Vertical flow constructed wetlands for the treatment of inorganic industrial wastewater

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Murdoch University
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I hereby declare that this thesis is my own account of my research and contains as its main content work that has not previously been submitted for a degree at any university.

Sergio Santos Domingos

Supervisors
Dr Stewart Dallas
Professor Goen Ho
Items derived from this study

1. Journal articles:


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Abstract

The focus of this thesis is primarily on nitrogen removal and secondarily on heavy metal accumulation in unsaturated and saturated vertical flow constructed wetlands (VFCWs) treating inorganic industrial wastewater. This thesis is divided into an experimental component and a case study component. Three research themes are presented within the scope of this thesis. The first theme involves the study of nitrification and denitrification and the characterisation of the respective bacterial communities in unsaturated and semi-saturated VFCWs. The identification of functional bacteria with the aid of polymerase chain reaction (PCR) based molecular techniques and the effect of salinity (NaCl) on these bacterial groups is also contained within this theme. The second theme is the use of low cost carbon sources to improve denitrification and nitrogen removal in saturated VFCWs. The third theme of this study is the performance of large scale VFCWs operating at CSBP Ltd, a chemical and fertiliser manufacturer based in Kwinana, Western Australia. The performance of the systems is assessed in regards to nitrogen and heavy metal removal. This theme also covers design and operational recommendations for improved nitrogen removal.

Laboratory scale VFCWs planted with *Schoenoplectus validus* were used to assess the impact of increasing salinity (up to 40gNaCl/L) on nitrification and on ammonia oxidising bacteria (AOB). Ammonia removal above 90% could be achieved in the fresh and saline wetlands when these were operated under a hydraulic loading rate of 11cm/d. This represented a removal rate in the order of 12gNH₃-N/m²/d. The gradual increase in salinity to 40gNaCl/L did not impact ammonia oxidation whereas the sudden increase (shock load) to 30gNaCl/L negatively impacted ammonia removal in the short term. Investigation of the microbial populations by terminal restriction fragment length polymorphism (T-RFLP) performed along with cloning and sequencing revealed that the increase in salinity selected for *Nitrosomonas* sp Nm 107-like (*Nitrosococcus mobilis*) and *Nitrosospira* sp 9SS1-like (*Nitrosospira multiformis*) AOB while other groups were eliminated or only present in very low proportions.

Nitrification and denitrification were further studied and the AOB and denitrifying bacterial (DB) community analysed in unplanted, fresh and saline, semi-saturated VFCWs dosed with acetic acid as carbon source. The semi-saturated design allowed nitrification to occur in the unsaturated sand layer and denitrification to occur in the saturated drainage layer where organic carbon was added, resulting in a high nitrogen removal. Nitrogen removal rates were on average 13.6gN/m²/d and 12.7gN/m²/d for the fresh and saline systems, respectively. Total nitrogen removal was significantly higher in the fresh system than in the saline system. The presence of salt, however, did not impact nitrate or COD removal and similar nitrate and COD concentrations were obtained in both wetlands. The gram-negative DB were also similar in both wetlands and dominated by representatives of the α and β-proteobacteria.
The feasibility of using carbon rich wastewater from a soft drink manufacturer (COD = 70,000mg/L), as exogenous carbon source to improve denitrification and nitrogen removal in saturated VFCWs treating high nitrate wastewater was tested. The addition of the carbon rich wastewater significantly increased nitrate removal from 23% to 65% and total nitrogen from 53% to 76%. Neither effluent ammonia nor effluent COD were affected by the addition of the carbon rich wastewater. Combining industrial wastewaters to improve treatability has proven to be cost effective and good example of industrial synergy with both economical and environmental benefits.

The case study covered the full scale treatment wetlands at CSBP Ltd. Firstly, heavy metal distribution, nitrogen removal performance and the AOB were analysed in the 1.3ha saturated surface VFCW, which has been operational since 2004. Secondly, the design rationale of two parallel nitrifying VFCWs, 0.8ha each, commissioned at CSBP Ltd in 2009 is described and the results from the first year of operation analysed.

The distribution of bioavailable Cu and Zn in the top sediment layer followed a horizontal profile with significantly higher concentrations near the inlet pipe than at the farthest location. The average total Cu concentration in the sediment at the 2m location has reached the 65mg/kg trigger value suggested by the Interim Sediment Quality Guidelines (ANZEEC 2000), indicating that increasing Cu levels could become toxic to plants and bacteria. From September 2008 to October 2009, the overall NH3-N and TN removal rates were 1.2gNH3-N/m²/d and 1.3gTN/m²/d, respectively. The 1.3ha wetland was operated in a sequencing batch mode, receiving highly fluctuating batch volumes and nitrogen concentrations. The majority of AOB sequences obtained were most similar to Nitrosomonas sp., while Nitrosospira sp. were less frequent.

The two VFCWs added to the treatment train in 2009 were designed assuming an NH3-N removal rate of 4.5gNH3-N/m²/d. Monitoring of the first year of data revealed that the cells operated under hydraulic and mass overloads. Ammonia oxidation was slightly higher than initially anticipated with the overall removal rate for the new cells being 5gNH3-N/m²/d. Since commissioning of the new cells ammonia discharges have been greatly reduced.

Overall, this thesis has demonstrated that vertical flow constructed wetlands can be effectively applied for the treatment of inorganic industrial wastewaters containing nitrogen. These systems have proven to harbour diverse salt tolerant nitrogen transforming bacteria, allowing them to operate reliably under varying salinities.
Abbreviations

AMO – ammonia monooxygenase
AOB – ammonia oxidising bacteria
BOD – biochemical oxygen demand
C – control
COD – chemical oxygen demand
CW – constructed wetland
DB – denitrifying bacteria
DO – dissolved oxygen
EC – electrical conductivity
FWS – free water surface
HF – horizontal flow
HLR – hydraulic loading rate
HRT – hydraulic retention time
NOB – nitrite oxidising bacteria
ORP – oxidation-reduction potential
OTU – operational taxonomic unit
PCR – polymerase chain reaction
SBR – sequencing batch reactor
SDW – soft drink manufacturer wastewater
SND – simultaneous nitrification and denitrification
T – treatment
TN – total nitrogen
TOC – total organic carbon
TP – total phosphorus
T-RFLP – terminal restriction fragment length polymorphism
TSS – total suspended solids
VF – vertical flow
VFCW – vertical flow constructed wetland
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Chapter 1:

Introduction

1.1 Industrial wastewaters

Rapid industrialisation in the last and current centuries has led to the generation of enormous amounts of liquid waste products. With the growth of industry, particularly the chemical industry, new problems appeared owing to the addition of new constituents into liquid waste streams (Doble and Kumar 2005; Callely et al., 1977). Industrial effluents were initially disposed of in the environment without treatment resulting in ecosystem damage and health problems. As people realised the detrimental environmental impacts and became more aware of the toxic effects that untreated industrial discharges could have, pressure on federal and local governments have resulted in the formulation of laws and guidelines which impose treatment procedures prior to effluent disposal (Doble and Kumar, 2005).

Most industries produce liquid wastes which require appropriate control and management. Under the current legislation, industrial effluents may be discharged either to sewer or to natural water courses providing they comply with the requirements of the appropriate statutory bodies (DEC, 1986; DEC, 2004a).

Three types of adverse effects on receiving water bodies are recognised. These are nutritional pollution, which can be either organic or inorganic; chemical pollution, which includes direct toxicity, pH and salinity alterations, and physical pollution, including changes in temperature, turbidity or surface properties of the water (Callely et al., 1977).

The choice for wastewater treatment systems will vary in relation to the pollutant to be removed. Treatment processes can be classified as physical, such as sedimentation and filtration; chemical, such as ion exchange, chemical oxidation or electrolysis; and biological, including microbial degradation and plant uptake. Most commonly applied to industrial and municipal wastewaters is the combination of all
processes. In the case of nitrogen laden wastewater, which is the major subject of this thesis, the usual approach is biological treatment.

Biological treatment of nitrogen rich wastewaters can be achieved in active conventional systems, such as activated sludge and sequencing batch reactors; these systems, however, have reasonably high energy requirements due to aeration. Nitrogen removal can also be achieved in passive natural systems such as ponds and constructed wetlands. Treatment in ponds and wetlands make use of energy flows naturally occurring in the water, sediments and plants, therefore, these systems have much lower energy requirements (Kadlec and Wallace, 2009).

1.2 Constructed wetlands for wastewater treatment

Constructed wetlands (CWs) for the purpose of wastewater treatment, also called treatment wetlands, can be defined as engineered or man made systems that utilise wetland plants, sediments and their associated microbial assemblages to treat an effluent or other water source (Vymazal, 2005). Constructed wetlands attempt to mimic natural wetlands and take advantage of their unique properties to convert or remove pollutants present in water.

Abundant water and temporary or prolonged saturation is the main characteristic of wetlands and this confers upon them properties that make them unique among other ecosystems on Earth (Kadlec and Wallace, 2009). Wetlands are considered regions of very high biological activity and productivity with swamps, marshes, mangroves and similar wetland formations comprising the most biologically productive ecosystems on the planet. Because of the high levels of biological and biochemical activity naturally occurring in wetlands they have the ability to convert common pollutants present in wastewaters into less harmful by-products (Kadlec and Wallace, 2009).

Historically natural wetlands have conveniently served as sewage discharge areas and continue to be used as discharge sites for treated and untreated wastewaters in many countries. The discharge of treated wastewater to natural wetlands can be
beneficial as long as the water is treated to a quality similar or superior to that of the receiving environment. Examples of beneficial discharges are those used to maintain environmental flows and natural water levels in currently drying rivers, lakes and wetland areas. The discharge of untreated or poorly treated wastewater in natural wetlands or other water bodies contributes to eutrophication, ecosystem failure and spread of diseases. The idea of using CWs as treatment systems, however, arose from the recognition that natural water bodies such as wetlands were capable of improving water quality. There are three main types of CWs for wastewater treatment and they are classified according to their hydrological regimes. The three types of wetlands will be described in detail in Chapter 2. The particular focus of this thesis is on vertical flow (VF) constructed wetlands (also termed VFCWs).

1.3 Benefits of using constructed wetlands

The energy requirements in terms of electricity/grid power to obtain effective treatment in constructed wetlands are usually quite low when compared to other treatment technologies. In most cases, electricity is only needed for pumping wastewater in or out of a wetland in the situations where gravity can not be applied. This reduced requirement for electricity is manly because of the natural environmental energies at work in wetlands, (Kadlec and Wallace, 2009). Table 1.1 shows the different energy requirements (Kw/hr/m^3) for different types of wastewater treatment plants to achieve a similar level of treatment.

Table 1.1: Energy requirements for different types of wastewater treatment plants (extracted from Kadlec and Wallace, 2009)

<table>
<thead>
<tr>
<th>System</th>
<th>Hydraulic load (m^3/d)</th>
<th>Energy use (Kw/hr/m^3)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Water Surface wetlands</td>
<td>3,786</td>
<td>0.11</td>
<td>Brix (1999)</td>
</tr>
<tr>
<td>Subsurface flow wetlands</td>
<td>3,786</td>
<td>0.11</td>
<td>Brix (1999)</td>
</tr>
<tr>
<td>Facultative lagoon + Rapid infiltration</td>
<td>3,786</td>
<td>0.11</td>
<td>Campbell and Ogden (1999)</td>
</tr>
<tr>
<td>Facultative lagoon + overland flow</td>
<td>3,786</td>
<td>0.16</td>
<td>Crites et al. (2006)</td>
</tr>
<tr>
<td>Aerated subsurface flow wetlands</td>
<td>5,500</td>
<td>0.16</td>
<td>Wallace et al. (2006)</td>
</tr>
<tr>
<td>Tidal flow wetlands (fill and drain)</td>
<td>1,000</td>
<td>0.18</td>
<td>Maciolek and Austin (2006)</td>
</tr>
<tr>
<td>Carrousel oxidation ditch</td>
<td>3,786</td>
<td>0.51</td>
<td>USEPA (1996)</td>
</tr>
<tr>
<td>Trickling filter + nitrogen removal</td>
<td>3,786</td>
<td>0.61</td>
<td>Crites et al. (2006)</td>
</tr>
<tr>
<td>Activated Sludge + nitrification</td>
<td>3,786</td>
<td>0.76</td>
<td>Campbell and Ogden (1999)</td>
</tr>
<tr>
<td>Extended aeration package plant</td>
<td>3,786</td>
<td>1.06</td>
<td>USEPA (1996)</td>
</tr>
<tr>
<td>Sequencing batch reactor</td>
<td>303</td>
<td>1.13</td>
<td>USEPA (1996)</td>
</tr>
<tr>
<td>Living machine</td>
<td>3,786</td>
<td>1.51</td>
<td>USEPA (1996)</td>
</tr>
</tbody>
</table>
Because wetlands are passive systems relying on physical, chemical and biological activities occurring in the water, macrophytes, substrates and their respective interfaces, they usually require a larger land area per volume of water to be treated than other conventional wastewater treatment systems. While wastewater spends only a few hours in conventional systems, the retention time is in the order of days in wetland systems. Once built, wetlands are very cheap to operate, requiring minimum maintenance and low ongoing costs.

Some of the main benefits of using treatment wetlands include (DLWC, 1998):

- lower capital and operating costs;
- greater intangible benefits (habitat creation, opportunities for community involvement and environmental education);
- greater potential stability and reliability.

According to Wallace (2010) some of the drivers for the uptake of constructed wetlands by industries are: the ability, or desire, to trade land for mechanical complexity, which results in substantially less maintenance and operator input; the recognition that industrial contamination is inherently long-term, therefore the operating cost savings offered by wetland systems presents a compelling value proposition for industry; the contamination source is in a remote or difficult to access situation, so the intrinsic stability of wetland ecosystem processes are favourable in terms of regulatory compliance.

Considerable research has been conducted into the different areas of constructed wetland for industrial wastewater treatment. Common themes may include influence of plants on treatment, types of media, types of wetland system and wastewater, pollutant removal mechanisms, and others. It has become clear that microbial processes dictate the performance of wetlands designed to treat nitrogen rich wastewaters. There is still a lack of knowledge concerning the nitrogen transforming microbial community composition within constructed wetlands treating fresh and saline industrial wastewaters. Monitoring how these systems perform in large scale industrial applications allows the dataset of systems operating in Australia to increase. Generating performance data is not only essential to wetland modelling and design but also demonstrates the efficacy of such technology.
1.4 Scope of this thesis

The main research question of this thesis is: can vertical flow constructed wetlands be used to effectively treat nitrogen rich inorganic industrial wastewaters? In order to answer this question, three research themes have been developed, all three themes revolve around the applicability of using VFCWs to treat nitrogen rich inorganic wastewater and stormwater runoff from CSBP Ltd, a chemical and fertiliser manufacturer located in Kwinana, Western Australia. The first theme is the study of nitrification and denitrification in these systems and the characterisation of the bacterial groups responsible for nitrogen transformations, in particular the ammonia oxidising bacteria (AOB) and denitrifying bacteria (DB) present in the substrate. As part of this study the effects of varying NaCl concentrations on nitrification and denitrification and respective bacterial groups were investigated. The second research theme is the use of high organic carbon content industrial wastewaters as low cost external carbon source to improve denitrification and nitrogen removal. The third theme is the performance assessment and optimisation of full scale systems operational at CSBP Ltd.

The specific objectives of this study were to:

- Verify the feasibility of using VFCWs to remove nitrogen from fresh and saline industrial wastewaters.
- Verify the impact of gradual and abrupt increases of NaCl on nitrification efficiency and on AOB in VFCWs.
- Characterise the AOB and DB communities in fresh and saline VFCWs supplemented with external carbon.
- Determine the suitability of low cost carbon sources to improve nitrate removal in VFCWs.
- Design VFCWs systems to achieve nitrification of up to 1600m$^3$/day of ammonia rich wastewater.
- Monitor nitrogen and heavy metal removal performance in a full scale VF wetlands and how heavy metals distribute in the sediment and plant tissue.
• Characterize the AOB community in a full scale VF wetland subject to metal accumulation.

The objectives above were addressed by conducting three major laboratory experiments and by having a full scale industrial wastewater treatment train, composed of wetland of various ages, as a case study. The experiments and case studies are elaborated in the chapters following the Literature Review (Chapter 2) as explained below.

Chapter 3 examines the hydraulic loading limits in terms of ammonia oxidation in intermittently fed VF wetlands operating at different salinities and attempts to characterise the AOB in these systems in relation to NaCl concentration, depth and season.

Because of the aerobic conditions prevailing in unsaturated VF wetlands these systems are very efficient in nitrifying but fail to denitrify and therefore present low or absent nitrate or total nitrogen removal capacity. A modification of the systems used in Chapter 3 is investigated in Chapter 4. These batch fed VF wetlands are unsaturated in the main filtering layer and are kept constantly saturated in the drainage layer where acetic acid is introduced as source of reducing power for denitrification. Nitrifying and denitrifying performance in saline and non saline conditions are examined and the respective bacterial groups characterised.

The suitability of using low cost organic carbon sources for denitrification is discussed in Chapter 5. External carbon sources such as methanol, ethanol, molasses or acetic acid can represent an extra cost in nitrogen removal systems; in this chapter high COD wastewater from a soft drink manufacturing plant was tested as a carbon source in saturated VF wetlands.

The performance of a large scale constructed wetland treating inorganic wastewater at CSBP is analysed in Chapter 6. A focus on how heavy metals distribute in the sediment and plant rhizome is given in this chapter. Chapter 7 describes nitrogen removal and the associated AOB in this system.
Chapter 8 describes the design rationale of two large scale nitrifying batch fed VF wetlands, their construction stages and NH$_3$-N removal performance for the first year of operation.

Chapter 9 is a summary of the insights obtained in this thesis. Conclusions and recommendations for further research are addressed.
Chapter 2:

Literature Review

2.1 Initial constructed wetland research

It was researcher Käthe Seidel from the Max Planck institute in Germany who conducted the first experiments in using wetland plants to improve water quality in the early 1950s (Seidel, 1955 in Vymazal, 2005). Seidel tested numerous innovative designs to treat a variety of wastewaters, including phenol, dairy and livestock wastewaters. Her systems included shallow tray-like ditches planted with macrophytes (wetland plants) and were initially called the hydrobotanical method (Seidel, 1965 in Vymazal, 2005). Later she incorporated different stages of treatment using sandy soils with high hydraulic conductivity planted with Phragmites australis. Primary sludge and sewage were firstly filtered vertically through an unsaturated sand bed and then the percolate flowed horizontally through an elimination bed (Seidel, 1965 in Vymazal, 2005). The systems tested by Käthe Seidel formed the basis of the three main types of constructed wetlands that are currently in use. The use of systems in combination forms what is now popularly known as hybrid wetlands (Seidel, 1955, 1976 in Vymazal, 2005).

2.2 Types of constructed wetlands

Constructed wetlands can be classified according to their hydrologic and water flow regimes. The three main types of constructed wetlands currently used are described below, in all cases a plastic or clay liner is usually used to prevent wastewater seepage or underground infiltration into the systems.

Free Water Surface (FWS) CWs mimic natural marshes having areas of open water and areas of macrophytes. Floating, submerged or emergent vegetation can be present in FWS wetlands. These systems are also called surface flow wetlands. They are
similar to aerobic ponds, usually shallow and having the water flowing through the stands of vegetation (Figure 2.1).

Horizontal subsurface flow (HF) CWs consist of gravel or coarse sediment beds planted with emergent macrophytes. The water is kept below the surface of the gravel and it flows horizontally through the media and plant roots from the inlet to the outlet area. These systems are also known as reed beds or root zone method (Figure 2.2).

Vertical Flow (VF) CWs consist of sand or gravel beds planted with emergent macrophytes. The water is distributed on the surface of the bed and then percolates through the media down to the outlet zone usually placed on the bottom of the bed (Figure 2.3). VF wetlands can also be operated in an up-flow manner, by inverting the position of inlet and outlet pipes, or in a tidal fashion, with fill and drain cycles.

FWS CWs allow for algal growth, ultraviolet disinfection and can have a combination of aerobic and anaerobic environments. The area for biofilm attachment and growth, however, is limited to the sediments on the bottom or leaves and stems of macrophytes. Due to the presence of the gravel or sand substrate subsurface flow wetlands act as filters and have a much higher surface area available for biofilm growth and present higher pollutant removal rates than surface flow wetlands. HF CWs are saturated systems being mainly anaerobic or anoxic and, therefore, present low capacity for oxidation processes such as nitrification or aerobic respiration. VF CWs, on the other hand, are usually unsaturated or only temporarily saturated (in the case of tidal flow or fill and drain wetlands) and therefore favour oxidation processes while reduction processes such as denitrification are only marginal. Different wetlands can be used in combination with one another (hybrid systems) to enhance removal of pollutants. For example it is common to see a VF wetland followed by a HF or FWS wetland, or a HF system followed by a VF with recirculation (Cooper, 1999).
Figure 2.1: Section view of a typical FWS wetland (Extracted from Kadlec and Knight, 1996).

Figure 2.2: Section view of a typical HF wetland with details of major components.

Figure 2.3: Section view of a typical VF wetland with details of major components (picture modified from Tilley et al., 2008).
2.3 Common uses of constructed wetlands

Constructed wetland technology can be applied to treat different wastewaters. As mentioned in section 2.1 they were initially used to treat phenol, dairy and livestock wastewaters as well as domestic effluents (Seidel, 1955 in Vymazal, 2005). Since the 1950’s constructed wetlands have been applied to treat an increasing variety of waters including domestic and municipal wastewaters, industrial wastewaters, agricultural field runoff, animal wastewaters including those from livestock, poultry, swine and aquaculture operations, landfill leachate, contaminated groundwater, urban stormwater runoff and also relatively clean waters from rivers and lakes as pre treatment for human supply schemes (Kadlec and Wallace, 2009).

While there is a vast body of literature discussing domestic and municipal wastewater treatment in constructed wetlands the focus of this section will be primarily on industrial effluents and stormwater runoff. For comprehensive information on domestic and municipal effluents the reader is directed to Dallas et al., (2004); Brix and Arias (2005); USEPA (2000) and Kadlec and Wallace (2009). Detailed information on the use of CWs for landfill leachate treatment is given in Bulc (2006), Johnson et al. (1999) and Vrhovsek et al. (2000).

2.3.2 Industrial wastewaters

Because of their versatility, CWs have been increasingly used to treat nearly all types of industrial effluents. They have been effectively applied to highly organic effluents from food processing facilities such as potato (Burgoon et al., 1999), sugar and ethanol (Salati et al., 1999), dairy (Mantovi et al., 2003), beverages, wine (Shepherd et al., 2001) and olive oil (Grafias et al., 2010), and to moderately low organic content waters from mines (O’Sullivan et al., 2004), chemical and fertiliser manufacturers (Domingos et al., 2007, 2008), tanneries (Calheiros et al., 2007) amongst others. Some of these industrial effluents can be very acidic or saline so specific media for pH control and careful selection of salt tolerant plants is required. Some examples
of constructed wetlands used for industrial wastewater treatment can be seen in Table 2.1 below:

Table 2.1: Examples of CWs treating industrial wastewater

<table>
<thead>
<tr>
<th>Low organic load</th>
<th>High organic load</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid coal mine drainage</td>
<td>Dairy effluents</td>
</tr>
<tr>
<td>Metal ores mine drainage</td>
<td>Winery effluents</td>
</tr>
<tr>
<td>Metallurgic industry</td>
<td>Meat processing</td>
</tr>
<tr>
<td>Refinery process waters</td>
<td>Potato processing</td>
</tr>
<tr>
<td>Paper and pulp wastewaters*</td>
<td>Tannery</td>
</tr>
<tr>
<td>Fertiliser and chemical</td>
<td>Olive oil</td>
</tr>
<tr>
<td>manufacturer</td>
<td>Sugar mill</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Low organic load</th>
<th>High organic load</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spain</td>
<td>Italy</td>
</tr>
<tr>
<td>Ireland</td>
<td>US/Italy</td>
</tr>
<tr>
<td>Argentina</td>
<td>Australia</td>
</tr>
<tr>
<td>U.S</td>
<td>Australia</td>
</tr>
<tr>
<td>U.S.</td>
<td>Australia</td>
</tr>
<tr>
<td>(Ramirez Masferrer, 2002)</td>
<td>(Mantovi et al., 2003)</td>
</tr>
<tr>
<td>(O'Sullivan et al., 2004)</td>
<td>(Shepherd et al., 2001; Masi et al., 2002)</td>
</tr>
<tr>
<td>(Maine et al., 2007)</td>
<td>(Kurup, 2007)</td>
</tr>
<tr>
<td>(Litchfield &amp; Schatz, 1989)</td>
<td>(Burgoon et al., 1999)</td>
</tr>
<tr>
<td>(Tettleton, et al., 1993)</td>
<td>(Calheiros et al., 2007)</td>
</tr>
<tr>
<td>(Domingos et al., 2007, 2008)</td>
<td>(Grafias et al., 2010)</td>
</tr>
<tr>
<td>(Domingos et al., 2007)</td>
<td>(Salati et al., 1999)</td>
</tr>
</tbody>
</table>

*Paper and pulp mill wastewater can have high organic load content.

2.3.3. Storm water runoff

Stormwater runoff is surface water resultant from rain events. Uncontrolled urban stormwater has been identified as a major contributor to non point source pollution of surface waters and shallow aquifers. Concentrations and loads of pollutants can range from high, in heavily developed industrialised and urban areas, to low in undeveloped areas and parkland. In order to address surface and ground water pollution stormwater management has gained special attention in recent years (Reed et al., 2005; DEC, 2004b). As a result, best management practices (BMPs) to control the quantity and quality of runoff have been implemented in Australia, Europe and United States (DEC, 2004b; Scholes et al., 1999; Kadlec and Wallace, 2009). These practices include the incorporation of pervious surfaces in urban landscapes, construction of swales, ponds and treatment wetlands.

The main type of constructed wetland applied in runoff treatment is FWS, which can be unlined to allow aquifer recharge or lined to prevent seepage to groundwater.
These systems favour settling of suspended solids and transformation of dissolved pollutants prior to discharge. Reed et al. (1995) state that stormwater wetlands usually include a combination of deep ponds and shallow marshes, additionally wet meadows and shrub areas can be present. Wetlands and ponds can also be used to collect and store stormwater during the rainy season and allow controlled discharges during dry periods.

The hydraulic characteristics and therefore design rationale of stormwater wetlands differ from other wetlands such as those treating domestic, municipal or industrial effluents. The main difference is that stormwater wetlands are event based systems receiving irregular flows as consequence of rain patterns and storm events. Wetlands treating domestic, municipal and industrial wastewaters, on the other hand, tend to have more regular inflows throughout the year. Flow variations in these systems either reflect human daily and seasonal water use patterns in domestic and municipal systems or process water usage in industrial systems.

The full scale VFCWs at CSBP Ltd, analysed in the case study component of this thesis (Chapters 6 to 8), are subject to more or less regular inflows generated at the production and processing facilities of the industry and also to irregular stormwater flows which vary according to the local climate. These regular and irregular flow conditions have implications on the performance of these systems; such implications will be further discussed in Chapters 6, 7 and 8.

2.4 Pollutant removal mechanisms in constructed wetlands

Removal of pollutants from wastewater is essential for the protection and maintenance of receiving aquatic environments. In order to avoid depreciation of water resources, regulating government agencies are setting increasingly more stringent pollutant levels in discharged waters. In an attempt to meet licensed discharge criteria the wastewater industry realises that the use of cost-effective and environmentally friendly technologies such as constructed wetlands play an important role in pollutant removal. With the ability to mimic nature and yet being an engineered system, key parameters such as hydraulic loading rate (HLR) and hydraulic retention time (HRT), pH,
wet and dry cycles, oxidation-reduction (redox) potential, among others, which dictate removal processes, can be easily regulated in order to enhance removal of one or more selected pollutants. It has been verified that the maturity of a CW system is an important factor influencing its removal efficiency – pollutant removal in wetlands is dependent on plant and microbial mediated reactions, plant and microbial communities take time to establish and reach steady state conditions. Because of this, it is common that new wetlands improve in pollutant removal performance as they age (Bulc, 2006).

The following sections will highlight some major pollutant removal mechanisms in constructed wetlands with a greater focus on nitrogen and heavy metals which are commonly found in inorganic industrial wastewaters.

2.4.1 Organic carbon (C)

While organic carbon is a major pollutant in municipal and food/beverage processing wastewaters, in wastewaters from chemical and fertiliser industries, carbon is usually lacking. The lack of carbon has major implications for nitrogen removal systems relying on the conventional denitrification pathway and it will be further discussed in the nitrogen section.

Carbon is not considered difficult to remove biologically and, in general, wetlands are efficient in the reduction of organic matter. Organic carbon compounds in wastewaters are usually quantified and monitored indirectly by different analytical techniques with the most common being:

Biochemical Oxygen Demand (BOD) is a measure of the oxygen consumed by microorganisms in the oxidation of organic matter. This test usually runs for 5 days and the final oxygen consumption is designated BOD₅.

Chemical oxygen demand (COD) is the amount of a chemical oxidant, usually potassium dichromate, required to oxidise the organic matter. This measure is larger than BOD because the strong oxidant oxidises more compounds than microorganisms do.
Total organic carbon (TOC) determination usually involves measuring the inorganic and organic carbon fractions present in a sample and then subtracting the inorganic component (i.e. CO₂) from the total.

Measurements of organic carbon can be made before or after filtration in 0.45μm pore size filter, resulting in total and dissolved COD, BOD or TOC.

**2.4.1.1 Organic Carbon reactions**

Treatment wetlands may receive large amounts of external organic carbon. Degradable carbon is decomposed or transformed by a variety of reactions depending on the conditions present in the wetland. Under aerobic conditions carbon compounds are oxidised via respiration by microorganisms with oxygen being the final electron acceptor. When no oxygen is available other electron acceptors are involved in decomposition. Under anoxic (no dissolved molecular oxygen is available) or anaerobic (no dissolved molecular nor bound oxygen) conditions carbon compounds are degraded via fermentation, denitrification, iron/sulfate reductions or methanogenesis. Important reactions are represented below (Kadlec and Wallace, 2009, Mitsch and Gosselink, 1993).

In aerobic conditions respiration prevails (glucose as substrate):

\[ C_6H_{12}O_6 + 6 O_2 \rightarrow 6 CO_2 + 6 H_2O \]

(2.1)

In anoxic or anaerobic conditions fermentation occurs:

\[ C_6H_{12}O_6 \rightarrow 2 CH_3CHOHCOOH \text{ (lactic acid)} \]

(2.2)

\[ C_6H_{12}O_6 \rightarrow 2 CH_3CH_2OH \text{ (ethanol)} + 2 CO_2 \]

(2.3)

Nitrate (NO₃⁻) reduction (denitrification) occurs in anoxic zones:

\[ C_6H_{12}O_6 + 4 NO_3^- \rightarrow 6 CO_2 + 6 H_2O + 2 N_2 + 4 e^- \]

(2.4)

Iron reduction occurs in anaerobic conditions (acetate as substrate):

\[ CH_3COO^- + 8 Fe^{3+} + 3 H_2O \rightarrow 8 Fe^{2+} + CO_2 + HCO_3^- + 2 H_2O + 8H^+ \]

(2.5)
Sulfate reduction occurs under anaerobic conditions (lactate and acetate as substrates):

\[
2 \text{CH}_3\text{CHOHCOO}^- + \text{SO}_4^{2-} + \text{H}^+ \rightarrow 2 \text{CH}_3\text{COO}^- + 2 \text{CO}_2 + 2 \text{H}_2\text{O} + \text{HS}^- \tag{2.6}
\]

\[
\text{CH}_3\text{COO}^- + \text{SO}_4^{2-} + 2 \text{H}^+ \rightarrow 2 \text{CO}_2 + 2 \text{H}_2\text{O} + \text{HS}^- \tag{2.7}
\]

Methanogenesis occurs in anaerobic conditions (acetate as substrate or CO₂ reduction):

\[
\text{CH}_3\text{COO}^- + 4 \text{H}_2 \rightarrow 2 \text{CH}_4 + \text{H}_2\text{O} + \text{OH}^- \tag{2.8}
\]

\[
4 \text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2 \text{H}_2\text{O} \tag{2.9}
\]

The contribution of each process to carbon removal is variable and will depend on the nature of the wastewater and the type of constructed wetland, for example in FWS wetlands all process may occur in similar proportions, aerobic respiration near the surface and in the water column and anoxic/anaerobic reactions on the bottom and sediments. In HF wetlands oxygen transfer is negligible, therefore it is likely that anoxic and anaerobic reactions will prevail. In unsaturated VF wetlands, however, aerobic processes dominate.

