Study of Bauxsol\textsuperscript{TM} pellets' efficiency in wastewater treatment and their biocompatibility with microbial communities

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Study of Bauxsol™ pellets' efficiency in wastewater treatment and their biocompatibility with microbial communities

Thesis submitted by

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A thesis presented in fulfilment of the requirements for the degree of Doctor of Philosophy in the School of Environment, Science and Engineering at Southern Cross University

2013
I certify that the work presented in this thesis is, to the best of my knowledge and belief, original, except as acknowledged in the text, and that the material has not been submitted, either in whole or in part, for a degree at this or any other university.

I acknowledge that I have read and understood the University's rules, requirements, procedures and policy relating to my higher degree research award and to my thesis. I certify that I have complied with the rules, requirements, procedures and policy of the University (as they may be from time to time).

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Date: 1st February 2013
Abstract

Municipal wastewater is treated by physical, biological and chemical processes to reduce its concentration of contaminants that may otherwise impact on public health and the environment. Biological and chemical processes are separated because chemicals used (e.g. alum for phosphate precipitation) may affect microbial activities, are costly and create hazardous waste. Bauxsol™ (seawater-neutralised red mud) could be used to overcome this issue as it has the ability to remove phosphate and trace-metals from contaminated waters. However, few studies have been undertaken in circum-neutral waters, such as wastewater, because pelletised Bauxsol™ produces a pH/alkalinity spike of the waters. Furthermore, no studies have investigated the biocompatibility of Bauxsol™ with microbial communities. Consequently, the aims of this doctorate thesis are to develop suitable Bauxsol™ pellets for circum-neutral water, to study their geochemical efficiency in wastewater treatment and their biocompatibility with environmental microbial communities.

Suitable cement-bound porous Bauxsol™ pellets were developed by testing different pre-production modified makeup waters (H₂O + CO₂ or NaHCO₃) and post-production curing baths (CO₂, NaHCO₃, or Mg/CaCl₂). The best result for reducing the alkalinity/pH spike was obtained from the Mg/CaCl₂ bath treatment (pH 8.05; alkalinity 35.4 mg/L).

Field-based experiments (microcosm columns over 1 month, mesocosm columns over 6 months, unplanted constructed wetland over 6 months) were conducted to determine the effectiveness of Bauxsol™ pellets to remove phosphate and trace-metals from wastewater and to assess the pellet’s biocompatibility. The results indicated that 10% of the phosphate was removed from the effluent in the microcosm columns (i.e. signs of saturation), 70% in the mesocosm columns and >95% in the constructed wetland. Moreover, Bauxsol™ pellets were loaded with up to 2 g/kg of Colwell P (available phosphate). Trace-metals were also effectively bound to the Bauxsol™ pellets. The observed short-lasting effluent’s pH spike was associated with the release of hydroxide from unreacted CaO in the cement binder (i.e. pellets production);
however, in the constructed wetland experiment the soil acted as a buffer delaying the appearance of the pH spike.

Using PCR/DDGE techniques the biocompatibility of Bauxsol™ pellets was confirmed. The results showed a distinct, well-adapted, and highly diverse microbial community attached to Bauxsol™ pellets. Functional PCR-amplification assays showed the presence of aerobic and anaerobic ammonia-oxidising bacteria and denitrifiers on Bauxsol™ pellets. Nitrogen removal (≤49% in mesocosm columns; <35% in constructed wetland) was most probably caused by geochemical binding and by biological activities (illustrated by isotopic N-assay).

In conclusion, this thesis has demonstrated the effectiveness of newly developed Bauxsol™ pellets in municipal wastewater treatment by combining biological and chemical processes.
Acknowledgements

I would like to sincerely thank my supervisors, Dr Malcolm Clark, Dr Tony Vancov and Dr Michel Aragno for their full support during my PhD. Their help in planning and designing this project, as well as executing the experiments and analysing the data was immensely valuable. I also thank them for their assistance in reviewing this thesis and preparing my journal publications. I also acknowledge the contribution of the late Dr David McConchie for planning and designing this project. In addition, I acknowledge Dr Dirk Erler for his work on nitrogen isotope assay.

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Finally I would like to thank my family and friends for their immense support throughout this journey.
List of publications

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Laure Despland

Date: 6th July 2012
Statement of contribution of others

I, Laure Despland, am the first author of each paper incorporated in this thesis. These papers were co-written with Dr Malcolm Clark, Dr Tony Vancov, and Dr Michel Aragno; Dr Dirk Erler also collaborated on the paper entitled “Nutrient and Trace-metal Removal by Bauxsol Pellets in Wastewater Treatment”. Consequently, I acknowledge the contribution of the co-authors in planning, executing and reviewing the experimental work, and in editing these papers.

Laure Despland

I, Dr Malcolm Clark as Principal Supervisor of Laure Despland at Southern Cross University, confirm that the statement of Laure Despland is true and correct. I can confirm that Laure executed the fieldwork, and laboratory work required to construct and complete the necessary experiments used to generate data for these papers. I also confirm that Laure took the leadership in data collection, data analysis, drafting the papers, and submitting these to the journals. I as principal supervisor assisted in the development of the intellectual ideas behind the experiments and experimental setup, provided direction towards data interpretation, and provided reviews and editorial comments on paper drafts. Hence, Laure has every claim that well in excess of 50% of the work conducted on any paper is by Laure’s hand alone.

Malcolm Clark

I, Dr Tony Vancov as Co-Supervisor of Laure Despland at Wollongbar Primary Industries Institute, confirm that the statement of Laure Despland is true and correct. I can confirm that Laure executed the fieldwork, and laboratory work required to construct and complete the necessary experiments used to generate data for these papers. I also confirm that Laure took the leadership in data collection, data analysis, drafting the papers, and submitting these to the journals. Laure undertook most of her work in my laboratory and as her supervisor was responsible for developing her research skill set including experimental design. Supervision extended to assisting in data interpretation and manuscript editing.

Tony Vancov
I, prof. Michel Aragno, as Co-Supervisor of Laure Despland at Neuchâtel University, confirm that the statement of Laure Despland is true and correct. I can confirm that Laure executed the fieldwork, and laboratory work required to construct and complete the necessary experiments used to generate data for these papers. I also confirm that Laure took the leadership in data collection, data analysis, drafting the papers, and submitting these to the journals. Laure undertook most of her work in Drs Clark and Vancov laboratories. As her co-supervisor, I participated for developing her research skill set including experimental design. Supervision extended to assisting in data interpretation and manuscript editing.

Michel Aragno

Date: 19th June 2012

I, Dr Dirk Erler as a co-author with Laure Despland confirm that the statement of Laure Despland is true and correct. I can confirm that Laure executed the fieldwork, and laboratory work required to construct and complete the necessary experiments used to generate data for these papers. I also confirm that Laure took the leadership in data collection, data analysis, drafting the papers, and submitting these to the journals. Laure undertook most of her work in my laboratory and as her supervisor was responsible for developing her research skill set including experimental design. Supervision extended to assisting in data interpretation and manuscript editing.

Dirk Erler

Date: 7th June 2012
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CHAPTER 1: INTRODUCTION

1.1. Overview

Waters impacted by anthropogenic activity (e.g. municipal wastewater) often require treatment to reduce contaminants concentrations before release because they may adversely impact public health and the environment. Conventionally, municipal wastewaters are treated in sewage treatment plants (STP) via a series of physical, biological and chemical processes (i.e. primary, secondary and tertiary treatment when carried out in sequence) (Liu and Liptak 2000; Hammer and Hammer Jr. 2005). An advanced treatment step (e.g. constructed wetlands) may be added at the end of conventional treatment to further improve water quality (Hammer 1991; Sundaravadivel and Vigneswaran 2001; Kadlec and Wallace 2009). Primarily, solids, oil and grease are removed by physical processes, while biological processes transform nitrogen (generally to nitrogen gas) and degrade many organic components (Bitton 1994; Liu and Liptak 2000; Hammer and Hammer Jr. 2005). The precipitation of phosphates and the destruction of disease-causing pathogenic microorganisms are completed using chemical treatments (e.g. alum dosing, ozonation) (Omoike and Vanloon 1999; Liu and Liptak 2000; Hammer and Hammer Jr. 2005). However, one of the challenges in wastewater treatment is that chemical and biological processes must be separated because many chemical reagents (e.g. alum for phosphate precipitation) may adversely impact on the microorganisms, including those involved in the nitrogen cycle, and the microbial activity (Madigan et al. 2012; Walker 2012). Moreover, chemical treatments are costly and create hazardous waste requiring disposal (Liu and Liptak 2000; Hammer and Hammer Jr. 2005). Consequently, there is a need to find a cost effective and environmentally friendly technology that combines chemical and biological processes during wastewater treatment.

Bauxsol™, seawater-neutralised red mud from the alumina refinery, is a complex mixture of minerals that are extremely fine grained particles, have a high surface to volume ratio, a moderate acid neutralising capacity (ANC), and high metal and phosphate binding capacities (e.g. McConchie et al. 1999; McConchie et al. 2000; Hanahan et al. 2004; Clark et al. 2008). Hence, because of these physico-chemical
characteristics, Bauxsol™ (slurry, powder or pellet forms) has been used in environmental remediation, especially for acidic contaminated soils and waters (McConchie et al. 2000; Genc-Fuhrman et al. 2004; Clark et al. 2006b; Lapointe et al. 2006; Clark et al. 2011). Although some studies have demonstrated the efficiency of Bauxsol™ to bind phosphate in circum-neutral waters (Hanahan et al. 2004; Akhurst et al. 2006; Clark et al. 2006a), powdered Bauxsol™ (the most commonly used form) is inefficient when used in a water filtration device due to a poor hydraulic conductivity (Clark et al. 2005). However, one way of overcoming this issue is to use porous pelletised forms of Bauxsol™ with a good hydraulic conductivity. Unfortunately, these pellets are produced with a cement binder that creates a significant pH/alkalinity spike in circum-neutral waters from the release of unreacted calcium oxide.

Given the above effects, very few studies on the geochemical binding capacity of Bauxsol™ pellets have been undertaken, especially for circum-neutral municipal wastewaters treatment (Clark et al. 2006a; Clark et al. 2008). Consequently, the development of some form of cured cement-bound porous Bauxsol™ pellets that minimises the intensity and/or duration of pH/alkalinity spike is required. In addition, no studies have been completed on the biocompatibility and interaction of Bauxsol™ with microbial communities, particularly those found in municipal wastewaters. Hence, determining the likely effects of these interactions is important to further expand the use and application of Bauxsol™ technology. Cured cement-bound porous Bauxsol™ pellets may provide a basis for new studies in wastewater treatment and a full investigation of their effect on microbial communities is warranted.

Consequently, the hypotheses of this thesis are that cured cement-bound porous Bauxsol™ pellets can be used in wastewaters treatment with a minimised pH/alkalinity spike, a high phosphate and metal binding capacity and an enhanced hydraulic conductivity. Moreover, such pellets can act as a biomass support particle (BSP) for the wastewater microbial community, enhancing microbial growth including microorganisms linked to nitrogen cycle. In producing and using these pellets as a wastewater treatment device, a sustainability loop can be closed: turning a
waste (red mud) into a product (cured porous Bauxsol™ pellets) to treat a waste (sewage) to produce clean water.

### 1.2. Aims & Objectives

The overall aim of this PhD thesis is to develop a cured cement-bound porous Bauxsol™ pellets, and to study their geochemical efficiency in wastewater treatment and their biocompatibility with wastewater microbial communities. Firstly, a suitable Bauxsol™ pellet requires development such that it may be used as a filter media for circum-neutral waters, without any substantial pH/alkalinity spike that may otherwise adversely affect microbiological populations and physico-chemical parameters of waters. Secondly, using field-based experiments (conducted at the South Lismore Sewage Treatment Plant, Australia), the ability of the Bauxsol™ pellets to remove phosphate and trace-metals from secondary treated effluent (pre-chemical dosing) has to be determined. Thirdly, the diversity and structure of bacterial communities attached to the Bauxsol™ pellets and suspended in the effluent, and the presence of microbial communities associated with nitrogen cycle have to be investigated.

To achieve the aim, a number of objectives can be identified. The first objective is to develop a general recipe to make Ordinary Portland Cement (OPC)-bound porous Bauxsol™ pellets that minimise pH/alkalinity spikes and improve hydraulic conductivity without affecting the high phosphate and metal binding capacity of pellets. To achieve this objective, experiments were performed to convert soluble alkalinity to insoluble forms by investigating, using different Bauxsol™ to cement ratios, pre-production modified makeup waters (H₂O + CO₂ or NaHCO₃) and post-production curing baths (CO₂, NaHCO₃, or Mg/CaCl₂).

The second objective is to field-test (microcosm experiment) at a sewage treatment plant these newly developed pellets to investigate their phosphate removal capacity and biocompatibility with environmental bacterial communities (via molecular techniques). The origin of the short-lasting pH spike observed in a laboratory experiment when using Bauxsol™ pellets in pH circum-neutral waters requires

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¹ Trace-metals removal and the investigation of the binding environments of Bauxsol™ pellets were not the focus of this thesis.
investigation. The first field-based experiment consisted of short PVC columns packed with cured OPC-bound porous Bauxsol™ pellets, sand pellets (cement control) or sand (overall control), subjected to an upward direction flow of secondary treated effluent for one month.

The third objective is to scale up the previous column field experiment to mesocosm size, where larger columns of cured OPC-bound porous Bauxsol™ pellets, or gravel (control) were upwardly fed with secondary treated effluent for a six-month trial period. This field experiment study determined the efficiency of Bauxsol™ pellets to remove nutrients and trace-metals from wastewater, and to act as a biomass support particle for microbial communities. Emphasis is placed on:

a) monitoring the short-lasting pH spike caused by the cement used during the Bauxsol™ pellets production;
b) quantifying total phosphorus and phosphate loadings removed from the effluent and bound to pellets;
c) measuring trace-metals loading trapped by the pellets;
d) determining the nitrogen removal processes occurring in the system;
e) investigating the structure and diversity of bacterial communities in liquid and solid phases using molecular techniques (PCR/DGGE); and
f) studying microbial communities associated with nitrogen cycling using PCR amplification of functional genes.

The fourth and final objective is to use Bauxsol™ pellets in an unplanted constructed wetland with a lower soil layer for the treatment of wastewater over a six-month period. Interactions between the Bauxsol™ pellets, soil, the secondary treated effluent and the existing microbial communities were examined. Temporal and spatial removal of phosphate, nitrogen, and trace-metals were also studied. Moreover, the structural differences of both attached and free-floating bacterial communities and the presence of microbial communities involved in the nitrogen cycle were investigated.
1.3. Thesis Layout

This PhD thesis incorporates refereed publications in accordance with the Southern Cross University policy. The first three experiments (objectives 1 to 3) have been subjected to peer-reviewed publication in international journals, or as fully refereed international conferences proceedings. Chapters 3 to 6 present these publications as they were published and a brief overview of the context is placed before each of these chapters to guide the reader.

Chapter 2 provides a comprehensive literature review and context of the project in relation to wastewater management, red mud, and the Bauxsol™ technology. The first part of the chapter focus on municipal wastewaters: history, composition, physical process treatments, biological process treatments (including the nitrogen and sulphur cycles), chemical process treatments (including the phosphorus cycle and pathogen removal), constructed wetland systems (including the use of solid materials as physico-chemical process treatment and biomass support particles), and the Australian guidelines for wastewater treatment. The second part of this chapter reviews the literature surrounding red mud (including red mud production, history, mineralogy, geochemical properties, modifications and applications) and the Bauxsol™ technology (including Bauxsol™ production, mineralogy, geochemical properties, and use as an environmental remediation agent).

Chapter 3 presents the developmental findings for the production of the cured cement-bound porous Bauxsol™ pellets (i.e. 1st objective). The article Minimising Alkalinity and pH Spikes from Portland Cement-Bound Bauxsol (Seawater-Neutralized Red Mud) Pellets for pH Circum-Neutral Waters has been published in Environmental Science and Technology journal (es9032138; 2010, 44 (6): 2119-2125).

Chapter 4 presents the results of the field experiment testing the microcosm columns (i.e. 2nd objective). The article Bauxsol™ Pellets: Bacterial Communities and Phosphate Removal in Wastewater Treatment has been published in the proceedings of the IWA World Water Congress & Exhibition, Montreal, 19–24 September 2010.
Chapter 5 presents the geochemical data of the field experiment testing the mesocosm columns (i.e. 3rd objective). The article *Nutrient and Trace-Metal Removal by Bauxsol Pellets in Wastewater Treatment* has been published in Environmental Science and Technology journal (es200934y; 2011, 45 (13): 5746-5753).

Chapter 6 presents the microbiology results of the field experiment testing the mesocosm columns (i.e. 3rd objective). The article *Diversity of Microbial Communities in an Attached-Growth System using Bauxsol™ Pellets for Wastewater Treatment* has been published in Science of the Total Environment journal (10.1016/j.scitotenv.2012.06.079; 433: 383-389).

Chapter 7 presents the findings of the field experiment performed on the constructed wetland canal (i.e. 4th objective). This chapter provides detailed material, result and discussion sections, as well as a conclusion.

Chapter 8 provides a general conclusion of the PhD thesis, drawing together the findings of chapters 3, 4, 5, 6 & 7. A summary briefly recalls the knowledge gaps found in the wastewater treatment and Bauxsol™ technology literature reviews, and present the major findings in the context of these gaps. The implications of these findings, and the further research associated with them (e.g. planted constructed wetland, decontamination of other wastewaters) are also discussed and a final conclusion closes this chapter.

Chapter 9 provides the appendices with a section on quality assurance/quality control procedures and the supplementary data collected from the different experiments, not otherwise presented in the attending papers. This chapter also provides the experimental details and data for the microbiology optimisation.

Chapter 10 provides a complete references list for the PhD thesis, pooling together all references used.
1.4. References


McConchie, D., Clark, M., Hanahan, C. and Fawkes, R. (1999). The use of seawater-neutralised bauxite refinery residues (red mud) in environmental remediation...


CHAPTER 2: LITERATURE REVIEW

2.1. Municipal wastewater management

2.1.1. History

When humankind established the first permanent settlement some 10,000 years ago, ecological and health impacts of generated wastes became an issue. Historical records show that the Mesopotamian Empire (3500–2500 BC) was the first to address sanitation issue with latrine drainage systems to remove wastes to cesspits (Cooper 2007; Lofrano and Brown 2010). Similarly, the Indus Valley Civilisation, known as the Harappan Civilisation (3300–1300 BC), showed remarkable wastewater management with an extensive knowledge and use of urban planning, and a strong emphasis on hygiene. By 2500 BC, Harappans developed what is believed to be the first urban sanitation system, where houses were mandatorily connected to drainage channels with a wastewater treatment (i.e. solids settlement) (Cooper 2007; Lofrano and Brown 2010). It appears that many modern technologies for water management were designed and developed by the Ancient Greeks (300 BC–AD 500), with private and public latrines connected to a sewer system that conveyed wastewaters to a collection basin outside the city. The wastewater was subsequently used to irrigate crops (Angelakis et al. 2005; Cooper 2007; Lofrano and Brown 2010). Yet another ancient civilisation excelling in water management was The Roman Empire (509 BC–AD 476). Not only did the Romans improve on older-style sewer and water pipe systems, they invented the first fully integrated water service. Water was collected and supplied via aqueducts and channels systems, and wastewater was then disposed of via complex storm and sewage system networks. To further improve hygienic conditions latrines, baths and water fountains were available to the public, even to the poorest citizens (Gibbon and Williams 1979; Cooper 2007; Lofrano and Brown 2010).

The sanitary “Dark Ages” began at the collapse of the Roman Empire (AD 476) and lasted through the Middle Ages, Renaissance and up to the 1800’s. During this time populations in major cities grew rapidly and the waste management was highly inadequate with untreated wastewater being discharged into rivers from which...
drinking water was being drawn. Moreover, washing in water was seen as bad for the body and soul considerably reducing overall hygiene (Hoy 1995; Cooper 2007; Lofrano and Brown 2010). This regression in sanitation and hygiene resulted in the spread of many diseases (Hoy 1995; Ziegler 2003; Byrne 2006; Cooper 2007; Sherman 2007; Lofrano and Brown 2010). For example, the plague pandemic from 1348–1350 (i.e. “The Black Death”) killed an estimated 30% to 60% of Europe’s population, and approximately 100 million people worldwide. The proliferation of rats and fleas carrying the pathogen responsible for the plague (*Yersinia pestis*) was facilitated by the lack of hygiene, and poor waste and wastewater management (Ziegler 2003; Byrne 2006; Cooper 2007; Sherman 2007; Madigan et al. 2012). Similarly, the London cholera outbreaks of 1832 and 1848–1849, which killed some 23,000 and 53,000 people respectively, were linked to sewage contaminating drinking water (Swanson 2001; Cooper 2007; Sherman 2007).

However, towards the end of the 19th century came an age of sanitary enlightenment, where an improved understanding of the environment drew attention to the importance of wastes and wastewater disposal. Septic tanks, filtration and trickling filters treatment occurred between 1860 and 1895. Moreover, the concept of a biochemical oxygen demand (BOD; uptake rate of dissolved oxygen by the biologically active microorganisms) was introduced in 1912 following the 8th report of the Royal Commission on Sewage Disposal (Royal Commission on Sewage Disposal 1912). The activated sludge process (i.e. suspended-growth system) was patented in 1913 and was used as a biological treatment for wastewater, oxidising organic matter to carbon dioxide, water, ammonia, and new cell biomass.

Chlorine was first used in 1854 to deodorize London’s wastewater, but by 1880, scientists began to understand the effects of chlorine on disease-causing pathogenic microorganisms and began using it to disinfect wastewater. Later the effects of ozone (1906) and ultraviolet light (1916) on these microorganisms was understood and these methods were also used to disinfect wastewater (Bitton 1994; Hammer and Hammer Jr. 2005; Lofrano and Brown 2010). Furthermore, water quality standards were established in the early 1900’s, but both World Wars and other political ideologies greatly impeded the implementation of wastewater treatment until 1950 (Lofrano and Brown 2010). However, between 1950 and 1960 constructed wetland systems and
rotating biological reactors systems were introduced as biological treatments for wastewater. Similarly, full scale sequencing batch reactors and membrane biological reactors were developed during the 1970’s and 1980’s and phosphorus removal was broadly implemented from 1980 (Bitton 1994; Hammer and Hammer Jr. 2005; Lofrano and Brown 2010). Further developments saw the membrane bioreactors (MBR) introduction in the 1990’s particularly in Europe; MBR is a combination of activated sludge bioreactor and a cross-flow membrane filtration (Cooper 2007; Lofrano and Brown 2010).

Despite many advances and technologies, currently in developing countries some 884 million people have limited access to safe drinking water and some 2.6 billion people live with poor sanitation (WHO and UNICEF 2008). Poor water sanitation is responsible for millions of deaths every year, where for example dysentery, the most deadly diseases caused by waterborne pathogens, kills an estimated 1.5 millions people per year (mostly children) (Rosemarin et al. 2008; WHO 2012). Consequently, to tackle these issues, the World Health Organisation has set a goal of 75% sanitation coverage worldwide by 2015 (WHO 2012).

2.1.2. Composition & issues

Wastewater is defined as any water that has been adversely affected in quality by anthropogenic activities from residential (e.g. houses, flats) and non-residential sources (e.g. offices, stores, restaurants, schools, hospitals, industries, farms). In industrial countries an average of 450 L of wastewater is generated per person per day (Bitton 1994; Liu and Liptak 2000; Hammer and Hammer Jr. 2005). Municipal wastewater, also referred as sewage, is contaminated with feces and urine where a single person can excrete between 100 and 500 g wet weight of feces and 1 to 1.3 L of urine per day (Bitton 1994). Commonly, municipal wastewater consists of >95% water by mass, but varies widely depending on the source (Bitton 1994; Liu and Liptak 2000; Hammer and Hammer Jr. 2005). Typically, municipal wastewater contains suspended solids and macro-solids, organic matter, inorganic matter (e.g. minerals, trace-metals, metalloids, oil and grease), nutrients (i.e. nitrogen and phosphorus), pathogenic and non-pathogenic bacteria, microorganisms and small

Untreated wastewater containing high concentrations of contaminants is harmful to human health and the environment, and it is desirable to treat this before release into the environment (Bitton 1994; Liu and Liptak 2000; Hammer and Hammer Jr. 2005). Natural and xenobiotic chemicals (e.g. trace-metals, pesticides) can cause major human health issues such as birth defects, cancers, neurological disorders, severe poisoning and even death (Liu and Liptak 2000; Alavanja et al. 2004; Wang et al. 2005b; Pepper et al. 2006; Khan et al. 2008; Walker 2012; WHO 2012). In addition, high nitrate and phosphate concentrations can lead to the eutrophication of aquatic environments such as lakes, rivers, and coastal ecosystems. During eutrophication, water quality can show very high concentrations of nutrients (often 350–1,500 mg/m$^3$ nitrogen and of 30–100 mg/m$^3$ of phosphorus; Smith et al. 1999), that stimulate the growth of algae and aquatic plants. This in turn induces a negative feedback system by depleting dissolved oxygen from the increased quantities of dead and decaying organic matter. As a result, aquatic life is strongly affected, potentially leaving a severely limited and perturbed ecosystem (Vitousek et al. 1997; Correll 1998; Smith et al. 1999; Howarth and Marino 2006; Schindler 2006; Brady and Weil 2008; Smith and Schindler 2009).

Consequently, municipal wastewater is treated to minimise its impact on public health and the environment. Conventionally, wastewater is directed to a sewage treatment plants (STP), where it goes through a series of separated or combined physical, biological and chemical processes (i.e. primary, secondary and tertiary treatments). Advanced treatment (e.g. constructed wetland) may be added as a polishing step to lower biological oxygen demand, suspended solids, phosphate, nitrogen, and pathogens concentrations to acceptable water quality standards (Liu and Liptak 2000; Hammer and Hammer Jr. 2005).

2.1.3. Physical process

During the physical processes (i.e. primary treatment phase) raw wastewater passes through a series of screen of bars, grit chambers, clarifiers and sedimentation tanks to
remove floating and settleable solids, including oil and grease. This gross primary separation process produces wastes in the form of macro-solids, organic-rich sludge, and scum (Liu and Liptak 2000; Hammer and Hammer Jr. 2005). These wastes are then sent for further treatment often including dewatering, by filtration, and anaerobic/aerobic digestion or composting (Bitton 1994).

2.1.4. Biological process

Biological processes (i.e. secondary and advanced treatment phases) use the inherent microbial activities (aerobic or anaerobic) in the wastewater to biologically degrade organic contaminants and transform ammonium ($\text{NH}_4^+$) to nitrite ($\text{NO}_2^-$) to nitrate ($\text{NO}_3^-$) and finally to the less toxic form nitrogen gas ($\text{N}_2$) (see section 2.1.4.2 for further details). Conventionally, bacteria are either attached to a support (i.e. attached-growth system: trickling filter, membrane bioreactor, rotating biological contactor, up/down-flow submerged filter, or constructed wetland), or suspended in the liquid phase (i.e. suspended-growth system: activated sludge, or surface-aerated basin) (Bitton 1994; Liu and Liptak 2000; Hammer and Hammer Jr. 2005).

2.1.4.1. Study of microbial communities

Studies of microbial communities are performed by both culture-dependent and culture-independent methods. Culture-dependent methods, although simple, cheap and sometimes useful, generally fail to provide sufficient microbial ecology details (i.e. composition, diversity, structure, and functions of the community). This failure is primarily because for any environment some 90–99% of the microorganisms present remain un-cultured under laboratory conditions (Ward et al. 1990; von Wintzingerode et al. 1997). Consequently, culture-independent methods have become a more reliable and powerful means to overcome this obstacle, particularly for microbial ecology studies. These DNA-based methods (i.e. molecular techniques) typically rely on polymerase chain reaction (PCR) amplification of genetic markers using different primer sets for ribosomal operon genes, or functional genes (von Wintzingerode et al. 1997; Madigan et al. 2012). For example, important functions (e.g. nitrogen transformation) may be determined and monitored by PCR assays of specific marker genes (Muyzer and Smalla 1998). Quantitative polymerase chain reaction (qPCR) is
used to amplify and simultaneously quantify the number of copies of specific genes or DNA targets present in a sample. Contrary to the classic PCR, qPCR quantification of targeted genes can provide information on the abundance of specific microorganisms (Madigan et al. 2012). This technique is often used to monitor bioremediation processes on contaminated sites.

After PCR amplification, DNA fragments can be separated using denaturing gradient gel electrophoresis (DGGE) technique according to their melting behaviour that is determined by the unique nucleotide sequence of the fragments. This DNA-based fingerprinting technique provides a unique bands pattern, or profile, which is considered representative of the bacterial community. However, an individual discrete band within the gel does not represent single species or even a single bacterial population, because amplified DNA fragments from related and non-related bacteria may co-migrate to a similar position on the gel (Muyzer et al. 1993; Muyzer et al. 1995; Muyzer and Smalla 1998; Fromin et al. 2002; Yu and Morrison 2004; Madigan et al. 2012). Specific imaging software (e.g. Carestream Molecular Imaging software version 5.0) locates the mobility and quantifies the intensity of each band making it possible to compare and describe microbial community structure, diversity and dynamics from a given ecosystem. Statistical derivatives such as Shannon diversity and equitability indices, dendrogram relationships, non-metric multi-dimensional scaling, and one-way analysis of similarity can then be determined to compare and contrast between communities (e.g. ePRIMER software version 6, Clarke and Gorley 2006).

Information about the phylogenetic affiliation of the microbial community members can be obtained by sequencing a DNA fragment isolated by PCR or DGGE (Muyzer et al. 1995; Muyzer and Smalla 1998; Madigan et al. 2012). The resulting sequences are aligned with and compared to known sequences stored in a nucleotide database. The quality of the results obtained by comparing 16S rDNA sequence can be relatively low as sequences from environmental samples often represent uncultured or novel microorganisms, and only partial and/or ambiguous sequences exist in the database (Madigan et al. 2012). Despite this, a phylogenetic tree can be built with the sequences retrieved to depict the relationship among community members.
Consequently, by employing PCR/DGGE method in wastewater treatment studies, significant changes in microbial community structure, composition, diversity and function may be observed, compared, and contrasted. The changes may be caused by variability of environmental parameters such as the use of different substrate materials (e.g. clay, gravel, zeolite, steel slag, and ceramic) and specific treatment systems (e.g. activated sludge, membrane bioreactor) (Boon et al. 2002; Stamper et al. 2003; Gomez-Villalba et al. 2006; Ivnitsky et al. 2007; Xiao et al. 2009; Wan et al. 2011; see studies for constructed wetlands in section 2.1.6.2).

2.1.4.2. Nitrogen cycle

Microorganisms associated with the global nitrogen cycle transform nitrogen between ammonium (NH$_4^+$), nitrite (NO$_2^-$), nitrate (NO$_3^-$), nitrogen gas (N$_2$), and organic nitrogen (Org. N) via different routes: fixation, assimilation, ammonification, nitrification, denitrification, and anammox (Figure 2.1). Because high concentrations of ammonia and nitrate are found in sewage, nitrification, denitrification and anammox are identified as the three main transformations occurring during wastewater treatment (Butcher 1992; Bitton 1994; Paul and Clark 1995; Schmitz 1996; O'Neill 1998; Liu and Liptak 2000; Brady and Weil 2008). Stable isotopes can be used to study various cell’s metabolic pathway such as microbial nitrogen transformation (Nielsen 1992; Madigan et al. 2012). Solids supporting a microbial community can be incubated in vials containing water mixed with $^{15}$NO$_3^-$, $^{15}$NH$_4^+$, or a combination of $^{15}$NH$_4^+$ + $^{14}$NO$_3^-$, and after a period of incubation $^{29}$N$_2$ and $^{30}$N$_2$ gas produced can be analysed by GC-IRMS and isotope ratio mass spectrometer. Production of $^{29}$N$_2$ where only $^{15}$NH$_4^+$ is added is indicative of nitrification, whereas production of $^{29}$N$_2$ where $^{15}$NH$_4^+$ + $^{14}$NO$_3^-$ is added indicates anammox process, and production of $^{30}$N$_2$ where $^{15}$NO$_3^-$ is added is a sign of denitrification (Nielsen 1992).
Nitrification is an aerobic two-step process involving Bacteria affiliated to the beta and gamma subdivisions of the Proteobacteria, and Archaea affiliated with the recently described phylum Thaumarchaeota (Brochier-Armanet et al. 2008; Madigan et al. 2012). Nitritation is the first step of nitrification process, which requires the presence and activity of two key enzymes, ammonium monooxygenase (AMO) and hydroxylamine-oxydase (HAO) to oxidise the ammonia into nitrite (NH$_3$ + 1.5O$_2$ → NO$_2^-$ + H$_2$O + H$^+$). Nitroso-bacteria (e.g. Nitrosomonas sp., Nitrosospira sp.) carry the functional gene amoA encoding for the catalytic $\alpha$-subunit of the AMO enzyme (Bitton 1994; Rotthauwe et al. 1997; Purkhold et al. 2000; Nicolaisen and Ramsing 2002; Madigan et al. 2012). Similarly, Archaea have been reported to carry the archaea homolog amoA gene encoding for the catalytic $\alpha$-subunit of the AMO enzyme and in some environments Archaea are extensively involved in the nitritation process (Schleper et al. 2005; Treusch et al. 2005; Hallam et al. 2006; Erguder et al. 2009).
Nitratation is the second step of the nitrification process, and is performed by *Nitrobacteria* (e.g. *Nitrobacter* sp., *Nitrospira* sp.) that possess the nitrite-oxidase (NO) enzyme to oxidise nitrite into nitrate ($\text{NO}_2^- + 0.5\text{O}_2 \rightarrow \text{NO}_3^-$) (Bitton 1994; Madigan et al. 2012). Factors controlling nitrification are pH (optimum 7.2–8.4), ammonia/nitrite concentration (i.e. *Nitrobacter* grows faster than *Nitrosomonas*), dissolved oxygen concentration (4.6 mg of O$_2$ to oxidise 1 mg of NH$_3$), temperature (optimum 15–35°C), the BOD/total Kjeldahl ratio (ratio <3), and the presence of possible inhibitors (e.g. trace-metals <5 mg/L; cyanide <20 mg/L; phenol <20 mg/L) (Bitton 1994; Madigan et al. 2012).

Ammonia-oxidising bacteria (AOB) have been identified using molecular techniques in a diverse range of environments such as in soils (Rotthauwe et al. 1997; Horz et al. 2000; Nicolaisen and Ramsing 2002; Shen et al. 2008; Tourna et al. 2008; Glaser et al. 2010; Yamamoto et al. 2010), liquid and solid phases of both fresh and saline waters (Rotthauwe et al. 1997; Horz et al. 2000; Nicolaisen and Ramsing 2002; Freitag and Prosser 2003; Cebron et al. 2004; Weidler et al. 2007; Sahan and Muyzer 2008; Santoro et al. 2008), and in wastewaters (Rotthauwe et al. 1997; Horz et al. 2000; Purkhold et al. 2000; Nicolaisen and Ramsing 2002; Park et al. 2002; Kim et al. 2011). The community diversity, composition and structure of AOB have been studied in a wide variety of wastewater treatment processes including activated sludge, trickling filters, membrane reactors, and constructed wetlands (Ottova et al. 1997; Gomez-Villalba et al. 2006; Siripong and Rittmann 2007; Xiao et al. 2009; Domingos et al. 2011; Kim et al. 2011; Wan et al. 2011). In addition, the effects of different substrate materials, system designs and spatial locations on AOB communities have also been examined (Ibekwe et al. 2003; Rowan et al. 2003; Park and Noguera 2004; Truu et al. 2005; Gorra et al. 2007). These studies show that the physico-chemical conditions (e.g. nutrient, organic matter and oxygen concentrations) select for different populations of AOB.

Ammonia-oxidising archaea (AOA) are believed to be as equally abundant as AOB in the environment. Consequently, AOA have been studied and found in many soil types (Leininger et al. 2006; Shen et al. 2008; Tourna et al. 2008; Glaser et al. 2010; Yamamoto et al. 2010), and in liquid and solid phases of both fresh and saline waters (Francis et al. 2005; Konneke et al. 2005; Wuchter et al. 2006; Weidler et al. 2007;
Herrmann et al. 2008; Sahan and Muyzer 2008; Santoro et al. 2008; Herrmann et al. 2009). In wastewaters, AOA populations were found to be highly diverse and closely related to AOA found in soils and sediments (Truu et al. 2005; Park et al. 2006).

2.1.4.2.2. Denitrification

Denitrification proceeds via a combination of several intermediate forms and reduces nitrate to nitrogen gas (NO$_3^-$ → NO$_2^-$ → NO → N$_2$O → N$_2$). This reduction requires key enzymes nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR) and nitrous oxide reductase (NOS) (Bitton 1994; Zumft 1997; Braker et al. 1998; Hallin and Lindgren 1999; Scala and Kerkhof 1999; Braker et al. 2000; Braker and Tiedje 2003; Throback et al. 2004; Wallenstein et al. 2006; Madigan et al. 2012). Denitrification is initiated by more than 50 different microbial genera (strict or facultative anaerobic bacteria) belonging to the various classes of the Proteobacteria (e.g. Pseudomonas sp., Paracoccus denitrificans, Thiobacillus denitrificans), which carry functional genes such as nirS, nirK, norB, or nosZ. Denitrification must be conducted in the absence of oxygen as O$_2$ competes with NO$_3^-$ as the final electron acceptor in respiration. Denitrification is also controlled by nitrate concentration (follows Monod-type kinetics), the presence of organic matter as electron donor (e.g. acetic acid, citric acid, and methanol), pH (optimum 7.0–8.5), and temperature (optimum >35°C) (Bitton 1994; Zumft 1997; Madigan et al. 2012).

Denitrifying bacteria have been identified in many environments (soils, freshwaters and sediments, marine waters and sediments) by targeting the different genes involved in the denitrification process (e.g. Braker et al. 1998; Scala and Kerkhof 1999; Braker et al. 2000; Throback et al. 2004). In wastewater treatment, the diversity, composition and structure of the denitrifying community have been studied in different systems including activated sludge, membrane bioreactor, batch biofilm reactor and submerged filter. Distinct populations of denitrifiers were found, indicating contrasting environmental and operational conditions (Gomez-Villalba et al. 2006; Xiao et al. 2009; Wan et al. 2011). Moreover, Kjellin et al. (2007), Dong & Reddy (2010), and Song et al. (2011) found that denitrifiers’ distribution in constructed wetlands strongly correlated with nitrogen concentration where the key population Pseudomonas sp. dominated.
In addition, in some environments simultaneous nitrification and denitrification, which are diametrically opposed (i.e. aerobic versus anaerobic process) have been reported by Robertson et al. (1988), Pochana & Keller (1999), Pochana et al. (1999), Holman & Wareham (2005), Sundberg et al. (2007) and Hocaoglu et al. (2011). These simultaneous joint processes are only found where anoxic micro-zones occur within media particles, thereby allowing the growth of denitrifiers, and oxic micro-zones prevail on the media surfaces, where nitrifiers may grow (Pochana and Keller 1999; Pochana et al. 1999; Gobat et al. 2004; Madigan et al. 2012). Consequently, these micro-environments (niches) are determined by their specific physico-chemical conditions and may support various physiological types of microorganisms. Moreover, microbial populations may change rapidly as diffusion of oxygen and nutrients in and around the media particles fluctuates (Gobat et al. 2004; Madigan et al. 2012).

2.1.4.2.3. Anammox

The anaerobic ammonium oxidation (anammox) process \((\text{NH}_4^+ + \text{NO}_2^- \rightarrow \text{N}_2 + 2\text{H}_2\text{O})\) is performed by a group of anaerobic, chemo-autotrophic bacteria belonging to a monophyletic branch within the phylum Planctomycetales. These include, to date, only five genera classified as Candidatus (Brocadia, Anammoxoglobus, Kuenenia, Scalindua, and Jettenia) (Mulder et al. 1995; van de Graaf et al. 1995; Strous et al. 1999a; Schmidt et al. 2003). Several enzymes including hydroxylamine-oxidase (HAO) and hydrazine oxidoreductase (HZO) are active during the anammox process (Jetten et al. 2001; Kuenen 2008; Schmid et al. 2008); however, the whole mechanism is yet to be described. To date anammox bacteria have not been isolated in pure cultures and consequently DNA-based methods targeting the 16S rRNA genes of Planctomycetes or the functional genes hzo are widely used (Quan et al. 2008; Schmid et al. 2008; Humbert et al. 2010; Li et al. 2010a; Li and Gu 2011).

The anammox process depends on optimum physiological parameters such as pH (6.7–8.3), nitrite concentration (inhibited if >0.1g/L of NO\(_2^-\) ), temperature (typically between 20–43°C), and/or the presence of inhibitors (e.g. acetylene, methanol) (van de Graaf et al. 1995; Strous et al. 1997; Strous et al. 1999b; Strous et al. 2002). In addition, anammox organisms are extremely slow growing (0.003/h growth rate; 10.6
days doubling time; Jetten et al. 2001), which means that observable anammox in wastewater systems may take many weeks to establish. However, the anammox process may be a critical process to establish and improve wastewater treatments, because it allows nitrogen removal from anoxic environments without requiring a reducing capacity (Kartal et al. 2010).

Anammox organisms have been found in many environments such as soils (Strous et al. 1999a; Humbert et al. 2010), liquid and solid phases of both fresh and saline waters (Thamdrup and Dalsgaard 2002; Trimmer et al. 2003; Risgaard-Petersen et al. 2004; Engstrom et al. 2005; Jensen et al. 2007; Li et al. 2010a; Hong et al. 2011; Li et al. 2011), and wastewaters (Mulder et al. 1995; van de Graaf et al. 1995; Strous et al. 1997; Jetten et al. 2001; van Dongen et al. 2001; Fux et al. 2002; Strous et al. 2002; Innerebner et al. 2007; Quan et al. 2008; Schmid et al. 2008; Li et al. 2009; Kartal et al. 2010). Two branches of the phylum Planctomycetales, Candidatus Brocardia anammoxidans and Candidatus Kuenenia stuttgartiensis, dominate wastewater treatment systems (Jetten et al. 2001; Innerebner et al. 2007). However, several unidentified sequences belonging to Planctomycetales have also been reported (Quan et al. 2008; Li et al. 2009), hence more studies on anammox in wastewater environments are required to fully assess the extent that these bacteria have.

2.1.4.3. Sulphur cycle

Sulphur is an essential element found in the composition of certain amino acids (cystine and methionine), co-factors (thiamine, biotin, and co-enzyme A), ferredoxins, and some enzymes (as –SH groups). Sulphur, depending on the environmental conditions (i.e. oxic or anoxic) may serve as an oxidising or reducing agent. Igneous rocks (e.g. pyrite, FeS$_2$) are the main pool of sulphur in terrestrial environment, while sulphates (SO$_4^{2-}$) are mainly found in ocean (Butcher 1992; Bitton 1994; Paul and Clark 1995; Liu and Liptak 2000).

In wastewater sulphur is mostly present as organic sulphur, found in feces and other organic matter, and as sulphate, found in urine (Bitton 1994; Liu and Liptak 2000). Organic sulphur is mineralised to hydrogen sulphide (H$_2$S) under anaerobic conditions; this step is also known as desulphurisation. Hydrogen sulphide, toxic to
plants and animals, may be assimilated by some anaerobic microorganisms, or oxidised to elemental sulphur ($S^0$) under aerobic conditions by *Thiobacillus thioparus* and under anaerobic conditions by photoautotrophs (e.g. *Chromatiaceae* – purple sulphur bacteria, *Chlorobiaceae* – green sulphur bacteria) and a chemoautotroph, *Thiobacillus denitrificans*. From there, $S^0$ may be assimilated within the cells of *Chromatiaceae* or outside the cells of *Chlorobiaceae*, or being further oxidised to $SO_4^{2-}$ by aerobic chemolithoautotroph sulphur-oxidising bacteria. Sulphur may also be oxidised to sulphate by heterotrophs (e.g. *Arthrobacter*, *Micrococcus*, *Bacillus*, *Pseudomonas*) in neutral and alkaline soils. Sulphate can be assimilated by aerobic microorganisms or reduced back to $H_2S$ by strict anaerobes sulphate-reducing bacteria (e.g. *Desulfovibrio*, *Desulflotomaculum*, *Desulfbacter*, *Thermodesulfobacterium*). This reduction constitutes the most important source of $H_2S$ in wastewater (Bitton 1994; Gobat et al. 2004; Madigan et al. 2012).

Dissolved hydrogen sulphide (correlates to its initial concentration, the water temperature, and the water pH) represents a major issue for STP as it is a colourless toxic gas with a rotten-egg odour. Moreover, sulphuric acid ($H_2SO_4$), formed by oxidation of $H_2S$ by sulphide-oxidising bacteria, is a major corrosive agent in distribution pipes. Consequently, ozone is often used as an oxidising agent to eliminate $H_2S$ (Butcher 1992; Bitton 1994; Liu and Liptak 2000; Hammer and Hammer Jr. 2005).

### 2.1.5. Chemical process

Chemical processes (i.e. tertiary treatment phase) commonly involve the use of metal salts to precipitate phosphate, and chlorine, ozone or ultraviolet light is used to kill potentially disease-causing pathogenic microorganisms. In addition, ozone and chlorine dioxide (hypochlorite) are utilized to control taste and odour problems, particularly in drinking water. Although widely use and very efficient, chemicals processes are costly, potentially create hazardous wastes that require disposal and, if not closely monitored, may adversely affect desirable microbial activities (Liu and Liptak 2000; Hammer and Hammer Jr. 2005).
2.1.5.1. Phosphorus cycle

Phosphorus, an essential macronutrient, is present in aquatic and terrestrial systems but is not found in significant quantities in the atmosphere. Phosphorus usually combines with other elements to form natural inorganic phosphorus compounds (e.g. apatites, calcium phosphate, aluminium phosphate, iron phosphate), anthropogenic inorganic phosphorus compounds (e.g. organophosphates in insecticides and pesticides), or organic phosphorus compounds (e.g. adenosine triphosphate (ATP), nucleic acids (DNA and RNA), phospholipids, phytates). Phosphorus in the natural environment is almost exclusively found as phosphate (PO$_4^{3-}$) in dissolved form or associated with particulate material. Between pH 2 and 7 H$_2$PO$_4$ is the dominant form, whereas HPO$_4^{2-}$ exists mainly between pH 7 and 12. Above pH 12, PO$_4^{3-}$ is prevailing, whereas below pH 2 H$_3$PO$_4$ is the dominant form (Brady and Weil 2008). The bioavailability of PO$_4^{3-}$ in the environment is regulated by complex exchanges between particulate material, microorganisms and/or solution via physical, chemical and biological processes. Phosphates in aquatic environments principally come from natural sources, but anthropogenic activities (e.g. sewage, use of pesticides in agriculture) substantially increase their concentration (Butcher 1992; Bitton 1994; Paul and Clark 1995; Schmitz 1996; O’Neill 1998; Liu and Liptak 2000).

