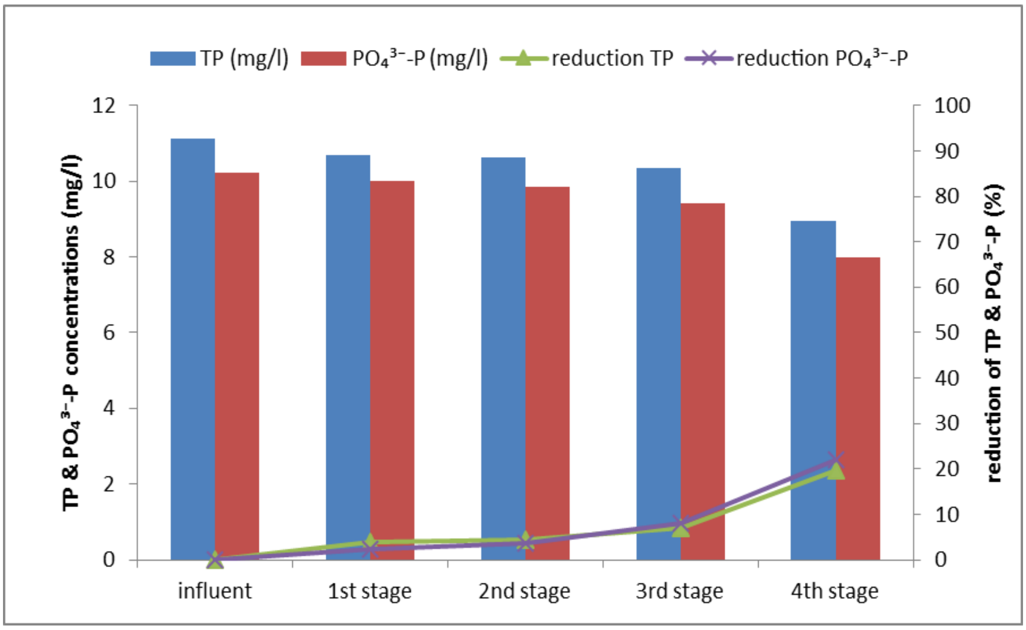


Figure 5.18 Typical phosphorus profile in individual stages: TP and PO_4^{3-}-P concentrations, reduction of the influent for both TP and PO_4^{3-}-P in each stage and the reduction of the individual stages for TP and PO_4^{3-}-P in phase 2

5.2.3.3.3 Phase 3

In this phase, the influents of TP and PO_4^{3-}-P were reduced by 11.5% and 16.3% from 11.29 mg/l to 9.99 mg/l and from 10.71 mg/l to 8.96 mg/l respectively. No significant reductions in the influent occurred in the first two stages for TP and PO_4^{3-}-P, with 1.2% and 0.6%, and 4.8% and 5.2 for TP and PO_4^{3-}-P in the first and second stages respectively. The main reduction occurred in the third stage with 26.1% and 28.6% for TP and PO_4^{3-}-P respectively. However, the effluents increased in the fourth stage; as shown in Figure 5.19, the reduction of TP and PO_4^{3-}-P were 11.5% and 16.3% respectively.

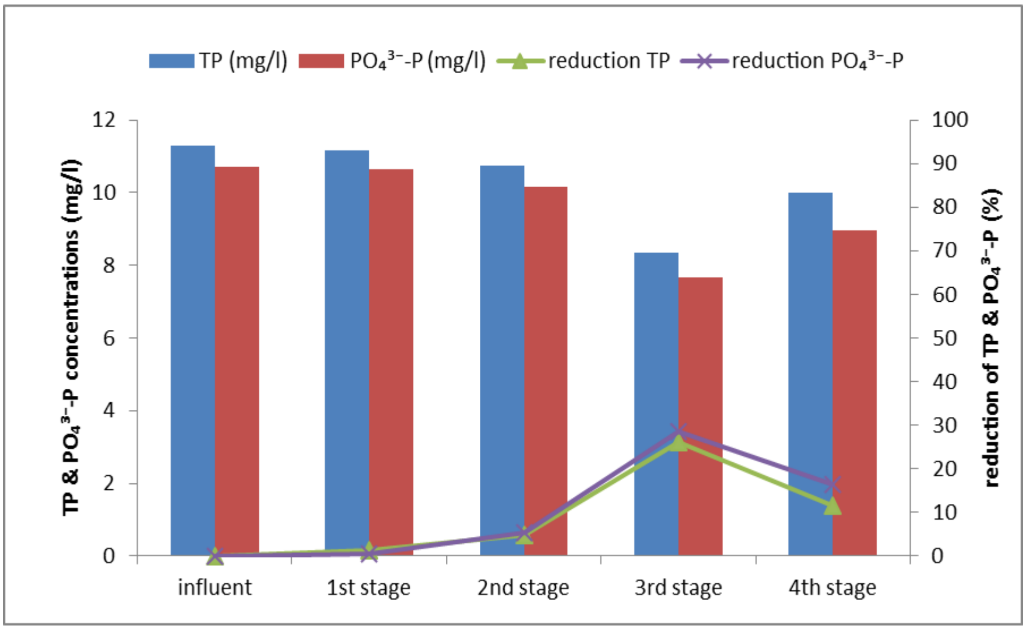


Figure 5.19 Typical phosphorus profile in individual stages: TP and PO_4^{3-}-P concentrations, reduction of the influent for both TP and PO_4^{3-}-P in each stage and the reduction of the individual stages for TP and PO_4^{3-}-P in phase 3

5.2.4 Mass balance

Mass balance was elucidated as discussed in Chapter 4 (section 4.2.4). Figure 5.20 and Table 5.2 shows the contributions of each stage to overall nitrogen removal with step-feeding strategy. The net mass flux of NH_4^+-N reduction (φNH_4^+-N, Re.) and NOx^--N (NO_2^--N + NO_3^--N) accumulation (φ NOx^--N, Acc.) in each stage is calculated according to Table 5.3. In addition, here, the calculation is based on the simplifying assumption that NH_4^+-N reduction is due to nitrification, thus the nitrogen loss by biomass assimilation and plant uptake is negligible (Stottmeister et al., 2003; Hu et al., 2012). The removal percentage of the influent NH_4^+-N in each stage is calculated as the φ NH_4^+-N, Re. of each stage divided by the influent NH_4^+-N loading as shown in Table 5.4.

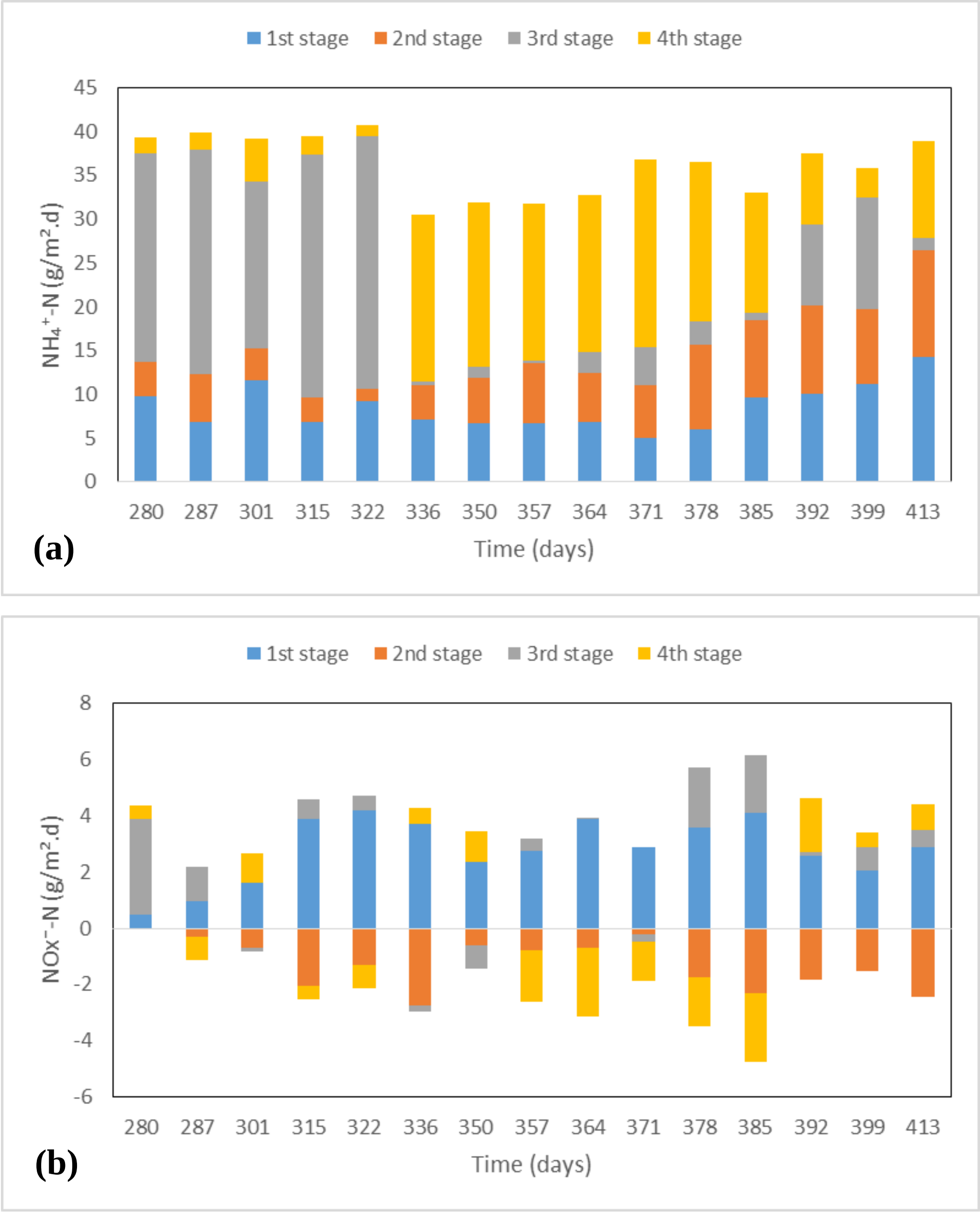


Figure 5.20 Mass balance for (a) NH_4^+-N and (b) NOx^--N accumulation in each stage

Figure 5.20 describes the contribution of individual stages to the overall NH_4^+-N and NOx^--N accumulation in each stage during the whole operation period. Step-feeding strategy was clearly affected the NOx^--N accumulation in the system. The NOx^--N accumulation was negative in stage 2 and 4 in most of the time, which specified that not only the nitrified N in the current stage is fully denitrified, but also certain amount of NOx^--N from the previous stage was reduced.

Table 5.2 Mass balance for the multistage system with step-feeding strategy (g/ m².d)

	1st Stage		2nd Stage		3rd Stage		4th Stage	
Phase 1 (80:20)	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent
NH_4^+-N	44.52	8.85	8.85	3.42	3.42	25.04	25.04	2.39
NOx^--N	0.03	2.22	2.22	-0.87*	-0.87*	1.14	1.14	-0.12
Phase 2 (70:30)	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent
NH_4^+-N	44.43	6.45	6.45	5.52	5.52	1.59	1.59	19.22
NOx^--N	0.019	3.12	3.12	-1.01*	-1.01*	-0.17*	-0.17*	-0.81*
Phase 3 (60:40)	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent
NH_4^+-N	44.4	10.19	10.19	9.87	9.87	5.39	5.39	10.89
NOx^--N	0.027	3.03	3.03	-1.97*	-1.97*	1.16	1.16	-0.17*

*indicated that denitrification step took place

Table 5.3 Mass flux of NH_4^+-N reduction (φNH4, Re.) and NOx^--N accumulation (φNOx-N, Acc.) in each stage (mg/d)

	Stages	**Phase 1 (S1:S3 = 80:20)**	**Phase 2 (S1:S3 = 70:30)**	**Phase 3 (S1:S3 = 60:40)**
φNH₄, Re.	**1**	$Q \times 0.8 \times (NH_4,in - NH_4,S_1)$	$Q \times 0.7 \times (NH_4,in - NH_4,S_1)$	$Q \times 0.6 \times (NH_4,in - NH_4,S_1)$
	2	$Q \times 0.8 \times (NH_4,S_1 - NH_4,S_2)$	$Q \times 0.7 \times (NH_4,S_1 - NH_4,S_2)$	$Q \times 0.6 \times (NH_4,S_1 - NH_4,S_2)$
	3	$Q \times (0.8 \times NH_4,S_2 + 0.2 \times NH_4,in - NH_4,S_3)$	$Q \times (0.7 \times NH_4,S_2 + 0.3 \times NH_4,in - NH_4,S_3)$	$Q \times (0.6 \times NH_4,S_2 + 0.4 \times NH_4,in - NH_4,S_3)$
	4	$Q \times (NH_4,S_3 - NH_4,S_4)$	$Q \times (NH_4,S_3 - NH_4,S_4)$	$Q \times (NH_4,S_3 - NH_4,S_4)$
φNOx-N,	**1**	$Q \times 0.8 \times (NOx,S_1 - NOx,in)$	$Q \times 0.7 \times (NOx,S_1 - NOx,in)$	$Q \times 0.6 \times (NOx,S_1 - NOx,in)$
	2	$Q \times 0.8 \times (NOx,S_2 - NOx,S_1)$	$Q \times 0.7 \times (NOx,S_2 - NOx,S_1)$	$Q \times 0.6 \times (NOx,S_2 - NOx,S_1)$

Acc.	3	$Q \times (NOx,S_3 - 0.8 \times NOx,S_2 - 0.2 \times NOx,in)$	$Q \times (NOx,S_3 - 0.7 \times NOx,S_2 - 0.3 \times NOx,in)$	$Q \times (NOx,S_3 - 0.6 \times NOx,S_2 - 0.4 \times NOx,in)$
	4	$Q \times (NOx,S_4 - NOx,S_3)$	$Q \times (NOx,S_4 - NOx,S_3)$	$Q \times (NOx,S_4 - NOx,S_3)$

S1:S3 (stage 1: stage 3) = distribution ratio of the step-feeding

Q: flow rate, l/d; Q = 6, $NOx^- \text{-N} = NO_2^- \text{-N} + NO_3^- \text{-N}$

NH_4,in, NOx,in: influent NH_4^+-N and NOx^--N, mg/l

NH_4,Si, NOx,Si : effluent NH_4^+-N and NOx^--N of each stage, mg/l; i=1-4

Table 5.4 Removal percentage of the influent NH_4^+-N in each stage

stages	1	2	3	4
% removal of NH_4^+-N inf.	φ NH_4^+-N, $Re._1$ / (NH_4,in × Q)	φ NH_4^+-N, $Re._2$ / (NH_4,S_1 × Q)	φ NH_4^+-N, $Re._3$ / (NH_4,S_2 × Q)	φ NH_4^+-N, $Re._4$ / (NH_4,S_3 × Q)

Q: flow rate, l/d; Q = 6,

NH_4,in: influent NH_4^+-N

NH_4,Si: effluent NH_4^+-N of each stage, mg/l; i=1-3

5.2.4.1.1 Phase 1

Table 5.5 shows the mass balance for the phase 1 period according to the simplified calculations in Table 5.3, whereas Table 5.6 shows the removal percentage of the influent NH_4^+-N in each stage according to the calculations in Table 5.4.

Table 5.5 Mass balance for phase 1 (mg/d)

Stages	1	2	3	4
φNH_4^+-N, Re.	55.58	21.5	215.95	18.78
φNOx-N, Acc.	13.97	-5.48	11.07	-0.94

Table 5.6 Removal percentage of the influent NH_4^+-N in each stage for phase 1

stages	**1**	**2**	**3**	**4**
% removal of NH_4^+-N inf.	15.9	7.7	85.2	33.2

From Table 5.5, it can be concluded that the net mass flux of NH_4^+-N reduction for the first, second, third and fourth stages is equal to 55.58, 21.5, 215.95 and 18.78 mg/d respectively, whereas NOx^--N accumulation for the first, second, third and fourth stages is equal to 13.97, -5.48, 11.07 and -0.94 mg/d respectively. On the other hand, from Table 5.6, the removal percentages of the influent NH_4^+-N in each stage were 15.9%, 7.7%, 85.2% and 33.2% for the first, second, third and fourth stages respectively.

5.2.4.2 Phase 2

Table 5.7 shows the mass balance for the phase 2 period according to the simplified calculations in Table 5.3, whereas Table 5.8 shows the removal percentage of the influent NH_4^+-N in each stage according to the calculations in Table 5.4.

Table 5.7 Mass balance for phase 2 (mg/d)

Stages	**1**	**2**	**3**	**4**
φNH_4^+-N, Re.	35.45	30.32	41.87	149.76
φNOx-N, Acc.	17.14	-5.54	3.76	-6.35

Table 5.8 Removal percentage of the influent NH_4^+-N in each stage for phase 2

stages	**1**	**2**	**3**	**4**
% removal of NH_4^+-N inf.	10.2	10.2	16.4	62.1

From Table 5.7, it can be concluded that the net mass flux of NH_4^+-N reduction for the first, second, third and fourth stages is equal to 35.45, 30.32, 41.87 and 149.76 mg/d respectively, whereas NOx^--N accumulation for the first, second, third and fourth stages is equal to 17.14, -5.54, 3.76 and -6.35 mg/d respectively. However, from Table 5.8, the removal percentages of the influent NH_4^+-N in each stage were 10.2%, 10.2%, 16.4% and 62.1% for the first, second, third and fourth stages respectively.

5.2.4.3 Phase 3

Table 5.9 shows the mass balance for the phase 3 period according to the simplified calculations in Table 5.3, whereas Table 5.10 shows the removal percentage of the influent NH_4^+-N in each stage according to the calculations in Table 5.4.

Table 5.9 Mass balance for phase 3 (mg/d)

Stages	**1**	**2**	**3**	**4**
φNH_4^+-N, Re.	48.02	46.51	105.38	85.5
φNOx-N, Acc.	14.28	-9.28	12.6	-1.36

Table 5.10 Removal percentage of the influent NH_4^+-N in each stage for phase 3

stages	**1**	**2**	**3**	**4**
% removal of NH_4^+-N inf.	13.8	17.3	55.1	57.5

From Table 5.9, it can be concluded that the net mass flux of NH_4^+-N reduction for the first, second, third and fourth stages is equal to 48.02, 46.51, 105.38 and 85.5 mg/d respectively, whereas NOx^--N accumulation for the first, second, third and fourth stages is equal to 14.28, -9.28, 12.6 and -1.36 mg/d respectively. However, from Table

5.10, the removal percentages of the influent NH_4^+-N in each stage were 13.8%, 17.3%, 55.1% and 57.5% for the first, second, third and fourth stages respectively.

For all phases 1, 2 and 3: from Tables 5.1, 5.2, 5.5, 5.7 and 5.9, it can be concluded that in the first stage, the nitrification process took place under an aerobic process; as a result, NH_4^+-N was converted to a small amount of NO_2^--N, and this increased the amount of NO_3^--N in the effluent. The effluent from the second stage, NO_2^--N increased slightly, whereas NO_3^--N decreased, which indicates that nitrification and denitrification happened simultaneously (the amount of NOx^--N removed via denitrification is greater than the amount of NOx^--N produced through the nitrification process, with -5.48, -5.54 and -9.28 mg/d for phase 1, 2 and 3 respectively). In the third stage, in which the step-feeding was introduced, NO_2^--N and NO_3^--N levels varied due to the ratio distribution in each phase and the amount of carbon source received; in phase 1, both NO_2^--N and NO_3^--N levels were increased indicating the nitrification process did not fully take place. However, in phase 2, NO_2^--N increased and NO_3^--N decreased indicating denitrification took place, while in phase 3, NO_2^--N decreased and NO_3^--N increased indicating nitrification took place. In the fourth stage, the nitrification and denitrification happened simultaneously (the amount of NOx^--N removed via denitrification is greater than the amount of NOx^--N produced through the nitrification process, with -0.94, -6.35 and -1.36 mg/d for phase 1, 2 and 3, respectively).

5.2.5 Statistical analysis

Table 5.11 shows the statistical analysis results for the comparison of the mean removal efficiencies between different step-feeding schemes. ANOVA revealed statistically significant differences between the three step-feeding schemes in the removal efficiencies for COD, NH_4^+-N, TN, and TP. Regarding COD, the mean removal efficiency with scheme 3 was significantly higher than with schemes 1 and 2 by 3.4% and 1.4% respectively (post hoc). The difference in COD removal might be because the inert parts of influent COD in schemes 1 and 2 were higher than in scheme 3, which could not be removed biologically (Hu et al., 2011). For TP, the mean removal efficiency with scheme 3 was significantly lower than with scheme 1 by 15.1%; however, the difference is statistically significant. The nitrification performance with scheme 2 was significantly lower than with scheme 1 by 15.4%. This may indicate that a long bed resting time (6 hrs) could improve and enhance the nitrification performance; the distribution ratio had significant impacts on nitrification performance as well. For TN, scheme 2 was significantly lower than 1 by 14.9%. This result detected that the step-feeding ratio has a major impact on nitrogen removal, which has been discussed in the previous sections.

Table 5.11 Statistical analysis results ($P<0.05$) for the comparison of mean removal efficiencies between different step-feeding schemes

	Scheme comparison		COD	NH_4^+-N	TN	TP
ANOVA	$1^{**}, 2^{***}, 3^{****}$	F	10.44^*	15.93^*	21.16^*	9.09^*
		Sig.	0.002	0.000	0.000	0.004
Post Hoc (Tamhane's T2)	1-2	Diff.	-2.01	15.42^*	14.92^*	7.13
		Sig.	0.184	0.007	0.000	0.259
	1-3	Diff.	-3.40^*	7.33	7.44	15.10^*
		Sig.	0.032	0.089	0.057	0.010
	2-3	Diff.	-1.38^*	-8.09	-7.48	7.97
		Sig.	0.050	0.116	0.065	0.123

*. The mean difference is significant at the 0.05 level.