2.4.2 Nitrogen (N)

Nitrogen is an essential nutrient for all living organisms, being present in the form of proteins, nucleic acids, adenosine phosphates and pigments. (Hagopian and Riley, 1998). Yet, nitrogen compounds are among the main pollutants of concern in wastewater because of their role in, (among with other nutrients such as phosphorus) contributing to eutrophication, favouring algal blooms, and depreciation of dissolved oxygen levels in receiving water bodies. Furthermore, unionised ammonia (NH₃) and nitrite (NO₂⁻) are toxic to fish and other aquatic organisms in low concentrations. In water and wastewater the most important inorganic forms of nitrogen are ammonium (NH₄⁺), NO₃⁻, NO₂⁻, and dissolved nitrogen gas (N₂). Organic nitrogen compounds in wastewaters originate from food, faeces and urine, these high molecular weight compounds (proteins, urea, amino acids) are proteolised and deaminated to ammonia. The overall multi step biochemical process by which ammonia is released from
organically bound N is called ammonification. The rate of ammonification is related to the rate of organic matter degradation but it occurs quickly in an aquatic environment. Ammonia nitrogen exists in aqueous solution as either unionised ammonia or the ammonium ion depending on the pH and temperature as described below (Crites and Tchobanoglous, 1998):

\[
NH_3 + H_2O \leftrightarrow NH_4^+ + OH^- \tag{2.10}
\]

Un-ionised ammonia is relatively volatile and can be removed from solution to the atmosphere via diffusion in a process called ammonia volatilisation. At higher pH and temperatures the equilibrium (eq 2.10) shifts to the left and more un-ionised ammonia is present in solution and likely to volatilise. Ammonia volatilisation plays a minor role in nitrogen removal in wetlands where neutral or acidic conditions prevail as little unionised ammonia is present.

Table 2.2 shows the oxidation states that elemental nitrogen and corresponding inorganic forms may assume. Proteinaceous nitrogen is present in the most reduced form (-3). The same is true for the nitrogen atom in ammonia and ammonium. For nitrite the oxidation state is +3 and for nitrate it is +5, the most oxidised state of N (Hagopian and Riley, 1998). Nitrite is an intermediate oxidation state between ammonia and nitrate and because of this intermediate energetic condition, nitrite is not chemically stable in the environment and is usually found only in low concentrations (Kadlec and Wallace, 2009).

<table>
<thead>
<tr>
<th>Species</th>
<th>Common name</th>
<th>oxidation state N</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₃</td>
<td>Anhydrous or free or un-ionized ammonia, ammonia gas</td>
<td>-3</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>Ammonium, ammonium ion, ionized ammonia</td>
<td>-3</td>
</tr>
<tr>
<td>NH₄OH</td>
<td>Ammonium hydroxide</td>
<td>-3</td>
</tr>
<tr>
<td>N₂H₄</td>
<td>Hydrazine</td>
<td>-2</td>
</tr>
<tr>
<td>NH₂OH</td>
<td>Hydroxylamine</td>
<td>-1</td>
</tr>
<tr>
<td>N₃⁻</td>
<td>Azide ion</td>
<td>-1/3</td>
</tr>
<tr>
<td>N₂</td>
<td>Nitrogen, nitrogen gas, dinitrogen gas</td>
<td>0</td>
</tr>
<tr>
<td>N₂O</td>
<td>Nitrous oxide, nitrous oxide gas, ‘laughing gas’</td>
<td>+1</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide, nitric oxide gas</td>
<td>+2</td>
</tr>
<tr>
<td>NO₂⁻</td>
<td>Nitrite ion</td>
<td>+3</td>
</tr>
</tbody>
</table>
Conventional nitrogen removal is usually achieved biologically by two distinct processes: autotrophic nitrification, the oxidation of ammonia to nitrate (Eqs 2.11, 2.12) and heterotrophic denitrification, the reduction of NO₂⁻ or NO₃⁻ into N₂ (Eqs 2.13-2.15). Other less conventional and recently understood nitrogen removal methods will be discussed later.

### 2.4.2.1 Nitrification

Nitrification has been typically referred to two separate strictly aerobic processes carried out by phylogenetically distinct chemoautotrophic bacterial groups as described below, although it is now recognised that heterotrophic nitrification (Keeney, 1973; Paul and Clark, 1996) and archaeal nitrification (Wuchter et al., 2006) occur and can be of global significance. The first step in nitrification, the oxidation of ammonia to nitrite (Eq 2.11), also called nitritation, is carried out by ammonia oxidising bacteria (AOB). Comparative 16S rRNA sequence analyses of cultured AOB revealed that members of this physiological group are confined to two monophyletic lineages within the class *Proteobacteria* (Purkhold et al., 2000). One group is affiliated with the gamma-subclass of the class *Proteobacteria* and includes *Nitrosococcus oceani*, the other group is affiliated within the beta-subclass of the *Proteobacteria* and includes the genera *Nitrosomonas* (including *Nitrosococcus mobilis*), *Nitrosospira, Nitrosolobus* and *Nitrosovibrio* (Purkhold et al., 2000). It has been suggested, however, that *Nitrosolobus* and *Nitrosovibrio* should be reclassified within the genus *Nitrosospira* (Head et al., 1993).

\[
\text{NH}_3 + 1.5\text{O}_2 \rightarrow \text{NO}_2^- + \text{H}_2\text{O} + \text{H}^+ + 84 \text{ kcal mol}^{-1} \quad (2.11)
\]

Note that 48g of oxygen are necessary for the oxidation of 14g of ammonia nitrogen (3.43gO₂/gNH₃-N) into nitrite.
Biochemically equation 2.11 is subdivided into two reactions (2.12 and 2.13) with hydroxylamine (NH$_2$OH) as an intermediate compound as represented and explained below (Kowalchuk and Stephen, 2001):

(A) $2H^+ + NH_3 + 2e^- + O_2 \rightarrow NH_2OH + H_2O$ (catalysed by AMO) \hspace{1cm} (2.12)

(B) $NH_2OH + H_2O \rightarrow HONO + 4e^- + 4H^+$ (catalysed by HAO) \hspace{1cm} (2.13)

(C) $2H^+ + 0.5O_2 + 2e^- \rightarrow H_2O$ (electron transport chain, proton motive force) \hspace{1cm} (2.14)

The first reaction (A) is catalysed by ammonia monooxygenase (AMO), a membrane bound enzyme and the second (B) is catalysed by hydroxylamine oxidoreductase (HAO), an enzyme associated with the periplasm. In reaction (C) two of the electrons (and H$^+$) generated by (B) pass via the electron transport chain to the terminal oxidase. The electron transport chain generates a proton motive force necessary for ATP production and therefore cell energy. Two moles of H$^+$ are released into the environment lowering the pH of the water. The sum of the three reactions is Eq 2.11 (Kowalchuk and Stephen, 2001). Nitrification of ammonia to nitrate consumes approximately 7.1g of alkalinity (as CaCo$_3$) for each gram of ammonia nitrogen nitrified (USEPA, 1993).

The nitrite produced is further oxidised by nitrite oxidising bacteria (NOB) in the second step of nitrification (Eq 2.15). NOB are comprised by the genera *Nitrobacter*, *Nitrospira*, *Nitrococcus* and *Nitrospina* (Watson *et al.*, 1986; Meicke *et al.*, 1989). The enzyme nitrite oxidoreductase, also know as nitrite dehydrogenase, catalyses nitrate formation (Hagopian and Riley, 1998).

\[ NO_2^- + 0.5O_2 \rightarrow NO_3^- + 17.8 \text{ kcal mol}^{-1} \] \hspace{1cm} (2.15)

During the oxidation of nitrite 16g of oxygen are required to oxidise 14g of nitrite nitrogen, (1.14gO$_2$/g NO$_2^-$-N). The final oxygen demand to fully nitrify 1g of NH$_3$-N is therefore 4.57g of O$_2$. Because of this high oxygen demand the elimination of ammonia is of great importance in wastewater treatment in order to prevent oxygen depletion in receiving waters.

All chemolithoautotrophs are characterised by the ability to utilise an inorganic chemical substrate (e.g. NH$_3$, NO$_2^-$) as a source of electrons for the immobilisation of
inorganic carbon (i.e. CO$_2$ or HCO$_3^-$) into biomass. They are also aerobic, usually employing O$_2$ as final electron acceptor. This sole energy source is used for carbon fixation, assimilation of monomers into precursor metabolites and subsequent polymerization of building blocks and macromolecules (Hagopian and Riley 1998). Bacteria derive only a small amount of energy from the combined processes of nitrification, only 0.17 g of dry weight biomass is produced when 1g of NH$_3$-N is oxidised (USEPA, 1993). The first exogenous reaction (2.11) releases more than four times more energy than the second (2.15) (Gibbs and Schiff, 1960).

2.4.2.2 Denitrification (dissimilatory nitrate reduction, nitrate dissimilation, nitrate respiration)

Under anoxic conditions and when easily biodegradable carbon is available heterotrophic organisms reduce NO$_3^-$ → NO$_2^-$ → NO → N$_2$O → N$_2$ according to equations below, acetate is used as the electron donor in this case (Third, 2003):

\[
8\text{NO}_3^- (aq) + 2\text{CH}_3\text{COOH} \rightarrow 8\text{NO}_2^- (aq) + 4\text{CO}_2 + 4\text{H}_2\text{O} \quad (2.16)
\]

\[
8\text{NO}_2^- (aq) + \text{CH}_3\text{COOH} + 2\text{H}_2\text{O} \rightarrow 8\text{NO} (g) + 2\text{CO}_2 + 8\text{OH}^- \quad (2.17)
\]

\[
8\text{NO} (g) + \text{CH}_3\text{COOH} \rightarrow 4\text{N}_2\text{O} (g) + 2\text{CO}_2 + 2\text{H}_2\text{O} \quad (2.18)
\]

\[
4\text{N}_2\text{O} (g) + \text{CH}_3\text{COOH} \rightarrow 4\text{N}_2 (g) + 2\text{CO}_2 + 2\text{H}_2\text{O} \quad (2.19)
\]

\[
8\text{NO}_3^- (aq) + 5\text{CH}_3\text{COOH} \rightarrow 4\text{N}_2 (g) + 10\text{CO}_2 + 6\text{H}_2\text{O} + 8\text{OH}^- \quad (2.20)
\]

The ability to denitrify is widespread among bacteria of unrelated systematic affiliations, most likely due to lateral gene transfer in evolution (Bothe et al., 2000). Also other organisms such as Archaea (Zumft, 1997), the mitochondria of some fungi (Kobayashi et al., 1996) and even Nitrosomonas (Bock et al., 1995) have been reported to denitrify. As seen in equation 2.20, alkalinity (OH-) is produced during denitrification.

Because of the opposing oxygen requirements for nitrification and denitrification, these processes are usually separated by space (activated sludge) or time (sequencing batch reactors) in conventional municipal wastewater treatment plants (Third, 2003). Denitrification requires an organic carbon source which is usually lacking at the end of the nitrification stage where organic matter has already been
oxidised. External carbon sources such as methanol, acetate or molasses are often added to the anoxic, post nitrification, phase of a treatment system in order to improve denitrification rates (Bernet et al., 1996). This practice however can incur in significant extra cost and requires careful dosing. Another option is the recycling of nitrified effluent to the initial anoxic zone where reducing power is present, but recycling is costly and also reduces the hydraulic capacity of the treatment system (Third, 2003).

The carbon (energy) requirements for denitrification (according to equation 2.20) are 2.68g of acetate per gram of nitrate nitrogen. For methanol and glucose, the requirements are 1.90g and 2.67g, respectively, per gram of nitrate nitrogen (Kadlec and Wallace, 2009). Gersberg et al. (1984) indicated an optimum carbon level of 2.3gBOD/gNO3-N while in terms of COD, Bernet et al. (1996) indicated a requirement of 2.85g COD/gNO3-N.

In soils and wetlands where both aerobic and anaerobic zones coexist nitrification and denitrification are known to occur simultaneously, in a process called simultaneous nitrification and denitrification (SND). The lack of organic carbon in anoxic zones of constructed wetlands where nitrate is present can represent a limiting factor for denitrification (Langergraber, 2001).

As previously mentioned, the addition of a commercially available carbon source to enhance denitrification can represent a critical cost to the treatment process. Lin et al. (2002) added fructose to the influent of experimental FWS CWs treating nitrate contaminated groundwater and verified higher nitrate removal after the addition of fructose. Fructose, however, is more expensive than ethanol and acetic acid; therefore, cheaper alternative carbon sources such as industrial by-products or wastes have been investigated, and are still the focus of recent research. Bernet et al. (1996) achieved high denitrification rates using wine distillery effluent as an example of an industrial carbon source. Singer et al. (2008) used solid cotton wool as primary carbon source for denitrification in a bioreactor treating water in a recirculating aquaculture system. Other solid options which have been used successfully are wood chips and wheat straw (Saliling et al., 2007). Biodiesel waste (glycerine – g-phase) has been also successfully applied in laboratory and full scale denitrifying systems (Bodík et al., 2009). In a more recent study, Lu et al. (2009) verified the effectiveness of glucose
to improve denitrification in HF CWs. The authors further recommended testing of cheap alternative carbon sources in wetlands.

2.4.2.3 Simultaneous nitrification and denitrification (SND)

Simultaneous nitrification and denitrification can occur in a treatment system, (vessel, reactor or wetland) under identical operating conditions. SND reduces or eliminates the need for separate reactors required in conventional treatment plants and therefore simplify the design of treatment systems resulting in savings in time and space (Keller et al., 1997; Third, 2003). Usually the key factor for achieving SND in treatment plants is the tight control of dissolved oxygen (DO) concentrations (or redox potential) which have to be maintained at a level to allow aerobic oxidation of organic matter by heterotrophs, nitrification by AOB and NOB and concurrent denitrification by heterotrophs. SND has been extensively studied in sequencing batch reactors (SBR), because SBRs consist of one single vessel that undergoes time-separated aerobic and anoxic phases they more easily allow DO control in the aeration phase as to permit part of the organic carbon to be consumed by denitrifying organisms during the aeration phase but also to permit organic carbon passing through to the anoxic phase, more favourable for denitrification. The opposing DO requirements of nitrification and denitrification mean that by lowering DO concentration to improve denitrification, nitrification rates are reduced. A DO concentration of 0.5mg/L in the aeration phase of a SBR has been shown to result in nitrogen removal via SND at a moderate rate (Pochana and Keller, 1999). Other factors affecting SND in SBRs are the organic carbon source and the sludge floc size (Third, 2003).

Wetland environments are more complex than those in biological nutrient removal systems in conventional wastewater treatment plants (WWTPs). Research on treatment wetlands over the last two decades has demonstrated that these systems differ and that WWTP results may not apply to wetlands (Kadlec and Wallace, 2009). Simultaneous nitrification and denitrification in constructed wetlands occur as there is a complicated spatial zonation within these systems, with oxygen gradients occurring in close proximity between surface waters and bottom sediments (Kadlec and Wallace
2009) in FWS wetlands, and also oxygen gradients occurring as a result of spatial and temporal variations in fill and drain vertical flow wetlands which create alternating unsaturated and saturated environments. A representation of the different nitrogen transformation pathways in a FWS wetland can be seen in Figure 2.4. Some of the pathways represented in the figure are explained in the following section.

![Figure 2.4: Representation of the nitrogen cycle in a FWS wetland (picture extracted from Kadlec and Wallace, 2009)](image)

**2.4.2.4 Other N removal pathways**

Other possible N transformation pathways in wetlands include anaerobic ammonia oxidation (anammox), which is the anaerobic conversion of nitrite and ammonium to nitrogen gas in the absence of organic carbon (Mulder *et al.*, 1995; Strous *et al.*, 1999) as demonstrated below. Identification via 16S rDNA sequencing has
shown that anammox bacteria belong to a deep branching planctomycete (Strous et al., 1999).

\[ 1\text{NH}_4^+ + 1.3\text{NO}_2^- \rightarrow 1.02\text{N}_2 + 0.26\text{NO}_3^- + 2\text{H}_2\text{O} \] \hspace{1cm} (2.21)

Because of the reduced carbon and oxygen requirements, less than half the oxygen and no carbon, as compared to conventional routes, anammox is particularly suited to the treatment of high strength industrial wastewaters in which the ammonium content is high and the organic carbon content very low (Mulder et al., 1995). The discovery of this alternative route has led to the development of other processes that make use of anammox such as SHARON-anammox (Single reactor High Activity Ammonia Removal Over Nitrite), CANON (complete autotrophic nitrogen removal over nitrate) and OLAND (Oxygen Limited Autotrophic Nitrification-Denitrification). In the SHARON-Anammox process partial nitritation is achieved in one vessel by washing out NOB with high temperatures and short residence times (< 1 day) and anaerobic elimination of nitrite by ammonia is achieved in a second vessel (Hellinga et al., 1998). The CANON process performs both partial nitritation and anammox in a single reactor simultaneously, under oxygen-limitation. The process relies on the simultaneous interaction of aerobic and anaerobic ammonium oxidisers (Third, 2003). The OLAND process makes use of the ability of *Nitrosomonas* to denitrify using ammonium as the electron donor and nitrite as the electron acceptor, together with their ability to perform anaerobic ammonium oxidation in the presence of gaseous NO₂ (Schmidt and Bock, 1997).

Macrophyte uptake of ammonium and nitrate represents an important nitrogen removal process for many wetland systems. The majority of the assimilated nitrogen is subsequently released in the water during the death and decay cycles, providing a “flywheel” effect in a nitrogen removal time series (Kadlec and Wallace, 2009). A small part of the nitrogen containing residues is permanently stored as part of the creation of new soil and sediment, hence accretion represents a burial process for nitrogen. Accretion (ammonia net removal in the order of 10gN/m²/yr) is only of importance in lightly loaded systems but has no importance in heavy loaded wetlands (Kadlec and Wallace, 2009). Plant harvesting with the objective to remove nitrogen from the system does not represent a cost effective solution, proving to be expensive and labour
intensive (Reed et al., 1995). Harvesting trials in wetlands result in quite low amounts of nitrogen recovered in the biomass, accounting for up to 16% of the total nitrogen removed by the systems (Reed et al., 1995). Potential removal for floating large leaved plants (Eichhornia, Pistia, Hydrocotyle) is in the order of 100-250gN/m²/yr, and 50-150gN/m²/yr for floating small leaved plants (Salvinia, Lemna, Azolla) (Kadlec and Wallace, 2009), such plants however, especially the large leaved ones are considered weeds in many countries including Australia and therefore, apart from their use in some tropical countries, they are not largely applied in constructed wetlands.

Ammonium can be adsorbed onto active sites of the soil matrix. Because of the positive charge of the ammonium ion, it is subject to cation exchange. Adsorbed ammonium is bound loosely to the substrate and can be released easily when water chemistry conditions change. In continuous flow systems adsorbed ammonium and ammonium in solution is assumed to be in equilibrium. In intermittently fed wetlands where the substrate is exposed to oxygen by periodic draining, ammonium can be quickly removed from solution by sorption and subsequently oxidised to nitrate. Ammonia oxidation frees available adsorption sites. Nitrate is negatively charged and does not bind to the substrate being washed out by subsequent rewetting. The rapid removal of ammonia by adsorption and its depletion on the adsorption sites during draining and resting forms the basis for intermittently fed and tidal (fill and drain) vertical flow wetlands (Kadlec and Wallace, 2009).

Fill and drain operated systems, such as VF wetlands, have fluctuating conditions where environmental parameters oscillate regularly. These systems can sustain rich, diverse and effective microbial communities that can make use of the lowest levels of nutrients and survive with changing conditions on various time levels (Chiesa et al., 1985; Stal, 1994; Irvine et al., 1997; Third, 2003).
2.4.2.5 Nitrogen transforming bacteria community analyses in wetlands

Nitrogen transformations in constructed wetlands are principally carried out by complex microbial communities which are associated with the substrate, macrophytes roots and incoming wastewater (Vymazal, 2005).

Early studies on constructed wetlands have inferred that microbial processes influence treatment performance in wetlands, based primarily on the knowledge of processes in other wastewater treatment systems (Faulwetter et al., 2009). Faulwetter et al. (2009) also point out that circumstantial evidence based on measurement of changes in influent vs effluent water chemistry has been commonly used to corroborate microbial activity taking place in wetlands. Qualitative and quantitative data on the specific microorganisms in action, however, is usually lacking and has only been the focus of more recent research.

Research in wetland biogeochemistry has predominantly focused on processes, such as denitrification and methanogenesis, for example, and less often on microbial communities or on specific microbial populations (Gutknecht et al., 2006). Investigating functional microbial communities, along with assessing their diversity and distribution is important not only because it contributes to the characterisation of constructed wetlands by identifying key players in pollutant transformation (Sleytr et al., 2009) but it also contributes towards our mechanistic understanding of wetland ecosystem functioning (Gutknecht et al., 2006) which can be used to overcome the “black box” approach that has been commonly applied to treatment wetlands used for water pollution control.

There are several different methods to investigate microbial composition and diversity in the environment. Classical methods to analyse microbial abundance in environmental samples involve the use of liquid or solid media (general or selective) to allow the growth of microbes initially present in the sample (Truu et al. 2009). The growth of microbes in plates under controlled conditions and subsequent colony counting (plate counting), or the growth of microbes in liquid medium with serial dilutions (most probable number –MPN) are classical culture based microbiological methods. The applicability of culture based techniques to assess microbial diversity is
limited because they only allow the evaluation of the abundance of about 1% to 10% of the total community. The majority of microorganisms are not culturable under laboratory conditions or grow too slowly to be cultured within a practical timeframe, therefore, these methods are regarded to underestimate the real abundance of these organisms (Torsvik and Ovreas, 2002; Truu et al., 2009). MPN techniques, nevertheless, have been used to investigate functional groups such as ammonia and nitrite oxidisers in planted HF wetlands (Truu et al., 2005) and sand filter studies (Bahgat et al., 1999).

With the advancement of molecular biology in the last two decades, culture-independent molecular techniques have permitted for problems relating to cultivating slow growing organisms (e.g. AOB) to be overcome. These DNA-based methods are advantageous because they can be used to assess nearly all forms of microorganisms, given there is published sequencing information on conserved areas of their genomes. The reason being is that DNA-based methods rely on polymerase chain reactions (PCR) to amplify genetic markers (i.e. a gene or DNA sequence with a known location on a chromosome and associated with a particular gene or trait) using different sets of primers for ribosomal genes (16S rDNA) and functional genes (Truu et al., 2009).

Microbial ecology studies have benefited from molecular techniques which permit identification of organisms down to species level through direct cloning and sequencing and the assessment of complex microbial communities through the use of denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) and fluorescent in-situ hybridisation (FISH). Bothe et al. (2000) reporting on the importance of molecular methods for nitrogen transforming bacteria state:

“We are starting to recognize that for nitrifiers and denitrifiers the real bacterial world is also much more complex and diverse than ever thought. Those bacteria which have been studied extensively in pure cultures are probably not the major `players’ in most natural habitats. Thus, molecular techniques have given access to a whole world of yet unknown nitrifying and denitrifying bacteria.”

PCR and cloning have now been extensively used to study AOB communities (Rotthauwe et al., 1997; Juretschko et al. 1998; Kowalchuk et al., 2000; Haleem et al.,
2000), NOB communities (Maixner et al., 2006, Dionisi et al. 2002; Cebron and Garnier, 2005) and denitrifying communities (Throback et al., 2004, Henry et al., 2006) in natural and engineered systems. Because the construction of clone libraries followed by sequencing are laborious, they have been routinely applied in conjunction to other community profiling methods such as DGGE and T-RFLP (Horz et al. 2000, Tietz et al., 2007).

The following section is a summary of studies characterising microbial communities in CWs. While general microbial studies are occasionally mentioned, the focus is on nitrogen transforming bacteria.

It is known that slow growing organism such as AOB often grow attached to surfaces in an extracellular polymeric matrix, or biofilm, which help them maintain a stable physiological state in the presence of inhibitory substances (acidic or saline conditions) and increase their recovery rate after a period of starvation for ammonium (Powell and Prosser 1992; Phillips et al., 1999; Gorra et al., 2007). The presence of surfaces for adherence may also select for specific strains of ammonia-oxidizing bacteria (Stehr et al. 1995; Phillips et al. 1999).

The diversity of the β-proteobacteria AOB was investigated in a rhizoremediation system treating municipal wastewater in Germany by Haleem et al. (2000). Cloning and sequencing of AOB associated with the roots of Phragmites australis and soil below the root system revealed that the majority of all sequences belonged to the Nitrosospira cluster and only a few sequences were affiliated with Nitrosomonas subcluster 7 (classification according to Stephen et al., 1996).

Truu et al. (2005) analysed the diversity and distribution of microbial communities in a sand HF wetland treating domestic wastewater. Methods used included PCR-DGGE (with universal 16S rDNA eubacterial and archaeal primers and AOB specific primers), and also MPN counts. They found significant differences in the spatial distribution of the microbial communities. In the case of general bacteria and specific AOB, the communities were very diverse and the most important factors affecting their distributions were related to depth and differences between the inflow and outflow wastewater characteristics. For the archaeal community, very high diversity was verified, with 20 to 30 dominant populations. Archaea was the only group
whose distribution followed a clear horizontal spatial pattern related to the wastewater flow. Nurk et al. (2005) found higher nitrifying activity occurring near the top of the wet area (water table) within a HF CW treating domestic wastewater.

In a 2.5 year old VF wetland treating mechanically settled municipal wastewater Tietz et al. (2007) found that only two AOB sequences dominated the different depths of the sand filter layer, independent of the season sampled. *Nitrosomonas* sp Nm107 and *Nitrosospira* sp NsP5 like sequences were found from the surface to a depth of 50cm (Tietz et al., 2007). Despite the small diversity of AOB present, efficient and stable nitrification occurred throughout the study and the AOB community did not seem to be influenced by strong temperature changes between summer and winter. The authors also point out to the importance that other groups, such archaea (crenoacheota), may have in ammonia oxidation and their possible contribution to stable nitrification occurring in VFCWs.

Gorra et al. (2007) analysed the composition of AOB in relation to different substrate materials in a wetland treating cheese factory effluent in the Alpine region of Italy. Similarly to what Haleem et al. (2000) found, *Nitrosospira* sequences dominated in the wetland. All AOB identified belonged to the *Nitrosospira* lineage whereas *Nitrosomonas* members were not detected. It was verified, however, that when averaged over the seasons, neither the presence of plants nor the different substrata (gravel, ground ceramic wastes, magnetite, zeolite, soil mixed with marble sand) present in the different components of the wetlands system selected for certain species of AOB, but the AOB community present in the zeolite component was most dissimilar to the other assemblages. Zeolite resulted in the best performance in regards to AOB activity over the seasons.

The denitrifying and nitrifying bacterial communities in the rhizosphere zone of a FWS CW used for wastewater polishing in Spain was studied by Ruiz-Rueda et al. (2009). DGGE analysis revealed that the structure of the denitrifying community in the rhizosphere differed from that of the bulk sediment. In regards to the AOB, the community was less complex and all retrieved sequences were related to *Nitrosomonas marina* and *Nitrosomonas ureae*, both present in the rhizosphere and bulk sediment. An interesting finding from the potential activity measurements was that potential
nitrification and nitrate reduction rates were higher in the rhizosphere samples when compared to the bulk sediment samples. The effect of plants in enhancing nitrogen removal in wetlands was demonstrated by the increased microbial activity around the rhizosphere. The presence of plants also impacted the structure of the bacterial community. It is known that treatment systems harbouring diverse microbial communities are more resistant to operational changes (Purkhold et al., 2000) while less diverse systems can be more susceptible to disturbances.

Sleytr et al. (2009) used terminal restriction length polymorphism (T-RFLP) to investigate the diversity of abundant bacteria in pilot and full scale VF constructed wetlands treating municipal wastewater, they verified that wetlands operated under similar conditions had bacterial communities with similar diversities, it was also verified that the soil around the rhizosphere of the plants *Phragmites australis* and *Miscanthus sinensis giganteus* harboured different bacterial communities. This finding helps corroborate the idea that plants have a significant role in selecting bacterial populations.

### 2.4.3 Heavy metals

Several metallic elements are essential micronutrients at very low concentrations, but when present in higher concentrations in water, some metals are toxic to sensitive organisms including humans. Most trace metals are known to accumulate in organisms in a process called bioaccumulation; bioaccumulation can then lead to biomagnification, a phenomenon in which increasing concentrations occur in consumers along the food chain (Kadlec and Wallace, 2009).

It is the periodic saturation of a wetland area that leads to the anaerobic conditions in the wetland soil under which typical wetland biogeochemical processes occur (Sheoran & Sheoran, 2006). In anoxic water conditions, decomposition of organic matter is by reduction and organic matter builds up on the surface of sediments. The resulting organic sediment surface is responsible for scavenging heavy metals from the wastewater (Walker and Hurl, 2002).
The concentration of heavy metal ions removed from solution in wetlands is determined by interacting processes of settling, sedimentation, sorption, co-precipitation, cation exchange, photodegradation, phytoaccumulation, biodegradation, microbial activity and plant uptake. The entire processes depend on each other and the reactions are also regulated by the composition of the substrate, sediment pH, nature of wastewater and composition of plant species. It is also known that the metal binding behaviour in wetland sediments can be modified by drying and flooding cycles because of changes in redox potential, pH, bacterial activity, sulphur speciation and organic ligands which typically accompany these events (Brant et al., 2003). Heavy metal removal mechanisms in wetlands can be classified as physical, chemical and biological; these processes are succinctly described below:

### 2.4.4.1 Physical processes (settling/sedimentation):

Settling and sedimentation are considered efficient physical processes for removing metals associated with particulate matter in the acid mine water (Kadlec & Knight, 1996). Sedimentation is a physical process occurring after other mechanisms which aggregate heavy metals into particles large enough to sink. These prior mechanisms are chemical reactions such as precipitation and co-precipitation (Walker and Hurl, 2002).

### 2.4.4.2 Chemical processes

Sorption: the transfer of ions from water to the soil, is the most important chemical removal processes in wetland soils. Sorption converts contaminants from the solution phase to the solid phase and may result in long term immobilization or short term retention of a wide range of heavy metals. Sorption includes adsorption and precipitation reactions.

Adsorption: heavy metals are adsorbed to the clay and organic matter in the sediment by electrostatic attraction, such attraction results either in cation exchange or
chemisorption. Cation exchange involves the physical binding of cations to the surface of clays and organic matter. Once metals are adsorbed on to humic or clay colloids they will remain there as metal atoms, their speciation however may change with time as the conditions in the sediment change, (Sheoran and Sheoran, 2006; Wiebner et al., 2005). The cation exchange capacity of soils is directly proportional to its organic matter and clay contents. Chemisorption represents a stronger a more permanent form of bonding than cation exchange.

More than 50 % of the metals can be easily adsorbed onto particulate matter in a wetland and thus be removed from the water component by sedimentation. Lead and copper tend to be adsorbed most strongly whereas zinc, nickel and cadmium are usually held weakly and hence likely to be more labile or bioavailable (Alloway, 1990).

Oxidation and hydrolysis of metals: Iron, aluminium and manganese can form insoluble compounds through hydrolysis and/or oxidation that occur in wetlands, such reactions lead to the formation of oxides, oxyhydroxides and hydroxides. Iron removal depends on pH, redox potential and presence of various anions (ITRC, 2003). Aluminium removal is governed by pH, it can precipitate as aluminium hydroxides at pH close to 5 (Hedin et al., 1994). Manganese removal can be accomplished by oxidation, which takes place at a pH close to 8. Bacteria play an important role in accelerating the oxidation of Mn$^{2+}$ to Mn$^{4+}$ (Stumm and Morgan, 1981).

Precipitation and co-precipitation: Precipitation and co-precipitation are important adsorptive mechanisms in wetland sediments. The formation of insoluble heavy metal precipitates is one of many factors limiting their bioavailability in aquatic ecosystems. Precipitation depends on the solubility product $K_{sp}$ of the metal, pH of the wetland and concentration of metal ions and anions involved (Sheoran and Sheoran, 2006).

Co-precipitation: heavy metals co-precipitate with secondary minerals in wetlands. Copper, nickel, zinc, chromium and manganese are co-precipitated in Fe oxides. Cobalt, iron and also nickel and zinc are co-precipitated in manganese oxides. Alkaline conditions are necessary for co-precipitation of cationic metals such as copper, zinc nickel and cadmium (Stumm and Morgan, 1981).
Metal carbonates: When the concentration of bicarbonate in water is high, heavy metals may form carbonates, which although not being as stable as sulphides, can still play an important role in initial trapping of metals (Sheoran and Sheoran, 2006).

Metal sulphides: Anaerobic conditions and appropriate substrate may promote the growth of sulphate reducing bacteria which generate hydrogen sulphide. Most heavy metals react with hydrogen sulphide to form highly insoluble metal sulphide solids that precipitate (Stumm and Morgan, 1981).