Phosphate removal in the aquatic environment is driven by adsorption and precipitation processes, which depend on the pH, the ionic speciation at the mineral surface, and the mineral surface area (Rhue and Harris 1999; Li and Stanforth 2000). Adsorption onto reactive mineral surface is the principle mechanism during the initial rapid phase of PO$_4^{3-}$ removal, hence oxides, hydroxides and oxyhydroxides of iron and aluminium appear to be the most reactive surfaces for PO$_4^{3-}$ adsorption (Rhue and Harris 1999). Hydroxyl groups (OH$^-$) at the mineral surfaces can be replaced by a PO$_4^{3-}$ anion during ligand exchange mechanism (i.e. principal adsorption mechanism) and covalent bonds are created to develop inner-sphere complexes between PO$_4^{3-}$ and metal ions of the solid material (Stumm and Morgan 1995). Often the ligand exchange mechanism is an irreversible process (i.e. hysteresis) as desorption happens very slowly. Chemisorption may also occur with the PO$_4^{3-}$ ion, forming an irreversible binding. However, chemisorption differs from ligand exchange mechanism as it involves electronic interaction between PO$_4^{3-}$ and metal ions of the solid material,
generates high heat, requires the presence of activation energy, and is restricted to a monolayer surface coverage (Rhue and Harris 1999). Following the adsorption mechanism (i.e. ligand exchange or chemisorption), a slow phase of sorption occurs where the \( \text{PO}_4^{3-} \) ion may be precipitated and diffused to internal crystal lattice defects of the solid (Froelich 1988; Rhue and Harris 1999), and it is during this phase that \( \text{PO}_4^{3-} \) ions can combine with additional free ions (e.g. Ca, Mg) to form a new 3D solid phase on the solid surface (Rhue and Harris 1999).

In wastewater treatment salts of iron (e.g. \( \text{FeCl}_3 \)) or aluminium [e.g. \( \text{Al}_2(\text{SO}_4)_3 \), \( \text{AlO(OH)} \)] are commonly used to precipitate with the \( \text{PO}_4^{3-} \) ion at pH 4 to 6.5 (Galarneau and Gehr 1997; Hano et al. 1997; Omoike and Vanloon 1999; Tanada et al. 2003). In addition, phosphorus may be removed by biological means but this represents a small percentage of phosphate removal. Unfortunately, the use of chemical precipitation often increases the cost of treatment, and generates large quantities of phosphate-hydroxide-rich sludge that require disposal (Liu and Liptak 2000; Hammer and Hammer Jr. 2005). Moreover, incomplete precipitation may result in leaching of phosphate in the system, whereas over dosing of iron and aluminium salts may also lead to leaching of these chemicals down stream and can seriously affect the microbial activities (Maurer and Boller 1999; Gobat et al. 2004; Madigan et al. 2012; Walker 2012). Furthermore, these phosphates removal chemicals (i.e. iron and aluminium salts) are typically acidic, which can lead to a reduction of effluent pH (Stumm and Morgan 1995; Liu and Liptak 2000; Hammer and Hammer Jr. 2005).

Consequently, alternate materials have been investigated for their phosphate removal capacity from natural and synthetic wastewaters by sorption mechanisms (see alternate substrates used in constructed wetlands in section 2.1.6.1). Roques et al. (1991) and Johansson (1999b) found that limestone has a sorption capacity of about 20 mg \( \text{PO}_4^{3-}\text{-P/g} \) with a P loading <25 mg/L. Marl used in a batch experiment and in a field experiment showed, respectively, up to 98% phosphate removal from solutions containing 0–10 mgP/L, and 37–52% phosphate removal in wastewater with an average P concentration of 0.82 mg/L (Szogi et al. 1997). Phoslock™, a lanthanum-modified bentonite, adsorbs phosphate (via chemisorption) up to 98% of 1 mg/L phosphate solutions (Haghseresht et al. 2009). A study by Johansson (1999a) has also
showed that blast furnace slag removed phosphate from synthetic wastewater (column lab experiment) over 95% (P loading of 10 mg/L). Hence, these studies would suggest that natural materials, industrial by-products and man-made materials can have substantial P-removal capacities.

2.1.5.2. Pathogens

Disease-causing pathogenic microorganisms, including viruses, bacteria, and protozoan parasites are killed using disinfectants such as chlorine, ozone and ultraviolet light (Bitton 1994; Liu and Liptak 2000; Hammer and Hammer Jr. 2005). The efficiency of the disinfectant depends strongly on dose rate, the contact time, chemical and physical interference (i.e. inorganic and organic nitrogenous compounds, iron, magnesium, and hydrogen sulphide), effluent temperature, and the type of microorganisms targeted. The decreasing resistance of microorganisms to disinfectants is as follows: protozoan cysts > bacterial spores > viruses > vegetative bacteria (Bitton 1994).

Free chlorine, especially as hypochlorous acid (HOCl), is very effective in deactivating pathogens, but less efficient towards viruses from their increased resistance. Free chlorine causes two types of damages in bacterial cells: disruption of cell permeability resulting in cell functions failure and eventually cell death, and damage to nucleic acids and enzymes inhibiting cell activities leading to the cell death. However, there are several disadvantages to using chlorine because of efficacy dependence on pH, and potential formation of toxic (mutagenic and carcinogenic) by-products (Bitton 1994; Liu and Liptak 2000; Hammer and Hammer Jr. 2005; Walker 2012). Consequently, chlorine dioxide is preferred to free chlorine, where chlorine dioxide disrupts protein synthesis in bacterial cells and quickly inactivates bacteria and viruses present in wastewaters (Bitton 1994).

Ozone is a powerful oxidising agent used to inactivate pathogenic microorganisms and to oxidise taste- and odour-causing compounds, particularly in drinking water. Ozone is a much more powerful oxidant than chlorine, because of the free radicals attack. Ozone affects the permeability, enzymatic activity and DNA of bacterial cell leading to the cell death. In addition, ozone is not affected by pH and does not interact
with ammonia, however, it is a very expensive process (Bitton 1994; Liu and Liptak 2000; Hammer and Hammer Jr. 2005).

Ultraviolet (UV) light radiation (260 nm) effectively inactivates microorganisms by damaging microbial DNA (thymine dimerisation, which blocks DNA replication). UV disinfection does not produce any known toxic by-product, has no taste and odour problems, does not use chemicals and only requires a small space for the system. However, UV disinfection is relatively costly and its efficiency can be significantly reduced by the water turbidity, and the built up of biofilm on the lamp surface. The determination of UV dose and potential photo-reactivation of UV-treated microbial pathogens are also an issue (Bitton 1994; Liu and Liptak 2000; Hammer and Hammer Jr. 2005).

2.1.6. Combined treatments: Constructed wetlands

A constructed wetland (CW) is a passive, virtually self-maintaining system that offers an economical and effective alternative to provide secondary and advanced treatments of wastewater (Hammer 1991; Sundaravadivel and Vigneswaran 2001; Kadlec and Wallace 2009). Typically, CWs are more suitable for small to medium size communities where sufficient land is available, but they may also be used as an advanced treatment for wastewater in municipal STPs. A CW consists of filtration/support substrate material (various types of soil, sand and/or gravel), aquatic plants (e.g. Typha latifolia, Phragmites australis) and an effluent stream (e.g. wastewater, stormwater). All three components, along with microorganisms, interact and provide a combined physical, chemical and biological process to improve the water quality by reducing BOD, and concentrations of suspended solids, nutrients, heavy metals, trace organic compounds, and pathogens (Watson et al. 1989; Hammer 1991; Reed et al. 1995; Sundaravadivel and Vigneswaran 2001; Imfeld et al. 2009; Kadlec and Wallace 2009; Imfeld et al. 2010). Two types of CWs are typically described in wastewater treatment: a subsurface-flow wetland and a surface flow wetland. For the most commonly used wetland (subsurface-flow), water flows vertically or horizontally through the medium just below the medium-air interface. For surface flow wetlands the effluent flows well above the medium surface, creating
a shallow body of water (Reed et al. 1995; Sundaravadivel and Vigneswaran 2001; Kadlec and Wallace 2009).

Designing a suitable CW is complex because all aspects of the system have to be carefully selected. Size and shape of the CW depends strongly on the initial nitrogen and phosphate loadings, but Reed et al. (1995) suggest an aspect ratio of 2:1 to 3:1 (i.e. aspect ratio between long-and-thin and square shape). In addition, the hydraulic retention time (HRT) in CW, which is defined by the following equation:

\[ \text{HRT} = \frac{(A \times y \times p)}{Q} \]

where \( A \) = surface area of canal, \( y \) = wetted depth, \( p \) = porosity of the medium, \( Q \) = flow rate;

is a crucial parameter, because contaminant removal rate is often dictated by the time the effluent spends in the CW. Typical contaminant removals in subsurface-flow wetland are: total suspended solids >90% after 2–3 days, BOD >90% after 3–5 days, total nitrogen >50% after 5–7 days, and faecal coliforms 99–99.9% after 7 days. Moreover, the first order kinetic model is used to estimates BOD and total nitrogen final concentration (C):

\[ C = C^* + (C_i - C^*) e^{-kt} \]

where \( C^* \) = background concentration, \( C_i \) = initial concentration, \( k \) = rate constant at 20°C, and \( t \) = hydraulic retention time (Hammer 1991; Reed et al. 1995; Sundaravadivel and Vigneswaran 2001; Rousseau et al. 2004; Vymazal 2007; Kadlec and Wallace 2009).

Total phosphorus (TP) is strongly removed by soil in surface flow wetland (up to 95%) (Vymazal 2007) but not in subsurface-flow wetland where gravel is commonly used, which only removes ~25% of P (P adsorption capacity of 36 mg/kg) (Mann 1997). TP removal to the substrate is a dominant process, as plants uptake is relatively low in both types of wetland (Hammer 1991; Reed et al. 1995; Tanner 1996; Kim and Geary 2001; Sundaravadivel and Vigneswaran 2001; Stottmeister et al. 2003; Vymazal 2007; Kadlec and Wallace 2009). However, one way to increase TP removal in subsurface-flow wetlands is to use alternate substrates that have significantly
higher phosphate removal and hydraulic capacities than gravel (see sections 2.1.6.1 and 2.2.5).

2.1.6.1. Alternate substrates for constructed wetlands

Many studies have investigated alternate substrates in CWs to determine their success at removing phosphate (and other contaminants) from wastewater (Table 2.1). Laterite, Maerl, steel-slag, and wollastonite appear to be the most efficient phosphate removal substrates (>90%), whereas Maerl, Saprist peat and Saprist peat/oil-shale ash sediments are the most efficient at nitrogen removal (>50%). Consequently, from these studies the most suitable substrates as an alternative to gravel in CWs appear to be Maerl and Saprist peat/oil-shale ash sediments. However, further field experiments should be undertaken with laterite, steel-slag and wollastonite to fully investigate their contaminants removal capacity. In addition, studies on the substrates biocompatibility are also necessary. Furthermore, many other potential substrates (e.g. siderites, oyster shells and modified oyster shell derivatives) (Yu et al. 2010) are available but they have not been fully investigated for wastewater.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Type of experiment</th>
<th>P loading</th>
<th>P results</th>
<th>Other results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dolomite (porosity 0.40; pH 9.3; Ca &amp; Fe &amp; Mg rich)</td>
<td>field exp.: vertical sub-surface flow CW (3 cells in series); vegetation Typha sp.</td>
<td>189 mg/m²/d</td>
<td>P removal: 44%</td>
<td>Life-time (estimation): 4.6 yrs</td>
<td>(Pant et al. 2001)</td>
</tr>
<tr>
<td>Calcite</td>
<td>field exp.: vertical sub-surface flow CW (2 cells)</td>
<td></td>
<td>P removal: 62%</td>
<td>Life-time: 3 mths</td>
<td>(Arias et al. 2003)</td>
</tr>
<tr>
<td>Laterite (Al &amp; Fe rich)</td>
<td>field exp.: CW; vegetation Phragmites australis and Phalaris arundinacea</td>
<td>1.5 mg/L</td>
<td>P removal: 96%</td>
<td>Al removal: 81%; Fe removal: 95%; Mn removal: 50%</td>
<td>(Wood and McAtamney 1996)</td>
</tr>
<tr>
<td>Anthracite</td>
<td>lab exp.: vertical flow CW</td>
<td></td>
<td>P removal: &gt;60%</td>
<td>TOC removal: 70%; BOD removal: 70%; TN removal: 20%</td>
<td>(Zhang et al. 2007)</td>
</tr>
<tr>
<td>Maerl (Ca &amp; Mg carbonate rich)</td>
<td>lab exp.: artificial CW; vegetation Phragmites australis</td>
<td>7.5 mg/L</td>
<td>P removal: 98%; P adsorption capacity (estimation): 1,184 mgP/kg</td>
<td>Life-time (estimation): 4 yrs; COD removal: 75%; TN removal: 59%; effluent pH: 7.6 (1/2 unit increase)</td>
<td>(Gray et al. 2000)</td>
</tr>
<tr>
<td>Steel-slag</td>
<td>lab exp.: vertical flow CW</td>
<td></td>
<td>P removal: &gt;90%</td>
<td>TOC removal: 20–30%; BOD removal: 70%; TN removal: 20%</td>
<td>(Zhang et al. 2007)</td>
</tr>
<tr>
<td>Bauxite (porosity 34%; pH 5.9)</td>
<td>lab exp.: vertical flow column</td>
<td>25–100 mg/L</td>
<td>P adsorption capacity: 350 mgP/kg</td>
<td></td>
<td>(Drizo et al. 1999)</td>
</tr>
<tr>
<td>Material</td>
<td>Lab experiment</td>
<td>Initial P concentration</td>
<td>P removal</td>
<td>P adsorption capacity</td>
<td>Life-time (estimation)</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>-----------------</td>
<td>--------------------------</td>
<td>-----------</td>
<td>-----------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>Shale (porosity 37.7%; pH 4.5)</td>
<td>lab exp.: vertical flow column</td>
<td>25–100 mg/L</td>
<td></td>
<td>P adsorption capacity: 730 mgP/kg</td>
<td>Life-time (estimation): 7–20 yrs depending on CW design</td>
</tr>
<tr>
<td>Wollastonite (porosity 0.54; Ca rich)</td>
<td>lab exp.: vertical flow column</td>
<td>3.4 mg/L</td>
<td></td>
<td>P removal: &gt;85%</td>
<td></td>
</tr>
<tr>
<td>Blast furnace slag (Ca &amp; Si rich)</td>
<td>lab exp.: batch</td>
<td>0–200 mg/L</td>
<td>P removal: 50%; P adsorption capacity: 44.2 gP/kg</td>
<td>Life-time in sub-surface vertical flow CW (estimation): 58.8 yrs if P conc. 8 mg/L, 470.4 yrs if P conc. 1 mg/L</td>
<td>(Sakadevan and Bavor 1998)</td>
</tr>
<tr>
<td>Zeolite</td>
<td>lab exp.: batch</td>
<td>0–200 mg/L</td>
<td>P removal: 50%; P adsorption capacity: 2.15 gP/kg</td>
<td>Life-time in sub-surface vertical flow CW (estimation): 11.1 yrs if P conc. 8 mg/L, 88.8 yrs if P conc. 1 mg/L</td>
<td>(Sakadevan and Bavor 1998)</td>
</tr>
</tbody>
</table>
2.1.6.2. Substrate as biomass support particle

In CWs, the substrate materials and roots from aquatic plants act as a biomass support particle (BSP) on which microorganisms may grow and develop as biofilms (Bitton 1994; Liu and Liptak 2000; Hammer and Hammer Jr. 2005; Madigan et al. 2012). Survival and growth of microorganisms are enhanced because the biofilm provides a protective defence, and resistance to physical forces and environmental changes. This allows cells to remain in the most favourable environment and to live in close association with each other. Furthermore, the development of rare microorganisms or those with slow growth rates (both responsible for microbial activities) is therefore possible within the biofilm.

Further enhancing biofilm and microbial population development is that a redox gradient typically forms in CW, where the bottom part becomes anoxic and the upper part remains oxic. Consequently, simultaneous aerobic and anaerobic microbial activities can occur throughout the CW and this allows simultaneous nitrification, denitrification and anammox processes. In addition, simultaneous nitrification, denitrification and anammox processes may occur from the creation of oxic and anoxic micro-zones in and around plant roots, and within porous substrates and associated biofilms (Hammer 1991; Bitton 1994; Reed et al. 1995; Sundaravadivel and Vigneswaran 2001; Tanner et al. 2002; Stottmeister et al. 2003; Gobat et al. 2004; Ragusa et al. 2004; Kadlec and Wallace 2009; Madigan et al. 2012).

Truu et al. (2009) and Faulwetter et al. (2009) found, surprisingly, that microbial community structure, composition and diversity in CW have rarely been studied. However, among the work done, Ragusa et al. (2004) showed that the biomass needed time (i.e. 100 days) to stabilise and this needs to be taken into account for newly constructed wetlands. The formation of the wetland microbial communities was also found to be a stochastic process in the initial stage (Baptista et al. 2008). Several studies clearly demonstrated that the physico-chemical conditions strongly influence microbial community. For example, Calheiros et al. (2009), Dang & Reddy (2010), and Li et al. (2010b) have shown that CW substrate materials changed bacterial community diversity and structure, because of the specific physico-chemical characteristics (e.g. pH, electrical conductivity, porosity, and organic matter content).
of the substrate material, and because of the subsequent changes to the effluent composition the substrate material imparts. Similarly, Kjellin et al. (2007) demonstrated that community diversity increased with decreased nutrient concentrations and increased effluent residence times. Furthermore, Imfeld et al. (2010) observed that the changes of the bacterial community structure in the aqueous phase of a CW treating contaminated groundwater correlated with a succession of oxic to anoxic conditions and the emergence of particular contaminants (e.g. vinyl chloride and ethene).

To further this, several studies have found higher microbial biomass, and greater microbial community diversity and activity in the upper layer of CWs. This depth effect is related to an increased carbon, nitrogen, oxygen, and organic matter supply in the upper layer, which was directly influenced by the rhizosphere, the water depth and the redox potential (Nguyen 2000; Ragusa et al. 2004; Nurk et al. 2005; Truu et al. 2005; Tietz et al. 2007; Krasnits et al. 2009; Iasur-Kruh et al. 2010; Lin et al. 2010). Similarly, Sleytr et al. (2009) found little variation in the structure of microbial communities over the depth of a vertical CW because the physico-chemical parameters were very similar at all depths. Finally, several microorganisms have been identified in constructed wetlands, including ammonia-oxidising bacteria and archaea, denitrifiers, and anammox bacteria (Shipin et al. 2005; Truu et al. 2005; Dong and Sun 2007; Krasnits et al. 2009; Iasur-Kruh et al. 2010).

2.1.7. Australian guidelines for municipal wastewater treatment

In Australia, as part of the National Water Quality Management Strategy (NWQMS), the Natural Resource Management Ministerial Council (NRMMC) has written five guidelines related to sewerage systems: acceptance of trade (industrial waste; 1994), effluent management (1997), use of reclaimed water (1999), sludge (biosolids) management (2004), and sewerage system overflows (2004) (Department of Sustainability, Environment, Water, Population and Communities 2012). The “Australian guidelines for sewerage systems - effluent management” provides a national standard for the adequate treatment of wastewater, and assists with managing and monitoring the treated effluent (National Water Quality Management Strategy 1997); Table 2.2 shows an adaptation of the guidelines.
The level of treatment required will depend on wastewater origin and the intended use of the effluent. A total of 20 different documents including policies, processes and guidelines developed by the NWQMS have been used to identify appropriate levels for effluent quality (Department of Sustainability, Environment, Water, Population and Communities 2012). Effluent can be reused (e.g. irrigation, aquaculture, industry), applied on land (e.g. evaporation ponds, evapotranspiration, infiltration), or discharged to coastal and/or inland waters. Land applications usually require secondary treatment, and in some cases an additional secondary treatment and/or tertiary treatment may be required. Effluent for discharge to coastal waters is at least primary treated, whereas discharge to inland waters requires a minimum of secondary treatment but usually includes additional secondary treatment and tertiary treatment (National Water Quality Management Strategy 1997).

Table 2.2: Australian guidelines for effluent quality following various treatments (primary treatment, secondary treatment, additional secondary treatment and tertiary treatment, and advanced wastewater treatment) in regards to biochemical oxygen demand (BOD), total suspended solids (TSS), total nitrogen (TN), total phosphorus (TP), E.Coli, oil and grease (O&G). All data are in mg/L except E.Coli, which is in cfu/100 mL. (Adapted from National Water Quality Management Strategy 1997).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BOD</th>
<th>TSS</th>
<th>TN</th>
<th>TP</th>
<th>E.Coli</th>
<th>O&amp;G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw (no treatment)</td>
<td>150–500</td>
<td>150–450</td>
<td>35–60</td>
<td>6–16</td>
<td>10⁷–10⁸</td>
<td>50–100</td>
</tr>
<tr>
<td>Primary treatment</td>
<td>120–250</td>
<td>80–200</td>
<td>30–55</td>
<td>6–14</td>
<td>10⁶–10⁷</td>
<td>30–70</td>
</tr>
<tr>
<td>Secondary treatment</td>
<td>20–30</td>
<td>25–40</td>
<td>20–50</td>
<td>6–12</td>
<td>10⁵–10⁶</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Additional secondary treatment and/or tertiary treatment</td>
<td>5–20</td>
<td>5–20</td>
<td>10–20</td>
<td>&lt;2</td>
<td>&lt;10⁷</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Advanced wastewater treatment</td>
<td>2–5</td>
<td>2–5</td>
<td>&lt;10</td>
<td>&lt;1</td>
<td>&lt;10³</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

2.2. Red Mud & Bauxsol™ Technology

2.2.1. Red mud history

In 1887, Karl Josef Bayer patented the Bayer process to replace the older method (i.e. sodium carbonate heating and carbon dioxide precipitation) to more efficiently extract alumina from bauxite ore for the textile industry (i.e. alumina was used as a mordant in dyeing cotton). The Bayer process involves the digestion of ground pre-desilicated (silica depleted) bauxite in a caustic soda solution at 145–250°C to convert alumina to a soluble sodium aluminate (NaAl(OH)₄), which was drawn off and Al(OH)₃ precipitated from the pregnant liquor.
The insoluble iron oxides in the bauxite constitute a waste called red mud (Hind et al. 1999; Clark et al. 2008; Klauber et al. 2011; Liu et al. 2011; Power et al. 2011). In 1892, the first Bayer plants were built and rapidly expanded in Germany, France, Italy and England to meet the demand for alumina as mordant and aluminium metal (Hall-Heroult process for smelting alumina to metal). With this rapid development came the problem of production and disposal of red mud (1 t of alumina produced generates between 1 and 6 t of red mud) (Hind et al. 1999; Kumar et al. 2006; Clark et al. 2008; Wang et al. 2008). It is estimated that by 1940 around 22 Mt of red mud had been created and this rapidly grew to 1 Gt in 1985. By 2007, estimated worldwide red mud storage was some 2.6 Gt and is increasing at a rate of 90–120 Mt/yr (Hind et al. 1999; Kumar et al. 2006; Power et al. 2011).

Prior to the 1970’s, two disposal methods were used with red mud waste: marine disposal where red mud was disposed directly into the sea, and lagooning where red mud was disposed into land-based ponds. However, in the early 1980’s, concerns were raised about possible red mud impacts on the environment because of physico-chemical properties, pH up to 13, high alkalinity of ~35 g/L, clay-like consistency, and its highly complex chemistry and mineralogy (Zhang et al. 2001; Kumar et al. 2006; Clark et al. 2008; Wang et al. 2008; Power et al. 2011; see also section 2.2.2 for further details). Subsequently, a new disposal method “dry-stacking” was developed, where red mud slurry is thickened to a paste before being stack in thin layers that are allowed to dewater and dry (Cooling 1988; Nikraz et al. 2007; Power et al. 2011). Although considered best practice for managing red mud, “dry-stacking” still poses a substantial threat to the environment and is costly (Dauvin 2010; Enserink 2010; Power et al. 2011).

In 1992, red mud was listed on the Basel Convention on the control of transboundary movements of hazardous wastes and their disposal (Basel Convention 1989). To address this issue, modifications to activate and/or neutralise red mud were developed and in most cases proved to be very successful. These modifications, where a manufacturing step can be shown, convert red mud from a hazardous waste to product status and are exempt from the Basel Convention (see also section 2.2.3 for further details).
2.2.2. Red mud mineralogy and geochemical properties

The composition of red mud depends on the origin of the bauxite ores, the operational procedures used in the alumina refinery, and the subsequent disposal of red mud. The main elements in red mud are iron oxides and oxyhydroxides (25–55%), aluminium oxides (15–30%), silica (5–15%), titanium oxides (5–15%), calcium oxide (1–15%), sodium oxide (1–8%), and other elements lost at high temperature (~10%) (Wang et al. 2008; Grafe and Klauber 2011; Grafe et al. 2011). Consequently, red mud contains minerals such as hematite, goethite, magnetite, boehmite, sodalite, cancrinite, tricalcium aluminate, gibbsite, diaspore, quartz, rutile, anastase, ilmenite, and calcite (Hind et al. 1999; Zhang et al. 2001; Wang et al. 2008; Grafe and Klauber 2011; Grafe et al. 2011).

The geochemical properties of red mud are: high alkalinity of ~35 g/L, pH of 10–13, electrical conductivity of 7.4 mS/cm, acid neutralising capacity of 0.94 mol H⁺/kg (pH 7) and 4.56 mol H⁺/kg (pH 5.5), fine particle size (0.1 to 200 μm with the vast majority <10 μm), surface area of 34.5–78.7 m²/g, bulk density up to 2.5 g/cm³, a point of zero charge at pH ~6.9, and largely insoluble (Roach et al. 2001; Zhang et al. 2001; Carter et al. 2008; Palmer and Frost 2009; Grafe and Klauber 2011; Grafe et al. 2011).

2.2.3. Red mud modifications and applications

The modifications, to activate and/or neutralise red mud, include treatment with acid (Koumanova et al. 1997; Apak et al. 1998; Genc-Fuhrman et al. 2004b; Cengeloglu et al. 2006; Li et al. 2006; Huang et al. 2008), heat (Altundogan and Tumen 2002; Li et al. 2006; Liu et al. 2007), seawater (McConchie et al. 1999; McConchie et al. 2002a; Genc et al. 2003; Genc-Fuhrman et al. 2004b; Akhurst et al. 2006; Carter et al. 2008; Palmer and Frost 2009), carbon dioxide (Nikraz et al. 2007; Khaitan et al. 2009), sulphur dioxide (Fois et al. 2007), bacteria (Vachon et al. 1994; Hamdy and Williams 2001; Krishna et al. 2005), gypsum (Ho et al. 1991; Wong and Ho 1993), or a combination of several treatments (Pradhan et al. 1998; Gupta et al. 2001; Genc-Fuhrman et al. 2004b; Genc-Fuhrman et al. 2004a; Liu et al. 2007; Huang et al. 2008; Johnston et al. 2010; Palmer et al. 2010; Tang et al. 2010). These modifications impact on the physico-chemical properties and mineralogy of red mud, and may
enhance or exacerbate trace-metal removals, alkalinity loss, and suitability as an environmental ameliorant.

Recent reviews by Klauber et al. (2011) and Liu et al. (2011) show that red mud and modified red mud have been successfully used for many different applications in construction (e.g. additive for cement, aggregate and bricks), in chemistry (e.g. as catalyst), in metallurgy (e.g. steel production, metals recovery from red mud), and in production of ceramics, coatings, plastics and pigments. Moreover, red mud and modified red mud have been effectively used in environmental remediation for contaminated waters, soils and air (Table 2.3; see also sections 2.2.4–2.2.6 for further details on seawater-neutralised red mud).
Table 2.3: Examples of red mud and modified red mud applications for contaminated waters, soils, and air.

<table>
<thead>
<tr>
<th>Application</th>
<th>Target</th>
<th>Effect</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>fluoride</td>
<td>sorption</td>
<td>(Cengeloglu et al. 2002; Tor et al. 2009)</td>
</tr>
<tr>
<td></td>
<td>nitrate</td>
<td>sorption</td>
<td>(Cengeloglu et al. 2006)</td>
</tr>
<tr>
<td></td>
<td>metals</td>
<td>sorption</td>
<td>(Apak et al. 1998; McConchie et al. 1999; Clark 2000; McConchie et al. 2000; Davies-McConchie et al. 2002; Genc et al. 2003; Clark et al. 2004; Genc-Fuhrman et al. 2004a; Genc-Fuhrman et al. 2004b; Munro et al. 2004; Genc-Fuhrman et al. 2005; Clark et al. 2006b; Lapointe et al. 2006; Genc-Fuhrman et al. 2007; Clark et al. 2009; Palmer et al. 2010; Clark et al. 2011a; Clark et al. 2011b; Despland et al. 2011)</td>
</tr>
<tr>
<td></td>
<td>organics</td>
<td>sorption</td>
<td>(Ho et al. 1991; Hamdy and Williams 2001; Gupta et al. 2004a; Gupta et al. 2004b; Wang et al. 2005a; Tor et al. 2006)</td>
</tr>
<tr>
<td></td>
<td>acid</td>
<td>neutralisation</td>
<td>(McConchie et al. 1999; McConchie et al. 2000; Davies-McConchie et al. 2002; Clark et al. 2004; Munro et al. 2004; Lapointe et al. 2006)</td>
</tr>
<tr>
<td>soil</td>
<td>metals</td>
<td>binding</td>
<td>(McConchie et al. 1999; McConchie et al. 2000; Lin et al. 2002; Maddocks et al. 2004; Maddocks et al. 2005; Garau et al. 2007; Garau et al. 2011)</td>
</tr>
<tr>
<td></td>
<td>acid</td>
<td>neutralisation</td>
<td>(McConchie and Clark 1999; McConchie et al. 1999; McConchie et al. 2000; Davies-McConchie et al. 2002; Lin et al. 2002; Maddocks et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>vegetation</td>
<td>amendment</td>
<td>(Summers et al. 1996; Davies-McConchie et al. 2002; Maddocks et al. 2004; Krishna et al. 2005; Wehr et al. 2006; Garau et al. 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sorption</td>
<td>(Jones et al. 2006; Fois et al. 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>binding</td>
<td>(Hutson and Attwood 2008)</td>
</tr>
</tbody>
</table>

### 2.2.4. Seawater-neutralisation: the Basecon™ procedure

Seawater neutralisation of red mud, better known as the Basecon™ procedure, has been developed by McConchie et al. (2002a; 2002b), and the subsequent end product of this neutralisation technique is called Bauxsol™ (Figure 2.2). The brine used during
the Basecon™ procedure is of >300 mg/L of calcium and >750 mg/L of magnesium (ideal ratio Mg:Ca is 14:1 molar), which converts the soluble alkaline to an insoluble form. Alkalinity in the residual liquor is <300 mg/L, which in turn lowers the basicity to a pH ≤9 (McConchie et al. 2002a; Clark et al. 2008). Magnesium precipitates with hydroxide and forms new minerals such as brucite, hydrotalcite, and/or additional boehmite and gibbsite, whereas calcium reduces the carbonate alkalinity by forming calcite and/or aragonite, and also forms hydrocalumite and p-aluminohydrocalcite (McConchie et al. 1999; McConchie et al. 2000; McConchie et al. 2002a; Hanahan et al. 2004; Carter et al. 2008; Clark et al. 2008; Clark et al. 2009; Palmer and Frost 2009). These precipitations are governed by the following 4 reactions:

$$\begin{align*}
6\text{MgCl}_2 + 2\text{Al(OH)}_4^- + 8\text{OH}^- + \text{CO}_3^{2-} + 12\text{Na}^+ & \rightarrow \text{Mg}_6\text{Al}_2(\text{CO}_3)(\text{OH})_{16} \cdot 4\text{H}_2\text{O} + 12\text{NaCl}; \\
\text{CaCl}_2 + 2\text{Al(OH)}_4^- + \text{CO}_3^{2-} + 2\text{Na}^+ & \rightarrow \text{CaAl}_2(\text{CO}_3)_2(\text{OH})_4 \cdot 3\text{H}_2\text{O} + 4\text{OH}^- + 2\text{NaCl}; \\
2\text{CaCl}_2 + \text{Al(OH)}_4^- + 3\text{OH}^- + 4\text{Na}^+ & \rightarrow \text{Ca}_2\text{Al(OH)}_7 \cdot 3\text{H}_2\text{O}(s) + 4\text{NaCl}; \\
2\text{CaCl}_2 + \text{MgCl}_2 + \text{Al(OH)}_4^- + 3\text{OH}^- + \text{CO}_3^{2-} + 6\text{Na}^+ & \rightarrow \text{Ca(OH)}_2(s) + \text{Mg(OH)}_2(s) + \text{CaCO}_3(s) + \text{Al(OH)}_3(s) + 6\text{NaCl}(aq).
\end{align*}$$

**Figure 2.2:** Summary of Bauxsol™ production procedure
In addition, the presence of organic matter in the bauxite results to the formation of oxalate, which during the seawater neutralisation allows the formation of whewellite, weddelite, and glushinkite; this is only at low concentrations (Clark 2000; Clark et al. 2008). Despite the creation of new minerals, the general mineralogy of Bauxsol™ remains similar to the red mud; however, important changes in geochemical properties are seen (McConchie et al. 1999; McConchie et al. 2000; Lin et al. 2002; McConchie et al. 2002a; Hanahan et al. 2004; Carter et al. 2008; Clark et al. 2008; Clark et al. 2009; Palmer and Frost 2009; Freire et al. 2012).

2.2.5. Bauxsol™ mineralogy and geochemical properties

Bauxsol™ is a complex mix of minerals with major constituents being hematite, boehmite, cancrinite, gibbsite, quartz, and sodalite, and minor constituents being anhydrite/gypsum, aragonite, brucite, calcite, hydrocalumite, hydrotalcite, p-aluminohydocalcite, diaspore, and portlandite (McConchie et al. 1999; McConchie et al. 2000; McConchie et al. 2002a; Hanahan et al. 2004; Carter et al. 2008; Clark et al. 2008; Clark et al. 2009; Palmer and Frost 2009). The concentration of major components and trace elements on Bauxsol™ varies depending on the origin of the bauxite, the operations parameters at the alumina refinery and the concentration of calcium and magnesium brines used to neutralise the red mud (McConchie et al. 2002a; Clark et al. 2008). Table 2.4 shows the typical proportion of major components and trace elements in Bauxsol™ derived from Weipa bauxite (McConchie et al. 1999).
Table 2.4: Average concentration of a) major components (%) and b) trace-metals (mg/kg) in Bauxsol™

<table>
<thead>
<tr>
<th>a) Major components (%)</th>
<th>b) Trace-metals (mg/kg)</th>
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<tbody>
<tr>
<td>FeO₃</td>
<td>30.12</td>
</tr>
<tr>
<td>Al₂O₃</td>
<td>23.26</td>
</tr>
<tr>
<td>SiO₂</td>
<td>16.59</td>
</tr>
<tr>
<td>Na₂O</td>
<td>7.4</td>
</tr>
<tr>
<td>TiO₂</td>
<td>6.66</td>
</tr>
<tr>
<td>CaO</td>
<td>3.17</td>
</tr>
<tr>
<td>CO₂</td>
<td>2.79</td>
</tr>
<tr>
<td>MgO</td>
<td>0.7</td>
</tr>
<tr>
<td>SO₂</td>
<td>0.19</td>
</tr>
<tr>
<td>P₂O₅</td>
<td>0.17</td>
</tr>
<tr>
<td>K₂O</td>
<td>0.13</td>
</tr>
<tr>
<td>MnO</td>
<td>0.04</td>
</tr>
<tr>
<td>Fe</td>
<td>Cr</td>
</tr>
<tr>
<td>Al</td>
<td>Ni</td>
</tr>
<tr>
<td>Si</td>
<td>V</td>
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<td>Na</td>
<td>Ce</td>
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<td>Ti</td>
<td>Sc</td>
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<td>Cr</td>
<td>Y</td>
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<td>Ni</td>
<td>Th</td>
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<tr>
<td>Cr</td>
<td>Nb</td>
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<td>Co</td>
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<td>Cu</td>
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<td>Zn</td>
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<td></td>
<td>La</td>
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<td></td>
<td>Nd</td>
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</table>

Bauxsol™ is commercially available in different forms (slurry, powder or pellet), and as a powder is of brick-red appearance dominated by extremely fine-grained particles with 90% under 10 µm; however, it remains largely non-dispersive and insoluble in water (McConchie et al. 2002a; Genc et al. 2003; Lapointe et al. 2006; Barbhuiya et al. 2010). Bauxsol™ powders have a high surface to volume ratio (≥100 m²/g) and a high charge to mass ratio (McConchie et al. 1999; McConchie et al. 2000; McConchie et al. 2002a), but the hydraulic conductivity of Bauxsol™ is very low (<1×10⁻⁶ cm/sec) and it is like clay when submerged into water (Clark et al. 2005). The plastic and liquid limits of Bauxsol™ have been determined at an average of 33% and 53%, respectively (McConchie et al. 2002a), and the specific gravity of Bauxsol™ has been reported at 2.75 (Barbhuiya et al. 2010).

When fully neutralised Bauxsol™ has a pH of 8.3 and its electrical conductivity (EC) has been reported at 22–53 mS/cm (Hanahan et al. 2004; Johnston et al. 2010). The study by Johnston et al. (2010) also reported a soluble total alkalinity (as CaCO₃) of 0.15 g/L, and an alkalinity speciation of <0.1 mg/L OH⁻, 1.37 mg/L CO₃²⁻, and 181.1 mg/L HCO₃⁻. However, because of the presence of high quantities of precipitated alkalinity (see equations in section 2.2.4) as hydroxy-carbonate minerals and fine crystalline minerals forming weak bases, Bauxsol™ possess a high acid neutralising
capacity (ANC). The acid neutralisation, initially rapid, increases over time and is dictated by the pH, ranging from 4–7 mol H⁺/kg at pH 7 to approximately 14 mol H⁺/kg at pH 5.5 (McConchie et al. 1999; McConchie et al. 2000; Lin et al. 2002; McConchie et al. 2002a; Hanahan et al. 2004; Maddocks et al. 2004; Clark et al. 2008).

The high metal binding capacity (>1,000 meq/kg) of Bauxsol™ increases over time, and is directly linked to the high ANC, high surface/volume ratio and high charge/mass ratio (McConchie et al. 1999; McConchie et al. 2000; McConchie et al. 2002a). Sodalite, alumina and hematite are the three key minerals with the ability to bind metals in Bauxsol™ (Dr. Richard Collins, personal communication, 2011; Clark et al. 2012). Metals are initially bound to the Bauxsol™ by adsorption and then by crystal growth via new mineral precipitation and intracrystalline diffusion. Desorption tests and toxicity characteristic leaching procedure (TCLP) tests have determined that the metals are irreversibly bound to Bauxsol™ (McConchie et al. 1999; Clark 2000; McConchie et al. 2000; Davies-McConchie et al. 2002; McConchie et al. 2002a; Genc et al. 2003; Clark et al. 2004; Hanahan et al. 2004; Munro et al. 2004; Clark et al. 2006b; Genc-Fuhrman et al. 2007; Clark et al. 2008; Clark et al. 2009; Clark et al. 2011a; Clark et al. 2011b).

The sequence of metals binding to available sites at the Bauxsol™’s surface varies depending on the metal concentration, the number of metals present, and the pH of the solution (McConchie et al. 1999; McConchie et al. 2000; Lin et al. 2002; Genc-Fuhrman et al. 2004a; Munro et al. 2004; Tillotson et al. 2005; Clark et al. 2006b; Genc-Fuhrman et al. 2007; Clark et al. 2009; Palmer et al. 2010; Clark et al. 2011b). However, different sequences of binding strength have been reported: Cr > Fe > Cu > As > Pb > Mn > Zn > Cd > Ni > Co (McConchie et al. 2000); Pb > Fe > Cr > Co > Cu > Ni = Zn > Cd > Mn (Lin et al. 2002); Cr > Fe > Pb > Cu > Zn > Cd = Ni > Mn = Co (Clark 2000); and Cu > Al > Cd > Fe > Zn > Pb > Mn (Clark et al. 2006b).

Bauxsol™ has a strong affinity for phosphate, and its phosphate binding capacity has been reported at 2% by mass (Clark et al. 2006a), with some 99% removal for 1.1 g/kg surface loadings or 50% removal for 2.6 g/kg surface loadings (Hanahan et al.
Despite this, Bauxsol™ was found to be unsuitable to remove phosphate in anoxic waters (Akhurst et al. 2004). Phosphate removal by Bauxsol™ has been identified as a combination of ligand exchange, chemisorption and surface precipitation mechanisms (Hanahan et al. 2004; Akhurst et al. 2006; Clark et al. 2006a; Clark et al. 2009). The efficiency of Bauxsol™ to remove phosphate from a solution diminishes as phosphate concentration decreases, which is also strongly influenced by pH at low phosphate concentration (<1 mg/L PO₄³⁻: 100% removal at pH 5.2, 60% removal at pH 9.0; Akhurst et al. 2006).

2.2.6. Bauxsol™ as an environmental remediation agent

Bauxsol™ (slurry, powder or pellet forms) has been successfully applied in field experiments to ameliorate waters and soils. Using Bauxsol™ technology sulphidic mine tailings, waste rock and acid mine drainage have been neutralised to pH ~8 and decontaminated from trace-metals such as aluminium, arsenic, cadmium, chromium, cobalt, copper, cyanide, iron, lead, manganese, nickel, and zinc by more than 90% (McConchie et al. 1999; McConchie et al. 2000; Davies-McConchie et al. 2002; McConchie et al. 2002a; Clark et al. 2004; Munro et al. 2004; Clark et al. 2006b; Lapointe et al. 2006). Bauxsol™ has also been successfully applied to acid sulphate soils, where soil pH increased significantly, heavy metals became irreversibly bound and unavailable, nutrient retention capacity of the soil was enhanced, and sulphide decomposition stopped (McConchie and Clark 1999; McConchie et al. 1999; McConchie et al. 2000; Lin et al. 2002). The treatment of stormwater runoff showed significantly reduced arsenic, cadmium, chromium, copper, nickel and zinc mobility (Genc-Fuhrman et al. 2005; Genc-Fuhrman et al. 2007). Whereas, radium contaminated groundwater was successfully treated below the detection limit (Clark et al. 2011a). In addition, field experiments performed by Virotec Company have shown over 80% phosphate removal from wastewater (Tillotson et al. 2005; Tillotson 2006; Clark et al. 2008).

An investigation by Barbhuiya et al. (2010; 2011a; 2011b) demonstrated that mixing Bauxsol™ with concrete successfully created a stronger and more durable concrete, particularly useful for construction of farm silage bins as these concretes are resistant
to acid and microbial attacks. Howe et al. (2011) showed that Bauxsol™ discharge liquors were 148 times less toxic than untreated red mud towards freshwater *Cladoceran* and 7,714 times less toxic to a marine amphipod. The high non-bioavailability of metals bound to Bauxsol™ has been demonstrated by Maddocks et al. (2005) using earthworms as an indicator (bioaccumulation lower than the threshold to cause mortality). Moreover, Brunori et al. (2005) showed that Bauxsol™ powder in contact with sea urchin embryo’s showed no toxic effect. Finally, Maddocks et al. (2004) also showed that a combination of Bauxsol™ and biosolids increased the biomass production on contaminated mine soil (i.e. reduced acidity and bound metals). Consequently, these studies suggest that Bauxsol™ is environmentally benign despite the source of the parent materials, and has many properties for the use in constructed wetlands.

### 2.3. References


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CHAPTER 3: BAXXSOL™ PELLET PRODUCTION

3.1. Overview

To successfully complete this project several experimental steps that followed in sequence are required. Firstly, a suitable Bauxsol™ pellet requires development such that it may be used as a filter media for circum-neutral waters. Such pellet development would minimise any substantial pH/alkalinity spikes that may otherwise adversely affect microbiological populations and physico-chemical parameters of waters (i.e. objective 1). The paper2 entitled “Minimising Alkalinity and pH Spikes from Portland Cement-Bound Bauxsol (Seawater Neutralized Red Mud) Pellets for pH Circum-Neutral Waters” presents the developmental findings for the production of the cured cement-bound porous Bauxsol™ pellets. Laboratory experiments were undertaken to neutralise or manipulate alkaline solutions to precipitate to insoluble forms by adjusting the Bauxsol™ to cement ratios, and using pre-production modified makeup waters and/or post-production curing baths. The best performing material of these trials was then selected and tested in wastewater treatment. Chapter 9 (Appendix 9.5) has additional information on this experiment.

3.2. Minimising Alkalinity and pH Spikes from Portland Cement-Bound Bauxsol (Seawater-Neutralized Red Mud) Pellets for pH Circum-Neutral Waters

3.2.1. Abstract

Bauxsol reagents (powder, slurry or pellet forms) are powerful tools in environmental remediation, and water and sewage treatment. However, when used in circum-neutral water treatments, cement-bound Bauxsol pellet produces a sustained pH and alkalinity spike due to the presence of unreacted CaO in the cement binder. This study developed a pellet treatment system to minimize the alkalinity/pH spike. The recipe for pelletisation consisted of Bauxsol powder, ordinary Portland cement (OPC), hydrophilic fumed silica, aluminum powder, a viscosity modifier and water. Several batches (including different ratios and sizes) were run using modified makeup waters

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2 This paper has been published in Environmental Science & Technology journal. The formatting of this journal requires the suppression of the symbol TM (trade mark) and uses US English (e.g. “z” instead of “s”).
(H₂O + CO₂ or NaHCO₃) or curing brines (CO₂, NaHCO₃, or Mg/CaCl₂). Alkalinity, pH stability and slake durability tests were performed on pellets before and/or after curing. The best result for reducing the alkalinity/pH spike was obtained from a MgCl₂, CaCl₂ bath treatment using a Bauxsol:cement ratio of 2.8:1 (pH 8.28; alkalinity 75.1 mg/L) for a 100 g batch or 2.45:1 (pH 8.05; alkalinity 35.4 mg/L) for a 1 kg batch. Although brine curing does provide a control on pH/alkalinity release, the pellets may still contain unreacted CaO. Therefore, a freshwater rinse of pellets before treating circum-neutral waters is recommended, as is the continued investigation of alternative pellet-binders.

3.2.2. Introduction

Bauxsol is a chemico-physically modified product derived from bauxite refinery residues, a waste of alumina refining using the Bayer process, and is different to the red mud it is derived from. Modification results in a pH decrease from ~13 to ~9 and conversion of soluble alkalinity to solid alkalinity. Bauxsol is a complex mix of minerals with major constituents including hematite, boehmite, gibbsite, sodalite, quartz, cancrinite and whewellite; while minor components include aragonite, brucite, calcite, diaspore, ferrihydrite, anhydrite/gypsum, hydrocalumite, hydrotalcite, p-aluminohydrocalcite and a few low solubility trace minerals (McConchie et al. 1999; Akhurst et al. 2006). However, the exact composition and geochemical character of a Bauxsol reagent depends on the origin of the bauxite, operational procedures used in the alumina refinery, and the concentration and balance of calcium and magnesium brine used for the red mud conversion (Genc-Fuhrman 2004).

Despite compositional variability, raw Bauxsol reagents are extremely fine-grained, typically 90% are less than 10 µm in diameter and have very high surface/volume ratios (up to and greater than 100 m²/g). Bauxsol reagents also have a high metal binding capacity (>1,500 meq/kg), moderate acid neutralizing capacity (from 4–7 moles H⁺/kg at pH 7, to approximately 14 moles H⁺/kg at pH 5; dependent on time and pH), are largely insoluble and are highly non-dispersive (McConchie et al. 1999; Genc-Fuhrman 2004; Lapointe et al. 2006; Clark et al. 2009). Bauxsol may undergo further physical and geochemical modification, such as acid treatments, blending with
additional mineral components and pelletisation (McConchie et al. 1999; McConchie and Clark 1999; Clark 2000).