**. Phase 1 (80:20).

***. Phase 2 (70:30).

****. Phase 3 (60:40).

5.2.6 Comparing the results of Chapter 5 with those of Chapter 4

In this chapter, the removal efficiencies were improved by applying the step-feeding strategy to the system (i.e. introducing external carbon to enhance the denitrification process). As for NH_4^+-N removal efficiency, the NH_4^+-N removal efficiency decreased between phase 1 (98.1%) and phase 2 (78.5%) in Chapter 4 due to temperature. By introducing the step-feeding strategy in Chapter 5, NH_4^+-N removal efficiency was improved (i.e. phase 1 (80:20) with 89.2%, phase 2 (70:30) with 73.8%, and phase 3 (60:40) with 81.9%), depending on the distribution ratio of the step-feeding, temperature, pH, DO, and C/N ratio. As for TN removal efficiency, the TN removal efficiency decreased from phase 1 (77.3%) to phase 2 (60.3%) in Chapter 4. In Chapter 5, the TN removal efficiency improved (i.e. phase 1 with 81.1%, phase 2 with 66.2%, and phase 3 with 73.6%), which is much better due to enhanced denitrification process as a result of introducing external carbon source to the system. Moreover, nitrogen removal rate in Chapter 5 was better than Chapter 4 (i.e. Chapter 5; phase 1 with 76.9 $g/m^3.d$, phase 2 with 62.7 $g/m^3.d$, and phase 3 with 69.7 $g/m^3.d$, whereas Chapter 4; phase 1 with 72.7 $g/m^3.d$, and phase 2 with 57.1 $g/m^3.d$). As for COD removal efficiency, the COD removal efficiency was dropped from phase 1 (97.3%) to phase 2 (87.5%) in Chapter 4. In Chapter 5, the COD removals efficiency were improved (i.e. phase 1 with 92.1%, phase 2 with 94.1%, and phase 3 with 95.4%), which is much better in the removal efficiency due to many influencing factors such as temperature, pH, DO, C/N ratio, etc. As for TP removal efficiency, the TP removal efficiency decreased from phase 1 (16.6%) to phase 2 (10.2%) in Chapter 4 as the system was not designed for phosphorus removal. In Chapter 5, TP removal efficiency improved depending on the distribution ratio of the step-feeding strategy (i.e. phase 1 with 26.7%, phase 2 with 19.6%, and phase 3 with 11.5%) which is much better removal efficiency.

5.3 Conclusion

This chapter has described the concept of using a step-feeding strategy in a CW system. It has also addressed the overall and the individual performance treatment efficiency in nutrients and organic matter (expressed as COD) removal from synthetic domestic wastewater. The results outlined within this chapter have improved the current scientific understanding of the step-feeding strategy. The key conclusions derived from the study are as follows:

- It was technically possible to use the step-feeding strategy to treat the synthetic domestic wastewater with high influent concentrations of nutrients and organic matter to improve and enhance the TN removal performance efficiency.
- Comparing the step-feeding performance at the three different distribution ratios (80:20, 70:30 and 60:40) for TN removal, 80:20 ratio (phase 1) was the optimal removal efficiency with 81.1%.
- Hydraulic loading rate (HLR) affected nitrogen removal processes.
- The optimal COD removal efficiency was obtained in the 60:40 distribution ratio (phase 3) with 95.4%, whereas the optimal TP and PO_4^{3-}-P removals efficiency was obtained in the 80:20 distribution ratio (phase 1) with 26.7% and 29.6% respectively.
- Sufficient bed resting time (6 hours) and introduce carbon source to the system (third stage) are revealed to be the key factors to preserve the efficient nitrification process and, support the denitrification process.
- Statistical analysis shows that the step-feeding ratio has a significant impact on organic matter and nutrients removals.

6 Investigation of Nitrogen Removal via CANON Process in a Single Stage Constructed Wetland System

6.1 Introduction

It is usually believed that a single stage constructed wetland (CW) system is incapable of achieving effective nitrogen removal because of its inability to provide alternative aerobic and anoxic conditions within the single stage (Vymazal et al., 2006; Vymazal, 2007; Kadlec and Wallace, 2008; Vymazal, 2010b). For instance, SFCWs, and HFSSCWs are limited in the nitrification step, whereas VFSSCWs are limited in the denitrification step (Vymazal, 2007). None of these types of CWs alone can deliver convenient environments for the two steps of the biological nitrogen removal process, that is, the nitrification and denitrification processes. Subsequently, hybrid or multistage systems are normally used and applied when TN removal is the main treatment goal (Cooper, 1999; Cooper et al., 1999; Vymazal, 2007; Seo et al., 2008).

Previous research studies into TFCWs systems have also revealed that a single stage TFCW is not able to achieve efficient TN removal (Green et al., 1997; Manios et al., 2002; Chan et al., 2008; Jia et al., 2010; Wu et al., 2011; Dong et al., 2012; Prost-Boucle and Molle, 2012; Hu et al., 2014b). In these research studies, the duration of contact and rest time or the size of the saturation and unsaturation zone is used to control the redox status in the bed. It was found that increasing the rest time (unsaturation zone) would improve and enhance the nitrification performance process, but would inhibit the denitrification process and vice versa. Therefore, a single stage TFCW is either a nitrification-limited system or a denitrification-limited system. The reason behind this problem is that in these studies, only one tide is generated within one fill and drain sequence thus leading to a limited supply of oxygen.

This chapter discusses how a single stage VFCW was adopted to follow the Completely Autotrophic Nitrogen Removal over Nitrite (CANON) process. As was illustrated in detail in Chapter 2 section 2.4.2.2., the CANON process requires a limited amount of oxygen for the process to take place; therefore, it requires a shorter unsaturated time and a longer saturation time in the bed, and that can be controlled by the number of tides and the duration of the saturated and unsaturated phases to weaken the oxygen supply in the system. Moreover, it is also expected that the influent inorganic carbon (IC) could be a substantial controlling factor for the CANON pathway, as IC is considered the carbon source for all the autotrophic microorganisms and may have an important influence on the nitrogen transformation pathway (Bagchi et al., 2010; Chen et al., 2012; Hu et al., 2014a; Ma et al., 2015).

Therefore, the overall aim of this chapter is to achieve a high rate of nitrogen removal through the CANON pathway in a single stage CW. To achieve this aim, the following key objectives were addressed:

- to investigate the effectiveness of the recirculation number (Rn) in creating a suitable environment for partial nitrification (PN) and Anammox for nitrogen removal to achieve the CANON process

- to investigate the effectiveness of different concentrations of IC on nitrogen removal in a single stage CW system to achieve the CANON process.

6.2 Results and discussion

The experiment was carried out for 11 months (from August 2015 to June 2016) including the start-up period. It was divided into two phases excluding the start-up period according to the Rn and IC concentrations, as shown in Table 3.2.

6.2.1 Redox potential (ORP) and dissolved oxygen (DO)

Over an 11-month period, real-time data were collected for monitoring and measuring purposes at every 10 minutes at two depths (see Figure 3.10). The first point, called the upper point, was at 20 cm beneath the surface of the distribution layer, whereas the second point, named the lower point, was at 50 cm below the surface of the distribution layer.

ORP and DO average data are shown in Figures 6.1 and 6.2 respectively. The figures show a proportional relationship between ORP and DO. This relationship illustrates that DO in both points (upper and lower) was less than 0.5 mg/l, which is considered as limited oxygen in the system because the unit was saturated almost continually. Therefore, ORP values were negative and remained negative at both points throughout the study period due to the microbial-mediated process. This has been shown to decrease the ORP level when the microbial processes are intense (Dušek et al., 2008). Negative ORP values also indicate that anoxic or anaerobic conditions were occurring as well.

Figure 6.1 presents the average ORP values at the upper and lower points in the CW system. ORP varied during the experimental period due to many factors that affect redox readings, such as temperature, depth of the probes inserted into the CW, concentrations of the synthetic wastewater, pH, DO, and microbial activities. Regarding Figure 6.2, which presents the average DO values at the upper and lower points in the CW system, the level of DO varied during the experimental period due to the consumption of the DO in the synthetic wastewater by the microbial community and the depth of the probes inserted into the CW. The maximum reading for DO was less than 0.5 mg/l, which is considered as a limited oxygen condition and as suitable for the PN. Consequently, the lower points have higher ORP and lower DO values than the upper points. One of the reasons for this is the closeness of the upper point to the surface, where oxygen can penetrate into the matrices bed of the CW for respiration. Therefore, the results of ORP and DO varied from one month to another because the system was driven by the physicochemical and biological factors (Szögi et al., 2004; Corbella et al., 2014).

In general, reducing aeration decreases the availability of the oxygen supply and consequently, decreases DO concentration. A corresponding decrease in ORP is expected as oxygen is a driving force of ORP (Wießner et al., 2005; Pennington and Walters, 2006; Białowiec et al., 2012). On the other hand, low DO and anoxic conditions reduce the nitrification rate; thus, the concentrations of nitrite and nitrate in the effluents were small, as shown in Tables 6.1 and 6.2. A small trace of nitrate production was detected during the operation of the experiment, which indicated that the activities of NOB were effectively suppressed. Therefore, NOB might be outcompeted during the competition for oxygen with AOB. It can also be said that an adequate oxygen supply induces the metabolic activity of AOB more than NOB. Moreover, Figure 6.3 demonstrated that tidal flow strategy was successfully applied during the operational period to achieve a limited oxygen supply that was suitable for PN in the system.

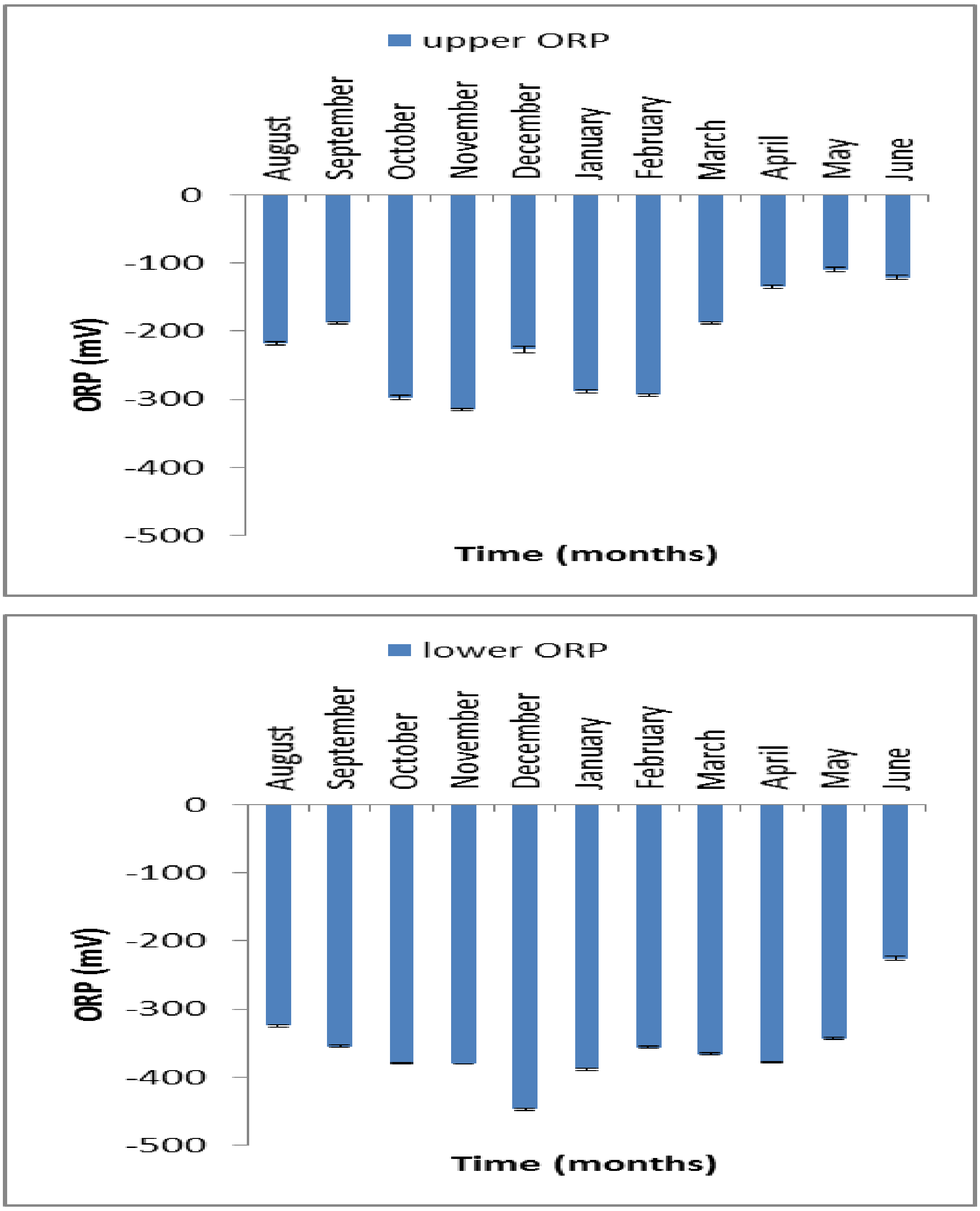


Figure 6.1 Average ORP values at (a) upper point and (b) lower point (error bars represent the standard error)

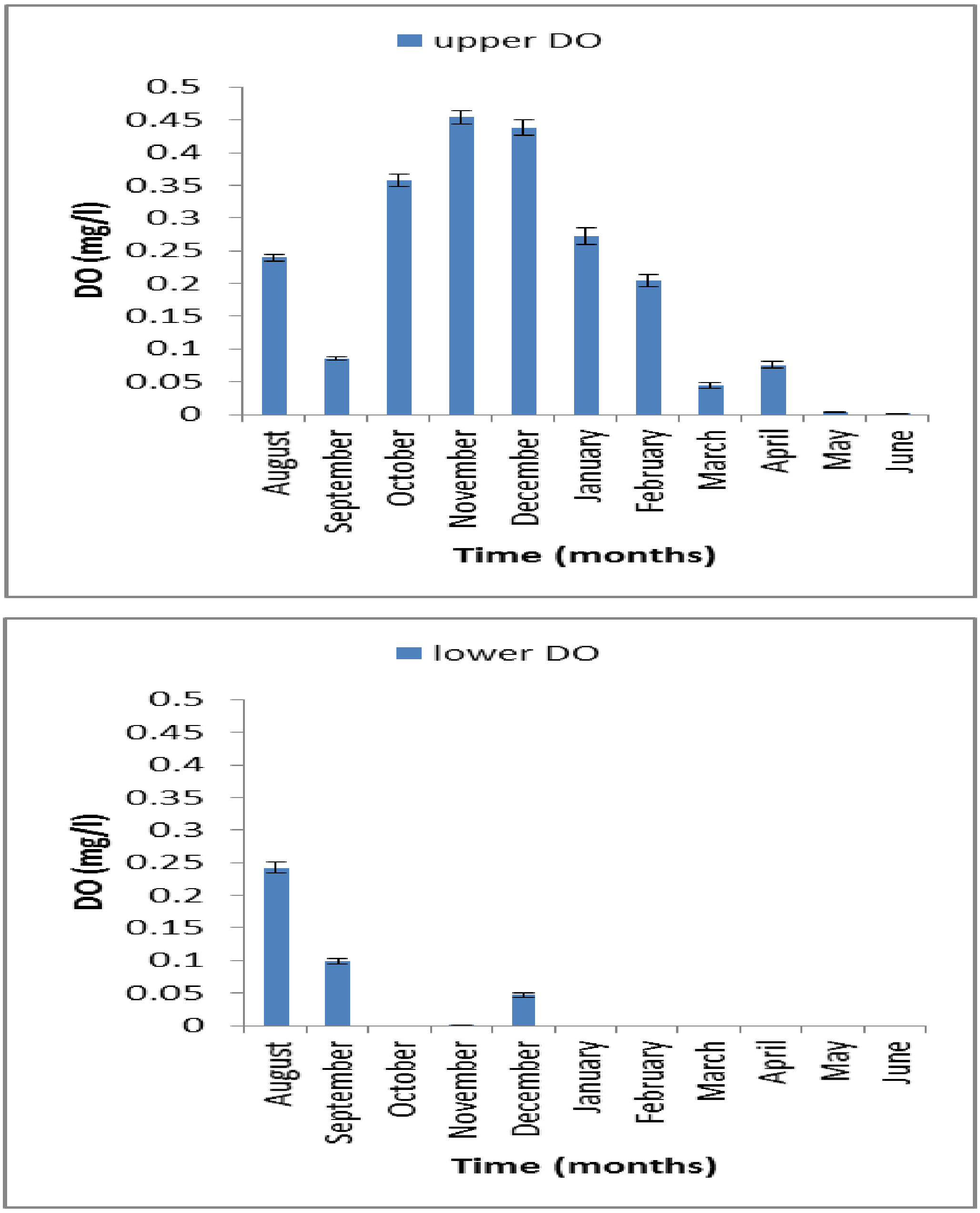


Figure 6.2 Average DO values at (a) upper point and (b) lower point (error bars represent the standard error)

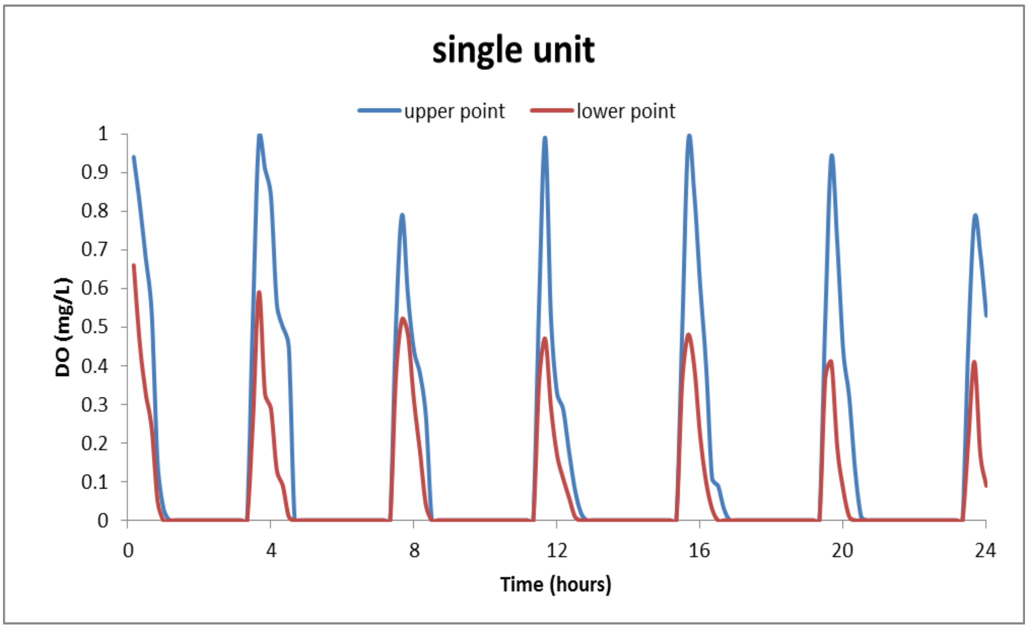


Figure 6.3 Tidal flow strategy during the filling and draining period

6.2.2 Overall treatment performance

For both operating strategies Rn and IC, the pH value used was approximately 8, which is the preferred pH for PN and Anammox processes, as a low pH value will prevent any further oxidation of ammonium, and in addition, the nitrite oxidizer will grow faster than the ammonium oxidizer; therefore it is preferable to have a pH of above 7 to inhibit NOB activities and to prompt AOB and Anammox bacteria activities and growth (Peng and Zhu, 2006; Yang et al., 2011; He et al., 2012; Li et al., 2013). Furthermore, when DO is less than 1 mg/l, the growth rate of AOB is 2.6 faster than NOB, as was observed in a similar study by (Tokutomi, 2004).

6.2.2.1 Effect of recirculation frequency (Rn) on nitrogen treatment performance

The effect of the recirculation frequency on overall treatment performance through the operational period is summarized in Table 6.1.

Table 6.1 Effect of the recirculation frequency on the overall treatment performance on a vertical single stage constructed wetland excluding the start-up period (between brackets represent standard deviation)

Phase 1 (Rn = 6 cycles/day)	**Influent**	**Effluent**	**Removal efficiency (%)**
pH	8.01 (±0.03)	7.63 (±0.05)	
Temperature*	18.21 (±0.81)	16.26 (±1.87)	
NH_4^+-N (mg/l)	14.69 (±0.11)	0.18 (±0.08)	**98.8**
NO_2^--N (mg/l)	0.003 (±0.001)	0.008 (±0.007)	
NO_3^--N (mg/l)	0	1.61 (±0.04)	
TN (mg/l)	16.79 (±0.15)	5.26 (±0.36)	**68.7**
Phase 2 (Rn = 4 cycles/day)	**Influent**	**Effluent**	**Removal efficiency (%)**
pH	8.03 (±0.03)	7.47 (±0.1)	
Temperature*	16.65 (±1.08)	12.29 (±3.16)	
NH_4^+-N (mg/l)	14.78 (±0.1)	1.25 (±0.32)	**91.5**
NO_2^--N (mg/l)	0.003 (±0.001)	0.011 (±0.007)	
NO_3^--N (mg/l)	0	1.71 (±0.08)	
TN (mg/l)	16.94 (±0.18)	4.37 (±0.73)	**74.2**
Phase 3 (Rn = 3 cycles/day)	**Influent**	**Effluent**	**Removal efficiency (%)**
pH	8.02 (±0.03)	7.41 (±0.08)	
Temperature*	15.1 (±1.23)	12.36 (±1.75)	
NH_4^+-N (mg/l)	14.81 (±0.07)	1.97 (±0.51)	**86.7**
NO_2^--N (mg/l)	0.003 (±0.001)	0.007 (±0.004)	
NO_3^--N (mg/l)	0	1.81 (±0.1)	
TN (mg/l)	16.96 (±0.16)	4.28 (±0.53)	**74.8**

Temperature*: Temperature of the sample

It is well-known that the NH_4^+-N removal rate increases with an increase in the recirculation number (Rn) and vice versa; this due to the aeration for the NH_4^+-N conversion, as the Rn acts as an oxygen supply for the system (Hu et al., 2014a). The influence of the recirculation frequency on the treatment performances of NH_4^+-N and TN removal is shown in Figures 6.4 and 6.5 respectively.