2.4.4.3 Biological processes

Plant uptake: The most widely recognised biological process in wetlands for metal removal is plant uptake. In emergent and surface floating wetland plants the main route for metal uptake is through roots, in the case of plants with completely submerged leaves or both floating and submerged leaves take up is through leaves and roots (Sheoran & Sheoran, 2006).

Bacterial metabolism: It is the bacterial metabolic processes that play the most significant role in removal of metals. Reduction of metals to non-mobile forms by microbial activity in wetlands has been reported. Metals like chromium, uranium, selenium and copper are removed through reduction by bacterial activity (Sheoran & Sheoran, 2006).

2.5 Treatment of saline wastewaters

Many industrial wastewaters are characterised by high salinity. Industries related to the production of chemicals, fertilisers and pharmaceuticals, food pickling, cheese manufacturing, seafood processing, tanning, oil and gas recovery and mining usually produce high inorganic salt and heavy metal concentrations in their wastewaters (Moussa et al., 2006, Lefebvre and Moletta, 2006).
Saline effluents are conventionally treated by physico-chemical processes as biological treatment can be strongly inhibited by NaCl (Lefebvre and Moletta, 2006). Physico-chemical treatments, however, are costly so alternative biological treatments have been the focus of recent research. Salt is known to reduce microbial activity and therefore constitutes a microbiological agent of stability (Lozach, 2001). High percentages of salt are known to compromise the correct operation of conventional aerobic WWTP. Ludzack and Norman (1965) found that Cl\(^{-}\) concentrations above 5-8ppt affected performance of aerobic processes. Biological treatment to remove carbon, nitrogen and phosphorus are feasible at high salt concentrations but adaptation of the biomass or use of halophilic (salt tolerant) organisms will dictate treatment performance (Lefebvre and Moletta, 2006).

Acclimation means the exposure of organisms to increasing concentrations of salt in order to obtain satisfactory effluent treatment. Salt tolerant organisms have to be present in the original conditions or introduced to the treatment system via inoculation, then slow increases in salinity permit such tolerant organisms to reproduce while non salt tolerant organism are eliminated. Abrupt increases in salinity usually cause performance disruptions which can be either permanent, if halophilic organisms are absent in the initial microbial biomass, or temporary, when halophilic organisms are initially present and the reduced performance represents the lag time their populations take to increase and consume substrate (Ludzack and Norman, 1965; Lefebvre and Moletta, 2006). It is usual to see a reduced pollutant removal rate in saline systems, the theory postulated here is that this is probably due to reduced richness and diversity of organisms capable of withstanding high salt concentrations and therefore niches that were previously occupied by some organisms remain vacant.

Panswad and Anan (1999) used salt “adapted” organisms in the anaerobic/anoxic/aerobic treatment system resulting in improved removal percentages of organic matter and nitrogen. They initially verified a 55% inhibition of nitrification at 30g/L NaCl, but after a few days normal activity was recovered. Campos et al. (2002) achieved efficient nitrification in an activated sludge unit treating saline wastewater with influent NH\(_4^+\)-N up to 3.3g/L. Once NH\(_4^+\)-N increased over 3.3g/L nitrification efficiency decreased and ammonium accumulated in the system. At the time of ammonium accumulation, total salt concentrations were 13.7g NaCl/L, 19.9g NaNO\(_3\)/L.
and 8.3 g Na₂SO₄/L. The authors attribute this decrease in efficiency to a combined effect of toxic salt and ammonium concentrations.

Cheng and Wong (2004) found that the gradual exposure of a continuous nitrifying activated sludge culture to chlorides up to 18.2 g Cl⁻/L resulted in better nitrification than a culture operated at a fixed chloride concentration. Beyond 18.2 g Cl⁻/L however nitrification became unstable. Dahl et al. (1997) concluded that nitrification can take place up to 20.0 g Cl⁻/L with a maximum nitrification rate of 2.0 mg N/g VSS/h. In a fluidised bed reactor with up to 34 g Cl⁻/L, Vredenbregt et al. (1997) could achieve nitrification on the condition that ammonia load was maintained at 15 mg NH₃/L/h.

The existence of extremely halophilic denitrifying bacteria has been known since the middle of the 1980’s when they had been isolated from different hypersaline environments and grown in anoxic conditions (Hochstein and Tomlinson, 1985) producing nitrite, nitrous oxide and nitrogen gas. Dincer and Kargi (1999) found that denitrification was more sensitive to salt increases than nitrification, in their study, denitrification was affected by salt concentrations higher than 2% and that the amount of salt to reduce the denitrification rate by 50% of that obtained in fresh water was equal to 15.2 g NaCl/L.

### 2.5.1 Wetlands treating saline wastewater

Most of the wetlands treating saline wastewaters are relatively new and therefore there is limited literature and data on constructed wetlands treating saline wastewaters. It is important to determine how high and fluctuating salinity affect plant health and bacterial community composition and function in a wetland environment. Understanding the effect of salinity is essential for the management of constructed wetlands subject to saline conditions and will help determining the applicability of such systems.

Brown et al. (1999) used different halophytes (salt-tolerant plants) in unsaturated biofilters to treat saline aquaculture effluents. It was verified that NaCl at
10g/L and 35g/L inhibited growth rate, nutrient removal and volume of water that all three plants could process. At higher salinities, systems planted with *Sueda* sp and *Salicornia* sp outperformed systems planted with *Atriplex* sp.

Calheiros *et al.* (2010) studied the diversity of bacterial communities in HF wetlands polishing saline (electrical conductivity (EC) = 17mS/cm, total dissolved solids (TDS) = 10,853mg/L) tannery wastewater. The wetlands were planted with *Arundo donax* and *Sarcocornia sp* and were monitored for one year. Both plants were resilient to the high salinity with the two wetlands performing similarly in terms of COD, BOD, total suspended solids (TSS), total phosphorus (TP) and NH₄⁺. However different plants played a significant role in shaping the microbial communities around the rhizosphere in each wetland, while seasons did not. The presence or absence of plants also has an effect in shaping the microbial assemblages in constructed wetlands treating domestic wastewater (Zhang *et al.*, 2010), furthermore the presence of plants enhances microbial activity and diversity, with plant species, root development and morphology appearing to be key factors influencing microbial-plant interactions (Gagnon *et al.*, 2007; Vymazal *et al.*, 2001).

Wu *et al.* (2008) used HF mangrove wetland microcosms containing sandy soils and planted with *Aegiceras corniculatum* to study the effect of different salinities (0, 15 and 30g NaCl/L) on pollutant removal performance during a four month experiment. Removal of dissolved organic carbon, ammonia-N and nitrate-N was significantly lower at 15g/L and 30g/L when compared to the control 0g NaCl/L. In spite of the salinity effect, all effluent samples from the wetland microcosms complied with the total nitrogen and phosphorus discharge standards set by the Hong Kong Government for Coastal Water Control Zones. The mangrove plant *Aegiceras corniculatum* grew best and had higher N uptake at 15g/L of NaCl. The suitability of mangrove HF wetlands to treat wastewater was therefore verified. Their microcosms however operated under a fixed NaCl concentration and were not subject to a gradual increase of salinity which would allow selection of halophytic microbial biomass.

Klomjek and Nitisoravut (2005) tested 8 different macrophytes in batch fed FWS wetlands under saline (EC= 14-16mS/cm) conditions. It was verified that Cattail (*Typha angustifolia*) and Asia crabgrass (*Digitaria bicornis*) could bear the saline conditions and
therefore were selected for a 97 day operational continuous FWS flow wetland under brackish conditions (EC = 4mS/cm). The wetland containing Asia crabgrass outperformed the cattail one in terms of BOD and SS removal. The FWS design however favoured extensive algal growth and deterioration of water quality in terms of SS and organic compounds in the effluent.

Gregory et al. (2010) used T-RFLP and cloning analyses to characterise the AOB community in recirculating biofilters treating saline wastewater (28 – 38g/L NaCl) from a commercial marine fish farm. Ammonium chloride was added to the wastewater in order to increase the concentrations of NH$_3$-N to between 600 – 2290mg/L. In their study salinity was kept constant throughout and did not affect ammonia removal but increasing NH$_3$-N concentrations higher than 600mg/L resulted in lower percentage removal. Total removal, as measured by reduction in concentration, however, increased. Interestingly, the higher concentrations of NH$_3$-N did not seem to present toxic effects to all AOB community members as ammonia oxidation was still high. The high concentration of substrate most likely exceeded the ammonia oxidation rate of the nitrifiers and therefore a higher retention time would be necessary to oxidise all NH$_3$-N (given that other requirements such as O$_2$, pH and alkalinity were fulfilled). At higher NH$_3$-N concentrations nitrite peaks were observed in the system, likely resultant from nitrite oxidation rates being exceeded. Elevated nitrite is also known to impair the ammonia mono-oxygenase activity of *Nitrosomonas europaea* (Stein and Arp, 1998). Their experiment did not have a control system operating at a fixed lower NH$_3$-N concentration so a firm conclusion that higher NH$_3$-N concentrations caused the changes in the bacterial community could not be drawn. Above 600mg NH$_3$-N/L a decrease in the number of species was observed as revealed by 16sRDNA based T-RFLP. The *amoA* based T-RFLP showed a succession of AOB with three major nitrosomonad groups dominating the biofilters: *Nitrosomonas* sp. lineage Nm 143 abundant initially at low ammonia concentrations, then *Nitrosomonas oligotropha/N. ureae* like sequences becoming more dominant at increasingly NH$_3$-N and lastly *N. aestuarii/N. marina* dominating towards the end of the experiment when NH$_3$-N concentrations were highest.
2.6 Summary

The potential use of vertical flow constructed wetlands for the treatment of saline and inorganic industrial wastewaters has not been fully investigated in Australia and the uptake of this technology by the industrial sector has been limited. The available literature has revealed that constructed wetlands are mechanically simple but biologically complex systems which require low inputs of energy and maintenance. When land is available, constructed wetlands can be a cost effective solution for the treatment of a wide range of industrial wastewaters.

Microbial processes dictate the performance of highly loaded wetlands and therefore microbial communities in these systems deserve further investigation. Vertical flow constructed wetlands can be operated in different hydrological conditions, ranging from unsaturated to fully saturated, and these conditions set the scene for specific microbial action. Another important point highlighted by the literature is the possible use of high carbon content wastewaters as carbon source to improve the treatability of nitrogen rich inorganic wastewaters.

The potential use of vertical flow wetlands to treat saline and inorganic wastewaters with high nitrogen content forms the basis of this thesis. The following chapters will describe laboratory and field scenarios where optimisation of processes naturally occurring in mangroves and salt marshes has been attempted in a controlled manner. Nitrogen removal mechanisms and the characterisation of key players in nitrogen transformation is the primary focus of this research.
Chapter 3:

Salinity impact on nitrification and on the ammonia oxidising bacterial community in vertical flow constructed wetlands treating inorganic industrial wastewater

3.1 Introduction

Constructed wetlands (CWs) are a cost effective solution for water and wastewater treatment (Vymazal, 2005) and have been increasingly used for treating a variety of industrial effluents (Green et al., 1997; Maine et al., 2006). Many industrial wastewaters, however, are characterised by high salt concentrations. Industries related to the production of chemicals, fertilisers, pharmaceuticals, food manufacturing, seafood processing, tanning, oil and gas recovery and mining usually produce wastewaters with high inorganic salt concentrations (Moussa, et al., 2006, Maine et al., 2006; Calheiros et al., 2007).

Most of the CWs treating saline wastewaters are relatively new and inhibition of bacterial function has not yet been reported to levels which prevent wetlands from performing their remediation functions e.g. removing nitrogen, pathogens, organic matter (BOD and COD) and other pollutants. It is important to determine how elevated and/or fluctuating salinity affects plant health, bacterial community composition and function in a wetland environment. Understanding the effect of salinity is essential for the management of constructed wetlands subject to saline conditions and it will assist in determining the applicability of such systems.

The current water restrictions in most Australian states (Chong et al., 2009), the development of water saving processes to reduce water demand and the contemplative use of sea water as an alternative water source for less noble applications, as for example its distribution for toilet flushing, currently common practice in Honk Kong (Wu et al., 2008) are likely to result in the production of higher strength and more saline effluents. Existing wastewater treatment facilities relying on biological treatment may, therefore, face the new challenge of maintaining discharge
standards while dealing with higher concentrations of salts and pollutants. Identifying the nitrifying bacteria strains which are tolerant to salinity will assist in building treatment systems which are more resistant to process upset. The objective of this chapter was to verify the effect of increasing salinity on nitrification and on the ammonia oxidising bacteria in vertical flow constructed wetlands (VFCWs).

3.2 Materials and Methods

3.2.1 Experimental set up

A total of nine laboratory scale VFCWs were prepared using 100mm diameter PVC pipe columns as shown in Figure 3.1. The columns were 1m high and filled with a 30cm drainage layer, consisting of 25cm of larger gravel (14mm-diameter) and a 5 cm intermediate layer of smaller gravel (5mm diameter), and a 45cm main filtering layer of fine sand (sand particle size distribution in Figure A.1, Appendix A). Rhizomes of native River Club Rush, *Schoenoplectus validus*, were planted in all columns. Systems were set up in triplicate and from November 2008 to July 2009 (phase I) the control (C) wetlands received only wastewater, the treatment 1 (T1) wetlands received wastewater containing 2.5g/L NaCl and treatment 2 (T2) wetlands received wastewater spiked with gradually increasing concentrations of NaCl up to 40g/L (Figure 3.1). In August 2009 (phase II), the C and T1 wetlands were subject to a NaCl shock load, with an abrupt increase in salinity to 35g/L. T2 wetlands, which had been receiving hypersaline wastewater, were relieved of the salt stress and started receiving wastewater without NaCl.

The wastewater used in this study consisted of inorganic wastewater produced by CSBP Ltd, a chemical and fertiliser manufacturer located in Kwinana, Western Australia. A batch of wastewater containing high NH$_3$-N was purposely selected and brought to the laboratory at the Environmental Technology Centre (Murdoch University) and stored at 4°C until further use (please refer to Table 3.2 in Results for wastewater characteristics). The choice for the concentrated batch of wastewater was based firstly on the convenience of being able to dilute it with tap water to achieve the desired influent concentration of NH$_3$-N and secondly on the financial and time savings
associated with the reduced number of trips for wastewater collection. Once diluted, the wastewater was then stored in 200L tanks which contained a 7 watt recirculation pump (Rain Master King 1F-Qmax=380L/hr) and a 20 watt submersible dosing pump (Aquapro AP-1000FP, Qmax=900L/hr). The pumps were connected to timers which turned on once a day. The recirculation pump operated for 20 minutes prior to any dosing event and the dosing pump operated for one minute delivering from 0.3 to 1.8L doses of influent into the wetland. The quick dosing event allowed flooding of the surface with subsequent percolation. The wetland outlet pipe on the bottom of the column consisted of a hose attached to a solenoid valve which had its spring mechanism modified to work under low pressure. The solenoid valve remained open for 45 minutes each day to allow complete draining of the effluent.

![Figure 3.1: A. Triplicate experimental wetland column schematic showing the sand filtering layer and gravel drainage layer. B. Photo of the 9 experimental wetlands and detail of C, T1 and T2.](image)

The wetlands were operated on a feed-stay-drain-rest mode with the following daily schedule: 9:30am to 9:31am - feed; 9:31am to 8:00am (following day) - stay; 8:00am to 8:45am - drain; 8:45am to 9:30am - rest empty.
The fine sand used had a high porosity of 0.42 and low hydraulic conductivity of 5.0m/d, typical of fine sands, according to Brassington (1988). The gravel component had a porosity of 0.45. When completely saturated to the surface of the sand the wetland could, theoretically (assuming an initial oven dry condition for the sand), hold 1574.8mL of water in the sand component and 1124.9mL in the gravel component according to equation 3.1 below. The total saturation volume for the system would therefore be 2669.7mL.

\[
\text{Substrate saturation} = \text{volume of substrate} \times \text{porosity} = W_A \times S_D \times p \quad (3.1)
\]

Where:

- \( W_A \) = wetland area = 83.323 cm\(^2\)
- \( S_D \) = substrate depth (sand = 45 cm and gravel = 30 cm)
- \( p \) = porosity (sand = 0.42; gravel = 0.45)

The field capacity of the sand substrate was measured as an indication of the water content remaining in the column after draining occurred. Field capacity was measured volumetrically (vol of water/vol of sand) and resulted in 0.38 or 38%, this means that after a draining event the sand could hold 38% of its volume in water (volume of sand \times 0.38 = 1424.8mL). While the sand layer of the column remained unsaturated and only subject to higher water content immediately after feeding, the gravel layer on the bottom worked as a reservoir remaining saturated for nearly the whole “stay” period. The column was emptied daily with the outlet valve remaining open for 45 minutes to allow complete drainage and oxygenation of the gravel layer. This operation mode was based on the work of Green et al. (1998) who used a siphon mechanism as passive air pump to increase nitrification in VF wetlands.

3.2.2 Wetland residence time and tracer test

The capacity of a CW to remove pollutants is directly associated with the time water stays within the system, so the contact time between pollutants in the water and the wetland’s substrate, microbial biofilms and rhizosphere dictates wetland performance. The simplest way to characterise the hydraulic behaviour of a wetland is by calculating its theoretical (or nominal) hydraulic residence time (HRT), (Liehr et al.,
The theoretical HRT is the average time that water stays in the system and can be calculated, assuming ideal plug flow conditions, according to:

\[ \tau_n = \frac{V}{Q_{avg}} \]  

(3.2)

Where:

- \( \tau_n \) = theoretical hydraulic residence time (d)
- \( V \) = wetland holding volume (L)
- \( Q_{avg} \) = average flow into wetland (L/d)

In the case of the unsaturated sand component of the VF wetlands, an approximation of the holding volume is given by the sand field capacity. In the case of the fine sand used the holding volume was approximately 1425mL. Assuming an average flow of 1000mL/d the theoretical HRT for the sand compartment is, according to equation 3.2, 1.425 day. Once drained from the sand compartment the water was retained in the gravel portion of the CW for another 22.5 hours (or 0.94 day) until the next drain event, when samples were collected. Therefore, the theoretical HRT of 2.365 days was assumed for these wetlands (1.425 day + 0.94 day).

Preferential flow paths, dead zones and mixing are known phenomena in CWs, therefore these systems are assumed to have non-ideal flows, behaving as a combination of plug flow reactors and completely mixed systems (continuous stirred tank reactor). Several completely mixed systems in series have also been used to describe the hydraulics of constructed wetlands (Kadlec and Wallace, 2009). Depending on mixing and preferential flow paths different parcels of water will remain in the system for different lengths of time and therefore a distribution of residence times can be expected.

Tracer tests can be carried out to determine the actual hydraulic characteristic of a wetland since the theoretical HRT occurs only in ideal plug flow systems, which is not a reality in constructed wetlands. Tracer studies can provide a more accurate representation of the hydraulics of a system by enabling the calculation of a mean HRT. Non-reactive fluorescent dyes or inorganic salts can be dissolved in water and added to the wetland influent as a slug. Measurement of the tracer through the system will in fact be a measurement of the movement of water in the wetland. NaCl and KCl can be
used as tracers in small scale constructed wetlands; rhodamine and bromide are also tracers of choice in large scale systems due to their relative non-reactivity (Kadlec and Wallace, 2009).

Tracer analysis can be conducted through the residence time distribution (RTD) curve. The average time of travel from the inlet to outlet is given by the mean of the tracer response curve and not by the peak in response (Kadlec et al., 1993). The RTD represents a probability density function of the amount of time that various fractions of fluid spend in a reactor (Levenspiel, 1972) and is calculated according to equation 3.3:

\[ f(t) = \frac{Q(t)C(t)}{\int_0^\infty Q(t)C(t)dt} \]  

(3.3)

Where:

- \( Q \) = Outflow, (L/d)
- \( C \) = Outlet concentration (g/L)
- \( t \) = Time since tracer injection (d)
- \( f(t) \) = RTD (residence time distribution) function (d-1)
- \( dt \) = Time increment between concentration measurements (d)

The mean tracer retention time (\( T \)) is presumed to be the actual mean retention time and is calculated from:

\[ T = \frac{1}{M_i} \int_0^\infty tQC \, dt \]  

(3.4)

Where:

- \( M_i \) = Mass of tracer in (g)
- \( Q \) = Outflow, (L/d)
- \( C \) = Outlet concentration (g/L)
- \( t \) = Time since tracer injection (d)
- \( dt \) = Time increment between concentration measurements (d)

The tracer used in this experiment consisted of NaCl and the effluent concentrations were calculated based on the measurements of electrical conductivity (EC). Twenty grams (20g) of NaCl were dissolved into one litre of water and added to the wetland in one feeding event. Effluent samples were monitored until the background EC values were measured, i.e., no more traces of the NaCl tracer were found.
3.2.3 Water sampling and analysis

Water samples were collected from the influent storage tank and from the wetlands’ outlet pipes for analysis. Samples were analysed for pH and oxidation-reduction potential (ORP) with a Hanna Instruments HI 9025 hand held meter, dissolved oxygen (DO) was measured with a Hanna Instruments HI 9145 hand held DO meter, and (EC) was measured with a Hanna Instruments HI 8733 hand held EC meter. Inorganic nitrogen forms were measured spectrophotometrically using a HACH DR 2800™ spectrophotometer and methods as follows: Ammonia-nitrogen was measured using the Nessler method (Method 8038), nitrate+nitrite-nitrogen was measured using the cadmium reduction method (Method 8039) and nitrite nitrogen was measured using the diazotization method (Method 8507). Samples were diluted and treated accordingly in order to be within the methods’ reading range and exclude interferences.

All wetlands initially received tap water and then diluted wastewater without NaCl for two months prior to the start of the intense monitoring phase, during this time the tracer experiments were conducted. When monitoring started, effluent samples from all wetlands were initially analysed for inorganic nitrogen forms, once it was verified that the triplicate systems were in fact performing as replicates then sampling effort decreased to one effluent sample from the triplicate being randomly selected for chemical analysis. Other parameters such as effluent DO, pH, EC, temperature and volume were always measured for all 9 wetlands.

3.2.4 Sediment sampling and analysis

Sand samples for bacterial analysis were collected from one randomly chosen wetland out of the triplicate C, T1 and T2 systems. Sampling locations were: surface, 5cm 15cm and 30 cm deep (Figure 3.2), and they all represented unsaturated, free draining sediment conditions. Only the gravel drainage layer remained saturated for extended periods of time, this gravel layer however was not sampled in this study. Sampling dates are represented in Table 3.1. Samples were collected by introducing the wider end of a sterile 1.0mL pipette tip into the sand and shaking the sand out into
a small zip lock bag; this procedure was repeated until 3.0g of sediment were collected. On the surface, the pipette tip was only introduced down to 1cm deep, and for the other locations it was introduced into the sampling ports located on the side of the columns. Once collected, samples were stored at -20°C until DNA extraction. DNA was extracted with the use of UltraClean® soil DNA isolation kit (Mobio Laboratories, CA, USA.) according to the manufacturer’s instructions for maximum yields. DNA was eluted in water and stored at -20°C for further use.

3.2.5 Data analysis

Statistical analyses were performed with MS Excel (MS Office 2007) and SPSS (SPSS statistics 17.0). Analysis of variance (one way ANOVA) was carried out in order to verify differences in effluent concentrations among control and treatments. Some sets of data had to be log10 transformed in order to satisfy ANOVA requirements of normality and homoscedasticity. Tukey’s post multiple comparison test was chosen to verify differences between pairs. The critical level for all statistical tests was $p \leq 0.05$. 

Figure 3.2: Detail of sediment sampling locations for bacterial analysis.
Table 3.1: Sediment sampling dates and depths for bacterial analysis from the C, T1 and T2 wetlands.

<table>
<thead>
<tr>
<th>date/depth</th>
<th>surface</th>
<th>5cm</th>
<th>15cm</th>
<th>30cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>10/12/08*</td>
<td>X</td>
<td>✓</td>
<td>✓</td>
<td>X</td>
</tr>
<tr>
<td>16/02/09</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26/03/09</td>
<td>X</td>
<td>✓</td>
<td>✓</td>
<td>X</td>
</tr>
<tr>
<td>22/06/09</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>05/08/09</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>04/09/09</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Samples from the T2 wetlands were not analysed by TRFLP on this date.

X = Indicates that clone libraries were constructed for the T2 wetlands.

3.2.6 Polymerase Chain Reactions (PCRs)

The primer pair amoA-1F (5’-GGG GTT TCT ACT GGT GGT) and amoA-2R (5’-CCC CTC KGS AAA GTC TTC TTC) which, respectively, target positions 332 to 349 and 802 to 822 of the open reading frame of the amoA gene sequence of *Nitrosomona europaea* was used for the amplification of a 491 base pair (bp) fragment of the *amoA* gene (Rotthauwe *et al.*, 1997). The *amoA* gene codes for the enzyme ammonia monooxygenase (AMO) which catalyses the oxidation of ammonia to hydroxylamine, the first step in ammonia oxidation to nitrite. This primer pair has been successfully used in characterising ammonia oxidisers belonging to the β-subclass Proteobacteria (Rotthauwe *et al.*, 1997). Because of this specificity of the primer, AOB belonging to γ-subclass Proteobacteria were not assessed in this study.

Amplifications were performed in 25μL reaction volumes for cloning purposes and 50μL reactions for T-RFLP analysis. Reactions contained 10 to 20ng of template DNA, 1x PCR buffer, 2.5 mM of MgCl₂, 0.2 mM of mixed dNTPs, 0.5μM of each primer 1.0 U of Taq DNA polymerase (Promega, Madison, Wi, USA). The following PCR cycles were used in a BioRad MyCycler thermal cycler: predenaturarion (5 min, 94°C), 30 cycles of denaturation (30 sec, 95°C), annealing (30sec, 55°C), extension (1 min, 72°C) and a final extension step (7 min, 72°C).

To verify the presence of the amplified gene 10μL samples of the PCR products were run on 1% agarose gel electrophoresis, stained with ethidium bromide and visualised under UV light. For the preparation of the cloning reactions and T-RFLP analysis PCR products were purified with the Wizard SV Gel and PCR clean up kit (Promega, Madison, Wi, USA).
3.2.7 Clone library

Cloning reactions were prepared according to the instructions in the PGEM-T easy Vector System (Promega, Madison, WI, USA) and contained 1μL of clean PCR product, 2.5μL of ligation buffer (2x), 0.5μL of ligase and 1μL of water. Ligations were performed overnight at 4°C. Transformations were performed using high transformation efficiency JM109 competent cells (Promega, Madison, WI, USA) according to the manufacturers’ instructions. Transformants were selected by ampicillin resistance and blue–white screening, with X-gal, was performed to identify clones with inserts. A total of 260 clones, subdivided in 5 libraries from the T2 wetlands, were selected for the amplification of the amoA gene and subsequent digestion with HaeIII and AluI restriction enzymes (REs) (Promega, Madison, WI, USA). Digestion of 10 μL of PCR product was conducted using 1.19μL of restriction enzyme buffer, 0.119μL of bovine serum albumin (BSA) and 0.297μL of HaeIII or AluI. Digestions were conducted for 2hrs at 37°C. Digested products were electrophoresed and visualised in 3% agarose gels using standard electrophoresis procedures. Clones were categorised into different operational taxonomic units (OTUs) according to their restriction fragment lengths (restriction fragment length polymorphism- RFLP).

3.2.8 T-RFLP

The amoA1F- 5’FAM labelled primer was used for PCR mixtures used for T-RFLP analysis. Clean PCR products were digested with AluI, HaeIII and TaqI REs, according to the manufacturers’ recommendations (Promega, Madison, WI, USA). One μl of each digest was added to 0.25μl LIZ 600 marker (Applied Biosystems, CA) and 9μl Hi-Di™ formamide (Applied Biosystems, CA, USA) and fragment analysis performed on an Applied Biosystems 3730 DNA sequencing system (SABC, Murdoch University). Resulting profiles were analysed using Genemapper software (Applied Biosystems, CA). Similarly to the categorisation in the clone library, different terminal restriction fragment lengths, measured in bp, were assigned to different operational taxonomic units (OTUs). T-RFLP profiles were normalised according to the constant percentage
Threshold calculation suggested by Sait et al. (2003). Peak areas that fell below the percentage threshold (ranging from 1 - 3% of the total peak area, depending on the profile) were therefore disregarded. Only Alul based T-RFLP profiles are presented in the results and discussion session, as they gave optimal species separation. Haelll and TaqI results can be seen in the Appendix B section.

### 3.2.9 Sequencing and phylogeny

Clones that contained *AmoA* gene which resulted in dissimilar cutting pattern were selected for growth and insert sequencing. Following overnight growth on LB medium with ampicillin, the selected clones were harvested and the plasmids extracted with the QIAGEN Spin Miniprep Kit according to the manufacturers instructions. Plasmid DNA was used for the sequencing reactions using the amoA forward primer and the big ABI Prism BigDye dideoxy terminators. An ethanol precipitation protocol recommended for BigDye versions 3.1 dye terminators was used for the post reaction purification of the DNA. Sequencing was conducted in an Applied Biosystems ABI 3730 sequencer at the State Agricultural Biotechnology Centre (SABC) at Murdoch University, Western Australia. Sequences were manually checked using BioEdit sequence alignment editor software (Hall, 1999) and submitted to a BLAST search (NCBI: [http://blast.ncbi.nlm.nih.gov](http://blast.ncbi.nlm.nih.gov)). Sequences in the GenBank database sharing the greatest similarities were selected and imported for multiple alignments using ClustalW2.0.12 software (Larkin et al., 2007). Phylogenetic trees were visualised with Treeview (Page, 1996) software. Unique clone sequences from this study have been published in GenBank under accession numbers JF682361-JF682371.
3.3 Results

3.3.1 Tracer experiments

Total recovery of the NaCl tracer was obtained during the experiment indicating the validity of the test. Twenty grams of NaCl were dissolved in 1.0 L of tap water and introduced in one feed event; the tracer was fully recovered (19.98g) after 5 days. The NaCl concentration and RTD curve can be seen in Figure 3.3 below. From equation 3.3 the actual retention time was calculated as 2.15 days, which is less than the theoretical 2.36 days assuming an average flow of 1.0L/d.

![Figure 3.3: A: Concentrations of NaCl measured at the outflow of cell 2 during tracer experiment. B: Response of cell 2 to a tracer impulse, retention time distribution (RTD curve) in cell 2.](image)

3.3.2 Plant health and biomass

Plant health was monitored visually and above ground biomass harvested at the end of the experiment (September 2009). Figure 3.4 shows the wetland columns photographed in June 2009 when the T2 wetlands were receiving 35g/L NaCl (EC=50mS/cm). It is evident that the increase in salinity affected plant growth. The T2 wetlands started presenting signs of stress such as yellowing and drying when EC was increased from 10mS/cm to 20mS/cm. T1 wetlands (7mS/cm) did not present visual signs of stress but plant density was lower than in the C wetlands. All wetlands had significant differences (p<0.05) in final aboveground biomass and a biomass gradient C>T1>T2 was verified (Figure 3.5). The leaves in the C and T1 wetlands started yellowing within 7 days from the start of the shock load phase and were completely yellow after
18 days. The plants in T2 wetlands, however, did not show any signs of recovery once the salt stress was relieved and remained dry until the end of the experiment in September 2009.

Figure 3.4: Wetland columns photographed in June 2009 showing varying plant densities. Note the decreasing density and health from the C→T1→T2.

Figure 3.5: Final mean above ground biomass (dry weight) of the C, T1 and T2 wetlands. Error bars represent 1 standard deviation, (n=3 for each group).
3.3.3 Water physical-chemical parameters

The wastewater supplied by CSBP is described in Table 3.2 below. This wastewater consisted of a concentrated batch and was diluted to achieve NH$_3$-N concentrations in the 100 - 160 mg/L range. Mean values of influent pH, temperature and DO for all systems for the duration of the experiment were 6.14, 21.8°C and 6.9 mg/L respectively.