Bauxsol-based technologies (powder, slurry or pellets) have been successfully applied to environmental remediation, and water and sewage treatment. McConchie et al. (1999) proved the efficiency of Bauxsol powder to remove metal and neutralize pH from acidic mine effluent and from acid sulfate soils. Genc-Fuhrman (2004) demonstrated the removal of arsenate from water using Bauxsol powder mixed with sand. Akhurst et al. (2006) and McConchie et al. (1999) showed that Bauxsol powder or slurry strongly adsorbs phosphate from aqueous solutions by a ligand exchange mechanism. Clark et al. (2003; 2005) developed porous cement-bound Bauxsol pellets (using hydrogen peroxide as a foaming agent) to overcome low hydraulic conductivity encountered when using Bauxsol powder as a filter device for mild acid mine drainage treatment. Lapointe et al. (2006) confirmed the efficiency of these pellets in permeable reactive barriers to treat acid rock drainage.

However, ordinary Portland cement (OPC) used as a binder in Bauxsol pellets often produces a sharp increase in water pH and alkalinity, especially when used in circum-neutral water treatments (Virotec International, personal communication, 2006). The pH and alkalinity loss from red mud has been modelled by Khaitan et al. (2009), and in general terms may be applied to Bauxsol reagents. However, the specificities of the model do not apply because of changes in mineralogy during Bauxsol formation, and that no tri-calcium aluminate has been observed in the red mud source material. Moreover, the pelletisation process adds excess CaO from the cement binder, which directly leads to the spike in pH and alkalinity (Dr Malcom Clark, personal communication, 2008). Therefore, the successful suppression of pH and alkalinity spike from OPC-bound materials has a broader application in concrete manufacture, especially where concretes are to be placed in pH or alkaline sensitive environments.

The aim of this paper is to investigate techniques to control pH/alkalinity spikes from Portland cement-bound Bauxsol pellet without affecting the performance of the pellet. Specifically, the objectives are to develop a general recipe to make Bauxsol pellets using OPC and to determine the efficiency of alkalinity conversion using modified makeup waters (H$_2$O + CO$_2$ or NaHCO$_3$) during the production of the pellets or using
curing baths (CO₂, NaHCO₃, or Mg/CaCl₂) postpellet production. The intended purpose of these pellets is deployment in the polishing phase of wastewater treatment. The porous Bauxsol pellets should strongly bond phosphates as well as improve hydraulic conductivity and provide a support matrix for existing natural microbial communities.

3.2.3. Methodology

The general recipe was an adaptation from Clark et al. (2003) and consisted of a mix of six ingredients: Bauxsol powder (<125 µm) with a poorly buffered reaction pH of 12, OPC (<63 µm), hydrophilic fumed silica (0.014 µm; 200±25 m²/g), aluminum powder (AG8, A. Van Lerberghe), Carbopol 940 (viscosity modifier), and Milli-Q water. Powdered aluminum reacts with water under alkaline conditions to form gibbsite [Al(OH)₃] and H₂ gas, which then foams the cement. It is a standard method of forming aerated concretes (De Chiffre and De Chiffre 1989). Three experimental trials were carried out for this work using different Bauxsol:cement ratios. Trial 1 investigated the effects of different makeup waters (H₂O + CO₂ or NaHCO₃) to determine whether alkalinity may be converted during pellet setting. Trial 2 examined the effect of curing brines (CO₂, NaHCO₃, or Mg/CaCl₂) on postproduction pellets to control alkalinity. Trial 3 focused on using scaled-up versions of the best performing brine cure and Bauxsol:cement ratios demonstrated in Trial 2.

All dry ingredients except the aluminum powder were mixed with the water until a smooth thick paste was achieved. The aluminum powder was quickly added, mixed (reaction occurs within a few minutes), and transferred to cylindrical molds. Molds of 50 mm diameter by 100 mm were used for the 100 g batches (Trials 1 & 2), and molds of 210 mm diameter by 190 mm were used for the 1 kg batches (Trial 3) (see Table 3.1 for summary of trial mix).
<table>
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<tr>
<th></th>
<th>Trial 1</th>
<th>Trial 2</th>
<th>Trial 3</th>
<th>Trial 4</th>
<th>Trial 5</th>
<th>Trial 6</th>
<th>Trial 7</th>
<th>Trial 8</th>
<th>Trial 9</th>
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<tbody>
<tr>
<td>ratio (Bauxsol: cement)</td>
<td>2.28:1</td>
<td>2.6:1</td>
<td>2.97:1</td>
<td>2.97:1</td>
<td>2.2:1</td>
<td>2.3:1</td>
<td>2.45:1</td>
<td>2.6:1</td>
<td>2.7:1</td>
</tr>
<tr>
<td>Bauxsol (g)</td>
<td>65</td>
<td>67.5</td>
<td>70</td>
<td>70</td>
<td>65</td>
<td>65.9</td>
<td>67.1</td>
<td>68.25</td>
<td>68.95</td>
</tr>
<tr>
<td>cement (g)</td>
<td>28.5</td>
<td>26</td>
<td>23.5</td>
<td>23.5</td>
<td>29.5</td>
<td>28.6</td>
<td>27.4</td>
<td>26.25</td>
<td>27.5</td>
</tr>
<tr>
<td>hydrophilic fumed silica (g)</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
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<tr>
<td>aluminum powder (g)</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
<td>-</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>carbopol (g/L)</td>
<td>13.7</td>
<td>13.7</td>
<td>13.7</td>
<td>13.7</td>
<td>4.325</td>
<td>4.325</td>
<td>4.325</td>
<td>4.325</td>
<td>4.325</td>
</tr>
<tr>
<td>makeup water</td>
<td>CO₂&lt;sup&gt;a&lt;/sup&gt; or NaHCO₃&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CO₂&lt;sup&gt;a&lt;/sup&gt; or NaHCO₃&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CO₂&lt;sup&gt;a&lt;/sup&gt; or NaHCO₃&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CO₂&lt;sup&gt;a&lt;/sup&gt; or NaHCO₃&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>curing process</td>
<td>steam&lt;sup&gt;c&lt;/sup&gt; or no steam</td>
<td>steam&lt;sup&gt;c&lt;/sup&gt; or no steam</td>
<td>steam&lt;sup&gt;c&lt;/sup&gt; or no steam</td>
<td>-</td>
<td>H₂O&lt;sup&gt;e&lt;/sup&gt; or Brine I&lt;sup&gt;f&lt;/sup&gt;</td>
<td>H₂O&lt;sup&gt;e&lt;/sup&gt; or Brine I&lt;sup&gt;f&lt;/sup&gt;</td>
<td>H₂O&lt;sup&gt;e&lt;/sup&gt; or Brine I&lt;sup&gt;f&lt;/sup&gt;</td>
<td>H₂O&lt;sup&gt;e&lt;/sup&gt; or Brine I&lt;sup&gt;f&lt;/sup&gt;</td>
<td>H₂O&lt;sup&gt;e&lt;/sup&gt; or Brine I&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Water saturated with carbon dioxide (CO₂); <sup>b</sup> Water saturated with 1 M NaHCO₃; <sup>c</sup> Thirty minutes at 130°C and at 210 kPa; <sup>d</sup> MilliQ-water; <sup>e</sup> 141 g/L of magnesium chloride hexahydrate (MgCl₂·6H₂O) and 42 g/L of calcium chloride dihydrate (CaCl₂·2H₂O); <sup>f</sup> 106 g/L of sodium bicarbonate (NaHCO₃); <sup>g</sup> Water bath saturated with carbon dioxide (CO₂).
The curing process was initiated after the samples had set: a few hours for Trial 1, seven days for Trial 2, and more than 10 days for Trial 3. Complete immersion of the set pellet material in a curing bath was required for Trials 2 and 3 during seven days (see Appendix 9.5, Figure 9.5 for further details). Samples were then washed in Milli-Q water for approximately 3 min and allowed to dry at room temperature for at least 24 h. Bauxsol pellet materials from Trials 2 and 3 were crushed to small pellets of 2–5 mm diameter. Larger pieces from Trial 3 only were preserved for slake durability testing.

**pH and Alkalinity Analyses of the Solids; Electrical Conductivity Washing Drop Test**

The pH was measured for each sample in Trial 1 using the APHA 2310 method (American Public Health Association 1998). For Trials 2 and 3, the initial and final pH and temperature of each curing bath were recorded as well as the pH and temperature of the washes. The alkalinity and speciation of the solution test was performed before and after the curing bath on each sample using the APHA 2320 method (American Public Health Association 1998) and the Alkalinity Calculator (USGS 2009).

After brine curing, a pH stability test was performed. The pH was recorded every minute for each sample until stabilization occurred (pH held for more than 2 min). Sample pH was again measured after 1, 3, 6, and 24 h. Additional pHs were taken at 72 h and at one week (168 h) for Trial 3. An “Electrical Conductivity washing drop” test for Trial 2 was performed on four samples over a 4 h period where 10 g of pellets were immersed in 50 mL of Milli-Q water. Electrical conductivity (EC) was recorded after 1 h and the water replaced. Analyses were undertaken following QA/QC procedures (Appendix 9.1, Table 9.1).

**Slake Durability Testing**

Material from Trial 2 (pellets of 2–5 mm diameter) and Trial 3 (pieces >2 cm diameter) were subjected to a slake durability test to determine resistance of the pellet to physical breakdown during abrasive wetting and drying cycles. A modification of the standard method of determining slake durability (ASTM-D 4644 (ASTM 1995); refer to Supporting Information for details) was carried out to calculate the slake
durability index (SDI). SDI refers to the percentage of mass retained: (dry mass after test/dry mass before test) × 100 (ASTM 1995).

3.2.4. Results

**Bauxsol Pellets Made with Modified Makeup Waters: Trial 1**
The use of modified makeup waters had little effect on reducing soluble pH of the pellet material. Saturated CO₂ water showed a faster foaming reaction rate of the mix following the addition of aluminum powder compared to mixes with NaHCO₃ saturated water (a few minutes vs 2 h). The final pH of the pellets was similar and unacceptable (between pH 11.5 and 11.8) regardless of the makeup water composition, the curing process (± steaming), or the Bauxsol:cement ratio. Controls samples without aluminum failed to incite mixture foaming.

**Bauxsol Pellets Cured in Saturated Bath: Trials 2 and 3**
Irrespective of the Bauxsol:cement ratio for samples prepared in Trial 2 (100 g batches), the reaction pH before curing was approximately 12.3 (Table 3.2). A slight trend to lower pHs with higher Bauxsol:cement ratios was noted. The pH of samples prepared for Trial 3 (1 kg batches) was approximately 11.3, except for Bauxsol:cement ratio 2.8:1 which recorded a pH 9.86 (Table 3.3).
Table 3.2: Alkalinity, Electrical Conductivity, and pH of the Different Bauxsol:Cement Ratios before and after Different Curing Processes, Trial 2 (100 g batch). Standard analytical error: pH ±0.01, EC ±0.2%, total alkalinity ±2 mg/L.

<table>
<thead>
<tr>
<th>Ratio</th>
<th>EC (µS) before bath</th>
<th>reaction pH before bath</th>
<th>total alkalinity as CaCO₃ (mg/L) before bath</th>
<th>curing process</th>
<th>EC (µS) after bath</th>
<th>reaction pH after bath</th>
<th>total alkalinity as CaCO₃ (mg/L) after bath</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2:1</td>
<td>7250</td>
<td>12.41</td>
<td>1412.5</td>
<td>H₂O</td>
<td>2680</td>
<td>11.42</td>
<td>349.4</td>
</tr>
<tr>
<td></td>
<td>7380</td>
<td>12.41</td>
<td>1412.1</td>
<td>Brine I (MgCl₂, CaCl₂)</td>
<td>17900</td>
<td>8.5</td>
<td>49.3</td>
</tr>
<tr>
<td></td>
<td>7360</td>
<td>12.41</td>
<td>1448.9</td>
<td>Brine II (NaHCO₃)</td>
<td>11250</td>
<td>10.72</td>
<td>6100.8</td>
</tr>
<tr>
<td>2.3:1</td>
<td>7400</td>
<td>12.43</td>
<td>1442.7</td>
<td>H₂O + CO₂</td>
<td>2760</td>
<td>10.65</td>
<td>596.1</td>
</tr>
<tr>
<td></td>
<td>7420</td>
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<td>1383</td>
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<tr>
<td></td>
<td>6970</td>
<td>12.38</td>
<td>1258.2</td>
<td>Brine II (NaHCO₃)</td>
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<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>7300</td>
<td>12.38</td>
<td>1264.8</td>
<td>H₂O + CO₂</td>
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<td>10.8</td>
<td>393</td>
</tr>
<tr>
<td></td>
<td>2.6:1</td>
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<td>12.32</td>
<td>H₂O</td>
<td>3840</td>
<td>11.44</td>
<td>475.4</td>
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<tr>
<td></td>
<td>7100</td>
<td>12.23</td>
<td>1243.5</td>
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<tr>
<td></td>
<td>7210</td>
<td>12.38</td>
<td>1340.2</td>
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<td>10.84</td>
<td>5021</td>
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<td></td>
<td>7250</td>
<td>12.35</td>
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<td>1156.1</td>
<td>H₂O</td>
<td>2850</td>
<td>11.67</td>
<td>418.5</td>
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<td></td>
<td>6970</td>
<td>12.38</td>
<td>1262</td>
<td>Brine I (MgCl₂, CaCl₂)</td>
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<td>8.37</td>
<td>38.2</td>
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<td></td>
<td>6820</td>
<td>12.34</td>
<td>1217.3</td>
<td>Brine II (NaHCO₃)</td>
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<td>6710</td>
<td>12.32</td>
<td>1122.9</td>
<td>H₂O + CO₂</td>
<td>2030</td>
<td>10.54</td>
<td>383.5</td>
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<tr>
<td>2.8:1</td>
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<td>12.31</td>
<td>1318.1</td>
<td>H₂O</td>
<td>3150</td>
<td>11.39</td>
<td>347.2</td>
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<td></td>
<td>6790</td>
<td>12.34</td>
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<td>Brine I (MgCl₂, CaCl₂)</td>
<td>18220</td>
<td>8.31</td>
<td>65.5</td>
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<td></td>
<td>6930</td>
<td>12.35</td>
<td>1251</td>
<td>Brine II (NaHCO₃)</td>
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<td>10.68</td>
<td>5136.9</td>
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<tr>
<td></td>
<td>6780</td>
<td>12.28</td>
<td>1239</td>
<td>H₂O + CO₂</td>
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<td>10.51</td>
<td>487.9</td>
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<td>H₂O</td>
<td>2770</td>
<td>11.53</td>
<td>350.3</td>
</tr>
<tr>
<td></td>
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<td>12.27</td>
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<td>Brine I (MgCl₂, CaCl₂)</td>
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<td>8.28</td>
<td>75.1</td>
</tr>
<tr>
<td></td>
<td>6270</td>
<td>12.3</td>
<td>1087.9</td>
<td>Brine II (NaHCO₃)</td>
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<td>10.65</td>
<td>8140.9</td>
</tr>
<tr>
<td></td>
<td>6170</td>
<td>12.27</td>
<td>1050.4</td>
<td>H₂O + CO₂</td>
<td>2381</td>
<td>10.55</td>
<td>547</td>
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</tbody>
</table>
Table 3.3: Alkalinity, Electrical Conductivity, Reaction pH of Four Different Bauxsol:Cement Ratios before and after Brine I (MgCl₂, CaCl₂) Curing Process, Trial 3 (1 kg batch). Standard analytical error: pH ±0.01, EC ±0.2%, total alkalinity ±2 mg/L.

<table>
<thead>
<tr>
<th>ratio (Brine I)</th>
<th>EC (µS) before bath</th>
<th>reaction pH before bath</th>
<th>total alkalinity as CaCO₃ (mg/L) before bath</th>
<th>EC (µS) after bath</th>
<th>reaction pH after bath</th>
<th>total alkalinity as CaCO₃ (mg/L) after bath</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.45:1</td>
<td>3896</td>
<td>11.11</td>
<td>285.2</td>
<td>11720</td>
<td>8.05</td>
<td>35.4</td>
</tr>
<tr>
<td>2.6:1</td>
<td>4144</td>
<td>11.16</td>
<td>216</td>
<td>14064</td>
<td>8.75</td>
<td>32</td>
</tr>
<tr>
<td>2.7:1</td>
<td>3832</td>
<td>11.56</td>
<td>506.8</td>
<td>15632</td>
<td>8.39</td>
<td>23.4</td>
</tr>
<tr>
<td>2.8:1</td>
<td>3880</td>
<td>9.86</td>
<td>132.8</td>
<td>12928</td>
<td>9.05</td>
<td>44.5</td>
</tr>
</tbody>
</table>

The Milli-Q water bath control showed that the reaction pH of Bauxsol pellets was approximately 11.53. Pellets cured in brine solutions and carbon dioxide bath displayed lower reaction pHs (Tables 3.2 and 3.3; Figures 3.1 and 3.2). The sodium bicarbonate and the carbon dioxide baths gave similar reaction pH values: 10.72 for bicarbonate cured materials and 10.62 for saturated CO₂-cured material. This approximates an 80% reduction in available hydroxide as pH decreases from 11.5 to 10.6. The greatest reduction of Bauxsol pellet reaction pH was recorded for Brine I (MgCl₂, CaCl₂) where an average pH of 8.4 (or a 99.9% reduction in available OH⁻) was found in Trial 2 and a pH of 8.6 for Trial 3 samples (Tables 3.2 and 3.3; Figures 3.1 and 3.2). Figure 3.2 also illustrates that the reaction pH for Trial 3 showed a general increase with higher Bauxsol:cement ratios, which was the reverse of the observed reaction pH of Trial 2 samples.

![Figure 3.1: Comparing reaction pH of the six different Bauxsol:cement ratios (2.2:1, 2.3:1, 2.45:1, 2.6:1, 2.7:1, 2.8:1) after different curing baths: H₂O, Brine I (MgCl₂, CaCl₂), Brine II (NaHCO₃), H₂O + CO₂; Trial 2 (100 g batch). Standard analytical error ±0.01.](image-url)
In general, the EC of the material was slightly more elevated for higher Bauxsol:cement ratios (Tables 3.2 and 3.3). However, Trial 2 showed a higher average EC of about 6900 µS (Table 3.2), whereas Trial 3 showed an EC of about 3900 µS (Table 3.3). Soluble EC decreased to about 3050 µS and 2415 µS for the Milli-Q water and CO₂ baths respectively, whereas EC increased to about 16370 µS and 10370 µS for the two respective brine baths (Table 3.2; Figure 3.3). The reason for the low EC of ratio 2.45:1 may be the product of a faulty probe reading. The EC also increased considerably in Trial 3 to an average of 13586 µS (Table 3.3).
The total alkalinity (as CaCO$_3$) for sample material before curing decreased with increasing Bauxsol content in Trial 2 but was not seen in Trial 3 where a Bauxsol:cement ratio 2.7:1 provided the highest alkalinity (Tables 3.2 and 3.3). The alkalinity ranged from 1023 mg/L (ratio 2.8:1) to about 1449 mg/L (ratio 2.2:1) in Trial 2 and from 133 mg/L to 507 mg/L in Trial 3 (Tables 3.2 and 3.3). The total alkalinity after the curing process (Tables 3.2 and 3.3; Figures 3.2 and 3.4) showed on average, a smaller increase in Trial 2 (+32%) and a decrease in Trial 3 (~88%). This did not follow the previous high ratio/low alkalinity observations. Brine II also showed a reduction in alkalinity but remained unacceptably high at an average of 5675 mg/L; the CO$_2$ bath showed an average alkalinity of about 470 mg/L. The Milli-Q water curing process gave a total alkalinity average of 392 mg/L, whereas, Brine I led to a substantial alkalinity reduction (from about 1254 mg/L before bath to about 71 mg/L after curing). Comparing the total alkalinity (as CaCO$_3$) of the four Bauxsol:cement ratios from Brine I, the lowest alkalinity was recorded for ratio 2.6:1 (38.2 mg/L) and the highest reading was found for the ratio 2.45:1 (132 mg/L). In Trial 3, the total alkalinity (as CaCO$_3$) was relatively low: 23.4 mg/L (ratio 2.7:1); 32 mg/L (ratio 2.6:1); 35.4 mg/L (ratio 2.45:1); and 44.5 mg/L (ratio 2.8:1) (Tables 3.2 and 3.3; Figures 3.2 and 3.4).
Figure 3.4: Comparing total alkalinity (CaCO$_3$) (mg/L) of the six different Bauxsol:cement ratios (2.2:1, 2.3:1, 2.45:1, 2.6:1, 2.7:1, 2.8:1) after different curing baths: H$_2$O, Brine I (MgCl$_2$, CaCl$_2$), Brine II (NaHCO$_3$), H$_2$O + CO$_2$; Trial 2 (100 g batch). Standard analytical error ±2 mg/L.

The pH stability test for the small batch showed that Bauxsol pH equilibrated after an average of 10 min for the Brine II (sodium bicarbonate), whereas pH equilibration took almost 15 min for the Milli-Q water bath and for the carbon dioxide bath. Brine I (MgCl$_2$, CaCl$_2$) took 12 min to equilibrate. The initial pH of the four different baths (8.4 Milli-Q water, 6.58 Brine I, 7.78 Brine II, 4.58 CO$_2$) rose after the curing process by 3.71 pH units in the Milli-Q water bath and by approximately 1.5 pH units in the three other baths (Supporting Information: Table 3.S3).

The pH stability test for the large batch (Trial 3) showed that the pH stabilised after 19 min for the Bauxsol:cement ratio 2.6:1, whereas it took 16 min, 5 min and 4 min for ratios 2.45:1, 2.8:1 and 2.7:1 respectively, to reach the stabilisation. The ratio 2.6:1 also provided the highest pH of 9.39, followed by the ratio 2.45:1 (pH 9.29), ratio 2.7:1 (pH 7.97) and ratio 2.8:1 (pH 7.88). However, after 1 h equilibration, the pH became constant with only minimal changes over a week equilibration (Table 3.4).
Table 3.4: pH Stability Test for Trial 2 (100 g batch) between Curing Baths: H₂O; Brine I (MgCl₂, CaCl₂); Brine II (NaHCO₃); H₂O + CO₂ and Trial 3 (1 kg batch) between the Four Bauxsol:Cement Ratios of Brine I (MgCl₂, CaCl₂). Standard analytical error ±0.01.

<table>
<thead>
<tr>
<th>Trial 2: curing process</th>
<th>Trial 3: Bauxsol:cement ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H₂O</td>
</tr>
<tr>
<td>pH at T=0</td>
<td>10.83</td>
</tr>
<tr>
<td>pH stable after “x” min</td>
<td>11.37</td>
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<tr>
<td>pH at T=1 h</td>
<td>11.32</td>
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<tr>
<td>pH at T=3 h</td>
<td>11.35</td>
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<tr>
<td>pH at T=6 h</td>
<td>11.39</td>
</tr>
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<td>pH at T=24 h</td>
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<td>pH at T=48 h</td>
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<td>pH at T=72 h</td>
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<tr>
<td>pH at T= 7 days</td>
<td>-</td>
</tr>
</tbody>
</table>

The electrical conductivity washing drop test showed a substantial reduction of EC (more than 10000 µS) for each ratio of Brine I (Trial 2) after the second wash (Supporting Information: Table 3.S2). The decline in soluble EC continued with increasing washes but the proportional change decreased, suggesting that a plateau for the EC will be reached, which is proportional to the solubility or diffusion rates of the matrix binder.

Slake durability test for Trial 2 samples using the modified method indicated that all the pelleted materials were considered as weak soil materials. All samples showed a loss of more than 50% of the original material for the Bauxsol:cement ratios 2.6:1, 2.7:1, and 2.2:1. A mass loss of 50% was recorded for the ratio 2.8:1, and a mass loss of 42% and 34% for the ratios 2.45:1 and 2.3:1, respectively, was noted. The same test showed a mass loss for each ratio in Trial 3 of approximately 45%.

3.2.5. Discussion

Bauxsol pellets made using OPC (but not additionally treated) provide alkalinity and pH spikes to waters in which they are immersed, making the treatment of circum-neutral waters difficult. The conversion of soluble alkalinity to insoluble form appears to be the best option for controlling alkalinity release. This paper investigated two modes of control: alkalinity conversion during the production of the pellets using modified makeup waters, and alkalinity conversion postproduction using curing.
brines. The second option was the most effective and may have applications for pretreating concrete structures to be placed in pH and alkaline sensitive systems.

**Bauxsol Pellets Made with Modified Makeup Waters: Trial 1**

The use of alkalinity modifiers during the production of the pellets was not appropriate because the addition of either NaHCO₃ or CO₂ to the makeup water had little impact on solubility (Table 3.1). The quantities of CO₂ and NaHCO₃ added to the mix were insufficient to eliminate excess hydroxide in the slurry (sodium bicarbonate only 12% of the Bauxsol weight). The foaming reaction kinetics with the sodium bicarbonate slowed down because the presence of bicarbonate lowered pH by consuming free hydroxide, thus, reducing the early stage attack on the aluminum and thereby potentially reducing the initial rise of the mixture and so acting as a set inhibitor (De Chiffre and De Chiffre 1989). Moreover, because hydroxide is required to affect set and curing, conversion of hydroxide at the slurry stage effected set strength. Typically when dissolved salts exceed 1000 ppm in makeup water for concrete, setting time and strength (28 days) were effected. In addition, the curing of cement materials relies on CaO and water, interacting with poorly crystalline silicates to develop Ca-silicates that bind the material (Bhatt 1995). In essence, the addition of NaHCO₃ and CO₂ to the makeup water had greater detrimental effects and was unable to control alkalinity.

**Bauxsol Pellets Cured in Saturated Bath: Trials 2 and 3**

Soaking of pellets in brines was found to be more effective in controlling pH and alkalinity. The pH and alkalinity results prior to the curing process suggest that unreacted cement in the pellet structure (as high as 43% of the OPC) (Goni et al. 2002) was the dominant contribution to the pH/alkalininity spike (higher cement loadings in lower Bauxsol:cement ratio), although some partially neutralized particles of Bauxsol may also had an effect. The data of Trial 2 (small batch) showed that the lower the Bauxsol:cement ratio, the greater the soluble alkalinity (Tables 3.2 and 3.3; Figures 3.2 and 3.4). However, absolute differences were minor, indicating that a substantial change in Bauxsol content produced only a small change in alkalinity. The relatively high total alkalinity of ratio 2.45:1 from Brine I may be explained by the surprisingly low EC reading. The observed trend of Trial 2 changed with the larger batches (Trial 3) (Table 3.3; Figure 3.2) and are best explained by the batch sizes
(possible inhomogeneity in the mixing). The larger sample sizes in Trial 3 required much longer curing times (approximately two months; seven days for Trial 2 batches), and may represent incomplete curing (Table 3.3; Figure 3.2). Also, differences in alkalinity seen in precured materials may reflect cementation processes becoming more fully developed in larger batches than in smaller samples, and therefore providing lower leaching materials.

Brine II (NaHCO$_3$) treatments of pellets demonstrate that alkalinity conversion follows the reaction pathway:

$$\text{NaHCO}_3 + \text{OH}^- \rightarrow \text{Na}^+ + \text{CO}_3^{2-} + \text{H}_2\text{O}$$  \hspace{1cm} (1)

This reaction allows for the conversion of high pH/alkalinity (hydroxide) to the highly soluble sodium carbonate form which has a reduced reaction pH compared to control samples. Hence for Brine II, the alkalinity and salt addition theory (Stumm and Morgan 1996; Evangelou 1998) suggest that only a reduction in soluble pH should be observed, whereas an increase of soluble alkalinity and EC should be found (Table 3.2; Figures 3.1, 3.3, and 3.4). The increase in soluble alkalinity is most likely due to the residual divalent carbonate present converted from a monovalent hydroxide (NaHCO$_3$). These effects (increase in alkalinity and EC, and slight reduction of soluble pH) were evident in the data (Table 3.2; Figures 3.1, 3.3, and 3.4). Hence Brine II does not produce the alkalinity conversion desirable for the treatment of circum-neutral waters.

The H$_2$O + CO$_2$ treatment is similar to Brine II except that extra salts are not added, rather bicarbonate is produced by:

$$\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{HCO}_3^- + \text{H}^+$$  \hspace{1cm} (2)

$$\text{HCO}_3^- + \text{H}^+ + 2\text{OH}^- \rightarrow \text{CO}_3^{2-} + \text{H}_2\text{O}$$ \hspace{1cm} (3)

Since no salts are added to the system, alkalinity conversions (eqs 2 and 3) and salt addition theory (Stumm and Morgan 1996; Evangelou 1998) suggest a reduction in reaction pH. However little or no change in EC or soluble alkalinity should be seen (Table 3.2; Figures 3.1, 3.3, and 3.4) (Khaitan et al. 2009). Hence, CO$_2$ neutralization does not covert soluble alkalinity but causes a change in alkalinity speciation (i.e. hydroxide alkalinity conversion to carbonate) that affects pH. This observation was
previously made in red mud neutralizations (Johnston et al. 2008; Khaitan et al. 2009). Although an EC change was made and a slight change in pH was observed, alkalinity of the cured materials was still high. Therefore, this does not provide a particularly suitable solution.

Brine I is a mixture of Ca and Mg chlorides which precipitate alkalinity through the Basecon™ technology (McConchie et al. 2002) by the following:

\[
6\text{MgCl}_2 + 2[\text{Al(OH)}_4]^- + 8\text{OH}^- + \text{CO}_3^{2-} + 12\text{Na}^+ \rightarrow \text{Mg}_6\text{Al}_2(\text{CO}_3\text{(OH)}_16\cdot 4\text{H}_2\text{O} + 12\text{NaCl} \quad (4)
\]

\[
\text{CaCl}_2 + 2[\text{Al(OH)}_4]^- + \text{CO}_3^{2-} + 2\text{Na}^+ \rightarrow \text{CaAl}_2(\text{CO}_3\text{(OH)}_4\cdot 3\text{H}_2\text{O} + 4\text{OH}^- + 2\text{NaCl} \quad (5)
\]

and

\[
2\text{CO}_3^{2-} + 2\text{OH}^- + \text{Ca}^{2+}, 2\text{Mg}^{2+} \rightarrow \text{MgCO}_3 + \text{CaCO}_3 + \text{MgOH}_2
\quad (6)
\]

Aluminum is deposited into solution above pH 8.5 as aluminate ion [Al(OH)_4]^- (Stumm and Morgan 1996; Evangelou 1998), therefore at higher pH (>pH 10) substantial aluminum may be mobilized from the pellet. To effectively influence alkalinity precipitation Ca and Mg chlorides must be added. Alkalinity conversions (eqs 4, 5 and 6) and salt addition theory (Stumm and Morgan 1996; Evangelou 1998) suggest a reduction in reaction pH and soluble alkalinity and an increase of EC (Table 3.2; Figures 3.1, 3.3, and 3.4). The data from Trials 2 and 3 confirmed this. However, the increase of reaction pH with increase of the Bauxsol:cement ratio in Trial 3 possibly came from the dilution of brine solution, which prevented the full neutralization reactions. This result may also reflect the increase in the volumes of the samples to be neutralized or a combination of the two. Johnston et al. (2008) showed that for the same ionic strength, the CaCl_2 and MgCl_2 are 9 and 40 times, respectively, more EC responsive than NaOH. The substantial increase in EC for Trial 2 (Table 3.2; Figure 3.3) was most likely caused by divalent Ca and Mg as residual interstitial brine. The washing data showed that by rinse four some 93% of the soluble salts were removed. Therefore rinsing brine cured pellets with a small quantity of fresh water prevents water EC increases.
During the pH stability test each mix ratio of Brine I and Brine II (Table 3.4) showed a rapid equilibration, which suggests that when all hydroxides have precipitated the minerals deposited are almost insoluble. Moreover, the pH remained stable even after a few days unlike the CO$_2$ neutralised materials of Khaitan et al. (2009). However, Trial 3 batches (Table 3.4) showed a steadier pH stability time, presumably due to the larger surface to volume ratios, thereby creating longer diffusion paths into the pellet material. Consequently the brine took more time to precipitate alkalinity. Explanations for the high pH (above 9 after 3h) of Bauxsol:cement ratios 2.6:1 and 2.8:1 include a fresh exposure of unreacted hydroxides ions from internal fracturing and pore activation to previously unexposed areas.

Because aluminum powder has been used as a foaming agent (De Chiffre and DeChiffre 1989), and in relatively large quantity (2% of cement mass or 0.5% of total dry weight), the slake durability index was poor. Yam (2006) found comparable results with 0.6% aluminum powder of cement mass. While small improvements in slake durability were observed in Trial 3, no discernable differences between the treatment brine or the Bauxsol:cement ratio were noted. The data suggests that the pellets produced in this work may have handling issues (i.e. breakage and size reductions leading to clogging and lower hydraulic conductivities). This is at odds with work reported by Clark et al. (2005), where <6% of the pellet material was lost using hydrogen peroxide as a foaming agent. Consequently, the current data would suggests that slake durability is mostly dependent on the cement set development, foaming agent used, Bauxsol loading rate (Bauxsol:cement ratio), and porosity of the foamed material.

This study demonstrates the possibility of producing Bauxsol pellets using OPC bonds with minimal pH/alkalinity spikes using magnesium and calcium chlorides curing bath. The best performing materials (total alkalinity <80 mg/L and pH <8.3) was obtained with Bauxsol:cement ratio 2.8:1 for Trial 2 (100 g batch) and ratio 2.45:1 for Trial 3 (1 kg batch). This work suggested that the use of a MgCl$_2$, CaCl$_2$ brine could be employed to condition the outside surfaces of concrete structures that are to be placed in pH and alkaline sensitive systems. This breakthrough is important as these new type of porous Bauxsol pellets can be used as phosphate binders and also as “biomass support particles” for the development of bacterial biofilms in circum-
neutral wastewater treatment without causing lasting pH/alkalinity spikes. Biogeochemical activities associated with microbial consortia in the biofilm may help further remediate wastewater. However, the pellets may still contain residual brine and/or unreacted CaO. Consequently, a freshwater rinse of pellets before treating circum-neutral waters is recommended, as is the continued investigation of alternative pellet binders.

3.2.6. Acknowledgments

The paper draws inspiration and builds on the previous work of the late Professor David McConchie, Southern Cross University, who saw potential where others saw none. This work was also supported by an Australian Research Council Grants LP0212037, and LP0056012.

3.2.7. Supporting Information

**Method description**

The pH and EC were recorded using a “TPS labCHEM-CP Benchtop Conductivity/TDS–pH/mV–Temperature”.

Alkalinity test was performed as follows: 5 g of pellets in 25 mL of MilliQ-water tumbling for 48 h for Trial 2 (100 g batch) and 100 g in 500 mL tumbling for 72 h for Trial 3 (1 kg batch); titration performed using a volume of 20 mL and 50 mL for Trials 2 and 3, respectively; pH and mL of 0.1N HCl used recorded at 10.2, 8.3, 7–8 and 4.5.

A modification of the standard method of determining slake durability – ASTM-D 4644 (ASTM 1995) – was carried out using a drum basket covered with 2 mm mesh (10% of the drum is solid support) turning at 40 rpm for 5 min in a bucket half full of tap water to abrade the material. Samples pieces were removed and oven dried at 65°C for 24 h and were then reweighed.
### Results

**Table 3.S1**: Alkalinity Speciation of Different Bauxsol:cement Ratios after Different Curing Baths – Trial 2 (100 g batch). Standard analytical error ±2 mg/L.

<table>
<thead>
<tr>
<th>ratio</th>
<th>curing process</th>
<th>total alkalinity as CaCO₃ (mg/L) after bath</th>
<th>hydroxide as OH⁻ (mg/L) after bath</th>
<th>carbonate as CO₃²⁻ (mg/L) after bath</th>
<th>bicarbonate as HCO₃⁻ (mg/L) after bath</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2:1</td>
<td>H₂O</td>
<td>349.4</td>
<td>44.9</td>
<td>127.1</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>Brine I (MgCl₂, CaCl₂)</td>
<td>49.3</td>
<td>0.1</td>
<td>2.0</td>
<td>55.8</td>
</tr>
<tr>
<td></td>
<td>Brine II (NaHCO₃)</td>
<td>6100.8</td>
<td>9.7</td>
<td>3333.1</td>
<td>625.6</td>
</tr>
<tr>
<td></td>
<td>H₂O + CO₂</td>
<td>596.1</td>
<td>7.3</td>
<td>299.4</td>
<td>91.5</td>
</tr>
<tr>
<td>2.3:1</td>
<td>H₂O</td>
<td>409.6</td>
<td>83.4</td>
<td>97.1</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>Brine I (MgCl₂, CaCl₂)</td>
<td>67.4</td>
<td>0.1</td>
<td>2.6</td>
<td>76.7</td>
</tr>
<tr>
<td></td>
<td>Brine II (NaHCO₃)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>H₂O + CO₂</td>
<td>393.0</td>
<td>10.5</td>
<td>195.8</td>
<td>43.4</td>
</tr>
<tr>
<td>2.45:1</td>
<td>H₂O</td>
<td>475.4</td>
<td>52.1</td>
<td>189.0</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>Brine I (MgCl₂, CaCl₂)</td>
<td>132.0</td>
<td>0.1</td>
<td>4.5</td>
<td>151.6</td>
</tr>
<tr>
<td></td>
<td>Brine II (NaHCO₃)</td>
<td>5021.0</td>
<td>13.4</td>
<td>2788.4</td>
<td>403.6</td>
</tr>
<tr>
<td></td>
<td>H₂O + CO₂</td>
<td>418.1</td>
<td>7.4</td>
<td>206.3</td>
<td>63.6</td>
</tr>
<tr>
<td>2.6:1</td>
<td>H₂O</td>
<td>418.5</td>
<td>77.1</td>
<td>113.4</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>Brine I (MgCl₂, CaCl₂)</td>
<td>38.2</td>
<td>0.0</td>
<td>1.2</td>
<td>44.0</td>
</tr>
<tr>
<td></td>
<td>Brine II (NaHCO₃)</td>
<td>3975.5</td>
<td>9.3</td>
<td>2149.7</td>
<td>442.3</td>
</tr>
<tr>
<td></td>
<td>H₂O + CO₂</td>
<td>383.5</td>
<td>5.5</td>
<td>182.6</td>
<td>76.6</td>
</tr>
<tr>
<td>2.7:1</td>
<td>H₂O</td>
<td>347.2</td>
<td>44.0</td>
<td>127.2</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>Brine I (MgCl₂, CaCl₂)</td>
<td>65.5</td>
<td>0.0</td>
<td>1.8</td>
<td>76.0</td>
</tr>
<tr>
<td></td>
<td>Brine II (NaHCO₃)</td>
<td>5136.9</td>
<td>8.7</td>
<td>2776.2</td>
<td>586.2</td>
</tr>
<tr>
<td></td>
<td>H₂O + CO₂</td>
<td>487.9</td>
<td>5.3</td>
<td>2341.1</td>
<td>99.8</td>
</tr>
<tr>
<td>2.8:1</td>
<td>H₂O</td>
<td>350.3</td>
<td>57.9</td>
<td>105.8</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>Brine I (MgCl₂, CaCl₂)</td>
<td>75.1</td>
<td>0.0</td>
<td>1.9</td>
<td>87.5</td>
</tr>
<tr>
<td></td>
<td>Brine II (NaHCO₃)</td>
<td>8140.9</td>
<td>8.3</td>
<td>4390.3</td>
<td>968.4</td>
</tr>
<tr>
<td></td>
<td>H₂O + CO₂</td>
<td>547.0</td>
<td>5.7</td>
<td>266.0</td>
<td>105.6</td>
</tr>
</tbody>
</table>

**Table 3.S2**: Changes in Electrical Conductivity of Four Bauxsol:cement Ratios (2.45:1, 2.6:1, 2.7:1, 2.8:1) of Brine I (MgCl₂, CaCl₂) recorded by a 1:5 Solid to Water Ratio Wash – Trial 2 (100 g batch). Standard analytical error ±0.2%.

<table>
<thead>
<tr>
<th>ratio (Brine I)</th>
<th>1&lt;sup&gt;st&lt;/sup&gt; wash 1 h EC (µS)</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; wash 2 h EC (µS)</th>
<th>3&lt;sup&gt;rd&lt;/sup&gt; wash 3 h EC (µS)</th>
<th>4&lt;sup&gt;th&lt;/sup&gt; wash 4 h EC (µS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.45:1</td>
<td>14980</td>
<td>4750</td>
<td>1909</td>
<td>976</td>
</tr>
<tr>
<td>2.6:1</td>
<td>14940</td>
<td>4580</td>
<td>1877</td>
<td>1039</td>
</tr>
<tr>
<td>2.7:1</td>
<td>15060</td>
<td>4730</td>
<td>1762</td>
<td>754</td>
</tr>
<tr>
<td>2.8:1</td>
<td>14510</td>
<td>4320</td>
<td>2028</td>
<td>948</td>
</tr>
</tbody>
</table>

79
Table 3.S3: Initial and Final Reaction pH of Four Curing Baths: H₂O, Brine I (MgCl₂, CaCl₂), Brine II (NaHCO₃), H₂O + CO₂ – Trial 2 (100 g batch). Standard analytical error ±0.01.

<table>
<thead>
<tr>
<th></th>
<th>H₂O</th>
<th>Brine I</th>
<th>Brine II</th>
<th>CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>initial reaction pH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- before bath</td>
<td>8.40</td>
<td>6.58</td>
<td>7.78</td>
<td>4.85</td>
</tr>
<tr>
<td>final reaction pH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- after bath</td>
<td>12.11</td>
<td>8.19</td>
<td>9.24</td>
<td>6.58</td>
</tr>
</tbody>
</table>

Table 3.S4: Alkalinity Speciation of Four Different Bauxsol:cement Ratios After Brine I Curing Bath (MgCl₂, CaCl₂) – Trial 3 (1 kg batch). Standard analytical error of total alkalinity ±2 mg/L.

<table>
<thead>
<tr>
<th>ratio (Brine I)</th>
<th>total alkalinity as CaCO₃ (mg/L) after bath</th>
<th>hydroxide as OH⁻ (mg/L) after bath</th>
<th>carbonate as CO₃⁻ (mg/L) after bath</th>
<th>bicarbonate as HCO₃⁻ (mg/L) after bath</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.45:1</td>
<td>35.4</td>
<td>0.0</td>
<td>0.5</td>
<td>42.1</td>
</tr>
<tr>
<td>2.6:1</td>
<td>32.0</td>
<td>0.1</td>
<td>2.0</td>
<td>34.7</td>
</tr>
<tr>
<td>2.7:1</td>
<td>23.4</td>
<td>0.0</td>
<td>0.7</td>
<td>26.9</td>
</tr>
<tr>
<td>2.8:1</td>
<td>44.5</td>
<td>0.2</td>
<td>4.9</td>
<td>43.7</td>
</tr>
</tbody>
</table>

3.2.8. Literature Cited


CHAPTER 4:MICROCOSM COLUMNS FIELD EXPERIMENT

4.1. Overview
The second step in the successful completion of this thesis was to test the newly developed cured cement-bound porous Bauxsol™ pellets (Chapter 3) in wastewater (i.e. objective 2). The subsequent paper entitled “Bauxsol™ Pellets: Bacterial Communities and Phosphate Removal in Wastewater Treatment” presents the results of a one-month field-based experiment using microcosm columns. The origin of the short-lasting pH spike, observed in the laboratory experiment (Appendix 9.4, Figure 9.2) when using the cured cement-bound Bauxsol™ pellets in these pH circum-neutral wastewaters, was examined. The biocompatibility of Bauxsol™ pellets with environmental bacterial communities was also investigated as a laboratory experiment (Appendix 9.4, Figures 9.3 & 9.4, Table 9.9) demonstrated a bactericide effect linked to the pH spike. In addition, the paper investigated the phosphate removal capacity of Bauxsol™ pellets. Chapter 9 (Appendix 9.6) has additional information on this experiment.

4.2. Bauxsol™ Pellets: Bacterial Communities and Phosphate Removal in Wastewater Treatment

4.2.1. Abstract
Experimental data detailing biocompatibility of cement-bound Bauxsol™ pellets with bacterial communities and pellet efficiency to remove phosphate from wastewater are presented in this paper. Bacterial community profiles of liquid samples using DGGE fingerprinting revealed major differences in bacterial diversity due to temporal event and small changes owing to treatment. Bio-mats grew on Bauxsol™ pellets demonstrating their biocompatibility with bacterial communities. Phosphate was effectively absorbed by formulated pellets until the point of saturation (672 bed volumes). The source of short-lasting pH spikes in the effluent was found to be associated with the release of hydroxide from unreacted CaO in the cement binder used in the pellets production. In essence, cement-bound Bauxsol™ pellets can be used as a support matrix for bacterial communities and as a phosphate binder in
wastewater treatment, providing a pH correction of the treated water in the initial stages.

4.2.2. Introduction

Wastewater is treated to minimise contaminants impacting on public health and the environment. Nitrifying and denitrifying bacteria play a major role in conversion of nitrogen in wastewater treatment. The former group convert ammonia to nitrate in the presence of oxygen whilst the latter reduce nitrate to gaseous nitrogen under anaerobic environments (Bitton 1994). When both nitrification and denitrification ($\text{NH}_4^+ + \text{NO}_2^- \rightarrow \text{N}_2 + 2\text{H}_2\text{O}$) are at play under anaerobic conditions, the process is referred to as anammox (Risgaard-Petersen et al. 2004). Phosphorus in wastewater is typically present as phosphates and is commonly removed through chemical precipitation using trivalent metal cations such as aluminium [$\text{Al}_2(\text{SO}_4)_3$] or iron ($\text{FeCl}_3$). However, precipitation can be both expensive and creates additional hazardous waste (Hammer et al. 2005).

Bauxsol™ (a commercially available seawater-neutralised bauxite refinery residue) is a complex mix of minerals predominantly composed of iron and aluminium oxy-hydroxides. The main characteristics are: finely grained (90% $<10 \, \mu\text{m}$ in diameter); very high surface to volume ratio (up to 100 $\text{m}^2/\text{g}$); high metal binding capacity ($>1,500 \, \text{meq/kg}$); moderate acid neutralising capacity; largely insoluble; and highly non-dispersive (McConchie et al. 1999; Lin et al. 2002; Clark et al. 2009). Bauxsol™ reagents have been effectively employed for environmental contamination issues and water treatment (McConchie et al. 1999; Genc-Fuhrman 2004; Akhurst et al. 2006; Lapointe et al. 2006). These studies have been carried out almost exclusively on acidic waters and/or solids. When used in circum-neutral water treatments, cement-bound Bauxsol™ pellets produce prolonged pH and alkalinity spikes due to the release of hydroxide from unreacted CaO in the cement binder. However, using a curing process ($\text{MgCl}_2$ & $\text{CaCl}_2$ bath) allows the cement pellets to be further treated to minimise the pH/alkalinity spikes (Despland et al. 2010). Consequently, these treated pellets can be used as a phosphate binder and as biomass support media for the development of bacterial biofilms in wastewater treatment without potentially causing long-lasting pH/alkalinity spikes.
Owing to the size and complexity of the bacteria kingdom, different methods are used to study the composition and the activities of bacterial communities. Although sometimes useful, traditional methods such as microscopy, surface-plate count, biochemical profiling and biomass estimation fail to provide sufficient detail about community members and/or their functions under environmental circumstances because greater than 90% of microorganisms remain uncultured (Ward et al. 1990). A number of culture-independent molecular approaches such as fingerprinting techniques have been developed with a view to providing comparative microbial community profiles of different environments, and to follow the behaviour of particular populations over time. One of the most powerful approaches used in exploring microbial diversity in nature is the analysis of bacterial 16S ribosomal RNA (rRNA) genes. 16S rDNA are readily amplified with polymerase chain reaction (PCR) using universal or specific primers. The products are either sequenced or separated by techniques such as denaturing gradient gel electrophoresis (DGGE) or some form of restriction fragment length polymorphism. DGGE is the preferred method and is capable of detecting sequence polymorphism(s) by separating the PCR amplified DNA fragments based on their melting behaviour (Muyzer et al. 1998; Madigan et al. 2006).