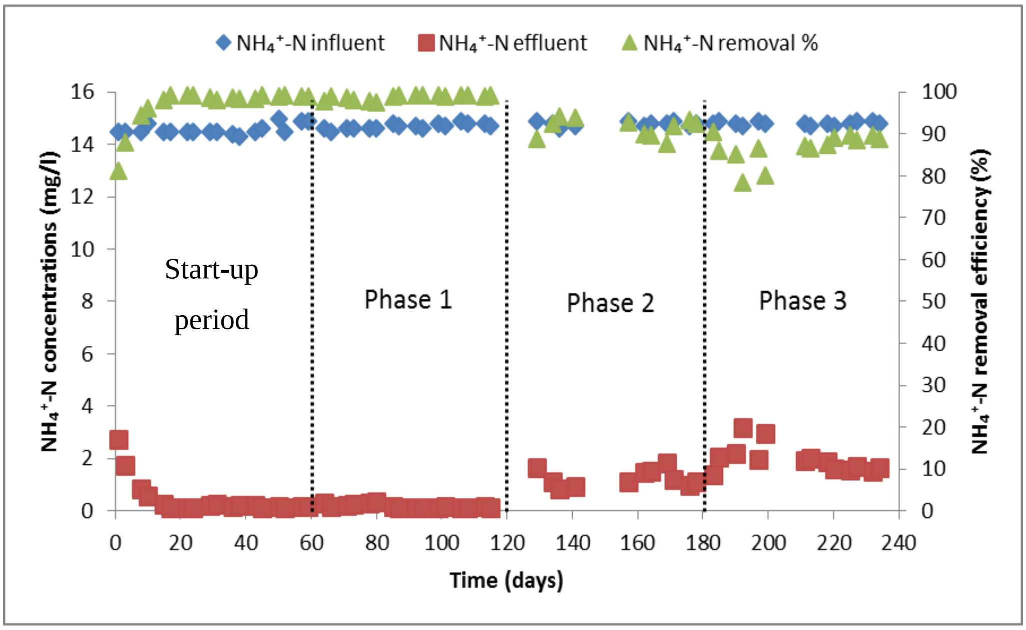


Figure 6.4 Treatment performances of NH_4^+-N removal under different recirculation frequency

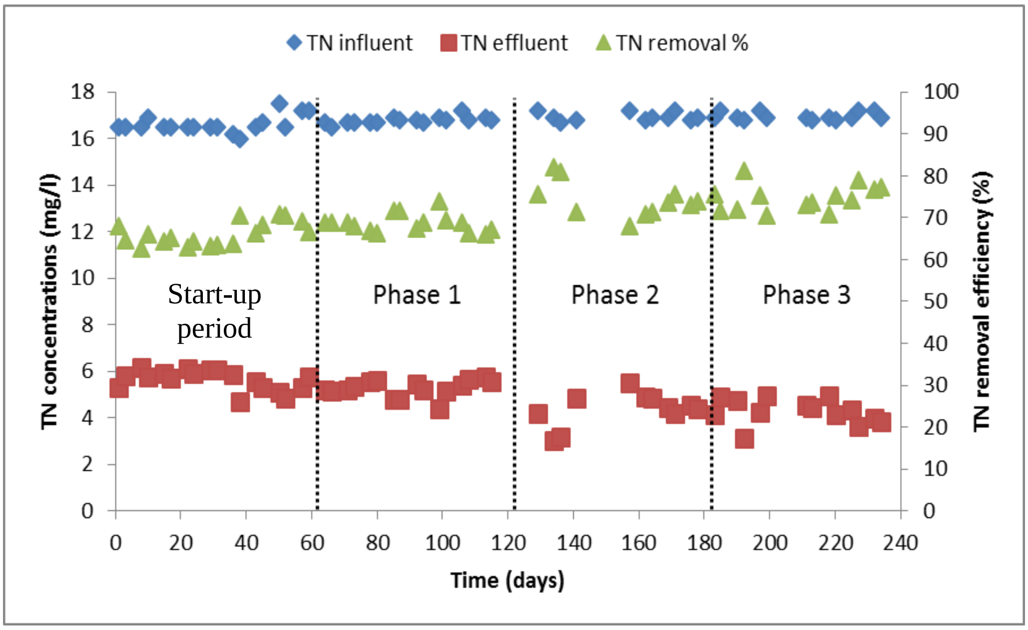


Figure 6.5 TN removal performances through the experimental period under different recirculation frequency

In the phase 1 period, NH_4^+-N and TN removal efficiency achieved 98.8% and 68.7% respectively when Rn = 6 times/day. When this number was reduced to 4 times/day, NH_4^+-N removal efficiency decreased to 91.5% in the phase 2 period, whereas TN removal efficiency increased to 74.2% due to the deficiency of the oxygen supply. In phase 3 period, NH_4^+-N removal efficiency was further reduced to 86.7% when the Rn decreased to 3 times/day, while TN removal efficiency increased slightly than phase 2 by 74.8%. Figure 6.6 illustrates the followed hypothesis (NAR values, section 3.3.3) to achieve the CANON route through the experimental period under different recirculation frequencies. However, the nitrogen removal rate for phase 1, 2 and 3 were 5.9 gN/m^3.d, 6.4 gN/m^3.d and 6.5 gN/m^3.d respectively. This study was higher in the nitrogen removal rate than other studies using single stage; 2.5 gN/m^3.d was achieved by Jia et al. (2010), and 2 gN/m^3.d achieved by Bahlo (2000). Also, similar results were achieved in other studies that used two stages TFCW; 5.3 gN/m^3.d was obtained by Korkusuz et al. (2004), and 6.2 gN/m^3.d by Langergraber et al. (2007). Generally, the removal rate varied between studies, mainly due to the HLR, HRT, and other conditions (i.e. temperature, DO, concentrations of the contaminants, etc.).

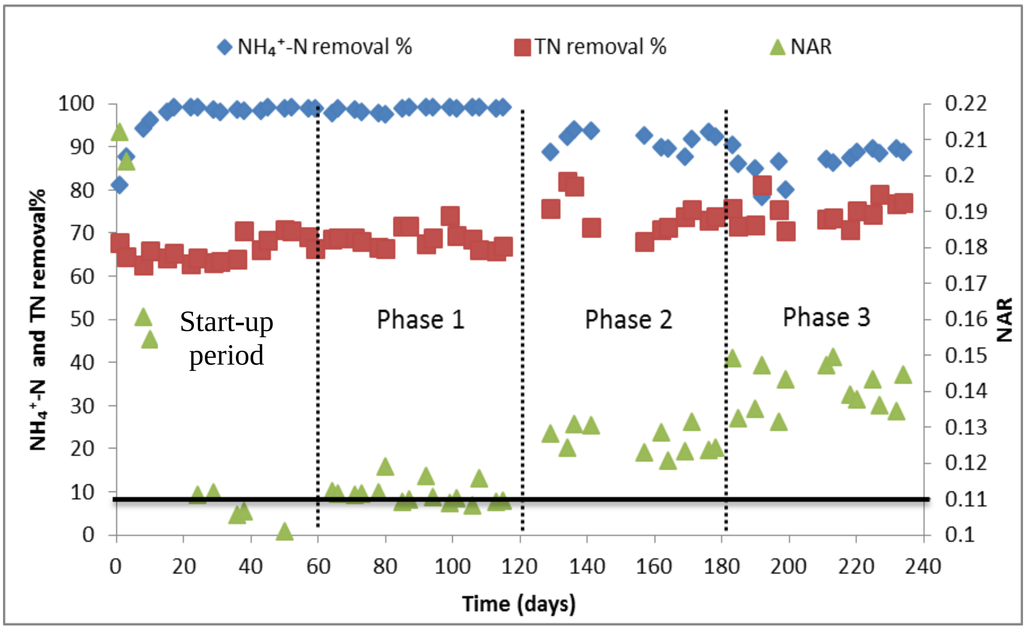


Figure 6.6 CANON route through the phases under different recirculation frequencies

Recirculation frequency affected the NAR values in the system. In the phase 1 period, the NAR values were almost steady around 0.11, which means that the system was following the CANON route according to the hypothesis in equation 3.1. Nonetheless, in both phases 2 and 3 periods, the NAR values increased with decreasing recirculation frequency. This implies that there was a failure to achieve the CANON route in the CW system in these phases.

Overall, Rn plays a role in the oxygen supply in the system, and Rn could be manipulated to control the oxygen supply in the TFCW. However, noticeable differences can be observed in performance across the three phases. The results indicate the effectiveness of the recirculation technique, and this is also in agreement with similar published work by other researchers (Prost-Boucle and Molle, 2012; Wen et al., 2013; Hu et al., 2014a).

6.2.2.2 Effect of inorganic carbon (IC) in achieving CANON process route

The effect of the IC on overall treatment performance through the operational period is summarized in Table 6.2.

Table 6.2 Effect of inorganic carbon on the overall treatment performance on a vertical single stage constructed wetland excluding the start-up period and Rn = 6 times/day (between brackets represent standard deviation)

Phase 1 (IC = 100 mg/l)	**Influent**	**Effluent**	**Removal efficiency (%)**
pH	8.03 (±0.03)	7.47 (±0.11)	
Temperature*	16.66 (±2.08)	13.88 (±1.77)	
NH_4^+-N (mg/l)	14.83 (±0.07)	0.11 (±0.04)	**99.3**
NO_2^--N (mg/l)	0.003 (±0.001)	0.007 (±0.003)	
NO_3^--N (mg/l)	0	1.63 (±0.05)	
TN (mg/l)	17 (±0.17)	6.1 (±0.63)	**64.1**
Phase 2 (IC = 200 mg/l)	**Influent**	**Effluent**	**Removal efficiency (%)**
pH	8.04 (±0.03)	7.49 (±0.06)	
Temperature*	17.61 (±1)	16.15 (±1.05)	
NH_4^+-N (mg/l)	14.81 (±0.08)	0.15 (±0.04)	**99**

NO_2^--N (mg/l)	0.003 (±0.001)	0.005 (±0.001)	
NO_3^--N (mg/l)	0	1.61 (±0.04)	
TN (mg/l)	16.99 (±0.18)	5.79 (±0.39)	**65.9**
Phase 3 (IC = 300 mg/l)	**Influent**	**Effluent**	**Removal efficiency (%)**
pH	8.02 (±0.03)	7.69 (±0.04)	
Temperature*	19.15 (±0.56)	19.13 (±1.68)	
NH_4^+-N (mg/l)	14.8 (±0.08)	0.19 (±0.04)	**98.7**
NO_2^--N (mg/l)	0.002 (±0.001)	0.004 (±0.002)	
NO_3^--N (mg/l)	0	1.61 (±0.01)	
TN (mg/l)	16.94 (±0.17)	4.92 (±0.13)	**71**

Temperature*: Temperature of the sample

The results showed that the effectiveness of high influent of IC could facilitate the CANON process route to be achieved in the system. The treatment performances for NH_4^+-N and TN removal efficiency under different IC concentrations are illustrated, in Figures 6.7 and 6.8 respectively with Rn = 6 times/day.

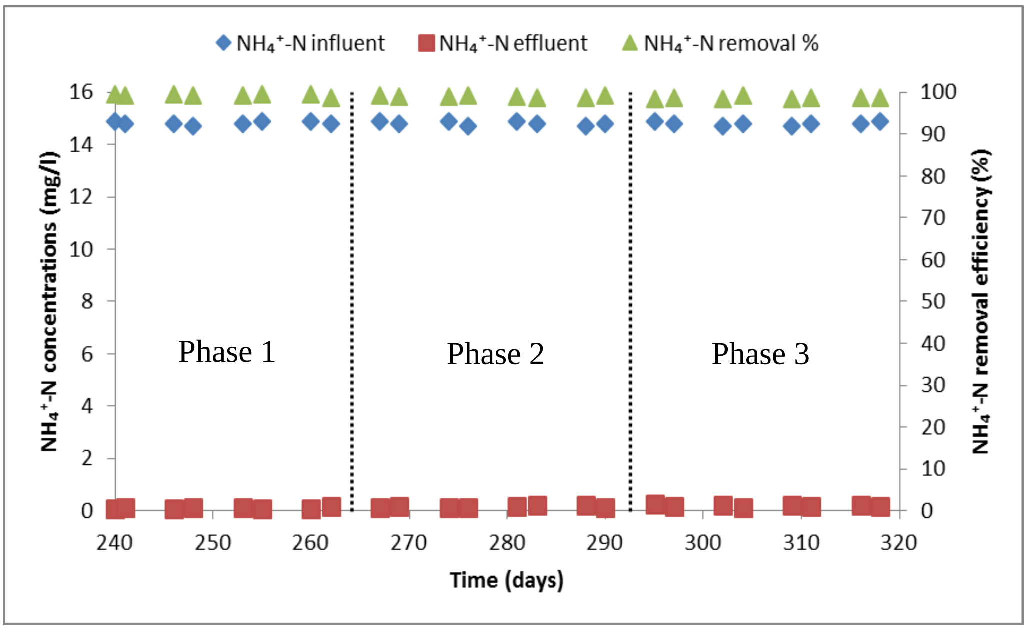


Figure 6.7 Treatment performances of NH_4^+-N removal under different inorganic carbon source concentrations

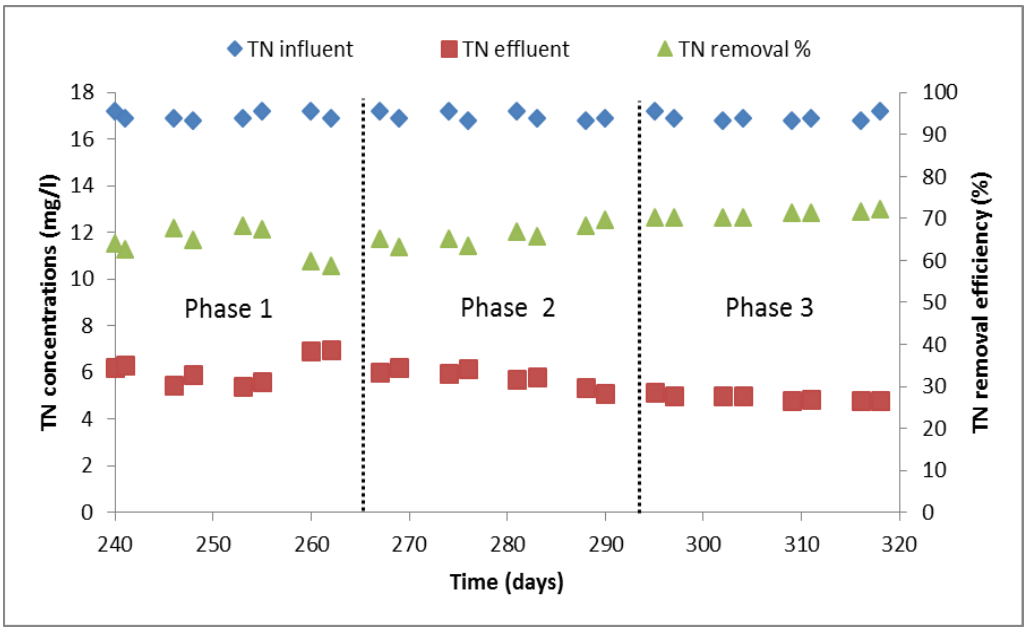


Figure 6.8 TN removal performances through the experimental period under different inorganic carbon source concentrations

In the phase 1 period, IC = 100 mg/l; NH_4^+-N removal efficiency was backed again to produce an increase to achieve 99.3% when Rn = 6 times/day and TN removal efficiency decreased to 64.1% (because in the previous phase, Rn = 3 times/ day, the last phase in the Rn strategy; NH_4^+-N and TN removal efficiencies were 86.7% and 74.8%, respectively). As expected, NH_4^+-N removal efficiency increased because of the enhanced oxygen supply to the system. In phase 2 period, IC was increased to 200 mg/l; the NH_4^+-N removal efficiency decreased slightly compared to phase 1 from 99.3% to 99%. In addition, TN increased slightly in the removal efficiency from 64.1% to 65.9%. In phase 3 period, IC was increased to 300 mg/l; the NH_4^+-N removal efficiency decreased slightly compared to phase 2 from 99% to 98.7%. Moreover, TN was improved by increasing the removal efficiency from 65.9% to 71%. Overall, IC has a negligible effect on NH_4^+-N removal in the system. However, the nitrogen removal rate for phase 1, 2 and 3 were 5.6 $gN/m^3.d$, 5.7 $gN/m^3.d$ and 6.1 $gN/m^3.d$ respectively. The nitrogen removal rates in this phase were lower than previous phase, which indicate that IC has a limited impact on TN removal in the system. Nevertheless, this study achieved higher nitrogen removal rate than other studies (Bahlo, 2000; Korkusuz et al., 2004; Jia et al., 2010). Figure 6.9 illustrates the followed hypothesis (NAR values) to achieve the CANON route through the experimental period under different IC concentrations.

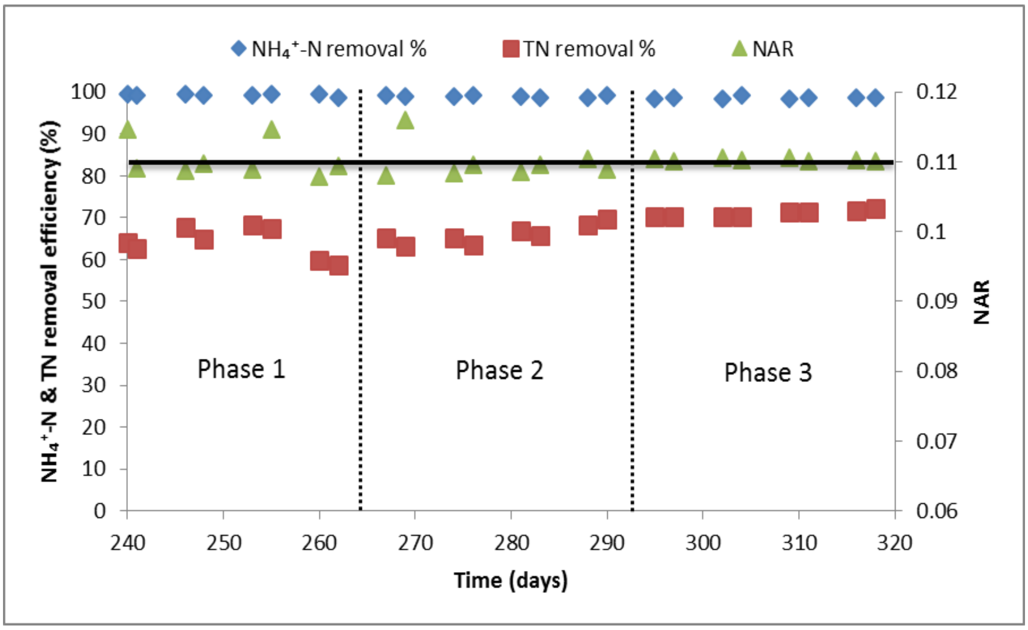


Figure 6.9 CANON route through the phases under different inorganic carbon concentrations

Inorganic carbon affected NAR values in the system, as illustrated in Figure 6.9. Almost all phases followed the CANON route according to Figure 6.9. In phase 1 period, NAR values were around 0.11, but TN removal efficiency fluctuated from 58.6% to 68.2%. In phase 2 period, NAR values almost around 0.11, and TN removal efficiency increased from 63.3% to 69.7%. In phase 3 period, NAR values were steady at 0.11, and TN removal efficiency was steady as well ≈71%. This indicates that high influent IC can prompt the CANON process in the system. In addition, it was also observed that TN removal efficiency was improved with the increase of the influent of IC concentrations. This can be used as a controlling influence in keeping and preserving the performance and stability of the CANON route. NAR values of around 0.11 indicate that there is Anammox activity in the system. If high NAR values close to 1 were detected in the system, it would mean that there was no Anammox activity in the system due to the fact that most of the influent NH_4^+-N will be transformed to NO_3^--N (full nitrification) (Hu et al., 2014a).

Achieving the CANON route can be explained by the impact of the substantial role of high IC on the transformation activities of autotrophic nitrogen microorganisms (Tokutomi et al., 2006; Guisasola et al., 2007; Tokutomi et al., 2010; Yang et al., 2010; Kimura et al., 2011; Hu et al., 2014a). Most significantly, previous research studies have shown that AOB activities could be significantly improved and enriched with high IC concentrations, whereas NOB activities were independent of the IC concentrations (Guisasola et al., 2007; Tokutomi et al., 2010). This gives a strong and robust tool to obtain and maintain PN (Tokutomi et al., 2006; Tokutomi et al., 2010). In addition, it was found that Anammox activities could be significantly supported with high IC concentrations (Yang et al., 2010; Kimura et al., 2011). Consequently, if high IC is applied, both PN and Anammox activities can be enhanced, whereas NOB activities remain the same; this provides a robust selective pressure to trigger the CANON process route. The outcomes from this research study substantially support this hypothesis.

6.2.3 Statistical analysis

Statistical analysis of the effluent quality between different recirculation numbers and inorganic carbon concentration efficiencies is summarized in Tables 6.3 and 6.4 respectively. ANOVA revealed statistically significant differences between different recirculation numbers and inorganic carbon concentrations in the removal efficiencies for NH_4^+-N and TN.

Regarding the recirculation number statistical analysis (post hoc) (Table 6.3), the nitrification performance where Rn = 6 times/day was significantly higher than when Rn = 4 times/day and Rn = 3 times/day by 7.2% and 12.1% respectively. Also Rn = 4 times/day was significantly higher than Rn = 3 times/day by 4.8%. This may indicate that more bed-resting time could improve and enhance the nitrification performance. For TN, Rn = 6 times/day was significantly lower than Rn = 4 times/day and Rn = 3 times/day by 5.6% and 6.1% respectively. This result reveals that recirculation numbers have a significant impact on nitrogen removal and the CANON route, as previously discussed (section 6.2.2.1).

Table 6.3 Statistical analysis results ($P<0.05$) for the comparison of mean removal efficiencies between different recirculation numbers (Rn)

	Recirculation Number (per day)		NH_4^+-N	TN
ANOVA	3^{**}, 4^{***}, 6^{***}	F	99.94	16.89
		Sig.	0.000	0.000
Post Hoc (Tamhane's T2)	3-4	Diff.	-4.84^{*}	0.55
		Sig.	0.001	0.979
	3-6	Diff.	-12.06^{*}	6.10^{*}
		Sig.	0.000	0.000
	4-6	Diff.	-7.22^{*}	5.55^{*}
		Sig.	0.000	0.004

*. The mean difference is significant at the 0.05 level.

**. Rn = 3 cycles / day

***. Rn = 4 cycles / day

****. Rn = 6 cycles / day

Regarding the inorganic carbon source statistical analysis (post hoc) (Table 6.4), the nitrification performance with IC = 300 mg/l was slightly lower than IC = 100 mg/l by 0.6%, as they were running with Rn = 6 times/day. This may indicate that inorganic carbon has negligible effect on the nitrification performance. For TN, IC = 300 mg/l was significantly higher than IC = 100 mg/l and IC = 200 mg/l by 6.8% and 5% respectively. This result shows that inorganic carbon concentrations only have limited impact on nitrogen removal and the CANON route, as previously discussed (section 6.2.2.2).