Table 3.2: Characteristics of the undiluted wastewater supplied by CSBP Ltd and used in this study.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>unit</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH$_3$-N</td>
<td>mg/L</td>
<td>3700</td>
</tr>
<tr>
<td>NO$_3$-N</td>
<td>mg/L</td>
<td>86.4</td>
</tr>
<tr>
<td>TP</td>
<td>mg/L</td>
<td>9.5</td>
</tr>
<tr>
<td>COD</td>
<td>mg/L</td>
<td>68.3</td>
</tr>
<tr>
<td>Al</td>
<td>mg/L</td>
<td>0.092</td>
</tr>
<tr>
<td>Ca</td>
<td>mg/L</td>
<td>61.0</td>
</tr>
<tr>
<td>Cd</td>
<td>mg/L</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Co</td>
<td>mg/L</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Cr</td>
<td>mg/L</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Cu</td>
<td>mg/L</td>
<td>0.205</td>
</tr>
<tr>
<td>Fe</td>
<td>mg/L</td>
<td>0.483</td>
</tr>
<tr>
<td>K</td>
<td>mg/L</td>
<td>26.3</td>
</tr>
<tr>
<td>Mg</td>
<td>mg/L</td>
<td>33.8</td>
</tr>
<tr>
<td>Mn</td>
<td>mg/L</td>
<td>0.035</td>
</tr>
<tr>
<td>Mo</td>
<td>mg/L</td>
<td>0.223</td>
</tr>
<tr>
<td>Ni</td>
<td>mg/L</td>
<td>0.142</td>
</tr>
<tr>
<td>Pb</td>
<td>mg/L</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>S</td>
<td>mg/L</td>
<td>2740</td>
</tr>
<tr>
<td>SO$_4$</td>
<td>mg/L</td>
<td>8220</td>
</tr>
<tr>
<td>Sn</td>
<td>mg/L</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>V</td>
<td>mg/L</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Zn</td>
<td>mg/L</td>
<td>3.11</td>
</tr>
</tbody>
</table>

Effluent pH, temperature and DO for all wetlands can be seen in Table 3.3 below:

Table 3.3: Mean (range) effluent values for pH, temperature and DO for the duration of the experiment. November 2008 to September 2009.

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>Temp (°C)</th>
<th>DO (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.35</td>
<td>(7.00 - 8.23)</td>
<td>21.26</td>
</tr>
<tr>
<td>T 1</td>
<td>7.37</td>
<td>(6.90 - 8.36)</td>
<td>21.31</td>
</tr>
<tr>
<td>T 2</td>
<td>7.35</td>
<td>(6.99 - 8.22)</td>
<td>21.19</td>
</tr>
</tbody>
</table>

*$n_c=227$, $n_{T1}=226$, $n_{T2}=233$
Even though known amounts of sea salt were added to the wastewater, salinity was ultimately monitored in terms of electrical conductivity (EC). Both control and treatment wetlands received ammonia nitrogen concentrations ranging from 100 to 160mg/L.

Data originated from inflows ranging from 0.7 to 1.0L/day, hydraulic loading rates (HLR) of 8.4 to 12cm/d were selected for comparative analysis. The hydraulic loading rates applied and influent and effluent nitrogen forms are presented in Table 3.4. This selection was made because flows below 0.7L/day were representative of a system operating under low flow and loading conditions which would result in an overestimated removal performance. Flows higher than 1.0L/day represented an overloaded system and resulted in the removal capacity being exceeded. Data originated from these low and high flows are presented and discussed in the load response section, later in this chapter (Section 3.3.4).
Table 3.4: Mean (standard deviation) concentration of inorganic nitrogen forms in and out of the vertical flow wetlands under different salt regimes. November 2008 to July 2009, NaCl (g/L): Control =0.0; T1=2.5; T2=up to 40.0. August 2009: Control =35.0; T1=35.0; T2=0.0.

<table>
<thead>
<tr>
<th>Period</th>
<th>Wetland</th>
<th>HLR</th>
<th>NH$_3$-N in</th>
<th>NH$_3$-N out</th>
<th>NO$_2$-N in</th>
<th>NO$_2$-N out</th>
<th>NO$_3$-N in</th>
<th>NO$_3$-N out</th>
<th>TN in</th>
<th>TN out</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nov-08 to</td>
<td>Control</td>
<td>11.0</td>
<td>(1.1)</td>
<td>106</td>
<td>(6.2)</td>
<td>1.18$^a$</td>
<td>(2.5)</td>
<td>7.5$^a$</td>
<td>(1.7)</td>
<td>135$^b$</td>
</tr>
<tr>
<td>Jul-09</td>
<td>T 1</td>
<td>10.6</td>
<td>(1.5)</td>
<td>104</td>
<td>(7.0)</td>
<td>0.91$^a$</td>
<td>(1.5)</td>
<td>7.2$^a$</td>
<td>(1.7)</td>
<td>107$^b$</td>
</tr>
<tr>
<td></td>
<td>T 2</td>
<td>10.4</td>
<td>(1.4)</td>
<td>104</td>
<td>(6.8)</td>
<td>1.35$^a$</td>
<td>(2.2)</td>
<td>6.4$^a$</td>
<td>(1.9)</td>
<td>100$^b$</td>
</tr>
<tr>
<td>Phase II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shock load</td>
<td>Control</td>
<td>10.6</td>
<td>(1.0)</td>
<td>149</td>
<td>(13)</td>
<td>8.14$^a$</td>
<td>(8.7)</td>
<td>2.9$^a$</td>
<td>(2.7)</td>
<td>156$^b$</td>
</tr>
<tr>
<td>Aug-09</td>
<td>T 1</td>
<td>10.5</td>
<td>(0.84)</td>
<td>150</td>
<td>(13)</td>
<td>3.73$^a$</td>
<td>(5.4)</td>
<td>2.7$^a$</td>
<td>(2.7)</td>
<td>144$^b$</td>
</tr>
<tr>
<td></td>
<td>T 2</td>
<td>10.8</td>
<td>(0.92)</td>
<td>150</td>
<td>-</td>
<td>0.43$^a$</td>
<td>(0.58)</td>
<td>1.36$^b$</td>
<td>-</td>
<td>157$^a$</td>
</tr>
</tbody>
</table>

HLR (cm/d); N compounds (mg/L); $^a,b$ different letters indicate significant difference from other wetland effluent ($p<0.05$) within same period.
### 3.3.3.1 Ammonia removal

Ammonia removal in the control wetlands was higher than 90% on all occasions from November 2008 to July 2009 when conductivity was at about 2.5mS/cm. In August/September 2009 a salt shock load was applied to the wetland, salinity was increased to the equivalent of seawater (approximately 35g/L of NaCl), when EC values reached 50mS/cm. During this period ammonia removal initially decreased to 73.8% but within one week removal was recovered and sustained over 91.6% until the end of the experiment (Figure 3.6).

Treatment 1 wetlands received only wastewater for one month and then NaCl was added to the influent tank to form a 2.5g/L solution (EC = 6 to 8.4mS/cm) in order to mimic brackish wastewater. From December 2008 to July 2009, T1 received brackish wastewater; during this period ammonia removal was higher than 96%. Similar to the C wetlands, salinity was abruptly increased to 35g/L (EC=50mS/cm) in the last month of monitoring, during the period ammonia removal only slightly decreased to 88% in one sampling event (two days after salinity increased) and was maintained between 91.6 - 99.9% removal for the remainder of the experiment (Figure 3.7).

Treatment 2 wetlands received gradually increasing salinity from raw wastewater (no NaCl added, EC=2.5ms/cm) to hypersaline wastewater (NaCl= 40g/L, EC=60mS/cm). Gradually increasing salinity did not affect ammonia removal as demonstrated in Figure 3.8. From November 2008 to July 2009 ammonia removal was maintained at a minimum of 95%, in only one event removal was 93% (Effluent NH₃-N = 9.0mg/L). As opposed to the shock load trial in the C and T1 wetlands, the T2 wetlands received wastewater without NaCl during August 2009. The sudden change from 40g/L NaCl to 0.0g/L NaCl did not affect ammonia removal, which was maintained at a minimum of 98%.
Figure 3.6: Ammonia nitrogen concentrations in and out of the Control vertical flow wetlands. Electrical conductivity indicated in the secondary Y axis.

Figure 3.7: Ammonia nitrogen concentrations in and out of the Treatment 1 vertical flow wetlands. Electrical conductivity indicated in the secondary Y axis.
Effluent ammonia nitrogen concentrations from the different wetlands were compared. One-way ANOVA indicated no significant differences ($p>0.05$) in NH$_3$-N concentrations among wetlands for the period of November 2008 to July 2009 (Figure 3.9). These results show that neither the maintenance of brackish influent in the T1 wetlands nor the gradual increase of salinity in T2 wetlands affect ammonia oxidation as compared to the control wetlands.

During the shock load experiment the sudden increase in influent salinity to 35g/L of NaCl in the C and T1 wetlands resulted in elevated effluent NH$_3$-N compared to the T2 wetlands which then received influent without salt (Figure 3.10). One-way
ANOVA (log10 transformed data to meet ANOVA requirements) indicated that concentrations were significantly higher ($p<0.05$) in the control and treatment 2 wetland samples than they were in the treatment 1 wetlands. These results indicate that the AOB which adapted to the gradual increase in salinity in the treatment 2 wetlands tolerated the abrupt change to 0.0g/L NaCl. Even though the T1 (brackish wastewater) wetlands had lower mean effluent NH$_3$-N concentration than the C wetland during the shock load experiment this difference was not significant as visible from the overlapping error bars.

The lower concentration of effluent NH$_3$-N (albeit not significantly lower) and higher percentage removal by T1 when compared to the C during the shock load experiment might be explained by the fact that T1 wetlands treated brackish wastewater for 7 months prior to the abrupt increase in salinity and therefore this would have selected for salt tolerant AOB which were more robust to unexpected salinity changes.

It is important to emphasize that although the C system had lower NH$_3$-N removal than the other wetlands during the shock load experiment, its performance was still exceptional with an overall 94% average removal for the period.

Results from this experiment showed that the gradual increase in salinity had no impact on the long term ammonia oxidation in vertical flow treatment wetlands and a sudden increase had only a short term impact on ammonia oxidation. A question
attempted to be answered in one of the following sections is: What are the ammonia oxidising bacteria capable of standing high salinities?

3.3.3.2 NO$_3$-N and TN

For the period of November 2008 to July 2009 NO$_3$-N and TN concentrations in the effluent of the C wetlands were significantly higher than in effluents from T1 and T2 wetlands. Given that conditions in the wetlands were unfavourable for either NH$_3$ volatilization (pH neutral) or denitrification (wastewater and sediments lacked sufficient organic carbon and had high DO content) NO$_3$-N and TN removal was anticipated not to happen and therefore influent and effluent TN values were expected to be similar if not equal.

Effluent TN was mainly constituted of NO$_3$-N due to complete nitrification. Interestingly and unexpectedly the average effluent concentration of TN in the control systems was higher (albeit not significantly higher, p>0.05) than the influent TN concentration. In order to confirm that the high NO$_3$-N effluent was not due to erroneous analysis the same lot of samples was subject to analysis by two independent laboratories which used different NO$_3$-N analysis methods. Results from the different analyses were similar and confirmed the high NO$_3$-N, ruling out the possibility of biased analysis. This was also verified in T1 and T2 wetlands in sporadic events; their overall effluent TN average however was not different from the influent TN.

During the shock load period no difference was verified in effluent NO$_3$-N and TN concentrations among the control, T1 and T2 systems.

3.3.3.3 NO$_2$-N

Effluent NO$_2$-N was highly variable and higher in the wetlands subject to saline conditions. Significant differences among NO$_2$-N concentrations were found in wetlands during the shock load experiment. The T2 wetlands receiving wastewater without salt produced lower NO$_2$-N effluents than the control and T1 systems which
received 35g/L NaCl. The presence of salt somehow affected nitrite oxidation as
demonstrated by the higher NO₂-N levels in the effluent of the systems receiving high
salinity.

### 3.3.4 Load response in the wetlands

To evaluate how the wetlands responded to different hydraulic and nitrogen
loads all data was included in the analysis. Results originating from very small and very
large flows were included and load response curves were drawn. The minimum flow
registered during phase I was 0.32L/d (HLR=3.8cm/d) and the maximum flow was
1.8L/d (HLR=21.6cm/d). During phase II (shock load), the minimum flow registered was
0.64L/d (HLR=6cm/d) and the maximum was 1.34L/d (HLR=17.3cm/d).

#### 3.3.4.1 Phase I: November 2008 to July 2009

A NH₃-N load-concentration chart is presented in Figure 3.11, which summarises
the effluent concentrations of NH₃-N as a response to influent loads. It is visible from
the chart that all VF systems produced effluents lower than 10mg/L NH₃-N when loads
were up to 12-13gNH₃-N/m²/day. When the daily load exceeded 13gNH₃-N/m²/day
effluent concentrations increased above 10mg/L NH₃-N. The load - concentration data is
very similar for the control and treatment wetlands indicating that the ammonia
oxidation rates were similar and even though they were subject to different salinities
these systems were behaving as replicates in terms of ammonia removal. The
exponential trend lines however suggest that the control system can tolerate overloads
more than the treatment systems, indicating a slightly higher ammonia oxidation rate
in the control system. The maximum operating loading rate for these VF wetlands
treating inorganic industrial wastewater with an influent NH₃-N up to 130mg/L was
therefore 13gNH₃-N/m²/day (4,745g/m²/year) if near complete nitrification (>90%
removal) is desired.
During Phase I, the overall average (± standard deviation) NH$_3$-N load removal rates for the C, T1 and T2 wetlands were 11.6g/m$^2$/day (± 2.7), 10.9g/m$^2$/day (± 3.3) and 11.1g/m$^2$/day (±2.5), respectively.

A similar pattern was observed with the hydraulic loading rate (HLR). As the HLR increased past 11cm/day (110L/m$^2$/day) effluent NH$_3$-N concentrations increased above 5mg/L and with HLRs higher than 12cm/day effluent NH$_3$-N were above 10mg/L (Figure 3.12). These results indicate that a HLR higher than 11cm/d exceeded the rate AOB could oxidise the 100-130mg/L NH$_3$-N present in the influent due to the reduced HRT and therefore reduced contact time of the NH$_3$-N with the AOB biofilms. The overlapping data points for the different wetlands reinforce the similar performance of the systems.
3.3.4.2 Phase II: August 2009 - Salt shock (C and T1) and salt relief (T2)

The hydraulic loading rate-concentration chart (Figure 3.13) indicates how adversely affected the C, and to a lesser extent, T1 wetlands were when salt was abruptly increased to 35g/L in the influent. Even low HLR of 5-10cm/day resulted in the effluent NH₃-N being higher in the C wetlands (Figure 3.13). T1 which initially received brackish wastewater was able to produce NH₃-N effluents lower than 2.0mg/L up to about 10cm/day but even at the highest HLR of 15.8cm/day effluent NH₃-N was below 15.0mg/L. The removal of the salt stress in T2 had no impact on ammonia oxidation resulting in very low NH₃-N effluents (<2.0mg/L) even at a HLR of 12cm/day.

The load-concentration data presented in Figure 3.14 also shows the direct impact of salt on non-salt acclimatised systems, as visible in the C wetlands (blue data points). The removal of the salt stress had no impact on ammonia removal suggesting that once acclimatised, systems can cope with varying salinities achieving NH₃-N effluents lower than 2.0 mg/L when NH₃-N load is 19g/m²/day and influent NH₃-N = 150.0mg/L.

During Phase II, the overall average (± standard deviation) NH₃-N load removal rates for the C, T1 and T2 wetlands were 15.0g/m²/day (± 3.2), 15.3 g/m²/day (±2.0) and 15.7g/m²/day (±1.4), respectively.
3.3.5 Ammonia oxidising bacteria (AOB) community

3.3.5.1 Clone libraries of the T2 CW:

Clone libraries were constructed from samples of the T2 wetlands for the December surface (70 clones) and 30 cm deep samples (53 clones), for the March surface (43 clones) and 30 cm deep samples (56 clones), and for the June surface samples (48 clones). No unspecific amplification occurred with the amoA primer pair.
Table 3.5 shows that the majority of clones obtained from the surface samples were most similar to *Nitrosomonas* sp Nm107 (AF272407) and *Nitrosospira* sp 9SS1 (DQ228455). Only in the December 2008 surface sample, when the system was under 2.5gNaCl/L, a sequence 99% similar to an uncultured bacterium clone isolated from an anoxic biofilm (AF202650) (Schmid et al., 2000), was found in low abundance, sequence analysis revealed that this bacterium is also closely related to *Nitrosomonas* sp Nm 107. In the 30cm deep samples, this same anoxic biofilm-like clone was abundant, comprising 70% of clones in December 2008, but its abundance decreased to 16% in March 2009 as salinity increased to 15g/L NaCl. Interestingly, the number of unique *amoA* clone OTUs, based on HaeIII RFLP, increased with depth, with a total of 6 different OTUs being found at the 30cm deep in March 09 when the system was subject to 15g/L NaCl.

Table 3.5: Percentage of occurrence (%) of individual clone OTUs in the T2 wetland at different depths and salinities.

<table>
<thead>
<tr>
<th>Clone OTUs</th>
<th>Similarity (%)</th>
<th>Closest relative (Accession Number)</th>
<th>Dec-08 2.5gNaCl/L</th>
<th>Mar-09 15gNaCl/L</th>
<th>Jun-09 35gNaCl/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>Surface</strong></td>
<td><strong>30 cm</strong></td>
<td><strong>Surface</strong></td>
<td><strong>30 cm</strong></td>
</tr>
<tr>
<td>1</td>
<td>99</td>
<td><em>Nitrosomonas</em> sp Nm107 (AF272407)</td>
<td>90</td>
<td>16</td>
<td>70</td>
</tr>
<tr>
<td>2</td>
<td>98</td>
<td><em>Nitrosospira</em> sp 9ss1 (DQ228455)</td>
<td>3</td>
<td>8</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>98</td>
<td><em>amoA</em> Anoxic biofilm clone S1 (AF202650)</td>
<td>7</td>
<td>70</td>
<td>18</td>
</tr>
<tr>
<td>4</td>
<td>93</td>
<td><em>Nitrosomonas</em> ureae (AJ388585)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>95</td>
<td><em>Nitrosomonas</em> oligotropha (AF272406)</td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>97</td>
<td><em>amoA</em> Anoxic biofilm clone S1 (AF202650)</td>
<td></td>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>

3.3.5.2 T-RFLP

Only results from the AluI based T-RFLP are reported here and further discussed in the following sections, this is because the AluI based T-RFLP resulted in optimal peak separation. Results of the HaeIII and TaqI based TRFLP are reported in the Appendix B section. The structure of the AOB community was analysed in regards to depth, time of the year (season) and salinity. T-RFLP analysis with AluI digestion produced 9 distinct OTUs in total, represented by peaks: 80bp, 104bp, 114bp, 215bp, 224bp, 360bp, 392bp, 405bp and 491bp.

OTUs were assigned taking into account information that has been previously published on T-RFLP of the *amoA* gene and also by cloning and sequencing. According
to the literature, some peaks have been claimed to be highly indicative of specific AOB groups, for example, Horz et al. (2000) indicated that the AluI-104bp fragment represented the “Plußsee” lineage and the AluI-224bp fragment represented the “Schöhsee” lineage (Rotthauwe et al., 1997, Horz et al., 2000). The 392bp fragment has been assigned to the Nitrosospira sp Ka4 and the 491bp is a signature of Nitrosospira sp strain Nsp1 and Nitrosospira briensis (Mintie et al., 2003). In this study a major 405bp T-RF has been identified as belonging to Nitrosomonas sp Nm107-like sequences (Nm104, Nitrosococcus mobilis) and peak 224bp has been assigned to Nitrosomonas ureae-like sequences. The identity of fragments 80bp, 114bp and 360bp could not be verified in this study and still require further validation.
Figure 3.15: Phylogenetic tree of partial amoA gene sequences of the 11 AOB clones obtained in this study and most similar sequences retrieved from GenBank. Neighbour joining analysis carried out using clustalW2.0.12. *Methylococcus capsulatus* strain BL4 (AF533666) selected as outgroup. Scale bar represents 10% nucleotide divergence. (Numbers in parentheses represent AluI, HaeIII and TaqI T-RFLs, respectively).
3.3.5.2.1 Control wetland

TRFLP analysis of AluI digests produced a few consistently prominent peaks in all samples. Peaks 405bp (all samples), 491bp (14 of 15 samples) and 224bp (12 of 15 samples) dominated the control wetlands in terms of occurrence (presence or absence) and relative abundance (% of each peak area in relation to total peak area in a profile). Some secondary peaks, 360bp (6 of 15 samples), 392bp (4 of 15 samples), 104bp (4 of 15 samples) and 215bp (1 of 15 samples) were present always in low abundance (Figure 3.16). It is interesting to see that the AOB community presented a few changes from December 2008 to the 5th of August 2009, even though the only changes in operating conditions during the time was increased NH₃-N in the influent (100 to 150mg/L) and normal seasonal temperature variation (26 – 15°C).

When only surface samples were compared (Figure 3.17) the change of T-RF composition among months is quite evident showing that the AOB community was not static along the year. In September 2009 when the system had been subject to 35g/L of NaCl only the 405bp and 491bp T-RFs were present and the 224bp disappeared, likely indicating a NaCl sensitive strain or strains.
Figure 3.16: Relative abundance of T-RFs generated by AluI digestion of the amoA gene retrieved from different depths of the control wetland. The percentage which is not shown in graphs corresponds to the sum of peaks that fell below the threshold (1-3%).
3.3.5.2.2 Treatment 1 wetland

Treatment 1 wetland received influent spiked with 2.5g/L NaCl from December 2008 to 5\textsuperscript{th} of August 2009. Changes in the abundance of T-RFs at different depths and months is presented in Figure 3.18. Again T-RFs 405bp and 491 bp predominated in all samples; fragment 224bp constantly decreased in abundance and nearly disappeared in Aug 2009 when the system was still under 2.5g/L NaCl at the time of sampling, this fragment was only present at 30cm deep and represented only 2% of the total peak area for that profile.

Less frequent and less abundant peaks were also present in T1, 104bp (1 of 15 samples), 114bp (2 of 15 samples), 215bp and 392bp (4 of 15 samples) and 360bp (5 of 15 samples), always representing less than 8% of the total peak area in any profile. Similarly to what was observed in the control, the community was not static and the proportion of members changed when the system was subject to a constant salinity of 2.5gNaCl/L.

The view of the surface samples is presented in Figure 3.19. Similar to what happened at the C the sudden increase in salinity eliminated other groups and selected for the 405bp and 491bp fragments. The higher relative abundance of fragments 405bp and 491bp on the surface of T1 throughout the experiment also suggest that these strains are more resistant to brackish and saline environments.
Figure 3.18: Relative abundance of T-RFs generated by AluI digestion of the amoA gene retrieved from different depths of the T1 wetland. The percentage which is not shown in graphs corresponds to the sum of peaks that fell below the threshold (1-3%).
Figure 3.19: Relative abundance of T-RFs generated by AluI digestion of the amoA gene retrieved from the surface of the T1 wetland. The percentage which is not shown in graphs corresponds to the sum of peaks that fell below the threshold (1-3%).

3.3.5.2.3 Treatment 2 wetland

Treatment 2 wetland was not sampled in December 2008 for microbial analysis as it was receiving the same NaCl concentration (2.5g/L) as T1 wetland at the time. A comparison of the AOB composition between 0g/L NaCl and 2.5g/L NaCl has been presented in the C and T1 wetlands.

The composition of the AOB at different depths when the wetland was subject to 10 and 40g/L NaCl is shown in Figure 3.20. Again, the predominant peaks were 405bp and 491bp (in all 11 of 11 samples) followed by peak 360bp (6 of 11 samples) which had low abundance throughout the study with a maximum of 3.3% of total peak area) and 224bp (5 of 11 samples). The 224bp fragment was abundant in the profiles at up to 10g/L NaCl, representing 22% of the total peak area at 15cm deep in March 2009, but nearly disappeared in August (40g/L NaCl) accounting for only 3.4 % of the total peak area at 30cm deep. The 392bp fragment was only present in 4 of the 11 samples, 3 of them being at the highest salinity. An 80bp fragment unique to T2 wetland appeared in low abundance (<2%) in the 30cm deep samples.

In all surface samples from 5 to 40g/L NaCl the 405bp and 491bp peaks predominated, interestingly the smaller 114bp and 392bp peaks were still present at hypersaline conditions.
Figure 3.20: Relative abundance of T-RFs generated by AluI digestion of the *amoA* gene retrieved from different depths of the T2 wetland. The percentage which is not shown in graphs corresponds to the sum of peaks that fell below the threshold (1-3%).

Figure 3.21: Relative abundance of T-RFs generated by AluI digestion of the *amoA* gene retrieved from the surface of the T2 wetland. The percentage which is not shown in graphs corresponds to the sum of peaks that fell below the threshold (1-3%).
3.4 Discussion

3.4.1 Plant health

*Schoenoplectus validus* can occur in fresh to brackish lakes and estuarine locations with EC varying from 0.6 to 12mS/cm (Hammer, 1986). *S. validus* was chosen in this study because it is a native species from the south west of Australia and rhizomes were readily available from the University campus. Furthermore, knowing the salinity threshold for plants growing in high nutrient concentrations was a desired outcome from an industrial wetland management/operation perspective.

During phase I of this experiment, *S. validus* did not present signs of stress in the T1 wetlands (EC =7mS/cm) but had significantly lower biomass that the C system (EC= 2.5mS/cm). Our results are in agreement with the statements of Wu *et al.* (2008) that most of the commonly used constructed wetland plants are freshwater species and are not suitable for treating wastewater containing salts or saline wastewater. We have demonstrated that *S. validus* is far from being an ideal candidate to be planted in VFCWs treating saline effluents, especially if high biomass is desired.

Calheiros *et al.* (2010) verified that *Arundo donax* and *Sarcocornia* sp were resilient to salinity (EC= 20mS/cm) in HF-CWs polishing tannery wastewaters. Wu *et al.* (2008) verified that the mangrove species *Aegiceras corniculatum* produced more biomass and up took more N and P at 15g NaCl/L rather than at 0 or 30g NaCl/L, being a potential candidate for CWs treating saline wastewaters. Other mangrove species should therefore be considered as candidates for CWs treating saline wastewaters.

3.4.2 Ammonia removal

The presence of salt did not affect the performance of the VFCWs in this study. The fresh, brackish and saline systems performed equally in terms of ammonia removal. The good performance of the saline wetlands is in accordance with what Calheiros *et al.* (2010) and Lefebvre *et al.* (2004) found when studying different systems treating saline tannery wastewaters. The death of plants in T2 did not seem to have any impact on performance.
Tanneries wastewater treatment systems have gained special attention in the last decade (Calheiros 2010, Lefebvre et al., 2004) because of the saline wastewaters generated by the pickling, chromium tanning and the soaking of hides and skins, which use large amounts of salt. Different to our high nitrogen and low carbon scenario, tannery wastewaters are usually characterised by high organic and chromium contents so treatment systems are focused at primarily removing these compounds. Calheiros et al. (2010) studied the bacterial diversity in polishing HF-CWs receiving wastewater with EC =20mS/cm. The high salt content of the wastewater did not affect treatment in the CWs and removal of COD, ammonium (NH4+) and total Kjeldahl nitrogen (TKN) ranged between 58 to 77%, 60 to 86% and 51 and 79%, respectively. It was verified that the CWs harboured a highly diverse bacterial community and that the presence of plants seemed to impact the distribution of bacterial groups (Calheiros et al., 2010).

Lefebvre et al. (2004) assessed the microbial diversity in soak liquor pits (72.3g/L NaCl), an activated sludge plant (6.9g/L NaCl), a sequencing batch reactor (52.5g/L NaCl) and an upflow anaerobic sludge blanket reactor (45.6g/L NaCl), they found that COD removal was similar to that of non saline systems and that microbial diversity was in the same range as that of non saline counterparts. They concluded that the halophytic sludge biodiversity allows the biological treatment of hypersaline wastewater to be of the same efficiency as that observed in fresh wastewater treatment systems, both aerobically and anaerobically.

As expected, when subject to a salt shock, ammonia removal was more affected in the fresh system than in the brackish system, indicating that a salt acclimatised AOB biofilm is less prone to failure in situations of sudden salinity variations, which are very likely to happen in industrial wastewater treatment systems. Panswad and Anan (1999) also verified that salt acclimated activated sludge units recovered from NaCl shock loads more rapidly than non acclimated ones. They also found that nitrogen transforming bacteria could rapidly adapt to environments with higher salt concentrations. This is also in accordance with our results, as demonstrated by the quick recovery in nitrification of the control system when subject to the shock load.

The average NH3-N load removal rates obtained during phases I and II of this study ranged from 10.9 to 15.7g/m²/day. These load removal rates are similar to the
ones reported by Kantawanichkul et al. (2009) in VF wetlands treating high strength wastewater under tropical climate. In their study, NH$_3$-N removal rates varying between 4.4 and 16.2g/m$^2$/day were obtained in planted systems. Higher removal rates occurred in parallel with higher influent loadings but, similar to what was observed here, effluent concentrations were also higher during these events.

### 3.4.3 Nitrate and total nitrogen

The formation of nitrate and inefficient nitrogen removal by the VFCWs was expected due to the lack of carbon and aerobic conditions maintained in the bed (Langergraber, 2001). The fact that effluent total nitrogen concentrations were higher than influent concentrations in the control system was rather unexpected. One possible explanation for the increase in concentration would be evapotranspiration which decreases the available volume and therefore concentrates the solution. This would only partially explain an increase in TN as the estimated evaporation was minimal (loss <5%) and could not account for the nearly 20% increase in TN concentration in the effluent. Another explanation would be mineralisation of organic nitrogen compounds present in the wastewater and wetland sediment via ammonification with subsequent nitrification and increase in NO$_3$-N effluent. But, because the wastewater used in this study was free of organic matter and the substrate consisted of clean beach sand and gravel free of nitrogenous organics the hypothesis of ammonification and subsequent nitrification contributing to higher TN effluent is not plausible. Besides, evapotranspiration and ammonification would be similar in the control and treatment systems, not explaining the higher concentration in the control wetlands.

An explanation for this phenomenon has not been formulated but it is believed that feed-stay-drain-rest operation might have partially contributed to such observation. Because of the positive charge of the ammonium ion, it is subject to cation exchange and thus capable of adsorbing to the substrate (Kadlec and Wallace, 2009). Part of the ionized ammonia from a batch may leave the solution to remain loosely sorbed to the sediment. During the resting period this sorbed ammonium can be
oxidised to nitrate. Because nitrate is not bound to the substrate it is washed out by the subsequent rewetting of the feed cycle. The tidal operation of the wetlands with a resting period associated with ammonium adsorption to sediments which remains in contact with the nitrifying biofilms under aerobic conditions may therefore have contributed to higher TN concentrations in the C wetlands. The presence of NaCl may result in fewer adsorption sites available for NH$_4^+$ to bind because Na$^+$, just like NH$_4^+$, binds to negative particles in the sediment. This “competition” for adsorption sites might explain why such higher NO$_3$-N values occurred in the control system without NaCl and not in the T1 and T2 systems with NaCl. Sands, however, have a very low cation exchange capacity, so this theory would only partially explain the increase in TN.

3.4.4 Nitrite formation

Nitrite oxidisers were somehow affected by increasing salinity as demonstrated by the accumulated nitrite in the wetlands subject to higher salinity and especially during the shock load phase. Although not significantly different, during the NaCl shock phase, nitrite concentrations were higher in the non salt adapted system (C) than in the salt adapted system (T1). This also demonstrates that salt acclimated nitrite oxidisers can more easily recover from a NaCl shock load. Moussa et al. (2006) also verified nitrite build up in SBR receiving increasing salt concentrations. Up to 95% inhibition of ammonia and nitrite oxidisers was verified in the reactors when salinity was 40gCl$^-$/L (66gNaCl/L).

One possible explanation for nitrite accumulation is the actual inhibition of nitrite oxidisers (Moussa et al., 2006); another explanation would be oxygen limitation under saline conditions (Campos et al., 2002) which leads to incomplete nitrification and nitrite accumulation. Salt affects oxygen solubility in water which can lead to limited oxygen availability (Moussa et al., 2006, van’t Riet and Tramper, 1991). The average effluent DO concentrations obtained in the saline systems (Table 3.3) corroborate the idea that there was enough oxygen for complete nitrification to occur in the VFCWs, therefore we can discard the possibility of low oxygen availability.
Chen et al. (2003) observed nitrite accumulation in their batch culture experiments and verified that only *Nitrobacter* species could survive salinities up to 10gCl-/L (16.5gNaCl/L), beyond 18gCl-/L no nitrite oxidisers could be observed. Differently, Moussa et al. (2006) found that, although both being present, *Nitrospira* rather than *Nitrobacter* dominated their SBRs at salinities up to 10gCl-/L. At salinities higher than 10gCl-/L, no nitrite oxidisers could be detected, but when the salt stress was relieved only *Nitrobacter* was recovered, indicating that this genus is more resistant to high salinities.

3.4.5 Impact on AOB

Our Alul T-RFLP results revealed that the surface of the control wetlands was dominated by 3 main groups of the 224bp, (*Nitrosomonas oligotropha/ureae-like*), 405bp (*Nitrosomonas* sp Nm107-like) and 491bp (*Nitrospira* sp 9SS1-like). Other groups such as the 360bp and 392bp were sporadically present in low percentages. An important observation is that the AOB community was not static under fairly constant operating conditions and varied throughout the experiment. When considering the different depths, from December 2008 to August 2009, group 224bp decreased at the 5cm, 15cm and 30cm locations. The step increases in ammonia concentrations (100 to 130 and slight temperature drop might have contributed to such change in their abundance. This 224bp group is closely related to *N. oligotropha and N. ureae*, (also *Nitrosomonas* sp Nm 47, *Nitrosomonas* sp NL7) which have been characterised as being salt sensitive (maximum salt tolerance 200mM NaCl) and having a low substrate affinity (K=1.9-4.2uM NH₃) (Koops et al., 2006).