This paper examines and reports on the origin of the short-lasting pH spike observed when using treated cement-bound Bauxsol™ pellets in pH circum-neutral waters. The capacity of the pellets to remove phosphate and their biocompatibility with wastewater bacterial communities is also discussed.

4.2.3. Methods

PVC columns (200 mm high x 50 mm diameter) were filled with cured ordinary Portland cement (OPC)-bound Bauxsol™ pellets (5-10 mm diameter) (Despland et al. 2010), OPC-bound Sand pellets (4-8 mm diameter), or acid-washed sand (0.5-2 mm diameter) and fed at 20 mL/min with updraft secondary treated effluent from a Sewage Treatment Plant (South Lismore, NSW, Australia; Appendix 9.2, Figure 9.1) for 28 days (Figure 4.1). Sand, commonly used as filtration/support substrate material, was selected as it easily mixed with the ingredients to produce sand pellets. Although the material weight was different (Sand control: 440 g; Sand pellets: 185 g;
Bauxsol™ pellets: 131 g), the volume of each column was constant (300 mL of solid). To avoid clogging, a nylon fibre was placed beneath the caps at both ends of each column and the system cleaned weekly to remove particulate build up (Appendix 9.6, Figure 9.6).

Liquid samples (300 mL) were collected from the column’s inlet (=influent) and outlet (= effluent) at predetermined bed volumes (Appendix 9.6, Table 9.10). Solids (randomly selected pellets or sand particles) were taken at the end of the experiment from the individual columns for analysis.

A pump failure occurred between samples T=17 and T=22 days (1,616 and 2,024 bed volumes, respectively). The system was restarted at T=21 days, hence there is a possible full four day non-flow period.

Figure 4.1: Microcosm experiment setting at the Sewage Treatment Plant, South Lismore (Australia) - Top: front and back views of the system with the columns (two Sand pellets; one Sand control; three Bauxsol™ pellets); Bottom: pumping from humus tank (2nd treated effluent); bucket, peristaltic pump and pH/EC, DO meters.
Physico-chemical parameters

Influent and effluent pH (APHA 4500 H⁺-b), electrical conductivity (EC) (APHA 2510-b) and temperature (APHA 2550-b) were recorded in-situ every four hours. Dissolved oxygen (APHA 2810-b) was only recorded for the influent (American Public Health Association 1998). In addition, pH and EC were checked for each liquid sample. Faulty pH and EC probes and meters were changed during the trial.

Nutrients determined for liquid samples included: total nitrogen (APHA 4500 N-c), total phosphorus (APHA 4500 P-h), nitrate (APHA 4500 NO₃⁻-f), nitrite (APHA 4500 NO₂⁻-c), ammonia (APHA 4500 NH₃-h) and orthophosphate (APHA 4500 P-g). For solid samples before and after the experiment: total nitrogen (%N; LECO CNS2000 Analyser), total carbon (%C; LECO CNS2000 Analyser), ammonia (1:10 KCl extract; APHA 4500 NH₃-h), nitrate (1:10 KCl extract; APHA 4500 NO₃⁻-f) and phosphate (1:10 KCl extract; Lechat QuickChem method 31-115-01-3-A) (American Public Health Association 1998; Lachat 1998). An alkalinity test was also performed on each liquid sample (20 mL) using the APHA 2320-b method (American Public Health Association 1998), and speciation was determined by an online Alkalinity Calculator (USGS 2009). In addition, pH was recorded for crushed solids (5 g in 25 mL of Milli-Q water). Analyses were undertaken following QA/QC procedures (Appendix 9.1, Table 9.1).

Microbiology

Surface-plate counting was used to determine colony forming units (cfu/mL) for liquid and crushed solid samples (2 g in 20 mL of 0.85% saline). Serial dilutions (100 µL of two dilutions) were spread-plated in triplicate on nutrient agar and incubated at 26°C. CFUs were counted after 72 hrs, 1 week and 2 weeks. Biomass was estimated for individual liquid and crushed solid samples using the bicinchoninic acid protein assay method (Smith et al. 1985).

Molecular analysis of bacterial communities was undertaken on liquid and randomly selected solids from individual columns. DNA extraction was performed as described by Bell et al. (2006) using 80 mL of liquid and approximately 650 mg of solids (Appendix 9.3, Tables 9.2–9.7). 50 ng of isolated DNA was subjected to PCR amplification, specifically targeting the V3 region of 16S rDNA with primers GC-
357F and Alexa labelled 518R-546 (Yu et al. 2004). PCR conditions were optimised at 94°C 5 mins; 35 cycles of 94°C 30 sec, 58°C 30 sec, 72°C 30 sec; and 72°C 10 mins (Appendix 9.3). PCR products were separated by DGGE, using a 6.5% (w/v) polyacrylamide gel with a gradient of 30-70% (150 V for 5 hrs at 60°C) (Appendix 9.3). Analyses were undertaken following QA/QC procedures (Appendix 9.1).

**Statistical analyses**
Data was statistically analysed using the ePRIMER software package (Clarke et al. 2006) to evaluate the diversity and similarity of liquid and solid samples regarding their physico-chemical parameters and their DGGE profiles.

### 4.2.4. Results and discussion

**Physico-chemical parameters**
Influent and effluent pHs were quite stable (5.6-7.0 and 6.0-7.3 pH, respectively). However, at the beginning of the experiment, pH spikes of 8.1 and 9.2 were recorded in Bauxsol™ and Sand pellet effluents (Figure 4.2). Because both pelletised systems produced pH spikes, the Bauxsol™ powder can be ruled out as a primary source of the spikes. The spikes were caused by the release of unreacted CaO from the cement binder used in the pellets production. However, the Bauxsol™ pellets utilised did minimise the spikes because of the curing process used during their production (Despland et al. 2010). A pH spike in the Sand pellet effluent (pH 8.3) was also observed at 2,024 bed volumes (after the system failure/restart) indicating substantial leachable alkalinity remained (Figure 4.2). This pH spike was not observed for the Bauxsol™ pellets suggesting that they were more in equilibrium with the water, and that either low solubility minerals, or the physical properties of the pellets dictated the alkalinity loss. The EC of influent and effluent ranged from 800-900µS except at the beginning where EC levels were recorded at 7,515 and 10,055 µS for Bauxsol™ and Sand pellet effluents, respectively (Appendix 9.6, Table 9.11). The lower pH/EC spike found in the Bauxsol™ pellet effluent compared to the Sand pellet effluent may be explained by the additional strength provided by the Bauxsol™, a reduced micro-permeability and the salt diffusion (Barbhuiya et al. 2010a; Barbhuiya et al. 2010b). Furthermore Bauxsol™ has a high metal binding capacity (>1500 meq/Kg) owing to
its high surface area and its composition which includes iron and aluminium oxy-hydroxides (McConchie et al. 1999).

![Figure 4.2: pH level in effluent (Sand control; Sand pellets; Bauxsol™ pellets) + influent - at predetermined bed volumes; thick grey error bar = ± standard error for Sand pellets (2 columns); thin black error bar = ± standard error for Bauxsol™ pellets (3 columns).](image)

Influent and effluent revealed an average of 250 mg/L of total alkalinity (as CaCO₃), although some spikes occurred at mid-experiment (445 mg/L to 780 mg/L; Appendix 9.6, Table 9.12). The weak alkalinity was primarily caused by the alkalinity speciation (with pH below 8.4): bicarbonate with trace concentrations of carbonates, but no free hydroxide.

Dissolved oxygen varied between 0.02-2.5 ppm in the influent, indicating a hypoxic environment. However, the environment in the columns was initially hyperoxic (i.e. air trapped inside the dry matrix of the pellets) and possibly became anoxic in the centre of the pellets over time due to bacterial accumulation and respiration. Moreover anoxia development and the potential reduction of iron from the Fe³⁺ oxidation state to Fe²⁺ (especially for Bauxsol™ pellets) by bacterial activity invariably raised the pH through the release of carbonate and hydroxide (Clark et al. 1998).

Influent orthophosphate concentrations were on average 5.6 mg/L. Orthophosphate concentration declined by 90% and 77% in both pelletised columns effluent and Sand control effluent respectively, after one bed volume (Figure 4.3; Appendix 9.6, Table
Saturation was recorded after 16 bed volumes for the Sand control column because only minimal precipitation of phosphate with quartz was possible. On the other hand, both pelletised systems plateaued at 10% phosphate reduction after 672 bed volumes (Figure 4.3; Appendix 9.6, Table 9.13), indicating that fast kinetic reactions were exhausted and only slower kinetic reactions were removing orthophosphate. Despite the apparent presence of more reaction sites in the Bauxsol™ (McConchie et al. 1999; Akhurst et al. 2006), the effectiveness of phosphate removal was similar for both types of pellets probably because of the formation of a coat on the surface of the Bauxsol™ pellets, preventing more removal. The pellet curing process may have affected the Bauxsol™ binding capacity (Despland et al. 2010). A pH test on crushed solids showed that Sand particles and Sand pellets had a pH less than the 8.4 carbonate endpoint limit (7.7 and 8, respectively) whereas Bauxsol™ pellets had a pH above the limit (8.6). This suggests further alkalinity release may still occur with the Bauxsol™ pellets.

The precipitation of phosphate on Al, Fe, Ca, Mg and Si oxides appears as the principal cause of phosphorus reduction, although some ligand exchange and surface adsorption occurred in the early stages. According to Li & Stanforth (2000) and Tanada et al. (2003), Al and Fe oxyhydroxides are among the most effective adsorbents for phosphate removal from solution. Akhurst et al. (2006) also found
similar results and suggests that phosphate adsorption might be increased when Bauxsol™ reagent is formed into porous pellets. At 2,024 bed volumes (after the system failure/restart) an increase of 80% orthophosphate in the Sand control effluent suggests that all orthophosphate removal achieved in the first few bed volumes was released. A similar spike (4% increase) was recorded in both pelletised effluent (Figure 4.3; Appendix 9.6, Table 9.13). The release most likely represents exchangeable fractions mobilised by changing geochemical conditions (anoxia development in stagnant waters). Furthermore, it suggests that Sand particles and Sand pellets are exchange site dominated, whereas Bauxsol™ pellets irreversibly bind orthophosphate most likely through precipitations reactions. The constant release of Ca from the leaching of the OPC pellet binder suggests that phosphate removal is as insoluble Ca$_3$(PO$_4$)$_2$ or CaHPO$_4$ for the observed effluent pHs.

Throughout the trial, equal concentrations of nitrate were found in both the influent and effluent (~20 mg/L). Likewise ammonia concentrations (5-10 mg/L) were uniform. Nitrite concentrations fluctuated between 0.15-0.6 mg/L in both pelletised effluents and influent, and between 0.05-0.4 mg/L in Sand control effluent (Appendix 9.6, Table 9.13). Cumulative data over the one-month trial showed that the level of ammonia concentrations in the effluent increased by 10%, whereas nitrate concentrations declined by 10%. Nitrite concentrations decreased on average by 45% in the Sand control effluent, and by 15% in Bauxsol™ pellet effluent, but increased by 10% in Sand pellet effluent. These results suggest nitrogen transformations, but as the hydraulic retention time was only 20 minutes, biological nitrogen removal was minimal. Physico-chemical bonds to the substrates were most likely to be the direct consequence of changes in ammonia, nitrite and nitrate in the effluent.

Chemical analysis of pelleted material (before and after experiment) showed that phosphate concentrations increased for each material: 4.78 to 13.76 mg/kg for Sand particles; 0.07 to 18.93 mg/kg for Sand pellets; 0.05 to 2.57 mg/kg for Bauxsol™ pellets (Appendix 9.6, Table 9.14). However, a mass balance of phosphorous removal (Figure 4.3) showed that much more phosphorous bonded in the Bauxsol™ pellets than what was extracted. Consequently, the technique used to assess the amount of phosphate on solids (1:10 KCl extract; QuickChem 31-115-01-3-A) was too weak to effectively attack the Bauxsol™ mineral structure and extract the totality of
phosphates bond on or in the Bauxsol™ pellets. As a result, the phosphate concentration on Bauxsol™ pellets has been underestimated. Phosphate was most effectively removed to the solids by a precipitation process and not by ligand exchange because of the inability to extract phosphorous back off Bauxsol™ pellets. Nitrate concentrations rose from 5.35 to 9.85 mg/kg for Sand pellets and from 7.02 to 41.58 mg/kg for Bauxsol™ pellets, but decreased from 61.32 to 1.49 mg/kg for Sand particles (Appendix 9.6, Table 9.14). Ammonia increased for each material: 4.53 to 13.57 mg/kg for Sand particles; 2.92 to 39.3 mg/kg for Sand pellets; 3.06 to 67.74 mg/kg for Bauxsol™ pellets (Appendix 9.6, Table 9.14). The percentage of TN and TC increased slightly for all solids (Appendix 9.6, Table 9.14). These results confirm the strong binding capacity of Bauxsol™ pellets, especially with highly soluble cations (ammonia). This observation has not been reported in other literature (e.g. McConchie et al. 1999; Genc-Fuhrman 2004; Akhurst et al. 2006; Lapointe et al. 2006).

Statistical analysis of the physico-chemical properties of the liquids showed 11% dissimilarity between samples. This difference was mainly (95%) attributed to the total phosphorus/orthophosphate concentrations and 5% to high pH. Therefore, TP/PO₄³⁻ and pH are key factors in the experiment. Overall, the pelletised columns effluents were more closely related to each other than to the Sand control effluent and/or influent. Statistical analysis of the solids based on the nutrients levels (not the composition of the material) revealed 80% similarity between Sand and Bauxsol™ pellets before experiment, between Sand pellets and Sand particles after experiment, and between Bauxsol™ pellets after experiment and Sand particles before experiment. The dissimilarity of Sand and Bauxsol™ pellets after the experiment indicates that Bauxsol™ is the core ingredient controlling Bauxsol™ pellet's efficiency.

**Microbiology**

CFU counts and biomass estimation were similar in all effluent samples and slightly lower than the influent (Appendix 9.6, Figures 9.7 & 9.8). In the early samples, Sand pellet effluent recorded low bacterial population numbers probably because of the high pH. High pH affects the activity of microbial enzymes and reduces the growth of bacteria (Bitton 1994). Exceptions were noted at T=22 days (higher CFUs in effluent), suggesting that the system failure and restart would have stripped some bio-
mats off the solid matrix and flushed them into the effluent stream. A drop in microbial counts mid-experiment is presumably associated with the lower pH levels of the influent (5.6), and/or a dilution factor caused by extra wastewater and heavy rainfall prior to sampling. These disturbances induced a specific reaction from individual Sand and Bauxsol™ pellets columns, as suggested by the standard deviation (Appendix 9.6, Figures 9.7 & 9.8), which is most probably due to the uniqueness of each pellet. CFUs counts and biomass estimation on solids showed that Sand particles recorded the highest readings ($6.03 \times 10^6$ cfu/mL; 445 µg/mL, respectively), whereas Bauxsol™ pellets registered the lowest readings ($1.43 \times 10^6$ cfu/mL; 201 µg/mL, respectively; Appendix 9.6, Table 9.15). The extra bacteria count on Sand particles may be explained by some effluent being accidentally mixed with the Sand particles sample. Furthermore, the high porosity and binding capacity of the Bauxsol™ may have prevented total release of the attached biomass.

DGGE profiles of 16S rDNA products from liquid samples (T=7 days) are presented in Figure 4.4a as an example (see all DGGE profiles in Appendix 9.6, Figures 9.9–9.13). Generally, effluent samples were found to have more bands (13 to 24) that those corresponding to influent samples (14 to 19). Band pattern anomalies (low counts) were noted particularly amongst mid-experiment samples, presumably owing to poor DNA yields. At T=22 days higher than average bands numbers were found and new bands corresponding to the solids’ profiles were visible in effluent DGGE profiles, suggesting flushing of bio-mat fragments into the liquid. Shannon diversity index ($H'$) and equitability index ($J'$) revealed that all samples had similar species richness and species evenness ($H'$: average of 2.6; $J' > 0.9$).

Non-metric multi-dimensional scaling (MDS) analysis of the liquids DGGE profiles showed a temporal diversity, suggesting a fluctuation of the bacterial communities in the influent (Figure 4.5). Communities’ profiles within a same sampling time revealed minor differences between the treatments: some influent and Sand control effluent dissimilar to pelletised columns’ effluent (Figure 4.5). This dissimilarity can be explained by the characteristic of the material (e.g. higher pellet binding capacity) and by the changes in physico-chemical parameters induced by the material itself (i.e. pH). These results are at odds with Calheiros et al. (2009) who found that bacterial communities’ composition in effluent changed more according to the substrate than
the temporal factor. However, that study was done on planted constructed wetland treating industrial wastewater over a three years period.

DGGE profiles of solids (Figure 4.4b) displayed 30, 25 and 24 bands for Sand particles, Sand and Bauxsol™ pellets, respectively. Statistical analysis showed 76% similarity between the solids (Appendix 9.6, Figure 9.14). Closer inspection of the Sand particles profile (lane 7, Figure 4.4b) revealed that the additional bands were similar to and in common with the liquid samples (lanes 0–6, Figure 4.4a). This evidence suggests that some liquid may have been inadvertently incorporated along with the Sand particles sample. New and unique band species common to all DGGE profiles from solids (denoted by arrows in Figure 4.4b), Shannon diversity (3.0) and equitability index (0.92) values suggest the formation of bio-mats on all three substrates. However, equitability within species (J’ index) failed to reveal dominance of any one particular species.
Figure 4.4: DGGE analysis of 16S rDNA gene products amplified from total DNA. a) Gel image of liquid samples collected at $T = 7$ days: lane 0 - influent, lane 1 - Sand control effluent, line 2 - Sand pellet effluent I, line 3 - Sand pellet effluent II, line 4 - Bauxsol™ pellet effluent I, line 5 - Bauxsol™ pellet effluent II, line 6 - Bauxsol™ pellet effluent III. b) Gel image of solid samples collected at the end of the experiment ($T = 28$ days) with arrows indicating new and unique bands: lane 7 - Sand particles, line 8 - Sand pellets I, line 9 - Sand pellets II, line 10 - Bauxsol™ pellets I, line 11 - Bauxsol™ pellets II, line 12 - Bauxsol™ pellets III. St = DNA marker standard isolate.
Figure 4.5: Non-metric multi-dimensional scaling (MDS) of bacterial communities generated by the analysis of liquids DGGE 16S rDNA patterns. Fourth root transform and the Bray-Curtis measure used. Samples displayed according to their sampling time (T = 0; 4 hours; 1, 3, 7, 10, 14, 17, 22, 24, and 28 days): * = Influent, × = Sand control effluent, ○ = Bauxsol™ pellet effluent, □ = Sand pellet effluent.

4.2.5. Conclusion

Cured cement-bound Bauxsol™ pellets are biocompatible with wastewater bacterial communities. None of the ingredients used in the manufacture of the pellets directly influence the microorganisms. However, disturbances such as pH spikes, dilution factor, system failure and restart, and short hydraulic retention time impact on the microbiology of the system. Bauxsol™ pellets also effectively remove phosphorus from wastewater by a precipitation process. Owing to the release of unreacted CaO from the cement binder used in the pellets production, which subsequently leads to pH spikes, it is highly recommended that the effluent’s pH be monitored and corrected in the initial stages. Further investigations are necessary to fully assess the efficiency of these Bauxsol™ pellets, adequately determine their saturation time, study the spatial and temporal bacterial communities’ diversity on/in the pellets and investigate biological nitrogen removal processes. Suggested work is a scale-up of this experiment either as larger columns, which is being undertaken, or as a horizontal flow wetland system. In the future Bauxsol™ pellets should offer ecological solutions for sewage water treatment.
4.2.6. References


CHAPTER 5: MESOCOSM COLUMNS FIELD EXPERIMENT (GEOCHEMISTRY)

5.1. Overview

Based on the findings from the microcosm field experiment (i.e. Bauxsol™ pellets are efficient in removing phosphate from wastewater; Chapter 4), the third step in the successful completion of this thesis was to scale-up the previous experiment to determine the efficiency of Bauxsol™ pellets to remove nutrients and trace-metals from wastewater (i.e. 3rd objective). The subsequent paper3 entitled “Nutrient and Trace-Metal Removal by Bauxsol Pellets in Wastewater Treatment” presents the geochemical data of this six months field-based experiment testing the mesocosm columns. The geochemical investigations of this experiment specifically report on the short-term pH spike caused by the cement used during the Bauxsol™ pellets production, the total phosphorus, phosphate, and trace-metal loadings bound to pellets from the effluent, and the nitrogen removal processes occurring in the system. Chapter 9 (Appendix 9.7) includes additional information on this experiment.

5.2. Nutrient and Trace-Metal Removal by Bauxsol Pellets in Wastewater Treatment

5.2.1. Abstract

In this study, Bauxsol pellets packed in PVC columns were used to remove nutrients and trace-metals from municipal wastewater during a 6 months field trial. Bauxsol pellet columns showed a high phosphate removal rate via precipitation of PO$_4^{3-}$ with Ca$^{2+}$ and Mg$^{2+}$ ions: at 90% in the first month; at 80% from the second to fifth months and at 60% in the sixth month. Pellet bound total phosphorus and Colwell phosphate were 7.3 g/kg and 2 g/kg, and are about 20 times the concentrations found in most fertile soils. Trace-metals in effluents were bound, probably irreversibly under the columns’ environmental conditions, to the Bauxsol minerals that have high surface area to volume ratios and high charge to mass ratios. Experimental results showed a

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3 This paper has been published in Environmental Science & Technology journal. The formatting of this journal requires the suppression of the symbol TM (trade mark) and uses US English (e.g. “z” instead of “s”).
complex nitrogen cycle operating within the Bauxsol pellet columns including anoxic nitrification, denitrification, and anammox processes. Although a transient pH spike, associated with the release of unreacted CaO from the cement binder used in the pellets, was observed, this may be readily corrected through post-treatment pH adjustment. Hence, the geochemistry of Bauxsol pellets can effectively remove and bind nutrients and trace-metals during wastewater treatment, and further research may show that saturated spent pellets can be used as fertilizer.

5.2.2. Introduction

Wastewaters contain high concentrations of phosphorus and nitrogen often leading to eutrophication with potential health hazards to both animal and humans. During the sewage treatment process, nitrogen is primarily removed by biological activities, whereas phosphorus is mostly removed by chemical precipitation (Hammer and Hammer 2005). In addition to nitrification and denitrification, nitrogen removal may occur by anammox process \(\text{NH}_4^+ + \text{NO}_2^- \rightarrow \text{N}_2 + 2\text{H}_2\text{O}\), which is mediated by a group of chemo-autotrophic anaerobes. Anammox is noteworthy because, unlike denitrification, it allows nitrogen removal in anoxic environments without requiring a reducing capacity (Mulder et al. 1995).

Free phosphorus primarily exists in wastewater as orthophosphates \(\text{o-PO}_4^{3-}\) and depending on pH, may be present as \(\text{H}_3\text{PO}_4^-\), \(\text{HPO}_4^{2-}\) or \(\text{PO}_4^{3-}\). Phosphate anions precipitate readily with soluble metal salts, such as \(\text{Al}_2(\text{SO}_4)_3 – \text{alum} \) – and \(\text{FeCl}_3\), forming metal-phosphate-hydroxo complexes. Lanthanum (La) impregnated bentonite Phoslock is also commonly used as a phosphate removal agent because the resulting \(\text{LaPO}_4\) is extremely insoluble (Haghseresht et al. 2009). However, use of chemical precipitation in wastewater treatments is an unsound practise because of the cost, the addition of new contaminants (sulfate, chloride and La ions), the requirement of strict dosing in proportion to the phosphate concentration (i.e. water may become toxic through excess soluble Al/Fe), and the creation of large quantities of phosphate-rich sludge deposits requiring disposal (Galarneau and Gehr 1997; Omoike and Vanloon 1999; Hammer and Hammer 2005). In addition, wastewaters contain trace-metals from industrial discharges and urban stormwater runoffs. Trace-metals are generally closely monitored and removed by sorption using metal-oxide-based sorbents,
because they cannot be broken-down (Genc-Fuhrman et al. 2007). Consequently, there is a desire to find cost-effective and environmentally beneficial materials to remove nutrients and trace-metals from wastewater.

Bauxsol, a modified bauxite refinery residue, is available as powder, slurry or pellets. Bauxsol reagents have been successfully applied to environmental remediation for acidic contaminated soils and waters (McConchie et al. 1999; Clark et al. 2005; Lapointe et al. 2006). Batch studies have also demonstrated the potential of Bauxsol to remove and bind phosphorus from pH-controlled waters (Hanahan et al. 2004; Akhurst et al. 2006); however, Bauxsol reagents (powders) are limited by their hydraulic conductivity. No studies to date have investigated the geochemical and biological removal of nutrients and trace-metals by pellets in circum-neutral waters, such as sewage effluent, although clear cost savings of Bauxsol powders in wastewater treatment have been made (Clark et al. 2008). Consequently, in a previous study Despland et al. (2010a) developed a porous Bauxsol pellets, using an ordinary Portland cement (OPC) binder, that characteristically posses a high surface to volume ratio (up to 100 m²/g); a high binding capacity (metals: >1,500 meq/kg; phosphorus: >2% by mass); moderate acid neutralizing capacity; are insoluble and highly non-dispersive (McConchie et al. 1999; Hanahan et al. 2004; Clark et al. 2006a). However, unreacted calcium oxide in the cement binder produces a pH/alkalinity spikes when pellets are placed in circum-neutral waters. To mitigate against this, a postproduction curing process using MgCl₂ and CaCl₂ precipitates most hydroxide alkalinity (Despland et al. 2010a; Despland et al. 2010b). The commercial value of Bauxsol pellets produced this way is about $US700 per ton.

The aim of this paper is to investigate the efficiency of cured OPC-bound porous Bauxsol pellets in wastewater treatment to remove nutrients and trace-metals. Specifically, the objectives are to monitor a possible pH spike caused by Bauxsol pellets, to quantify total phosphorus and phosphate loadings removed from the effluent and bound to pellets, to determine the nitrogen removal processes occurring in the system, and to measure trace-metals loadings trapped by pellets over a 6 months period.
5.2.3. Experimental section

PVC columns (130 cm high \( \times \) 10 cm diameter) were filled with 10 L of OPC-bound Bauxsol pellets (5–10 mm diameter; total weight of 5 kg; experimental columns) (Despland et al. 2010a) or ordinary basalt gravel (5–10 mm diameter; total weight of 13.5 kg; control column) (Appendix 9.7, Figure 9.15). Gravel was selected as it is the most commonly used filtration/support substrate material in wastewater treatment. Secondary treated effluent from the South Lismore Sewage Treatment Plant NSW Australia (Appendix 9.2, Figure 9.1) was fed into the columns in an upward direction at 15 mL/min to provide a hydraulic retention time (HRT) of approximately 11 h. Particulate clogging of columns was prevented by placing polyester fibre beneath the caps at both ends of the each column and by a regular cleaning and maintenance of the system.

At predetermined bed volumes (Appendix 9.7, Table 9.16), liquid samples (150–1200 mL) were collected from column inlet, midway column and outlets during the 6 months trial. At the end of the experiment, the columns were opened vertically and the solids (Bauxsol pellets and Gravel) were sampled at 0 (bottom; inlet), 25, 50, 75, 105 and 130 (top; outlet) cm points along the column.

The pH (APHA 4500 H\(^+\)-b), electrical conductivity (EC) (APHA 2510-b) and temperature (APHA 2550-b) were recorded in-situ every six hours at the column inlets and outlets using a Conductivity-TDS-pH-Temperature meter (TPS, WP-81). Individual liquid samples were also tested for dissolved oxygen (DO) (APHA 2810-b) using a Dissolved Oxygen-Temperature meter (TPS, WP-82Y) (American Public Health Association 1998). Alkalinity determinations (APHA 2320-b) were conducted using 20 mL inlet and outlet waters samples using a potentiometric titrator (Metrohm, Titrando 856), whilst alkalinity speciation was determined by the fixed endpoint method using the online Alkalinity Calculator (USGS 2009).

Concentrations of total phosphorus (TP) (APHA 4500 P-h), orthophosphate (PO\(_4^{3-}\)) (APHA 4500 P-g), ammonia (NH\(_3^+\)) (APHA 4500 NH\(_3\)-h), nitrite (NO\(_2^-\)) (APHA 4500 NO\(_2^-\)-c), nitrate (NO\(_3^-\)) (APHA 4500 NO\(_3^-\)-f), total nitrogen (TN) (APHA 4500 N-c), and calcium (Ca) (APHA 3120 ICPOES) were assessed for column inlet,
midway point and outlet liquid samples (American Public Health Association 1998). Solids samples (before and after experiment at each distance) were analysed for: TP and Colwell phosphorus (Rayment and Higginson 1992), NH$_4^+$ and NO$_3^-$ (1:10 KCl extract APHA 4500), trace-metals such as silver (Ag), arsenic (As), lead (Pb), cadmium (Cd), chromium (Cr), copper (Cu), manganese (Mn), nickel (Ni), selenium (Se), zinc (Zn), mercury (Hg), iron (Fe) and aluminum (Al) (Nitric/HCl digest-APHA 3120 ICPMS) (American Public Health Association 1998). Analyses were undertaken following QA/QC procedures (Appendix 9.1, Table 9.1).

Nitrogen assays were performed at the end of the trial on solids with a range of stable isotope amendments (mixture of $^{15}$N and $^{14}$N) in 20 mL glass vials with screw cap lids with Teflon septa. Gravel solids or Bauxsol pellets (triplicate) were incubated with deoxygenated tap water and with $^{15}$NO$_3^-$, $^{15}$NH$_4^+$, or a combination of $^{15}$NH$_4^+$ + $^{14}$NO$_3^-$. In addition, vials containing Bauxsol pellets and the same three N combinations were mixed with methanol (MeOH) at 15 mg/L. Unamended controls vials were also included. At 0, 1, 3 and 5 days vials were analysed after stopping microbial activity with 0.5 mL of ZnCl$_2$ (50% w/v). After 5 days incubation, 5 mL helium headspace was introduced into the remaining vials. The headspace was subsequently analysed by GC-IRMS (Thermo Trace GC Ultra) interfaced to the isotope ratio mass spectrometer (Thermo Finnigan GC Combustion III). All preparations and incubations were performed in an anoxic glove bag. Production of $^{29}$N$_2$ in treatments where only $^{15}$NH$_4^+$ is added is indicative of nitrification, whereas production of $^{29}$N$_2$ in treatments where $^{15}$NH$_4^+$ + $^{14}$NO$_3^-$ is added indicates anammox process.

The concentration of $^{29}$N$_2$ (i.e. $^{15}$N–$^{14}$N) was calculated using equation (1) - similar equation for $^{30}$N$_2$ (i.e. $^{15}$N–$^{15}$N) - derived by Nielsen (1992)

\[
C^{29}N_2 = \left( \frac{29}{\text{All}} A_s - \frac{29}{\text{All}} A_c \right) \times C \div W \tag{1}
\]

Where $C^{29}N_2$ = concentration of $^{29}$N$_2$ [µmol/g]; $29A_s$ = area of $^{29}$N$_2$ peak for the sample from GC-IRMS; $\text{All}A_s$ = total area of N$_2$ peak for the sample; $29A_c$ = area of $^{29}$N$_2$ peak for the control; $\text{All}A_c$ = total area of N$_2$ peak for the control; C = total
The denitrification rate was calculated using equation (2) - derived by Nielsen (1992)

\[
D_{nt} = \left( \frac{R^{29}N_2}{2 \times R^{30}N_2} \right) + \left( R^{29}N_2 + 2R^{30}N_2 \right)
\]

Where \(D_{nt}\) = rate of denitrification [\(\mu\text{mol/g/d}\)]; \(R^{29}N_2\) = production’s rate of \(^{29}\text{N}_2\); \(R^{30}N_2\) = production’s rate of \(^{30}\text{N}_2\); \(R^{29}N_2\) and \(R^{30}N_2\) were calculated as the slope of the linear increase in concentration over time.

5.2.4. Results and discussion

**pH spike and stabilisation**

Although the pH of the gravel column’s outlet was similar to the inlet (pH 6.3–7.2), a pH spike (10.7) was recorded in the Bauxsol columns’ outlets during the first 15 bed volumes (~1 week). The pH fell to 9.5 after another 15 bed volumes (~2 weeks) and slowly decreased to 8.5 over the next 100 bed volumes (~2 months). A further 260 bed volumes (~4 months) saw the pH decline to 7.8 – Figure 5.1. This pH spike resulted from the release of unreacted calcium oxide in the cement binder. However, the spike was short-lived because most of the cement derived hydroxide was converted to low solubility hydroxyl-carbonates during pellet curing (Despland et al. 2010a); carbonate/hydroxide buffer limit of pH 10.2. Similar results (including pH spike causes), reported by Despland et al. (2010b) using microcosm studies, show the pH spike was slightly greater, and takes longer to decline in the current study, presumably because of column scale-up, i.e. larger column and pellet volumes (10 L vs 300 mL in the small-scale). A freshwater rinse of the pellets pretreatment and/or a pH correction post-treatment may prevent pH spike in circum-neutral waters.
Similarly an EC spike recorded after the first bed volume (≈11 h) in the Bauxsol columns’ outlets (14000 µS) decreased to 965 µS after 15 bed volumes (≈1 week), and then mirrored the Gravel column outlets and inlets EC (≈700 µS) (Appendix 9.7, Figure 9.16). The observed EC spike correlated with the calcium spike in Bauxsol columns’ outlet samples (1730 mg/L) is attributed to the release of unreacted CaO (cement binder) in the effluent, i.e. a rise of calcium greatly increases EC (Stumm and Morgan 1996). However, a small spike of alkalinity as OH⁻ (9 mg/L) and as CO₃²⁻ (80 mg/L) found in the Bauxsol column outlets during the first 30 bed volumes (≈2 weeks) (Appendix 9.7, Table 9.17), suggests that inlet waters are poorly buffered and small changes in concentration have large effects in physicochemical measure such as pH and EC.

During this study, water temperature ranged from 24 to 29°C, whilst dissolved oxygen (DO) ranged from 0.5 to 3 mg/L at the inlets, and from 1.5 to 3 mg/L at column outlets. This disparity in DO is partially attributed to the presence of trapped air in sampling taps during the initial stages, although for Bauxsol pellet columns DO concentration differences may reflect the high porosity of pellets. Moreover, greater porosity and surface area to volume ratio of Bauxsol pellets may have provided additional anoxic micro-zones within the columns; similar effects were also found in a small scale column experiment (Depland et al. 2010b).
Trace-metals bound to solids
Although trace-metal concentrations in sewage effluent are typically low, an assessment of their accumulation on the substrates is important. Bauxsol pellets and Gravel naturally contain trace-metals (Appendix 9.7, Table 9.19). The metal concentrations in the Bauxsol pellets reflect the average composition of Bauxsol powder for As, Pb, Cd, Al and Ni (McConchie et al. 1999). However, Cu, Mn and Zn are higher, whereas Cr and Fe are lower in pellet formulations. These differences are from the use of OPC-cement in pellet production, where Cu, Mn, and Zn are enhanced by OPC either as contaminants or changing surface chemistries brought about by a rise in pH, whereas Cr and Fe concentrations are diluted by the cement mass in the pellets (McConchie et al. 2000; Munro et al. 2004; Clark et al. 2009).

Trace-metal scans from Bauxsol and Gravel columns solids at 0 (inlet), 25, 50, 75, 105 and 130 (outlet) cm after 6 months of operation, generally showed increases in metal concentrations on Bauxsol pellets but a decline for Gravel samples (Table 5.1). Gravels lost approximately 76% of the initial concentrations of Ag; 20–23% of initial Cu, Mn, Fe and Al; and 13–17% of the initial Pb, Cd, Cr, Ni, Se and Zn. These losses are most likely caused by chemical weathering of minerals surfaces and loss of weakly bound trace-metals, especially from reactive olivine surfaces. Increased metal binding capacity of Bauxsol pellets reflects the high surface to volume ratio and high charge to mass ratio of Bauxsol (McConchie et al. 2000). Compared to findings by Clark et al. (2009) and by Genc-Fuhrman et al. (2004; 2007) trace-metal binding capacity of the Bauxsol pellets are quite high even after 6 months of field trials.
### Table 5.1: Bound Trace-Metal Content of Mesocosm Substrates after 6 Months Accumulation As Measured by Aqua Regia Digest

<table>
<thead>
<tr>
<th>Metal</th>
<th>Bp 0cm</th>
<th>Bp 25cm</th>
<th>Bp 50cm</th>
<th>Bp 75cm</th>
<th>Bp 105cm</th>
<th>Bp 130cm</th>
<th>Gc 0cm</th>
<th>Gc 25cm</th>
<th>Gc 50cm</th>
<th>Gc 75cm</th>
<th>Gc 105cm</th>
<th>Gc 130cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>silver</td>
<td>0.88 ±0.10</td>
<td>0.16 ±0.02</td>
<td>0.19 ±0.02</td>
<td>0.12 ±0.02</td>
<td>0.15 ±0.05</td>
<td>0.26 ±0.13</td>
<td>-0.30</td>
<td>-0.37</td>
<td>-0.39</td>
<td>-0.39</td>
<td>-0.40</td>
<td>-0.40</td>
</tr>
<tr>
<td>arsenic</td>
<td>5.37 ±0.54</td>
<td>1.93 ±0.60</td>
<td>5.75 ±1.15</td>
<td>4.32 ±1.36</td>
<td>4.41 ±1.45</td>
<td>3.60 ±1.52</td>
<td>0.04</td>
<td>0.32</td>
<td>-0.26</td>
<td>0.25</td>
<td>-0.18</td>
<td>0.00</td>
</tr>
<tr>
<td>lead</td>
<td>14.53 ±1.32</td>
<td>6.52 ±1.14</td>
<td>12.82 ±3.06</td>
<td>10.01 ±3.09</td>
<td>5.50 ±1.78</td>
<td>6.03 ±4.37</td>
<td>-0.03</td>
<td>-0.12</td>
<td>-0.48</td>
<td>-0.62</td>
<td>-0.48</td>
<td>-0.72</td>
</tr>
<tr>
<td>cadmium</td>
<td>0.20 ±0.12</td>
<td>0.01 ±0.01</td>
<td>0.12 ±0.17</td>
<td>0.27 ±0.16</td>
<td>0.02 ±0.03</td>
<td>0.02 ±0.18</td>
<td>-0.01</td>
<td>-0.04</td>
<td>-0.02</td>
<td>0.12</td>
<td>-0.03</td>
<td>-0.01</td>
</tr>
<tr>
<td>chromium</td>
<td>85.72 ±15.59</td>
<td>33.92 ±5.11</td>
<td>53.96 ±22.14</td>
<td>33.90 ±18.71</td>
<td>41.99 ±12.26</td>
<td>44.79 ±4.76</td>
<td>-1.64</td>
<td>-2.66</td>
<td>-3.54</td>
<td>-2.88</td>
<td>-2.74</td>
<td>-3.17</td>
</tr>
<tr>
<td>copper</td>
<td>28.01 ±1.05</td>
<td>8.99 ±3.03</td>
<td>14.18 ±3.30</td>
<td>10.82 ±3.84</td>
<td>12.76 ±5.99</td>
<td>5.45 ±3.64</td>
<td>-0.45</td>
<td>-5.46</td>
<td>-6.56</td>
<td>-9.18</td>
<td>-5.82</td>
<td>-7.43</td>
</tr>
<tr>
<td>manganese</td>
<td>244.09 ±11.06</td>
<td>89.04 ±15.86</td>
<td>84.48 ±12.01</td>
<td>56.69 ±13.94</td>
<td>50.41 ±21.37</td>
<td>49.71 ±14.45</td>
<td>-107.61</td>
<td>-190.73</td>
<td>-271.54</td>
<td>-297.20</td>
<td>-207.38</td>
<td>-259.35</td>
</tr>
<tr>
<td>nickel</td>
<td>13.25 ±0.66</td>
<td>6.06 ±0.76</td>
<td>7.59 ±0.72</td>
<td>6.71 ±1.48</td>
<td>4.40 ±0.69</td>
<td>3.97 ±1.65</td>
<td>-2.55</td>
<td>-4.04</td>
<td>-7.81</td>
<td>-8.69</td>
<td>-7.57</td>
<td>-6.32</td>
</tr>
<tr>
<td>selenium</td>
<td>0.40 ±0.03</td>
<td>0.24 ±0.05</td>
<td>0.30 ±0.08</td>
<td>0.15 ±0.04</td>
<td>0.21 ±0.04</td>
<td>0.13 ±0.04</td>
<td>-0.27</td>
<td>-0.18</td>
<td>-0.21</td>
<td>-0.02</td>
<td>0.01</td>
<td>-0.09</td>
</tr>
<tr>
<td>mercury</td>
<td>0.09 ±0.02</td>
<td>0.05 ±0.00</td>
<td>0.07 ±0.01</td>
<td>0.05 ±0.01</td>
<td>0.06 ±0.01</td>
<td>0.08 ±0.02</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>iron*a</td>
<td>4.05 ±0.22</td>
<td>1.40 ±0.30</td>
<td>2.62 ±1.08</td>
<td>1.41 ±0.82</td>
<td>2.02 ±0.53</td>
<td>2.20 ±0.36</td>
<td>-1.22</td>
<td>-1.03</td>
<td>-1.88</td>
<td>-1.71</td>
<td>-1.69</td>
<td>-1.97</td>
</tr>
<tr>
<td>aluminum*a</td>
<td>1.71 ±0.22</td>
<td>0.80 ±0.10</td>
<td>1.10 ±0.35</td>
<td>0.67 ±0.25</td>
<td>0.84 ±0.14</td>
<td>0.94 ±0.17</td>
<td>-0.15</td>
<td>-0.22</td>
<td>-0.43</td>
<td>-0.50</td>
<td>-0.31</td>
<td>-0.38</td>
</tr>
</tbody>
</table>

Bp = Bauxsol pellets, Gc = Gravel; 0cm = inlet, and 130cm = outlet (intermediate distances are also provided). All data are mg/kg except iron*a and aluminum*a, which are in weight %; ± standard error for Bp. Negative values represent a loss in mass relative to an earlier condition.
The results indicate that Ag, Cr, Cu, Mn, Ni, Se, Fe and Al are preferentially bound to Bauxsol in the first quarter of the columns, whereas As, Pb, Cd, Zn and Hg were found more evenly distributed along the column length. This may be explained by the geochemical structure of Bauxsol pellets and the competition of metals for available sites. Metals are initially bound to the Bauxsol by surface charge attracting dissolved metals and then by crystal growth via new mineral precipitation and intracrystalline diffusion (McConchie et al. 2000; Hanahan et al. 2004; Clark et al. 2005; Clark et al. 2009). Recent synchrotron data (Dr. Richard Collins, personal communication, 2011) and isotopic exchange experiments (Clark et al. 2011) supports these observations.

Metal removal by Bauxsol is very complex and not yet fully understood as it is a complex combination, surface charging, pH, thermodynamic and kinetic solubility aspects. Moreover, different metal removal mechanisms may be at play as the composition of the effluent changes overtime. Furthermore, Genç-Fuhrman et al. (2007) reported that trace-metal speciation is pH-dependent and concluded that the metal removal processes changes with time. Clark et al. (2009) and McConchie et al. (1999) also showed that bound metal leaching from Bauxsol decreases as loaded samples are allowed to age. Consequently, it is unlikely that many of the trace-metals accumulated on Bauxsol pellets will leach under natural conditions if applied to soils. Several studies (e.g. Munro 2004; Clark et al. 2006b; Clark et al. 2009) demonstrate the long term fate of bound trace-metals on the Bauxsol matrix.

**Phosphate removal**

Phosphate concentrations in the inlet ranged from 3.0 to 9.2 mg/L. Bauxsol pellet columns effectively eliminated phosphate from the effluent (Appendix 9.7, Table 9.18). During the first 60 bed volumes (≈1 month), >95% of the orthophosphate was removed and subsequently stabilised at around 80% for the next 240 bed volumes (≈5 months), before falling to 60% for the remaining 60 bed volumes (≈6 months; Figure 5.2). These results are below the Australian concentrations for tertiary treated effluent (postchemical precipitation) (National Water Quality Management Strategy 1997). No significant phosphate removal was observed in the Gravel column; phosphate binding was transitory and eventually leached away.
Analyses of Bauxsol pellets following 6 months exposure showed substantially higher TP binding and Colwell P (available phosphate) than the Gravel. Most of the bound TP was observed on the first quarter of the Bauxsol columns (7.3 g/kg) with the remaining three-quarters binding 3.3 g/kg, gradually declining to 1.5 g/kg at the outlet (Figure 5.3). Phosphate was bound evenly along the first half of the columns (~2 g/kg) and then dropped to 1.6 g/kg in the second half (Figure 5.3). Contrary to this, Gravel samples showed little phosphate binding and even signs of TP loss (Figure 5.3; Appendix 9.7, Table 9.19).

Figure 5.2: Percentage removal of orthophosphate in the columns’ outlets (Bauxsol pellets and Gravel).
Observed differences in phosphate binding capacity between Gravel and Bauxsol pellets reflects that Bauxsol has a recorded phosphate binding capacity of ≈2% by mass (Clark et al. 2006a). According to Akhurst et al. (2006) the adsorption of PO$_4^{3-}$ by Bauxsol increases with decreasing pH (i.e. change in dominant phosphate speciation, which facilitate the electrostatic and chemical attraction of phosphate ions). In the present study, phosphate speciation for inlet water is predominantly H$_2$PO$_4^-$ at pH 6.3–7, but as the effluent passes through the Bauxsol column the speciation changes to HPO$_4^{2-}$ with minor amounts of H$_2$PO$_4^-$ at pH of 8–10 (Stumm and Morgan 1996). Accordingly, this should result in reduced phosphate removals at the beginning of this experiment. However, excess Ca$^{2+}$ and Mg$^{2+}$ ions (i.e. Bauxsol pellets curing process) present in our system precipitates the HPO$_4^{2-}$ to form MgHPO$_4$ and CaHPO$_4$. Additionally, it is thought that the HPO$_4^{2-}$ and NH$_4^+$ may also be precipitated as struvite (MgNH$_4$PO$_4$) (Babic-Ivancic et al. 2002); however, confirmation by X-ray diffraction (XRD) is difficult because of the dominance of the Bauxsol mineralogy. The investigation of the speciation and binding environments requires very sophisticated techniques (e.g. synchrotron EXAFS and XANES; not available for this thesis). Based on present and past geochemical studies (Hanahan et al. 2004; Clark et al. 2006a; Clark et al. 2009) it appears that the main phosphate removal mechanism proceeds via precipitation of PO$_4^{3-}$ with Ca and Mg ions, therefore back flushing of the material is not possible.
Bauxsol pellets also demonstrated equivalent or greater P-removal rates compared to other materials under similar experimental conditions (i.e. bed volumes, pH, and initial phosphate concentration). Studies on alum showed between 60 and 80% P-removal (Galarneau and Gehr 1997; Omoike and Vanloon 1999). wollastonite used by Brooks et al. (2000) and zeolite used by Sakadevan and Bavor (1998) demonstrated P-removal around 50%. Laterite used by Wood and McAtamney (1996) in a batch system and a constructed wetland showed a P-removal at 85% and 95%, respectively. Gray et al. (2000) found in their pilot-scale constructed-wetland using Maerl material (dead deposits of calcareous red algae) 98% P-removal. Blast furnace slag mixed with sand in the Johansson study (1999) revealed similar results to Bauxsol pellets. Comparing to Akhurst et al. study (2006), Bauxsol pellets are superior to Bauxsol powder (<80% P-removal). This difference can partially be explained by a lower Bauxsol dosage used in the Akhurst study (2006) - up to 5 g/L of Bauxsol powder VS estimated 300 g/L of Bauxsol powder used in the pellets production.