Table 6.4 Statistical analysis results ($P<0.05$) for the comparison of mean removal efficiencies between different inorganic carbon concentrations (IC)

	Inorganic carbon source (mg/l)		NH_4^+-N	TN
ANOVA	100**, 200***, 300****	F	8.47	15.88
		Sig.	0.002	0.000
Post Hoc (Tamhane's T2)	100-200	Diff.	0.26	-1.80
		Sig.	0.197	0.592
	100-300	Diff.	0.58*	-6.81*
		Sig.	0.003	0.003
	200-300	Diff.	0.31	-5.01*
		Sig.	0.152	0.001

*. The mean difference is significant at the 0.05 level.

**. IC = 100 mg/l

***. IC = 200 mg/l

****. IC = 300 mg/l

6.3 Conclusion

This chapter has discussed the overall treatment performance of the single VFCW. It has also addressed the impact of different operational strategies (recirculation number (Rn) and inorganic carbon (IC)) on the system. Moreover, statistical analysis has been discussed to illustrate the impact of Rn and IC on the CANON process and nitrogen removal. The results outlined within this chapter have improved the current scientific understanding of nitrogen removal via the CANON process in a single VFCW. The key conclusions derived from the study are as follows:

- Implementation of the downflow tidal flow mode, a shorter bed resting time (long saturation time), and an intermittent recirculation strategy can produce a continual limited oxygen condition to facilitate the CANON route in a tidal flow constructed wetland (TFCW).

- Recirculation number (Rn) plays a key role for oxygen supply in the system, and Rn can be manipulated to control the oxygen supply in the TFCW to achieve a suitable environment for the partial nitrification (PN) process.
- High influent inorganic carbon (IC) concentration can significantly improve the activity of both ammonia-oxidizing bacteria (AOB) and Anammox bacteria. However, it has no effect on nitrite-oxidizing bacteria (NOB), and this provides a strong selective pressure to trigger the CANON route.
- Nitrate accumulating ratio (NAR) values can be affected by both Rn and IC concentrations.
- Statistical analysis shows that Rn and IC have a significant impact on both nitrogen removal and the CANON route.

7 Microbial Community

7.1 Introduction

Microorganisms play an important role in the mineralization and degradation of OM and the cycling of nutrients in a CW system. Nitrogen transformations in a CW system are generally carried out by complex microbial communities which are related to the macrophyte roots, media substrate, and influent wastewater (Vymazal, 2005). In addition, Faulwetter et al. (2009) indicated that the microbial activity taking place in wetland systems is based on the water chemistry measurements in the influent and effluent of the wetlands. There is usually a lack of qualitative and quantitative data information regarding microorganism activities, but recently more research studies have been focusing on such activities (Zhi et al., 2015). Examining the microbial communities functioning and evaluating their diversity and distribution is significant to identifying the key players in the characterisation and pollutant transformation of CWs, and in understanding the mechanistic function of the wetland ecosystem (Gutknecht et al., 2006), which can be used to cope with the black box process that has usually been used to control the wastewater in wetland systems (Sleytr et al., 2009).

With the development of and improvements in molecular biology in recent years, culture-independent molecular techniques have been able to overcome the problems relating to cultivating slow-growing organisms (e.g. ammonia-oxidizing bacteria, AOB). DNA-based methods are beneficial because they can be used to assess almost all microorganism forms by giving sequencing information on the areas of their genomes. DNA-based methods depend on polymerase chain reactions (PCR) to amplify genetic markers (i.e. a gene or DNA sequence) by using various sets of primers for ribosomal genes (16S rDNA) and functional genes (Truu et al., 2009). PCR has now been widely applied to studying AOB communities - the microorganisms responsible for converting ammonium (NH_4^+-N) to nitrite (NO_2^--N) (Dong and Reddy, 2012; Yan et al., 2016); nitrite-oxidizing bacteria (NOB) communities - the microorganisms responsible for converting nitrite (NO_2^--N) to nitrate (NO_3^--N) (Maixner et al., 2006; Wang et al., 2016c); denitrifying communities - the microorganisms responsible for converting nitrate (NO_3^--N) to dinitrogen gas (N_2)

(Chen et al., 2014; Wang et al., 2016b); and Anammox bacteria communities - the microorganisms responsible for converting ammonium (NH_4^+-N) directly to dinitrogen gas (N_2) (Yapsakli et al., 2011; Chen et al., 2016) in engineered and natural systems.

Because variable nitrogen forms exist in a CW system, the overall dominant microbial communities related to nitrogen removal are bacterial groups like *Betaproteobacteria* and *Gammaproteobacteria* for the nitrification process (Purkhold et al., 2000; Faulwetter et al., 2009), bacterial groups like *Enterobacter* and *Micrococcus* for the denitrification process (Gómez-Villalba et al., 2006; Meng et al., 2014), and planctomycete-like bacteria *Candidatus Brocadia anammoxidans* for the anaerobic ammonium oxidation (Anammox) process (Schmid et al., 2000; Chen et al., 2016). The main aim of this chapter is to investigate and profile the structure of the microbial communities (i.e. characteristics and composition) and their impact on and contribution to organic matter and nitrogen removal from the system.

7.2 Results and discussion

The columns (stages) were named to run the analysis as 1L: lower point of the first column; 1U: upper point of the first column; 2L: lower point of the second column; 2U: upper point of the second column;, 3L: lower point of the third column; 3U: upper point of the third column; 4L: lower point of fourth column; and 4U: upper point of fourth column for the multistage of CWs system, whereas for the single stage CW system, the names were 5L: lower point of the single column; and 5U: upper point of single column.

7.2.1 Richness and diversity of bacteria communities

All points of the CWs obtained sequence readings were in the range of 104118–191610. Richness and evenness comparison was made of biodiversity in five columns for the CWs. The Chao1 indices were used to estimate the biodiversity richness (Zhao et al., 2015), whereas the Shannon index was used to indicate the diversity of the microbial communities (Logan and Regan, 2006).

For the multistage CW system, the Chao1 estimator ranged from 930 (1L) to 1460 (3L). In general, the microbial communities in the lower point at the third stage indicated the highest biodiversity richness, whereas the lower point at the first stage presented the lowest biodiversity richness. This result indicates that the higher richness of the microbial community was due to the carbon source supplied at that stage (step-feeding), as reported in (Chen et al., 2015). The Shannon index estimator ranged from 3.6 (1L) to 5.4 (4L and 4U). This result indicates that the highest diversity of the microbial community was at the fourth stage at both lower and upper points, whereas the lowest diversity of the microbial community was at the first stage at the lower point. For the single stage CW system, the Chao1 and Shannon index estimators ranged from 950 (5L) to 1030 (5U) and 4 (5L) to 4.6 (5U) respectively. This result indicates that the upper point had a higher biodiversity richness and greater diversity of the microbial community than the lower point.

7.2.2 Comparative analysis and taxonomic identification of bacterial communities

7.2.2.1 Heat map

The heat map shows the differently abundant OTUs in all points of the multistage and single stage CW systems to the family level (Figure 7.1). The samples were arranged on the x-axis using the UniFrac similarity method (method to compute differences between microbial communities). The colour intensity scale is related to the relative abundance of OTUs among all points of the CW. Colour (light red to dark red to black) indicates the relative abundance (high to medium to low). To evaluate further the differences in the microbial community structure in all columns of both CW systems, Principal Coordinates Analysis (PCoA) and taxonomic identification of the bacterial community were applied.

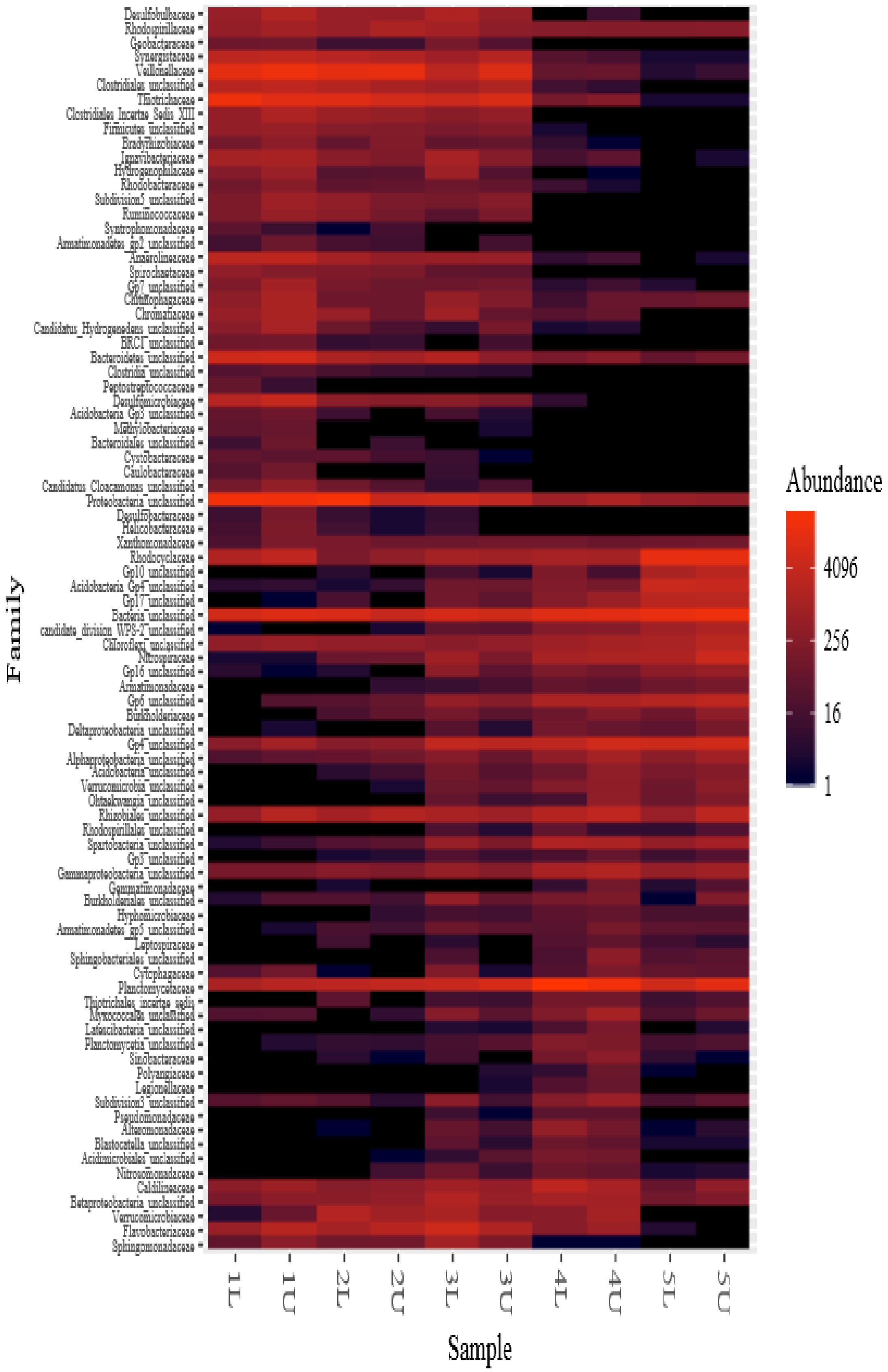


Figure 7.1 Heat map of the dominant OTUs in all columns to the family level

7.2.2.2 Principal Coordinates Analysis (PCoA)

PCoA was used to observe the similarities in the microbial communities in both lower and upper points in the multistage and single stage CW systems, as shown in Figure 7.2. For the multistage CW system, the results revealed that the profile of the microbial communities in the lower and upper points at the first stage were similar, because the observed identical points plot on each other. The same results were observed at the second and fourth stages, where the profiles of the microbial communities were similar in the lower and upper points in each individual column. However, the profile of the microbial communities at the third stage was significantly different between the lower and upper points due to the far distance between the points inside the circle, as shown in Figure 7.2. For the single stage CW system, the profiles of the microbial communities in the lower and upper points were similar as the observed identical points plot on each other.

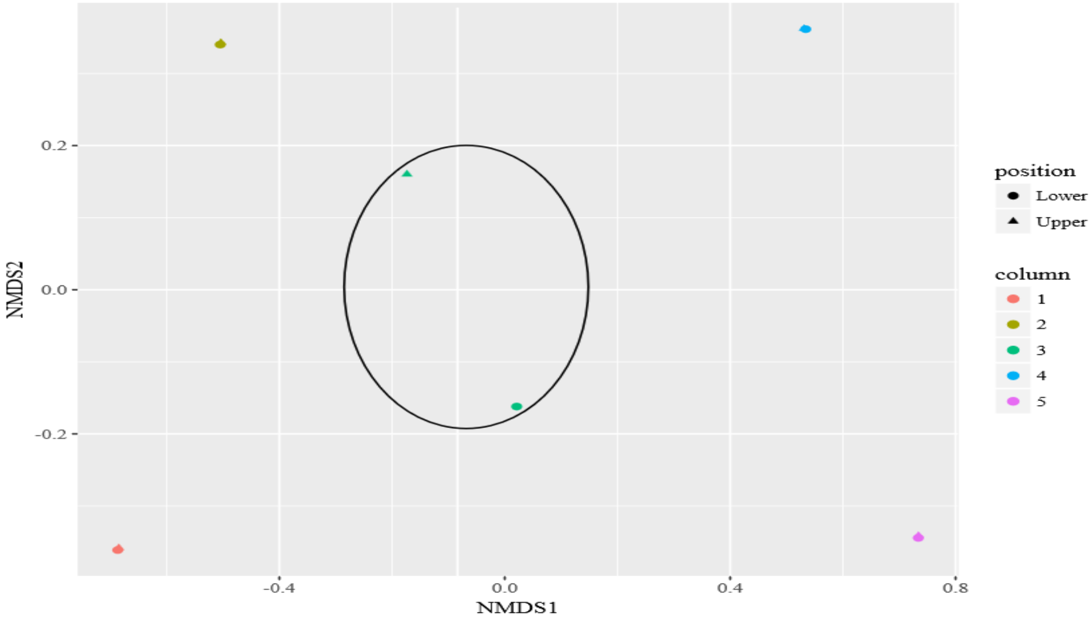


Figure 7.2 Principal Coordinates Analysis (PCoA) plot

7.2.2.3 Taxonomic identification of bacterial communities

To identify the phylogenetic differences in the compositions of the bacterial community structures in both CW systems, relative bacterial community abundance was described at the phylum, class, and genus levels, as shown in Figures 7.3, 7.4, and 7.5 respectively.

7.2.2.3.1 Phylum level

At the phylum level, a total of 24 phyla were found in both 1L and 1U of the CW, 26 and 24 phyla in 2L and 2U respectively, 26 and 25 phyla in 3L and 3U respectively, 20 and 19 phyla in 4L and 4U respectively, and 18 and 20 phyla in 5L and 5U respectively. For the multistage CWs system, the most dominant phylum at the first stage in 1L was Proteobacteria, accounting for 58.9% of the bacterial sequencing, followed by Firmicutes with 15.5%, Bacteria unclassified with 6.7%, Bacteroidetes with 6.4%, and Planctomycetes with 3.8%, whereas the most dominant phylum in 1U was Proteobacteria, accounting for 49.7% of the bacterial sequencing, followed by Firmicutes with 16.5%, Bacteria unclassified with 8.1%, Bacteroidetes with 7.3% and Planctomycetes with 6.3%. For the second stage, the most dominant phylum in 2L was Proteobacteria with 56.6% of the bacterial sequencing, followed by Firmicutes with 14.4%, Bacteria unclassified with 10.8%, Planctomycetes with 5.2%, and Verrucomicrobia with 4.2%, whereas the most dominant phylum in 2U was Proteobacteria, accounting for 45.8% of the bacterial sequencing, followed by Firmicutes with 20.9%, Bacteria unclassified with 6.9%, Bacteroidetes with 5.2%, and Planctomycetes with 4.5%. For the third stage, the most dominant phylum in 3L was Proteobacteria with 49.3% of the bacterial sequencing, followed by Bacteroidetes with 16%, Bacteria unclassified with 10.8%, Planctomycetes with 5.2%, and Acidobacteria with 4.3%, whereas the most dominant phylum in 3U was Proteobacteria, accounting for 43.3% of the bacterial sequencing, followed by Firmicutes with 15.2%, Bacteria unclassified with 9.7%, Planctomycetes with 5.4%, and Bacteroidetes with 4%. For the fourth stage, the most dominant phylum in 4L was Proteobacteria with 50% of the bacterial sequencing, followed by Planctomycetes with 19.4%, Bacteria unclassified with 12.9%, Chloroflexi with 6.8%, and Acidobacteria with 5.1%, whereas the most dominant phylum in 4U was Proteobacteria, accounting for 33.1% of the bacterial sequencing, followed by Planctomycetes with 22.4%, Bacteria unclassified with 14.6%, Acidobacteria with 8.6%, and Chloroflexi with 4.9%. For the single stage, the most dominant phylum in 5L was Proteobacteria with 48.2% of the bacterial sequencing, followed by Bacteria unclassified with 19.7%, Acidobacteria with 17%, Planctomycetes with 7.1%, and Chloroflexi with 2.2%, whereas the most dominant phylum in 5U was Proteobacteria, accounting for 37.1% of the bacterial sequencing, followed by Bacteria unclassified with 20.7%, Acidobacteria with 16.4%, Planctomycetes with 13.4%, and Nitrospirae with 3.5%. Seven phyla were the common phyla observed at the lower and upper points in the multistage CW system: Proteobacteria, Bacteria unclassified, Bacteroidetes, Planctomycetes, Chloroflexi, Verrucomicrobia, and Acidobacteria.

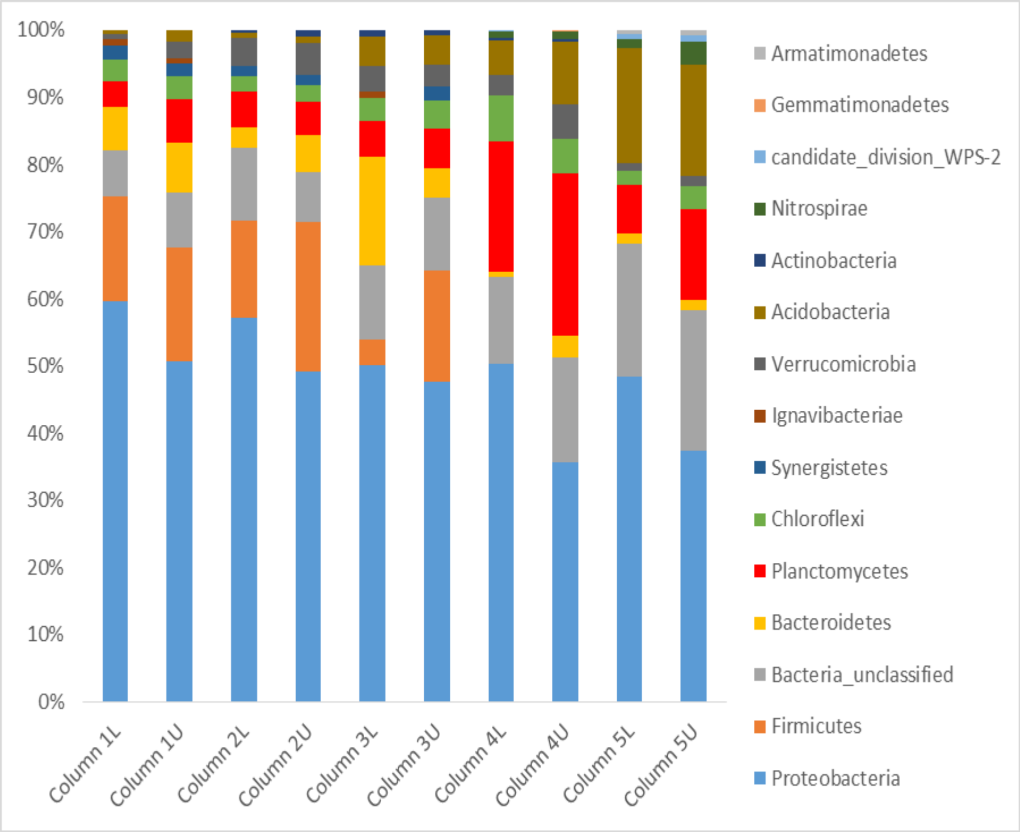


Figure 7.3 Relative abundances of the bacterial communities of the CWs at the phylum level

Overall, the observed abundance phyla in this study were similarly widely distributed in natural wetlands (Wang et al., 2012b; Ligi et al., 2014) and CWs (Ahn et al., 2007; Gorra et al., 2007). All the columns had similar abundant phyla with differences in the percentages in their lower and upper points. Proteobacteria was the most dominant phylum in all points of multistage and single stage CW systems. For the multistage CW system, the top four dominant phyla were Proteobacteria, Planctomycetes, Firmicutes, and Bacteroidetes. The dominant Proteobacteria phylum is substantially linked to the carbon and nitrogen (nitrification process) cycle, which has been reported in natural wetlands, CWs, and activated sludge (Faulwetter et al., 2009; Ye et al., 2011; Ansola et al., 2014). As a result, reductions occurred in each stage for the organic matter and ammonium in the system. The Planctomycetes phylum can conduct

Anammox metabolism (Zhu et al., 2011), which can contribute to ammonium removal in vertical flow systems (Liu et al., 2013a). As a result, the Planctomycetes phylum contributes to ammonium removal in each stage, as it was detected in all stages. However, it was detected at a high percentage in the fourth stage of the system at both lower and upper points; this is because the low concentrations of organic matter in the fourth stage were received from the third stage (Chapter 5). Bacteria in the Firmicutes phylum are chemotrophic bacteria that feature and play a significant role in the organic degradation (Yuan et al., 2016) and denitrification processes (Xu et al., 2016). It was observed that this phylum was dominant in the first three stages of the system. As a result, a major reduction in organic carbon occurred in these stages, and the denitrification process took place within these stages according to the mass balance (Chapter 5, section 5.2.4). Bacteria in the Bacteroidetes phylum have the ability to produce the biodegradation of the organic pollutants (Sack et al., 2011), which have the highest percentage of abundance at the third stage compared to all the stages as step-feeding was supplied in this stage to provide an external organic source. Therefore, there was significant organic matter removal at this stage. For the single stage CW, the top four dominant phyla were Proteobacteria, Planctomycetes, Acidobacteria, and Chloroflexi. As mentioned above, Proteobacteria phyla play a significant role in organic matter and nitrogen (nitrification process) removal in the system. Planctomycetes phyla are related to Anammox bacteria, which, as has already been discussed, transform ammonium to dinitrogen gas by using nitrite as an electron acceptor. Briefly, this system was almost constantly saturated with limited oxygen (Dissolved oxygen (DO) <0.5 mg/l) to follow and achieve CANON process. CANON process is two process that occur in a single unit; partial nitrification process (limited oxygen) and Anammox process (anoxic/anaerobic condition). The existence of the Planctomycetes phyla confirmed the presence of Anammox bacteria in the system and indicated that they played a significant role in achieving the CANON process in this study. Acidobacteria phyla have the ability to oxidize the aerobic ammonia (Elshahed et al., 2007) and play a part in organic carbon precipitation (Chen et al., 2015). Chloroflexi phyla have a potential role in nutrient removal processes (He et al., 2016) and carbon decomposition (Chen et al., 2015).