As salinity increased the proportion of the 224bp fragment decreased in both the T1 and T2 CWs. Interesting however was the fact that this fragment was abundant in the 5cm-30cm samples of the T2 wetlands at 10gNaCl/L and was still present (albeit at very low abundance of 3.3%) in the 30cm sample at 40gNaCl/L.

Moussa et al. (2006) found that *Nitrosomonas europaea* and *Nitrosococcus mobilis* (*Nitrosomonas* sp Nm107) were abundant at higher salinities but only *N. europaea* was present at 40g Cl⁻/L and also after the salt stress was removed, indicating
that other groups were completely eliminated by such high salinity. At 0gCl/L *Nitrosomonas oligotropha* was the dominant species followed by *N. mobilis* and then equally by *N. europaea* and *Nitrosospira* sp. *Nitrosospira* sp and *N. oligotropha* were present in relatively high numbers at salinities up to 10gCl/L but could not tolerate further salt increases.

In a saline nitrifying batch culture, the dominant AOB species shifted from *N. europaea* and *N. eutropha* to *Nitrosococcus mobilis* when salinity was increased from 10gCl/L to 18gCl/L (Chen *et al.*, 2003). Chen *et al.* (2003) found no *Nitrosospira* in either the fresh or saline batch cultures. In a marine aquaculture biofilter receiving low ammonia concentrations, amongst ten different nitrifying populations, only the AOB *Nitrosomonas* sp Nm143 and *Nitrosomonas* marina cluster 2, and the NOB *Nitrospira marina* were quantitatively relevant Foesel *et al.* (2008). The authors suggest niche differentiation as potential explanation for the coexistence of the two dominant AOB populations: *Nitrosomonas marina*, possibly, representing a high affinity/low activity population, which is active under normal low ammonium concentrations and the second being a low affinity/high activity population, possibly represented by *Nitrosomonas* sp. Nm143, which thrives under high ammonium concentrations.

The increase in salinity contributed to the dominance of *Nitrosomonas* sp Nm107-like (405bp) and *Nitrosospira* sp 9ss1-like (491bp) while *Nitrosomonas oligotropha/ureae-like* (224bp), which had a high abundance initially, was nearly eliminated. *Nitrosospira* sp 9ss1 (491bp) usually presented higher abundance on the superficial samples (surface, 5cm deep), independent of salinity. *Nitrosospira* members are usually found in unsaturated natural soils (agricultural, forest) but have also been detected in fresh and saline systems (Koops *et al.*, 2006; Freitag *et al.*, 2006; Bernhard *et al.*, 2005). The conditions on the surface of the sediment in the VFCWs might have contributed to the success of *Nitrosospira* sp-like bacteria over other less favoured groups. The surface of VFCWs is characterised by a periodic lower water content due to draining and evaporation (Langergraber, 2001), higher O2 availability due to atmospheric diffusion into the biofilms and temporary high substrate concentrations due to intermittent inflow. These facts suggest that *Nitrosospira* sp 9ss1 (*Nitrosospira multiformis*) members are likely to have high substrate affinity. The results from this experiment also confirm the high salt tolerance of *Nitrosospira* members.
Ward et al. (2000) found that in the hypersaline (74 to 88gNaCl/L) Mono Lake, California, USA, most AOB sequences were closely related to *N. europaea*, and *N. eutropha*. This is surprising as cultured *N. europaea* and *N. eutropha* do not have a salt requirement and have shown to be inhibited by higher salinities. Our analyses have revealed that *Nitrosomonas* sp Nm107-like (*Nitrosococcus mobilis*) sequences (AluI=405bp), which belong to the *N. europaea* lineage (cluster 7), dominated at lower and higher salinities, indicating the environmental versatility of this group. The same was observed for *Nitrosospira multiformis*-like sequences (AluI= 491bp) which were abundant from low to high salinity environments.

Although AOB diversity was not directly measured in this study, a loss in the number of OTUs, as revealed by TRFLP, was verified as salinity increased above 2.5gNaCl/L. Berhnard et al. (2005) verified a loss in AOB diversity as a consequence of increased salinity in an estuary system in Massachusetts, USA. They also verified a decrease in seasonal variability in the community as salinity increased. The same phenomenon was observed during this experiment, especially in the surface samples; furthermore the AOB community was very stable and active at salinities up to 40gNaCl/L.

### 3.5 Conclusions

The VFCWs tested in this study received inorganic industrial wastewater with average influent concentrations of 103.8 to 106.5 mg NH₃-N /L. When operating under a HLR of 11cm/d, ammonia removal above 90% could be achieved, representing a load removal rate in the order of 12gNH₃-N /m²/d. Such removal could be achieved in the control (0gNaCl/L), T1 (2.5gNaCl/L) and T2 (up to 40gNaCl/L) wetlands. When the HLR and NH₃-N loads were higher than 11cm/d and 12gNH₃-N/m²/d, respectively, ammonia removal decreased in all wetlands with the C and T1 demonstrating a higher removal capacity than the T2 wetlands.

A gradual increase in influent salinity up to 40gNaCl/L did not impact ammonia oxidation in VFCWs whereas a sudden increase (shock load) in salinity to 30gNaCl/L, however, negatively impacted ammonia removal in the short term. This impact was
higher in the control system than in the brackish system, indicating the higher resilience of salt acclimated AOB.

Nitrite accumulated in the systems subject to higher salinity and in this study the effect is believed to be directly caused by salt affecting the activity of NOB, rather than partial nitrification due to limited oxygen availability.

The increase in salinity selected for *Nitrosomonas* sp Nm 107-like (*Nitrosococcus mobilis*) and *Nitrosospira* sp 9SS1-like (*Nitrosospira multiformis*) AOB while other groups were eliminated or only present in very low proportions. The wetlands subject to brackish wastewater were less affected by a sudden increase in salinity than the control, freshwater wetlands, corroborating the importance of acclimating systems (i.e. allowing time for resistant populations to build up) in order to cope with increasing stress factors such as salinity. The removal of the salt stress from the wetlands that were previously subject to hypersaline conditions did not affect nitrification, demonstrating the versatility of the salt adapted AOB community in VFCWs.
Chapter 4:

Nitrification and denitrification in semi-saturated sand filters: Effect of salinity and dosing of external carbon

4.1 Introduction

Vertical flow constructed wetlands (VFCWs) have been shown to achieve total nitrification (>95%) of fresh and saline inorganic wastewaters when operated at a HLR up to 13cm/d and an ammonia loading rate up to 15g/m²/d. Nitrate removal is not favourable due to the aerobic conditions of the VFCW filter and the lack of organic carbon in the wastewater and substrate.

In this chapter, two of the VFCWs columns used in Chapter 3 were modified in order to incorporate a saturated zone in the drainage layer on the bottom as per suggestions of Langergraber et al. (2009) for TN removal. In addition, a concentrated liquid carbon source was introduced into this saturated zone as reducing power for denitrification. The performance of the modified design was analysed in terms of ammonia and nitrate removal in controlled (without NaCl) and treatment conditions (30gNaCl/L), additionally, NO₂-N, COD, DO, Redox were monitored and the ammonia oxidising bacteria (AOB) and denitrifying bacteria (DB) communities inhabiting the substrate characterised.

Denitrification, the biological stepwise reduction of NO₃-N and NO₂-N to the gaseous compounds nitric oxide (NO), nitrous oxide (N₂O) and ultimately dinitrogen (N₂) is carried out by a diverse group of prokaryotes under anoxic conditions (Ahn, 2006). Denitrifiers play an important role in controlling global N fluxes. In marine ecosystems denitrifiers control the availability of N for primary producers, therefore influencing the productivity of oceans. Also of concern is the potential contribution of denitrifiers to global warming through the release of N₂O from incomplete denitrification (Corredor et al., 1999). Denitrifiers are responsible for N losses in agricultural soils and are considered the primary players in N removal from wastewaters (Magalhaes et al., 2008).
Determining how environmental factors affect the distribution and control the dynamics of denitrifiers is of extreme importance for understanding the ecosystem-level controls on the biogeochemical process of denitrification (Magalhaes et al., 2008).

4.2 Materials and Methods

4.2.1 Experiment set up

Two of the VFCW columns used as control wetlands in Chapter 3 had their plants removed and outlet pipe arrangement raised in order to create a 30cm deep saturated zone on the bottom of the systems as demonstrated in Figures 4.1 and 4.2. For this experiment, both columns remained unplanted and therefore the choice for calling them sand filters rather than wetlands, which typically refer to planted systems. The sediment and gravel media were kept unchanged as to provide an AOB colonised environment. While the top 45 cm of sand remained unsaturated to favour nitrification, the saturated conditions on the bottom would help reduce oxygen transfer and promote anoxic conditions for denitrifiers.

This experiment was conducted between November 2009 and February 2010 and prior to its start both columns were fed with fresh and occasional brackish (EC= 20mS/cm) flows for two months. Concentrated inorganic wastewater was collected from CSBP Ltd in Kwinana and brought to the laboratory to be diluted with tap water until the desired nitrogen concentration was achieved (Please refer to Table 3.2 in Chapter 3 for undiluted wastewater characteristics). Once diluted, the wastewater was placed in the storage tank. A submersible pump was placed in the wastewater storage tank and connected to a timer to feed between 0.7L and 1.1L of wastewater to the column in a one minute interval on a daily basis. The control (C) column received wastewater only and the treatment (T) column received wastewater spiked with 30gNaCl/L.
4.2.1.1 Carbon dosing

Because of the low organic carbon content of the wastewater acetic acid was introduced as a carbon source into the top of the saturated zone by a peristaltic pump. The peristaltic pump was connected to the same timer as the wastewater dosing pump so both pumps ran for one minute at the same time on a daily basis. This arrangement would allow the nitrified wastewater to mix with the just injected acetic acid as it percolated down from the unsaturated to the saturated zone. The COD equivalence of
1g of glacial acetic acid = 1.07g COD. Glacial acetic acid was diluted to 5% using distilled water to prepare a 53,500mg/L COD (53.5mg/mL) solution. The peristaltic pump delivered 10mL of this solution (535mg COD) to each column. The influent wastewater had an average COD of 33.83 mg/L (±8.13 std dev) and TN content of 165.5 mg/L, therefore the initial influent COD:N ratio is 0.2. By adding 535mg of COD to the column the final COD concentration would rise to 568.83mg/L and COD:N = 3.4 if we assume that one litre of wastewater was added to the column. Wastewater inflows, however, were not constant and ranged from 0.7 to 1.1L/day, influent COD therefore varied from 812.6 to 517.1mg/L. In one event both columns received 20mL of the 5% acetic acid solution due to a timer malfunction and in another event the T system received approximately 5mL of acetic acid due to a blockage in the feeding tube.

4.2.2 Water sampling and analysis

Influent and effluent samples were collected and analysed for pH, temperature, DO, ORP, EC, NH₃-N, NO₃-N and NO₂-N according to the methods described in Chapter 3. Total nitrogen (TN) is reported as the sum of NH₃-N, NO₂-N and NO₃-N. COD was measured spectrophotometrically using a HACH DR 2800™ spectrophotometer following the potassium dichromate reactor digestion method (Hach method: 8000) with dilution and addition of mercuric sulphate to eliminate Cl⁻ interference.

Wastewater characteristics are shown in Table 4.1 (Results section). Both filters received the same wastewater with the only differences being the addition of salt in the T system and slight variations in the COD dosed into the saturated zone. A NaCl tracer test was conducted, according to the method described in Chapter 3, in order to determine the actual HRT.

4.2.3 Sediment sampling and analysis:

Sediment samples were collected for bacterial analysis from both filters at 15cm (unsaturated) and 45 cm (saturated-carbon injection point) deep at day 44. The AOB was characterised at the 15cm depth and the DB at the 45cm depth. Collection of 3.0g
of sediment was performed through the sampling ports by inserting the wider side of a sterile pipette tip into the sediment, the sediment contained within the pipette tip was transferred into sterile tubes which were then stored at -20°C until DNA extraction. DNA extraction was performed according to the protocol described in Chapter 3. Eluted DNA was stored at -20°C until further use.

4.2.4 PCR and T-RFLP of AOB

The AOB specific pair amoA-1F 5'-FAM (6-carboxyfluorescein) labelled primer (5'-GGG GTT TCT ACT GGT GGT) and amoA-2R (5'-CCC CTC KGS AAA GCC TTC TTC) (Rotthauwe et al., 1997) targeting a 491 base pair (bp) region of the amoA gene was used in the PCR. Amplifications were performed in 50µL reactions and PCR cycles have been described by Siripong and Rittmann (2007) with annealing at 55°C. PCR products of the expected size were purified with the Wizard SV Gel and PCR clean up kit (Promega, Madison, Wi). Purified PCR products were digested with Alul and TaqI restriction enzymes (RE) according to the manufacturers’ recommendations (Promega, Madison, Wi). One µl of each digest was added to 0.25 µl LIZ600 marker (Applied Biosystems, CA) and 9 µl Hi-Di™ formamide (Applied Biosystems, CA) and fragment analysis performed in an Applied Biosystems 3730 DNA sequencer (SABC, Murdoch University). Resulting profiles were analysed using Genemapper software (Applied Biosystems, CA). T-RFLP profiles were normalised according to the constant percentage threshold (Sait et al., 2003). Peak areas that fell below the percentage threshold (ranging from 1 - 3% of the total peak area, depending on the profile) were therefore disregarded.

4.2.5 PCR and Clone Libraries of DB

The DB community was analysed by using the nosZ gene as a molecular marker, the nosZ gene encodes for the enzyme nitrous oxide reductase, which catalyses the reduction of the greenhouse gas N₂O to N₂ in the denitrification pathway (Enwall and Hallin, 2009). The nosZ gene was recently shown to have the greatest level of
congruence with 16S rRNA-based taxonomic classification (Jones et al. 2008). The primer pair nosZ1F (5’ WCS YTG TTC MTC GAG AGG CAG) and nosZ1R (5’-ATG TCG ATC ARC TGV KCR TTY TC), degenerated sites (W = AT, S = CG, Y = CT, M = AC, R = AG, K = GT, and V = ACG) published by Henry et al. (2006) was used to amplify a 259bp fragment of the nosZ gene. Henry et al. (2006) noted that these nosZ primers were not able to amplify the nosZ gene from Bacillus strains, indicating that such primers were probably specific only for the nitrous oxide reductase from gram-negative bacteria.

Amplifications were performed in 25µL reaction volumes for cloning purposes and. Reactions contained 10 to 20ng of template DNA, 1x PCR buffer, 2.5 mM of MgCl2, 0.2 mM of mixed dNTPs, 0.5µM of each primer 1.0 U of Taq DNA polymerase (Promega, Madison, Wi).

Cloning reactions and transformations were performed according to the instructions in the PGEM-T easy Vector System and JM109 competent cells (Promega, Madison, Wi). Positive clones from the libraries were selected for the amplification of the nosZ gene and subsequent digestion with HaeIII RE according to the manufacturers’ instructions. Digested products were electrophoresed and visualised in 3% agarose gels. Different restriction fragment patterns were assigned to different operational taxonomic units (OTUs).

4.2.6 Sequencing and phylogeny

Clones that contained nosZ genes which resulted in dissimilar cutting patterns were selected for insert sequencing in an Applied Biosystems ABI 3730 sequencer, (SABC, Murdoch University). Sequences in the GenBank database (NCBI: http://blast.ncbi.nlm.nih.gov) sharing the greatest similarities were selected for multiple alignments using ClustalW2.0.12 software (Larkin et al., 2007). Phylogenetic trees were visualised with Treeview software (Page, 1996). Sequences from this chapter will be deposited in GenBank.
4.2.7 Data analysis

Microsoft Excel was used to tabulate data and graphs. Student t-test was performed between effluent concentration data from the C and T system in order to evaluate the effect of salt in treatment efficiency. The significance level for all tests was \( p \leq 0.05 \).

4.3 Results and discussion

4.3.1 Influent

Influent pH was neutral to slightly acidic, temperature ranged from 24 to 26°C and nitrogen forms were on average \( \text{NH}_3\text{-N} = 158\text{mg/L} \), \( \text{NO}_3\text{-N} = 6.75 \) and \( \text{NO}_2\text{-N} = 0.18\text{mg/L} \) and varied only slightly during storage (Table 4.1). Flows were kept between 0.7 and 1.1 L/day with an average of 0.83L/d, this resulted in an average COD in the saturated zone of 704mg/L (COD:N= 4.26) and 687mg/L (COD:N=4.15) for the C and T systems, respectively. The average HLR of both systems was 10cm/d and the actual HRT was calculated as 2.03 days according to a NaCl tracer study.

4.3.2 Effluent

Effluent pH for the C and T was neutral and just slightly, but not significantly, higher than the influent pH. Temperature was on average 26°C for both systems, which is within the optimum range for biological nitrogen removal systems. DO dropped due to nitrification and excess COD from the acetic acid addition, concentrations were similar in both C and T, ranging from 0 to 3.15mg/L and 0 to 2.85mg/L, respectively.
Table 4.1: Mean values, ± standard error, of parameters analysed from the inlet and outlet samples of the control and treatment systems and calculated COD dosed into the saturated zone. $n_{\text{influent}}=3$, $n_{\text{effluent}}=38$.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Effluent</th>
<th>% Removal</th>
<th>Treatment</th>
<th>Effluent</th>
<th>% Removal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow (L/d)</td>
<td>0.83 ± 0.01</td>
<td>0.83 ± 0.01</td>
<td>0.83 ± 0.01</td>
<td>0.83 ± 0.01</td>
<td>0.83 ± 0.01</td>
<td>0.83 ± 0.01</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>25 ± 1</td>
<td>25.9 ± 0.38</td>
<td>25 ± 1</td>
<td>25.9 ± 0.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC (mS/cm)</td>
<td>1.77</td>
<td>2.44 ± 0.02</td>
<td>50.2</td>
<td>50.9 ± 0.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>6.94 ± 0.22</td>
<td>7.09 ± 0.03</td>
<td>6.94 ± 0.22</td>
<td>7.07 ± 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DO (mg/L)</td>
<td>6.76 ± 0.27</td>
<td>0.91 ± 0.12</td>
<td>6.76 ± 0.27</td>
<td>0.82 ± 0.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ORP (mV)</td>
<td>-11.0 ± 17</td>
<td>-42.1 ± 18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH$_3$-N (mg/L)</td>
<td>158 ± 3.6</td>
<td>7.78 ± 1.1</td>
<td>95</td>
<td>158 ± 3.6</td>
<td>16.5 ± 1.32</td>
<td>90</td>
</tr>
<tr>
<td>NO$_2$-N (mg/L)</td>
<td>0.18 ± 0.08</td>
<td>5.27 ± 0.65</td>
<td>0.18 ± 0.08</td>
<td>4.73 ± 0.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO$_3$-N (mg/L)</td>
<td>6.75 ± 0.53</td>
<td>14.6 ± 3.7</td>
<td>6.75 ± 0.53</td>
<td>16.1 ± 2.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TN (mg/L)</td>
<td>165 ± 4.2</td>
<td>27.5 ± 4.2</td>
<td>83</td>
<td>165 ± 4.2</td>
<td>37.3 ± 3.9</td>
<td>77</td>
</tr>
<tr>
<td>COD (mg/L)</td>
<td>704 ± 18</td>
<td>128 ± 14</td>
<td>82</td>
<td>687 ± 23</td>
<td>140 ± 13</td>
<td>79</td>
</tr>
<tr>
<td>COD:N ratio</td>
<td>4.26 ± 0.11</td>
<td>8.71 ± 1.49</td>
<td>4.15 ± 0.14</td>
<td>5.79 ± 0.92</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.3.2.1 NH$_3$-N

Even though the mean effluent NH$_3$-N concentration was significantly lower ($p<0.05$) in the C filter, ammonia removal was high in both systems with an average of 95% removal for the C and 90% removal for the T. The minimum of 70% removal in the T filter occurred during the first week of experiment monitoring just after an increase in salinity from 20mS/cm to 50mS/cm, removal quickly increased afterwards indicating the nitrifying capacity of systems operated at high salinity (Figure 4.3) and reinforcing what had been previously demonstrated in Chapter 3.

![Figure 4.3: Time course of system performance for ammonia nitrogen removal.](image-url)
4.3.2.2 NO$_3$-N

Effluent nitrate concentrations were higher than influent concentrations due to efficient nitrification of the 158mg/L NH$_3$-N (Table 4.1). Effluent NO$_3$-N was high during the first week of operation when ammonia conversion to nitrate was occurring efficiently but denitrification was only minor most likely due to growth of the heterotrophic denitrifying populations as a result of the introduction of acetic acid and the still predominantly aerobic conditions. NO$_3$-N fluctuated during the period of study (Figure 4.4) but means were not statistically different. Variations in NO$_3$-N concentrations in the systems seemed to be only related to the dose of COD applied. In carbon limited, nitrate rich wetlands the rate of denitrification will depend strongly on the rate of carbon supply (Hume et al., 2002; Kadlec and Wallace, 2009).

![Figure 4.4: Time course of effluent nitrate concentrations of the C and T filters.](image)

4.3.2.3 NO$_2$-N

Effluent NO$_2$-N was maintained below 15mg/L but exceeded 20mg/L in one occasion after an abrupt fluctuation in the dosed COD and subsequent DO concentrations; the average effluent NO$_2$-N was 5.27mg/L and 4.73mg/L for the C and T, respectively (Table 4.1). Nitrite accumulation was not expected and was likely to be resultant from partial nitrification due to low O$_2$ conditions present in the bottom layer of the filter or incomplete denitrification, or rather, the combination of both. NO$_2$-N concentrations were similar in both systems showing that salt had no impact on nitrite transformation processes. Itokawa et al. (2001) demonstrated that at a COD:N ratio of 3.5 or lower, 20 to 30% of the influent nitrogen was emitted from the bioreactors in
the form of N\textsubscript{2}O. N\textsubscript{2}O production from incomplete denitrification is undesirable as N\textsubscript{2}O is a powerful greenhouse gas. It was suggested that this N\textsubscript{2}O emission at lower COD/N ratio was due to endogenous denitrification with NO\textsubscript{2}-N. Furthermore lower COD:N ratios resulted in NO\textsubscript{2}-N build up and it was verified that NO\textsubscript{2}-N reduction rates were lower than NO\textsubscript{3}-N reduction rates. Almeida et al. (1995) suggested that NO\textsubscript{3}-N reduction and NO\textsubscript{2}–N reduction in denitrification were in competition for electron supply, where NO\textsubscript{3}-N is used preferably over NO\textsubscript{2}-N as electron acceptor. Oh et al. (1999) demonstrated that NO\textsubscript{2}−–N was built up during denitrification when electron donor supply was insufficient in their SBR-type denitrifying bioreactor. They concluded that NO\textsubscript{3}-N reduction out-competed NO\textsubscript{2}−–N reduction for limited electron supply.

4.3.2.4 TN

Significant average reductions of 83% and 77% in TN were achieved in the C and T systems, respectively. Effluent concentrations of TN were significantly different between systems and this was a direct reflection of the differing NH\textsubscript{3}-N concentrations present as a component of TN. Similarly to NO\textsubscript{3}-N, TN concentrations were higher initially, when no carbon was available, and then dropped as the experiment progressed with the addition of acetic acid in the saturated zone (Figure 4.5). Decreases in the TN % removal in the T system were clearly associated with decreases in the COD:N ratio rather than with the presence of salt.

![Figure 4.5: Time course of system performance for total nitrogen removal.](image-url)
The load vs concentration chart presented in Figure 4.6 indicates that when TN influent loads were between 14 and 17 gN/m²/d, effluent values were, in most cases, maintained below 20mg/L for the C filter. For the same influent load, effluent values were around 20 to 32mg/L for the T system, indicating the lower removal capacity of the system. The removal of TN was high in both systems and comparable to what Laber et al. (1997) obtained in a two stage VFCW where methanol was intermittently added to the second stage VF bed, which remained saturated. Their removal was in the order of 78% for TN and 82% for inorganic nitrogen. In our case, TN removal rates were on average 13.6gN/m²/d and 12.7gN/m²/d for the C and T systems, respectively. For the C system, elimination rates ranged from 4.5 to 17.0gN/m²/d and for the T system elimination rates varied from 2.2 to 15.37gN/m²/d. The upper range values are very high as compared to the literature. The higher removal rates reported here must have been resultant from the excess organic substrate available for denitrifiers.

Langergraber et al. (2009) when studying a two stage VFCW, treating settled municipal effluent, loaded at mean nitrogen rate of 4.84gN/m²/d verified a 53% TN removal and a mean elimination rate of 2.6gN/m²/d, reaching a maximum elimination rate of 4.07g/m²/d. Such elimination rates were considered very high by the authors for CWs treating municipal effluents without recirculation or carbon dosing. The strategies for the high nitrogen removal achieved by their system was the use of coarser sand (2-3.2mm) in the main filtering layer of the first stage to allow some organic matter to pass through the filter without being mineralised and the incorporation of a saturated section in the drainage layer on bottom of the first stage VF wetland which permitted

![Figure 4.6: TN load versus concentration chart.](image-url)
denitrification to occur given that enough organic matter was present. Following this first stage semi-saturated wetland, a subsequent unsaturated VFCW with fine sand in the main filtering layer was used to guarantee further NH₃-N and organic matter oxidation. A similar semi-saturated design was adopted in this study to allow nitrification and denitrification to occur within the same system. While a direct comparison is not possible due to the different natures of municipal (organic) and industrial (inorganic) wastewaters and by the fact that an external carbon source was added to the column in this study it is plausible to assume that the saturated drainage layer contributes to denitrification given that carbon is present.

4.3.2.5 COD

The filters were operated without acetic acid dosing prior to monitoring. Effluent COD in both systems was significantly lower than the dosed COD into the saturated zone. Removal ranged from 47 to 95% in the C and 30 to 94% in the T system. No difference was found in COD removal between the C and T filters (see Table 4.1). Two accidental misdosages of acetic acid occurred in this experiment, one on day 43 when double the volume (and therefore double the mass) of acetic acid was accidently dosed into both filters and then a peak in effluent COD was noticed on days 44 and 45. The other was on day 51 when only approximately half of the volume of acetic acid was dosed into the T filter, bringing COD concentration to 407mg/L (Figure 4.7).

Figure 4.7: Time course of dosed and effluent COD concentrations in the C and T filters.
Because the influent pump did not deliver the same volume of wastewater daily, inflows and concentrations of COD resultant from the dosing of acetic acid into the saturated zone varied slightly. The dosed COD was kept high enough to fulfil the stoichiometric requirements for complete denitrification of the 165mg/L TN. The dosing of acetic acid occurred simultaneously with the influent feeding to allow mixing of the acetic acid injected on the top of the saturated zone with the nitrified effluent percolating from the unsaturated to the saturated zone. It was evident that part of the nitrate and COD left the anoxic saturated zone without being utilised by heterotrophic denitrifying bacteria; this is most likely due to insufficient contact time and some degree of mixing in the saturated zone.

The calculated COD:N ratios into the saturated zone ranged from 3.25 to 7.55 in the C and 2.46 to 8.71 in the T filter. Effluent COD:N ratio ranged from 0.73 to 40.82 and 0.61 and 20.84 in the C and T systems, respectively. COD:N ratios were on average higher in the effluent than in the influent indicating that there would still be potential for denitrification to occur had more contact time been allowed.

The addition of another VFCW would permit further removal of COD and NH$_3$-N increasing the final quality of the effluent as it has been previously demonstrated by Langergraber et al. (2009). Although feasible in laboratory scale systems, an extra VFCW may prove prohibitive in large scale applications such as in industrial premises, as it would represent additional area requirements and costs.

### 4.3.3 Bacterial communities

#### 4.3.3.1 AOB community

The AOB community was analysed by AluI and TaqI based T-RFLP at 15cm in the C and T systems (Figure 4.8). The AluI based T-RFLP resulted in three distinct peaks in total, namely 224, 405 and 491bp. The AluI based T-RFLP revealed all three peaks in the C system; peak 491bp was most abundant followed by 405 and 224bp. In the T system, peak 405bp dominated and peak 224 only accounted for 6% of the total normalised peak area. The 2244bp was initially regarded to belong to non salt tolerant
Nitrosomonas sp, as demonstrated by their near complete exclusion in the surface layers of the wetlands subject to higher salinities (Chapter 3). The TaqI based TRFLP revealed a total of three T-RFLs, these fragments were 212, 219 and 281bp long. In the C system both 219bp and 281bp fragments were highly abundant, accounting for 56% and 44% of the total peak area, respectively. In the T system, the highest abundance was of the 219bp fragment (67%) followed by the 281bp (31%) and a small contribution of 212bp fragment.

The control system had a similar distribution of Nitrosomonas sp (AluI 405bp, TaqI 219bp) and Nitrosospira sp (AluI 491bp and TaqI 281bp) while the treatment system favoured the presence of Nitrosomonas sp over that of Nitrosospira sp.

![Diagram of relative abundance of AluI digested amoA T-RFs at 15cm depths for the Control (C) and Treatment (T) systems. 224bp - Nitrosomonas sp. 405bp - Nitrosomonas sp/ N. mobilis. 491bp - Nitrosospira sp/Nitrosomonas nitrosa. B - Relative abundance of TaqI digested amoA T-RFs at 15cm depth for the Control (C) and Treatment (T) systems. 212bp - undetermined. 219bp - Nitrosomonas sp. 281bp-Nitrosospira sp. Percentages of peaks below the threshold have been excluded.]

Ammonia removal was high in both systems, demonstrating that nitrification was not inhibited by salinity. As previously demonstrated in Chapter 3, Nitrosomonas sp and Nitrosospira sp were abundant members of the AOB community in the filtering layer of saline systems. Ammonia removal was low initially in the T system but steadily
increased during the first two weeks, this pattern was probably a result of the shift in salinity from 13g/L NaCl to 30g/L NaCl which caused some of the less salt tolerant AOB strains to be removed and the remaining strains to reproduce and fill niches previously occupied by others. The results of the Alul and TaqI based T-RFLP were contrasting for sample T15; while the Alul digestion did not produce any of the 491bp-\textit{Nitrosospira} sp peak, the TaqI digestion revealed that \textit{Nitrosospira} sp was quite abundant (281bp, accounting for approximately 30% of total peak area) in this sample.

\textbf{4.3.3.2 Denitrifying bacterial community}

In total, 126 \textit{nosZ} clones were selected for restriction fragment length polymorphism (RFLP) analysis following HaeIII digestion. Out of the 126 clones picked, 66 were from the control filter library and 60 from the treatment filter library. The RFLP analysis generated 13 distinct RFL patterns (OTUs), and clones representing individual OTUs were selected for sequencing. Both control and treatment systems presented clones related to the Alphaproteobacteria and Betaproteobacteria (Figure 4.9). In general, the sequences obtained in this study presented low similarity to other sequences of cultured denitrifiers published in the GenBank database (80%-93% similarity). Slightly higher similarities were found to uncultured bacterial clones. Some of the sequences from clones that had distinct RFL presented similarity to the same organism in the GenBank as it can be seen in Table 4.2. In the control library 33\% of clones were most similar to \textit{Burkholderia pseudomallei}, 15\% to \textit{Thiobacillus denitrificans} and 13.6\% to \textit{Azospirillum largimobile}. The treatment library revealed that 30\% of the clones were most similar to \textit{Thiobacillus denitrificans}, 28\% similar to \textit{Azospirillum sp. TSO41-3} and 13\% similar to \textit{Ralstonia solanacearum}.

Apart from clones se7 and se8, most similar to \textit{Ruegeria pomeroyi} and \textit{Nisaea denitrificans}, respectively, being only present in the control system and clone se4A12, related to \textit{Alicycliphilus denitrificans} being only found in the treatment filter, there was no clear distinction between the C and T filters in relation to the distribution of clones indicating that salinity in the T system had no or little effect in shaping the denitrifying community. As mentioned earlier, the fact that the filters had been used in the
A previous experiment and received occasional flows of saline effluent (13g/L NaCl) in the two months prior to the start of the experiment could have had a greater effect in selecting salt tolerant organisms in both systems, so that when salinity dropped to 0g/L in the control system or increased to 30g/L in the treatment system the denitrifying community was already composed of salt tolerant organisms and therefore masking possible differences in the bacterial composition between the freshwater system and the saline system.

Table 4.2: Percentage of occurrence and phylogenetic affiliation of nosZ clones from the control and treatment filters.