Analysis of solids showed that most of the TP was trapped on the Bauxsol pellets, presumably as fine particulates (Figure 5.3), and the Colwell extractions of Bauxsol pellets confirms high concentrations of available phosphates (~2 g/kg), some 20 times the concentrations found in most fertile soils (Peverill et al. 1999). Consequently, spent or waste Bauxsol pellets could be used as P-based supplementary (low-level) fertilizer. However, further studies are warranted to analyse the effects on plants, soil and groundwater when using spent Bauxsol pellets as fertilizer. This work demonstrates that Bauxsol pellets are superior to Bauxsol powders with respect to P in that 2 g/kg loadings at 80–95% removal rate are higher than those of Hanahan et al. (2004) - 1.1 g/kg loadings at 99% removal rate and 2.6 g/kg loadings at 50% removal rate. It is likely that tobermorite-like (Yu et al. 2010) and hydrated-Ca-silicate minerals present in the cement binders of the pellets (Barbhuiya et al. 2010) account for these findings.

**Nitrogen removal processes**

Predominantly controlled by biological activities, which are dependent on environmental factors, the nitrogen cycle is also influenced by some physicochemical processes. As shown in Table 5.2a, the inlet concentrations of ammonia, nitrite and
Nitrate fluctuated greatly over time due to changes of raw sewage composition and other environmental factors (e.g. pH) that subsequently affect microorganism activities and nitrogen removal. Nitrate concentrations were consistently lower in both Bauxsol and Gravel outlets. Whereas nitrite data tended to have higher concentrations in Bauxsol and Gravel outlets, however lower concentrations were recorded after 150 bed volumes (≈10 weeks) in Gravel outlets. Ammonia concentrations in Bauxsol outlets were close to inlet concentrations up until 150 bed volumes (≈10 weeks) and subsequently dropped down. The Gravel outlets displayed very low ammonia concentrations during the first 15 bed volumes (≈7 days) and from 120 bed volumes (≈8 weeks). Overall, total nitrogen (TN) decreased by 31% in Bauxsol columns outlets and by 34% in Gravel column’s outlet between 60 and 360 bed volumes (≈1 to 6 months), suggesting that particulate filtration by the substrates was an important N removal mechanism (Table 5.2a). Although the reduction of TN is reasonably high, increasing the hydraulic retention time (HRT) may achieve better results because of increased contact times between the effluent and the biofilms.
Table 5.2: a) \( \text{NH}_4^+ , \text{NO}_2^- , \text{NO}_3^- , \text{TN} \) Concentration [mg/L] in Inlets, Bauxsol Pellets (Bp) Columns Outlets, Gravel (Gc) Column's Outlets at 1, 60, 180 and 360 Bed Volumes; b) \( \text{NH}_4^+ \) and \( \text{NO}_3^- \) Concentration [mg/Kg] on Solids before Experiment (Raw) and Bound on Substrates (Bp = Bauxsol Pellets; Gc = Gravel) after 6 Months Experiment at Different Length along the Columns (0cm = inlet; 130cm = outlet); ± Standard Error for Bp.

<table>
<thead>
<tr>
<th>Samples port</th>
<th>Inlet</th>
<th>Bp outlet</th>
<th>Gc outlet</th>
<th>Inlet</th>
<th>Bp outlet</th>
<th>Gc outlet</th>
<th>Inlet</th>
<th>Bp outlet</th>
<th>Gc outlet</th>
<th>Inlet</th>
<th>Bp outlet</th>
<th>Gc outlet</th>
</tr>
</thead>
<tbody>
<tr>
<td>ammonia</td>
<td>13.04</td>
<td>14.88 ±0.97</td>
<td>2.16</td>
<td>10.93</td>
<td>9.32 ±1.11</td>
<td>13.05</td>
<td>13.70</td>
<td>6.77 ±0.13</td>
<td>5.00</td>
<td>1.17</td>
<td>0.04 ±0.00</td>
<td>0.58</td>
</tr>
<tr>
<td>nitrite</td>
<td>0.24</td>
<td>1.02 ±0.09</td>
<td>0.64</td>
<td>0.44</td>
<td>1.13 ±0.10</td>
<td>0.80</td>
<td>0.25</td>
<td>1.21 ±0.11</td>
<td>0.06</td>
<td>0.12</td>
<td>1.20 ±0.11</td>
<td>0.58</td>
</tr>
<tr>
<td>nitrate</td>
<td>17.56</td>
<td>9.66 ±1.98</td>
<td>13.85</td>
<td>12.47</td>
<td>7.45 ±0.75</td>
<td>6.10</td>
<td>20.90</td>
<td>20.23 ±0.37</td>
<td>10.80</td>
<td>12.03</td>
<td>9.44 ±0.44</td>
<td>8.71</td>
</tr>
<tr>
<td>total nitrogen</td>
<td>35.10</td>
<td>30.60 ±0.10</td>
<td>21.60</td>
<td>27.30</td>
<td>18.60 ±0.17</td>
<td>20.10</td>
<td>37.80</td>
<td>30.33 ±0.73</td>
<td>17.70</td>
<td>16.56</td>
<td>11.22 ±0.49</td>
<td>9.95</td>
</tr>
</tbody>
</table>

b) Solids

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Before experiment</th>
<th>Bound after experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>raw Bp</td>
<td>raw Gc</td>
</tr>
<tr>
<td>ammonia</td>
<td>1.38</td>
<td>0.19</td>
</tr>
<tr>
<td>nitrate</td>
<td>14.38</td>
<td>0.79</td>
</tr>
</tbody>
</table>
Analyses of solids after the trial showed a substantial load of bound nitrate on Bauxsol pellets (17.3 mg/kg close to the inlet and 61.8 mg/kg close to the outlet), as opposed to ≈2.5 mg/kg on the Gravel; also some 5.6 and 2.9 mg/kg of ammonia was bound to Bauxsol pellets and Gravel, respectively (Table 5.2b; Appendix 9.7, Table 9.19). Removal of ammonia in the Gravel column outlet in the initial phase of the experiment probably arises from struvite (MgNH₄PO₄) precipitation i.e. NH₄⁺ combines with phosphates and Mg from the reactive olivine (Mg, Fe)₂SiO₄ contained in the basalt Gravel. Ammonia precipitation through struvite formation in Bauxsol columns may well have occurred, however high Ca²⁺ concentrations (>10 mg/L) interfere with struvite formation (Babic-Ivancic et al. 2002) and phosphate was most likely bound as MgHPO₄ and CaHPO₄.

N-assay experiments revealed that denitrification was active in both Gravel and Bauxsol pellets incubations but higher in Bauxsol pellets incubations; after 24 h the production of ¹⁵N–N₂ was negligible, so all rate calculations were made for the first 24 h (Figure 5.4). This difference may be explained by the greater porosity and surface to volume ratio of the Bauxsol pellets that could have provided additional anoxic micro-zones within the column. Denitrification of the added NO₃⁻ in the Bauxsol pellets incubation accounted for up to 1.5% without, and 5.5% with MeOH in the incubations. The addition of MeOH most likely provided a source of labile dissolved organic carbon for denitrifiers and also led to the consumption of any available O₂, further increasing the availability of anoxic microzones. The increased denitrification from added MeOH may provide an improvement to wastewater treatment using Bauxsol pellets.
The presence of $^{29}$N$_2$ detected in all the $^{15}$NH$_4^+$ incubations, points to the presence of nitrification producing $^{15}$NO$_3^-$ that has mixed with $^{14}$NO$_3^-$ from the nitrification of internally mineralised of $^{14}$NH$_4^+$. In the incubations amended with $^{15}$NH$_4^+$ + $^{14}$NO$_3^-$, nitrification will also have produced $^{15}$NO$_3^-$. However, this would have mixed with pool of available $^{14}$NO$_3^-$ and diluted the $^{29}$N$_2$ signal produced via denitrification. Hence, the presence of nitrification indicates a small oxygen contamination in the incubations. Although the incubations were carried out in an anoxic glove box, only a very small concentration (5 µM O$_2$) is required to facilitate nitrification. Interestingly, the presence of $^{29}$N$_2$ increased in the Bauxsol pellets incubations amended with MeOH. Consequently, the MeOH consumed available O$_2$ leading to nitrification under anoxic conditions. Anoxic nitrification can occur in the presence of electron acceptors other than O$_2$ such as manganese oxides (Hulth et al. 1999) and iron oxides (Straub et al. 2001). Metal scans (Table 5.1) show high levels of Mn bound to the Bauxsol pellets. Moreover recent synchrotron work showed that Mn binds to the hematite and sodalite minerals in the Bauxsol and a partial oxidation consistent with Mn(IV) occurs (Dr. Richard Collins, personal communication, 2011). Consequently, this Mn(IV) would be a suitable electron acceptor, as well as the iron-rich Bauxsol pellets (McConchie et al. 1999). In addition, high nitrates loadings on Bauxsol pellets in the last quarter of the columns (i.e. nitrification is proceeding along the length of the column) also seems to support this hypothesis, although further investigations are necessary.
The presence of anammox was detected only in Bauxsol pellet incubations with MeOH (Figure 5.4). This is counter-intuitive given that MeOH is a known inhibitor of anammox (Jensen et al. 2007). However, if it is considered that MeOH resulted in the consumption of available O₂ at the surface of the Bauxsol pellets, then it is possible that anoxia may have been prevalent in microzones within the pellets, and consequently, these conditions may have favoured anammox. The cycling of N is clearly complex within the Bauxsol pellet columns and further investigations are also warranted, in particular the use of molecular biology to isolate different bacterial processes and pathways.

This study demonstrates that OPC-bound Bauxsol pellets used to filter municipal secondary treated wastewater successfully removed effluent nutrients and trace-metals. The short-lived pH spike resulting from the release of unreacted CaO from the cement binder used in pellet production should be monitored and corrected post-treatment during the initial stages of column establishment. Bauxsol pellets showed a large capacity to bind phosphate during the course of the 6 months trial, and were yet to reach saturation, however further studies are required to determine pellet saturations. Furthermore, experimental results indicated that a complex nitrogen cycle may be functioning within the Bauxsol pellets columns, i.e. anoxic nitrification, denitrification and anammox processes associated with nutrient binding. Further chemical and biological investigations (i.e. molecular biology) are required to elucidate and confirm these processes. Spent Bauxsol pellets (waste from columns), providing supporting data from further investigations, may be suitable for use as fertilizer because they contain high concentrations of plant available phosphate (≈2 g/kg), but only small loadings of trace-metals, which under normal environmental conditions (pH 5–8) appear irreversibly bound.

5.2.5. Acknowledgements

The paper draws inspiration and builds on the previous work of the late Professor David McConchie, Southern Cross University, who saw potential where others saw none. This work was also supported by an Australian Research Council Grant LP0056012, and the Stipend provided to Dr Clark from CRC-CARE.
5.2.6. References


CHAPTER 6: MESOCOSM COLUMNS FIELD EXPERIMENT (MICROBIOLOGY)

6.1. Overview
The findings from the microcosm field experiment, which showed that Bauxsol™ pellets were biocompatible with environmental bacterial communities (Chapter 4), allowed the 4th step in the successful completion of this thesis. A determination on the efficiency of Bauxsol™ pellets to act as a biomass support particle for microbial communities (i.e. objective 3) was made simultaneously with the geochemical treatment of wastewater (Chapter 5) in a mesocosm. The subsequent paper entitled “Diversity of Microbial Communities in an Attached-Growth System using Bauxsol™ Pellets for Wastewater Treatment” presents the microbiology results of this six months field-based experiment. The microbiology investigations of this experiment specifically report on the structure and diversity of the bacterial communities suspended in the liquid phase and attached to the solid phase, and the presence of microbial communities associated with nitrogen cycling. Chapter 9 (Appendix 9.7) includes additional information on this experiment.

6.2. Diversity of Microbial Communities in an Attached-Growth System using Bauxsol™ Pellets for Wastewater Treatment

6.2.1. Abstract
Columns of Bauxsol™ pellets were used in a field experiment as biomass support particle for wastewater microbial communities. The attached microbial community structure was analysed using denaturing gradient gel electrophoresis (DGGE), targeting the 16S rDNA gene’s V3 region. DGGE profiles showed that the type and composition of support particles used (i.e. Bauxsol™ pellets or gravel) had a significant impact on the attached bacterial communities (64% dissimilarity). In addition, ecological indices revealed a more heterogeneous bacterial community structure on the Bauxsol™ pellets. TOC/TN ratios post-experiment (6.5–9.3) suggested a good level of biological activity (i.e. active biofilm) in the Bauxsol™ columns. Moreover, Bauxsol™ pellets were mostly made of inorganic carbon, suggesting insoluble carbonate biomineralisation. Polymerase chain reaction (PCR)
amplification of specific marker genes (i.e. bacterial and archaeal *amoA* genes, *nosZ* gene, and *hzo* gene) were used to identify the presence of attached bacterial communities associated with nitrogen transformation. The results along with geochemical data (i.e. up to 50% nitrogen removal) revealed co-existence of ammonia-oxidising bacteria, denitrifiers, and anammox organisms. This study conclusively demonstrates that microbial communities are well-adapted to Bauxsol™ pellets and bacterial communities involved in the nitrogen cycle are present.

6.2.2. 1. Introduction

Biological nitrogen transformations are commonly achieved in wastewater treatment via an attached-growth system (i.e. trickling filter, membrane bioreactor, rotating biological contactor, and up/down-flow submerged filter) or a suspended-growth system (i.e. activated sludge). In attached-growth systems, microorganisms generally grow and form biofilms on biomass support particles (BSP) such as gravels, plastics, sand, and activated carbon material (Bitton 1994). Attached-growth systems offer many advantages over suspended-growth systems such as reduced size, control and maintenance simplicity, a robustness to extreme changes, and odour and noise minimisation (Bitton 1994; Madigan et al. 2012).

Bauxsol™ technology (i.e. a commercially available seawater-neutralised bauxite refinery residue) is another potential BSP for attached-growth, not previously described. Bauxsol™ possesses excellent physicochemical characteristics: high surface to volume ratio (up to 100 m²/g); high binding capacity (metals: >1,500 meq/kg; phosphorus: >2% by mass); moderate acid neutralising capacity; and it is insoluble and highly non-dispersive (McConchie et al. 1999; Hanahan et al. 2004). Several studies report the utility and effectiveness of Bauxsol™ in environmental remediation of soil and water, particularly those contaminated by acids and heavy metals (McConchie et al. 1999; Genc-Fuhrman et al. 2004; Clark et al. 2011). Previously, Despland et al. (2010a) described the development of porous Bauxsol™ pellets to potentially remove phosphate and trace-metals whilst functioning as a BSP in wastewaters. To date only one study has reported on the biocompatibility of Bauxsol™ with environmental bacterial communities in circum-neutral waters such
as sewage (Despland et al. 2010b) but few details on the interactions of porous Bauxsol™ pellets with bacterial communities were presented. Biological activities linked to the nitrogen cycle have been reported on porous Bauxsol™ pellets in a previous study (Despland et al. 2011), however, the presence of microorganisms involved in nitrogen cycle has yet to be confirmed.

The study of microbial communities, such as in attached-growth systems, is usually performed by culture-dependent and to a lesser degree by recent culture-independent methods. Techniques that do not rely on culturing, such as those based on DNA, have become a powerful means to describe microbial diversity and functionality in given environments over time and space and/or in relation to perturbations and management practices. Denaturing gradient gel electrophoresis (DGGE), a DNA fingerprinting method, allows rapid assessment of microbial community diversity and structure, where profiles (i.e. banding patterns) are generated according to the melting behaviour of amplified fragments of 16S ribosomal genes (16S rDNA). A profile is considered representative of the whole bacterial community, however, an individual discrete band on the gel does not represent a single species or even a single bacterial population because amplified DNA fragments from related and non-related bacteria might co-migrate (Muyzer and Smalla 1998; Madigan et al. 2012). Several studies have used the DDGE technique to describe microbial communities in wastewater treatment. Calheiros et al. (2009) reported the use of 16S rDNA DGGE in a constructed wetland system to investigate the effect of support particle types on the diversity, structure and dynamics of microbial communities. Wan et al. (2011) showed the effect of contrasting environmental and operational conditions on the diversity and dynamic of bacterial populations in a membrane bioreactor and an activated sludge system. Imfeld et al. (2010) also demonstrated via PCR/DGGE the changes of the bacterial community structure with a succession of hydrochemical conditions in a constructed wetland.

Some of the more important functional structures of an ecosystem may be determined and monitored via PCR analysis of known microbial genes. For instance, bacterial communities growing on BSP may transform nitrogen via several different routes – nitrification (i.e. nitritation and nitratation), denitrification and/or anammox processes.
Bacteria affiliated with beta and gamma Proteobacteria subdivisions, and Archaea allied with Thaumarchaeota (Brochier-Armanet et al. 2008) possess ammonium monoxygenase subunit A (amoA) genes (Purkhold et al. 2000; Schleper et al. 2005) which are routinely used as functional markers for nitritation (i.e. ammonia to nitrite oxidation). Several wastewater studies have revealed the presence of both bacterial and archaeal amoA genes (Park et al. 2006; Kim et al. 2011). More than 50 distinctively different Proteobacteria possess functional genes (e.g. nirK, nirS, nosZ and norB), encoding for enzymes involved in denitrification (Zumft 1997). Some of these functional marker genes have been successfully applied to wastewater studies (Wan et al. 2011). Anaerobic ammonium-oxidising (anammox) bacteria belong to a monophyletic branch within the phylum Planctomycetes and include only five genera classified as Candidatus (Strous et al. 1999). Given that anammox bacteria have not been isolated in pure cultures, the reported studies – including those on wastewater – are molecular DNA based, targeting the 16S rRNA genes of Planctomycetes or the functional gene hydrazine dehydrogenase hzo (Quan et al. 2008; Li et al. 2010a).

The current paper aims to evaluate the efficiency of porous Bauxsol™ pellets to act as a biomass support particle by studying the attached microbial communities in a submerged up-flow wastewater filter experiment. Specifically, the objectives of this study are to investigate the impact of the support used (i.e. Bauxsol™ pellets or gravel particles) on the structure, diversity, and richness of bacterial communities attached to the solids and suspended in the liquid phase using PCR/DGGE methods. The presence of microbial communities associated with nitrogen-cycling is also assessed through PCR amplification of functional genes – amoA (nitritation), nosZ (denitrification) and hzo (anammox) – to confirm biological activities found in an isotopic N-assay analysis performed on both supports used in this experiment (Despland et al. 2011).

6.2.3. 2. Materials and methods

2.1 Experimental design and sampling

The experimental design, described by Despland et al. (2011), consisted of three PVC columns (1300 mm high × 100 mm diameter) filled with porous Ordinary Portland Cement (OPC)-bound Bauxsol™ pellets (5–10 mm diameter; Despland et al. 2010a), and a column filled with basalt gravel (5–10 mm diameter) as a control (Appendix
Polyester fibre was placed underneath the caps to prevent particulate clogging. Gravel was selected as it is the most commonly used filtration/support substrate material in wastewater treatment. All columns were fed in an upward direction at 15 mL/min with secondary treated wastewater from the South Lismore Sewage Treatment Plant (NSW, Australia) (Appendix 9.2, Figure 9.1) to provide a hydraulic retention time (HRT) of approximately 11 h. Secondary treated wastewater, preferred to raw or primary treated wastewaters because they contain a high concentration of sediments that may clog the system, was used as a feeding substrate and as an inoculum source (i.e. Bauxsol™ pellets and gravel were autoclaved pre-experiment). The only source of oxygen available inside the columns was the dissolved oxygen present in the wastewater.

Liquid fraction was sampled (150 to 1200 mL) at predetermined times (T = 0 = HRT, T = 1 day, 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 6 weeks, 8 weeks, 10 weeks, 12 weeks, 14 weeks, 16 weeks, 18 weeks, 20 weeks, 22 weeks, and 24 weeks; Appendix 9.7, Table 9.16) from column inlets, mid-column, and column outlet ports over a six month period. Bauxsol™ pellets and gravel particles (i.e. solid fraction) were sampled post-experiment along the column length at 0 (bottom; inlet), 25, 50, 75, 105 and 130 (top; outlet) cm.

2.2 Liquid and solid physicochemical analysis

The pH (APHA 4500 H⁺-b), and dissolved oxygen (DO) (APHA 2810-b) were recorded for all column inlet and outlet samples using a conductivity-TDS-pH-temperature meter (TPS, WP-81) and a dissolved oxygen-temperature meter (TPS, WP-82Y) (American Public Health Association 1998). Analysis of biochemical oxygen demand (BOD) (APHA 5210-b), chemical oxygen demand (COD) (APHA 5220-d), ammonium (NH₄⁺) (APHA 4500 NH₃-h), nitrite (NO₂⁻) (APHA 4500 NO₂⁻-c), nitrate (NO₃⁻) (APHA 4500 NO₃⁻-f), total nitrogen (TN) (APHA 4500 N-c), and organic nitrogen [calculation TN – (NH₄⁺ + NO₂⁻ + NO₃⁻)] was undertaken on the column inlet and outlet samples (American Public Health Association 1998). Solid samples (Bauxsol™ pellets and gravel particles) were analysed pre- and post-experiment for percentage TN, total carbon (TC) and total organic carbon (TOC) (LECO CNS2000 Analyser) (American Public Health Association 1998); TOC/TN
ratio (i.e. C/N ratio) and inorganic carbon (IC; TC – TOC) were also calculated. Analyses were undertaken following QA/QC procedures (Appendix 9.1, Table 9.1).

2.3 DNA extraction; PCR/DGGE on bacterial 16S rDNA

The molecular analysis of the bacterial communities was conducted on all liquid and solid samples, with a bead beating DNA extraction using 80 mL of liquid or approximately 600 mg of solids. Liquid samples were centrifuged and the pellet obtained was resuspended in 500 µL phosphate buffer saline and transferred to a bead beating microtube. The solid samples were directly placed in a bead beating microtube without any phosphate buffer saline. The bead beating DNA extraction protocol described by Bell et al. (2006) and Vancov & Keen (2009) was then followed for both liquid and solid samples (Appendix 9.3, Tables 9.2 – 9.7). Bacterial communities were profiled using DGGE separation of PCR amplified fragments corresponding to the 16S rDNA gene’s V3 region (Table 6.1; Appendix 9.3). All PCR amplifications were performed in a GeneAmp PCR system 9700 (Applied Biosystems). PCR of the V3 16S rDNA region was performed in a total volume of 50 µL containing 5 µL of 10× Dream taq Buffer (with 20 mM MgCl₂; Fermentas), 0.4 µL of BSA (20 mg/mL; Sigma), 1 µL of deoxynucleotide triphosphate mix (10 mM; Astral Scientific), 1 µL of MgCl₂ (50 mM; Gibco), 1 µL of each forward (20 µM) and reverse (25 µM) primer (Invitrogen), 0.4 µL of Dream taq (5 U/µL; Fermentas) and 20 ng/µL of DNA template. PCR products of 16S rDNA were then separated by DGGE electrophoresis using the CBS Scientific system (USA). Conditions for electrophoresis were: 150 V for 5 h at 60°C, using a 6.5% (w/v) polyacrylamide gel with a 30–70% gradient prepared in accordance with the manufacturer’s (CBS Scientific, USA) instructions (Appendix 9.3). Incorporated in each gel was a standard DNA marker prepared from four bacteria colonies isolated from the secondary-treated wastewater used in this study. Analyses were undertaken following QA/QC procedures (Appendix 9.1).
Table 6.1: Summary of PCR primer sets and setting conditions used on liquid and solid samples

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Targeted site</th>
<th>Use</th>
<th>PCR setting</th>
<th>Reference example</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC-357F/546 Alexa-518R</td>
<td>Bacterial 16SrDNA gene’s V3 region</td>
<td>Bacterial communities profiling (DGGE)</td>
<td>94°C for 5 min; 40 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s; and a final extension at 72°C for 10 min</td>
<td>Yu and Morrison (2004)</td>
</tr>
<tr>
<td>amoA-1F/amoA-2R-TC</td>
<td>Ammonia-oxidising bacteria with amoA gene</td>
<td>Functional PCR</td>
<td>94°C for 4 min; 30 cycles of 94°C for 1 min, 58°C for 30 s, 72°C for 45 s; and a final extension at 72°C for 10 min</td>
<td>Wan et al. (2011)</td>
</tr>
<tr>
<td>amoA-23F/amoA-616R</td>
<td>Ammonia-oxidising archaea with amoA gene</td>
<td>Functional PCR</td>
<td>94°C for 4 min; 30 cycles of 94°C for 1 min, 58°C for 30 s, 72°C for 45 s; and a final extension at 72°C for 10 min</td>
<td>Saha and Muyzer (2008)</td>
</tr>
<tr>
<td>nosZF/nosZ1622R</td>
<td>Denitrifier with nosZ gene</td>
<td>Functional PCR</td>
<td>94°C for 4 min; 35 cycles of 94°C for 1 min, 55°C for 30 s, 72°C for 45 s; and a final extension at 72°C for 10 min</td>
<td>Wan et al. (2011)</td>
</tr>
<tr>
<td>hzo1F1/hzo1R2</td>
<td>Anammox bacteria with hzo gene</td>
<td>Functional PCR</td>
<td>94°C for 4 min; 35 cycles of 94°C for 1 min, 50°C for 30 s, 72°C for 45 s; and a final extension at 72°C for 10 min</td>
<td>Li et al. (2010a)</td>
</tr>
</tbody>
</table>

DGGE gels were photographed using an Image Station 2000 MM system (Kodak, USA) and analysed with Carestream Molecular Imaging software version 5.0 (Carestream Health Inc., USA). The floating point values method was used to find band mobility (double checked using the profile option of the software) and peak intensity (excluded if intensity <2). Relative band mobility was calculated from gels using bands corresponding to DNA marker and then aligned. Percentage relative band intensity was then calculated for each sample.

2.4 Statistical analyses of DGGE bacterial communities’ profiles and of geochemical data

DGGE data sets (i.e. profiles) were statistical analysed using ePRIMER software (version 6; Clarke and Gorley 2006). Shannon diversity index (H’), equitability index (J’), and richness (i.e. total number of bands) were calculated from the DGGE profiles to compare Bauxsol™ pellets and gravel bacterial communities’ structure. A dendrogram (group average clustering from Bray-Curtis similarities on fourth roots-transformed) was built to identify samples generating similar profiles. The dendrogram was further examined using the similarity profile (SIMPROF) permutation test to look for statistical evidence of genuine clusters within samples that were a priori unstructured. Where applicable, DGGE profiles and geochemical data
were subjected to one-way analysis of similarity (ANOSIM) to statistically test for a significant difference between the samples (R value close to 1 indicates dissimilarity between samples).

2.5 Functional PCR-amplification of genes involved in nitrogen transformation

DNA extracted from Bauxsol™ pellets and gravel at 0, 75 and 130 cm, as well as from the column inlets at the beginning and end of the experiment, were subjected to functional PCR amplification targeting specific genes involved in nitrogen transformation (Table 6.1; Appendix 9.3, Table 9.8). Functional gene amplifications were performed in a total volume of 25 µL containing 50 ng/µL of DNA template and reagents as described for 16S rDNA PCRs. PCR products were electrophoresed at 60 V for at least 60 min on 2% agarose gels containing GelRed™ Nucleic Acid Gel Stain (Biotium, USA). Positive and negative controls were incorporated to each gel to ensure the results were not caused by PCR artefacts. Gels were photographed using an Image Station 2000 MM system (Kodak, USA) and visualised with Carestream Molecular Imaging software version 5.0 (Carestream Health Inc., USA).

6.2.4. 3. Results and Discussion

3.1. Physicochemical parameters

The physicochemical parameters of this experiment have been presented by Despland et al. (2011; Chapter 5). Briefly, the study showed that the pH of Bauxsol™ outlets spiked at 11 during the first week, and then reduced to 9 at week six, to slowly stabilising around 8.3 after 12 weeks. In contrast, gravel outlets recorded a steady pH at 6.9±0.1 over the six month trial. Dissolved oxygen was recorded at the column inlets (1.87±0.16 mg/L), Bauxsol™ outlets (2.08±0.09 mg/L) and gravel outlets (1.92±0.12 mg/L). Bauxsol™ pellets were directly used to bind phosphate (>70%) and trace-metals present in the wastewater. Moreover, the concentration of available phosphate (Colwell P) on Bauxsol™ pellets rose from 48 mg/kg pre-experiment to 1904 mg/kg post-experiment, whereas it only increased from 10 to 54 mg/kg on gravel particles.

Nitrate concentration on Bauxsol™ pellets increased from 14 to 60 mg/kg, suggesting that nitrogen was partially removed via geochemical binding. Similarly, some
nitrogen removal was attributed to geochemical precipitation in the gravel column, where ammonia combined with phosphate and magnesium from the reactive olivine contained in the gravel, to possibly form struvite. Additionally, Despland et al. (2011) showed that nitrogen removal was also caused by biological activities, where denitrification, nitrification and anammox processes were identified by isotopic N-assay using stable isotope amendments (mixture of $^{15}$N and $^{14}$N) performed on the Bauxsol™ pellets and gravel post-experiment.

3.2 Structure of free-floating bacterial communities
The structure of the free-floating bacterial communities was studied to evaluate the impact of effluent composition variability and the support particle used. The results showed a statistically significant temporal diversity ($R = 0.974$ with $p<0.1\%$; Fig. 6.1) and spatial diversity (e.g. Fig. 6.1, lanes 1–2–4, I–II–III) between DGGE profiles of 16S rDNA products from liquid samples (see all DGGE profiles in Appendix 9.7, Figures 9.17–9.24). This was also corroborated by the difference in richness ($R$; number of bands) and diversity index ($H'$; Table 6.2). The temporal and spatial differences were probably caused by wastewater inlet composition variability and subsequent changes of effluent induced by the column support material (e.g. alkaline environment in Bauxsol™ columns, changes in nutrient concentrations along the columns; Fig. 6.2 B, C and D). Imfeld et al. (2010) found similar results in their study, showing that the changes in the bacterial community in the aqueous phase of a constructed wetland system coincided with changes in the water conditions (oxic to anoxic environment).
**Figure 6.1:** DGGE analysis of 16S rDNA gene products amplified from liquid samples collected at T = 3 days (1–5) and T = 8 weeks (I–IV). Lane 1/I – inlet, lane 2/II – gravel outlet, lane 3 – gravel mid-column, lane 4/III – Bauxsol™ pellets outlets, lane 5/IV – Bauxsol™ pellets mid-column; St = DNA marker from wastewater isolates.
Table 6.2: Richness (R; number of bands), Shannon diversity index ($H'$) and equitability index ($J'$) calculated from DGGE profiles analysis of 16 rDNA gene products amplified from liquid samples (inlets, mid-columns, outlets) between 0 and 1, 1 and 2, 2 and 3, 3 and 4, 4 and 5, and 5 and 6 months. ± standard error.

<table>
<thead>
<tr>
<th>Time (months)</th>
<th>Sample</th>
<th>R</th>
<th>$H'$</th>
<th>$J'$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-1</td>
<td>Inlet</td>
<td>16 ±2</td>
<td>2.61 ±0.13</td>
<td>0.96 ±0.01</td>
</tr>
<tr>
<td></td>
<td>Bauxsol™ outlet</td>
<td>19 ±1</td>
<td>2.73 ±0.07</td>
<td>0.93 ±0.01</td>
</tr>
<tr>
<td></td>
<td>Gravel outlet</td>
<td>18 ±2</td>
<td>2.70 ±0.09</td>
<td>0.94 ±0.01</td>
</tr>
<tr>
<td>1-2</td>
<td>Inlet</td>
<td>17 ±1</td>
<td>2.70 ±0.07</td>
<td>0.96 ±0.01</td>
</tr>
<tr>
<td></td>
<td>Bauxsol™ outlet</td>
<td>16 ±4</td>
<td>2.53 ±0.22</td>
<td>0.94 ±0.02</td>
</tr>
<tr>
<td></td>
<td>Gravel outlet</td>
<td>20 ±0</td>
<td>2.83 ±0.05</td>
<td>0.94 ±0.02</td>
</tr>
<tr>
<td>2-3</td>
<td>Inlet</td>
<td>19 ±0</td>
<td>2.84 ±0.02</td>
<td>0.96 ±0.01</td>
</tr>
<tr>
<td></td>
<td>Bauxsol™ outlet</td>
<td>19 ±3</td>
<td>2.69 ±0.15</td>
<td>0.92 ±0.01</td>
</tr>
<tr>
<td></td>
<td>Gravel outlet</td>
<td>20 ±1</td>
<td>2.80 ±0.03</td>
<td>0.94 ±0.02</td>
</tr>
<tr>
<td>3-4</td>
<td>Inlet</td>
<td>18 ±1</td>
<td>2.76 ±0.06</td>
<td>0.95 ±0.01</td>
</tr>
<tr>
<td></td>
<td>Bauxsol™ outlet</td>
<td>20 ±4</td>
<td>2.79 ±0.12</td>
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</tr>
<tr>
<td></td>
<td>Gravel outlet</td>
<td>19 ±0</td>
<td>2.83 ±0.00</td>
<td>0.96 ±0.00</td>
</tr>
<tr>
<td>4-5</td>
<td>Inlet</td>
<td>14 ±4</td>
<td>2.40 ±0.40</td>
<td>0.94 ±0.02</td>
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<tr>
<td></td>
<td>Bauxsol™ outlet</td>
<td>19 ±1</td>
<td>2.60 ±0.07</td>
<td>0.89 ±0.01</td>
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<tr>
<td></td>
<td>Gravel outlet</td>
<td>16 ±4</td>
<td>2.56 ±0.21</td>
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</tr>
<tr>
<td>5-6</td>
<td>Inlet</td>
<td>11 ±3</td>
<td>2.19 ±0.31</td>
<td>0.92 ±0.02</td>
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<td>Bauxsol™ outlet</td>
<td>15 ±2</td>
<td>2.50 ±0.12</td>
<td>0.93 ±0.00</td>
</tr>
<tr>
<td></td>
<td>Gravel outlet</td>
<td>13 ±1</td>
<td>2.42 ±0.06</td>
<td>0.94 ±0.00</td>
</tr>
</tbody>
</table>
Figure 6.2: Nitrogen concentration (mg/L) in the inlet (— — —), Bauxsol™ effluent at mid-column (—□—), Bauxsol™ outlet (—■—), gravel effluent at mid-column (—○—), and gravel outlet (—●—) between 0 and 1 month, 1 and 2 months, 2 and 3 months, 3 and 4 months, 4 and 5 months, and 5 and 6 months. A) Total nitrogen, B) ammonia (NH$_4^+$), C) nitrite (NO$_2^-$), and D) nitrate (NO$_3^-$). ± standard error.
3.3 Structure of attached bacterial communities

The structure of the attached bacterial communities was studied to evaluate the impact of support particle used. The results from the DGGE community profiles of Bauxsol™ pellets and gravel particles analysis (Fig. 6.3) showed that the support particle types had a significant impact ($R = 0.991$ with $p<0.2\%$) on the structure of the attached bacterial community, following 6 months of continual operation. The difference in profiles indicated the selection of specific bacterial communities by the support particle physicochemical characteristics and contrasting environmental conditions. These findings have also been reported in several other studies (Calheiros et al. 2009; Li et al. 2010b; Addison et al. 2011).

![Figure 6.3: DGGE analysis of 16S rDNA community profiles of DNA isolated from column solids collected at the end of the 6 month experiment along the column’s length: Bauxsol™ pellets (left; a–f) and Gravel (right; A–F). Lane a/A – 0cm (near inlet, bottom column), lane b/B – 25cm, lane c/C – 50 cm, lane d/D – 75 cm, lane e/E – 105 cm, lane f/F – 130 cm (near outlet, top column); St = DNA marker from wastewater isolates. Arrows indicate new and unique bands compared to the liquid DGGE profiles (Fig. 6.1).]

The microporous structure of Bauxsol™ pellets could provide steeper redox and nutrient diffusion gradients, which would impact on the bacterial community
structure. Moreover, the differences can also be explained by the completely different nature of the microenvironments generated by the interactions between biota and the support particle, including the effluent composition (e.g. pH, nutrients concentrations; Fig. 6.2 B, C and D), and TN and TC concentrations on the support particles (Table 6.3). In fact, Bauxsol™ pellets compared to gravel pre- and post-experiment had a much higher percentage of TC, which was mostly made of inorganic carbon (90%; Table 6.3). This result probably indicates insoluble carbonate biomineralisation with Bauxsol™, linked to alkaline pH within the column (i.e. CaCO$_3$ precipitate above pH 8.4) and CO$_2$ production by respiration. Moreover, Bauxsol™ pellets showed an increase in TC of more than 60% and in TOC of around 100% post-experiment; no significant changes on gravel particles were observed (Table 6.3). The changes in TOC and TN concentrations affected the C/N ratio (TOC/TN) of Bauxsol™ pellets (increased from 3 up to 9.3). It is generally admitted that, in a soil litter, C/N ratios (TOC/TN) between 6 and 25 are indicative of a nitrogen-rich litter, with easy access to decomposers. Higher values are typical of litters with poor decay potential, therefore resisting biological degradation. Lower values may reveal excessively high N concentrations, which may be toxic to biota (e.g. biomethanization processes). Consequently, the C/N ratio of 9.3 found in Bauxsol™ pellets may be interpreted as a good level of biological activity (i.e. active biofilm) in Bauxsol™ columns (Gobat et al. 2004; Brady and Weil 2008).

<table>
<thead>
<tr>
<th>Time</th>
<th>Material</th>
<th>Distance</th>
<th>TC</th>
<th>TOC</th>
<th>IC</th>
<th>TN</th>
<th>C/N ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-</td>
<td>Bauxsol™</td>
<td>0 cm</td>
<td>1.59</td>
<td>0.15</td>
<td>1.44</td>
<td>0.05</td>
<td>3.0</td>
</tr>
<tr>
<td>Pre-</td>
<td></td>
<td>25 cm</td>
<td>2.54</td>
<td>0.39</td>
<td>2.15</td>
<td>0.06</td>
<td>6.5</td>
</tr>
<tr>
<td>Pre-</td>
<td></td>
<td>50 cm</td>
<td>2.60</td>
<td>0.32</td>
<td>2.28</td>
<td>0.04</td>
<td>8.0</td>
</tr>
<tr>
<td>Pre-</td>
<td></td>
<td>75 cm</td>
<td>2.70</td>
<td>0.28</td>
<td>2.41</td>
<td>0.03</td>
<td>9.3</td>
</tr>
<tr>
<td>Pre-</td>
<td></td>
<td>105 cm</td>
<td>2.67</td>
<td>0.24</td>
<td>2.42</td>
<td>0.03</td>
<td>8.0</td>
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<tr>
<td>Pre-</td>
<td></td>
<td>130 cm</td>
<td>2.54</td>
<td>0.26</td>
<td>2.28</td>
<td>0.03</td>
<td>8.7</td>
</tr>
<tr>
<td>Post-</td>
<td>Gravel</td>
<td>0 cm</td>
<td>0.14</td>
<td>0.10</td>
<td>0.04</td>
<td>0.02</td>
<td>5.0</td>
</tr>
<tr>
<td>Post-</td>
<td></td>
<td>25 cm</td>
<td>0.04</td>
<td>0.04</td>
<td>0.00</td>
<td>0.01</td>
<td>4.0</td>
</tr>
<tr>
<td>Post-</td>
<td></td>
<td>50 cm</td>
<td>0.03</td>
<td>0.00</td>
<td>0.03</td>
<td>0.01</td>
<td>0.0</td>
</tr>
<tr>
<td>Post-</td>
<td></td>
<td>75 cm</td>
<td>0.03</td>
<td>0.02</td>
<td>0.01</td>
<td>0.01</td>
<td>2.0</td>
</tr>
<tr>
<td>Post-</td>
<td></td>
<td>105 cm</td>
<td>0.02</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>1.0</td>
</tr>
<tr>
<td>Post-</td>
<td></td>
<td>130 cm</td>
<td>0.02</td>
<td>0.00</td>
<td>0.02</td>
<td>0.01</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Table 6.3: Total carbon (%), total organic carbon (%), inorganic carbon (%), total nitrogen (%), and carbon/nitrogen ratio (i.e. TOC/TN) in Bauxsol™ pellets and gravel particles pre- and post-experiment (6 months) at different lengths along the columns (0 cm = inlet; 130 cm = outlet).
ANOSIM results for the DGGE solids revealed that the richness (i.e. R; number of bands), Shannon diversity index (H’) and equitability index (J’) were greater for the Bauxsol™ pellets compared to the gravel (R: 39.8±1.3 versus 26.7±1.1; H’: 3.40±0.04 versus 2.85±0.05; J’: 0.92±0.1 versus 0.87±0.1; Table 6.4), which may be interpreted as a more microheterogenous bacterial community growth on the Bauxsol™ pellets. Presumably, the larger surface area of Bauxsol™ pellets (~60% porosity; up to 100m²/g surface to volume ratio; McConchie et al. 1999), and the most likely presence of anoxic microzones in Bauxsol™ pellets may provide additional ecological niches (i.e. higher H’) for attached-growth bacteria development.

**Table 6.4**: Richness (R; number of bands), Shannon diversity index (H’) and equitability index (J’) calculated from DGGE profiles analysis of 16 rDNA gene products amplified from solid samples (Bauxsol™ pellets and gravel) after a six month trial at different lengths along the columns (0 cm = inlet; 130 cm = outlet).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Distance (cm)</th>
<th>R</th>
<th>H’</th>
<th>J’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bauxsol™</td>
<td>0</td>
<td>37</td>
<td>3.25</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>38</td>
<td>3.41</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>44</td>
<td>3.49</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>42</td>
<td>3.44</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>105</td>
<td>42</td>
<td>3.47</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>130</td>
<td>36</td>
<td>3.35</td>
<td>0.94</td>
</tr>
<tr>
<td>Gravel</td>
<td>0</td>
<td>23</td>
<td>2.64</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>30</td>
<td>3.00</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>27</td>
<td>2.92</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>28</td>
<td>2.94</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>105</td>
<td>24</td>
<td>2.78</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>130</td>
<td>28</td>
<td>2.84</td>
<td>0.85</td>
</tr>
</tbody>
</table>

The bacterial communities attached to the Bauxsol™ pellets and gravel particles were clustered into two distinct groups (Fig. 6.4; separation at 36% similarity). A closer examination of spatial variability along the Bauxsol™ column length showed communities clustering into two major sub-groups (~60% similarity), which correlated strongly with distance from the inlet (proximal 0 and 25 cm; distal 50, 75, 105 and 130 cm). Gravel treatment also showed similar groupings along column length at ~58% similarity (proximal 0, 25, and 50 cm; distal 75, 105, and 130 cm). However, further attempts to differentiate these groups using SIMPROF test failed. The spatial variability is more difficult to explain than the variability between the two supports, however, it may be related to the changes in nutrients concentrations along
the length of the columns (Table 6.3). For example, nitrite concentration was lower in the first half of the columns (0 – 65 cm) than the second half of the columns (65 – 130 cm; Table 6.3) and this may have a significant impact on the structure of the bacterial community.

Figure 6.4: Dendrogram of attached bacterial communities at different lengths along the columns (0 cm = inlet; 130 cm = outlet) after six month trial. Fourth-root transformed DGGE data sets were used in clustering Bray-Curtis similarities. Bp = Bauxsol™ pellets; Gc = Gravel. Dark line = significantly different; light line = SIMPROF test showing that these samples cannot be statistically differentiated.

3.4 Bacterial communities functions to transform nitrogen

Reductions of BOD (inlet 29±4 mg/L; Bauxsol™ outlet 11±2 mg/L, gravel outlet 9±2 mg/L) and COD (inlet 82±7 mg/L; Bauxsol™ outlet 34±3 mg/L, gravel outlet 36±3 mg/L) concentrations over 6 months of operation, highlighted the establishment of biochemical activities and resulting BOD concentrations were within Australian guidelines (National Water Quality Management Strategy 1997). The appearance of new and unique bands on the solid DGGE profiles (bottom half of the gels; arrow in Fig. 6.3) probably correspond to and signify the development of biofilms. Presumably these biofilms harbour bacteria populations involved in important biogeochemical functions within the columns.

Establishment of biogeochemical activities in columns may be partly responsible for the reduction of nitrogen concentrations in Bauxsol™ outlets (0 – 1 month 11%, 1 – 2 months 16%, 2 – 3 months 23%, 3 – 4 months 30%, 4 – 5 months 54%, and 5 – 6
months 47%) and in gravel outlets (0 – 1 month 29%, 1 – 2 months 12%, 2 – 3 months 32%, 3 – 4 months 29%, 4 – 5 months 53%, and 5 – 6 months 52%; Fig. 6.2 A). However, as described in a previous study (Despland et al. 2011), TN removal may also be partially explained by strong removal of ammonia via struvite precipitation in the gravel column, and by nitrate binding to the Bauxsol™ matrix via ligand exchange in Bauxsol™ columns. Removal of organic nitrogen (average of 47% removal) is best explained by organic matter breakdown by microorganisms into ammonium (i.e. mineralisation). Although TN was equally removed in both Bauxsol™ and gravel outlets, Bauxsol™ pellets present additional advantages such as efficient phosphate (>70%) and trace-metal binding (Despland et al. 2011).

The presence of ammonia-oxidising bacteria, denitrifiers and anammox organisms were seen in both systems by PCR-amplification of marker genes involved in nitrogen transformation (Table 6.5; Appendix 9.7, Figure 9.25). This analysis also highlighted the difference between the suspended and attached bacterial communities. Although the presence of such genes does not mean intense activity, the isotopic N-assay using stable isotope amendments (mixture of $^{15}$N and $^{14}$N) previously undertaken on Bauxsol™ pellets and gravel particles used in this study showed indications of biological activities linked to nitrogen transformation (Despland et al. 2011). The coexistence of nitrifiers, denitrifiers, and anammox bacteria in a particular wastewater treatment system has been previously reported by Xiao et al. (2009).