7.2.2.3.2 Class level

In class levels, a total of 52 and 57 bacterial class were found in 1L and 1U of the CW respectively, 59 bacterial class in both 2L and 2U, 60 and 59 bacterial class in the 3L and 3U respectively, 53 and 55 bacterial class in 4L and 4U respectively, and 45 and 44 bacterial class in 5L and 5U respectively. For the multistage CW system, the most dominant bacterial class at the first stage in 1L was Proteobacteria unclassified, accounting for 24.2% of the bacterial sequencing, followed by Gammaproteobacteria with 22.2%, Negativicutes with 12.6%, Bacteria unclassified with 6.7%, and Betaproteobacteria with 6.3%, whereas the most dominant bacterial class in 1U was Proteobacteria unclassified, accounting for 15.4% of the bacterial sequencing, followed by Negativicutes with 12.8%, Gammaproteobacteria with 12.3%, Betaproteobacteria with 10.4% and Bacteria unclassified with 8.1%. For the second stage, the most dominant bacterial class in 2L was Proteobacteria unclassified with 26.6% of the bacterial sequencing, followed by Gammaproteobacteria with 13.5%, Negativicutes with 11.2%, Bacteria unclassified with 10.8% and Betaproteobacteria with 8.8%, whereas the most dominant bacterial class in 2U was Gammaproteobacteria, accounting for 17.9% of the bacterial sequencing, followed by Negativicutes with 17.8%, Alphaproteobacteria with 13.2%, Betaproteobacteria with 8.1%, and Planctomycetia with 7.5%. For the third stage, the most dominant bacterial class in 3L was Betaproteobacteria with 22.3% of the bacterial sequencing, followed by Gammaproteobacteria with 11.5%, Bacteria unclassified with 10.8%, Flavobacteriia with 8.7%, and Alphaproteobacteria with 8.4%, whereas the most dominant bacterial class in 3U was Gammaproteobacteria, accounting for 15.8% of the bacterial sequencing, followed by Betaproteobacteria with 12.7% Negativicutes with 11.2%, Planctomycetia with 10.4%, and Bacteria unclassified with 9.7%. For the fourth stage, the most dominant bacterial class in 4L was Bacteria unclassified with 50% of the bacterial sequencing, followed by Planctomycetia with 12.9%, Betaproteobacteria with 8.7%, Alphaproteobacteria with 5.9%, and Caldilineae with 5.7%, whereas the most dominant bacterial class in 4U was Betaproteobacteria, accounting for 28.4% of the bacterial sequencing, followed by Planctomycetia with 17.3%, Bacteria unclassified with 14.6%, Alphaproteobacteria with 7.7%, and Acidobacteria Gp4 with 5.9%. For the single stage, the most dominant bacterial class in 5L was Betaproteobacteria with 39.9% of the bacterial sequencing, followed by

Bacteria unclassified with 19.7%, Acidobacteria Gp4 with 10.5%, Planctomycetia with 7.1%, and Alphaproteobacteria with 4.5%, whereas the most dominant bacterial class in 5U was Betaproteobacteria, accounting for 25.4% of the bacterial sequencing, followed by Bacteria unclassified with 20.7%, Planctomycetia with 13.4%, Acidobacteria Gp4 with 9% and Alphaproteobacteria with 7.6%.

In general, all multistage CWs had similar abundant bacterial classes with different percentages in their lower and upper points, whereas the single stage had also similar abundant bacterial classes in their lower and upper points with different percentages. The common bacterial class observed at the lower and upper points in multistage CWs system were five bacterial classes: Gammaproteobacteria, Bacteria unclassified, Betaproteobacteria, Planctomycetia, and Alphaproteobacteria.

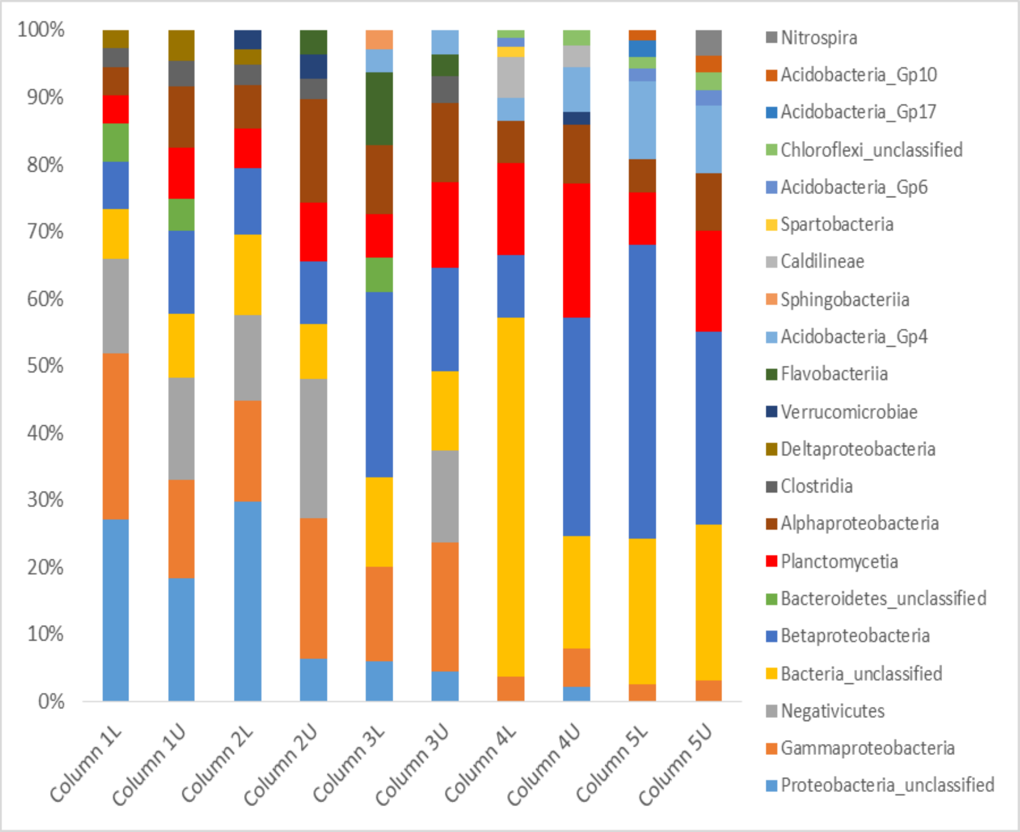


Figure 7.4 Relative abundances of the bacterial communities of the CWs at the class level

7.2.2.3.3 Genus level

In genus level, a total of 126 and 162 genera were found in 1L and 1U of the CW respectively, 143 and 140 genera in both 2L and 2U respectively, 167 and 155 genera in 3L and 3U respectively, 130 and 135 genera in 4L and 4U respectively, and 103 genera in both 5L and 5U. For the multistage CW system, the most dominant genera at the first stage in 1L were NA (which are complicated to classify), accounting for 24.2% of the bacterial sequencing, followed by *Thiothrix* with 20.3%, *Desulfomicrobium* with 1.7%, *Azonexus* with 1.3%, and *Thauera* with 1.2%, whereas the most dominant genera in 1U were NA, accounting for 15.4% of the bacterial sequencing, followed by *Thiothrix* with 7.7%, *Desulfomicrobium* with 2.5%, *Azonexus* with 2.4% and *Pseudoxanthomonas* with 1.7%. For the second stage, the most dominant genera in 2L were NA with 26.5% of the bacterial sequencing, followed by *Thiothrix* with 9%, *Hydrogenophaga* with 2.9%, *Pseudoxanthomonas* with 2.3% and *Azonexus* with 1.7%, whereas the most dominant genera in 2U were NA, accounting for 17.8% of the bacterial sequencing, followed by *Pseudoxanthomonas* with 7.8%, *Thiothrix* with 7.3%, *Azonexus* with 2.9%, and *Hoeflea* with 1.7%. For the third stage, the most dominant genera in 3L were NA with 14.9% of the bacterial sequencing, followed by *Azonexus* with 4.2%, *Thiothrix* with 4%, *Flavobacterium* with 3.9%, and *Pseudoxanthomonas* with 2.7%, whereas the most dominant genera in 3U were NA, accounting for 11.1% of the bacterial sequencing, followed by *Thiothrix* with 9.7%, *Azonexus* with 5.7% *Pseudoxanthomonas* with 3.2%, and *Gemmobacter* with 2%. For the fourth stage, the most dominant genera in 4L were NA with 26.2% of the bacterial sequencing, followed by *Planctomyces* with 18.2%, *Pirellula* with 2.8%, *Azonexus* with 1.6%, and *Nitrospira* with 1%, whereas the most dominant genera in 4U were NA, accounting for 14.6% of the bacterial sequencing, followed by *Planctomyces* with 11.6%, *Azonexus* with 6.5%, *Pirellula* with 1.5%, and *Nitrospira* with 1%. For the single stage CW, the most dominant genera in 5L were NA with 19.7% of the bacterial sequencing, followed by *Thauera* with 12.1%, *Zoogloea* with 5.4%, *Azoarcus* with 3.6%, and *Planctomyces* with 1.6%, whereas the most dominant genera in 5U were NA, accounting for 20.7% of the bacterial sequencing, followed by *Thauera* with 10.2%, *Nitrospira* with 3.5%, *Planctomyces* with 2.3% and *Zoogloea* with 2.2%.

In general, all the multistage CWs had similar abundant genera with difference percentages in their lower and upper points, whereas the single stage CW had similar abundant genera in their lower and upper points with different percentages. The most commonly observed genera at the lower and upper points in the multistage CW systems was *Azonexus*.

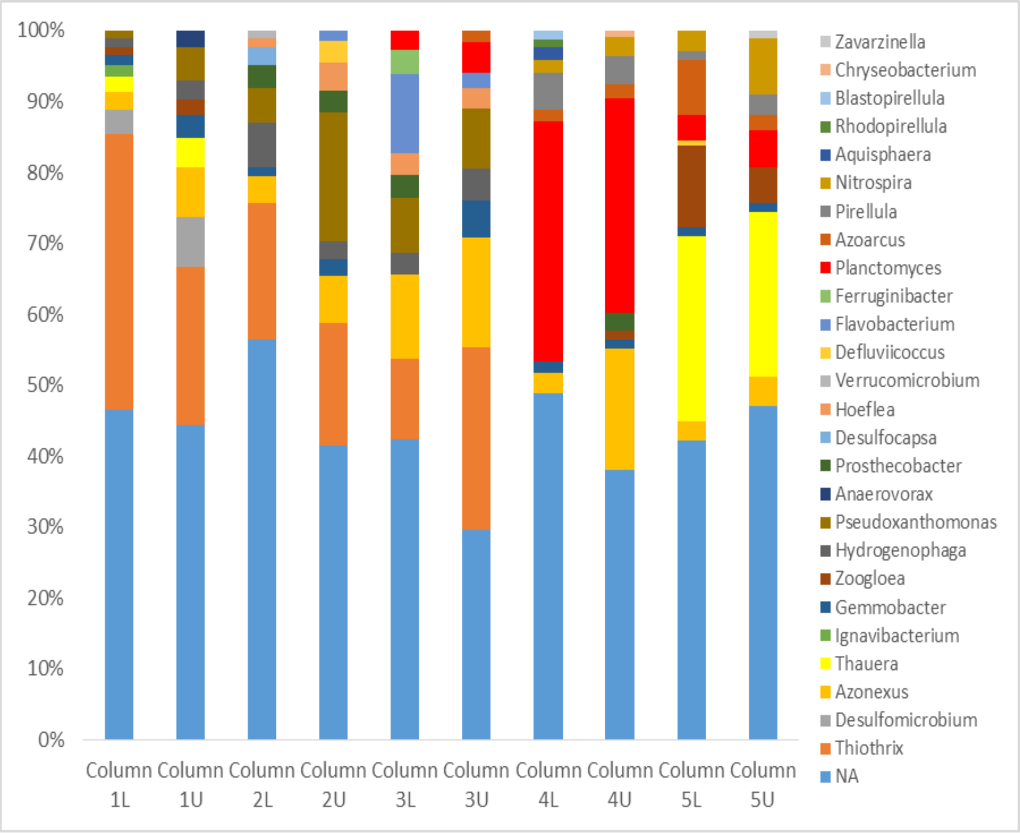


Figure 7.5 Relative abundances of the bacterial communities of the CWs at the genus level

For the multistage CW system, the top four dominant genera were *Thiothrix*, *Azonexus*, *Pseudoxanthomonas*, and *Planctomyces*. *Thiothrix* genera are an oxygen demand bacteria that could survive in the lower CW media that are planted with *Phragmites australis* (Liu et al., 2016), which were detected at the first, second, and third stages of the CW system. *Azonexus* genera comprise denitrifying bacteria which have the ability to convert nitrate into nitrogen gas (Zheng et al., 2016), and this genera existed in all the stages at the lower and upper points. *Pseudoxanthomonas* genera are related to the Proteobacteria phyla (Sànchez-Melsió et al., 2009), which play a significant role in carbon and nitrogen (nitrification process) removal at the first, second, and third stages of the system. The *Planctomyces* genera are related to the Anammox bacteria, which play a potential role in nitrogen removal. It was detected at a high percentage in the fourth stage of the system at both lower and upper points, this is because the low concentrations of organic matter in the fourth stage were received from the third stage (Chapter 5). To illustrate this point (explained in Chapter 3, section 3.3.1), when a high concentration of organic matter is applied, Anammox can be inhibited (Jin et al., 2012). This is because in such a case, nitrite will be utilized predominantly by heterotrophic denitrifiers (i.e. *Azonexus, Azoarcus,* etc.), leaving very little or none at all for the Anammox bacteria. This is why the *Planctomyces* gene existed in a high percentage due to low concentrations of organic matter. For the single stage CW system, the top four dominant genera were *Thauera*, *Zoogloea*, *Nitrospira*, and *Planctomyces*. The *Thauera* genera are related to heterotrophic denitrifying bacteria, which existed in the activated sludge system (Chen et al., 2015). It has the highest abundance in the system, which indicated that the system has limited oxygen (DO readings were below 0.5 mg/l according to the DO probes). This is because the system was almost continually saturated (anoxic/anaerobic condition). The *Zoogloea* and *Nitrospira* genera were considered to play a significant role in the organic matter and nitrogen (nitrifying activity) removal in the system respectively (Yuan et al., 2016). The *Planctomyces* genera thrives under alkaline conditions (Wang et al., 2016a). It was detected and expected in this system, because its influent pH value was 8 (alkalinity) and the system had anoxic/anaerobic conditions that favour Anammox bacteria. The limited oxygen in the system may have resulted in the abundance of bacteria related to denitrifying and Anammox bacteria (Han and Li, 2016), thus leading to the decrease in nitrogen concentrations.

7.2.2.4 Statistical differences between column 1 vs column 4

To enhance and further our understanding, statistical analysis was carried out to investigate the differences in microbial communities between the first and fourth stages in a multistage CW system, where a statistically significant difference ($p < 0.05$) was considered. *Anaerovorax*, *Desulfobulbus*, *Levilinea*, *Thiobacillus*, and *Novosphingobium* genera were found to be more abundant in the first stage than in the fourth stage. However, *Planctomyces*, *Pirellula*, *Nitrospira*, *Haliea*, and *Litorilinea* genera were found to be more abundant in the fourth stage than in the first stage. The greater number of genera of microbial communities in each stage is mainly due to the variable operational conditions.

Based on 16S rRNA gene sequence analysis for the first stage, the *Anaerovorax* genera belong to the class Clostridia of the phylum Firmicutes. *Anaerovorax* have the ability to degrade nitrogen compounds and release ammonia from the system (Tu et al., 2014). The *Desulfobulbus* genera belong to the class Deltaproteobacteria of the phylum Proteobacteria. *Desulfobulbus* usually exist in activated sludge or alum sludge (Doherty et al., 2015). Moreover, this bacterium was responsible for nitrogen removal in the system (Wang et al., 2016b). The *Levilinea* genera also belong to the class Anaerolineae of the phylum Chloroflexi. *Levilinea* are an oxygen respiration bacteria that have the ability to degrade the organic matter in the system (Li et al., 2016). The *Thiobacillus* genera belong to the class Betaproteobacteria of the phylum Proteobacteria. *Thiobacillus* are common species that exist in several environments including sewage treatment facilities (Zhai et al., 2016). They are an autotrophic denitrifying bacteria, which convert nitrate to nitrite to nitrous oxide and finally to nitrogen gas under limited carbon source (Park et al., 2015). The *Novosphingobium* genera belong to the class Alphaproteobacteria of the phylum Proteobacteria. *Novosphingobium* genera are common species that exist in the planted wetland system, and might account for the release of aromatic compounds from plant decay or root exudates (Chen et al., 2015). This suggests that the dense presence of *Phragmites australis* in the first stage has a potential effect on the microbial community selection in CWs (Ansola et al., 2014). Overall, these genera were responsible for organic matter and nitrogen removal in the first stage of the multistage CW system.

Based on 16S rRNA gene sequence analysis for the fourth stage, both the *Planctomyces* and the *Pirellula* genera belong to the class Planctomycetia of the phylum Planctomycetes. The *Planctomyces* and *Pirellula* genera are species of Anammox bacteria. They contribute to the ammonium removal of vertical flow systems (Liu et al., 2013a). Moreover, their abundance was related to the low organic carbon source in this stage (Jin et al., 2012). The *Nitrospira* genera belong to the class Nitrospira of the phylum Nitrospirae. *Nitrospira* are involved in the nitrification process, which can oxidize nitrite to nitrate (Zhai et al., 2016). The *Haliea* genera belong to the class Gammaproteobacteria of the phylum Proteobacteria. In this stage, *Haliea* reduced nitrate to nitrite as denitrification took place according to the mass balance (Chapter 5, section 5.2.4), and also degraded polymers to provides organic and energy sources for the system (Lucena et al., 2010). The *Litorilinea* genera belong to the class Caldilineae of the phylum Chloroflexi. *Litorilinea* are chemo-organotrophic bacteria that have the ability to produce carbon degradation in the system (Kale et al., 2013). Overall, these genera were responsible for organic matter and nitrogen removal in the fourth stage of the multistage CW system.

7.3 Conclusion

This chapter has discussed the profile structure and composition of the microbial communities and their impact on and contribution to organic matter and nitrogen removal in both multistage and single stage CW systems. The results outlined within this chapter have improved the current scientific understanding of the microbial communities in wetland systems. Illumina MiSeq was used to investigate and determine the profile structure and composition of the microbial communities for two CW systems, which were designed to remove organic matter and nitrogen from synthetic wastewater. In general, all microbial communities were related to organic matter and nitrogen removal for both systems. Chao1 and the Shannon index were used to estimate the richness and diversity of bacteria communities, whereas the heat map was used to show the differently abundant OTUs, and Principal Coordinates Analysis (PCoA) was used to observe the similarity in the microbial community in both systems. The dominant phyla in this study were similar to those of previous studies that had used wetland systems, given that in this study, the microbial communities structure profiles were similar to those found within real wetland systems. For the multistage CW system, the dominant phyla were Proteobacteria, Planctomycetes, Firmicutes, and Bacteroidetes. For the single stage CW system, the dominant phyla were Proteobacteria, Planctomycetes, Acidobacteria, and Chloroflexi. Moreover, statistical analysis was used to investigate the significant differences in microbial communities between the first stage (start of the system) and fourth stage (end of the system). The presence of these phyla and their relation to nitrogen removal in both CW systems confirmed that biological transformation was the biggest influence and effect on nitrogen behaviour in the CW systems.

8 Conclusion and Recommendation

8.1 Summary

Nitrogen in wastewater is an important contaminant causing eutrophication in surface water and pollution in ground water, and therefore leading to deterioration in water quality, which affects environmental and human health. Consequently, standard regulations have been legislated for and established regarding wastewater effluents discharge. Nutrient (nitrogen and phosphorus) removal becomes one of the fundamental choices for wastewater treatment systems. Nowadays, there are several wastewater treatment system technologies that can remove nutrients and organic matter as well. The conventional wastewater treatment system (activated sludge) is considered a costly system due to the high demand for equipment, energy, and labour, and so it may not be practical or suitable for small villages and communities. A constructed wetland (CW) system is an excellent alternative technology for various types of wastewater treatment in small villages and communities, due to it being easy to operate and cost effective, its reduced energy consumption and environmental friendliness (green system), and the production of quality effluents.

The main aim of this study was to further our mechanistic understanding of nitrogen removal in such systems to examine the engineering aspects (hydraulic loading rate (HRL), hydraulic retention time (HRT) and pollutant loading) and determine the pathways and mechanisms involved in nitrogen removal in an engineered wetland system. This research focused on nitrogen removal in the vertical flow constructed wetland (VFCW) system. Two CW designs were investigated and both demonstrated the potential capacity of the novelty tidal flow strategy to treat synthetic domestic wastewater through a CW system. The first design was a multistage system (four stages), and the second design was a single stage system.

Overall, several published papers have shown that the applications of a CW system to treat domestic wastewater were convenient for nitrogen removal. Consequently, the results from this research agree with those of several previous studies and suggest that CWs have the ability to reduce the concentrations of nitrogen and organic matter with the participation of a tidal flow strategy from synthetic domestic wastewater.