<table>
<thead>
<tr>
<th>Clones</th>
<th>Occurrence (%)</th>
<th>Accession no.</th>
<th>Closest organism in GenBank</th>
<th>Max similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone SE1A</td>
<td>13.3</td>
<td>AL646053.1</td>
<td>Ralstonia solanacearum</td>
<td>93%</td>
</tr>
<tr>
<td>clone SE2</td>
<td>15.2</td>
<td>CP000116.1</td>
<td>Thiobacillus denitrificans ATCC 25259</td>
<td>85%</td>
</tr>
<tr>
<td>clone SE3</td>
<td>9.1</td>
<td>AB542277.1</td>
<td>Azospirillum sp. TSA19</td>
<td>89%</td>
</tr>
<tr>
<td>cone se3A</td>
<td>14.4</td>
<td>CP000116.1</td>
<td>Thiobacillus denitrificans ATCC 25259</td>
<td>84%</td>
</tr>
<tr>
<td>clone SE4 D03</td>
<td>33.3</td>
<td>CP001408.1</td>
<td>Burkholderia pseudomallei MSHR346</td>
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<tr>
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<td>Alicycliphilus denitrificans</td>
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</tr>
<tr>
<td>clone SE5 E03</td>
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<td>CP000116.1</td>
<td>Thiobacillus denitrificans ATCC 25259</td>
<td>87%</td>
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<tr>
<td>clone SE5 B12</td>
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<td>Nisaea denitrificans</td>
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<td>Burkholderia pseudomallei MSHR346</td>
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<tr>
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<td>Ralstonia solanacearum</td>
<td>93%</td>
</tr>
<tr>
<td>clone SE10</td>
<td>6.1</td>
<td>AL646053.1</td>
<td>Ralstonia solanacearum</td>
<td>93%</td>
</tr>
</tbody>
</table>

Many denitrifiers are salt tolerant; Osaka et al. (2008), when studying the effect of acetate on denitrification efficiency and on the microbial community in anoxic reactors operated under increasing salinities, found that most of the clones at salt free conditions were related to the family Rhodocyclaceae of the Betaproteobacteria (genera Azoarcus and Thauera), to the genus Halomonas and to the phylum Bacteriodetes. The diversity of bacteria at 10% NaCl was less than that at 0% NaCl and most of the clones were related to the genera Halomonas and Marinobacter of the gammaproteobacteria. Osaka et al. (2008), however, used general bacterial 16SrDNA primers targeting a wide range of bacterial groups.

Ruiz-Rueda et al. (2008) when studying the nitrifying and denitrifying bacterial communities in free water surface constructed wetlands verified that the presence of plants had a significant effect in the denitrifying community with the nosZ genotypes from the rhizosphere samples being different from the bulk sediment samples. More diverse communities were found around roots when compared to the bulk sediment.
clear seasonal variation in *nosZ* genotypes was also found in the sediments and rhizosphere sites within the FWS wetland, most likely explained by a combination of factors such as plant effects and management practices including hydraulic and nutrient loadings as postulated by Kjellin *et al.* (2007). Kjellin *et al.* (2007) verified an increase in the diversity of denitrifiers (*nosZ* gene) with a longer HRT due to decreasing nitrogen and carbon content in the FWS CW.

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**Figure 4.9:** Neighbour-Joining Phylogenetic tree of the *nosZ* fragments and NosZ gene from reference organisms. Fragments of 193 nucleotides were used to calculate the tree.
Edmonds et al. (2009) verified no major changes in the bacterial community structure as a response to seawater amendment in low salinity tidal sediments. Their study focused on the activity and diversity of bacteria present in the pore space of sediment samples subject to increasing salinity. The increase in salinity resulted in biogeochemical changes in relation to the carbon mineralisation pathways with an evident decrease in methanogenesis and a final increase and establishment of sulfate reduction conditions. Surprisingly, however, was the fact that there were no differences in the composition of the bacterial and archaeal communities between the control and the saline amended sediments. It was proposed that shifts in gene expression and regulation may drive carbon mineralisation rates and metabolic activity as a result of seawater intrusion rather than the bacterial community becoming more “marine-like” through time. A similar result was obtained in our study, the composition of the nitrifying community varied slightly with an evident predominance of *Nitrosomonas* sp over *Nitrosospira* sp in the treatment system but the complete exclusion of *Nitrosospira* sp was not verified. In the case of denitrifiers, the percentage of groups in the clone libraries varied but no groups were fully excluded at the higher salinity. It has been suggested that other parameters such as NO$_3$-N and COD availability might have a stronger effect in shaping denitrifying communities (Magalhaes et al., 2008).

Magalhaes et al. (2008) found significant differences in the composition of denitrifiers that inhabit different intertidal environments of the Douro river estuary, Portugal. There was a strong relationship between the relative abundance of some organisms and denitrification rate and NO$_3$-N availability. Most of the clones retrieved by their libraries were contained within a cluster which included *Silicibacter pomeroyi* and *Paracoccus denitrificans.*
4.4 Conclusions

The unplanted semi saturated VFCW design tested, which allowed nitrification to occur in the unsaturated sand layer and denitrification to occur in the saturated drainage layer where organic carbon was added, resulted in a high nitrogen removal rates. Nitrogen removal rates were on average 13.6gN/m$^2$/d and 12.7gN/m$^2$/d for the control (freshwater) and treatment (saline) systems, respectively.

Total nitrogen removal was significantly higher in the control system than in the treatment system. The difference in nitrogen removal was attributed to the significantly lower ammonia removal obtained in the treatment system (90% in the T against 95% in the C), indicating that nitrification was the limiting step in nitrogen removal in the T filter. The presence of salt, however, did not impact nitrate or COD removal and similar nitrate and COD concentrations were obtained in both C and T filters.

The ammonia oxidising β-proteobacteria community was dominated by representatives of the genera *Nitrosomonas* and *Nitrosospira* in both systems and this is similar to what was observed in Chapter 3, indicating that members that had been previously selected in the preceding experiment were maintained in the filter. At 30gNaCl/L, there was a predominance of *Nitrosomonas* sp (*Nitrosomonas mobilis*) over *Nitrosospira*.

The gram-negative denitrifying bacteria were similar in the control and treatment filters and dominated by representatives of the α-proteobacteria and β-proteobacteria. The sequences obtained in this study had low similarity to other publicly available sequences from cultured organisms available in GenBank (80-93% similarity) and could represent novel organisms.

Semi saturated vertical flow sand filters can harbour complex bacterial communities capable of achieving nitrification and denitrification and therefore representing an alternative technology in nitrogen removal from both saline and fresh industrial wastewaters.
Chapter 5:

Combining industrial wastewaters to improve nitrogen removal in constructed wetlands: Effect of external carbon on denitrification

5.1 Introduction

Nitrate nitrogen is an important parameter to be measured in water and wastewater. When released to lakes, rivers and coastal areas nitrate constitutes a main risk for eutrofication and depreciated water quality; when present in drinking water systems, nitrate and nitrite constitute a public health concern, mainly related to infant methemoglobinemia (“blue baby syndrome”) and carcinogenesis (particularly gastric cancer) (USEPA, 1993). In anoxic conditions denitrifying bacteria reduce nitrate to nitrogen gas, as previously explained in Chapter two. Denitrification can be illustrated in a simplified form by equation 5.1:

$$5C + 4NO_3^- + 2H_2O \rightarrow 2N_2 + 4HCO_3^- + CO_2$$

When nitrogen is present in nitrate form, nitrogen removal via denitrification is generally rapid and complete when organic matter is available; however, denitrification is affected by several parameters, predominantly by a suitable organic carbon source as electron donor, anoxic conditions, pH and temperature (Crites and Tchobanoglous, 1998).

For wastewaters containing organic carbon the addition of external carbon for denitrification is not usually necessary but in the case of inorganic effluents the addition of carbon is needed. Common carbon sources for denitrification include methanol, acetic acid, ethanol or molasses. These conventional carbon sources, however, may add a prohibitive cost in operation, especially in the case of inorganic wastewaters produced by large chemical and fertiliser manufacturing facilities, where large volumes coincide with high nitrate concentrations. The use of less conventional organic carbon sources, such as plant matter or industrial by products has been previously investigated (Bernet et al., 1996; Skrinde and Bhagat, 1982; Constantin and

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1 This chapter was presented at the International Conference on Integrated Water Management. Murdoch University, Perth, Western Australia. 2-5 February 2011.
Fick 1997; Ingersoll and Baker, 1998; Domingos et al., 2009) and shown to fulfil the requirements of denitrification, resulting in economical and environmental benefits.

The theoretical stoichiometric COD: NO₃-N ratio for denitrification is 2.86 g CDO : 1g NO₃-N (USEPA, 1993). Carrera et al. (2004) when using ethanol as carbon for denitrification in a biological nitrogen removal system calculated the theoretical COD:N ratio as 4.2 g COD : g N. However, experimentally, the COD:N ratio for complete denitrification was found to be 7.1 ± 0.8 g COD : g N. Their experiment showed that there was a loss of about 39% of the COD added which was consumed by oxidation (aerobic respiration) and not by denitrification. Gersberg et al. (1983, 1984) also illustrated that the addition of COD should be higher than the theoretical ethanol to nitrogen ratios required for denitrification due to losses of the carbon fraction to aerobic decomposition.

It is important that the feasibility of an industrial organic carbon source be tested in laboratory systems prior to scaling up. This is because the nature of the carbon source determines the route of nitrate reduction (Bernet et al., 1996). Dissimilatory nitrate reduction to ammonia can occur in media with low dissolved oxygen and can be in competition with denitrification (Akunna et al., 1993; Bernet et al., 1996). Testing the electron donor may prevent the undesirable nitrate reduction to ammonia.

The work presented in this chapter is a complement to the experiments of Domingos et al. (2009) which tested woodchips and ethanol as possible carbon sources for denitrification in wetlands. Here, the aim was to test the feasibility of using soft drink manufacturer wastewater (SDW) as a possible carbon source for a large scale denitrifying saturated surface vertical flow constructed wetland receiving high concentrations of nitrate currently in operation at CSBP Ltd. The two objectives of the study were to: 1- verify the impact of adding soft drink manufacturer wastewater to CSBP wastewater on ammonia, nitrate and total nitrogen removal in wetland mesocosms and 2- assess the behaviour of the soft drink manufacturer wastewater during storage under different conditions.
5.2 Materials and Methods

5.2.1 Wetland experiment

Saturated vertical flow systems were used in order to simulate the existing wetland cell at CSBP Ltd, Kwinana. The main difference between the experimental system and the large scale one at CSBP was the lack of woodchips and of a well developed litter mat on the surface of the sand in the experimental system. The mesocosms consisted of open top 200L barrels with a 5cm drainage layer of 14mm diameter gravel and a 40 cm main filtering layer of fine beach sand (porosity = 0.3) (Figure 5.1).

Fig. 5.1: A: Experimental set up showing standing water on the surface and layers of sand and gravel. B: Wetland on the left is the control system receiving only CSBP wastewater. Wetland on the right is the treatment receiving CSBP wastewater plus SDW. C: Theoretical behaviour of a given 15L parcel of water “X” travelling through the wetland assuming ideal plug flow, no mixing, conditions.
Both systems were planted with native River Club Rush (*Schoenoplectus validus*) and received CSBP wastewater for 2 months prior to the start of the experiment. The wetlands were manually batch loaded every second day with 15L of wastewater, resulting in an approximate 6 day HRT. The batch loading operation (Figure 5.1C), consisting of filling 15L and withdrawing approximately 15L (less due to evapotranspiration) every 2 days simulated the large scale system at CSBP with the maintenance of standing water on the surface and saturated conditions in the substrate (fully described in Chapter 6, section 6.2.1).

CSBP wastewater was pumped from the containment pond into storage tanks and then brought to the Environment Technology Centre (ETC) Murdoch University and stored in the laboratory for use during the experiment. The SDW originated at the soft drink manufacturing plant consisted mainly of a variety of soft drinks, juices and water and had a COD = 70,000 mg/L and pH = 3.00. This wastewater was brought to the ETC and stored at 4°C during the experiment.

Monitoring started on the 19/03/2008 and finished on the 06/05/2008. The initial dosing with SDW was 50mL/15L and then 75mL/15L, these concentrations were chosen based on the COD of the SDW and a desired C:N ratio of 5 to 7. (Ingersoll and Baker, 1998; Carrera *et al.*, 2004). In terms of concentration these values correspond to 3.3 and 5.0 parts per thousand (ppt) of SWD. The control system (C) was fed solely with CSBP wastewater and the treatment system (T) was fed CSBP wastewater dosed with SDW; the actual COD:N ratio in the T system ranged from 2.8 to 5.

### 5.2.2 Storage trials

Two storage trials were conducted in order to verify the behaviour of the SDW when stored in tanks. COD and water characteristics (solids formation, smell) were monitored. For this purpose three litre capacity plastic containers were filled with two litres of SDW, in all cases container tops were left ajar to allow for gas exchange. The first trial compared COD behaviour in two tanks; one tank had an aeration system
(blower) while the other did not have any aeration (Figure 5.2A). The second trial was conducted in a single tank which was provided with a submersible pump in order to keep water moving in the tank.

5.2.3 Water analysis

Influent and effluent samples were collected and analysed according to the following methods: ammonia nitrogen was determined by an ion selective electrode (Thermo Scientific) according to APHA (1998). Nitrate nitrogen was determined colorimetrically with the cadmium reduction method according to APHA (1998). COD was also analysed colorimetrically with the potassium dichromate method with a HACH test kit.

5.3 Results and Discussion

5.3.1 Storage Trials

The first trial started on the 21/03/08 and finished on the 21/04/08. The two tanks were filled up and left unattended for 3 days. After 3 days a mould layer became apparent on the surface of the liquid in both tanks (Figure 5.2B). On the 3rd day an air blower (Resun Air Pump, AC-1000, capacity 2L/min) was installed with the aeration tube and diffuser placed in the tank. This provided intermittent aeration (with a timer set to one hour on/one hour off) and therefore oxygen and water movement in the tank. As a result of the water movement the mould layer sank and did not form again until the end of the experiment (Figure 5.2C). The mould layer on the control tank however stayed on the surface of the liquid until day 30.
Figure 5.2: A. Containers holding 2L of SDW. Note the aeration pipe going into the container on the right. B. Scum (mould) layer formed on the surface after 3 days. C. Scum layer sank and did not form again once aeration started.
COD decreased during storage in both tanks, after 30 days there was no significant difference in COD between the tanks, with a reduction in the order of 12,000mg/L (Figure 5.3). The main issue in storage was the strong decaying odour released by the wastewater. Even having a pH = 3, microbial colonization of the liquid and consequent odour developed quickly after day one. The high temperatures during week one (Figure C.1, Appendix C) also contributed to the strong smell which soon led to colleagues complaining about the experiment in the greenhouse.

The second storage trial started on the 23/04/08 and finished on the 06/05/08. A submersible pump (Rain Master, King F1, fountain pump, Hmax 0.09m, Qmax 380L/hr) was placed in the tank and was left running continuously for the duration of the experiment. The constant disturbance of the surface did not allow for mould to form on it. Air temperatures during trial 2 were lower (Figure C.1, Appendix C) and the odour from the tank was less intense. Similar to what happened during the first trial, COD decreased during storage, varying from 64,950mg/L in day one to 56,300mg/L in day 12.

![COD behaviour of SDW stored in tanks during a 30-day storage trial. Columns in blue represent the tank with aeration; columns in violet represent the control tank.](image-url)

Figure 5.3: COD behaviour of SDW stored in tanks during a 30-day storage trial. Columns in blue represent the tank with aeration; columns in violet represent the control tank.
5.3.1.1 Implications of storage for CSBP

While some degradation of carbon (COD) occurs during storage, this should not pose a problem for the purpose of dosing the wetland given the highly concentrated nature of the SDW. Strong odour is an issue during storage, especially in warm weather conditions. Aspects such as tank location at CSBP and presence of vents for proper ventilation of the headspace in the tank have to be considered. Surface aeration or another form of mechanical agitation may be required to avoid mould formation on the surface of the wastewater and predominance anaerobic/fermentative conditions during storage. The practicability of such aerators/agitators in large scale tanks however has to be analysed by CSBP.

5.3.2 Wetland trial

All influent and effluent results from the experiment can be found in Table C.1 (Appendix C).

5.3.2.1 Ammonia removal

In both systems ammonia was completely removed (removal >99.5%) and no significant difference was found between average effluent concentrations for the treatment and control. Results can be seen in Figure 5.4.

Conditions in both systems were favourable for nitrification, with the addition of COD in the treatment system having no negative impact on the conversion of ammonia to nitrate even when the system received up to 40mg/L of NH$_3$-N. It is assumed that the conditions on surface and superficial sediments were aerobic and sufficient HRT was allowed for complete ammonia removal.
Figure 5.4: Ammonia nitrogen concentrations in and out of the wetlands. Influent concentration was the same for both systems. Effluent ammonia was similar in both systems and only above 1.0mg/L in one occasion for the treatment wetland.

5.3.2.2 Nitrate removal

Nitrate removal in the saturated VFCWs was enhanced by the addition of SDW to CSBP wastewater as can be seen in Figure 5.5. As the experiment progressed nitrate removal increased in the T system, where SDW was added, indicating efficient denitrification in the presence of carbon. The average DO concentration in the effluent of the T system was 0.3mg/L indicating anoxic conditions favourable for denitrification. The lack of COD in the control system, however, did not permit consistent nitrate removal throughout the experiment. The average DO concentration in the effluent of the C system was 1.0mg/L. During the last 20 days of the experiment, concentrations of nitrate in the effluent were higher than in the influent, evidencing that nitrification was the main process occurring and denitrification was only marginal. Given the short duration of the experiment it is believed that the systems did not reach steady state conditions, nonetheless the general trend of how the system would operate under steady state conditions could be verified.
Figure 5.5: Nitrate concentrations in and out of the wetlands. Influent concentration was the same for both systems.

5.3.2.3 Total Nitrogen Removal

Given that all ammonia was removed, nitrate was the single nitrogen form in the effluent; as a result total nitrogen and nitrate concentrations in the effluent were equivalent (Figures 5.5 and 5.6). Total nitrogen removal increased from 47% in the C to 76% in the T system. Effluent total nitrogen and nitrate from the first week of monitoring (three initial data points, 26/03/2008 to 01/04/2008) are likely resultant from longer retention times prior to the intensive monitoring phase. Had the experiment been allowed to proceed for a longer period, it is believed that the difference in removal would have become even more evident, with nitrate accumulating in the C system and it being further removed in the T system.
5.3.2.4 COD removal

Samples from the C system were not analysed for COD as frequently as they were for the T system samples. Influent COD in the treatment system ranged from 278 to 481mg/L with an average of 378mg/L while in the control system COD ranged from 64 to 140mg/L and the average was 93mg/L. Effluent COD averaged 99mg/L in the treatment system and 92mg/L in the control.

As seen in Figure 5.7, COD input from the SDW was consumed during the 6 days HRT. Removal of COD in the T system was in the order of 74%, bringing effluent concentrations close to those in the C (CSBP wastewater). COD in the control system remained the same after 6 days HRT; removal was only 3.0% and there were no significant differences between the influent and effluent concentrations. COD values of CSBP wastewater (as pumped from the containment pond) measured in this study (ranging 64 to 140 mg/L) were higher than those previously measured by CSBP (data not shown) and also those measured during the previous ethanol and woodchip study conducted in November-December 2007 (38-79mg/L) (Domingos et al., 2009).
5.3.2.6 Implications of dosing for CSBP

The addition of 3.3mL to 5.0mL of SDW with a COD of 70,000 mg/L to 1.0L final volume of CSBP (3.3:1000 to 5:1000) wastewater increased CSBP’s wastewater COD from an average of 95 to 378mg/L. This addition corresponds to 5L of SDW/m³ of CSBP wastewater (5:1000) with a target nitrate concentration of 60-70 mg/L to be removed.

Assuming that SDW COD concentrations remain similar to the ones used in this study (= 70,000mg/L) the following recommendation (Table 5.1) is advisable for CSBP in regards to future dosing of SDW into the large scale saturated surface vertical flow wetlands receiving nitrified inorganic effluent.

Table 5.1 Recommended dosing regime of SDW into CSBP’s saturated VFCW assuming different scenarios.

<table>
<thead>
<tr>
<th>TN to be removed (mg/L)</th>
<th>Ideal COD (mg/L)</th>
<th>Dosing to achieve ideal COD (L/m³)</th>
<th>Daily SDW dosing (m³) considering Qi = 1000m³/d</th>
<th>Daily SDW dosing (m³) considering Qi = 2000m³/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10</td>
<td>70</td>
<td>0-0.5*</td>
<td>0 – 0.5</td>
<td>0 – 1.0</td>
</tr>
<tr>
<td>10 - 20</td>
<td>140</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>20 - 30</td>
<td>210</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>30 - 40</td>
<td>280</td>
<td>3</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>40 - 50</td>
<td>350</td>
<td>4</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>50 - 60</td>
<td>420</td>
<td>5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>&gt;60</td>
<td>490</td>
<td>5**</td>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>

Qi = flow into the wetland. *= varying according to COD of CSBP wastewater. ** = 5L/ m³ to be set as a maximum dosing concentration.
5.4. Conclusions and Outlook

The use of SDW as a carbon source to enhance denitrification and total nitrogen removal in saturated wetland systems proved to be a feasible option for CSBP. The average 2 to 3 m$^3$/day (Monday to Friday) of SDW currently generated at the soft drink manufacturer product destruction facility would not be enough to keep COD:NO$_3$-N at ideal levels at CSBP if influent NO$_3$-N >40 mg/L and Qi =1000 m$^3$/d or NO$_3$-N >20 mg/L and Qi=2000 m$^3$/d.

Wastewater flows and nitrate concentrations at CSBP are predicted to be in the upper range of the scenarios considered, that is, Qi= 2000 m$^3$/d and NO$_3$-N = 40 to 60 mg/L, therefore larger volumes or more concentrated SDW would be necessary. Given the positive outcome of this experiment the possibility of implementing diversion systems at the soft drink manufacturing plant to further intercept and collect high strength wastewater before it is diluted with other streams and discharged to sewer has been discussed and recommended. Because the industries are not next door neighbours, pumping of SDW via a pipeline is not possible. Negotiations on the logistic and on-site storage of the SDW were underway at the time this chapter was concluded.

Monitoring influent and effluent COD concentrations has shown to be a critical exercise in managing nitrate and total nitrogen removal in the saturated VFCWs. This activity has been recommended in order to ensure efficient control of SDW dosing and monitoring of the final effluent COD so current discharge licences are not exceeded.
Chapter 6:

Heavy metals in a constructed wetland treating industrial wastewater: Distribution in the sediment and rhizome tissue

6.1 Introduction

The wetland focus of this study was designed to treat nitrogen rich wastewater from CSBP Limited manufacturing plant in Kwinana, Western Australia. CSBP produces Ammonia, Ammonium Nitrate, Nitric Acid, Compound Nitrogen and Phosphorous Fertilisers, Superphosphate, and Chlor-alkali products. As part of their operation the plant generates different types and amounts of effluent, the main sources are cooling tower blow-down water, combined wastewater pumped from production facilities and large volumes of stormwater runoff restricted to the winter months. Wastewater originated at different locations in the plant is firstly pumped into a containment pond and from there to the constructed wetland which was built in 2004.

Because the CSBP constructed wetland displayed varying plant densities in November 2006/January 2007 with lower densities next to the inlet distribution pipe and increasing densities with distance from the inlet pipe this study was conducted to verify if heavy metal concentrations in the sediment could be affecting plant health and consequently distribution in the wetland. After this study was conducted the vegetation recovered and by March 2007 presented vigorous growth in all areas of the wetland showing that the vast die off was a mere normal seasonal effect rather than a shock load of pollutants. The sediment and plant sampling and analyses however resulted in valuable data and information about this wetland system. In treatment wetlands it is common for pollutants, especially metals and suspended solids, to accumulate nearest the inlet pipe (Kadlec and Knight, 1996). In horizontal flow wetlands one can expect a horizontal decreasing pattern of pollutants from the inlet to the outlet (Headley et al., 2005; Cooper et al., 2005). In vertical flow (downflow) wetlands a decreasing vertical profile from top to bottom can be expected with solids usually accumulating on the surface and pollutants being converted in the first 10 cm of the medium (Molle et al., 2005; Kayser and Kunst, 2005).

1 This chapter is published as Domingos et al. (2009). Water Science & Technology, 60(6). pp. 1425-1432.
6.2 Study Site and Methods

6.2.1 Wetland description

The constructed wetland at CSBP is 135 m long x 95 m wide (~ 1.3 ha); it has a vertical flow design and a 1m deep sand substrate with a void ratio of approximately 0.3. The influent distribution pipe is laid centrally on the surface of the sand (Figure 6.1) and the effluent drainage pipes are laid underneath the sand column on the surface of the liner, drainage pipes are covered with gravel and wrapped in geotextile fabric. The vegetation consists of River Club-rush (*Schoenoplectus validus*). The wetland can be described as a saturated surface vertical flow system and it is operated in a sequencing batch (feed - stay - drain) mode (Figure 6.1). The sand medium is kept constantly saturated with the water level varying between the sand surface and 0.3m above it (Figure 6.2:A). The wetland is never fully drained and draining stops when the water level reaches the surface of the sand. One batch cycle usually takes 3 days, day one - feed, day two- stay and day three - drain. It is important to highlight that batch volumes and residence times are highly variable and mainly dictated by rain events and wastewater production. Batch volumes range from 300 to 3,000m3, with an average of 1740m3/batch.

The wetland is equipped with flow meters and automated composite samplers so every time the system is filled or drained samples are collected from the inlet and outlet pipes. Samples are analysed for pH, conductivity, ammonia, nitrate, total nitrogen, total phosphorous and a wide range of metals routinely (Table 6.1), analyses are conducted according to the Standard Methods (APHA, 1998).
Figure 6.1: Photo of the constructed wetland system at CSBP. The limestone rocks seen in the centre are covering the inlet distribution pipe.

Figure 6.2: A- Section view of the wetland showing central inlet pipe on top of sand layer and outlet drainage pipes on the bottom of wetland. B- Plan view of the wetland showing transects with sediment and plant sampling locations.

6.2.2 Sediment and plant sampling

Sediment sampling was carried out along three transects positioned lengthwise in the wetland with the inlet pipe being located in the middle of the transect. Each transect was placed 23.5 m away from one another and from the sides of the wetland. There were 8 sampling points on each transect, two at 2 m, two at 20 m, two at 40 m and two at 60 m distance from the inlet pipe (Figure 6.2:B). There were a total of 24
sediment samples and 18 plant samples, there were no plants located on the 2m sampling points.

On each sampling point the top 7cm of sediment was collected by means of a 50mm pipe which was introduced into the sediment to a depth of 7cm (Figure 6.3). With a shovel introduced laterally down to the bottom of the sampling pipe the sediment column was held in the pipe and transferred to plastic zip lock bags for later analysis. Before introducing the pipe into the sediment, most of the plant debris was removed from the top sediment carefully so the sludge layer was not disturbed. On the same sampling station, within a 0.2 m radius from where the sediment was collected, a live green plant sample was collected. Once collected, leaves were cut off and discarded and sediments were washed off of the root system and the rhizomes packed for later analysis. Sediment and plant samples were taken to the CSBP soil and plant laboratory for analysis immediately after collection.

Figure 6.3: Schematic view of top soil collection methodology.

6.2.3 Sediment and plant analysis

For plant analysis rhizome material was digested in nitric acid using a Milestone microwave. Sediment analysis was carried out with the DTPA extraction method (Lindsay and Norvell, 1978) and with the Aqua Regia digestion method. The Aqua Regia extraction method (digestion with 3:1 mixture of hydrochloric and nitric acids) is a stronger method of extraction than the DTPA and it corresponds to the total fraction of metals in the sediment, the DTPA method corresponds to the parcel of metals that are potentially bioavailable. Copper, zinc, manganese, calcium, magnesium, sodium, iron,
potassium, phosphorus, sulphur and boron were measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES) (McQuaker et al., 1979).

6.2.4 Data analysis

Data from the different locations were grouped in 2m, 20m, 40m and 60m distances from the inlet, for plants the 2m location was not present. One way analyses of variance (ANOVA) with distance from the inlet pipe were used to assess the significance of spatial differences in sediment and rhizome Cu and Zn concentration. Tukey’s post multiple comparison was chosen to verify differences between pairs. If the raw concentration data did not satisfy the homoscedasticity (homogeneity of variance) and normality requirements for ANOVA then the concentration data were log10 transformed to meet the assumptions of homoscedasticity and normality. If still after transformation data did not meet ANOVA requirements, then the nonparametric statistic test Kruskal-Wallis was performed with the Man-Whitney test performed to identify differences between pairs. The critical level for all statistical tests was p ≤ 0.05. Statistical analyses were conducted with Minitab (Minitab software, 2006).

6.3 Results and Discussion

6.3.1 Water quality

Influent and Effluent quality parameters from August 2004 to June 2007 are briefly described in Table 6.1 below. These results are based on 436 influent and 427 effluent samples collected and analysed by CSBP during the period. The estimated detection limits (DL) are given in the table. Metals such as Cd, Co, Cr and Pb had concentrations below the detection limits (ICP-AES) in more than 90% of influent and effluent samples and therefore are not presented.

It is evident from the table that the average percentage removal for metals is just an unrefined indication of performance, the true performance is higher than the one shown as it can be seen by the high percentage of effluent samples whose metal
concentrations were brought to below detection limits (e.g. in 88% of the 427 effluent samples analysed Cu concentrations were below the detection limit).

Table 6.1: Water quality parameters in and out of the constructed wetland at CSBP. Only values > DL included.

<table>
<thead>
<tr>
<th></th>
<th>Influent</th>
<th>Effluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>mg/L</td>
<td>mg/L</td>
</tr>
<tr>
<td>NH₃-N</td>
<td>mg/L</td>
<td>mg/L</td>
</tr>
<tr>
<td>NO₃-N</td>
<td>mg/L</td>
<td>mg/L</td>
</tr>
<tr>
<td>N tot</td>
<td>mg/L</td>
<td>mg/L</td>
</tr>
<tr>
<td>P tot</td>
<td>mg/L</td>
<td>mg/L</td>
</tr>
<tr>
<td>Al</td>
<td>mg/L</td>
<td>mg/L</td>
</tr>
<tr>
<td>Cu</td>
<td>mg/L</td>
<td>mg/L</td>
</tr>
<tr>
<td>Fe</td>
<td>mg/L</td>
<td>mg/L</td>
</tr>
<tr>
<td>Mn</td>
<td>mg/L</td>
<td>mg/L</td>
</tr>
<tr>
<td>Mo</td>
<td>mg/L</td>
<td>mg/L</td>
</tr>
<tr>
<td>Ni</td>
<td>mg/L</td>
<td>mg/L</td>
</tr>
<tr>
<td>Zn</td>
<td>mg/L</td>
<td>mg/L</td>
</tr>
</tbody>
</table>

**Average:**
- Influent: 7.54, 43.8, 13.6, 11.3, 0.202, 0.186, 1.100, 0.096, 0.036, 0.050, 0.242
- Effluent: 7.53, 36.1, 8.7, 46.2, 6.6, 0.138, 0.168, 0.964, 0.068, 0.033, 0.050, 0.194

**Minimum:**
- Influent: 2.54, 2.3, 0.4, 6.2, 1.3, 0.042, 0.021, 0.050, 0.021, 0.026, 0.026, 0.024
- Effluent: 6.00, 0.2, 0.5, 4.6, 0.2, 0.026, 0.022, 0.040, 0.021, 0.026, 0.050, 0.021

**Maximum:**
- Influent: 9.07, 192.0, 146.1, 336.1, 101.0, 1.830, 1.540, 4.900, 0.728, 0.091, 0.110, 1.524
- Effluent: 8.29, 135.0, 99.6, 185.6, 25.6, 0.548, 0.238, 1.610, 0.157, 0.057, 0.050, 0.510

**Median:**
- Influent: 7.55, 36.0, 8.7, 46.2, 6.6, 0.138, 0.168, 0.964, 0.068, 0.033, 0.050, 0.194
- Effluent: 7.53, 32.0, 8.1, 42.1, 5.2, 0.050, 0.044, 0.149, 0.046, 0.030, 0.050, 0.056

**Avg removal (%)**
- Cu: 17.4%
- Zn: 10.0%

**DL below DL (%)**
- Cu: 0%
- Zn: 0%

**6.3.2 Metal distribution in the sediment**

Due to their higher concentrations in the sediment when compared to other elements analysed and possible toxic effect on the wetland plants the focus in this study is primarily on copper and zinc. Copper and zinc concentrations are higher near the inlet pipe (2m) and decrease outwards, with the lowest concentrations at the 60m location (Table 6.2). The DTPA is a weaker method of extraction than the Aqua Regia method, while the prior is used for estimating the potential bioavailability of metals in sediments the latter is more representative of the total concentration of metals in the sediment.