**Table 6.5**: Presence (+) or absence (−) of bands on gel electrophoresis of functional PCR amplification products targeting bacterial nitritation (amoA AOB genes), archael nitritation (amoA AOA genes), bacterial denitrification (nosZ genes), and anaerobic-ammonia oxidising (anammox) bacteria (hzo genes); positive and negative controls used. Solid samples (Bauxsol™ pellets and gravel particles) taken post-experiment (i.e. 6 months) at 0, 75, and 130 cm along the column length; Liquid samples (Inlet) taken during the trial at T = 1 day and 22 weeks.

<table>
<thead>
<tr>
<th></th>
<th>amoA AOB</th>
<th>amoA AOA</th>
<th>nosZ</th>
<th>hzo</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bauxsol™</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 cm</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>75 cm</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>130 cm</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Gravel</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 cm</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>75 cm</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>130 cm</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Inlet</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>22w</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>
Ammonia-oxidising bacteria (AOB) presence in Bauxsol™ pellets at a depth of 75 and 130 cm, and on gravel at 130 cm was revealed by functional PCR amplification of bacterial \( \text{amoA} \) genes. This finding, along with ammonia diminution, nitrite increase in column outlets (Fig. 6.2 B & C), and the oxic environment (DO inlet 1.87±0.16 mg/L, DO Bauxsol™ outlets 2.08±0.09 mg/L, DO gravel outlets 1.92±0.12 mg/L), confirms the active nitrification found by the isotopic N-assay (Despland et al. 2011), and therefore implies a nitrification process in both systems. AOB organisms were absent at a depth of 0 cm in Bauxsol™ and gravel columns (Table 6.5), suggesting competition for \( \text{NH}_4^+ \) with heterotrophic fast growing microorganisms (Hanaki et al. 1990). It is hypothesised that the presence of organic substrates that are easy to metabolisable may inhibit AOB organisms; however, no studies to date have found a clear explanation for that. No ammonia-oxidising archaea (AOA) carrying \( \text{amoA} \) genes were found in any of the samples (Table 6.5). The ‘young’ age of the column system (6 months) most likely prevented detection of AOA because they are very slow growing organisms. AOA are well adapted to wastewaters (Park et al. 2006) and it is expected that Bauxsol™ packed columns will harbour AOA organisms only after a longer operation time.

The presence of \( \text{nosZ} \) PCR gene products signified bacterial denitrifiers in both Bauxsol™ (0 and 130 cm) and gravel columns (0, 75 and 130 cm; Table 6.5); no \( \text{nosZ} \) PCR gene products were found in any of the inlets (1d and 22w; Table 6.5). Nitrate concentration decreased in the outlets (Fig. 6.2 D) and positive \( \text{nosZ} \) gene PCRs confirmed the denitrification activity found by isotopic N-assay (Despland et al. 2011). Collectively, these findings prove that denitrification is occurring. However, because denitrifiers are facultative aerobes, the denitrification must be occurring in anoxic microzones within formed biofilms (Gobat et al. 2004; Madigan et al. 2012). Analogous anoxic microzones are most likely present in Bauxsol™ pellets; however, the exact location of such microzones in the pellets has yet to be determined. The populations of denitrifiers may have been different between the two support particles because pH was slightly alkaline in the Bauxsol™ columns (pH 9.06±0.27) and neutral in the gravel columns (pH 6.89±0.04) (Zumft 1997).

Similarly, \( hzo \) genes carried by anaerobic-ammonia oxidising (anammox) bacteria were detected in all DNA extracted from the solid samples, but not from the column
inlets (Table 6.5). Being strictly anaerobic organisms, anammox bacteria were expected to be growing in the anoxic microzones previously mentioned. However, the anammox population in both systems was probably small as they are slow growers, and therefore their activity was presumably difficult to be detected in these relatively young systems; the geochemical data (Fig. 6.2 B & C) and isotopic N-assay (Despland et al. 2011) support this view.

6.2.5. 4. Conclusions
Data presented in this study demonstrate that environmental bacterial communities can grow on porous Bauxsol™ pellets. Moreover, DGGE profiles showed that the type of support particle used had a significant impact on the attached bacterial communities. The microporous structure of the Bauxsol™ pellets provided steeper redox and nutrient diffusion gradients, and the completely different nature of the microenvironments generated by the interactions between biota and the support particle impacted on the bacterial community structure. Ecological indices also revealed a more heterogeneous bacterial community structure on Bauxsol™ pellets compared to gravel particles. In addition, TOC/TN ratios of 6.5–9.3 post-experiment may indicate good biological activity (i.e. active biofilm) in Bauxsol™ columns. Geochemical data and functional PCR amplifications showed that a complex nitrogen cycle is at play within the Bauxsol™ columns (i.e. geochemical binding, and nitrification, denitrification, and anammox processes) because of oxic and anoxic zones within the biofilms and/or pellets. Further detailed studies are required to confirm these findings and to characterise bacterial species involved in nitrogen transformation. Finally, these preliminary results are encouraging and provide some confirmation that Bauxsol™ pellets are a useful biomass support particle for wastewater treatment.

6.2.6. Acknowledgements
We would like to thank Dr Rebecca McIntosh for her proof reading work. We also acknowledge the help of the anonymous reviewers who provided us with helpful comments to improve the manuscript.


CHAPTER 7: CONSTRUCTED WETLAND CANAL FIELD EXPERIMENT

7.1. Overview

The success of the mesocosm field experiment allowed the undertaking of the final step to successful completion of this thesis. The findings of the mesocosm columns experiment suggest that Bauxsol™ pellets may be useful treating wastewater in a simple unplanted horizontal constructed wetland system. Bauxsol™ pellets were very efficient in binding phosphate and trace-metals from wastewater, and they were suitable as biomass support particles for environmental bacterial communities including groups linked to nitrogen cycle. Constructed wetlands (CW; see Chapter 2, section 2.1.6), especially subsurface-flow constructed wetlands, are commonly used in a post-wastewater treatment circuit to further physically, chemically and biologically remove contaminants (Kadlec and Wallace 2009). The material (e.g. gravel, soil) and plant roots used in a CW provide considerable reactive surface area for adsorption of complexing ions (e.g. phosphate, trace-metals), and a physical support for microorganisms to develop as biofilms (Bitton 1994; Liu and Liptak 2000; Hammer and Hammer Jr. 2005; Madigan et al. 2012).

The aims of this experiment are to test Bauxsol™ pellets as an alternate material in an unplanted constructed wetland system, and to investigate the interactions between the pellets, the soil, the secondary treated effluent and the bacterial communities. The specific objectives are: to study the temporal and spatial removal of phosphate, nitrogen, and trace-metals; to investigate the structural differences of both attached and free-floating bacterial communities; and to assess the presence of microbial communities involved in the nitrogen cycle.

7.2. Materials and methods

7.2.1. Experiment setting & sampling

An unplanted horizontal flow wetland (Figure 7.1) was designed as slightly-U-shaped, rectangular canal made of PVC (300 cm long × 50 cm wide × 25 cm deep). The
experimental canal was filled up with a 5 cm deep lower layer of clay soil and a 5 cm deep upper layer of Bauxsol™ pellets (5–10 mm diameter; Despland et al. 2010a, Chapter 3), while the control canal was filled with a 5 cm deep lower layer of clay soil and a 5 cm deep upper layer of basalt gravel (5–10 mm diameter). Gravel was selected as it is the most commonly used filtration/support substrate material in constructed wetland. The canals were unplanted and filled with 2 × 5 cm layers to simplify the system and facilitate data interpretation (i.e. plants are active in contaminants removal and the roots act as biomass support; a deeper layer means more variability between the samples). The soil layer (Appendix 9.1) was introduced in both canals to simulate a wetland ecosystem, such as the one used at the South Lismore Sewage Treatment Plant NSW Australia (Appendix 9.2, Figure 9.1), and to act as a source of bacterial populations.

Secondary treated effluent from the South Lismore Sewage Treatment Plant NSW Australia was first pumped into a 200 L drum and then delivered to both control and experimental canals by a filtered/solenoid system at 16 mL/min (i.e. same inlet water for both canals; Figure 7.2). This effluent acted as a second source of bacterial populations and provided feeding substrates for bacteria. Holes were drilled just above the Bauxsol™/gravel upper layer at the distal end of both canals, which allowed water to discharge. To ensure an equal distribution of the secondary treated effluent in both layers (i.e. the hydraulic conductivity was much higher in the upper layer) and utilisation of the treatment materials, canals were fitted with an alternated T- and U-shaped baffles system across the flow (Figures 7.1, 7.3 & 7.4). No tracer was used to measure the flow between the two layers, however, water was found at the bottom of the soil layer, confirming the effectiveness of the baffles system. Piezometers, with an opening on the side (covered by fine mesh to prevent solid infiltration), were installed mid-canal (1.5 m) and in close proximity (2.8 m) to the distal end of each canal to sample water from the lower and upper layers (Figures 7.1 & 7.3). Double layers of shade cloth was placed above and around the canals to prevent algae growth (without causing complete darkness; Figure 7.1), and regular cleaning and maintenance of the system was undertaken to prevent particulate clogging.
Figure 7.1: Canals setting: empty canal with baffles and piezometers system (top left); lower soil layer (top right); experimental canal with upper Bauxsol™ pellets layer (bottom left); control canal with upper gravel layer (bottom right).
Figure 7.2: Details of the effluent delivery: pump taking secondary treated effluent from the humus tank (top left) and bringing it into the green bin (top right and bottom left); effluent delivered in the canals by a solenoid system at 16 mL/min (centre bottom and bottom right).

Figure 7.3: Side and above views of the canals system, showing the baffles system and the piezometers for water sampling in both upper layer (i.e. Bauxsol™ or gravel) and lower layer (i.e. soil).
A slope of 0.25° allowed the effluent to run by gravity at a hydraulic retention time (HRT) of approximately 5 days \[ HRT = \frac{A \times y \times p}{Q}; \text{where } A = \text{surface area of canal}, y = \text{wetted depth}, p = \text{porosity (i.e. void space) of the material (Bauxsol™ pellets } \sim 60\%, \text{gravel } \sim 50\%, \text{soil } \sim 30\%), Q = \text{flow rate}. \] This HRT was chosen because typical contaminants removal in CW is around 5 days; sufficient contact time between the contaminants and the biofilm attached to the support particles is needed to ensure adequate microbial activities (Hammer 1991; Reed et al. 1995; Rousseau et al. 2004; Kadlec and Wallace 2009). Furthermore, the 5 days HRT ensured that the quality of the outlets was within Australian guidelines for advanced wastewater treatment (National Water Quality Management Strategy 1997, Chapter 2, Table 2.2).

Liquid samples were taken after equilibration (i.e. water flowing just at the upper layer–air interface) and then at regular intervals \( T = 1, 2, 4, 6, 8, 12, 16, 20, \) and 24 weeks; Appendix 9.8, Table 9.20). Water samples (1,200 mL) were collected from the inlet (i.e. secondary treated effluent), the experimental and control canal outlets, as well as from the piezometers in both lower and upper layers (150 mL; Figure 7.3).
Piezometers were first emptied and allowed to re-fill prior to sampling to ensure sample freshness. Note that the waters from the piezometers refer to the water of the Bauxsol™ layer and water of the soil layer in the experimental canal, and water of the gravel layer and water of the soil layer in the control canal.

Solid samples were taken as small sediment cores of 2 cm diameter extracted at 0.6 (±0.15), 1.3 (±0.15), 1.8 (±0.15) and 2.5 (±0.15) m from the proximal end of the canals in the upper Bauxsol™ pellets and gravel layers, in the interface layer (i.e. between Bauxsol™ pellets and soil in the experimental canal, or between gravel particles and soil in the control canal), and in the lower soil layers from both canals (Figure 7.4). Duplicate cores at each distance were combined, using one sample from the canal edge and one from the mid-line of the canal (Figure 7.4). Core voids were re-filled with fresh materials (original material: soil – kept in a bucket in the dark – for the lower layer, Bauxsol™ pellets or gravel for the upper layer) washed with Milli-Q water to avoid any pH/alkalinity spikes. Samples were first taken at 0.15 m above the distance (+0.15 m) to avoid any disturbance, if any, from the refilled core voids.

7.2.2. Physico-chemical parameters analysis

Inlet, experimental outlet, control outlet, waters of the Bauxsol™ layer and soil layer from the experimental canal, and waters of the gravel layer and soil layer from the control canal were tested for their physico-chemical parameters. All liquid samples were analysed for pH (APHA 4500 H⁺-b), electrical conductivity (EC) (APHA 2510-b), temperature (APHA 2550-b), and concentration of dissolved oxygen (DO) (APHA 2810-b), total nitrogen (TN) (APHA 4500 N-c), total Kjeldahl nitrogen (TKN) (TN – NO₃), ammonium (NH₄⁺) (APHA 4500 NH₃-h), nitrite (NO₂⁻) (APHA 4500 NO₂⁻-c), nitrate (NO₃⁻) (APHA 4500 NO₃⁻-f), total phosphorus (TP) (APHA 4500 P-h), and orthophosphate (PO₄³⁻) (APHA 4500 P-g) (American Public Health Association 1998). Inlet, experimental outlet and control outlet were also analysed for concentration of biochemical oxygen demand (BOD) (APHA 5210-b), calcium (Ca) (APHA 3120 ICPOES), magnesium (Mg) (APHA 3120 ICPOES), and redox potential (American Public Health Association 1998). Moreover, at experiment completion, inlet and experimental and control outlets were analysed for concentration of total
organic carbon (TOC) (APHA 5310-b), dissolved organic carbon (DOC) (APHA 5310-b), and faecal coliform count (APHA 9222-d) (American Public Health Association 1998).

Bauxsol™ pellets and soil particles of the experimental canal (proximal (0.6 m) and distal (2.5 m) ends), and gravel and soil particles of the control canal (proximal (0.6 m) and distal (2.5 m) ends) were analysed for concentration of: TP and Colwell phosphorus (Rayment and Higginson 1992), NH$_4^+$ and NO$_3^-$ using a 1:10 KCl extract (APHA 4500). Pre- and post-experiment solids from both the proximal and distal ends of each layer of each canal were also analysed for concentration of TN, total carbon (TC), total organic carbon (TOC) using LECO CNS2000 Analyser, inorganic carbon (IC; calculation of: TC – TOC), C/N ratio (calculation of: TOC/TN), and concentration of trace-metals including silver (Ag), arsenic (As), lead (Pb), cadmium (Cd), chromium (Cr), copper (Cu), manganese (Mn), nickel (Ni), selenium (Se), zinc (Zn), mercury (Hg), iron (Fe) and aluminium (Al) using a Nitric/HCl digest (APHA 3120 ICPMS) (American Public Health Association 1998). All tests were performed under quality assurance certification from the National Association of Testing Authorities (NATA) in the Southern Cross Environmental Analysis Laboratory (Appendix 9.1, Table 9.1)

7.2.3. DNA extraction; PCR/DGGE on 16S rDNA genes; Functional PCR amplification of genes involved in nitrogen cycle

DNA extraction was performed on liquids and solids following the method described in Chapter 6 and Appendix 9.3. DGGE separation of PCR amplified fragments corresponding to the 16S rDNA gene’s V3 region was undertaken on extracts containing >20 ng/µL (i.e. all inlets, available liquids and solids from the 4$^{th}$, 5$^{th}$ and 6$^{th}$ month, and soil pre-experiment). The details of the PCR/DGGE setting and primer set used can be found in Chapter 6, Table 6.1. DNA extracted from Bauxsol™ pellets and soil particles in the experimental canal (proximal end (0.6 m) at 5 months and distal end (2.5 m) at 6 months), and gravel and soil particles from the control canal (proximal end (0.6 m) at 5 months and distal end (2.5 m) at 6 months), as well as DNA extracted from inlets at 0, 5, and 6 months, were subjected to functional PCR amplification targeting specific genes involved in nitrogen transformation (bacterial
and archaeal amoA genes, nosZ genes, and hzo genes). Details of the PCR setting and primer sets used can be found in Chapter 6, Table 6.1. Details on the optimisation of DNA extraction, PCR/DGGE method and functional PCR assay can be found in the Appendix 9.3, Tables 9.2–9.8. Analyses were undertaken following QA/QC procedures (Appendix 9.1).

7.2.4. Statistical analysis
Sample richness (i.e. average total number of bands), Shannon diversity index (H’) and equitability index (J’) were calculated from liquid and solid DGGE data sets using ePRIMER (version 6) software package (Clarke and Gorley 2006) to compare bacterial communities between the experimental and control canals and within the canals layers. Non-metric multi-dimensional scaling (MDS) analysis on fourth-roots transformed data and Bray–Curtis similarity matrix was used to identify similarity between samples according to sampling time, space (i.e. distance from the canal’s inflow) and treatment (i.e. Bauxsol™ pellets, gravel, soil). DGGE profiles were also subjected to one-way analysis of similarity (ANOSIM) to statistically test whether there was a significant difference between samples (R value close to 1 indicates dissimilarity between samples).

7.3. Results

7.3.1. Geochemistry

7.3.1.1. pH, EC, redox potential, DO & temperature
Inlet pH was stable during the entire trial (6.8±0.1), while experimental canal outlet showed an increased pH reading over time (0–1 month 8.3±0.4; 5–6 months 9.6±0.1; Figure 7.5 A). The data also showed an elevated pH in the waters of the Bauxsol™ and soil layers, where up until month 4 solution pH was higher at mid-canal (1.5 m) by at least one pH unit compared to the distal end of the canal (2.8 m). After 4 months a shift was observed with higher pH recorded towards the distal end of the canal (Figure 7.5 A). In contrast, the pH in the control canal outlet was stable at 7.4±0.1 (Figure 7.5 B), and pH readings in the waters of the control canal were consistent in
both gravel and soil layers (7.2±0.1). Moreover, no major difference was observed between mid-canal (1.5 m) and the distal end of the canal (2.8 m).

**Figure 7.5**: pH recorded between 0 and 1 month, 1 and 2 months, 2 and 3 months, 3 and 4 months, 4 and 5 months, and 5 and 6 months in A) experimental canal: inlet (– * –), experimental outlet (– ■ –), at mid-canal (1.5 m) in the Bauxsol™ layer waters (histogram vertical line) and in the soil layer waters (histogram horizontal line), at distal end of the canal (2.8 m) in the Bauxsol™ layer waters (histogram vertical dash) and in the soil layer waters (histogram horizontal dash); in B) control canal: inlet (– * –), control outlet (– ○ –), at mid-canal (1.5 m) in the gravel layer waters (gray histogram vertical line) and in the soil layer waters (gray histogram horizontal line), at distal end of the canal (2.8 m) in the gravel layer waters (gray histogram vertical dash) and in the soil layer waters (gray histogram horizontal dash). ± standard error.

Electrical conductivity readings were consistent in the inlet, and the control canal outlet (683±29 μS and 744±43 μS, respectively), and no major differences were observed between the gravel and soil layers waters (Figure 7.6 B). An EC spike was seen in the experimental canal outlet over the first four months (11,610±1,286 μS).
This EC spike was not only found in the waters from the Bauxsol™ layer but also in the waters from the soil layer. Moreover, the spike was more pronounced at the distal end of the canal (2.8m) (Figure 7.6 A).

**Figure 7.6:** Electrical conductivity (EC; μS) recorded between 0 and 1 month, 1 and 2 months, 2 and 3 months, 3 and 4 months, 4 and 5 months, and 5 and 6 months in A) experimental canal: inlet (−∗−), experimental outlet (−■−), at mid-canal (1.5 m) in the Bauxsol™ layer waters (histogram vertical line) and in the soil layer waters (histogram horizontal line), at distal end of the canal (2.8 m) in the Bauxsol™ layer waters (histogram vertical dash) and in the soil layer waters (histogram horizontal dash); in B) control canal: inlet (−∗−), control outlet (−○−), at mid-canal (1.5 m) in the gravel layer waters (gray histogram vertical line) and in the soil layer waters (gray histogram horizontal line), at distal end of the canal (2.8 m) in the gravel layer waters (gray histogram vertical dash) and in the soil layer waters (gray histogram horizontal dash). ± standard error.

Dissolved oxygen concentration in the inlet was at 2.2±0.3 mg/L during the 24 weeks (Figure 7.7), although two spikes were recorded at 3.4±0.2 mg/L during week 1 and 16. DO concentration in the experimental canal outlet was recorded at an average of
4.8±0.4 mg/L (Figure 7.7 A), with spikes at 7.4 and 6.2 mg/L at week 1 and 6, respectively. Up until 2 months, DO concentration was higher in the waters of the Bauxsol™ layer than in the waters of the soil layer. However, after 2 months DO was similar in the waters of both layers (3.6±0.2 mg/L), suggesting an equilibration coupling between the two layers. Moreover, no major difference was observed between the waters of the mid-canal (1.5 m) and the distal end of the canal (2.8 m) (Figure 7.7 A), suggesting a near homogeneous gradient in DO from effluent entry to discharge. On average, the DO concentration was around 3.5±0.2 mg/L in the outlet of the control canal and no difference in DO was observed between the waters from the gravel and soil layers in the control canal. However, some differences were observed between the waters at mid-canal (1.5 m) and distal end of the canal (2.8 m) (Figure 7.7 B).
Figure 7.7: Dissolved oxygen (DO) concentration (mg/L) recorded between 0 and 1 month, 1 and 2 months, 2 and 3 months, 3 and 4 months, 4 and 5 months, and 5 and 6 months in A) experimental canal: inlet (— * —), experimental outlet (—■—), at mid-canal (1.5 m) in the Bauxsol™ layer waters (histogram vertical line) and in the soil layer waters (histogram horizontal line), at distal end of the canal (2.8 m) in the Bauxsol™ layer waters (histogram vertical dash) and in the soil layer waters (histogram horizontal dash); in B) control canal: inlet (— * —), control outlet (— ○ —), at mid-canal (1.5 m) in the gravel layer waters (gray histogram vertical line) and in the soil layer waters (gray histogram horizontal line), at distal end of the canal (2.8 m) in the gravel layer waters (gray histogram vertical dash) and in the soil layer waters (gray histogram horizontal dash). ± standard error.

Redox potential for the inlet, and control canal outlet were recorded at around +189±2 mV, whereas for the experimental canal outlet the redox potential was around +209±9 mV over the first four months and then dropped to around +175±3 mV. Data showed that the average temperature in the inlet, experimental canal outlet and control canal outlet was similar and increased over time. The temperature rose from approximately 16.9±0.7°C (0–1 month) to 27.1±1.9°C (5–6 months), owing to seasonal change (i.e. beginning of the experiment in winter, end of the experiment in summer).
7.3.1.2. Calcium & magnesium

A calcium spike averaging 1,737±135 mg/L over the first three months was seen in the experimental canal outlet (average of 35 mg/L in the inlet and control canal outlet). Similarly, magnesium concentration was higher in the experimental canal outlet (26±4 mg/L) compared to the inlet and control canal outlet (~10 mg/L in both).

7.3.1.3. Phosphorus in liquid phase

Total phosphorus (TP) concentration in the inlet was mostly made of orthophosphate (average of 87%) and fluctuated from 3 mg/L to 7.7 mg/L (Figures 7.8 & 7.9). In the experimental canal, TP was almost completely removed to the substrate material in the Bauxsol™ layer with an average removal rate of 95% over the entire trial at both mid-canal (1.5 m) and distal end of the canal (2.8 m) (Figures 7.8 A, 7.9 A & 7.10). In contrast, the control canal had much lower TP removal (average of 53%), where TP was mostly removed in the distal end of the canal (2.8 m) in both gravel and soil layers (Figures 7.8 B, 7.9 B & 7.10).
Figure 7.8: Total phosphorus (TP) concentration (mg/L) recorded between 0 and 1 month, 1 and 2 months, 2 and 3 months, 3 and 4 months, 4 and 5 months, and 5 and 6 months in A) experimental canal: inlet (– * –), experimental outlet (–■–), at mid-canal (1.5 m) in the Bauxsol™ layer waters (histogram vertical line) and in the soil layer waters (histogram horizontal line), at distal end of the canal (2.8 m) in the Bauxsol™ layer waters (histogram vertical dash) and in the soil layer waters (histogram horizontal dash); in B) control canal: inlet (– * –), control outlet (–○–), at mid-canal (1.5 m) in the gravel layer waters (gray histogram vertical line) and in the soil layer waters (gray histogram horizontal line), at distal end of the canal (2.8 m) in the gravel layer waters (gray histogram vertical dash) and in the soil layer waters (gray histogram horizontal dash). ± standard error.
Figure 7.9: Orthophosphate (PO$_4^{3-}$) concentration (mg/L) recorded between 0 and 1 month, 1 and 2 months, 2 and 3 months, 3 and 4 months, 4 and 5 months, and 5 and 6 months in A) experimental canal: inlet (− ● −), experimental outlet (− ■ −), at mid-canal (1.5 m) in the Bauxsol™ layer waters (histogram vertical line) and in the soil layer waters (histogram horizontal line), at distal end of the canal (2.8 m) in the Bauxsol™ layer waters (histogram vertical dash) and in the soil layer waters (histogram horizontal dash); in B) control canal: inlet (− ● −), control outlet (− ○ −), at mid-canal (1.5 m) in the gravel layer waters (gray histogram vertical line) and in the soil layer waters (gray histogram horizontal line), at distal end of the canal (2.8 m) in the gravel layer waters (gray histogram vertical dash) and in the soil layer waters (gray histogram horizontal dash). ± standard error.
7.3.1.4. Phosphorus in solid phase

Comparisons of TP and Colwell P concentrations pre-experiment and at a specific period (i.e. 0–1, 1–2, 2–3, 3–4, 4–5, and 5–6 months) on Bauxsol™ pellets and soil particles in the experimental canal, and on gravel particles and soil particles in the control canal are presented in Table 7.1; pre-experiment data can also be found in the Appendix 9.8, Table 9.21. Bauxsol™ pellets and soil particles in the experimental canal showed major differences in TP and Colwell P binding over time and distance (Table 7.1). In fact, TP and Colwell P were predominantly bound to Bauxsol™ pellets at the proximal end (0.6 m) of the canal and the uptake increased with time from 23±42 mg/kg to 764±134 mg/kg for TP and from 56±34 mg/kg to 619±79 mg/kg for Colwell P. The soil particles in the experimental canal showed only moderate binding, increasing over time and similar at both ends of the canal (up to 111 mg/kg for TP and 23 mg/kg of Colwell P) suggesting the bulk of the P removal was by the active Bauxsol™ component. In contrast, gravel particles in the control canal showed a high TP loss (up to 2 g/kg or 0.2%) but the soil particles in this control canal had a much higher TP loading (up to 944 mg/kg) than the soil particles in the experimental canal.
Table 7.1: Comparison between materials (i.e. Bauxsol™ pellets and soil particles in the experimental canal; gravel particles and soil particles in the control canal) pre-experiment and at a specific period (i.e. 0–1, 1–2, 2–3, 3–4, 4–5, and 5–6 months) = accumulation or release\(^1\); total phosphorus and Colwell P concentration (mg/kg) at the proximal end (0.6 m) and distal end (2.5 m) of each canal.

**A: Total phosphorus (mg/kg)**

<table>
<thead>
<tr>
<th>Canal</th>
<th>Distance</th>
<th>Material</th>
<th>0-1 mth</th>
<th>1-2 mths</th>
<th>2-3 mths</th>
<th>3-4 mths</th>
<th>4-5 mths</th>
<th>5-6 mths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental</td>
<td>proximal end (0.6m)</td>
<td>Bauxsol™</td>
<td>23 ±42</td>
<td>178 ±20</td>
<td>380 ±183</td>
<td>582 ±20</td>
<td>616 ±14</td>
<td>764 ±134</td>
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<tr>
<td></td>
<td></td>
<td>Soil</td>
<td>-1 ±18</td>
<td>12 ±23</td>
<td>-8 ±25</td>
<td>43 ±26</td>
<td>70 ±1</td>
<td>71 ±1</td>
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<tr>
<td></td>
<td>distal end (2.5m)</td>
<td>Bauxsol™</td>
<td>-43 ±27</td>
<td>-1 ±27</td>
<td>27 ±69</td>
<td>102 ±6</td>
<td>70 ±38</td>
<td>36 ±4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soil</td>
<td>17 ±14</td>
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<td>48 ±64</td>
<td>74 ±38</td>
<td>42 ±6</td>
<td>41 ±7</td>
</tr>
<tr>
<td>Control</td>
<td>proximal end (0.6m)</td>
<td>Gravel</td>
<td>-1232 ±289</td>
<td>-900 ±170</td>
<td>-802 ±385</td>
<td>-337 ±80</td>
<td>-537 ±280</td>
<td>-775 ±42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soil</td>
<td>363 ±200</td>
<td>368 ±152</td>
<td>218 ±20</td>
<td>408 ±170</td>
<td>552 ±25</td>
<td>673 ±147</td>
</tr>
<tr>
<td></td>
<td>distal end (2.5m)</td>
<td>Gravel</td>
<td>-944 ±254</td>
<td>-867 ±201</td>
<td>-928 ±219</td>
<td>-591 ±118</td>
<td>-557 ±84</td>
<td>-755 ±115</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soil</td>
<td>211 ±85</td>
<td>146 ±40</td>
<td>168 ±51</td>
<td>156 ±39</td>
<td>262 ±68</td>
<td>318 ±12</td>
</tr>
</tbody>
</table>

**B: Colwell P (mg/kg)**

<table>
<thead>
<tr>
<th>Canal</th>
<th>Distance</th>
<th>Material</th>
<th>0-1 mth</th>
<th>1-2 mths</th>
<th>2-3 mths</th>
<th>3-4 mths</th>
<th>4-5 mths</th>
<th>5-6 mths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental</td>
<td>proximal end (0.6m)</td>
<td>Bauxsol™</td>
<td>56 ±34</td>
<td>191 ±47</td>
<td>331 ±47</td>
<td>420 ±43</td>
<td>501 ±39</td>
<td>619 ±79</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soil</td>
<td>9 ±4</td>
<td>15 ±3</td>
<td>7 ±6</td>
<td>5 ±4</td>
<td>13 ±5</td>
<td>17 ±0</td>
</tr>
<tr>
<td></td>
<td>distal end (2.5m)</td>
<td>Bauxsol™</td>
<td>-13 ±8</td>
<td>-12 ±12</td>
<td>4 ±2</td>
<td>-16 ±19</td>
<td>-14 ±21</td>
<td>1 ±6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soil</td>
<td>10 ±2</td>
<td>16 ±2</td>
<td>22 ±1</td>
<td>16 ±7</td>
<td>13 ±4</td>
<td>11 ±5</td>
</tr>
<tr>
<td>Control</td>
<td>proximal end (0.6m)</td>
<td>Gravel</td>
<td>1 ±2</td>
<td>6 ±6</td>
<td>17 ±2</td>
<td>21 ±2</td>
<td>21 ±2</td>
<td>22 ±3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soil</td>
<td>4 ±3</td>
<td>1 ±1</td>
<td>6 ±6</td>
<td>15 ±4</td>
<td>15 ±4</td>
<td>14 ±4</td>
</tr>
<tr>
<td></td>
<td>distal end (2.5m)</td>
<td>Gravel</td>
<td>1 ±1</td>
<td>6 ±5</td>
<td>11 ±5</td>
<td>8 ±2</td>
<td>9 ±0</td>
<td>10 ±1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soil</td>
<td>4 ±2</td>
<td>6 ±2</td>
<td>4 ±0</td>
<td>4 ±0</td>
<td>12 ±8</td>
<td>17 ±2</td>
</tr>
</tbody>
</table>

\(^1\)negative values represent a loss in mass relative to an earlier condition
7.3.1.5. Trace-metals

Trace-metal concentrations in the inlet were low: silver (0.001 mg/L), arsenic (0.001 mg/L), lead (0.001 mg/L), cadmium (0.001 mg/L), chromium (0.003 mg/L), copper (0.007 mg/L), manganese (0.064 mg/L), nickel (0.012 mg/L), selenium (0.001 mg/L), zinc (0.064 mg/L), mercury (0.0005 mg/L), iron (0.150 mg/L) and aluminium (0.008 mg/L).

Comparative analyses of solids pre- and post-experiment in the experimental canal indicated that As, Pb, Cd, Cr, Cu, Ni, Se, Zn, and Al were mostly bound to Bauxsol™ pellets rather than soil particles, whereas Mn was bound equally between both materials (Table 7.2; pre-experiment data can be found in the Appendix 9.8, Table 9.21). Trace-metals were evenly bound along the canal’s length, except Pb and Cr that accumulated mostly at the distal end (2.5 m), and Cu that accumulated mostly at the proximal end (0.6 m). In contrast, the control canal revealed that gravel lost all trace-metals except Cr and some Al. Moreover, soil particles in the control canal did not bind trace-metals (except Mn and Zn) similar to the soil found in the experimental canal (Table 7.2).
Table 7.2: Comparison between materials (i.e. Bauxsol™ pellets and soil particles in the experimental canal; gravel particles and soil particles in the control canal) pre-experiment and post-experiment (i.e. 24 weeks) = accumulation or release\(^1\); trace-metals concentration (mg/kg; except iron and aluminium which are in weight %) at the proximal end (0.6 m) and distal end (2.5 m) of each canal.

| Material | Experimental | | Control | | | Canal |
| --- | --- | --- | --- | --- | --- | --- | --- |
| | Proximal end (0.6 m) | Distal end (2.5 m) | Proximal end (0.6 m) | Distal end (2.5 m) | | Distance |
| | Bauxsol™ | Soil | Bauxsol™ | Soil | Gravel | Soil | Gravel | Soil | Material |
| Silver | 0.0 | 0.0 | -0.1 | 0.0 | -0.1 | 0.0 | -0.1 | 0.0 |
| Arsenic | 7.6 | 0.4 | 5.6 | 0.4 | -0.3 | -0.1 | -0.5 | 0.2 |
| Lead | 7.2 | 1.3 | 13.0 | 1.4 | -0.9 | -0.8 | -1.3 | -0.2 |
| Cadmium | 0.4 | 0.1 | 0.5 | 0.1 | 0.0 | 0.1 | 0.0 | 0.0 |
| Chromium | 48.8 | 17.9 | 88.9 | 23.6 | 8.9 | -7.1 | 7.2 | 7.4 |
| Copper | 17.4 | 0.7 | 8.8 | 0.6 | -4.5 | -2.3 | -6.6 | -1.1 |
| Manganese | 140.6 | 116.4 | 102.3 | 101.9 | -116.9 | 256.2 | -180.3 | 197.2 |
| Nickel | 6.9 | -2.6 | 7.3 | 0.1 | -1.0 | -7.0 | -6.1 | -3.1 |
| Selenium | 0.7 | 0.2 | 0.4 | 0.2 | 0.2 | -0.2 | 0.0 | 0.2 |
| Zinc | 58.6 | 17.2 | 60.6 | 16.7 | 0.4 | 35.0 | -12.8 | 20.3 |
| Mercury | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Iron | -1.2 | -1.5 | 0.6 | -1.4 | -2.2 | -1.5 | -3.1 | -1.5 |
| Aluminium | 1.6 | 0.4 | 2.1 | 0.5 | 0.5 | -0.6 | -0.1 | 0.1 |

\(^1\) negative values represent a loss in mass relative to an earlier condition
7.3.1.6. Nitrogen in liquid phase

The average total nitrogen concentration was at 25±2.3 mg/L in the inlet (a drop was recorded after month 3), while TN was recorded at 18.4±1.2 mg/L in the experimental canal outlet and at 17.9±1.3 mg/L in the control canal outlet (Figure 7.11). An exception was noted between 4 and 5 months, where a lower TN concentration was recorded in the inlet compare to both canals outlets. Overall TN reduction rate was ~35% in both canals outlets until 3 months, and ~13% after 5 months. In the experimental canal, TN concentration was significantly higher in the Bauxsol™ layer waters for the first month; however, there was no major difference between mid-canal (1.5 m) and the distal end of the canal (2.8 m) (Figure 7.11 A). TN concentration was fairly similar between the waters from both control canal layers, and in addition, up until 2 months the concentration was significantly higher at mid-canal (1.5 m) (Figure 7.11 B).
Figure 7.11: Total nitrogen (TN) concentration (mg/L) recorded between 0 and 1 month, 1 and 2 months, 2 and 3 months, 3 and 4 months, 4 and 5 months, and 5 and 6 months in A) experimental canal: inlet (– * –), experimental outlet (– ■ –), at mid-canal (1.5 m) in the Bauxsol™ layer waters (histogram vertical line) and in the soil layer waters (histogram horizontal line), at distal end of the canal (2.8 m) in the Bauxsol™ layer waters (histogram vertical dash) and in the soil layer waters (histogram horizontal dash); in B) control canal: inlet (– ○ –), control outlet (– □ –), at mid-canal (1.5 m) in the gravel layer waters (gray histogram vertical line) and in the soil layer waters (gray histogram horizontal line), at distal end of the canal (2.8 m) in the gravel layer waters (gray histogram vertical dash) and in the soil layer waters (gray histogram horizontal dash). ±standard error.

Total Kjeldahl nitrogen (TKN) concentration, effectively represent the organic nitrogen, ammonia and ammonium, was recorded at 7.8±1.1 mg/L in the inlet during the first month and after 4 months; a spike to 19.7±0.5 mg/L was observed between months 1 and 4 (Figure 7.12). TKN was more or less consistent during the 6 months trial in both canals outlets (3.8±0.9 mg/L in the experimental canal outlet and 3.1±0.7 mg/L in the control canal outlet; Figure 7.12 A & B). Moreover, in the experimental canal, TKN was slightly higher in the waters of the Bauxsol™ layer; and at mid-canal
(1.5 m) before 4 months (Figure 7.12 A). In the control canal, no major difference was observed between waters from the gravel and soil layers and between mid-canal (1.5 m) and the distal end of the canal (2.8 m) (Figure 7.12B).

**Figure 7.12:** Total Kjeldahl nitrogen (TKN) concentration (mg/L) recorded between 0 and 1 month, 1 and 2 months, 2 and 3 months, 3 and 4 months, 4 and 5 months, and 5 and 6 months in A) experimental canal: inlet (− *−), experimental outlet (−□−), at mid-canal (1.5 m) in the Bauxsol™ layer waters (histogram vertical line) and in the soil layer waters (histogram horizontal line), at distal end of the canal (2.8 m) in the Bauxsol™ layer waters (histogram vertical dash) and in the soil layer waters (histogram horizontal dash); in B) control canal: inlet (− *−), control outlet (−○−), at mid-canal (1.5 m) in the gravel layer waters (gray histogram vertical line) and in the soil layer waters (gray histogram horizontal line), at distal end of the canal (2.8 m) in the gravel layer waters (gray histogram vertical dash) and in the soil layer waters (gray histogram horizontal dash). ±standard error.

Ammonium (NH₄⁺) concentration varied greatly from 2.5 to 16.9 mg/L N/NH₄⁺ in the inlet during the 6 months (Figure 7.13). In the experimental canal outlet ammonium concentration was recorded at 5.3±0.5 mg/L N/NH₄⁺ during the first month, and then
decreased <1 mg/L N/NH$_4^+$ after 3 months. Ammonium was slightly higher in the waters of the Bauxsol™ layer and at mid-canal (1.5 m) (Figure 7.13 A). In the control canal, the ammonium concentrations were close to zero in almost all waters (i.e. outlet, gravel and soil layer waters), except in the waters at mid-canal between 2 and 5 months (Figure 7.13B). Overall, both canals removed almost all ammonium from the inlet, particularly from month 2 onwards.

**Figure 7.13:** Ammonium (NH$_4^+$) concentration (mg/L) recorded between 0 and 1 month, 1 and 2 months, 2 and 3 months, 3 and 4 months, 4 and 5 months, and 5 and 6 months in A) experimental canal: inlet (−∗−), experimental outlet (−■−), at mid-canal (1.5 m) in the Bauxsol™ layer waters (histogram vertical line) and in the soil layer waters (histogram horizontal line), at distal end of the canal (2.8 m) in the Bauxsol™ layer waters (histogram vertical dash) and in the soil layer waters (histogram horizontal dash); in B) control canal: inlet (−∗−), control outlet (−○−), at mid-canal (1.5 m) in the gravel layer waters (gray histogram vertical line) and in the soil layer waters (gray histogram horizontal line), at distal end of the canal (2.8 m) in the gravel layer waters (gray histogram vertical dash) and in the soil layer waters (gray histogram horizontal dash). ± standard error.
Nitrite (NO$_2^-$) concentrations were around 1.0±0.2 mg/L N/NO$_2^-$ in the inlet during the first month, and around 0.4±0.1 mg/L N/NO$_2^-$ thereafter (Figure 7.14). In the experimental canal outlet, nitrite concentrations were above 1.5 mg/L N/NO$_2^-$ until 4 months (spike to 9.6 mg/L N/NO$_2^-$ at week 6), and around 0.3±0.1 mg/L N/NO$_2^-$ thereafter. No major difference in nitrite concentrations was observed between the waters of the Bauxsol™ and soil layers or between mid-canal (1.5 m) and the distal end of the canal (2.8 m) (Figure 7.14 A). In contrast, the control canal outlet had nitrite concentrations <0.2 mg/L N/NO$_2^-$ after 1 month. Between 0 and 1, 2 and 3, and 3 and 4 months nitrite concentrations were significantly higher in mid-canal water samples (1.5 m); however no differences were observed between the waters of the gravel and soil layers (Figure 7.14 B).
Figure 7.1: Nitrite (NO$_2^-$) concentration (mg/L) recorded between 0 and 1 month, 1 and 2 months, 2 and 3 months, 3 and 4 months, 4 and 5 months, and 5 and 6 months in A) experimental canal: inlet (– • –), experimental outlet (–■–), at mid-canal (1.5 m) in the Bauxsol™ layer waters (histogram vertical line) and in the soil layer waters (histogram horizontal line), at distal end of the canal (2.8 m) in the Bauxsol™ layer waters (histogram vertical dash) and in the soil layer waters (histogram horizontal dash); in B) control canal: inlet (– * –), control outlet (–○–), at mid-canal (1.5 m) in the gravel layer waters (gray histogram vertical line) and in the soil layer waters (gray histogram horizontal line), at distal end of the canal (2.8 m) in the gravel layer waters (gray histogram vertical dash) and in the soil layer waters (gray histogram horizontal dash). ± standard error.

Nitrate (NO$_3^-$) concentrations averaged 12.6±0.9 mg/L N/NO$_3^-$ in the inlet (Figure 7.15). Up until 2 months, the nitrate concentration in the experimental canal outlet was at 10.3±0.8 mg/L N/NO$_3^-$ (i.e. lower than the inlet); however, from month 3 it increased and averaged 14.6±1.2 mg/L N/NO$_3^-$ (i.e. higher than the inlet). Moreover, nitrate concentration was slightly higher in the waters of the Bauxsol™ layer until 3 months. No major differences were seen between water samples at mid-canal (1.5 m) and distal end of the canal (2.8 m) (Figure 7.15 A). Similar to the experimental canal.
outlet, the control canal outlet displayed a nitrate concentration around 11.3±1.2 mg/L N/NO$_3^-$ until 2 months (i.e. lower than the inlet); however, from 3 months it increased and averaged 16.1±1.4 mg/L N/NO$_3^-$ (i.e. higher than the inlet). Moreover, in this canal a significant difference was found between mid-canal (1.5 m) and the distal end of the canal (2.8 m), and between the waters of the gravel and soil layers (Figure 7.15 B).

**Figure 7.15**: Nitrate (NO$_3^-$) concentration (mg/L) recorded between 0 and 1 month, 1 and 2 months, 2 and 3 months, 3 and 4 months, 4 and 5 months, and 5 and 6 months in A) experimental canal: inlet (– * –), experimental outlet (–■–), at mid-canal (1.5 m) in the Bauxsol™ layer waters (histogram vertical line) and in the soil layer waters (histogram horizontal line), at distal end of the canal (2.8 m) in the Bauxsol™ layer waters (histogram vertical dash) and in the soil layer waters (histogram horizontal dash); in B) control canal: inlet (– * –), control outlet (–○–), at mid-canal (1.5 m) in the gravel layer waters (gray histogram vertical line) and in the soil layer waters (gray histogram horizontal line), at distal end of the canal (2.8 m) in the gravel layer waters (gray histogram vertical dash) and in the soil layer waters (gray histogram horizontal dash). ±standard error.
7.3.1.7. Nitrogen in solid phase

Comparisons of ammonium and nitrate concentrations pre-experiment and at a specific period (i.e. 0–1, 1–2, 2–3, 3–4, 4–5, and 5–6 months) on Bauxsol™ pellets and soil particles in the experimental canal, and on gravel particles and soil particles in the control canal are displayed in Table 7.3; pre-experiment data can also be found in the Appendix 9.8, Table 9.21. The data showed that ammonium in the experimental canal was mostly bound to soil particles at the proximal end (0.6 m), increasing over time from 41±21 mg/kg N/NH$_4^+$ (0–1 month) to 100±14 mg/kg N/NH$_4^+$ (5–6 months). Bauxsol™ pellets also showed some ammonium binding, but it was much lower than for soil particles and was equally distributed along the length of the canal (4.1 mg/kg N/NH$_4^+$ up to 37.1 mg/kg N/NH$_4^+$; Table 7.3 A). In the control canal, ammonium was also mostly bound by soil particles at the proximal end (0.6 m), increasing over time from 39±24 mg/kg N/NH$_4^+$ (0–1 month) to 124±17 mg/kg N/NH$_4^+$ (5–6 months). In addition, gravel particles also bound some ammonium, mostly at the proximal end (0.6 m), up to 78.0 mg/kg N/NH$_4^+$ (Table 7.3 A).

Nitrate was strongly bound to Bauxsol™ pellets at both ends of the experimental canal (up to 597.0 mg/kg N/NO$_3^-$), while soil particles did not bind nitrate (Table 7.3 B). In the control canal nitrate was not heavily bound (Table 7.3 B).
Table 7.3: Comparison between materials (i.e. Bauxsol™ pellets and soil particles in the experimental canal; gravel particles and soil particles in the control canal) pre-experiment and at a specific period (i.e. 0–1, 1–2, 2–3, 3–4, 4–5, and 5–6 months) = accumulation or release; ammonium and nitrate concentration (mg/kg) at the proximal end (0.6 m) and distal end (2.5 m) of each canal.

A: Ammonium (mg/kg)

<table>
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<th>1-2 mths</th>
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<th>3-4 mths</th>
<th>4-5 mths</th>
<th>5-6 mths</th>
</tr>
</thead>
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<tr>
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<td>54 ±3</td>
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B: Nitrate (mg/kg)

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<td>29 ±28</td>
<td>45 ±12</td>
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<td>126 ±49</td>
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<td>0 ±0</td>
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<td>2 ±1</td>
<td>1 ±0</td>
<td>0 ±0</td>
</tr>
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<td>2 ±0</td>
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Notes: negative values represent a loss in mass relative to an earlier condition.
7.3.1.8. TC, TN, TOC, IC, C/N ratio

Concentration of total carbon (TC), total organic carbon (TOC), inorganic carbon (IC), total nitrogen (TN), as well as C/N ratio (i.e. TOC/TN) on materials pre- and post-experiment (i.e. 24 weeks) at both proximal and distal end of each canal can be found in Table 7.4. These data showed that pre-experiment Bauxsol™ pellets were mostly made of inorganic carbon (90% of the total carbon; Table 7.4), and that the C/N ratio was relatively low (1.9). In contrast, soil particles pre-experiment was mostly made of organic carbon (95% of the total carbon), and the C/N ratio was much higher (20.6). Meanwhile, pre-experiment gravel particles had very low concentration of TC and TN (<0.05%).