For both experiments, gravels were used as the main substrate media and for the distribution and drainage layers with different sizes. Samples were collected from the influent and effluent of each stage. Samples were measured *in situ* for pH and temperature, whereas COD, NO_2^--N, NO_3^--N, NH_4^+-N, TN, TP, and PO_4^{3-}-P were analysed in the laboratory. Each stage was instrumented with ORP and DO probes for monitoring in real-time at different depths. *Phragmites australis* was planted in each stage.

8.2 Conclusion

The results outlined within this research have improved the current scientific understanding of nitrogen removal using a VFCW system with a tidal flow strategy. Based on the results and findings of this research and on the previous discussions, the most important conclusions resulting from this research are summarised as follows.

8.2.1 Multistage constructed wetland (Chapter 4)

This is the first design. This experiment was conducted using four identical vertical stages connected in series to investigate and determine the mechanisms and pathways of nitrogen removal. The system was applied with the innovation of a tidal flow strategy with 2 hours saturated and 6 hours unsaturated. It was clearly demonstrated that with a shorter saturated time and longer unsaturated time, the CW system was more efficient in the removal of various pollutants due to the enhanced oxygen supply into the CW. The experiment was conducted based on the seasonal temperature and was divided into two phases. In phase 1, the temperature range was between 15 and 25 ˚C, whereas in phase 2, it was between 5 and 15 ˚C. Overall, the results from this research suggest that there was a clear and significant seasonal variation in the removal of contaminants in the CW system, with a higher efficiency performance removal in phase 1 than in phase 2. The start-up period was from May to July 2015. For phase 1, the average values of removals for the monitored parameters in the CW system from

August to October 2015 were as follows: COD 97.3%, NH_4^+-N 98.1%, TN 77.3%, TP 16.6%, and PO_4^{3-}-P 17.9%. For phase 2, the average values of removals for the monitored parameters in the CW system from November 2015 to January 2016 were as follows: COD 87.5%, NH_4^+-N 78.5%, TN 60.3%, TP 10.2%, and PO_4^{3-}-P 11.8%. The nitrogen removal rate for phase 1 and 2 were 72.7 $gN/m^3.d$ and 57.1 $gN/m^3.d$ respectively, where HLR and HRT affects nitrogen removal. In addition, the results of the correlation statistical analysis from the SPSS software showed that temperature, ORP, and DO are the key influencing factors for the removal performance efficiencies of organic matter and nitrogen. Moreover, because of the limited denitrification step at the third and fourth stages due to the high NO_3^--N effluents, a modification process was needed to overcome this issue. A step-feeding strategy was introduced to the system by supplying an external carbon source from the influent tank to enhance the denitrification step (Chapter 5).

8.2.2 Multistage constructed wetland (Chapter 5)

This research was a continuation of the previous research (Chapter 4), where the denitrification step was limited at the third and fourth stages due to the depleted carbon source that was detected within the first two stages for both phases 1 and 2 from the performance of the individual stages analysis. As a result, high NO_3^--N were produced. Because the denitrification step depends on the available carbon source, in order to reach and achieve a satisfactory TN removal performance, step-feeding was introduced to the nitrified effluents at third stage to enhance and improve TN removal. It was shown to be technically possible to use the step-feeding strategy to treat the synthetic domestic wastewater with high influent concentrations of nutrients and organic matter to improve and enhance the TN removal performance efficiency. In this research, three phases were applied each with a different distribution ratio for the step-feeding strategy (phase 1; 80:20, phase 2; 70:30 and phase 3; 60:40). Overall, the results from this research suggest that step-feeding had a significant impact on the removal of contaminants in the CW system. For phase 1, the average values of removals for the monitored parameters in the CW system were as follows: COD 92.1%, NH_4^+-N 89.2%, TN 81.1%, TP 26.7%, and PO_4^{3-}-P 29.6%. For phase 2, they were COD 94.1%, NH_4^+-N 73.8%, TN 66.2%, TP 19.6%, and PO_4^{3-}-P 22%. For phase 3, COD 95.4%, NH_4^+-N 81.9%, TN 73.6%, TP 11.5%, and PO_4^{3-}-P 16.3%. The nitrogen removal rate for phase 1, 2 and 3 were 76.9 gN/m³.d, 62.7 gN/m³.d and 69.7 gN/m³.d respectively. Comparing the step-feeding performance at the three different distribution ratios for TN removal, phase 1 was the best in TN efficiency removal with 81.1%. As a result, HLR affected the nitrogen removal processes. The optimal COD removal efficiency was obtained in phase 3 with 95.4%, whereas the optimal TP and PO_4^{3-}-P removal efficiency was obtained in phase 1 with 26.7% and 29.6% respectively. Sufficient bed resting time (6 hours) and introducing a carbon source to the system (third stage) were revealed to be the key factors to preserve the efficient nitrification process and support the denitrification process in the system. Overall, the efficiency performance of the system with a step-feeding strategy improved contaminant removal in all parameters compared to the previous research (Chapter 4).

8.2.3 Single stage constructed wetland (Chapter 6)

This is the second design of this research. This experiment was applied to investigate the CANON process. The CANON process requires limited oxygen for the process to take place. Therefore, it requires a shorter unsaturated time and longer saturation time in the bed; this can be controlled by the number of tides and the duration of the saturated and unsaturated phases to weaken the oxygen supply in the system. Moreover, it is also expected that the influent inorganic carbon (IC) could be a substantial controlling factor for the CANON pathway, as IC is considered the carbon source for all the autotrophic microorganisms and may have an important influence on the nitrogen transformation pathway. The experiment was divided into two phases. The aim of phase 1 was to investigate the effect of the recirculation number (Rn) on the system with different Rn (phase 1 = 6 times/day, phase 2 = 4 times/day, and phase 3 = 3 times/day). Rn played a key role in introducing an oxygen supply into the system, and it could be manipulated to control the oxygen supply in the VFCW to achieve a suitable environment for a partial nitrification (PN) process. The aim of phase 2 was to investigate the influent inorganic carbon (IC) on the system with different IC concentrations (phase 1 = 100 mg/l, phase 2 = 200 mg/l, and phase 3 300 mg/l). A high IC concentration could significantly improve the activity of both ammonia-oxidizing bacteria (AOB) and Anammox bacteria, while having no effect on nitrite-oxidizing bacteria (NOB), and this provides a strong selective pressure to trigger the CANON route. The results outlined within this research have improved the current scientific understanding of nitrogen removal via the CANON process in a single VFCW. Moreover, the hypothesis that followed by nitrate accumulating ratio (NAR) to verify the achievement of the CANON process could be affected by both Rn and IC concentrations. Overall, the results from this research suggest that Rn and IC have a significant impact on both nitrogen removal and the CANON route through the statistical analysis. For Rn, phase 1, the average values of removals for the monitored parameters in the CW system were as follows: NH_4^+-N 98.8%, and TN 68.7%. For phase 2, they were NH_4^+-N 91.5%, and TN 74.2%, and for phase 3, they were NH_4^+-N 86.7%, and TN 74.8%. The nitrogen removal rate for phase 1, 2 and 3 were 5.9 $gN/m^3.d$, 6.4 $gN/m^3.d$ and 6.5 $gN/m^3.d$ respectively. Regarding IC concentrations, in phase 1, the average values of removals for the monitored parameters in the CW system were as follows: NH_4^+-N 99.3%, and TN 64.1%. For phase 2, they were NH_4^+-N 99%, and TN 65.9%, and for phase 3, they were NH_4^+-N 98.7%, and TN 71%. The nitrogen removal rate for phase 1, 2 and 3 were 5.6 $gN/m^3.d$, 5.7 $gN/m^3.d$ and 6.1 $gN/m^3.d$ respectively. Overall, when Rn = 6 times/day, the CANON process was achieved according to NAR values, and when Rn was decreased, the CANON process was not achieved. As a result, Rn = 6 times/day was chosen for the next phase (different IC concentrations). Almost all the phases of different IC concentrations meant the CANON process was achieved. Consequently, manipulating Rn and IC concentrations could achieve the CANON process in the system and plays a role in a nitrogen removal rate of the system.

8.2.4 Microbial communities (Chapter 7)

The two CW designed systems were inoculated with activated flocs obtained from the aeration basin of a local domestic wastewater treatment plant for about 2 weeks to provide seed microorganisms on the substrate media (gravels) for both multistage and single stage CW systems. The main goal of this research was to identify the profile structure and the composition of the microbial communities for both systems. In general, Chao1 and the Shannon index were used to estimate the richness and diversity of bacteria communities, whereas a heat map was used to show the differently abundant OTUs, and PCoA was used to observe the similarities in the microbial communities in both systems. However, all microbial communities were related to organic matter and nitrogen removal. The dominant phyla in this study were similar to those in previous studies that had been used in wetland systems, given that the microbial communities structure profiles in this study were similar to those found within real wetland systems. For the multistage CW system, the dominants phyla were Proteobacteria, Planctomycetes, Firmicutes, and Bacteroidetes. For the single stage CW system, the dominant phyla were Proteobacteria, Planctomycetes, Acidobacteria, and Chloroflexi. Moreover, statistical analysis was used to investigate the significant differences in microbial communities between the first stage (start of the system) and fourth stage (end of the system). The presence of these phyla and their relation to the nitrogen removal in both CW systems confirmed that biological transformation was the biggest influence and effect on nitrogen behaviour in the CW systems.

8.3 Recommendation for future work

According to the findings and discussion of this research, the following recommendations for further future work to enhance the findings of this research are made:

- It is recommended that the continuous measurement of oxidation-reduction potential (ORP) and dissolved oxygen (DO) in the CW systems be investigated further, which might increase our understanding of the nitrogen transformation process in the novel tidal flow CW system.

- For better prediction of nitrogen removal in future work, modelling software is considered an excellent assessment tool to simulate the process of nitrogen removal and predict the influence of the operational conditions on the performance of the CW systems. The prediction results from the modelling software could improve and increase our understanding of the nitrogen transformation in CW systems.

- For microbial communities, it is recommended that samples be taken at the beginning of the experiment and at specific times throughout (monthly) till the end of the experiment to track the evolution and composition of the microbial communities, and their contribution to and impact on nitrogen removal.

- For multistage CWs system, it is recommended that the total nitrogen (TN) in each stage be measured instead of the influent and effluent of the final stage (fourth stage) to investigate further the mechanisms and pathways of nitrogen removal in the system in each stage.

- For a multistage CW system with a step-feeding strategy, from the findings of this applied strategy, it is recommended that the hydraulic retention time (HRT) be increased in the third and fourth stages to create anoxic/anaerobic conditions in the system with the supply of an external carbon source from the influent tank, thus enhancing and improving the performance efficiency of the denitrification process and consequently, increasing the total nitrogen (TN) removal efficiency in the system.

- For single stage CW system, from the findings results of this experiment, it is recommended that the recirculation number (Rn) be lower than (Rn = 6 times/day) with different inorganic carbon (IC) concentrations to investigate the total nitrogen (TN) removal in the system and the CANON route. In addition, because of the significant role of IC in the CANON route, it would be interesting to use limestone for providing IC in a cost-effective way as a substrate media option in the system.

Finally, by improving and enhancing knowledge through implementing these abovementioned recommendations, it will be possible to expand the application fields of constructed wetland systems and give better instructions to the engineers to take advantage of the benefits from the wastewater treatment of the constructed wetland system option in comparison to other wastewater treatment systems.

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Appendix

Analytical methods

Removal efficiency = $\frac{influent-effluent}{influent} * 100$

Contribution of the reduction =$\frac{amount\ of\ 1st\ stage\ -amount\ of\ 2nd\ stage}{influent-effluent\ of\ last\ (4th) stage} * 100$,

where amount of NH_4^+-N or COD

Accumulation of NO_3^--N = $\frac{NO_3^- - N\ \ for\ 2nd\ stage\ -NO_3^- - N\ of\ 1st\ stage}{NH_4^+ - N\ of\ 1st\ stage\ -\ NH_4^+ - N\ of\ 2nd\ stage} * 100$

Nitrogen removal rate (gN/m³.d) = $\frac{(influent-effluent)\left(\frac{mg}{l}\right) * Q\ (\frac{l}{d})}{Aera\ (\mathrm{m}^2) * HeigHt\ (m)} / 1000$

Data for Chapter 4

NH_4^+-N (mg/l) removal

Days	**Influent**	**1st stage**	**2nd stage**	**3rd stage**	**4th stage**	**Removal %**
7	25.6	22.3	20.4	18.7	17.8	30.468
14	19.8	15.3	12.5	10.68	8.53	56.919
21	32.7	22.9	17	14.6	11.7	64.220
28	47.2	35	26.5	20.2	15.4	67.372
35	46.3	36.2	22.9	16.9	11.9	74.298
42	44.6	28.1	18.1	15.7	9.57	78.542
49	44.8	31.4	19	9.94	6.09	86.406
56	45.1	24.8	10.9	6.67	4.03	91.064
63	47.2	26.5	13.3	7.02	5.13	89.131
avg.	**39.255**	**26.944**	**17.844**	**13.378**	**10.016**	**70.935**
std.	**10.471**	**6.614**	**5.081**	**4.983**	**4.656**	**19.159**
84	58	31.2	9.63	3.4	0.57	99.017
91	58	29.9	7.67	1.12	0.64	98.896
98	58.8	34.3	11.5	5.62	1.67	97.159
105	57.2	36.7	14.2	6.08	1.29	97.744
112	58	34.3	9.69	2.25	0.73	98.741
119	58	35.4	10.6	2.88	0.54	99.068
126	58	40.6	17.3	3	0.63	98.913
133	56.6	38.7	16	5.81	1.1	98.056
140	57.8	42.3	15.6	4.78	1.2	97.923
147	57.4	41.6	13.2	4.2	1.33	97.682
154	57.2	41.4	22.9	4.89	0.97	98.304
161	57.2	43.6	16	4.66	1.1	98.076
168	58	48.9	25.1	7.49	1.63	97.189
175	57.8	46.3	25.6	7.41	2.2	96.193
avg.	**57.714**	**38.942**	**15.356**	**4.542**	**1.114**	**98.069**
std.	**0.541**	**5.578**	**5.743**	**1.876**	**0.487**	**0.841**
182	57.8	45.7	23	7.2	3.4	94.117
189	58	43.8	21.9	8.3	4.2	92.758
196	58	44.3	25.8	11.5	3.4	94.137
203	58	44.8	28.5	16.6	7.2	87.586
217	57.8	42.1	24.3	15.3	6	89.619
231	57.8	44.3	28	21.2	17.7	69.377
245	58.8	54	45.7	38.3	29.6	49.659
259	58.8	52.9	43.9	40.8	28.3	51.870
avg.	**58.125**	**46.4875**	**30.1375**	**19.9**	**12.475**	**78.531**
std.	**0.426**	**4.425**	**9.339**	**12.958**	**11.1735**	**18.980**

NO_2^--N (mg/l) conversion

Days	Influent	1st stage	2nd stage	3rd stage	4th stage
7	0.108	0.05	0.006	0.007	0.005
14	0.12	0.007	0.004	0.006	0.006
21	0.1	0.007	0.009	0.006	0.008
28	0.019	0.007	0.007	0.006	0.015
35	0.017	0.007	0.006	0.006	0.006
42	0.021	0.006	0.007	0.006	0.02
49	0.005	0.005	0.007	0.004	0.007
56	0.004	0.004	0.006	0.005	0.01
63	0.11	0.001	0.006	0.026	0.017
avg.	**0.056**	**0.01044**	**0.00644**	**0.008**	**0.01044**
std.	**0.05132**	**0.01496**	**0.00133**	**0.00680**	**0.00550**
84	0.01	0.006	0.02	0.42	0.33
91	0.007	0.001	0.025	0.43	0.237
98	0.009	0.004	0.036	0.538	0.503
105	0.006	0.002	0.018	0.421	0.427
112	0.01	0.006	0.009	0.261	0.394
119	0.017	0.001	0.014	0.266	0.384
126	0.007	0.001	0.018	0.376	0.48
133	0.007	0.003	0.011	0.495	0.55
140	0.012	0.003	0.011	0.277	0.502
147	0.035	0.002	0.013	0.3	0.531
154	0.057	0.001	0.005	0.23	0.526
161	0.049	0.005	0.011	0.288	0.546
168	0.054	0.007	0.009	0.309	0.554
175	0.027	0.005	0.009	0.292	0.512
avg.	**0.02192**	**0.00335**	**0.01492**	**0.35021**	**0.46257**
std.	**0.01898**	**0.00213**	**0.00806**	**0.09586**	**0.09535**
182	0.019	0.003	0.007	0.273	0.455
189	0.018	0.002	0.006	0.281	0.447
196	0.02	0.001	0.041	0.545	0.548
203	0.009	0.007	0.009	0.31	0.559
217	0.005	0.011	0.01	0.234	0.429
231	0.032	0.004	0.006	0.004	0.052
245	0.056	0.005	0.006	0.001	0.005
259	0.019	0.008	0.009	0.004	0.007
avg.	**0.02225**	**0.00512**	**0.01175**	**0.20650**	**0.31275**
std.	**0.01581**	**0.00335**	**0.01192**	**0.19282**	**0.24609**

NO_3^--N (mg/l) conversion

Days	**Influent**	**1st stage**	**2nd stage**	**3rd stage**	**4th stage**
7	0	0	0	0	0.8
14	0	0	0	0	1.1
21	0	0	0	0.8	1.1
28	0	0	0.8	1.1	1.1
35	0	0	0	1.1	1.1
42	0	0.8	0	1.1	1.1
49	0	1.4	1.1	1.1	1.1
56	0	1.6	2.2	2.2	2.2
63	0	2.3	1.1	1.1	1.1
avg.	**0**	**0.67777**	**0.57777**	**0.94444**	**1.18888**
std.		**0.88850**	**0.78226**	**0.66164**	**0.39193**
84	0	0.8	1.1	3.6	2.5
91	0	1.1	2.3	2.5	2.5
98	0	1.1	2.5	3.6	3.6
105	0	2.2	2.2	3.6	3.6
112	0	3.6	2.3	2.5	2.5
119	0	4.9	0.8	2.2	2.5
126	0	3.8	0.5	3.6	3.4
133	0	7	0.6	3	4
140	0	6.4	0.3	2.3	2.4
147	0	7.7	0.6	2.7	4.1
154	0	6.3	0.4	3.6	4.8
161	0	4.3	0.6	3.4	4.4
168	0	5.4	0.5	2.8	4.9
175	0	4.3	0.5	3.4	4.1
avg.	**0**	**4.20714**	**1.08571**	**3.05714**	**3.52142**
std.		**2.26323**	**0.83652**	**0.54308**	**0.90569**
182	0	2.2	0.6	4.1	4.2
189	0	2.6	0.6	5.1	6.1
196	0	2.8	0.6	7.5	6.2
203	0	5.4	0.6	3.4	5.8
217	0	5.9	0.7	3.5	5.6
231	0	8	1.1	0.9	1.8
245	0	9.8	1.4	1.7	0.8
259	0	10	1.2	1.7	0.9
avg.	**0**	**5.8375**	**0.85**	**3.4875**	**3.925**
std.		**3.18116**	**0.32950**	**2.14372**	**2.38252**

TN (mg/l) removal

Days	**Influent**	**1st stage**	**2nd stage**	**3rd stage**	**4th stage**	**Removal %**
7	25.9	NM	NM	NM	18.4	28.957
14	23.2	NM	NM	NM	12.03	48.146
21	37.5	NM	NM	NM	16.6	55.733
28	48.8	NM	NM	NM	19.9	59.221
35	47.1	NM	NM	NM	16.3	65.392
42	44.9	NM	NM	NM	14	68.819
49	45.9	NM	NM	NM	14.1	69.281
56	46.3	NM	NM	NM	12.73	72.505
63	48.4	NM	NM	NM	12.4	74.380
avg.	**40.888**	**NM**	**NM**	**NM**	**15.162**	**60.270**
std.	**9.855**	**NM**	**NM**	**NM**	**2.786**	**14.484**
84	61.9	NM	NM	NM	13.51	78.174
91	61.8	NM	NM	NM	12.31	80.080
98	62.6	NM	NM	NM	12.91	79.376
105	61.3	NM	NM	NM	13.2	78.466
112	61.7	NM	NM	NM	13.4	78.282
119	61.7	NM	NM	NM	12	80.551
126	61.6	NM	NM	NM	13.7	77.759
133	60.9	NM	NM	NM	12.3	79.802
140	61.5	NM	NM	NM	13.1	78.699
147	61.4	NM	NM	NM	15.5	74.755
154	61.3	NM	NM	NM	14.9	75.693
161	61.3	NM	NM	NM	15.2	75.203
168	61.9	NM	NM	NM	16.2	73.828
175	61.5	NM	NM	NM	18.1	70.569
avg.	**61.6**	**NM**	**NM**	**NM**	**14.023**	**77.261**
std.	**0.398**	**NM**	**NM**	**NM**	**1.736**	**2.840**
182	61.5	NM	NM	NM	19	69.105
189	61.9	NM	NM	NM	19.3	68.820
196	61.9	NM	NM	NM	20.1	67.528
203	61.9	NM	NM	NM	21.3	65.589
217	61.5	NM	NM	NM	21.6	64.878
231	61.5	NM	NM	NM	30.4	50.569
245	62.6	NM	NM	NM	30.9	50.638
259	62.6	NM	NM	NM	34	45.686
avg.	**61.925**	**NM**	**NM**	**NM**	**24.575**	**60.342**
std.	**0.455**	**NM**	**NM**	**NM**	**6.109**	**9.657**