*Bioavailable copper and zinc:* Copper concentration data had to be log₁₀ transformed to satisfy ANOVA requirements. One-way ANOVA indicated significant differences among distances (P<0.05) with Tukey’s post multiple comparison test showing that the difference was between the 2m and the 60m locations.

Zinc concentration data did not meet ANOVA requirements even after log₁₀ transformation therefore the nonparametric Kruskal-Wallis test for medians was used. No statistical difference (P>0.05) was found in zinc concentrations among the locations studied. However, when the Mann-Whitney test was performed between the 2m and 60m locations the result was that medians differed significantly (P= 0.453).
Table 6.2: Mean (standard deviation) metal concentration in the sediment -dry weight- sampled at different distances from the inlet pipe within CSBP constructed wetland. Means are based on six observations for each location. Samples were taken in January 2007.

<table>
<thead>
<tr>
<th>Method</th>
<th>Metal</th>
<th>2 m</th>
<th>20 m</th>
<th>40 m</th>
<th>60 m</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTPA extraction</td>
<td>Cu (mg/kg)</td>
<td>30.2 (17.1)</td>
<td>19.6 (6.5)</td>
<td>11.2 (3.8)</td>
<td>10.3 (10.1)</td>
</tr>
<tr>
<td></td>
<td>Zn (mg/kg)</td>
<td>60.4 (33.7)</td>
<td>47.4 (9.0)</td>
<td>40.3 (5.8)</td>
<td>26.1 (21.9)</td>
</tr>
<tr>
<td></td>
<td>Mn (mg/kg)</td>
<td>3.6 (1.9)</td>
<td>2.2 (0.8)</td>
<td>1.7 (0.3)</td>
<td>2.5 (1.8)</td>
</tr>
<tr>
<td></td>
<td>Fe (mg/kg)</td>
<td>25.5 (14.2)</td>
<td>19.9 (6.6)</td>
<td>14.9 (3.8)</td>
<td>29.1 (8.8)</td>
</tr>
</tbody>
</table>

| AQUA REGIA extraction | Cu ppm | 92.7 (71.8) | 28.4 (7.1) | 22.4 (6.3) | 25.7 (6.6) |
|                       | Zn ppm | 198.5 (143.8) | 77.8 (14.6) | 78.6 (17.1) | 52.6 (56.3) |
|                       | Cd ppb | 499.8 (361.7) | 196.0 (26.2) | 200.4 (32.0) | 168.4 (136.4) |
|                       | Co ppb | 1226.0 (603.0) | 552.5 (209.2) | 435.4 (57.7) | 581.0 (177.5) |
|                       | Ni ppb | 6555.7 (3681.8) | 2887.0 (294.5) | 2651.3 (264.5) | 3747.0 (1367.4) |
|                       | As ppb | 4192.2 (1523.2) | 2423.0 (260.3) | 2332.7 (209.6) | 2322.2 (614.0) |
|                       | Mo ppb | 940.0 (646.7) | 196.5 (39.9) | 174.0 (48.7) | 416.8 (297.2) |
|                       | Se ppb | 165.5 (78.3) | 106.5 (20.8) | 107.5 (40.5) | 120.6 (55.8) |
|                       | Pb ppb | 4366.4 (3337.8) | 1203.1 (256.5) | 1052.7 (103.8) | 1531.9 (1142.7) |

These results imply that the significant higher concentrations of bioavailable copper in the sediment near the inlet pipe might represent a more stressful environment for the plants which could in turn result in the evident pattern of lower density, and smaller size of plants near the inlet pipe and higher density and bigger size of plants around the 60m location.

Total copper and zinc: The Aqua Regia extraction method resulted in higher concentrations of Cu and Zn. In this case no statistical analysis was conducted but the same pattern of higher mean concentrations near the inlet pipe and lower mean concentrations towards the 60m location was verified (Figures 6.4 and 6.5).
6.3.3 Sediment quality guidelines

Because of the difference in the extraction capacity of metal from the sediment given by different methods it is important that before any comparison be made an evaluation of the extraction methods used to derive the values of sediment quality guidelines must be carried out. Current sediment quality guidelines use values that are based on the total concentration of metals rather than their bioavailable fractions; such
values are comparable to the Aqua Regia values presented in this study. Background concentrations of Cu and Zn in the local beach sand are expected to be low but no sampling occurred in the surrounding of the wetland.

For Australia and New Zealand the trigger values for Cu and Zn in sediments are based on modified values presented by Long et al. (1995) from the US National Oceanic and Atmospheric Administration (NOAA) database. The sediment quality guidelines were published as part of the Australia and New Zealand guidelines for fresh and marine water quality, ANZECC (2000). The Low (trigger-value) and High Interim Sediment Quality Guidelines (ISQG) values for Cu and Zn are 65-270 mg/kg and 200-410 mg/kg respectively. The mean total concentrations of Cu and Zn for the 2m location are 92.7 ppm and 198.5 ppm respectively, so we can conclude that Cu concentrations at the 2m location are of greater concern than Zn, as Cu concentrations have already past the guidelines’ trigger value while Zn is still, but just, below the trigger value set by the ANZECC (2000) guidelines for sediment quality.

The Canadian Sediment Quality Guidelines for Protection of Aquatic Life, CCME (2001) has more conservative values, suggesting for Cu an interim freshwater sediment quality guideline (ISQG) value of 35.7 mg/kg and a probable effect level (PEL) of 197 mg/kg. For Zn these values are ISQG = 123 mg/kg and PEL = 315 mg/kg.

Although heavy metal values for the CSBP wetland are not above the ISQG high values, attention should be paid to the rate that metals accumulate in the sediment so high ISQG values are not overtaken. It is also important to note that there is a great variability in metal tolerance among plant species, with some species presenting toxicity symptoms at lower concentrations than others. Schoenoplectus and Typha spp can be more tolerant than other species (Dunbabin and Bower, 1992).

The same pattern of higher values near the inlet pipe and lower values on the other locations was observed for Cd, Ni, As, Mo, Se and Pb. However, the concentrations of these elements are low and far below the trigger values - low ISQG, proposed by the ANZECC (2000) guidelines.

Premi and Cornfield (1969) verified no impact of copper and zinc on ammonification and nitrification in an experiment with soil incubation even when these
elements were applied in water at the highest concentrations of 10,000 ppm. Cela and Summer (2002), however, found that more than 3.8mg water-extractable Cu/Kg soil was inhibitory for nitrification, whereas less than 2mg/kg was safe for nitrification to occur. The values obtained from the water extraction method for metal used by Cela and Summer are not comparable to the ones obtained here since the extraction methods are totally different. The impact of metals on nitrification was not quantified here.

6.3.4 Metal distribution in the rhizome tissue

Results of the plant tissue analysis are presented in Table 6.3. Only healthy plants were collected for analysis. The overall average concentration of copper in rhizome tissue for the wetland was 793.7 (± 554.1) mg/kg and for zinc it was 571.2 (± 221.1) mg/kg.

The nonparametric Kruskal-Wallis test was used to test the hypothesis of different rhizome copper and zinc concentrations in the wetland. Copper and zinc concentrations in the rhizome tissue did not vary significantly (P > 0.05) among different locations in the wetland.

Table 6.3: Mean (standard deviation) metal concentration in the rhizome tissue sampled at different distances from the inlet pipe within CSBP constructed wetland. Means are based on 4 observations for each location. Samples were taken in January 2007.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>20m</th>
<th>40m</th>
<th>60m</th>
</tr>
</thead>
<tbody>
<tr>
<td>NITROGEN</td>
<td>1.21 (0.72)</td>
<td>1.69 (0.65)</td>
<td>1.86 (0.71)</td>
</tr>
<tr>
<td>PHOSPHORUS</td>
<td>0.37 (0.10)</td>
<td>0.36 (0.04)</td>
<td>0.48 (0.21)</td>
</tr>
<tr>
<td>COPPER</td>
<td>541.98 (310.24)</td>
<td>564.20 (134.04)</td>
<td>1102.00 (1008.62)</td>
</tr>
<tr>
<td>ZINC</td>
<td>513.96 (104.68)</td>
<td>576.62 (116.43)</td>
<td>519.56 (322.21)</td>
</tr>
<tr>
<td>MANGANESE</td>
<td>44.38 (12.19)</td>
<td>45.30 (4.11)</td>
<td>52.05 (15.42)</td>
</tr>
<tr>
<td>IRON</td>
<td>2559.08 (1301.97)</td>
<td>2393.33 (481.07)</td>
<td>3840.78 (2939.96)</td>
</tr>
<tr>
<td>NITRATE</td>
<td>42.00 (3.92)</td>
<td>54.50 (18.34)</td>
<td>52.00 (44.42)</td>
</tr>
<tr>
<td>BORON</td>
<td>16.80 (2.50)</td>
<td>16.73 (1.50)</td>
<td>16.75 (3.31)</td>
</tr>
</tbody>
</table>
On a dry weight basis, macrophyte roots and rhizomes can have a much higher concentration than the sediments where they are located. It is also known that in emergent macrophytes, as it is the case of *S. validus*, the roots and rhizomes accumulate significantly greater concentrations of metals than stems and leaves (Cardwell *et al.*, 2002). When the concentrations of Cu and Zn were analysed in rhizome and leaf tissues of *S. validus* (data not shown) sampled from the CSBP treatment wetland and a non-treatment wetland located at the Environmental Technology Centre/Murdoch University no significant difference was found between concentrations of Cu and Zn in rhizome and leaf tissue from the CSBP plants, however the rhizomes from the non-treatment wetland plants presented significant higher concentrations of Cu and Zn than the leaves.

In a study conducted by Dunbain and Bowmer (1992) using constructed wetlands to treat industrial wastewaters containing metals it was found that *Schoenoplectus* and *Typha* spp. can be more tolerant than other species and that metal tolerance is a function of plant phenology, vigour and growth as well as metal speciation and aquatic chemistry. Also, high nutrient concentrations, as found in the CSBP wetland, could increase the toxicity tolerance of macrophytes (Manios *et al.*, 2003).

Copper rhizome concentrations here are much higher than those reported by Murray-Gulde *et al.* (2005) for *Schoenoplectus californicus* planted in a wetland receiving copper contaminated wastewater, in her study copper concentrations in the roots ranged from 9.34 (±5.14) to 51.32 (±32.13) mg/kg.

The significant higher concentrations of bioavailable metals in the sediment where plants are not present (2m) compared to the lower concentrations in sediment where plants grow more vigorously (60m) suggest that plants uptake and store considerable amounts of metals from the sediment indicating that *S. validus* plays an important role in removing and immobilizing heavy metals at the CSBP wetland. The rate which these metals leach out of the decaying plant matter and re-enter the water however is unknown and would be focus of further investigation.

Ebbs and Kochian (1997) working with *Brassica sp.* in phytoremediation for contaminated soils report concentration in roots in excess of 10 000 mg/kg Zn and for Cu ranging from 750 to 1500 mg/kg. Studying metal accumulation in some
macrophytes from polluted urban streams in Southeast Queensland, Cardwell et al. (2002) verified that roots of *S. validus* contained 76.6 mg/kg of Cu and up to 1568 mg/kg of Zn, on a dry weight basis. In this study, the overall concentration of Cu is similar to that of Zn in the rhizome, with higher values for Cu than for Zn being observed; these results differ from the pattern of higher Zn accumulation reported in the literature.

### 6.4 Conclusions

Whether Cu and Zn concentrations at the CSBP wetland may reach toxic levels to plants and bacteria is still unknown. Further research to characterise and verify the distribution of functional bacterial groups, such as ammonia oxidising bacteria, in this wetland will be presented in Chapter 7. What has been verified in this chapter is that the surface component of the wetland favours sedimentation and binding of metals to the organic matter and, the sediment which tend to be anoxic with reducing conditions act as a sink for metals. The distributions of bioavailable Cu and Zn in the top sediment layer follow a horizontal profile. Concentrations of these metals in sediments near the inlet pipe (2m) are significantly higher than in sediments at the farthest location (60m).
Chapter 7:

Nitrogen removal and ammonia-oxidising bacteria in a vertical flow constructed wetland treating inorganic wastewater

7.1 Introduction

Vertical flow constructed wetlands (VFCWs) have proven to be an efficient alternative in the treatment of ammonium contaminated wastewater (Brix and Arias, 2005). Due to their oxygen transfer capabilities VFCWs can harbour a diverse nitrifying community in their filtering layer (Tietz et al., 2007). Bacterial nitrification plays a major role in ammonium removal from wastewater therefore characterising the nitrifying bacterial communities and their distribution within a wetland system is important.

Ammonia oxidation is thought to be the main limiting factor for the conversion of ammonia to nitrate in most systems as nitrite concentrations in the environment are usually low (De Boer et al., 1990). Knowledge of the ammonia oxidising bacterial (AOB) community structure and composition may contribute to process optimisation of systems relying on conventional nitrification as first step for nitrogen removal. It is known that systems with higher diversity are less prone to failure (Purkhold et al., 2000) however little research has focused on microbial analysis of wetlands (Faulwetter et al., 2009).

Nitrifying bacterial communities have been characterised in many engineered systems such as membrane bioreactors (Wittebolle et al., 2008), sequencing batch reactors (Wittebolle et al., 2008), activated sludge plants (Siripong and Rittmann, 2007), VFCWs (Tietz et al., 2007) and chloraminated drinking water supply plants (Regan et al., 2002). AOB are ubiquitous and have been isolated from soils, fresh, brackish and marine waters and sediments, salt lakes and other environments (Kowalchuk & Stephen, 2001; Koops et al., 2006).

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The objective of this study was to assess nitrogen removal performance and to characterise the AOB community in the superficial sediment layers of the wetland at CSBP Ltd., where heavy metal accumulation had been previously verified (Chapter 6). The potential contribution of ammonia-oxidising archaea (Crenarchaeota) and heterotrophic nitrifiers in ammonia removal in the wetland is recognised, their characterisation however was not within the scope of this study.

7.2 Materials and Methods

7.2.1 Wastewater and wetland description

Cooling tower blowdown and stormwater runoff from CSBP’s Kwinana site is directed to its wastewater containment system. The inorganic wastewater has an average pH=7.5, COD = 50mg/L and a COD:N ratio ≤ 1. Most of the influent nitrogen is in the form of NH$_3$-N. In 2004 CSBP constructed a pilot nutrient stripping wetland (wetland cell 1) to treat part of this wastewater prior to discharge. This wetland was designed to treat up to 500m$^3$/day of wastewater with a targeted 50% nitrogen removal. In order to handle larger volumes of wastewater produced two new batch-fed VF wetlands (namely cells 2 and 3), 8000m$^2$ each, were incorporated into the treatment train in July 2009. Cells 2 and 3 were placed between the containment pond and wetland cell 1 so from August 2009 wetland cell 1 started receiving nitrified effluent from cells 2 and 3. This study analysed the performance of wetland cell 1 from September 2008 to October 2009.

CSBP’s wetland cell 1 is 135m long x 95m wide planted with Schoenoplectus validus and has a 1m deep fine sand substrate. At the time of construction a 10cm layer of woodchips was placed in the middle of the sand substrate with the goal to provide a carbon source for denitrification. The influent distribution pipe is located centrally on the surface of the sand and the effluent drainage pipes located underneath the sand column on the surface of a plastic liner, drainage pipes are covered with gravel and wrapped in geotextile fabric. The system is a saturated surface VFCW operated in a sequencing batch mode with approximate cycle times of 12hr –feed, 0 to 24hr –stay, 12hr –drain. The sand medium is kept constantly saturated with the water level varying
between the sand surface and 0.3m above it (Figure 7.1). The wetland is never fully drained and draining stops when the water level reaches the surface of the sand, at that time filling of another batch starts. Batch volumes and residence times are highly variable due to uneven wastewater production and storm events. Batches ranging from 31 to 5333m$^3$ were recorded during this study period with an average inflow of 1109m$^3$/day. With a total holding volume of 7695m$^3$, an average theoretical hydraulic retention time (HRT) of 6.9 days can be assumed. Heavy metal removal performance for this wetland has been described in Chapter 6 (Domingos et al., 2009).

![Diagram](image-url)  

**Figure 7.1:** A: Schematic view of the treatment train at CSBP Ltd. The asterisk between the containment pond and cell 1 indicates where the new cells were built (July 2009). 1 and 2 indicate water sampling points. B: Cross-section view of the wetland.

### 7.2.2 Water, sediment and microbial sampling and analyses

The wetland is equipped with flow meters and automated composite samplers in the inlet and outlet pipes. Samples are analysed for pH, conductivity, ammonia, nitrate, ammonia + nitrate= total nitrogen (TN), total phosphorous (TP) and a wide range of metals according to Standard Methods (APHA, 1998) in the CSBP laboratory.

For microbial analysis samples were collected from the very surface layer, <1cm deep, (sludge) and from a 10 cm depth where the sediment consisted of clear sand (sand). Sediment was taken by inserting a graded plastic pipe to the desired depth and then collecting approximately 10.0g of sludge and sand material in zip-lock bags. Sampling was conducted at 3 locations in the wetland in August 2008, September 2008, February 2009 and August 2009, samples from the same depth were grouped as sludge or sand. DNA was extracted with the use of UltraClean® soil DNA isolation kit (Mobio Laboratories, CA,) according to the manufacturer’s instructions, eluted in water and stored at -20°C until use.
7.2.3 Polymerase Chain Reactions (PCRs)

The AOB specific primer pair amoA-1F (5’-GGG GTT TCT ACT GGT GGT) and amoA-2R (5’-CCC CTC KGS AAA GCC TTC TTC) (Rotthauwe et al., 1997) targeting a 491 base pair (bp) region of the amoA gene was used in the PCR. Amplifications were performed in 25µL reaction volumes for cloning purposes and 50µL reactions for T-RFLP analysis. Reactions contained 10 to 20ng of template DNA, 1x PCR buffer, 2.5 mM of MgCl₂, 0.2 mM of mixed dNTPs, 0.5µM of each primer 1.0 U of Taq DNA polymerase (Promega, Madison, Wi). PCR cycles used have been described by Siripong and Rittmann (2007) with annealing at 55°C. PCR products of the expected size were purified with the Wizard SV Gel and PCR clean up kit (Promega, Madison, Wi), for further use.

7.2.4 Clone library

Cloning reactions and transformations were performed according to the instructions in the PGEM-T easy Vector System and JM109 competent cells (Promega, Madison, Wi). A total of 136 positive clones from the August and September 2008 samples were selected for the amplification of the amoA gene and subsequent digestion with HaeIII and AluI RE according to the manufacturers’ instructions. Digested products were electrophoresed and visualised in 3% agarose gels. Different restriction fragment patterns were assigned to different operational taxonomic units (OTUs).

7.2.5 T-RFLP

The amoA-1F- 5’FAM labelled primer was used to generate PCR products for T-RFLP. Purified PCR products were digested with Alu I, HaeIII and TaqI restriction enzymes (RE) according to the manufacturers’ recommendations (Promega, Madison, Wi). One µl of each digest was added to 0.25 µl LIZ600 marker (Applied Biosystems, CA) and 9 µl Hi-Di™ formamide (Applied Biosystems, CA) and fragment analysis performed in an Applied Biosystems 3730 DNA sequencer (SABC, Murdoch University). Resulting
profiles were analysed using Genemapper software (Applied Biosystems, CA). T-RFLP profiles were normalised according to the constant percentage threshold (Sait et al., 2003). Peak areas that fell below the percentage threshold (ranging from 1 - 3% of the total peak area, depending on the profile) were therefore disregarded.

### 7.2.6 Sequencing and phylogeny

Clones that contained *amoA* genes which resulted in dissimilar cutting patterns were selected for insert sequencing. Sequences in the GenBank database (NCBI: [http://blast.ncbi.nlm.nih.gov](http://blast.ncbi.nlm.nih.gov)) sharing the greatest similarities were selected for multiple alignments using ClustalW2.0.12 software (Larkin et al., 2007). Phylogenetic trees were visualised with Treeview software (Page, 1996). Sequences from this study have been deposited in GenBank under accession numbers HQ541435-HQ541447.

### 7.3 Results and discussion

#### 7.3.1 Wetland performance

Total monthly flows, hydraulic loading rates (HLR) and nutrient loads were highly variable but followed the climatic pattern of south-west Western Australia, with the highest flows and loads occurring around the rainy winter months (June to October) when large volumes of stormwater runoff carry dissolved nitrogen and phosphorus compounds into the wetland, and the lowest flows and loads occurring during the drier months (November to May) (Table 7.1).

From September 2008 to July 2009 influent TN was predominantly NH$_3$-N while NO$_3$-N concentrations were lower. Ammonia removal during this period was highest (> 40% load removed) between December 2008 and May 2009 when HLRs were the lowest (< 7cm/d). The same pattern was observed for TN removal with the exception of March 2009 when no TN load removal occurred. NO$_3$-N removal varied greatly during the whole period. NO$_3$-N removal as reported, in terms of influent vs effluent loads, does not reflect the total removal capacity of the system as it does not account for the NO$_3$-N fraction produced by nitrification within the wetland. If we assume that the NH$_3$-
N removed was converted to NO$_3$-N by nitrification and sum this fraction to the influent NO$_3$-N then a greater NO$_3$-N removal can be accepted.

This speculative assumption however relies on classical nitrification-denitrification and does not consider other alternative removal pathways such as anaerobic ammonium oxidation (anammox) and complete autotrophic nitrogen removal over nitrite (CANON) which may happen in wetland sediment environments with high ammonia and low oxygen and carbon concentrations as demonstrated by Sun and Austin (2007).

Due to flow meter works, influent flows and loads into wetland cell 1 could not be calculated for June and July 2009, effluent loads in these months however were the highest recorded, indicating that the system was likely overloaded (Figure 7.2). From August 2009 onwards cell 1 started receiving nitrified effluent from cells 2 and 3, as visible in Figure 7.2 the proportion of NO$_3$-N increased in the effluent as NH$_3$-N decreased. There was further removal of ammonia in the wetland cell 1 but nitrate was not removed efficiently resulting in low TN removal in August and September 2009. It is believed that the low COD:N ratio of the wastewater is unfavourable to denitrify the larger nitrate loads. The presence of woodchips in the sand (added at the time of construction, 2004) and the seasonal availability of plant litter are the only organic carbon sources in the wetland.

![Figure 7.2: Total monthly loads of influent TN (■), effluent NH$_3$-N and NO$_3$-N (stacked columns) and average inflow (∆).](image-url)
Table 7.1: Monthly total flows, average concentrations, HLRs, loads and removal rates from September 2008 to October 2009.

<table>
<thead>
<tr>
<th>DATE</th>
<th>sample</th>
<th>Total Flow (m³/month)</th>
<th>HLR (cm/d)</th>
<th>pH</th>
<th>Concentration (mg/l)</th>
<th>Load applied (g/m²/day)</th>
<th>Load Removal rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NH₃-N</td>
<td>NO₃-N</td>
<td>TN</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(g/m²/d)</td>
<td>(%)</td>
<td>(g/m²/d)</td>
</tr>
<tr>
<td>Sept-08</td>
<td>Inf</td>
<td>55,319</td>
<td>14.4</td>
<td>7.7</td>
<td>93.6</td>
<td>21.1</td>
<td>114.7</td>
</tr>
<tr>
<td></td>
<td>Eff</td>
<td>54,763</td>
<td>7.6</td>
<td></td>
<td>49.8</td>
<td>19.1</td>
<td>69.0</td>
</tr>
<tr>
<td>Oct-08</td>
<td>Inf</td>
<td>63,100</td>
<td>15.9</td>
<td>7.5</td>
<td>51.7</td>
<td>10.0</td>
<td>61.7</td>
</tr>
<tr>
<td></td>
<td>Eff</td>
<td>62,518</td>
<td>7.4</td>
<td></td>
<td>43.8</td>
<td>8.8</td>
<td>52.5</td>
</tr>
<tr>
<td>Nov-08</td>
<td>Inf</td>
<td>33,570</td>
<td>8.7</td>
<td>7.6</td>
<td>26.4</td>
<td>14.4</td>
<td>40.8</td>
</tr>
<tr>
<td></td>
<td>Eff</td>
<td>20,710</td>
<td>7.6</td>
<td></td>
<td>38.8</td>
<td>12.9</td>
<td>51.7</td>
</tr>
<tr>
<td>Dec-08</td>
<td>Inf</td>
<td>11,577</td>
<td>2.9</td>
<td>7.7</td>
<td>43.4</td>
<td>22.6</td>
<td>66.0</td>
</tr>
<tr>
<td></td>
<td>Eff</td>
<td>9,835</td>
<td>7.7</td>
<td></td>
<td>18.0</td>
<td>20.9</td>
<td>39.0</td>
</tr>
<tr>
<td>Jan-09</td>
<td>Inf</td>
<td>22,865</td>
<td>5.8</td>
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<td>32.8</td>
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</tr>
<tr>
<td></td>
<td>Eff</td>
<td>20,640</td>
<td>7.5</td>
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<td>20.4</td>
<td>13.0</td>
<td>33.3</td>
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<td>5.7</td>
<td>7.2</td>
<td>37.4</td>
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<td></td>
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<td>19.1</td>
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<td>3.8</td>
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<td>32.8</td>
<td>13.2</td>
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<tr>
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<td>19.0</td>
<td>23.3</td>
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<td>24,449</td>
<td>6.4</td>
<td>7.5</td>
<td>29.8</td>
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<td>47.1</td>
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<tr>
<td></td>
<td>Eff</td>
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<td>7.6</td>
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<td>20.1</td>
<td>22.6</td>
<td>42.8</td>
</tr>
<tr>
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<td>21,432</td>
<td>5.4</td>
<td>7.7</td>
<td>80.3</td>
<td>33.2</td>
<td>113.5</td>
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<tr>
<td></td>
<td>Eff</td>
<td>27,601</td>
<td>7.8</td>
<td></td>
<td>45.7</td>
<td>29.4</td>
<td>75.1</td>
</tr>
<tr>
<td>Jun-09</td>
<td>Inf</td>
<td>57,071</td>
<td>7.6</td>
<td>7.5</td>
<td>73.6</td>
<td>33.1</td>
<td>106.7</td>
</tr>
<tr>
<td></td>
<td>Eff</td>
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<td>7.6</td>
<td></td>
<td>54.3</td>
<td>43.6</td>
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<td>Jul-09</td>
<td>Inf</td>
<td>57,071</td>
<td>8.6</td>
<td>7.5</td>
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<td>25.0</td>
<td>88.9</td>
</tr>
<tr>
<td></td>
<td>Eff</td>
<td>57,071</td>
<td>8.6</td>
<td></td>
<td>64.0</td>
<td>25.0</td>
<td>88.9</td>
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<tr>
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<td>13.8</td>
<td>8.1</td>
<td>28.0</td>
<td>28.6</td>
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<td>7.7</td>
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<td>32.6</td>
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<td>16.6</td>
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<td>11.0</td>
<td>32.1</td>
<td>43.4</td>
</tr>
<tr>
<td></td>
<td>Eff</td>
<td>63,883</td>
<td>7.5</td>
<td></td>
<td>6.0</td>
<td>34.6</td>
<td>40.6</td>
</tr>
<tr>
<td>Oct-09</td>
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<td>53,147</td>
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<td>6.0</td>
<td>38.6</td>
<td>44.8</td>
</tr>
<tr>
<td></td>
<td>Eff</td>
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<td>7.8</td>
<td></td>
<td>6.0</td>
<td>33.6</td>
<td>34.7</td>
</tr>
<tr>
<td>Influent a</td>
<td></td>
<td>avg 36,521</td>
<td>9.4</td>
<td>7.5</td>
<td>39.8</td>
<td>23.2</td>
<td>63.0</td>
</tr>
<tr>
<td></td>
<td>max</td>
<td>63,883</td>
<td>16.6</td>
<td>8.8</td>
<td>220.0</td>
<td>205.5</td>
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<td>min</td>
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<td>2.9</td>
<td>6.2</td>
<td>0.03</td>
<td>0.1</td>
<td>1.8</td>
</tr>
<tr>
<td>Effluent b</td>
<td></td>
<td>avg 38,846</td>
<td>7.6</td>
<td>7.8</td>
<td>30.9</td>
<td>24.7</td>
<td>55.6</td>
</tr>
<tr>
<td></td>
<td>max</td>
<td>66,517</td>
<td>7.8</td>
<td></td>
<td>130.0</td>
<td>71.4</td>
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<td></td>
<td>min</td>
<td>9,835</td>
<td>7.3</td>
<td></td>
<td>0.0</td>
<td>1.9</td>
<td>10.8</td>
</tr>
</tbody>
</table>

Overall removal rate:

- NH₃-N: 1.2
- NO₃-N: 0.1
- TN: 4.2
- TP: 1.3
- avg: 25.4
- max: 0.2
- min: 39.9

n=312, b=n=301, c considers total load in whole period (excludes June and July 2009)
The higher percentages of TN removed between November 2008 and May 2009 (except March 2009) are likely to result from a combination of factors such as 1) lower flows and HLRs which mean smaller batches and therefore increased HRT; 2) lower TN loads into the wetland; 3) higher plant litter availability as carbon source for denitrification, as it has been verified the above ground biomass of *Schoenoplectus validus* is mostly dry at the end of winter and forms a thick mat over the surface of the sand during the summer months; 4) higher temperatures (water temperature not recorded, but Perth’s average 12.7°C in winter and 24.4°C in summer).

Total load removal for the 12 month period (excluding June and July 2009) was in the order of 39% for NH$_3$-N, accounting for 5,679kg of NH$_3$-N removed (removal rate = 1.21g/m$^2$/d). TN removal was 25% and accounted for 6,067kg of TN removed (removal rate = 1.30 g/m$^2$/d). The removal of TP was also significant with 40% of the load or 739kg of phosphorus removed over a 9 month period (removal rate = 0.21g/m$^2$/day) (see Table 7.1). The performance of cell 1 in terms of TN removal is similar to other free water surface wetlands treating municipal wastewaters with similar TN influent loads (our study = 1,788g/m$^2$/yr) and TN influent concentrations >50mg/L (Kadlec and Wallace, 2009). Cell 1 was designed to treat up to 500m$^3$/day with a targeted 50% TN removal so the 25% TN removal achieved during the period when the flow was on average 1109m$^3$/day is reasonable considering the system was mostly operated under hydraulic overloads.

### 7.3.2 AOB analysis by clone library

A total of 80 amoA clones were selected from the sludge samples and 56 clones from the sand samples (PCR products from August and September 2008). Sequences were closely associated with *Nitrosomonas nitrosa*, *Nitrosococcus mobilis* (*Nitrosomonas* sp) and *Nitrosospira multiformis* (Figure 7.3). Sequences from the sludge and sand were similar with the only exception being a *Nitrosospira*-like sequence present in the sand but not in the sludge. Three unique clone OTUs were most similar to uncultured organisms, clone S1-sand having 99% similarity to anoxic biofilm clone S1 (GenBank:AF202650.1) isolated from a trickling filter biofilm (Schmid *et al.*, 2000) and
clones S8-slime and S9-sand which formed independent OTUs according to HaeIII digestion but were both 99% similar to uncultured bacterium clone AS-AOB-60 (GenBank:GU327814.1) sampled from saline wastewater under low dissolved oxygen. The clone library originated from the sludge samples revealed that 51% of the clones were closely related to *Nitrosomonas* sp. Nm107 (AF272407.1) and 32% related to *Nitrosomonas nitroa* Nm 90 (AF272404.1). From the sand samples, 84% of clones were closely related to *Nitrosomonas nitroa* and only 2% associated to *Nitrosospira multiformis* (DQ228454.1). Similarly to our findings, Tietz *et al.* (2007) verified that the most abundant sequences from VFCWs receiving municipal effluent were related to *Nitrosomonas europaea/*“Nitrosococcus mobilis” and *Nitrosospira* sp.

### 7.3.3 AOB analysis by T-RFLP

The *amoA* based, AluI digest T-RFLP fingerprint revealed four discernible peaks at 224, 360, 405 and 491bp. The HaeIII digest revealed 7 discernible peaks at 138, 165, 175, 224, 312, 434 and 491bp and the TaqI fingerprints revealed 3 peaks at 212, 219 and 283bp (Figure 7.4).

There was a higher proportion peaks likely to result from *Nitrosomonas* sp. (AluI-491bp, 405bp and 224bp, HaeIII-165bp and 224bp, TaqI-219bp) than *Nitrosospira* sp (AluI-491bp, HaeIII-175 and TaqI-283bp) in all sludge and sand fingerprints. An exception was the predominance of a *Nitrosospira* sp. peak in the sludge sample from February 2009. Peak AluI-491bp may result from either *Nitrosomonas nitroa* Nm90 or *Nitrosospira* sp lineages, so the use of HaeIII or TaqI was necessary to differentiate between them. *N. nitroa* results in a HaeIII-165bp and TaqI-48bp peak while *Nitrosospira* sp results in HaeIII-175bp and TaqI-283bp.
Figure 7.3: Phylogenetic tree constructed for partial amoA gene sequences of the 13 unique clones obtained in this study and most similar sequences retrieved from GenBank. Neighbour-joining analysis carried out using ClustalW2.0.12. *Methylococcus capsulatus* strainBL4 (AF533666) as outgroup. Scale bar represents 10% nucleotide divergence.