Bauxsol™ pellets post-experiment showed an increase in TOC, particularly on the pellets located at the proximal end (0.6 m) of the experimental canal (1.43%), while TN decreased slightly. Consequently, C/N ratio significantly increased on Bauxsol™ pellets post-experiment [C/N ratio of 20.4 proximal end (0.6 m) and 14.2 distal end (2.5 m)]. Moreover, soil particles in the experimental canal recorded increases of TOC, IC and TN along the length of the canal, and consequently the C/N ratio reduced from 20.6 to 13.5. In contrast, the control canal data showed very little change for the gravel particles, while soil particles recorded decreased TC at the proximal end (0.6 m) and a slight increase at the distal end (2.5 m). Consequently, C/N ratio on soil particles from the control canal lowered from 20.6 to an average of 14.5 (Table 7.4).
Table 7.4: Total carbon (TC), total organic carbon (TOC), inorganic carbon (IC), total nitrogen (TN), and carbon/nitrogen ratio (i.e. TOC/TN ratio) pre- and post-experiment (24 weeks) at canal’s proximal end (0.6 m) and at distal end of the canal (2.5 m): on Bauxsol™ pellets and soil particles from the experimental canal, and on gravel particles and soil particles from the control canal. All data are in %, except the ratio.

<table>
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<td></td>
<td>(0.6 m)</td>
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<td>(2.5 m)</td>
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<td>Gravel</td>
<td>Soil</td>
<td>Bauxsol™</td>
<td>Soil</td>
<td>Bauxsol™</td>
<td>Soil</td>
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<tr>
<td>TC</td>
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<td>2.36</td>
<td>3.26</td>
<td>2.19</td>
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<tr>
<td>TOC</td>
<td>0.15</td>
<td>0.01</td>
<td>2.27</td>
<td>1.43</td>
<td>2.92</td>
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<td>IC</td>
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<td>0.02</td>
<td>0.11</td>
<td>0.07</td>
<td>0.22</td>
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<td>20.6</td>
<td>20.4</td>
<td>13.3</td>
<td>14.2</td>
<td>13.6</td>
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</tbody>
</table>
Biochemical oxygen demand (BOD) fluctuated from 5 to 60 mg/L in the inlet, whereas BOD was almost always <5 mg/L in both experimental and control canals outlets during the entire trial (Figure 7.16). Consequently, the BOD concentration was reduced by 84±5% in both canals.

![Figure 7.16](image)

**Figure 7.16**: Biochemical oxygen demand (BOD) concentration (mg/L) recorded between 0 and 1 month, 1 and 2 months, 2 and 3 months, 3 and 4 months, 4 and 5 months, and 5 and 6 months in the inlet (– * –), experimental outlet (–■–), and control outlet (–○–). ± standard error.

A decrease in total organic carbon (TOC) and dissolved organic carbon (DOC) concentrations in both experimental and control canals outlets was observed at the end of the trial, where the inlet concentrations of TOC and DOC were 9.3 mg/L and 7.0 mg/L, the experimental canal outlet concentrations of TOC and DOC were 3.6 mg/L and 3.4 mg/L, and the control canal outlet concentrations of TOC and DOC were 5.4 mg/L and 5.3 mg/L. Similarly, Bauxsol™ pellets had a greater effect in reducing faecal coliforms counts at 24 weeks, where the inlet counts was 3,600 cfu/100 mL, the experimental canal outlet count was 70 cfu/100 mL, and the control canal outlet count was 500 cfu/100 mL.
7.3.2. Microbiology

7.3.2.1. Free-floating bacterial communities

Non-metric multi-dimensional scaling (MDS) analysis of liquid DGGE profiles of 16S rDNA amplified products showed temporal variation between inlets (Figure 7.17; see all liquid DGGE profiles in Appendix 9.8, Figures 9.26–9.33). In addition, significant differences (R = 0.326, p<0.1%) in bacterial community structure between inlet and waters from both layers in the experimental and control canals were observed (Figure 7.17, circle showing a well-defined cluster of the experimental canal waters). Moreover, both canals showed some differences between the waters at mid-canal (1.5 m) and the distal end of the canal (2.8 m). Differences between the waters from both treatment and soil layers (i.e. Bauxsol™ and soil layers in the experimental canal, and gravel and soil layers in the control canal) were more pronounced for the control canal. These findings were also corroborated by the DGGE profile’s richness (i.e. number of bands), Shannon diversity index, and equitability index (Table 7.5).

Figure 7.17: Non-metric multi-dimensional scaling (MDS) of free-floating bacterial communities generated by the analysis of liquids DGGE 16S rDNA gene products. Fourth-root transform and Bray-Curtis matrix used. Samples are displayed according to their sampling time (0 to 24w) and location [i.e. mid-canal – 1.5 m (= half) and distal end of the canal – 2.8 m (= out)]: inlet (★), Bauxsol™ layer waters in the experimental canal (△), soil layer waters in the experimental canal (▲), gravel layer waters in the control canal (□), soil layer waters in the control canal (■).
Table 7.5: Profile’s richness (R; number of bands), Shannon diversity index (H’) and equitability index (J’) calculated from DGGE profiles analysis of 16S rDNA gene products amplified from total DNA extracted from liquid samples at 16, 20 and 24 weeks: inlet, experimental canal outlet, control canal outlet, Bauxsol™ layer and soil layer waters in the experimental canal (mid-canal 1.5 m, and distal end of the canal 2.8 m), gravel layer and soil layer waters in the control canal (mid-canal 1.5 m, and distal end of the canal 2.8 m).

<table>
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<th>J’</th>
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7.3.2.2. Attached bacterial communities

Increased profile richness (i.e. number of bands) over time (Table 7.6) on the Bauxsol™ pellets profiles, and significant temporal differences (R = 0.335, p<0.1%) within the profiles of the Bauxsol™ pellets (Figure 7.18 A & B) were observed (see all solid DGGE profiles in Appendix 9.8, Figures 9.34–9.45). The pre-experiment soil profile was more similar to the soil profile from the experimental canal at 6 months
than the soil profile at 4 months (Figure 7.18 A, B & D). Moreover, similar bands’ dominance at 4 months on DGGE profiles of Bauxsol™ and soil layers in the experimental canal (Figure 7.18 A) and higher Shannon diversity index at the interface (Table 7.6) were apparent. A lower equitability index was also recorded on Bauxsol™ pellets and the soil particles after 6 months (Table 7.6). Distinct profiles between Bauxsol™ and soil layers, and much stronger differences in profile richness along the length of the experimental canal were observed from 6 months (Figure 7.18 B; Table 7.6).

In addition, the bacterial community developing on gravel particles in the control canal was different to the bacterial community growing on Bauxsol™ pellets in the experimental canal (Figure 7.18 B & C). However, DGGE analysis of solids from the control canal showed greater similarity between profiles of the gravel and soil layers at 6 months (Figure 7.18 C). Moreover, the equitability index in the control canal increased over time (Table 7.6).
Table 7.6: Profile’s richness (R; number of bands), Shannon diversity index (H’) and equitability index (J’) calculated from DGGE profiles analysis of 16S rDNA gene products amplified from total DNA extracted from solid samples at 16, 20 and 24 weeks. **A) Experimental canal:** Bauxsol™ pellets, mix of Bauxsol™ pellets/soil particles (= interface), soil particles. **B) Control canal:** gravel particles, mix of gravel particles/soil particles (= interface), soil particles. Data are presented as <1.5m (= first half of the canal, 0.6 m + 1.3 m) and >1.5m (= second half of the canal, 1.8 m + 2.5 m).

**A: Experimental canal**

<table>
<thead>
<tr>
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<th>J'</th>
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**B: Control canal**

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</tr>
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</tr>
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Figure 7.18: DGGE analysis (triplicate) of 16S rDNA gene products amplified from total DNA extracted from solids samples. A) at 4th month in the experimental canal (Bauxsol™ and soil layers); B) at 6th month in the experimental canal (Bauxsol™ and soil layers); C) at 6th month in the control canal (gravel and soil layers); D) soil pre-experiment. St = DNA marker standard from wastewater isolates.

7.3.2.3. Functional genes presence

Aerobic ammonia-oxidising bacteria (AOB) carrying amoA genes were found in the experimental canal on Bauxsol™ pellets at 5 and 6 months along the length of the canal, and on soil particles at 5 months (proximal end). In contrast, AOB were only present on gravel particles at 5 months in the proximal end of the control canal (Table 7.7; Appendix 9.8, Figure 9.46). Moreover, AOB were also present on soil pre-experiment and in the inlets (0 and 5 months; Table 7.7). Ammonia-oxidising archaea (AOA) carrying amoA genes were evident on pre-experiment soil, in soil particles in the experimental canal (6 months, distal end), in soil in the control canal (5 months, proximal end), and on gravel particles at 6 months (distal end; Table 7.7).

Denitrifiers carrying nosZ genes were found in the experimental canal on Bauxsol™ pellets along the length of the canal at 5 and 6 months, and in the control canal on gravel particles at 5 months (proximal end; Table 7.7). Moreover, denitrifiers were also present in the inlets (0 and 5 months), but not on the pre-experiment soil. Anaerobic ammonia-oxidising bacteria (anammox) carrying hazo genes were attached
to Bauxsol™ pellets at 6 months (distal end) in the experimental canal, and on gravel particles at 5 months (proximal end) in the control canal (Table 7.7). In addition, anammox were also observed in the inlet at 6 months, but not on pre-experiment soil.
Table 7.7: Presence (+) or absence (−) of bands on gel electrophoresis of functional PCR amplification products targeting bacterial nitritation (*amo*A OAB genes), archaean nitritation (*amo*A AOA genes), bacterial denitrification (*nos*Z genes), and anaerobic-ammonia oxidising (anammox) (*hz*o genes). a) Liquid samples: inlet taken at T = 0, 5 months, and 6 months. b) Solids samples taken at 5 months (proximal end of the canal) and at 6 months (distal end of the canal): soil pre-experiment, Bauxsol™ pellets and soil particles from the experimental canal, gravel and soil particles from the control canal.

a) Liquid

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<th>6 months</th>
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<td>+</td>
<td>−</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>amo</em>A AOA</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>nos</em>Z</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>hz</em>o</td>
<td>−</td>
<td>−</td>
<td>+</td>
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b) Solids

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<th>6 months at 2.5 m</th>
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<tbody>
<tr>
<td><em>amo</em>A AOB</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
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<td>−</td>
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</table>
7.4. Discussion

7.4.1. Geochemical explanation

7.4.1.1. pH, EC & Ca spike

The pH spikes recorded in the experimental canal outlet (Figure 7.5 A) are caused by the release of unreacted calcium oxide from the cement binder used in the production of the pellets (Despland et al. 2010b; Despland et al. 2011). However, the soil layer in this canal acted as a buffer, lowering the water pH at the distal end of the canal (2.8 m) during the first 4 months and thereby delaying the appearance of an outlet pH spike; at similar bed volumes in Despland et al. (2011) study (Chapter 5) the pH was some 2 pH units higher. Moreover, the lower pH recorded mid-canal (1.5 m) after 4 months (Figure 7.5 A) is an indication that pellets are, at least in the first half of the canal, free of unreacted calcium oxide. Consequently, rinsing the pellets with freshwater as a pre-treatment and/or correcting the pH post-treatment would remedy the pH spike. Similarly, the observed EC spike (Figure 7.6 A) correlated with the calcium spike (1,737±135 mg/L) in the experimental canal outlet is attributed to the release of unreacted calcium oxide (i.e. a rise of calcium strongly affect the EC).

7.4.1.2. Phosphorus removal

The outstanding TP removal in the experimental canal (>95%, Figure 7.10; under the Australian guidelines for advanced wastewater treatment (National Water Quality Management Strategy 1997; Chapter 2, Table 2.2) is explained by the high phosphate binding capacity of Bauxsol™ (Hanahan et al. 2004; Akhurst et al. 2006; Clark et al. 2006a; Despland et al. 2011). Other studies using different materials in constructed wetland systems have reported phosphate removal rates >85% [e.g. laterite (Wood and McAtamney 1996), Maerl (Gray et al. 2000), steel-slag (Zhang et al. 2007), and wollastonite (Brooks et al. 2000)]. However, as the current study shows, Bauxsol™ pellets present other advantages such as high trace-metal binding (McConchie et al. 1999; McConchie et al. 2000; McConchie et al. 2002; Despland et al. 2011, Chapter 5) and provide a suitable biomass support framework (Despland et al. 2012, Chapter 6).
Similar to the findings of Akhurst et al. (2006), Hanahan et al. (2004), and Clark et al. (2006a; 2009) for Bauxsol™ powders, phosphate removal by Bauxsol™ pellets in this current study was most likely a combination of ligand exchange, chemisorption and surface precipitation, with abundant Ca$^{2+}$ and Mg$^{2+}$ present in the Bauxsol™ pellet matrix forming MgHPO$_4$ and CaHPO$_4$. However, confirmation by X-ray diffraction (XRD; Appendix 9.8, Figure 9.47) is difficult because of the dominance of the Bauxsol™ mineralogy, and more sophisticated techniques (e.g. synchrotron) are required to further investigate the speciation and binding environments. Moreover, the data show that the majority of P was removed by the Bauxsol™ layer and that saturation of Bauxsol™ pellets has not occurred (Figures 7.8 A & 7.9 A; Table 7.1 A). Hence, the underlying soil in the experimental canal will be less likely to saturate with P thereby potentially extending the canal life. An estimation based on the extrapolating bed volume from the mesocosm column experiment (Despland et al. 2011, Chapter 5) suggests that an unplanted Bauxsol™ pellets constructed wetland will remove >50% of the TP for at least five years. In addition, the high concentration of Colwell P found on the Bauxsol™ pellets towards the end of the experiment (Table 7.1 B) suggests that it may be a potential fertilizer, and saturated pellets may have reuse options.

The difference between control and experimental soil TP binding capacity can be explained by the lower pH (7.4±0.2; Figure 7.5 B) found in the soil layer of the control canal (i.e. dominant phosphate species as H$_2$PO$_4^-$), which facilitates electrostatic and chemical attraction (Brady and Weil 2008), and by higher concentration of phosphorus reaching the soil layer (Rhue and Harris 1999). Despite the higher phosphate removal by the soil layer of the control canal, the overall phosphorus removal from the control canal outlet quickly declined as saturation occurred because gravel particles could not bind phosphorus, demonstrated by high TP loss (Table 7.1 B). This loss was probably due to chemical weathering and weak binding (i.e. ion exchange forming outer-sphere complex, which incidentally is highly reversible; Rhue and Harris 1999). Consequently, as the soil is the only active component in P removal in the control canal, the longevity of this canal is severely reduced because the soil layer is likely to saturate with P rapidly.
7.4.1.3. Trace-metals binding

Bauxsol™ has a high trace-metal binding capacity (>1,500 meq/kg) from high surface to volume ratio (100 m²/g; McConchie et al. 1999; McConchie et al. 2000; McConchie et al. 2002), and the pellets used indicate that this high trace-metal binding capacity has been preserved (Table 7.2). Genc-Fuhrman et al. (2007) and Despland et al. (2011, Chapter 5) obtained similar binding capacities for trace-metals using Bauxsol™-coated sand and Bauxsol™ pellets.

It is believed that surface charge initially attracts dissolved metals and then a combination of adsorption and new mineral precipitation occurs on mineral surfaces. These surface deposits may be intracrystalline diffusion and/or recrystallisation and lead to renewed metal binding as surface sites are made available; this also leads to increased irreversibility of binding (e.g. Clark et al. 2009). The chemisorption reaction at the surfaces of the oxide components of Bauxsol™ may also explain the adsorption affinity of trace-metals (McConchie et al. 1999; McConchie et al. 2000; Genc et al. 2003; Hanahan et al. 2004; Clark et al. 2006b; Genc-Fuhrman et al. 2007; Clark et al. 2008; Clark et al. 2009; Clark et al. 2011a; Clark et al. 2011b; Clark et al. 2012). However, the investigation of the speciation and binding environments requires very sophisticated techniques (e.g. synchrotron EXAFS and XANES), which were not available to this project. Metal removal by Bauxsol™ is very complex and not yet fully understood as it is a complex combination, surface charging, pH, thermodynamic and kinetic solubility aspects. Moreover, the concentration/dissolutions of species in the solid are very minor (<1%) and consequently, XRD will not resolve these secondary phase minerals.

Furthermore, trace-metals on Bauxsol™ pellets are irreversibly bound and highly unlikely to leach (McConchie et al. 1999; Clark 2000; McConchie et al. 2000; Davies-McConchie et al. 2002; McConchie et al. 2002; Genc et al. 2003; Hanahan et al. 2004; Munro et al. 2004; Clark et al. 2006b; Clark et al. 2008; Clark et al. 2009; Clark et al. 2011a; Clark et al. 2011b). Since there is such a strong affinity for trace-metals by Bauxsol™ powders and Bauxsol™ pellets few metals are likely to remain soluble until saturation occurs. Moreover, there needs to be sufficient supply of the trace-metals to the solids, which appear relatively low in the inlet (mostly <0.01
mg/L). Consequently, the observed small changes in Ag, Hg and Fe concentrations on Bauxsol™ pellets (Table 7.2) were most probably due to analytical variability. In contrast, the weaker trace-metals binding on soil particles in the control canal (Table 7.2) may be explained by leaching of trace-metals from the gravel layer (i.e. chemical weathering of minerals surfaces and weak binding of trace-metals).

Trace-metal binding on soil particles in the experimental canal was weak because of low concentrations reaching this layer (Table 7.2). However, trace-metals removal by experimental soil was slightly higher than in the soil layer of the control canal, probably because of specific environmental conditions (i.e. pH of the water of the soil layer at 8.4±0.1; Figure 7.5 A) (Brady and Weil 2008).

7.4.1.4. Nitrogen removal

Geochemical processes, rather than biological, may explain part of the nitrogen removal in both canals outlets (Figure 7.11). The high removal rate of ammonium (Figure 7.13) may in part be due to geochemical binding as showed by the loading of ammonium found on the soil particles in both canals (Table 7.3 A). Efficient binding of NH$_4^+$ to soil particles is due to the high affinity of the soil organomineral complexes (Gobat et al. 2004; Brady and Weil 2008). Moreover, the higher removal rate of ammonium found in the control canal outlet (Figure 7.13 B) may be attributed to struvite (MgNH$_4$PO$_4$) precipitation [i.e. NH$_4^+$ combines with PO$_4^{3-}$ and Mg$^{2+}$ from the reactive olivine contained in the basalt gravel (Babic-Ivancic et al. 2002; Despland et al. 2011, Chapter 5)]. Struvite precipitation was probably weaker in the experimental canal as high calcium concentration interfered with struvite formation (Babic-Ivancic et al. 2002) and phosphate was predominantly bound as MgHPO$_4$ and CaHPO$_4$. Clark et al. (2008) found for sewage effluents that struvite precipitation could account for a 50% reduction in NH$_4^+$.

Nitrate removal in the experimental canal outlet (Figure 7.15 A) before 12 weeks may partially be explained by strong nitrate bound on Bauxsol™ pellets (Table 7.3 B). It may be hypothesised that the binding was via a ligand exchange mechanism, but to date Bauxsol™ has not been studied for its nitrate binding capacity. However, Dr Malcolm Clark (personal communication, 2012) suggests that equilibrium between
nitrate and Bauxsol™ can establish regardless of the nitrate concentration, which removes some 16% of soluble NO$_3^-$.

7.4.2. Geochemistry influencing microbiology and vice-versa

7.4.2.1. Free-floating bacterial communities

The observed differences between DGGE profiles of the free-floating bacterial community (Figure 7.17 & Table 7.5; Appendix 9.8, Figures 9.26–9.33) were mostly attributed to shifts of band dominance and relative intensity variations of common bands. The variability of the composition of wastewater at the inlet (e.g. ammonium and nitrate concentrations; Figures 7.13 & 7.15) and subsequent changes of water conditions imparted by the materials in the canals (e.g. alkaline pH, spatial and temporal changes in ammonium and nitrate concentrations in the experimental canal, Figures 7.5 A, 7.13 A & 7.15 A; spatial and temporal variability of nitrite and nitrate concentrations in the control canal, Figures 7.14 B & 7.15 B) were probably the main factors impacting on the free-floating microbial communities. Imfeld et al. (2010) reached a similar conclusion in their study, showing that the changes in water conditions (i.e. oxic to anoxic environment) impacted on the bacterial community from the aqueous phase of a constructed wetland.

7.4.2.2. Attached bacterial community

The results of the DGGE analysis of the 16S rDNA amplified products from solid samples in the experimental canal (Figure 7.18; Appendix 9.8, Figures 9.34–9.45) showed a distinct and well-adapted bacterial community attached to the Bauxsol™ pellets; this community has developed from both the inlet and the soil pre-experiment bacterial communities (i.e. similar bands’ dominance at 4 months on DGGE profiles of Bauxsol™ and soil layers; Figure 7.18). The temporal differences within Bauxsol™ pellet profiles may be a sign of species’ specificity and could show a shift in the ecological strategy of the bacterial community from a r-strategy (i.e. rapid colonisation of new environment by species with a high rate of reproduction) to a K-strategy (i.e. specific and competitive species well adapted to the environment; Gobat et al. 2004; Madigan et al. 2012).
The move to a K-strategy may also explain the difference in profile’s richness found along the canal’s length at 6 months (Table 7.6 A). The bacterial adaptation seen in the soil layer of the experimental canal (i.e. increase temporal similarity of profiles from soil in the canal and pre-experiment soil; Figure 7.18) may be further explained by Bauxsol™ layer influencing the geochemistry of the underlying soil layer (e.g. alkaline pH; Figure 7.5 A). Ragusa et al. (2004) showed that microbial biomass took up to 100 days (~3 months) to stabilize in constructed wetlands, indicating that recently established wetlands, like the one in this experiment, require time to build the bacterial communities. Moreover, the higher Shannon diversity index at the interface (Table 7.6A) suggests an ecotonal diversity, which is typically seen when two adjacent ecological systems (i.e. Bauxsol™ pellet layer and soil layer) possessing specific physico-chemical and microbial characteristics are interacting with each other (Gobat et al. 2004). Although bacterial community was attached to the gravel and soil particles in the control canal (Figure 7.18), no real ecotonal diversity was observed at the interface (Table 7.6) probably because of greater geochemical similarity between the two materials.

In addition, the distinct growth of the bacterial community between the Bauxsol™ pellet layer and soil layer at 6 months (i.e. different DGGE profiles; Figure 7.18), and the divergence observed between bacterial communities attached to Bauxsol™ pellets and gravel particles may be explained by differences in ecological niches characterised by the material’s geochemistry directly influencing the micro-environmental conditions (Tables 7.1–7.4; Appendix 9.8, Table 9.21, Figures 9.48–9.52). Several studies have shown that specific physico-chemical characteristics of the material and subsequent changes to the effluent composition had a significant impact on bacterial community diversity and structure (Calheiros et al. 2009; Dong and Reddy 2010; Li et al. 2010). The microporosity of Bauxsol™ pellets (Appendix 9.8, Figures 9.48 & 9.52) suggests that the pellets may provide steeper redox and nutrient diffusion gradients, which would impact on the bacterial community structure. Moreover, other studies have shown that CW depth impacted on microbial community diversity because of different supply rates of carbon, nitrogen, oxygen and organic matter (Nguyen 2000; Ragusa et al. 2004; Nurk et al. 2005; Truu et al. 2005; Tietz et al. 2007; Krasnits et al. 2009; Iasur-Kruh et al. 2010; Lin et al. 2010).
7.4.2.3. Biological activities

Post-experiment Bauxsol™ pellets at the distal end of the canal (2.5 m) consisted mostly of inorganic carbon (90%; Table 7.4), indicating insoluble carbonate biomineralisation with Bauxsol™, linked to an alkaline pH where CaCO₃ may precipitate above pH 8.4 consuming respiration produced CO₂. Moreover, the C/N ratio (i.e. TOC/TN) found on soil pre-experiment (20.6), and Bauxsol™ pellets and soil particles in the experimental canal post-experiment (17.3±3.1 and 13.4±0.1, respectively; Table 7.4) suggests good biological activity (i.e. active biofilm); in a soil litter C/N ratios between 6 and 25 are indicative of a nitrogen-rich litter, which are easy access to decomposers (Gobat et al. 2004; Brady and Weil 2008). In addition, the C/N ratios on Bauxsol™ pellets were higher than the C/N ratio (i.e. 9.3) found in a previous study by Despland et al. (2012, Chapter 6) on Bauxsol™ pellets, and this suggests an influence from the soil layer in the experimental canal. Similarly, the biological activity in the control canal would appear quite good, despite the low C/N ratio found in the gravel layer (1.3) because of the underlying active soil layer (C/N ratio averaging 14.5; Table 7.4).

Further evidence for biochemical activities is the improvement in water quality that also show decreases in TKN (Figure 7.12), faecal coliforms, TOC and DOC concentrations in both canals outlets, as well as a reduction in BOD concentrations (84±5%; Figure 7.16). These BOD concentrations are within Australian guidelines (National Water Quality Management Strategy 1997; Chapter 2 Table 2.2), and are well above other studies in CW: Krasnits et al. (2009) 70%, Iasur-Kruh et al. (2010) 50%; Calheiros et al. (2009) 35%. Furthermore, the reduction of faecal coliforms, TOC and DOC was more pronounced in the experimental canal, suggesting better biochemical activities.

7.4.2.4. Presence of microorganisms linked to nitrogen transformation

Biological activities may imply that the microorganisms present are involved in nitrogen transformation (i.e. changes in ammonium, nitrite and nitrate concentrations along the canals length over time; Figures 7.13–7.15) and total nitrogen removal (up to 35% in both canals; Figure 7.11). However, to examine this hypothesis more
analysis, such as quantitative PCR (qPCR) analysis, is needed because the presence of a particular gene does not necessarily signify intense activity.

The results of the PCR-amplification of bacterial amoA genes (Table 7.7) suggest that AOB came from the inlet and soil pre-experiment. Spatial diversity of AOB within the experimental canal may occur in response to changes in environmental condition (e.g. alkaline pH, temporal changes in ammonium concentrations; Figures 7.5 A & 7.13 A). These findings are supported by many studies on the distribution of AOB populations in constructed wetlands (Ibekwe et al. 2003; Rowan et al. 2003; Park and Noguera 2004; Truu et al. 2005; Gorra et al. 2007). It is likely that Bauxsol™ pellets provide more favourable niches than the gravel, and therefore a better stability for an AOB ecological guild, because of higher porosity and surface to volume ratio (Appendix 9.8, Figures 9.48 & 9.52). This observation appears to be supported by studies on adaptation of AOB to different substrates (Truu et al. 2005; Gorra et al. 2007). In contrast, the irregular presence of AOB in the control canal suggests competition for NH$_4^+$ with heterotrophic fast growing microorganisms (Hanaki et al. 1990). Furthermore, it is also hypothesised that the presence of readily metabolisable organic substrates may inhibit AOB organisms; however, no studies to date have been able to explain this phenomenon (Dr Michel Aragno, personal communication, 2012).

PCR-amplification of archaeal amoA genes (Table 7.7) suggests that AOA came from the pre-experiment soil. However, the relatively low presence of AOA in both canals may be explained by the young age of the system (i.e. 6 months) and by the very slow growth rate of these microorganisms (Erguder et al. 2009). Consequently, it suggests that Bauxsol™ pellets used in CW would potentially harbour substantial populations of AOA after longer operational periods. More mature CWs have been reported to contain a larger number of AOA populations (Truu et al. 2005; Park et al. 2006).

PCR-amplification of hzo genes (Table 7.7) indicates that anammox entered the CW from the inlet, which is surprising as anammox bacteria are strictly anaerobic and the DO concentration in the inlet was at 2.2±0.3 mg/L (Figure 7.7). However, it is possible that the perturbation involved when the soil was initially transferred into the canals may have disturbed the soil anammox population. Anammox organisms have
very slow growth rates (0.003/h growth rate, 10.6 days doubling time; Jetten et al. 2001), and therefore this can explain the low concentrations detected. Consequently, a more mature Bauxsol™ pellets CW may well harbour an important anammox population, similar to those show by Shipin et al. (2005) and Dong & Sun (2007).

Similar to anammox, PCR-amplification of nosZ genes (Table 7.7) suggests that denitrifier populations were introduced by the inlet wastewater. DO concentrations of 4.8±0.4 and 3.5±0.2 mg/L (Figure 7.7) in the experimental and control canals, respectively, suggest a constant diffusion of oxygen at the air-upper layer interface, and consequently, it is likely that the denitrifiers present, if active, used oxygen before nitrate as electron acceptor. However, the redox potential reading was surprisingly low (around +190 mV) for aerobic respiration and activity, and this may also explain the higher DO concentrations found in the canals outlets.

The co-existence of strict aerobic microorganisms (i.e. AOB), facultative anaerobic microorganisms (i.e. denitrifiers) and strict anaerobic microorganisms (i.e. anammox bacteria) attached to Bauxsol™ pellets in the experimental canal implies the presence of anoxic microzones at the centre of the pellets or biofilm that allow anaerobic processes, because the environment was oxic. Similarly, anoxic microzones of the biofilm growing on gravel allowed the co-existence of AOB, denitrifiers and anammox bacteria in the control canal. Similar conclusions were drawn by Despland et al. (2012, Chapter 6). Furthermore, the presence of oxic and anoxic zones in and around solid particles is well documented (Pochana and Keller 1999; Pochana et al. 1999; Gobat et al. 2004; Madigan et al. 2012), and several studies have reported the co-existence of aerobic and anaerobic bacteria in CWs (Shipin et al. 2005; Truu et al. 2005; Dong and Sun 2007; Krasnits et al. 2009; Iasur-Kruh et al. 2010). Consequently, the presence of such a relationship with Bauxsol™ pellets is not particularly surprising, but coupled with the phosphate and trace-metal binding would tend to confirm that pelletised Bauxsol™ makes an ideal and effective material for constructed wetlands.
7.5. Conclusion

This experiment demonstrated the complex interactions between Bauxsol™ pellets, soil particles, secondary treated effluent, and the existing microbial communities in an unplanted constructed wetland system. Furthermore, it showed that after some adaptation time, the bacterial community growing in the upper Bauxsol™ pellets layer and in the lower soil layer were distinct from each other because of differences in geochemistry and micro-environmental conditions. The co-existence of aerobic and anaerobic ammonia-oxidising bacteria and denitrifiers attached to Bauxsol™ pellets, and aerobic ammonia-oxidising bacteria and archaea attached to soil particles, showed the potential for a complex biological nitrogen cycle. However, qPCR analysis is required to confirm the activities of these microorganisms. Coupled with nitrogen cycling, the strong geochemical processes that generate >95% phosphate removal, and nitrate, ammonium and trace-metals binding make Bauxsol™ pellets an ideally suited alternative to conventional constructed wetland materials. Moreover, the high P-removal rates generated large amount of Colwell P bound to Bauxsol™ pellets, which potentially make saturated pellets an excellent fertiliser, thereby increasing their reuse value, reducing waste production, and increasing sustainability.

7.6. References


CHAPTER 8: CONCLUSION

8.1. Summary

It is of great importance to treat wastewaters generated by anthropogenic activities because they typically contain high concentrations of contaminants, which places our environment and public health at risk if left untreated. Traditionally, municipal wastewater is treated at a sewage treatment plant (STP) following a series of physical, biological and chemical processes (primary, secondary and tertiary treatments) (Liu and Liptak 2000; Hammer and Hammer Jr. 2005). To add further complexity and cost, biological and chemical processes must be separated because most chemical reagents (e.g. alum to precipitate phosphate) may severely impact on microorganisms and microbial activities (Madigan et al. 2012; Walker 2012). Consequently, several alternate materials have been used to remove phosphate from wastewaters [e.g. laterite (Wood and McAtamney 1996), Maerl (Gray et al. 2000), wollastonite (Brooks et al. 2000), marl (Szogi et al. 1997), blast furnace slag (Johansson 1999), Phoslock™ (Haghseresht et al. 2009)] with varying degree of success. However, none of these studies investigated microbial communities attached to the materials and therefore a lack of information on combined biological/chemical system for wastewater treatment exists. Hence, there is a need to study and find a cost effective and environmentally friendly technology to treat wastewater, combining biological and chemical processes.

Bauxsol™, a modified bauxite refinery residue, has been successfully used over the past decade in environmental remediation programs for acid contaminated soils and waters owing to its physico-chemical characteristics (e.g. high metals and acid neutralising capacities, high surface to volume ratio, highly non-dispersive) (e.g. McConchie et al. 1999; McConchie et al. 2000; Genc-Fuhrman et al. 2004; Clark et al. 2006; Clark et al. 2011a). Bauxsol™ has also been shown to be effective in binding phosphate (Hanahan et al. 2004; Akhurst et al. 2006). However, few field studies in circum-neutral waters have been undertaken because of the limited hydraulic conductivity of powdered Bauxsol™ and the production of sharp pH/alkalinity spike from unreacted calcium oxide in cement-bound Bauxsol™ pellets. Moreover, no studies to date have investigated the biocompatibility and interaction of Bauxsol™
with environmental microbial communities. Consequently, this thesis investigated a new research direction on Bauxsol™ technology and developed a new cost-effective and environmentally beneficial cured cement-bound porous Bauxsol™ pellets to treat municipal wastewater. These pellets bind phosphate and trace-metals from the sewage and act as a biomass support particle for the existing microbial communities, some of which are associated with nitrogen cycle.

The laboratory study (Chapter 3) developed a recipe and protocol for the production of cured cement-bound porous Bauxsol™ pellets with minimal pH and alkalinity spike using magnesium and calcium chloride in a curing bath to convert soluble alkalinity to an insoluble form. This new type of pellet has an improved hydraulic conductivity and still possesses a high phosphate and metals binding capacity. The use and choice of magnesium and calcium to change the chemistry of the pellets is similar to the Basecon™ neutralisation technique used to affect the alkalinity of the red mud during conversion to Bauxsol™ (McConchie et al. 2002). Hence, soluble alkalinity, resulting high pH, and aluminium mobility are stopped with the formation of complex magnesium and calcium aluminium hydroxy-carbonates, brucite, magnesites, and calcites.

Most importantly, recent micro-CT scanning of the pellets showed an excellent porosity well interconnected on the macro- and micro-scale within the 10 mm pellets (Appendix 9.8, Figure 9.52). Consequently, the pellets developed for this project are highly suitable for the treatment of circum-neutral waters such as municipal wastewater as they provide a high surface area for geochemical reactions to occur on. In addition, they are also a potential biomass support particle with multiple micro-environments allowing a wide range of bacterial growth. The production and properties of Bauxsol™ pellets are summarised in Figure 8.1.
Bauxsol™ powder
+ ordinary Portland cement
+ hydrophilic fumed silica
+ aluminium powder
+ water with viscosity agent

Mix, dry and cure in magnesium/calcium chloride brine

Bauxsol™ pellets
(10 mm Ø)

- high hydraulic conductivity
- high interconnected porosity
- high phosphate binding capacity
- high surface/volume ratio
- potential use as biomass support particle
- high metals binding capacity

Figure 8.1: Summary of production and properties of Bauxsol™ pellets
The microcosm field-based experiment using short-columns to treat wastewater for one month (Chapter 4) confirmed that the pellets developed in Chapter 3 are effective as a filter device for municipal wastewater treatment. The developed Bauxsol™ pellets showed that phosphate was removed mostly by precipitation processes. The experiment also showed that the pellets during the treatment only produced a short-lasting pH/alkalinity spike from some residual unreacted calcium oxide in the cement binder. Moreover, the Bauxsol™ pellets were biocompatible with environmental bacterial communities and none of the ingredients used in the production directly affected the microorganisms. The findings of this chapter indicate that scaling up the experiment to larger volumes and hydraulic residence time was warranted, because some of the effects observed in the microcosm were too short to be fully investigated.

The scaled-up field-based experiment using larger columns (mesocosm) to treat municipal wastewater for six months (Chapter 5) was undertaken and confirmed the successful removal of nutrients and trace-metals from secondary treated effluent. Phosphate was removed by over 70% from the effluent possibly by a precipitation process forming magnesium phosphate (boberrite or newbervite) and/or calcium phosphate (tuite or moneitite) and/or magnesium ammonium phosphate (struvite). However, despite the precipitation process, the analysis demonstrated that a large quantity of the phosphate although immobilised from the water remained plant available (2 g/kg after six months).

Moreover, the Bauxsol™ pellets in this scaled-up experiment acted exceptionally well as a biomass support particle for existing microbial communities in the wastewater. An observably distinct and highly diverse microbial community was attached to Bauxsol™ pellets and showed good sign of biological activities. There was strong evidence for the co-existence of aerobic ammonia-oxidising bacteria, anaerobic ammonia-oxidising (anammox) bacteria, and denitrifiers within and on the Bauxsol™ pellets. Presumably, the activity of these mixed microbial populations was partially responsible for nitrogen removal (≤ 49%); however, confirmation of such microbial activity is warranted using quantitative polymerase chain reaction analysis. The presence of both aerobic and anaerobic organisms implied oxic and anoxic microzones within the Bauxsol™ pellets. The interconnected porosity of the pellets
(Appendix 9.8, Figure 9.52) tends to confirm the existence of such microzones. Finally, the geochemical and microbiological findings from this experiment summarised in Figure 8.2 suggest that Bauxsol™ pellets may be used in more complex wastewater treatment systems such as a constructed wetland.
Treated effluent: > 70% phosphate removal and ≤ 49% nitrogen removal (over 6 months)

Bound Total Phosphorus: 1.62 g/Kg
Bound Colwell P (available phosphate): 1.62 g/Kg

- Ammonia-oxidising bacteria
- Denitrifiers
- Anammox organisms

Bound Total Phosphorus: 7.29 g/Kg
Bound Colwell P (available phosphate): 2.13 g/Kg

- Ammonia-oxidising bacteria
- Anammox organisms

Municipal wastewater (secondary treated effluent)

Figure 8.2: Summary of the main findings of the mesocosm columns field experiment
The field-based experiment using Bauxsol™ pellets as a treatment layer (on top of a soil layer) in a young unplanted constructed wetland system for six months (Chapter 7) confirmed the efficiency of Bauxsol™ pellets to treat wastewater in more complex systems. Strong interactions between the pellets, the underlying soil layer, the effluent and microbial communities had positively influence contaminants removal from the wastewater, particularly in the further suppression of the short-lasting pH/alkalinity spike from the pellets.

Phosphate was removed by more than 95% over the six months, significantly greater than the mesocosm column, without any sign of saturation. Nitrogen removal, despite being lower than the mesocosm column, was significant (<35%) and caused by geochemical binding and potential biological activities. Bauxsol™ pellets had also changed the environmental conditions in the wetland, improving the underlying soil’s geochemical binding capacity. Moreover, a distinct and well-adapted microbial community had developed within the Bauxsol™ pellet layer owing to the specific geochemical characteristics of the pellets, and the presence of multiple niches and interconnected porosity. Bacteria associated with the nitrogen cycle (aerobic and anaerobic ammonia-oxidising bacteria and denitrifiers) were also identified on Bauxsol™ pellets, further confirming the presence of oxic and anoxic microzones within the pellets, whereas ammonia-oxidising bacteria and archaea populated the soil particles. Consequently, the findings of this experiment demonstrate the formation of a bio-geochemically active ecosystem during wastewater treatment. Figure 8.3 summarises the main geochemical and microbiological findings, and identified processes occurring within the unplanted wetland system.
Figure 8.3: Summary of the main findings of the constructed wetland canal field experiment
8.2. Implications & Further Research

The Bauxsol™ pellets developed in this study have several advantages over Bauxsol™ powder and previous Bauxsol™ pellets, because not only they have a naturally high phosphate and trace-metal binding capacity, they have a minimal pH/alkalinity spike, an improved hydraulic conductivity, and they are biocompatible with environmental microbial communities. These pellets are consequently very effective in combining chemical and biological processes to treat municipal wastewater. The phosphate removal rate obtained with Bauxsol™ pellet [equivalent or better than conventional and alternate substrates used in wastewater treatments (e.g. Johansson 1999; Omoike and Vanloon 1999; Brooks et al. 2000; Gray et al. 2000; Haghseresht et al. 2009)] may well influence the Australian guidelines for advanced wastewater treatment (National Water Quality Management Strategy 1997), currently standing at <1 mg/L; a number five times higher than Bauxsol™ pellets treated effluent.

Clearly the scope and timing of this thesis did not allow for full exploration of the wetland system to 12, 18 or 24 months. Consequently, additional work in municipal wastewater treatment by Bauxsol™ pellets should involve a long-term full size planted constructed wetland system to further assess the interactions of Bauxsol™ pellets, the effluent, the soil, the plants and microbial communities and to determine if nitrogen removing bacterial populations could further establish and provide long-term nitrogen removal. A full size experiment would also provide economic data on cost-saving using Bauxsol™ pellets instead of a conventional system for municipal wastewater treatment, and useful indication of whether the system becomes P-saturated before N-removal efficiency can be successfully achieved. Furthermore, the preliminary findings of this thesis strongly recommend additional genetic works such as the cloning/sequencing technique and quantitative polymerase chain reaction method to identify the bacteria communities attached to Bauxsol™ pellets and quantify biological activities linked to the nitrogen cycle.

In addition, Bauxsol™ pellets, adapted to municipal wastewaters (providing a pH correction in the initial stage), may well be effective in other circum-neutral contaminated waters such as industrial wastewater, groundwater, surface water, and...
stormwater. Repeating existing studies (e.g. Szogi et al. 1997; Robb et al. 2003; Munro et al. 2004; Clark et al. 2006; Genc-Fuhrman et al. 2007; Zhang et al. 2010; Clark et al. 2011a) by using Bauxsol™ pellets on these types of waters may show that these new pellets can be more effective than alternate materials and that they can be used beyond municipal wastewater treatment. The suggested research deficiencies and discrepancies identified by this work will assist in further evaluating the P-saturation time of Bauxsol™ pellets and should include further research on the reuse of phosphate saturated Bauxsol™ pellets as a soil fertilizer.

Other further works should also focus on the pellet’s production to increase the reuse of cheap waste material. For example, fumed silica (i.e. highly purified waste material from iron smelting) used in the pellet’s production to provide some additional strength could be replaced by sugar cane fly ashes (i.e. waste from sugar cane refinery) as they contain very high silica concentration (50–70%: Clark et al. 2011b; Clark et al. 2012; Appendix 9.8, Table 9.22). The use of this economic and environmentally friendly fly ash as a feedstock for geopolymers and cement products is currently studied. Sugar cane (Saccharum officinarum) accumulates amorphous silica within cells typically at an average rate of 1.51% SiO₂ (Hodson et al. 2005), and Parr et al. (2009) suggest that silica (phytolith) content for varietals varies from 1.3–2.6%. Consequently, when this high silica organic matter is burnt it results in an ash much higher in silica than other boiler ashes. Moreover, the plant acts as a natural filter, such that many trace metals are excluded from accumulation. The additional benefit of using fly ash for the pellet’s production is that it includes some 20% mass of un-burnt activated carbon, which would improve pellet’s performance; Freire et al. (2012) have shown that minor components in complex mixes may have a disproportionate effect in overall geochemical behaviour of modified red mud.

8.3. Final Conclusion

This thesis conclusively demonstrated the effectiveness of a newly developed Bauxsol™ pellet to bio-geochemically treat municipal wastewater. Bauxsol™ pellets were not only successful in binding phosphate and trace-metals from the effluent, allowing the removal of a costly chemical dosing step in sewage treatment, but they also provided biocompatible niches for the development of specialised microbial
communities. Consequently, Bauxsol™ pellets can provide combined secondary and tertiary treatment to municipal wastewater, promoting biological processes and eliminating the use of chemicals for phosphates precipitation.

This research demonstrated the use of wastes as resources and provided a solution to several costly and potentially hazardous waste treatment issues within a closed feedback loop: a waste (bauxite residue) is turned into a product (porous Bauxsol™ pellets) to treat a waste (wastewater) and create another product (treated water), and all for the benefit of public health and the environment.