COD (mg/l) removal

Days	Influent	1st stage	2nd stage	3rd stage	4th stage	Removal %
7	460	249	220	185	138	70
14	793	557	353	199	112	85.876
21	619	372	281	200	172	72.213
28	692	311	173	138	101	85.404
35	697	319	185	107	71.7	89.713
42	699	315	182	102	72.6	89.613
49	698	445	176	101	50.7	92.736
56	755	483	141	73.2	43.1	94.291
63	755	382	94.2	60.8	53.4	92.927
avg.	**685.333**	**381.444**	**200.577**	**129.555**	**90.5**	**85.864**
std.	**98.108**	**97.519**	**76.563**	**53.522**	**43.806**	**8.910**
84	691	382	57.9	35.1	18.3	97.351
91	697	381	53.4	32.9	17.8	97.446
98	680	372	54.4	31.8	15.9	97.661
105	685	190	59.8	33	16	97.664
112	714	70.9	53.2	30.3	14.4	97.983
119	705	153	45.1	29.8	17.3	97.546
126	715	360	59.2	39.7	18.7	97.384
133	699	277	63.7	32.1	14.3	97.954
140	702	394	69.5	43.3	21.4	96.951
147	724	291	54.5	41.4	23.3	96.781
154	698	378	92.2	32.1	18.9	97.292
161	731	386	46.9	27.9	15.7	97.852
168	723	469	69.5	47.8	25.1	96.528
175	720	477	83.2	50.6	27.3	96.208
avg.	**706**	**327.207**	**61.607**	**36.271**	**18.885**	**97.329**
std.	**15.521**	**117.904**	**13.252**	**7.079**	**3.998**	**0.534**
182	714	476	86.2	53.6	31.7	95.560
189	721	371	98.3	66.2	40.2	94.424
196	734	383	101	71.9	46.7	93.637
203	704	466	116	79.4	50.2	92.869
217	685	358	95.5	71.2	54.1	92.102
231	711	500	123	97	71	90.014
245	733	592	400	300	203	72.305
259	731	621	339	299	217	70.314
avg.	**716.625**	**470.875**	**169.875**	**129.787**	**89.237**	**87.453**
std.	**16.877**	**98.955**	**124.820**	**105.458**	**75.480**	**10.232**

TP (mg/l) removal

Days	Influent	1st stage	2nd stage	3rd stage	4th stage	Removal %
7	10.62	16.16	22.63	24.91	30.45	
14	10.62	13.96	18.06	21.56	25	
21	7.75	8.89	11.39	14.05	16.88	
28	10.74	11.74	13.62	18.29	24.28	
35	11.53	12.74	13.77	17.13	23.76	
42	10.76	11.57	13.62	17.67	27.16	
49	10.8	12.78	12.95	14.4	19.39	
56	10.58	10.85	10.35	13.2	12.4	
63	9.55	10.26	13.64	13.2	16.37	
avg.	**10.327**	**12.105**	**14.447**	**17.156**	**21.743**	
std.	**1.090**	**2.135**	**3.722**	**4.025**	**5.826**	
84	9.65	9.12	6.69	4.49	6.72	30.363
91	10.34	8.85	9.74	6.69	7.88	23.791
98	9.49	9.53	8.92	6.84	8.06	15.068
105	9.63	8.71	8.11	6.13	7.34	23.780
112	10.04	9.35	8.74	5.53	7.27	27.590
119	9.42	8.09	7.46	5.79	7.56	19.745
126	10.02	10.49	9.35	10.27	8.71	13.073
133	10.2	12.42	9.89	10.4	8.82	13.529
140	10.41	7.73	4.79	5.86	7.46	28.338
147	9.7	8.79	4.04	5.78	7	27.835
154	10.34	6.77	9.41	9.23	9.59	7.253
161	10.76	9.85	8.12	10.53	10.21	5.112
168	11.54	9.35	7.72	11.42	11.33	1.820
175	10.27	11.97	11.13	10.87	10.19	0.779
avg.	**10.120**	**9.357**	**8.148**	**7.852**	**8.44**	**16.614**
std.	**0.570**	**1.509**	**1.947**	**2.452**	**1.412**	**10.368**
182	11.57	13	7.89	14.11	10.82	6.482
189	10.64	10.18	10.76	13.58	9.16	13.910
196	10.49	9.61	5.12	8.39	9.59	8.580
203	10.37	9.58	6.97	7.47	9.29	10.415
217	10.48	9.42	6.44	7.22	8.88	15.267
231	10.21	9.29	6.43	7.57	8.57	16.063
245	9.81	9.48	8.67	8.13	9.34	4.791
259	9.92	9.54	8.73	8.2	9.32	6.048
avg.	**10.441**	**10.013**	**7.631**	**9.331**	**9.372**	**10.214**
std.	**0.543**	**1.235**	**1.763**	**2.820**	**0.660**	**4.421**

PO_4^{3-}-P (mg/l) removal

Days	Influent	1st stage	2nd stage	3rd stage	4th stage	Removal %
7	10.5	15.5	21.3	24.4	28.5	
14	10.4	12.8	16	19.2	21.9	
21	10.6	11.4	14.7	18	21.5	
28	10.3	11.1	12.9	17.5	22.2	
35	10.1	10.8	11.5	14.5	20.1	
42	9.88	10.61	12.46	16.3	25.88	
49	9.92	11.7	11.86	13.16	18.23	
56	9.85	9.97	9.52	12.1	11.36	
63	8.81	9.43	12.48	12.07	14.93	
avg.	**10.04**	**11.478**	**13.635**	**16.358**	**20.511**	
std.	**0.537**	**1.795**	**3.416**	**3.983**	**5.228**	
84	8.1	8.7	5.31	3.93	5.98	26.296
91	8.79	7.52	8.28	5.69	6.7	23.777
98	8.06	8.29	7.49	5.88	6.6	18.114
105	8.59	7.75	6.81	5.08	6.23	27.474
112	8.53	8.04	7.42	4.75	6.17	27.667
119	8.2	6.88	6.12	5.04	6.43	21.585
126	8.72	8.92	7.95	8.63	7.4	15.138
133	8.55	10.1	7.66	8.15	6.91	19.181
140	9.98	7.21	3.51	5.38	6.82	31.663
147	8.25	7.47	3.43	4.91	5.95	27.879
154	9.94	6.3	8.75	8.58	8.92	10.262
161	9.89	9.01	7.51	9.67	9.38	5.157
168	10.59	8.62	7.14	10.46	10.41	16.699
175	9.75	11.3	9.87	9.64	9.5	2.564
avg.	**9**	**8.292**	**6.947**	**6.842**	**7.392**	**17.888**
std.	**0.853**	**1.313**	**1.832**	**2.224**	**1.503**	**10.069**
182	10.63	11.91	7.3	12.95	9.71	5.655
189	10.1	10	10.1	12.6	9.05	10.396
196	10	9.32	4.52	7.41	8.92	10.800
203	9.88	9.16	6.75	6.98	8.96	9.312
217	10	9.02	5.87	6.41	8.23	17.700
231	9.68	9.01	6.38	7.19	8.26	14.669
245	8.34	8.06	7.37	6.91	7.24	13.189
259	8.43	8.11	7.42	6.97	7.52	10.795
avg.	**9.634**	**9.321**	**6.962**	**8.432**	**8.491**	**11.838**
std.	**0.820**	**1.224**	**1.603**	**2.702**	**0.831**	**3.045**

pH

Days	Influent	1st stage	2nd stage	3rd stage	4th stage
7	7.51	7.56	7.57	7.57	7.58
14	7.56	7.73	7.89	7.86	7.96
21	7.48	7.36	7.43	7.4	7.55
28	7.28	7.37	7.55	7.51	7.61
35	7.32	7.2	7.4	7.5	7.6
42	7.26	7.25	7.34	7.39	7.6
49	7.32	7.34	7.31	7.38	7.42
56	7.31	7.34	7.46	7.4	7.55
63	7.34	7.1	7.1	7.08	7.38
avg.	**7.375**	**7.361**	**7.45**	**7.454**	**7.583**
std.	**0.110**	**0.188**	**0.216**	**0.205**	**0.163**
84	7.43	7.4	7.21	7.43	7.74
91	7.5	7.4	7.3	7.42	7.46
98	7.48	7.77	7.1	7.34	7.71
105	7.5	7.63	7.16	7.5	8.04
112	7.47	7.71	7.2	7.42	7.7
119	7.49	7.66	7.2	7.41	7.71
126	7.38	7.44	7.22	7.31	7.69
133	7.52	7.48	7.26	7.35	7.56
140	7.51	7.39	7.26	7.38	7.64
147	7.51	7.41	7.17	7.32	7.63
154	7.53	7.38	7.2	7.31	7.65
161	7.53	7.43	7.19	7.32	7.62
168	7.48	7.29	7.22	7.28	7.65
175	7.53	7.32	7.21	7.27	7.59
avg.	**7.49**	**7.479**	**7.207**	**7.361**	**7.670**
std.	**0.042**	**0.150**	**0.048**	**0.066**	**0.128**
182	7.54	7.37	7.22	7.25	7.44
189	7.52	7.47	7.21	7.25	7.47
196	7.51	7.49	7.21	7.25	7.49
203	7.5	7.48	7.29	7.34	7.4
217	7.6	7.31	7.22	7.05	7.41
231	7.44	7.42	7.34	7.63	7.64
245	7.49	7.67	7.42	7.75	7.83
259	7.48	7.76	7.57	7.9	7.98
avg.	**7.51**	**7.496**	**7.31**	**7.427**	**7.582**
std.	**0.046**	**0.149**	**0.129**	**0.295**	**0.216**

Temperature of samples (˚C)

Days	Influent	1st stage	2nd stage	3rd stage	4th stage
7	16.1	14.5	17.3	19.5	19.7
14	16.3	14.6	17.6	19.7	19.8
21	16.4	14.7	17.6	19.8	19.9
28	16.4	18.3	20.1	20.9	18.9
35	15.5	17.2	19.1	20.5	21.7
42	14.8	14.3	18.5	21.3	24
49	20.8	21.2	21.9	22.6	23
56	20.3	21.5	24.6	24.6	27.6
63	24.2	24.7	26	27.8	26.5
avg.	**17.866**	**17.888**	**20.3**	**21.855**	**22.344**
std.	**3.152**	**3.814**	**3.198**	**2.759**	**3.157**
84	15.9	16.5	17.8	19.5	21.3
91	18.5	18.2	19	19.9	20
98	18.5	19	20.1	23.2	24.1
105	18.5	19.6	21.9	23.7	24
112	16.1	17.7	17.4	18.7	19.5
119	16.8	18.2	19.3	20	20.3
126	15.6	15.3	17.3	19.1	21.2
133	15.5	15.7	17.4	18.1	20.5
140	15.1	16.5	17	17.3	18.6
147	15.7	16.3	18.5	19.9	20.9
154	17.8	18.6	19.7	19.9	20.8
161	13.5	14	16.8	17.3	17.2
168	14.3	15.2	17.9	18.4	18
175	14.6	15	15.4	15.9	16.1
avg.	**16.171**	**16.842**	**18.25**	**19.35**	**20.178**
std.	**1.632**	**1.713**	**1.641**	**2.118**	**2.262**
182	14.8	14.5	15.4	15.8	15.6
189	14.6	14.3	16.2	16.4	16.5
196	14.8	14	17.3	17.4	16.1
203	11.8	11.4	14.1	14	14.7
217	13.6	14.4	15.2	15.2	15.1
231	15.1	15.4	17	16.8	16.2
245	11.4	10.1	12.9	12.8	13
259	7	6	9.3	10	10.2
avg.	**12.887**	**12.512**	**14.675**	**14.8**	**14.675**
std.	**2.769**	**3.178**	**2.613**	**2.454**	**2.123**

Data for Chapter 5

NH_4^+-N (mg/l) removal

Days	Influent	1st stage	2nd stage	3rd stage	4th stage	Removal %
280	58.8	46	40.9	9.81	7.38	87.448
287	58.8	49.9	42.8	9.11	6.55	88.860
301	57.2	42.1	37.2	12.3	5.92	89.650
315	57.8	48.8	45.3	8.93	6.19	89.290
322	58.8	46.7	44.9	7.07	5.5	90.646
avg.	**58.28**	**46.7**	**42.22**	**9.444**	**6.308**	**89.179**
std.	**0.742**	**3.012**	**3.314**	**1.890**	**0.711**	**1.170**
336	57.4	48.2	43	42.4	17.4	69.686
350	58.8	50.1	43.3	41.7	17	71.088
357	57.8	49	40.1	39.7	16.2	71.972
364	58.8	49.8	42.5	39.4	15.9	72.959
371	58	51.5	43.6	37.9	9.8	83.103
avg.	**58.16**	**49.72**	**42.5**	**40.22**	**15.26**	**73.761**
std.	**0.622**	**1.239**	**1.401**	**1.821**	**3.110**	**5.358**
378	58.8	51	38.4	34.9	11	81.292
385	57.8	45.3	33.6	32.5	14.5	74.913
392	58.8	45.6	32.4	20.3	9.8	83.333
399	58	43.4	32.3	15.6	11.2	80.689
413	57.2	38.6	22.6	20.7	6.27	89.038
avg.	**58.12**	**44.78**	**31.86**	**24.8**	**10.554**	**81.853**
std.	**0.687**	**4.464**	**5.745**	**8.411**	**2.962**	**5.090**

NO_2^--N (mg/l) conversion

Days	Influent	1st stage	2nd stage	3rd stage	4th stage
280	0.057	0.014	0.015	0.042	0.053
287	0.049	0.005	0.007	0.01	0.011
301	0.019	0.006	0.008	0.008	0.01
315	0.017	0.012	0.014	0.015	0.028
322	0.054	0.007	0.008	0.022	0.013
avg.	**0.0392**	**0.0088**	**0.0104**	**0.0194**	**0.023**
std.	**0.01957**	**0.00396**	**0.00378**	**0.01374**	**0.01828**
336	0.03	0.009	0.013	0.021	0.053
350	0.038	0.007	0.007	0.006	0.027
357	0.018	0.004	0.007	0.006	0.01
364	0.02	0.005	0.003	0.006	0.011
371	0.017	0.005	0.004	0.004	0.048
avg.	**0.0246**	**0.006**	**0.0068**	**0.0086**	**0.0298**
std.	**0.00909**	**0.002**	**0.00389**	**0.00698**	**0.02014**
378	0.012	0.003	0.008	0.002	0.144
385	0.048	0.004	0.005	0.001	0.082
392	0.032	0.001	0.006	0.003	0.177
399	0.028	0.003	0.008	0.002	0.083
413	0.054	0.003	0.004	0.001	0.092
avg.	**0.0348**	**0.0028**	**0.0062**	**0.0018**	**0.1156**
std.	**0.01670**	**0.00109**	**0.00178**	**0.00083**	**0.04279**

NO_3^--N (mg/l) conversion

Days	Influent	1st stage	2nd stage	3rd stage	4th stage
280	0	0.7	0.7	5.1	5.7
287	0	1.3	0.9	2.5	1.4
301	0	2.1	1.2	1	2.4
315	0	5.1	2.4	3.3	2.7
322	0	5.5	3.8	4.5	3.4
avg.	**0**	**2.94**	**1.8**	**3.28**	**3.12**
std.		**2.21540**	**1.29807**	**1.62849**	**1.61152**
336	0	4.9	1.3	1	1.7
350	0	3.1	2.3	1.2	2.6
357	0	3.6	2.6	3.2	0.8
364	0	5.1	4.2	4.2	1
371	0	3.8	3.5	3.2	1.3
avg.	**0**	**4.1**	**2.78**	**2.56**	**1.48**
std.		**0.86313**	**1.11669**	**1.39570**	**0.71203**
378	0	4.7	2.4	5.2	2.8
385	0	5.4	2.4	5.1	1.8
392	0	3.4	1	1.2	3.5
399	0	2.7	0.7	1.8	2.4
413	0	3.8	0.6	1.4	2.5
avg.	**0**	**4**	**1.42**	**2.94**	**2.6**
std.		**1.06536**	**0.90664**	**2.02928**	**0.62048**

TN (mg/l) removal

Days	Influent	1st stage	2nd stage	3rd stage	4th stage	Removal %
280	62.6	NM	NM	NM	13.5	78.434
287	62.6	NM	NM	NM	12.9	79.392
301	61.3	NM	NM	NM	10.9	82.218
315	61.5	NM	NM	NM	11.8	80.813
322	62.6	NM	NM	NM	9.7	84.504
avg.	**62.12**	**NM**	**NM**	**NM**	**11.76**	**81.072**
std.	**0.661**	**NM**	**NM**	**NM**	**1.525**	**2.394**
336	61.4	NM	NM	NM	23.3	62.052
350	62.6	NM	NM	NM	22.3	64.376
357	61.5	NM	NM	NM	21.2	65.528
364	62.6	NM	NM	NM	20.3	67.571
371	61.9	NM	NM	NM	17.8	71.243
avg.	**62**	**NM**	**NM**	**NM**	**20.98**	**66.154**
std.	**0.578**	**NM**	**NM**	**NM**	**2.106**	**3.474**
378	62.6	NM	NM	NM	17.3	72.364
385	61.5	NM	NM	NM	20.3	66.991
392	62.6	NM	NM	NM	15.1	75.878
399	61.9	NM	NM	NM	16.5	73.344
413	61.3	NM	NM	NM	12.5	79.608
avg.	**61.98**	**NM**	**NM**	**NM**	**16.34**	**73.637**
std.	**0.605**	**NM**	**NM**	**NM**	**2.868**	**4.651**

COD (mg/l) removal

Days	Influent	1st stage	2nd stage	3rd stage	4th stage	Removal %
280	710	252	173	86	58.9	91.704
287	724	372	244	96.1	73.9	89.792
301	689	434	310	72.7	52.7	92.351
315	736	468	414	45.3	38.3	94.796
322	705	440	376	67.1	56.9	91.929
avg.	**712.8**	**393.2**	**303.4**	**73.44**	**56.14**	**92.114**
std.	**18.019**	**86.366**	**97.569**	**19.389**	**12.783**	**1.791**
336	714	514	298	96.6	31.7	95.560
350	692	502	265	179	41.2	94.046
357	723	474	248	174	45.6	93.692
364	734	439	294	183	47.6	93.514
371	703	501	201	96.3	43.4	93.826
avg.	**713.2**	**486**	**261.2**	**145.78**	**41.9**	**94.128**
std.	**16.452**	**30.074**	**39.505**	**45.144**	**6.183**	**0.823**
378	711	474	146	82.6	25.7	96.385
385	724	295	95.5	70.3	32.5	95.511
392	690	280	76.4	56.3	37.2	94.608
399	730	408	111	55.6	35.9	95.082
413	723	318	56.4	52.1	32.4	95.518
avg.	**715.6**	**355**	**97.06**	**63.38**	**32.74**	**95.421**
std.	**15.883**	**83.012**	**34.175**	**12.798**	**4.461**	**0.656**

TP (mg/l) removal

Days	Influent	1st stage	2nd stage	3rd stage	4th stage	Removal %
280	10.67	6.34	8.34	4.08	7.41	30.552
287	10.84	9.9	8.83	3.58	8.6	20.664
301	11.23	11.36	11.92	9.35	8.8	21.638
315	11.15	10.38	11.49	3.74	7.18	35.605
322	9.97	10.37	10.47	3.85	7.47	25.075
avg.	**10.772**	**9.67**	**10.21**	**4.92**	**7.892**	**26.707**
std.	**0.502**	**1.935**	**1.583**	**2.483**	**0.748**	**6.301**
336	10.48	10.21	9.72	6.04	8.5	18.893
350	11.29	10.84	10.46	11.12	9.7	14.083
357	11.27	10.62	10.53	12.09	8.05	28.571
364	11.79	10.65	12.35	12.34	9.4	20.271
371	10.84	11.13	10.09	10.1	9.1	16.051
avg.	**11.134**	**10.69**	**10.63**	**10.338**	**8.95**	**19.574**
std.	**0.496**	**0.336**	**1.014**	**2.560**	**0.670**	**5.578**
378	11.2	10.45	9.7	9.6	10.21	8.839
385	10.8	10.2	9.08	9.76	8.7	19.444
392	11.49	11.72	11.03	5.73	10.72	6.701
399	11.1	11.7	12.74	5.13	9.79	11.801
413	11.84	11.68	11.22	11.47	10.51	11.233
avg.	**11.286**	**11.15**	**10.754**	**8.338**	**9.986**	**11.524**
std.	**0.395**	**0.758**	**1.427**	**2.762**	**0.799**	**4.830**

PO_4^{3-}-P (mg/l) removal

Days	Influent	1st stage	2nd stage	3rd stage	4th stage	Removal %
280	9.59	5.57	8.16	3.01	6.27	34.619
287	9.98	9.14	8.35	2.89	7.97	20.140
301	9.79	10	10.2	8.25	7.51	23.289
315	9.93	9.88	9.62	3.21	6.56	33.937
322	9.75	9.75	10.3	2.94	6.22	36.205
avg.	**9.808**	**8.868**	**9.326**	**4.06**	**6.906**	**29.638**
std.	**0.154**	**1.873**	**1.013**	**2.345**	**0.789**	**7.364**
336	9.97	9.65	9.24	5	7.74	22.367
350	10.4	10.3	10.2	10.6	8.3	20.192
357	10.2	9.69	9.65	10.9	7.05	30.882
364	10.3	9.87	11.1	11.2	8.26	19.805
371	10.3	10.5	9.1	9.4	8.53	17.184
avg.	**10.234**	**10.002**	**9.858**	**9.42**	**7.976**	**22.036**
std.	**0.163**	**0.379**	**0.815**	**2.563**	**0.592**	**5.250**
378	10.7	10.2	9.48	9.36	9.18	14.205
385	10.3	10.05	8.67	8.41	7.74	24.854
392	10.8	11.5	10.3	5.46	8.88	17.777
399	10.5	11	12	4.32	9.2	12.380
413	11.23	10.5	10.3	10.7	9.79	12.822
avg.	**10.706**	**10.65**	**10.15**	**7.65**	**8.958**	**16.339**
std.	**0.350**	**0.597**	**1.235**	**2.678**	**0.756**	**5.175**

pH

Days	Influent	1st stage	2nd stage	3rd stage	4th stage
280	7.34	7.65	7.52	7.1	7.21
287	7.3	7.53	7.31	7.14	7.28
301	7.57	7.42	7.43	7.56	7.37
315	7.36	7.5	7.48	7.12	7.37
322	7.39	7.43	7.47	7.17	7.39
avg.	**7.392**	**7.506**	**7.442**	**7.218**	**7.324**
std.	**0.104**	**0.092**	**0.080**	**0.192**	**0.076**
336	7.44	7.33	7.38	7.61	7.22
350	7.39	7.17	7.36	7.73	7.37
357	7.41	7.34	7.62	7.89	7.38
364	7.34	7.21	7.53	7.91	7.24
371	7.34	7.21	7.53	7.91	7.24
avg.	**7.384**	**7.252**	**7.484**	**7.81**	**7.29**
std.	**0.043**	**0.077**	**0.110**	**0.134**	**0.078**
378	7.39	7.21	7.45	7.64	7.1
385	7.34	7.21	7.47	7.67	7.15
392	7.31	7.17	7.32	7.34	7.11
399	7.34	7.02	7.24	7.15	7.27
413	7.35	7.15	7.17	7.45	7.17
avg.	**7.346**	**7.152**	**7.33**	**7.45**	**7.16**
std.	**0.028**	**0.078**	**0.130**	**0.215**	**0.067**