In their TaqI based T-RFLP Horz *et al.* (2000) verified that all amoA sequence types which grouped within the genes *Nitrosospira* showed an OTU of 283bp, indicating the high specificity of the TaqI-283bp OTU to *Nitrosospira* spp. A TaqI-219bp OTU was assigned to members of the *Nitrosomonas* genus, indicative of *Nitrosomonas europaea*, *Nitrosomonas eutropha*, *Nitrosomonas halophila* and *Nitrosococcus (Nitrosomonas) mobilis*. *Nitrosomonas nitrosa*–like sequences have a TaqI-48bp peak also indicative of other lineages and was only differentiated by CfoI based T-RFLP which generated a
specific 119bp OTU. Even though *N. nitrosa*-like sequences were common in both sand and sludge samples, the expected TaqI-48bp peak could not be verified in our TaqI based profiles due to noise in peaks smaller than 50bp, its correspondent 165bp and 491bp peaks were common in the HaeIII and AluI profiles, respectively. A TaqI-212bp peak was present in the August and September 2008 samples but no sequence data from clones was retrieved to help assigning its identity.

![Figure 7.4: Relative abundance of T-RFs in the CSBP wetland based on TaqI digestion of amoA PCR products. T-RFs: 212bp=(undetermined), 219bp=*Nitrosomonas* sp, 283bp=*Nitrosospira* sp.](image)

Purkhold *et al.* (2000) verified that Nitrosomonads were responsible for ammonia oxidation in wastewater treatment plants and that *Nitrosospira* were only sporadically present. *Nitrosomonas* spp have a lower substrate affinity but higher maximum activity than *Nitrosospira* spp. therefore *Nitrosospira* spp would be more likely to predominate in low ammonia environments (Schramm *et al.*, 1996) and may be better at withstanding physicochemical variations (Purkhold *et al.*, 2000). However, these findings are just indicative as other studies of systems receiving low ammonia waters (Qin *et al.*, 2007; Regan *et al.*, 2002) revealed that Nitrosomonas-like sequences were predominant over *Nitrosospira*-like sequences. Our T-RFLP results support the sequence data. There was a predominance of *Nitrosomonas* sp over *Nitrosospira* sp peaks in the sludge and sand profiles, the *Nitrosospira* sp peak only dominated in the February 2009 sample.
Nitrosomonas nitrosa strains have relatively high affinity constants for ammonia (Koops et al., 2006) and are regarded as having an ecological versatility which allows them to survive in a wide range of substrate conditions (Qin et al., 2007). Nitrosomonas nitrosa like sequences have often been isolated from systems with high ammonia concentration such as industrial wastewater treatment plants (Dionisi et al., 2002) and at the same time from low ammonia environments such as freshwater estuaries (Cebron et al., 2003) and a submerged biofilm for drinking water pre-treatment in China (Qin et al., 2007). Nitrosomonas nitrosa-like sequences were abundant in the wetland cell 1 which receives highly variable NH3-N concentrations, corroborating the claims of ecological versatility for this group.

Wastewater treatment systems with a high AOB diversity have increased nitrification resistance against perturbation while a monoculture in terms of AOB in a treatment system might render its nitrification efficiency more susceptible (Purkhold et al., 2000). We have verified that a VFCW receiving inorganic industrial wastewater can sustain a diverse AOB community in its sediments, therefore representing a resilient and robust treatment technology for ammonia removal.

7.4 Conclusions

The CSBP wetland cell 1 proved to be efficient in reducing NH3-N, TN and TP from wastewater prior to its discharge, nitrogen loads and removal performance follow the climatic pattern of south-west Western Australia, with higher loads and lower removal in the rainy winter and lower loads and higher removal in the dry summer. Ammonia removal rates ranged from 0.6 to 3.3g/m2/day with an overall removal rate of 1.2g/m2/d. The TN removal rate of 1.3g/ m2/d is similar to rates reported for free water surface wetlands. The construction of clone libraries in conjunction with T-RFLP revealed a diverse AOB community dominated by Nitrosomonas sp members and by less abundant Nitrosospira sp. No clear patterns in the composition of the community were observed in relation to depth or time of the year. In August 2009 cell 1 started receiving nitrified effluent from the two new VFCWs, since then its objective has been to achieve denitrification. The addition of more woodchips to cell 1 and dosing of high carbon content wastewaters from nearby industries have been recommended and will be discussed in chapter 8.
Chapter 8:

Design and monitoring of vertical flow constructed wetlands for ammonia removal: Performance in the first year of operation

8.1 Introduction

This chapter describes the design rationale and results of the first year of operation of large scale vertical flow constructed wetlands (VFCWs) built at CSBP Ltd in 2009 as part of its stormwater and wastewater treatment system upgrade. Increased wastewater production and confirmation that the pilot wetland cell built in 2004 (described in chapters 6 and 7) provided long term nitrogen removal and ancillary benefits such as phosphorus and heavy metal removal led CSBP to approve the expansion of its wetland treatment system. The main purpose of the wetland system is to remove nitrogen from the wastewater prior to its discharge. Taking this into account and considering that influent nitrogen is predominantly ammonia, the proposed design opted for the conventional nitrification and denitrification approach. Given the opposing DO requirements of nitrification and denitrification, it was decided that each process should be kept spatially separate for ease of operation and to allow optimisation of each step independently, without affecting the other.

It has been demonstrated that unsaturated or intermittently loaded VF wetlands can achieve complete nitrification (>95% NH\textsubscript{3}-N removal) of fresh and saline wastewaters due to their oxygen transfer capabilities and diverse nitrifying communities inhabiting the sand substrate (Chapters 3 and 4). For that reason, and following the recommendations of Domingos et al. (2007), two fill and drain VFCWs operating in parallel, with full draining and resting, were chosen as the nitrifying component of the system. Two options were considered for the denitrifying component, the first option was to maintain the existing wetland cell as a primary treatment and then have another saturated surface VF wetland, following the two parallel VF wetlands, built as the last denitrifying cell; a carbon source would be introduced to this last cell. This option was considered as the existing wetland proved to have a high heavy metal removal capacity (Table 6.1, Chapter 6), potentially

\textsuperscript{1}Part of this chapter is published as Domingos et al. (2011). Water, 38(3). pp103-104.
preventing the new cells from receiving toxic concentrations of heavy metals or from being subject to metal accumulation. The second option was to place the two new VF wetlands as the primary nitrifying component and have the existing cell last, as the denitrifying wetland where a carbon source would be introduced. The first option would result in a larger area and therefore a higher treatment performance, with 4 wetland cells in total (approximately 4.4ha). Limited land availability and high construction costs, however, revealed the plan unfeasible. The second option, with two additional cells to be built, resulted in being more economically viable and was therefore chosen by CSBP Ltd.

The next sections will describe some aspects of the design and construction of the new VF CWs. Following these descriptive sections, results from the first year of operation will be shown and the performance of the new cells and of the treatment system as a whole will be analysed. The addition of carbon into the denitrifying wetland will also be discussed.

8.2 Materials and methods

8.2.1 The proposed design

8.2.1.1 Nitrifying vertical flow wetlands

8.2.1.1.1 Area

The values presented in Table 8.1 were used for design purposes; they were based on the existing monitoring data available from CSBP at the time (2004-2006):

<table>
<thead>
<tr>
<th>Q_{in} = 1600m^3/d</th>
<th>Daily Load</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH_3-N_{in} = 45mg/L</td>
<td>72kg/d</td>
</tr>
<tr>
<td>NO_3-N_{in} = 15mg/L</td>
<td>24kg/d</td>
</tr>
<tr>
<td>TN_{in} = 60mg/L</td>
<td>96kg/d</td>
</tr>
</tbody>
</table>

Based on results from a mesocosms wetland experiment, partially described in Domingos et al. (2007) full nitrification of CSBP inorganic wastewater was achieved in...
VF wetlands operated in parallel in a fill and drain mode with a maximum HLR = 10cm/d. An ammonia removal rate of 4 to 5gNH₃-N/m²/d was achieved in the experiment and this value was therefore used for design purposes.

Considering removal rates in the order of 4 to 5gNH₃-N/m²/d and a daily loading of 72kgNH₃-N/d (72,000g/d) the estimated nitrifying area ranged between 14,400m² to 18,000m². A total area of 16,000m² was then recommended assuming a removal rate of 4.5gNH₃-N/m²/day. The total area would be divided in two 8,000m² cells, each cell to receive 1600m³ every second day. A sketch of the recommended upgrade plan for the wetlands can be seen in Figure 8.1 below.

![Figure 8.1: Proposed wetland sizes and treatment train for CSBP.](image)

### 8.2.1.1.2 Depth and Media

A 45cm main filtering layer composed of local sand and a 30 cm layer composed of gravel or HiSmelt slag (depending on availability) were recommended (Figure 8.2). Assuming an approximate porosity of 0.3 for the local sand and 0.4 for the gravel, each 8000m² cell would hold approximately 2040m³ in its substrate.
8.2.1.1.3 Retention time and operation schedule

Assuming a flow of 1600m$^3$/day and a pumping/piping system able to deliver and withdraw this volume in 12 hours the recommended operation schedule for the two vertical flow wetlands working in parallel would be as demonstrated in Table 8.2 below. The complete Fill – Stay – Drain cycle for each cell would take 48 hours to complete.

<table>
<thead>
<tr>
<th>2 cells = 36 hours HRT</th>
<th>8,000m$^2$</th>
<th>8,000m$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day/cell</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>1  Am Fill (1600m$^3$)</td>
<td>Stay</td>
<td></td>
</tr>
<tr>
<td>Pm</td>
<td></td>
<td>Drain</td>
</tr>
<tr>
<td>2  Am Stay</td>
<td></td>
<td>Fill (1600m$^3$)</td>
</tr>
<tr>
<td>Pm Drain</td>
<td></td>
<td>Stay</td>
</tr>
<tr>
<td>3  Am Fill (1600m$^3$)</td>
<td>Stay</td>
<td></td>
</tr>
<tr>
<td>Pm</td>
<td></td>
<td>Drain</td>
</tr>
<tr>
<td>4  Am Stay</td>
<td></td>
<td>Fill (1600m$^3$)</td>
</tr>
<tr>
<td>Pm Drain</td>
<td></td>
<td>Stay</td>
</tr>
<tr>
<td>5  Am Fill (1600m$^3$)</td>
<td>Stay</td>
<td></td>
</tr>
<tr>
<td>Pm</td>
<td></td>
<td>Drain</td>
</tr>
</tbody>
</table>
8.2.1.1.4 Distribution/collection pipes

The distribution pipes located on the top of the VF cells could be based on the one used in the existing cell with orifices distributed on the side facing the sediment. The pipe should be placed on, and covered by limestone rocks (Figure 8.3). The perforated collection pipes placed on the bottom could also follow the same design as those used for the first cell, being covered by gravel and geo-textile fabric to prevent blockage. Riser “aeration” pipes (as per Cooper, 1996) should be connected to the drainage pipes to allow gas exchange during filling and draining.

![Figure 8.3: Detailed scheme of the inlet distribution pipe sitting on and covered by limestone rocks and drainage pipe on the bottom.](image)

8.2.1.1.5 Liner

A high density polyethylene (HDPE) membrane liner covered by geotextile was recommended for the two new cells, the geotextile membrane was to protect the HDPE membrane.

8.2.1.1.6 Plants

Different plant species should be favoured instead of one single species as pollutant removal processes and system resilience would be enhanced with increased
plant diversity. Candidate native Rushes/Sedges species commonly available from WA nurseries include (Water and Rivers Commission, 2000). A density of 6 - 9 plants/m² was suggested to estimate cost.

- **Schoenoplectus validus** - can be considered due to its tolerance to brackish and saline waters. However it prefers shallow water and permanently wet conditions.
- **Baumea articulatta** – grows well in sand and bears brackish water but alike *S. validus* prefers permanently wet conditions.
- **Baumea juncea** – grows well in sand, tolerates brackish water and temporary dry conditions.
- **Eleocharis acutta** – grows well in sand, tolerates saline water and temporary dry conditions.
- **Isolepis nodosa** – grows well in sand, tolerates saline water and temporary dry conditions.
- **Juncus kraussi** – grows well in sand, tolerates saline water but alike *S. validus* prefers permanently wet conditions.
- **Juncus pallidus** – grows well in sand, tolerates brackish water and temporary dry conditions.

### 8.2.1.2 Denitrifying saturated vertical flow wetland

Since the existing wetland cell had proven to be efficient in nitrate removal, the last wetland cell was proposed to be 16,000m² and follow the same design of the existing wetland (Figure 8.1). The construction of this last denitrifying wetland proved to be unfeasible due to limited space and funds. In case further treatment is needed due to increasing wastewater production or stricter licenses to discharge, CSBP can revisit the design and follow up with construction of this fourth cell.

### 8.2.2 The constructed system

The construction of the new VF cells was completed in July 2009. Figures 8.4 to 8.8 show different stages of construction. The final design and treatment train is represented in Figure 8.9.
The new VF wetlands used a HDPE liner covered by geotextile and incorporated a 20cm drainage layer of blast furnace slag covering the drainage pipes on the bottom (Figure 2A), this layer consisted of 15cm of 14mm slag on the bottom and an 5cm intermediate layer of 7mm slag on top (to avoid sand wash down into the slag layer). In an example of industrial synergy, CSBP has used slag from Kwinana neighbour HiSmelt (slag being a by-product of HiSmelt’s smelting activities). The sand used for the 45cm main filtering layer was locally available from the site. The simple falling head method described by Grant and Griggs (2001) to determine the suitability of sands for intermittent sand filters resulted in a grant time \( t_g \) equal to 116 (±13) seconds, higher than the acceptable 15 -100 seconds. The local sand (grain size 0.02-0.5mm, grain size distribution also listed in Appendix A), was used because of the low TSS and BOD contents of CSBP’s wastewater and to the fact that cell one had been operational without clogging issues. This fine sand, however, would not be suitable for VF wetlands treating wastewaters with a BOD and TSS content due to clogging potential. The combination of the local carbonaceous sand and the furnace slag was to provide good phosphorus retention capacity and alkalinity to support nitrification in the VF wetlands.

The inlet pipe has several spreaders to allow even distribution of water across the surface (Figure 8.7 and 8.8). Seedlings were sourced from a local nursery and included native Schoenoplectus sp, Juncus sp and Isolepis sp. (At the time this thesis
was completed full aerial photographic coverage of the construction stages could be
scrolled at www.nearmap.com by skipping through the different dates on top of the
picture, on the link: http://www.nearmap.com/?ll=-32.236989,115.761781&z=18&t=k&nmd=20090124)

Figure 8.5: Drainage layer taking shape, spreading the 14mm blast furnace slag to cover
perforated drainage pipes in cell 2 (March 2009).

Figure 8.6: Levelling of the intermediate 7mm blast furnace slag on top of the 14mm layer in
cell 3. Note “air” pipes which connect to drainage system under the slag coming up in the
centre and on the batter at the far end (March 2009).
Figure 8.7: Cell 2 being tested at CSBP. Alternate fill and drain operation is visible on the two VF parallel cells (far end) just prior to planting (May 2009). Note the transverse vegetation strips on the saturated VF wetland as a result of removal of the top sludge layer to overcome clogging (photo by CSBP).

Figure 8.8: Detail of inlet distribution pipe in cell 3. Note limestone rocks covering the inlet distribution pipe and protruding “aeration” pipes along the centre and batters (November 2009).
8.2.3 Operation

The two new cells, approximately 8,000m$^2$ each, added to the existing 7 year old, 12,000m$^2$, saturated surface VF wetland, expanding the total wetland area to 2.8ha. The upgraded treatment train, represented in Figure 8.9, also includes a containment pond which serves as an equalisation and settling basin. From the containment pond water is alternately pumped into the parallel VF wetlands. Rather than intermittently fed free-draining systems, these cells operate in a sequencing batch (fill and draw) mode. Batches would be ideally up to 1,600m$^3$/day but they can be quite variable depending on rainfall and wastewater production. Operation is usually 12hr – filling, 12hr-full, 12 hr-draining and 12hr-resting empty. While one cell is filling the other one is emptying and resting and vice versa. The nitrified effluent from the parallel VF cells is then pumped into the 7 year old saturated-surface VF system which had woodchips incorporated in the substrate during construction and on the top of the sediment in 2010. A dosing system for the supply a liquid organic carbon source has also been installed in this last denitrifying cell.

![Figure 8.9: Treatment train at CSBP Ltd showing the two new VF wetlands operating in parallel and the 7 year old saturated surface VF wetland. The circle with the letter C going into the saturated VF cell indicates the addition of high carbon content wastewater into the wetland to favour nitrate removal. Numbers 1, 2 and 3 indicate sampling points.](image)

After four years of continuous operation (2004-2008) the saturated VF wetland presented signs of surface clogging (slow draining of the wetland due to sludge accumulation) (Figure 8.10). In December 2008, this problem was overcome by fully draining the wetland and mechanically removing, by small bobcat, the accumulated sludge and exposing the clear sand underneath over about 50% of the area. Some plant sacrifice was expected with this operation hence the striped vegetation pattern seen
on Figure 8.7. Within a year vegetation had recovered in the cleared areas. A recommendation to avoid or postpone clogging in the saturated VF wetland was to drop the water level to below the surface of the sand to allow drying and oxidation of the accumulated sludge on a monthly basis.

Plant mortality was high in the new cells but survivors grew vigorously in the first year. Even though both VF wetlands are identical in terms of construction and operation, plant development and coverage was much higher in cell 3 than in cell 2 for no evident reason. Further sediment analysis would have to be carried out to clarify why plant development was impacted in cell 2.

8.2.4 Water sampling and analysis

Influent and effluent flows were measured and influent and effluent samples were collected and analysed for inorganic nitrogen forms, pH and temperature for the period of July 2009 to June 2010. During July and August 2009, flow meter works were taking place as part of the commissioning of the new VF cells, these months will
therefore not be included in the analysis of the load base performance. Water analyses were conducted according to the methods described in Chapter 6.

8.3 Results and discussion

8.3.1 Performance

Flow measurements revealed that in 65% of the time (202 out of the 309 days when flows were registered into the wetlands, from 1 Aug 2009 to 24 Jun 2010) flows were higher than the 1600m$^3$/d used in the design. Figure 8.11 shows that daily flows were highly variable, with peaks more frequently occurring during the wetter months (March to August).

Figure 8.11: Daily influent flow to the VF wetlands. Flow meter location represented by number 1 in Figure 8.9. Dotted line at 1600m$^3$ indicates flow used in design.

Only 41% of influent samples (144 of 347 samples analysed for NH$_3$-N) were lower or equal to the 45mg/L NH$_3$-N considered for the design. In terms of load, only 39% of influent loads (122 of 309) were lower than the 72kg NH$_3$-N /d used in the design (Figure 8.12). This shows that most of the time the wetland cells operated under higher loading conditions than originally anticipated.
Despite the high hydraulic and mass loadings the VF cells removed ammonia exceptionally well during the first year of operation. Ammonia removal, based on influent and effluent concentrations, varied from 19% to 85% with an average 59% removal (Table 8.2). The lowest removal was in the first month of operation following commissioning, this low initial removal was expected and it has also been observed in the laboratory experimental systems. Based on the performance data, approximately two months were necessary for ammonia oxidising bacteria populations to grow, colonise the media and attain equilibrium in the presence of excess NH$_3$-N substrate. No inoculum (or seed) was used and colonisation relied on AOB originally present in the local sand, planted seedlings and incoming wastewater.

Loads in and out could not be accurately measured for July and August 2009 due to flow meter works. The load based removal of ammonia varied between 61 and 89% with an overall removal of 68%. The areal removal rates ranged from 2.4 to 8.37g/m$^2$/d and the overall removal rate was 4.94g/m$^2$/d. This is equivalent to 23,451Kg of NH$_3$-N being removed in 297 days considered. The overall NH$_3$-N removal rate of 4.94g/m$^2$/d was satisfactorily close to the 4.5g/m$^2$/d assumed for design purposes.
Table 8.2. Total loads and average concentrations in and out of the VF wetlands during the first year of operation.

<table>
<thead>
<tr>
<th>Month</th>
<th>NH\textsubscript{3}-N Loads</th>
<th>NH\textsubscript{3}-N Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Influent (Kg)</td>
<td>Effluent (Kg)</td>
</tr>
<tr>
<td>Jul-09</td>
<td>78.8</td>
<td>64.0</td>
</tr>
<tr>
<td>Aug-09</td>
<td>57.1</td>
<td>28.2</td>
</tr>
<tr>
<td>Sep-09</td>
<td>1787</td>
<td>634</td>
</tr>
<tr>
<td>Oct-09</td>
<td>57.1</td>
<td>28.2</td>
</tr>
<tr>
<td>Nov-09</td>
<td>1787</td>
<td>634</td>
</tr>
<tr>
<td>Dec-09</td>
<td>57.1</td>
<td>28.2</td>
</tr>
<tr>
<td>Jan-10</td>
<td>3285</td>
<td>1031</td>
</tr>
<tr>
<td>Feb-10</td>
<td>3285</td>
<td>1031</td>
</tr>
<tr>
<td>Mar-10</td>
<td>3285</td>
<td>1031</td>
</tr>
<tr>
<td>Apr-10</td>
<td>3285</td>
<td>1031</td>
</tr>
<tr>
<td>May-10</td>
<td>3285</td>
<td>1031</td>
</tr>
<tr>
<td>Jun-10</td>
<td>3285</td>
<td>1031</td>
</tr>
<tr>
<td>Overall</td>
<td>34402</td>
<td>10951</td>
</tr>
</tbody>
</table>

A load - concentration chart for NH\textsubscript{3}-N is presented in Figure 8.13. The NH\textsubscript{3}-N load in is the monthly overall and concentration out is the monthly mean. The 10 data points represent months September 2009 to June 2010. A positive correlation was observed between NH\textsubscript{3}-N load in and NH\textsubscript{3}-N concentration out. This information could be used to control effluent concentrations out of cells 2 and 3, for example if a maximum effluent NH\textsubscript{3}-N concentration of 15mg/L is desired, then the load applied should be below 5g/m\textsuperscript{2}/d.

![Load - concentration chart for NH\textsubscript{3}-N](image)

Figure 8.13: Load - concentration plot for NH\textsubscript{3}-N in the new VF CWs at CSBP from September 2009 to June 2010.
The same strong positive correlation was verified between ammonia applied and ammonia removed (Figure 8.14). Load removed values were expected to plateau when subject to the higher loads applied, indicating the total removal capacity of the wetland; this however was not verified during the monitoring period.

Figure 8.14: Load applied - load removed plot for NH₃-N from September 2009 to June 2010.

The effect of the new VF cells in terms of NH₃-N oxidation in the whole process can be seen in Figure 8.15. Within three months of commissioning the pattern of higher NH₃-N and lower NO₃-N in the final effluent was permanently reversed. From September 2009 the final effluent NH₃-N dropped to <2.0 mg/L in several occasions indicating efficient nitrification in cells 2 and 3 and further removal in cell 1. As a result TN in the effluent was mainly composed of NO₃-N, therefore the need to reduce it to N₂ in the saturated VF. As the influent lacks organic carbon (influent COD=50mg/L) and whatever available initially gets oxidised in the first stage VFs, removal of NO₃-N by denitrification can only be achieved with the introduction of external carbon to cell 1.

In a one-off exercise acetic acid (1g Ac. acid = 1g COD) was dosed into cell one at a COD:NO₃-N ratio = 5 for approximately three weeks during May/June 2010, this addition resulted in NO₃-N dropping to 2.7mg/L, the lowest concentrations achieved in the whole period studied, July 2009 - June 2010 (see Figure 8.15). The monthly average fell from 70mg/L in May to 12 mg/L in June, when acetic acid was dosed. Cost, however, makes this practice prohibitive. Parallel to the acetic acid trial, in June-July
2010, woodchips were added to the surface of cell one as a long term slow release carbon source.

![Graph showing nitrogen concentrations](image)

**Figure 8.15:** Daily monitoring of N concentrations in the influent (cont. pond) and final effluent of the saturated VF wetland systems (sampling points 1 and 3). Influent TN is predominantly NH\(_3\)-N. Acetic acid addition at COD:NO\(_3\)-N = 5 indicated by the bracket (May/June 2010).

As result of the successful use of wastewater from the soft drink manufacturer as carbon source for denitrification in saturated VF wetlands (Chapter 5), CSBP installed a 60m\(^{3}\) storage tank on site in April 2011. This tank has allowed CSBP to receive and store larger amounts of high carbon wastewater and then dose it into the wetland in a controlled manner. Ethylene glycol waste (spent motor vehicle coolant, COD=400,000–600,000mg/L) has also been tested and demonstrated to be, as was the soft drink wastewater, a promising carbon source.

As visible from Figure 8.15 influent concentrations of TN (NH\(_3\)-N + NO\(_3\)-N) are highly variable and can be quite high in isolated events; the capacity of the wetlands in handling these events has been demonstrated. The expansion of the total wetland area has also resulted in increased storage and buffering capacity prior to discharge via the SDOOL. The total cost for the new cells was AU$2.1million (2009), this included all
construction and planting costs, integration to the existing control systems and installation of new on-line monitoring and automatic sampling stations.

8.4 Conclusions

This study has shown that large scale VF wetlands operated on a fill and draw mode can be successfully applied for the removal of ammonia from inorganic industrial wastewater. An overall removal of 68% was achieved for cells 2 and 3 during the study period; the equivalent areal removal rate was 5gNH$_3$-N/m$^2$/d, slightly higher than the 4.5gNH$_3$-N/m$^2$/d assumed for design purposes. Ammonia removal in the new cells was reasonable considering they operated under higher hydraulic and mass loadings than originally expected. Nitrate and, therefore, total nitrogen removal in the cell 1 could be efficiently improved with the introduction of external carbon sources to the wetland. Industrial synergies realised during this study, such as the use of slag in the substrate and use of high carbon content wastewaters (soft drink, ethylene glycol), proved to be very important and should serve as example to future projects, resulting in potential environmental and economical benefits.
Chapter 9:

Conclusions and further research

This research found that vertical flow constructed wetlands with sand substrate as main filtering layer:

- Can be applied to remove ammonia from saline and fresh inorganic wastewaters under intermittent or batch loading conditions.
- Remove ammonia irrespective of the presence of plants corroborating that removal is mainly dictated by microbial processes.
- Support diverse ammonia oxidising bacterial communities under fresh, brackish and hypersaline (40gNaCl/L) conditions.
- Can accomplish nitrification in the unsaturated main filtering layer and denitrification in the drainage layer given that the latter is kept saturated and a carbon source is introduced. Efficient denitrification and diverse denitrifying bacterial communities were verified in fresh and saline systems. This is of particular importance in a water management context. Saline water sources can be used in processes that do not require freshwater; treatability of inorganic saline wastewater streams has been demonstrated in VF wetlands.
- Can achieve nitrification and denitrification of inorganic wastewaters when operated in a saturated surface sequencing batch mode and when an external carbon source is introduced. Wastewater from a soft drink manufacturer proved to be a cost effective source of carbon to improve denitrification in the saturated VF cells.
- Can achieve heavy metal removal and store metals in the sludge layer when operated in a saturated surface sequencing batch mode. Heavy metals accumulated at higher concentrations in the sediment closer to the inlet pipe.
- Represent a cost effective system for nitrogen removal in large scale industrial applications. The systems have a reasonably simple operation which can be suited to optimise nitrification (intermittent feeding or fill and drain) or denitrification (saturated surface).
Considering the findings and limitations of this thesis, further research should focus on:

- Verifying the presence and role of ammonia oxidising archaea, anammox and heterotrophic nitrifiers in wetlands.
- Understanding the effects of salinity on nitrite oxidising bacteria.
- Testing mangrove and other salt tolerant plant species in VF wetlands.
- Improving nitrification and denitrification of inorganic wastewaters in a single wetland cell by controlling the level of saturation and carbon input.
References


Maciolek D.J., Austin D.C. (2006). Low energy biological nitrogen removal by cation exchange, thin film oxygen transfer, and heterotrophic nitrification in sequencing-batch, packed bed...


Appendices

Appendix A: Particle size and permeability of sand

Particle size distribution and permeability of the sand used in this study. (Data provided by Parsons Brinkerhoff. CSBP / BP Kwinana Nitrogen Stripping Wetland Preliminary Design Report. June 2003).

![Particle size distribution and uniformity coefficient graph]

Uniformity Coefficient, \( Cu = \frac{D_{60}}{D_{10}} \).
From graph, \( D_{60} = 0.22 \text{mm} \) and \( D_{10} = 0.1 \)
Then \( Cu = 2.2 \)

Figure A1: Particle size distribution and uniformity coefficient of the sand used in the experimental and field components of this thesis.
Figure A2: Permeability of the sand used in the experimental and field components of this thesis.
Appendix B: Results from TaqI and HaeIII based T-RFLP of the amoA gene

Results of the TaqI based T-RFLP

Control wetland

Figure B.1: Relative abundance of T-RFs generated by TaqI digestion of the amoA gene retrieved from different depths of the control wetland. The percentage which is not shown in graphs corresponds to the sum of peaks that fell below the threshold (1-3%). No results were obtained from the omitted depths (December 2008: 15 and 30cm and March 2009: 15cm).
Figure B.2: Relative abundance of T-RFs generated by TaqI digestion of the amoA gene retrieved from different depths of the T1 wetland. The percentage which is not shown in graphs corresponds to the sum of peaks that fell below the threshold (1-3%).
Treatment 2 wetland

Figure B.3: Relative abundance of T-RFs generated by TaqI digestion of the amoA gene retrieved from different depths of the T2 wetland. The percentage which is not shown in graphs corresponds to the sum of peaks that fell below the threshold (1-3%).
Comparison of Surface samples from the Control, Treatment 1 and Treatment 2 wetlands

Figure B.4: Relative abundance of T-RFs generated by TaqI digestion of the amoA gene retrieved from the surface of the Control, Treatment 1 and Treatment 2 wetlands. The percentage which is not shown in graphs corresponds to the sum of peaks that fell below the threshold (1-3%).
Results of the HaeIII based T-RFLP

Control wetland

Figure B.5: Relative abundance of T-RFs generated by HaeIII digestion of the *amoA* gene retrieved from different depths of the control wetland. The percentage which is not shown in graphs corresponds to the sum of peaks that fell below the threshold (1-3%). No results were obtained from the omitted depth (March 2009: 30cm) and for August 2009.
Treatment 1 wetland

Figure B.6: Relative abundance of T-RFs generated by HaeIII digestion of the *amoA* gene retrieved from different depths of the T1 wetland. The percentage which is not shown in graphs corresponds to the sum of peaks that fell below the threshold (1-3%). No results were obtained for August 2009.

Treatment 2 wetland

Figure B.7: Relative abundance of T-RFs generated by HaeIII digestion of the *amoA* gene retrieved from different depths of the T2 wetland. The percentage which is not shown in graphs corresponds to the sum of peaks that fell below the threshold (1-3%). No results were obtained for August 2009.
Comparison of Surface samples from the Control, Treatment 1 and Treatment 2 wetlands

Figure B.8: Relative abundance of T-RFs generated by HaeIII digestion of the amoA gene retrieved from the surface of the Control, Treatment 1 and Treatment 2 wetlands. The percentage which is not shown in graphs corresponds to the sum of peaks that fell below the threshold (1-3%).
Appendix C. Complementary results for Chapter 5

Table C.1. Results of the chemical analysis of water samples collected during the trial testing the feasibility of soft drink wastewater (SDW) as possible carbon source to improve nitrogen removal in constructed wetlands. All concentration values in mg/L.

<table>
<thead>
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<th>DATE</th>
<th>NO$_3^-$N</th>
<th>NH$_3$-N</th>
<th>TN</th>
<th>COD SDW</th>
<th>COD control</th>
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<td>EFF</td>
<td>INF</td>
<td>EFF</td>
<td>INF</td>
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<td>Control</td>
<td>SDW</td>
<td>Control</td>
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<td>26/03/2008</td>
<td>67</td>
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<td>22</td>
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<td>47.70</td>
<td>40.00</td>
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<td>Removal %</td>
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<td>65.4</td>
<td>99.8</td>
<td>99.6</td>
<td>46.7</td>
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Removal %
Figure C.1: Daily air temperature variation during the SDW storage experiment. Data obtained from the Murdoch University Meteorological Station.