Temperature of samples (˚C)

Days	Influent	1st stage	2nd stage	3rd stage	4th stage
280	10.6	9.6	11.9	13	12.9
287	11.3	10.5	12.1	12.7	12.6
301	11.5	11.3	14.8	14.1	14
315	10.6	11.8	15.6	14.4	15.8
322	12.3	12	12.3	14.4	15.3
avg.	**11.26**	**11.04**	**13.34**	**13.72**	**14.12**
std.	**0.709**	**0.991**	**1.727**	**0.810**	**1.416**
336	11.3	11.6	13.1	17.6	16.8
350	12.9	14.5	17	18.6	19.6
357	11.8	12.8	16.8	15.1	15.5
364	13	13.4	17.6	19.4	18.7
371	13	13.4	17.6	19.4	18.7
avg.	**12.4**	**13.14**	**16.42**	**18.02**	**17.86**
std.	**0.796**	**1.057**	**1.889**	**1.792**	**1.668**
378	14.8	15.3	17.8	18.3	18
385	15.9	17.4	20.2	22.5	21.7
392	18.8	21	24.4	25.8	24.4
399	19.6	19.3	21.9	26.5	25.3
413	18.2	18.4	20.9	23.5	23.3
avg.	**17.46**	**18.28**	**21.04**	**23.32**	**22.54**
std.	**2.026**	**2.127**	**2.411**	**3.246**	**2.871**

Data for Chapter 6

NH_4^+-N (mg/l) removal

	Rn phase		
Days	**NH_4^+-N influent**	**NH_4^+-N effluent**	**Removal %**
1	14.5	2.71	81.310
3	14.5	1.76	87.862
8	14.5	0.82	94.344
10	14.8	0.55	96.283
15	14.5	0.26	98.206
17	14.5	0.12	99.172
22	14.5	0.11	99.241
24	14.5	0.1	99.310
29	14.5	0.21	98.551
31	14.5	0.27	98.137
36	14.4	0.18	98.750
38	14.3	0.22	98.461
43	14.5	0.23	98.413
45	14.6	0.13	99.109
50	15	0.17	98.866
52	14.5	0.11	99.241
57	14.9	0.15	98.993
59	14.9	0.17	98.859
avg.	**14.577**	**0.459**	**96.848**
std.	**0.189**	**0.689**	**4.756**
64	14.6	0.32	97.808
66	14.5	0.167	98.848
71	14.6	0.21	98.561
73	14.6	0.26	98.219
78	14.6	0.31	97.876
80	14.6	0.34	97.671
85	14.8	0.15	98.986
87	14.7	0.12	99.183
92	14.7	0.11	99.251
94	14.6	0.12	99.178
99	14.8	0.12	99.189
101	14.7	0.17	98.843
106	14.9	0.13	99.127
108	14.8	0.12	99.189
113	14.8	0.15	98.986
115	14.7	0.11	99.251
avg.	**14.687**	**0.181**	**98.762**
std.	**0.108**	**0.080**	**0.557**
129	14.9	1.65	88.926
134	14.8	1.12	92.432
136	14.6	0.86	94.109

141	14.7	0.91	93.809
157	14.9	1.09	92.684
162	14.7	1.49	89.863
164	14.8	1.53	89.662
169	14.8	1.82	87.702
171	14.9	1.2	91.946
176	14.7	0.97	93.401
178	14.8	1.12	92.432
avg.	**14.781**	**1.250**	**91.537**
std.	**0.098**	**0.320**	**2.149**
183	14.8	1.4	90.540
185	14.9	2.07	86.107
190	14.8	2.21	85.067
192	14.7	3.16	78.503
197	14.9	1.98	86.711
199	14.8	2.94	80.135
211	14.8	1.9	87.162
213	14.7	1.99	86.462
218	14.8	1.86	87.432
220	14.7	1.62	88.979
225	14.8	1.54	89.594
227	14.9	1.69	88.657
232	14.9	1.53	89.731
234	14.8	1.67	88.716
avg.	**14.807**	**1.968**	**86.705**
std.	**0.073**	**0.514**	**3.503**
IC phase			
Days	**NH_4^+-N influent**	**NH_4^+-N effluent**	**Removal %**
239	14.9	0.08	99.463
241	14.8	0.12	99.189
246	14.8	0.09	99.391
248	14.7	0.12	99.183
253	14.8	0.11	99.256
255	14.9	0.08	99.463
260	14.9	0.07	99.530
262	14.8	0.18	98.783
avg.	**14.825**	**0.106**	**99.283**
std.	**0.070**	**0.0354**	**0.241**
267	14.9	0.1	99.328
269	14.8	0.15	98.986
274	14.9	0.14	99.060
276	14.7	0.1	99.319
281	14.9	0.17	98.859
283	14.8	0.2	98.648
288	14.7	0.2	98.639
290	14.8	0.1	99.3243

avg.	**14.812**	**0.145**	**99.021**
std.	**0.083**	**0.042**	**0.290**
295	14.9	0.24	98.389
297	14.8	0.18	98.783
302	14.7	0.23	98.435
304	14.8	0.1	99.324
309	14.7	0.23	98.435
311	14.8	0.18	98.783
316	14.8	0.19	98.716
318	14.9	0.18	98.791
avg.	**14.8**	**0.191**	**98.707**
std.	**0.075**	**0.044**	**0.304**

NO_2^--N (mg/l) conversion

Rn phase		
Days	**NO_2^--N influent**	**NO_2^--N effluent**
1	0.005	0.34
3	0.004	0.25
8	0.004	0.15
10	0.002	0.19
15	0.003	0.17
17	0.003	0.009
22	0.001	0.009
24	0.003	0.04
29	0.004	0.026
31	0.004	0.07
36	0.003	0.013
38	0.004	0.016
43	0.005	0.052
45	0.003	0.01
50	0.003	0.041
52	0.003	0.006
57	0.004	0.018
59	0.002	0.009
avg.	**0.00333**	**0.07883**
std.	**0.00102**	**0.09894**
64	0.004	0.005
66	0.004	0.007
71	0.003	0.006
73	0.004	0.007
78	0.004	0.007
80	0.003	0.004
85	0.004	0.034
87	0.002	0.007
92	0.003	0.006
94	0.004	0.005
99	0.002	0.005
101	0.002	0.004
106	0.003	0.011
108	0.002	0.006
113	0.003	0.007
115	0.003	0.005
avg.	**0.00312**	**0.00787**
std.	**0.00080**	**0.00716**
129	0.004	0.007
134	0.003	0.008
136	0.002	0.004
141	0.003	0.004

157	0.002	0.013
162	0.003	0.025
164	0.004	0.019
169	0.003	0.016
171	0.002	0.01
176	0.003	0.006
178	0.002	0.006
avg.	**0.00281**	**0.01072**
std.	**0.00075**	**0.00679**
183	0.003	0.018
185	0.002	0.004
190	0.003	0.01
192	0.003	0.006
197	0.003	0.006
199	0.002	0.003
211	0.002	0.006
213	0.003	0.006
218	0.002	0.008
220	0.003	0.006
225	0.002	0.007
227	0.003	0.006
232	0.003	0.011
234	0.002	0.007
avg.	**0.00257**	**0.00742**
std.	**0.00051**	**0.00367**
	IC phase	
Days	**NO_2^--N influent**	**NO_2^--N effluent**
239	0.003	0.005
241	0.002	0.009
246	0.003	0.013
248	0.002	0.011
253	0.002	0.004
255	0.003	0.005
260	0.003	0.006
262	0.002	0.006
avg.	**0.0025**	**0.00737**
std.	**0.00053**	**0.00324**
267	0.003	0.004
269	0.002	0.005
274	0.003	0.006
276	0.002	0.006
281	0.003	0.006
283	0.002	0.004
288	0.003	0.006
290	0.002	0.005
avg.	**0.0025**	**0.00525**

std.	**0.00053**	**0.00088**
295	0.003	0.006
297	0.002	0.003
302	0.001	0.002
304	0.002	0.006
309	0.001	0.002
311	0.001	0.002
316	0.003	0.005
318	0.002	0.004
avg.	**0.00187**	**0.00375**
std.	**0.00083**	**0.00175**

NO_3^--N (mg/l) conversion

Rn phase		
Days	**NO_3^--N influent**	**NO_3^--N effluent**
1	0	2.5
3	0	2.6
8	0	2.2
10	0	2.2
15	0	2.2
17	0	1.1
22	0	1.1
24	0	1.6
29	0	1.6
31	0	1.4
36	0	1.5
38	0	1.5
43	0	1.3
45	0	1
50	0	1.5
52	0	1.4
57	0	1.1
59	0	1.2
avg.	**0**	**1.61111**
std.		**0.50630**
64	0	1.6
66	0	1.6
71	0	1.6
73	0	1.6
78	0	1.6
80	0	1.7
85	0	1.6
87	0	1.6
92	0	1.7
94	0	1.6
99	0	1.6
101	0	1.6
106	0	1.6
108	0	1.7
113	0	1.6
115	0	1.6
avg.	**0**	**1.6135**
std.		**0.04031**
129	0	1.7
134	0	1.7
136	0	1.8
141	0	1.8

157	0	1.7
162	0	1.7
164	0	1.6
169	0	1.6
171	0	1.8
176	0	1.7
178	0	1.7
avg.	**0**	**1.70909**
std.		**0.08006**
183	0	2
185	0	1.7
190	0	1.7
192	0	1.7
197	0	1.7
199	0	1.7
211	0	1.9
213	0	1.9
218	0	1.8
220	0	1.8
225	0	1.9
227	0	1.8
232	0	1.8
234	0	1.9
avg.	**0**	**1.80714**
std.		**0.09972**
	IC phase	
Days	**NO_3^--N influent**	**NO_3^--N effluent**
239	0	1.7
241	0	1.6
246	0	1.6
248	0	1.6
253	0	1.6
255	0	1.7
260	0	1.6
262	0	1.6
avg.	**0**	**1.625**
std.		**0.04629**
267	0	1.6
269	0	1.7
274	0	1.6
276	0	1.6
281	0	1.6
283	0	1.6
288	0	1.6
290	0	1.6
avg.	**0**	**1.6125**

std.		**0.03535**
295	0	1.62
297	0	1.61
302	0	1.6
304	0	1.62
309	0	1.6
311	0	1.61
316	0	1.61
318	0	1.62
avg.	**0**	**1.61125**
std.		**0.00834**

TN (mg/l) removal

Rn phase			
Days	**TN influent**	**TN effluent**	**Removal %**
1	16.5	5.3	67.878
3	16.5	5.83	64.666
8	16.5	6.17	62.606
10	16.9	5.74	66.035
15	16.5	5.89	64.303
17	16.5	5.73	65.272
22	16.5	6.1	63.030
24	16.5	5.9	64.242
29	16.5	6.07	63.212
31	16.5	6.04	63.393
36	16.2	5.85	63.888
38	16	4.7	70.625
43	16.5	5.57	66.242
45	16.7	5.29	68.323
50	17.5	5.12	70.742
52	16.5	4.85	70.606
57	17.2	5.32	69.069
59	17.2	5.76	66.511
avg.	**16.622**	**5.623**	**66.166**
std.	**0.363**	**0.433**	**2.776**
64	16.7	5.22	68.742
66	16.5	5.13	68.909
71	16.7	5.19	68.922
73	16.7	5.34	68.023
78	16.7	5.55	66.766
80	16.7	5.61	66.407
85	16.9	4.81	71.538
87	16.8	4.77	71.607
92	16.8	5.47	67.440
94	16.7	5.21	68.802
99	16.9	4.39	74.023
101	16.8	5.13	69.464
106	17.2	5.38	68.720
108	16.8	5.67	66.25
113	16.9	5.75	65.976
115	16.8	5.53	67.083
avg.	**16.787**	**5.259**	**68.670**
std.	**0.15**	**0.364**	**2.200**
129	17.2	4.19	75.639
134	16.9	3.04	82.011
136	16.7	3.17	81.017
141	16.8	4.82	71.309
157	17.2	5.49	68.081

162	16.8	4.91	70.773
164	16.9	4.85	71.301
169	16.9	4.44	73.727
171	17.2	4.2	75.581
176	16.8	4.53	73.035
178	16.9	4.41	73.905
avg.	**16.936**	**4.368**	**74.208**
std.	**0.180**	**0.726**	**4.232**
183	16.9	4.12	75.621
185	17.2	4.88	71.627
190	16.9	4.74	71.952
192	16.8	3.14	81.309
197	17.2	4.24	75.348
199	16.9	4.97	70.591
211	16.9	4.53	73.195
213	16.8	4.43	73.630
218	16.9	4.93	70.828
220	16.8	4.16	75.238
225	16.9	4.34	74.319
227	17.2	3.61	79.011
232	17.2	3.98	76.860
234	16.9	3.85	77.218
avg.	**16.964**	**4.28**	**74.770**
std.	**0.159**	**0.527**	**3.128**
	IC phase		
Days	**TN influent**	**TN effluent**	**Removal %**
239	17.2	6.2	63.953
241	16.9	6.31	62.662
246	16.9	5.44	67.810
248	16.8	5.91	64.821
253	16.9	5.38	68.165
255	17.2	5.61	67.383
260	17.2	6.93	59.709
262	16.9	6.99	58.639
avg.	**17**	**6.09625**	**64.139**
std.	**0.169**	**0.628**	**3.641**
267	17.2	6	65.116
269	16.9	6.21	63.254
274	17.2	5.98	65.232
276	16.8	6.15	63.392
281	17.2	5.7	66.860
283	16.9	5.79	65.739
288	16.8	5.34	68.214
290	16.9	5.12	69.704
avg.	**16.987**	**5.786**	**65.938**
std.	**0.180**	**0.386**	**2.240**

295	17.2	5.14	70.116
297	16.9	5.01	70.355
302	16.8	4.98	70.357
304	16.9	5.01	70.355
309	16.8	4.8	71.428
311	16.9	4.85	71.301
316	16.8	4.77	71.607
318	17.2	4.8	72.093
avg.	**16.937**	**4.92**	**70.952**
std.	**0.168**	**0.133**	**0.741**

pH

	Rn phase	
Days	**pH influent**	**pH effluent**
1	8	7.37
3	8	7.58
8	8	7.4
10	8	7.56
15	8	7.56
17	8	7.61
22	8	7.57
24	8	7.6
29	8	7.5
31	8	7.54
36	8.14	7.63
38	8	7.58
43	8	7.64
45	8	7.54
50	8	7.7
52	8	7.66
57	8.09	7.74
59	8	7.61
avg.	**8.012**	**7.577**
std.	**0.038**	**0.091**
64	8	7.66
66	8	7.72
71	8	7.64
73	8	7.64
78	8	7.68
80	8	7.63
85	7.98	7.6
87	8.04	7.66
92	8.07	7.62
94	7.97	7.57
99	8.03	7.59
101	8.05	7.72
106	8.02	7.6
108	8.01	7.54
113	8.04	7.64
115	8	7.6
avg.	**8.013**	**7.631**
std.	**0.026**	**0.049**
129	7.99	7.42
134	8.02	7.44
136	8.03	7.37
141	8.05	7.46
157	8	7.48

162	8.11	7.47
164	8.03	7.5
169	8.05	7.72
171	8.01	7.56
176	8.05	7.4
178	8	7.4
avg.	**8.030**	**7.474**
std.	**0.033**	**0.097**
183	8	7.42
185	7.99	7.4
190	8.02	7.46
192	7.99	7.25
197	8	7.28
199	7.99	7.42
211	8.03	7.46
213	8.03	7.42
218	8.02	7.4
220	8.01	7.42
225	7.99	7.31
227	7.99	7.42
232	8.05	7.55
234	8.11	7.46
avg.	**8.015**	**7.405**
std.	**0.033**	**0.078**
	IC phase	
Days	**pH influent**	**pH effluent**
239	8.03	7.36
241	8	7.5
246	8.05	7.56
248	8	7.36
253	8.08	7.41
255	8.07	7.69
260	8.02	7.47
262	8	7.43
avg.	**8.031**	**7.472**
std.	**0.032**	**0.111**
267	8.01	7.49
269	8.03	7.42
274	8.04	7.47
276	8.02	7.43
281	8.1	7.57
283	8.04	7.44
288	8.01	7.58
290	8.03	7.53
avg.	**8.035**	**7.491**
std.	**0.028**	**0.062**

295	8.07	7.65
297	8.01	7.71
302	8.01	7.72
304	8.01	7.61
309	8	7.7
311	8.01	7.68
316	8	7.71
318	8.07	7.71
avg.	**8.022**	**7.686**
std.	**0.029**	**0.038**

Temperature of samples (˚C)

Rn phase		
Days	**Temperature influent**	**Temperature effluent**
1	23.7	19.5
3	20.2	23.2
8	23.1	22
10	21.1	19.1
15	23.4	18.1
17	21.3	19.8
22	23.2	19.8
24	21.8	18.6
29	20.5	19.7
31	20.5	20.6
36	19.4	19.2
38	19.7	17.1
43	19.5	18.6
45	19	19.6
50	18.7	18
52	17.9	19.1
57	17.9	18.3
59	18.2	16.8
avg.	**20.505**	**19.283**
std.	**1.919**	**1.557**
64	18.9	17
66	18.4	16.9
71	17.9	15.1
73	18.1	16.9
78	17.7	17.9
80	20	17.4
85	17.8	17.6
87	18.5	18.1
92	17.8	16.3
94	19.1	18.6
99	18	17.7
101	17.6	13
106	19	16.4
108	18.4	12.3
113	17.7	13.9
115	16.4	15.1
avg.	**18.206**	**16.262**
std.	**0.812**	**1.873**
129	17.2	13.2
134	17.1	16.4
136	16.8	15.6
141	18.1	11.2
157	16.1	11.6

162	17.1	8.2
164	17.1	9.4
169	15.1	6.7
171	14.3	14.5
176	17.1	14.9
178	17.2	13.5
avg.	**16.654**	**12.290**
std.	**1.084**	**3.159**
183	14.5	12.1
185	16.4	14
190	14.1	9.6
192	15.5	12.7
197	15.6	10.7
199	15.3	12.8
211	13.6	11.7
213	18.3	10.9
218	15.4	10.8
220	15.2	12.3
225	14.9	13.3
227	15.1	11.2
232	13.7	15.3
234	13.8	15.6
avg.	**15.1**	**12.357**
std.	**1.233**	**1.750**
	IC phase	
Days	**Temperature influent**	**Temperature effluent**
239	16.2	14.3
241	15.4	12.2
246	16.7	17.6
248	21.3	13.7
253	15.1	14.8
255	14.6	13.5
260	17.1	12.5
262	16.9	12.4
avg.	**16.662**	**13.875**
std.	**2.079**	**1.770**
267	17.2	16.5
269	16.2	15.1
274	19.1	17.4
276	18	16
281	17.4	16.7
283	16.5	14.8
288	17.8	15.2
290	18.7	17.5
avg.	**17.612**	**16.15**
std.	**1.002**	**1.046**

295	18.7	17.5
297	18.3	18.4
302	19.8	21.9
304	19.7	21.6
309	19.6	18.6
311	18.6	17.7
316	19.1	18.6
318	19.4	18.7
avg.	**19.15**	**19.125**
std.	**0.563**	**1.679**

ORP and DO probes were maintenance monthly by cleaning and calibrating them.

ORP probe

Specifications:

- Dimensions: 12 x 120 mm
- 3 m cable with BNC connector
- Temp. Range: 0°C to 80°C
- Gel-filled Plastic Body
- Platinum-Rod
- Protective Skirt
- Reference System: Ag/AgCl System in a saturated KCl solution / gel with approximately 200 mV offset against the Hydrogen reference system (Eh = E measured + approx. 200 mV)

Operating Instructions:

Preparation:

1. Remove the protective bottle from the sensing element and save it for future storage.
2. Clean any salt deposits from the exterior of the electrode by rinsing with distilled water.
3. Connect the electrode to the Meter or Analyser.
4. ORP measurements are temperature dependent, however there is no temperature compensation available

Maintenance:

1. **General cleaning:**

- For non-organic contamination, soak the electrode in 0.1 M HCl for 10 minutes, rinse with distilled water.
- For organic or oily contamination, soak the electrode for 10 minutes in a 1:10 dilution of household detergent.
- After one of the above cleaning procedure, soak the electrode in saturated KCl solution for 3 hours.
- The ORP electrodes contain an Ag/AgCl reference that may become clogged in solutions that contain silver complexing or binding agents such as TRIS, proteins and sulfides. Frequent cleaning may be required when measuring solutions that contain these agents.

2. **Checking Electrode Operation:**

- Place the electrode into pH7 solution saturated with Quinhydrone, the reading should be 86±15 mV.
- Place the electrode into pH4 solution saturated with Quinhydrone, the reading should be 263±15 mV.
- Alternatively the electrode can be checked in ZoBell’s solution, the reading should be 238 mV at 25°C.

DO probe

Specifications:

1. **Dimensions:**

- Electrode body: 12 mm diameter
- Electrode head: 16 mm diameter
- Total length: 150 mm
- Operating Current Range:
- 0 to 10 µA (Micro Ampere)
- Output at zero oxygen:
- less than 1% saturation

2. **Membrane type:**

- Teflon

3. **Cable length:**

- 3 m

4. **Standard connector:**

- 4 pin Mini-DIN
- Polarisation Voltage: 800 mV

Short Operating Instructions:

1. **Calibration:**

- Usually a 1-point Calibration is sufficient: 100 % D.O in air saturated water. The zero-drift is very low and zero-calibration can be omitted
- Connect the electrode to the Dissolved Oxygen Monitor and switch on to allow the electrode to polarise for approximately 15 minutes.
- Follow the instructions of the meter manual or follow the instructions on the screen when using a D.O. Monitoring software.
- For a short-time store the Sensor with the supplied Storage bottle filled with distilled water.
- For long-time storage remove the membrane assembly and the electrolyte, rinse everything with distilled water and blot dry.

2. **Maintenance:**

- To inspect and clean the electrodes and replace the electrolyte (filling solution), remove the membrane assembly and clean the silver anode and the platinum cathode with a very fine polishing cloth and rinse with distilled water and alcohol.