8.4. References


CHAPTER 9: APPENDICES

9.1. Quality assurance/Quality control

9.1.1. Geochemical and microbial analyses control

Quality assurance and quality control procedures were implemented to ensure the validity of the data from this study. Geochemical analyses were undertaken by the Environmental Laboratory Analysis at Southern Cross University, Lismore, Australia; a NATA (National Association of Testing Authorities) accredited laboratory (Lab. Acc. No. 14960). Table 9.1 describes the QA/QC procedures followed for each geochemical analysis undertaken on liquid and solid samples during this study.
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<td>2</td>
<td>1 in 10</td>
<td>4</td>
<td>0.01 mg/L</td>
</tr>
<tr>
<td>Orthophosphate</td>
<td>0 to 2.0 mg/L</td>
<td>7</td>
<td>2</td>
<td>1 in 10</td>
<td>4</td>
<td>0.005 mg/L</td>
</tr>
<tr>
<td>Faecal coliforms</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>&lt;1 mg/L</td>
</tr>
<tr>
<td>Calcium</td>
<td>0 to 200 mg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>1</td>
<td>&lt;0.05 mg/L</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0 to 200 mg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;0.05 mg/L</td>
</tr>
<tr>
<td>Silver</td>
<td>0 to 1 mg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;0.001 mg/L</td>
</tr>
<tr>
<td>Aluminium</td>
<td>0 to 100 mg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;0.05 mg/L</td>
</tr>
<tr>
<td>Arsenic</td>
<td>0 to 1 mg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;0.001 mg/L</td>
</tr>
<tr>
<td>Cadmium</td>
<td>0 to 1 mg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;0.001 mg/L</td>
</tr>
<tr>
<td>Chromium</td>
<td>0 to 1 mg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;0.001 mg/L</td>
</tr>
<tr>
<td>Copper</td>
<td>0 to 1 mg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;0.001 mg/L</td>
</tr>
<tr>
<td>Iron</td>
<td>0 to 100 mg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;0.05 mg/L</td>
</tr>
<tr>
<td>Manganese</td>
<td>0 to 10 mg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;0.001 mg/L</td>
</tr>
<tr>
<td>Nickel</td>
<td>0 to 1 mg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;0.001 mg/L</td>
</tr>
<tr>
<td>Lead</td>
<td>0 to 1 mg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;0.001 mg/L</td>
</tr>
<tr>
<td>Selenium</td>
<td>0 to 1 mg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;0.001 mg/L</td>
</tr>
<tr>
<td>Zinc</td>
<td>0 to 1 mg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;0.001 mg/L</td>
</tr>
<tr>
<td>Mercury</td>
<td>0 to 20 µg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;0.0005 mg/L</td>
</tr>
<tr>
<td><strong>Solid</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrate</td>
<td>0 to 5.0 mg/L</td>
<td>6</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>0.1 mg/kg</td>
</tr>
<tr>
<td>Ammonium</td>
<td>0 to 5.0 mg/L</td>
<td>6</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>0.1 mg/kg</td>
</tr>
<tr>
<td>Total nitrogen</td>
<td>0 to 20 %</td>
<td>2</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>0.02%</td>
</tr>
<tr>
<td>Total carbon</td>
<td>0 to 50 %</td>
<td>2</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>0.01%</td>
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<tr>
<td>Total organic carbon</td>
<td>0 to 50 %</td>
<td>2</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>0.01%</td>
</tr>
<tr>
<td>Total phosphorus</td>
<td>0 to 200 ppm</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;50 mg/kg</td>
</tr>
<tr>
<td>Colwell P</td>
<td>0 to 2 mg/L</td>
<td>7</td>
<td>2</td>
<td>1 in 10</td>
<td>1</td>
<td>5 mg/kg</td>
</tr>
<tr>
<td>Phosphate (KCl extract)</td>
<td>0 to 5 mg/L</td>
<td>6</td>
<td>2</td>
<td>1 in 10</td>
<td>1</td>
<td>1 mg/L</td>
</tr>
<tr>
<td>Silver</td>
<td>0 to 1 mg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;1 mg/kg</td>
</tr>
<tr>
<td>Arsenic</td>
<td>0 to 1 mg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;2 mg/kg</td>
</tr>
<tr>
<td>Cadmium</td>
<td>0 to 1 mg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;0.5 mg/kg</td>
</tr>
<tr>
<td>Chromium</td>
<td>0 to 1 mg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;2 mg/kg</td>
</tr>
<tr>
<td>Copper</td>
<td>0 to 1 mg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;1 mg/kg</td>
</tr>
<tr>
<td>Manganese</td>
<td>0 to 10 mg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;1 mg/kg</td>
</tr>
<tr>
<td>Nickel</td>
<td>0 to 1 mg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;1 mg/kg</td>
</tr>
<tr>
<td>Selenium</td>
<td>0 to 1 mg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;1 mg/kg</td>
</tr>
<tr>
<td>Zinc</td>
<td>0 to 1 mg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;1 mg/kg</td>
</tr>
<tr>
<td>Mercury</td>
<td>0 to 20 µg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;0.1 mg/kg</td>
</tr>
<tr>
<td>Aluminium</td>
<td>0 to 100 mg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;50 mg/kg</td>
</tr>
<tr>
<td>Iron</td>
<td>0 to 100 mg/L</td>
<td>3</td>
<td>2</td>
<td>1 in 10</td>
<td>2</td>
<td>&lt;50 mg/kg</td>
</tr>
</tbody>
</table>

*in large batches of samples EAL does duplicate analysis on every tenth sample or 10% of the samples*
Several parameters (pH, EC, temperature, and DO) were checked in-situ at the time of sampling. All meters and probes were manufactured by TPS Pty Ltd, Australia. The pH probe (range 0–14, resolution 0.01, accuracy ±0.01) and EC probe (k=1 sensor; range 0–10 ppK, resolution 0.05% of range, accuracy ±0.2% of full scale of range) were calibrated before each measurement, while the temperature probe (range –10.0°C to +120.0°C, resolution 0.1°C, accuracy ±0.2°C) was only calibrated once. Two pH buffers (6.88 and 4.00) were used to calibrate the pH probe (slope above 96%). A zero calibration (air) and a standard calibration using a conductivity standard solution of 2.6 mS/cm were used to calibrate the EC probe. Attached to a separate meter the DO probe (range 0–20 ppm, resolution 0.01 ppm, accuracy ±0.2% of full scale of range) was also calibrated before each measurement. After coordinating the temperature of the DO meter with the temperature of the pH/EC meter, a zero calibration was performed by placing the DO probe in 100 mL of distilled water containing 2 g of sodium sulphite. Subsequently, an air calibration was done (slope above 96%). The membrane of the DO probe was regularly checked and replaced if broken.

Microbial analyses (more details in Chapters 4, 5 & 7, and Appendix 9.3) were carried out in a certified microbiology/PC2–genetic laboratory at the Department of Primary Industries Wollongbar, Australia; ISO9001 accredited, OGTR and Australian/New Zealand standards for microbiology and safety. Bound to this accreditation, all equipments are serviced and calibrated by appropriately qualified technicians. This also extends to the methodology, most of them are controlled documents and there are managements systems to ensure that the data is appropriately managed and analysed. The department also set up QA/QC systems and this laboratory is duty bound to comply. A database contains the risk assessments and the safe work method statements. The laboratory also conforms to quarantine standards and all strains (including genetic constructs) are registered on the DPI database, which is updated annually.

9.1.2. Experimental design and sampling procedures

The microcosm column field experiment (Chapter 4) was designed as a 3–2–1 matrix (i.e. three Bauxsol™ pellets columns, two sand pellets columns, one sand column)
because of restricted number of channels on the peristaltic pump (i.e. six channels available). This experiment was run over one month and included 11 sampling times. The mesocosm columns field experiment (Chapters 5 & 6) was designed as a 3–1 matrix (i.e. three Bauxsol™ pellets columns, one gravel column) as it focused on the efficiency of Bauxsol™ pellets to treat wastewater. A multichannel peristaltic pump was used to ensure an identical flow rate for each column. This experiment was run over six months and included 17 sampling times.

During these two columns experiments, the system was cleaned weekly to avoid a failure and was checked several times a week – including flow rate’s check. The system was also kept under a double shade cloth to avoid undesirable rise in temperature inside the columns. Liquid samples were taken approximately at the same time during the day and a new bottle was used for each sample. Geochemical analyses were undertaken once on each sample, and the results were combined for the columns replicates. DNA extractions were done in triplicate on each sample, and the results were then combined. Liquid samples were brought to the Environmental Laboratory Analysis (EAL) at Southern Cross University within two hours of sampling. DNA extraction procedure was started on fresh sample within three to four hours of sampling. DNA extracts were frozen and kept for future analyses (i.e. PCR/DGGE).

At the end of the microcosm experiment, once liquid samples were taken, the six columns were opened and solids were placed in individual zip plastic bags. All samples were taken to the EAL within two hours of sampling and DNA extraction procedure was started on fresh sample within three to four hours of sampling. DNA extracts were frozen and kept for future analyses (i.e. PCR/DGGE). At the end of the mesocosm experiment, once liquid samples were taken, the four columns were brought back to Southern Cross University and opened along the length using an electric saw. A ruler was place next to the columns to ensure an accurate sampling at 0, 25, 50, 75, 105 and 130 cm. Solid samples were taken with a spoon that was sterilised with 100% ethanol between each sample. Solids were place in individual plastic containers. Extra samples were taken, placed in plastic bags and kept frozen for possible future analyses. Samples were at the EAL within three to four hours of the end of the experiment and DNA extraction procedure was started within four to
five hours of sampling. DNA extracts were frozen and kept for future analyses (i.e. PCR/DGGE).

The constructed wetland canal field experiment (Chapter 7) was designed as one experimental canal and one control canal. Canals were built in a U-shaped form (segments of up to one meter long) by Pacific West Corporation. Segments were assembled as instructed by the manufacturer using a Quick Bond glue specifically designed for PVC plastic. Canals were placed on levelled galvanised steel benches. To ensure a 0.25° slope, PVC plates of 4.5 mm thickness were placed under the canals. A spirit level was used to ensure that the canals were perfectly levelled. Subsequently, holes were drilled at the distal end of the canals for the outlet.

Water was pumped, using a powerful pump that did not require any maintenance over the six months trial, from the humus tank to a specifically designed bin possessing an on/off switch floater. The bin’s inlet was wrapped in a fine mesh to avoid clogging. A filter and solenoid system connected to a T-shaped tap (i.e. each subdivision of the tap was located just above each canal) was used at the bottom of the bin (for more details see Figures 7.1 & 7.2, Chapter 7). The flow rate was monitored weekly and corrected if necessary. The filter inside the solenoid was cleaned monthly, and the canals were checked at least twice a week. To comply with the Occupational Health & Safety guidelines of the Sewage Treatment Plant South Lismore, pipes from and to the humus tank were buried and the solenoid main switch panel was protected from the weather. Double shade cloth was placed above the canals in a reverse V-shape to ensure partial darkness, and partial protection against rain and animals.

Acrylic piezometers were placed in the empty canals in two sets of two to ensure that samples could be taken across the width (for more details see Figures 7.1 & 7.3, Chapter 7). Fresh soil was dug from the wetland located at the STP and transfer within the hour into the canals to form the lower layer. Gravel material, previously autoclaved to ensure no microorganisms were present, was placed in the control canal as an upper layer, and Bauxsol™ pellets were placed in the experimental canal as an upper layer. Subsequently, the experiment was started.
Inlet and outlet waters were taken in 1 L new plastic bottles, whereas liquid from the piezometers at mid-canal (1.5 m) and the distal end of the canal (2.8 m) were taken in 500 mL new plastic bottles by mixing liquid from the two piezometers (i.e. two piezometers in the soil layer and two piezometers in the upper Bauxsol™/gravel layer; see Figure 7.3, Chapter 7). A syringe connected to a hose was used for the sampling in the piezometers. It was ensure that the same syringe was used for the same piezometer each time, and that it was rinsed with MilliQ water before each sampling. Solid samples were taken using an apple corer (i.e. this ensures a good core, where the lower, the interface, and the upper layers were distinctly visible) that was thoroughly clean with MilliQ water and 100% ethanol to avoid crossed contamination between samples. Two cores were taken at each distance (one close to the edge and one close to the median line; see Figure 7.4, Chapter 7) and the material from each layer mixed to avoid bias. Solid samples were placed in individual plastic containers. Core voids were filled with fresh pre-washed material (i.e. soil, gravel or Bauxsol™ pellets). A marker was placed on the refilled voids to ensure that no further sample would be taken from there.

Geochemical analyses were undertaken once on each sample, while DNA extractions were done in triplicate on each sample, and the results were then combined. Liquid and solid samples were delivered to the EAL within two to three hours of sampling and DNA extraction procedure was started the same day. DNA extracts were kept frozen until future analyses (i.e. PCR/DGGE). At the end of the experiment extra liquid and solid samples were taken and kept at −20°C for possible additional analyses.
9.2. South Lismore Sewage Treatment Plant

Lismore Shire is located in the northeast of New South Wales, Australia and has a population of approximately 45,000 people. There are two municipal sewage treatment plants (STP) in Lismore city. South Lismore STP can currently provide full treatment for flows up to 2,000 L/s (i.e. 22,000 equivalent persons). The plant has conventional primary, secondary and tertiary treatments, as well as an advanced treatment system that consists of 13 hectares of constructed wetlands (Figure 9.1) (Lismore City Council 2012).
Figure 9.1: South Lismore Sewage Treatment Plant. Conventional plant with primary (red), secondary (blue), tertiary (yellow) and advanced (pink) treatments. (Retrieved from Google Earth 17 May 2012).
9.3. Microbiology optimisation

9.3.1. Overview

The DNA extraction procedure was optimised because Bauxsol™ pellets showed signs of interference with the bead beating technique as described by Bell et al. (2006). In addition, the choice of the DNA extraction method is critical because it may significantly influence future analysis (i.e. polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) methods) (von Wintzingerode et al. 1997; de Lipthay et al. 2004; Kowalchuk 2004; Carrigg et al. 2007; Feinstein et al. 2009). PCR reaction conditions (i.e. number of cycles and amount of DNA template; annealing temperature for functional PCR) were also optimised for each experiment (von Wintzingerode et al. 1997; Kowalchuk 2004; Carrigg et al. 2007). Finally, the percentage of denaturant concentration (i.e. gradient) and of acrylamide for the DGGE was adjusted for each experiment (Muyzer et al. 1993; Muyzer and Smalla 1998; Rochelle 2001; Kowalchuk 2004). Because DGGE is both reproducible and robust, no significant differences between the samples’ replicates were found (Carrigg et al. 2007); therefore replicates were used on some samples only.

9.3.2. DNA extraction

Equipments:
- Eppendorf 5810R centrifuge (Eppendorf, Germany)
- FastPrep FP120 bead-beater (Bio 101, Lajolla, CA)
- 2 mL screw cap free standing tubes (Astral Scientific, Australia)
- 1.5 mL micro test tubes (Eppendorf, Germany)
- Bio Rad electrophoresis system (Bio Rad, USA)
- Image Station 2000 MM system (Kodak, USA)

Reagents:
- 1 mm glass beads (Saint Gobain, USA)
- 0.1 mm glass beads (Sigma Aldrich, USA)
- Phosphate Buffered Saline (PBS): 8 g NaCl; 0.2 g KCl; 1.44 g Na₂HPO₄; 0.24 g KH₂PO₄, complete to 1 L pH 7.4
- 0.1 M Sodium Phosphate Buffer pH 7.0: 57.7 mL Na₂HPO₄ + 42.3 mL NaH₂PO₄ (= 1 M), dilute to 0.1 M
- Lysis Buffer: 1% SDS, 0.5% Extran®, 1% PVP 40, 50 mM EDTA, 100 mM Tris-HCl
- Bovine Serum Albumin (BSA) 100 mg/mL (Sigma, USA)
- 7.5 M Ammonium Acetate
- Polyethylene Glycol (PEG) Buffer: 20% polyethylene glycol 8000, 2.5 M NaCl
• 70% ethanol
• Sterile RNase and DNase free water (Invitrogen Life Technologies, USA)
• Agarose (Amresco, USA)
• Orange G.L. dye: 50% glycerol + orange G.L powder
• Standard λDNA/Hind III fragments ladder (GibcoBRL Life Technologies, USA)
• Ethidium bromide (EMD Chemicals Inc., USA)

Original Protocol:
• Add 0.8 g of 1 mm glass beads and 0.3 g of 0.1 mm glass beads to a 2 mL screw capped tube. Autoclave and dry prior to use.
• Put 300 mg of solids in a bead tube. Add 500 µL of PBS and briefly vortex. Spin for 2 mins at 13 krpm and discard the supernatant.
• Add 520 µL of sodium phosphate buffer and 81 µL of lysis buffer to each tube and beat in the beater for 30 secs at power 5.5 m/s.
• Spin the tube for 5 mins at 13 krpm and decant the supernatant into a fresh 1.5 mL micro-tube (~ 600 µL).
• Add 6 µL of 100 mg/mL solution of BSA (final concentration 1 mg/mL); invert the tube 3 times to mix. Add half of the volume (~300 µL) of 7.5 M ammonium acetate; invert the tube 3 times to mix. Place the tube on ice for at least 15 mins. Spin for 5 mins at 13 krpm.
• Transfer supernatant (~710 µL) into a fresh 1.5 mL micro-tube. Discard pellet.
• Add 1.1% volumes (~785 µL) of PEG buffer; invert the tube 3 times to mix. Incubate 30 to 60 mins at 37ºC or leave overnight at 4ºC wrap in aluminum foil.
• Spin 60 mins at 13 krpm at 4ºC. Discard supernatant. Wash pellet twice with 200 µL of 70% ethanol. Dry under vacuum for 3 mins.
• Resuspend DNA pellet in sterile RNase and DNase free water⁴. Leave overnight at 4ºC to resuspend or gently pipette up and down. Combine the replicates.
• Control the quality of DNA extract on 1% agarose gel (standard λDNA/Hind III fragments ladder incorporated into the gel), mix 6 µL of DNA extract + 2 µL of orange G.L. dye, run the gel for 45 mins at 60 V and stain for 20 mins in ethidium bromide. Take a picture using the Kodak image station.
• Store DNA at -20ºC.

The first step of the optimisation followed the original protocol and the bead beating technique with Bauxsol™ pellets, soil, and different cultures (in duplicate; Table 9.2). All cultures gave a positive result (i.e. band on agarose gel), as well as the soil and Bauxsol™ pellets mixed with the soil. The second step consisted of checking a pH spike using the original phosphate buffer and other substitute’s buffers with the bead beating technique (in duplicate; Bauxsol™ pellets mixed with secondary treated

⁴ The amount of water was determined by the size of the pellet in each tube: tiny pellet = 5 µL; very small = 15 µL; small = 20 µL; small/medium and medium/small = 30 µL; medium = 40 µL; medium/large = 50 µL; large/medium = 60 µL; large = 80 µL.
effluent from the STP South Lismore). The pH was recorded in the supernatant just after the beating step (Table 9.3) and results showed a pH ranging from 6.0 to 9.5. During the third step, substitutes to PBS wash buffer and phosphate buffer were used following the original protocol and the bead beating technique. Bauxsol™ pellets were resuspended in *E.Coli* 10419 culture and used as sample (in duplicate; Table 9.4). No DNA was extracted using the different substitutes to PBS wash buffer.

Because of inconstancies in the results, a microwave oven technique (45 secs, maximum power) was trialled instead of the bead beating technique. Three different tests were done: microwave only, microwave replacing the bead beating in the original protocol, and microwave and PEG precipitation. Phosphate buffer and lysis buffer were used at different stages of the procedures. Bauxsol™ pellets mixed overnight at 37°C in 15 mL *E.Coli* 10419 culture were used as samples (Table 9.5). No DNA was extracted using the microwave oven technique.

The sonication technique (Unisonics sonicator, Australia) was trialled as a replacement of the bead beating technique in the original protocol. Using Bauxsol™ pellets mixed overnight at 37°C in 15 mL *E.Coli* 10419 culture as samples, no band was visible on the agarose gel following the sonication technique (Bauxsol™ pellet from the overnight mix + 4 mL 0.85% saline, sonication for 60 secs or 150 secs, remove Bauxsol™ pellet, spin 10 mins at 4 krpm, discard supernatant, resuspend pellet in 520 µL phosphate buffer pH 7.0 + 81 µL lysis buffer, then follow the original protocol). Similar results were found using 520 µL of *Staphylococcus aureus* ATCC 25923 culture. Consequently, trials were undertaken combining sonication and microwave oven or sonication and bead beating techniques following the sonication technique protocol mentioned above (Table 9.6). No DNA was extracted using the combined sonication–microwave oven and sonication–bead beating techniques.

After considering the different DNA extraction methods and buffers, it appears that the best technique to extract DNA from Bauxsol™ pellets is the bead beating. However, some further optimisations were required.
### Table 9.2: Step 1: original protocol and the bead beating technique. + indicates a band on agarose gel; – indicates no band on agarose gel.

<table>
<thead>
<tr>
<th>Samples</th>
<th>DNA extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil from wetland system (South Ballina bypass construction March 2008); 2 types of samples (~200 mg)</td>
<td>+</td>
</tr>
<tr>
<td>Bauxsol™ pellets alone (~200 mg)</td>
<td>–</td>
</tr>
<tr>
<td>Bauxsol™ pellets + Soil (total ~200 mg)</td>
<td>+</td>
</tr>
<tr>
<td>Culture AS12-4 (bacterial culture resuspended in 10 mL nutrient broth): 500 µL</td>
<td>+</td>
</tr>
<tr>
<td>Bauxsol™ pellets + culture AS12-4 (~200 mg Bauxsol™ pellets + 200 µL culture mixed for 1 hr prior testing)</td>
<td>–</td>
</tr>
<tr>
<td>Bauxsol™ pellets resuspended in culture AS12-4 (Bauxsol™ pellets washed in PBS, remove supernatant; 1 mL culture spin 3 mins at 13 krpm, remove supernatant, resuspended in 520 µL P buffer; transfer in tube with the pellets)</td>
<td>–</td>
</tr>
<tr>
<td>Bauxsol™ pellets mixed in secondary treated effluent from STP South Lismore</td>
<td>–</td>
</tr>
<tr>
<td>E.Coli culture (NCTC E.Coli 10419; nutrient broth)</td>
<td>+</td>
</tr>
<tr>
<td>Bauxsol™ pellets in 15 mL E.Coli culture overnight at 37°C. Use the Bauxsol™ pellets only</td>
<td>–</td>
</tr>
<tr>
<td>Bauxsol™ pellets resuspended in E.Coli culture (Bauxsol™ pellets washed in PBS, remove supernatant; 1 mL E.Coli culture spin 3 mins at 13 krpm, remove supernatant, resuspended in 520 µL P buffer; transfer in tube with the pellets)</td>
<td>–</td>
</tr>
<tr>
<td>Rodococcus culture (1 mL/tube)</td>
<td>+</td>
</tr>
<tr>
<td>Bauxsol™ pellets resuspended in Rodococcus culture (Bauxsol™ pellets washed in PBS, remove supernatant; 1 mL Rodococcus culture spin 3 mins at 13 krpm, remove supernatant, resuspended in 520 µL P buffer; transfer in tube with the pellets)</td>
<td>–</td>
</tr>
<tr>
<td>Staphylococcus aureus ATCC 25923 culture</td>
<td>+</td>
</tr>
</tbody>
</table>

### Table 9.3: Step 2: Substitute’s buffers in replacement of phosphate buffer in the original protocol with the bead beating technique. Bauxsol™ pellets mixed with secondary treated effluent from the STP South Lismore as sample. pH of supernatant after the beating step.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM sodium phosphate buffer pH 7.0: 520 µL</td>
<td>9.5</td>
</tr>
<tr>
<td>100 mM sodium phosphate buffer pH 5.8: 520 µL</td>
<td>8.5</td>
</tr>
<tr>
<td>100 mM Tris-HCl buffer pH 4.5: 520 µL</td>
<td>9.5</td>
</tr>
<tr>
<td>100 mM Tris-HCl pH 4.5 (500 µL) + 100 mM CaCl₂ (55 µL) + 100 mM MgCl₂ (30 µL)</td>
<td>8</td>
</tr>
<tr>
<td>100 mM sodium phosphate buffer pH 5.8 (500 µL) + 100 mM CaCl₂ (55 µL) + 100 mM MgCl₂ (30 µL)</td>
<td>7</td>
</tr>
<tr>
<td>1 M acetic acid pH 2.5 (85 µL) + 100 mM Tris-HCl pH 4.5 (500 µL)</td>
<td>8</td>
</tr>
<tr>
<td>1 M acetic acid pH 2.5 (85 µL) + 100 mM sodium phosphate buffer pH 5.8 (500 µL)</td>
<td>6</td>
</tr>
<tr>
<td>100 mM Tris-HCl (500 µL) + 100 mM CaCl₂ (55 µL) + 100 mM MgCl₂ (30 µL)</td>
<td>8</td>
</tr>
<tr>
<td>100 mM sodium phosphate buffer pH 5.8 (500 µL) + 100 mM CaCl₂ (55 µL) + 100 mM MgCl₂ (30 µL)</td>
<td>7</td>
</tr>
</tbody>
</table>
**Table 9.4:** Step 3: Substitutes to PBS wash buffer and phosphate buffer. Bauxsol™ pellets resuspended in E.Coli 10419 culture as sample. – indicates no band on agarose gel.

<table>
<thead>
<tr>
<th>Wash and Buffer</th>
<th>DNA extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>wash: PBS</td>
<td>–</td>
</tr>
<tr>
<td>buffer: 10 mM CaCl₂ + 50 mM MgCl₂ + 100 mM sodium phosphate buffer pH 5.8: 520 µL of solution</td>
<td>–</td>
</tr>
<tr>
<td>wash: PBS</td>
<td>–</td>
</tr>
<tr>
<td>buffer: 10 mM CaCl₂ + 50 mM MgCl₂ + 100 mM sodium phosphate buffer pH 5.8: 520 µL of solution</td>
<td>–</td>
</tr>
<tr>
<td>wash: PBS</td>
<td>–</td>
</tr>
<tr>
<td>buffer: 1 M acetic acid (40 µL) + 100 mM sodium phosphate buffer pH 5.8 (480 µL)</td>
<td>–</td>
</tr>
<tr>
<td>wash: PBS</td>
<td>–</td>
</tr>
<tr>
<td>buffer: 100 mM sodium acetate: 520 µL</td>
<td>–</td>
</tr>
<tr>
<td>wash: PBS</td>
<td>–</td>
</tr>
<tr>
<td>buffer: 100 mM KH₂PO₄ + 100 mM NaOH: 520 µL of solution</td>
<td>–</td>
</tr>
<tr>
<td>wash: PBS</td>
<td>–</td>
</tr>
</tbody>
</table>
| buffer: 100 mM sodium phosphate buffer pH 7.0: 520 µL | –

*E.Coli culture 10419 only shows a band on agarose gel.*

**Table 9.5:** Microwave oven technique (45 secs, maximum power): only, following the original protocol, or with PEG precipitation. Bauxsol™ pellets mixed overnight at 37°C in 15 mL E.Coli 10419 culture as sample. – indicates no band on agarose gel.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Protocol</th>
<th>DNA extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.560 µL phosphate buffer pH 7.0</td>
<td>microwave only</td>
<td>–</td>
</tr>
</tbody>
</table>
| | microwave instead of bead beating in the original protocol | –
| | microwave, spin 5 mins at 13 krpm, transfer supernatant in fresh micro-tube, add PEG (1.1 × of the volume) | – |
| 1.560 µL phosphate buffer pH 7.0; after microwave + 81 µL lysis buffer | microwave only | – |
| | microwave instead of bead beating in the original protocol | – |
| | microwave, spin 5 mins at 13 krpm, transfer supernatant in fresh micro-tube, add PEG (1.1 × of the volume) | –
| 1.560 µL phosphate buffer pH 7.0 + 245 µL lysis buffer then microwave | microwave only | – |
| | microwave instead of bead beating in the original protocol | – |
| | microwave, spin 5 mins at 13 krpm, transfer supernatant in fresh micro-tube, add PEG (1.1 × of the volume) | –

*Staphylococcus aureus ATCC 25923 culture does not show a band.*

**Table 9.6:** Combined techniques: sonication & microwave oven or sonication & bead beating. Bauxsol™ pellets mixed overnight at 37°C in 15 mL E.Coli 10419 culture as sample. – indicates no band on agarose gel.

<table>
<thead>
<tr>
<th>Sonication</th>
<th>Microwave</th>
<th>Bead beating</th>
<th>DNA extract</th>
</tr>
</thead>
</table>
| 60 secs    | 30 secs   | n/a          | –
|            | 45 secs   | n/a          | –
| 150 secs   | 30 secs   | n/a          | –
|            | 45 secs   | n/a          | –
|            | 60 secs   | n/a          | –
|            | n/a       | 30 secs at 5.5 m/s | –
| 300 secs   | 45 secs   | n/a          | –
|            | 60 secs   | n/a          | –
|            | n/a       | 30 secs at 5.5 m/s | –

*E.Coli 10419 culture does not show a band*
Bead beating conditions were optimised for DNA extraction from a liquid sample (Table 9.7). A solution was prepared by mixing 270 mL of media (0.85% saline + 0.5 g/L yeast extract) and 30 mL secondary treated effluent. Three sets of sample were prepared: a) 20 mL of the solution (pH 7.7), b) 20 mL of the solution and ~100 Bauxsol™ pellets (~30 g; pH 10.8), and c) 20 mL of the solution and ~30 g of Bauxsol™ powder AAA (pH 9.6). The mixes were shaken overnight at 22°C. Different techniques (centrifuge or filter) were assessed to concentrate the microorganisms present in the liquid. Then the original protocol was followed, changing the amount of beads (0.8 g of 1 mm diameter beads + 0.3 g of 0.1 mm diameter beads; 0.4 g of 1 mm diameter beads + 0.1 g of 0.1 mm diameter beads) and the beating power (4 m/s for 10 or 20 secs; 4.5 m/s for 10 or 20 secs; 5.5 m/s for 20 secs). The best combination was the McKartney bottles (4 × 20 mL liquid centrifuge; 0.4 g of 1 mm diameter beads + 0.1 g of 0.1 mm diameter beads; 4.5 m/s for 20 secs).
<table>
<thead>
<tr>
<th>Protocol</th>
<th>Samples</th>
<th>DNA extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>spin 1.5 mL at 9 krpm for 5 mins; discard supernatant, resuspend pellet in 500 µL PBS, transfer in bead tube (0.8 g 1 mm, 0.4 g 0.1 mm); follow original protocol (beating at power 5.5 m/s for 20 secs)</td>
<td>no addition</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Bauxsol™ pellets added</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Bauxsol™ powder added</td>
<td>–</td>
</tr>
<tr>
<td>spin 3 × 10 mL at 4 krpm for 10 mins; discard supernatant, resuspend pellet in 1 mL PBS, transfer in bead tube (0.8 g 1 mm, 0.4 g 0.1 mm); follow original protocol (beating at power 5.5 m/s for 20 secs)</td>
<td>no addition</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Bauxsol™ pellets added</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Bauxsol™ powder added</td>
<td>–</td>
</tr>
<tr>
<td>vacuum filter; wash filter with 1 mL PBS, transfer in bead tube (0.8 g 1 mm, 0.4 g 0.1 mm); follow original protocol (beating at power 5.5 m/s for 20 secs)</td>
<td>no addition</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Bauxsol™ pellets added</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Bauxsol™ powder added</td>
<td>–</td>
</tr>
<tr>
<td>spin 250 mL at 5 krpm 10 mins; discard supernatant, wash in 3 mL PBS in micro-tube, spin 2 mins at 13 krpm, remove supernatant, transfer pellet in ~200 µL phosphate buffer in bead tube (0.4 g 1 mm, 0.1 g 0.1 mm); follow original protocol (beating at power 4 m/s for 10 secs)</td>
<td>no addition</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Bauxsol™ pellets added</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Bauxsol™ powder added</td>
<td>–</td>
</tr>
<tr>
<td>prefILTER (Whatman #2); wash filter with 1 mL PBS, transfer in bead tube (0.8 g 1 mm, 0.4 g 0.1 mm); follow original protocol (beating at power 5.5 m/s for 20 secs)</td>
<td>no addition</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Bauxsol™ pellets added</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Bauxsol™ powder added</td>
<td>–</td>
</tr>
<tr>
<td>~80 mL of liquid through filter/syringe 0.22 µm nitrocellulose, wash filter with 1 mL PBS, transfer in bead tube (0.8 g 1 mm, 0.4 g 0.1 mm); follow original protocol (beating at power 4.5 m/s for 10 secs)</td>
<td>no addition</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Bauxsol™ pellets added</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Bauxsol™ powder added</td>
<td>–</td>
</tr>
<tr>
<td>spin 90 mL at 10 krpm for 10 mins, discard supernatant, resuspend pellet in 1 mL PBS, transfer in bead tube (0.4 g 1 mm, 0.1 g 0.1 mm); follow original protocol (beating at power 4 m/s for 20 secs)</td>
<td>no addition</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Bauxsol™ pellets added</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Bauxsol™ powder added</td>
<td>+</td>
</tr>
<tr>
<td>spin 4 × 20 mL in McKartney bottles at 4 krpm for 25 mins; discard supernatant, resuspend pellet in 500 µL PBS and transfer in two micro-tubes; spin 2 mins at 13 krpm, discard supernatant, resuspend pellet in 520 µL P buffer, transfer in bead tube (0.4 g 1 mm, 0.1 g 0.1 mm) with 81 µL lysis buffer; follow original protocol (beating at power 4.5 m/s for 20 secs)</td>
<td>no addition</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Bauxsol™ pellets added</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Bauxsol™ powder added</td>
<td>+</td>
</tr>
</tbody>
</table>

The bead beating technique was also optimised for DNA extraction from a solid sample. About 500 mg of Bauxsol™ pellets from the set of sample prepared for the liquid sample extraction optimisation (see above) were subjected to the bead beating technique following the original protocol without the PBS wash (i.e. PBS strongly interferes with the Bauxsol™ pellet mineralogy). Similar to the liquid sample extraction optimisation, three settings of beads and beating power were tested: 0.8 g of 1 mm diameter beads + 0.3 g of 0.1 mm diameter beads at power 5.5 m/s for 20 secs; 0.4 g of 1 mm diameter beads + 0.1 g of 0.1 mm diameter beads at power 4 m/s for 20 secs; and 0.4 g of 1 mm diameter beads + 0.1 g of 0.1 mm diameter beads at power 4.5 m/s for 30 secs. All combination were successful (i.e. band on the agarose gel), but the best result was with the combination of a small amount of beads (0.4 g of
1 mm diameter beads + 0.1 g of 0.1 mm diameter beads) and the beating power at 4.5 m/s for 30 secs.

Consequently, two optimised DNA extraction protocols (liquid and solid) were developed for Bauxsol™ pellets used to treat wastewater.

**Liquid DNA extraction protocol:**

- Transfer 4×20 mL of liquid in McKartney bottles (4 bottles; under laminar flow).
- Spin bottles 25 mins at 4 krpm; discard the supernatant.
- Add 500 µL of PBS to each McKartney bottle and resuspend the pellet.
- Transfer the mix into a 1.5 mL micro-tube (2 McKartney bottles/micro-tube).
- Spin for 2 mins at 13 krpm; discard the supernatant. The pellet can be kept overnight at 4ºC.
- Add 520 µL of sodium phosphate buffer (0.1 M, pH 7.0) to each micro-tube and resuspend the pellet. Transfer the mix in a bead beating tube (2 mL screw cap, 0.4 g of 1 mm glass beads and 0.1 g of 0.1 mm glass beads; autoclave and dry prior to use).
- Add 81 µL of lysis buffer.
- Place the tubes in the bead beater and beat for 20 secs at power 4.5 m/s.
- Spin the tube for 5 mins at 13 krpm and decant the supernatant into fresh 1.5 mL micro-tubes (~600 µL).
- Add 6 µL of 100 mg/mL solution of BSA (final concentration 1 mg/mL); invert the tubes 3 times to mix. Add half of the volume (~300 µL) of 7.5 M ammonium acetate; invert the tubes 3 times to mix. Place the tubes on ice for at least 15 mins. Spin for 5 mins at 13 krpm.
- Transfer supernatant (~710 µL) into fresh 1.5 mL micro-tubes. Discard pellet.
- Add 1.1% volumes (~785 µL) of PEG buffer; invert the tubes 3 times to mix. Incubate 30 to 60 mins at 37ºC or leave overnight at 4ºC wrap in aluminum foil.
- Spin 60 mins at 13 krpm at 4ºC. Discard supernatant. Wash pellet twice with 100 µL 70% ethanol (200 µL if the pellet is large). Dry under vacuum for 3 mins.
- Resuspend DNA pellet in sterile RNase and DNase free water. Leave overnight at 4ºC to resuspend or gently pipette up and down. Combine the replicates.
- Control the quality of DNA extract on 1% agarose gel (standard λDNA/Hind III fragments ladder incorporated into the gel), mix 6 µL of DNA extract + 2 µL of orange G.L. dye, run the gel for 45 mins at 60 V and stain for 20 mins in ethidium bromide. Take a picture using the Kodak image station.
- Store DNA at -20ºC.

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5 The amount of water was determined by the size of the pellet in each tube: tiny pellet = 5 µL; very small = 15 µL; small = 20 µL; small/medium and medium/small = 30 µL; medium = 40 µL; medium/large = 50 µL; large/medium = 60 µL; large = 80 µL.
Solid DNA extraction protocol:
- Put ~ 600 mg of solids (i.e. Bauxsol™ pellets, sand pellets, sand, gravel, or soil) in a bead tube (2 mL screw cap; triplicate) with 0.4 g of 1 mm glass beads and 0.1 g of 0.1 mm glass beads (autoclave and dry prior to use).
- Add 520 μL of sodium phosphate buffer (0.1 M, pH 7.0).
- Add 81 μL of lysis buffer
- Place the tubes in the bead beater and beat for 30 secs at power 4.5 m/s.
- Spin the tube for 5 mins at 13 krpm and decant the supernatant into fresh 1.5 mL micro-tubes (~600 μL).
- Add 6 μL of 100 mg/mL solution of BSA (final concentration 1 mg/mL); invert the tubes 3 times to mix. Add half of the volume (~300 μL) of 7.5 M ammonium acetate; invert the tubes 3 times to mix. Place the tubes on ice for at least 15 mins. Spin for 5 mins at 13 krpm.
- Transfer supernatant (~710 μL) into fresh 1.5 mL micro-tubes. Discard pellet.
- Add 1.1% volumes (~785 μL) of PEG buffer; invert the tubes 3 times to mix. Incubate 30 to 60 mins at 37°C or leave overnight at 4°C wrap in aluminum foil.
- Spin 60 mins at 13 krpm at 4°C. Discard supernatant. Wash pellet twice with 100 μL 70% ethanol (200 μL if the pellet is large). Dry under vacuum for 3 mins.
- Resuspend DNA pellet in sterile RNase and DNase free water. Leave overnight at 4°C to resuspend or gently pipette up and down. Combine the replicates.
- Control the quality of DNA extract on 1% agarose gel (standard λDNA/Hind III fragments ladder incorporated into the gel), mix 6 μL of DNA extract + 2 μL of orange G.L. dye, run the gel for 45 mins at 60 V and stain for 20 mins in ethidium bromide. Take a picture using the Kodak image station.
- Store DNA at -20°C.

9.3.3. DNA quantification & PCR

DNA was quantified following the method described by Angersbach & Earp (2004) using the PicoGreen dsDNA quantification kit (Molecular Probes, USA). A total of 1 μL of DNA was mixed with 199 μL of diluted PicoGreen (1:200 in TE 1× buffer) in a black Greiner 96-well microlitre plate (Greiner Bio-one, Germany). A DNA standard curve was prepared from a known quantity of bacteriophage λDNA stocks (supplied with the kit) and also mixed with diluted PicoGreen to a total volume of 200 μL. After a few minutes in the dark, the plate was read for fluorescence in a Fluorostar fluorometer (BMG Labtech, GmbH, Germany) with an excitation filter of 485 nm and an emission filter of 520 nm. DNA quantification was then calculated by the instrument as ng of DNA per μL.
PCR amplification of the bacterial 16S rDNA gene’s V3 region (primers: GC-357 F and 546 Alexa-518R; Invitrogen Life Technologies, USA) was undertaken in a GeneAmp PCR system 9700 (Applied Biosystems) with the following conditions: 94°C for 5 mins; 30 to 40 cycles of 94°C for 30 secs, 58°C for 30 secs, 72°C for 30 secs; 72°C for 10 mins; and 4°C for ∞. PCR was performed in a total volume of 50 µL containing 5.0 µL of 10× Dream taq Buffer (with 20 mM MgCl₂; Fermentas), 0.4 µL of BSA (20 mg/mL; Sigma), 1 µL of deoxynucleotide triphosphate mix (10 mM; Astral Scientific), 1 µL of MgCl₂ (50 mM; Gibco), 1 µL of each forward (20 µM) and reverse (25 µM) primer (Invitrogen), 0.4 µL of Dream taq (5 U/µL; Fermentas) and 10–100 ng/µL of DNA template.

The optimisation for the microcosm columns field experiment (Chapter 4) was done using the following samples: influent at T = 1d (100 ng/µL, 50 ng/µL, and 10 ng/µL), Bauxsol™ pellets effluent at T = 24d (50 ng/µL), sand particles (100 ng/µL) and Bauxsol™ pellets (10 ng/µL). All tests were done in duplicate in 50 µL reaction following the standard 16S rDNA PCR protocol, changing the number of cycles only (94°C for 5 mins; 30 or 35 cycles of 94°C for 30 secs, 58°C for 30 secs, 72°C for 30 secs; 72°C for 10 mins; and 4°C for ∞). The result indicated no major difference between the amounts of DNA template used but a better result was obtained with 35 cycles.

The optimisation for the mesocosm columns field experiment (Chapter 6) was undertaken using the following samples: inlet at T = 0 (10 ng/µL) and at T = 3d (10 and 20 ng/µL); gravel one-quarter-column effluent at T = 3d (10 and 20 ng/µL), gravel half-column effluent at T = 3d (10 and 20 ng/µL), gravel three-quarter-column effluent at T = 3d (10 and 20 ng/µL), gravel outlet at T = 1d, 2w (10 ng/µL) and at T = 3d (10 and 20 ng/µL); Bauxsol™ pellets one-quarter-column effluent at T = 3d (10 and 20 ng/µL), Bauxsol™ pellets half-column effluent at T = 1d, 4w (10 ng/µL) and at T = 3d, 6w (10 and 20 ng/µL), Bauxsol™ pellets three-quarter-column effluent at T = 3d (10 and 20 ng/µL), Bauxsol™ pellets outlet at T = 1d, 1w (10 ng/µL) and at T = 3d, 6w (10 and 20 ng/µL). All tests were done in 50 µL reaction following the standard 16S rDNA PCR protocol, changing the number of cycles only: 94°C for 5 mins; 35 or
40 cycles of 94°C for 30 secs, 58°C for 30 secs, 72°C for 30 secs; 72°C for 10 mins; and 4°C for ∞. The best result was obtained with 40 cycles.

The optimisation for the constructed wetland canal field experiment (Chapter 7) was done using 20 ng/µL of DNA template in a 50 µL reaction on the following samples: inlet T = 16w; Bauxsol™ piezometer outlet T = 16w; gravel piezometer outlet T = 16w; Bauxsol™ pellet solid at 0.6 m T = 20w; soil from the experimental canal at 1.3 m T = 20w; gravel solid at 1.3 m T = 24w; soil from the control canal at 0.6 m T = 20w. The standard 16S rDNA PCR protocol was used changing the number of cycles only: 94°C for 5mins; 35 or 40 cycles of 94°C for 30 secs, 58°C for 30 secs, 72°C for 30 secs; 72°C for 10 mins; and 4°C for ∞. The best result was seen with 40 cycles.

Functional PCR amplification targeting specific genes involved in nitrogen transformation (ammonia-oxidising bacteria with amoA gene: amoA-1F/amoA-2R-TC; ammonia-oxidising archaea with amoA gene: amoA-23F/amoA-616R; denitrifier with nosZ gene: nosZ1F/nosZ1622R; anammox bacteria with hzo gene: hzocl1F1/hzocl1R2; Invitrogen Life Technologies, USA) was performed in a GeneAmp PCR system 9700 (Applied Biosystems) under the following conditions: 94°C for 4 mins; 25 to 35 cycles of 94°C for 30 secs, 45 to 59°C for 30 secs, 72°C for 45 secs; 72°C for 10 mins; and 4°C for ∞. PCR was performed in a total volume of 25 µL containing 5.0 µL of 10× Dream taq Buffer (with 20 mM MgCl₂; Fermentas), 0.4 µL of BSA (20 mg/mL; Sigma), 1 µL of deoxynucleotide triphosphate mix (10 mM; Astral Scientific), 1 µl of MgCl₂ (50 mM; Gibco), 1 µl of each forward (20 µM) and reverse (20 µM) primer (Invitrogen), 0.4 µL of Dream taq (5 U/µL; Fermentas) and 5 to 100 ng/µL of DNA template.

All optimisations were undertaken using DNA extracts from soil samples (Wollongbar, Australia) and Bauxsol™ pellets and gravel solids from the mesocosom columns field experiment. Different conditions of annealing temperature, number of cycles and amount of DNA template were trialled for the four different functional PCR amplifications (Table 9.8).
Table 9.8: Optimisation of functional PCR amplification targeting specific genes involved in nitrogen transformation. Different annealing temperature, number of cycles, and amount of DNA template used were tested.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Annealing temperature</th>
<th>Number of cycles</th>
<th>DNA template (ng/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bacterial amoA gene</td>
<td>57°C; 58°C; 59°C</td>
<td>25×; 30×; 35×</td>
<td>10; 20; 50; 100</td>
</tr>
<tr>
<td>archaeal amoA gene</td>
<td>57°C; 58°C; 59°C</td>
<td>25×; 30×; 35×</td>
<td>10; 20; 50; 100</td>
</tr>
<tr>
<td>nosZ gene</td>
<td>53°C; 54°C; 55°C</td>
<td>25×; 30×; 35×</td>
<td>5; 10; 20; 50</td>
</tr>
<tr>
<td>hzo gene</td>
<td>45°C; 50°C</td>
<td>25×; 30×; 35×</td>
<td>50</td>
</tr>
</tbody>
</table>

Bacterial and archaeal amoA genes were best amplified using 50 ng/μL of DNA template with the following condition: 94°C for 4 mins; 30 cycles of 94°C for 30 secs, 58°C for 30 secs, 72°C for 45 secs; 72°C for 10 mins; and 4°C for ∞. The nosZ gene was best amplified using 50 ng/μL of DNA template with the following condition: 94°C for 4 mins; 35 cycles of 94°C for 30 secs, 55°C for 30 secs, 72°C for 45 secs; 72°C for 10 mins; and 4°C for ∞. Finally the best amplification of the hzo gene was found using 50 ng/μL of DNA template with the following condition: 94°C for 4 mins; 35 cycles of 94°C for 30 secs, 50°C for 30 secs, 72°C for 45 secs; 72°C for 10 mins; and 4°C for ∞.

9.3.4. DGGE

PCR products of 16S rDNA were separated by DGGE electrophoresis (150 V for 5 hrs at 60°C) using the CBS Scientific system (USA). Polyacrylamide gels were prepared by mixing 40% acrylamide/Bis [37.5:1] (Amresco, USA), 50× TAE buffer, formamide (Amresco, USA), urea (Spectrum Chemical mfg corp.), TEMED (Amresco, USA), ammonium-persulfate, and Milli-Q water. The amount of the reagents depends on the denaturant concentration and the percentage acrylamide desired.

Incorporated in each gel was a standard DNA marker prepared from four bacteria colonies isolated on nutrient agar (Oxoid Nutrient Broth CM001 and Oxoid Agar bacteriological no.1 LP011) from secondary-treated effluent of the STP South Lismore. Isolate DNA was extracted as follows: a small amount of the culture isolate (i.e. using the end of a tip) was mixed in 50 μL of sterile water DNA/RNA free. The micro-tube was placed in a microwave oven for 2 mins at maximum power. The
supernatant was spun for 1 min at 13 krpm and then transferred in a fresh micro-tube. PCR was run at 94°C for 5 mins; 30 cycles of 92°C for 30 secs, 58°C for 30 secs, 72°C for 30 secs; 72°C for 10 mins; and 4°C for ∞ using 1 µL of the each isolate and the reagents as described in the section 9.3.3. PCR products were separated by DGGE electrophoresis (150 V for 5 hrs at 60°C) on a 6.5% acrylamide gel with 30–70% denaturant gradient. Four of the nine isolates were pooled together to constitute the standard DNA marker. One isolate was used as a positive control for each run.

DGGE gels were photographed using a Image Station 2000 MM system (Kodak, USA) and analysed with Carestream Molecular Imaging software version 5.0 (Carestream Health Inc., USA). The floating point values method was used to find band mobility (double checked using software’s profile option) and peak intensity (excluded if intensity under 2). Relative band mobility was calculated from gels using bands corresponding to the DNA marker and then aligned. Percentage relative band intensity was then calculated for each sample.

For all three experiments (Chapters 4, 6, & 7) DGGE setting was optimised using the samples from the PCR optimisation (section 9.3.3) on the following mixtures: 7.5% acrylamide, 30–70% gradient; 6.5% acrylamide, 30–70% gradient; 8.0% acrylamide, 40–60% gradient; 7.5% acrylamide, 40–60% gradient; 6.5% acrylamide, 40–60% gradient; and 6.0% acrylamide, 40–60% gradient. The gels were run at 150 V and 60°C for 5 hrs and included positive and negative controls. The results showed that the best gel was 6.5% acrylamide with a denaturant gradient of 30–70%. 
9.4. Effects of Bauxsol™ on bacterial growth (laboratory experiment)

9.4.1. Overview
A surface-plate technique was used to understand how the pH spike, observed with Bauxsol™ pellets when used in circum-neutral waters, influences the growth of bacterial communities.

9.4.2. Method
Media was prepared mixing a saline solution (0.85%) and 0.5 g/L yeast extract. Inoculum (secondary treated effluent from South Lismore STP) was mixed with the media at a ratio of 1:9 in Erlenmeyer flasks (100 mL), all in triplicate: Control (C) flasks didn’t have any addition; raw Bauxsol™ (rB) flasks had 3 g of fully neutralised Bauxsol™ powder (AAA grade); Bauxsol™ pellets (Bp) flasks had 10 pellets (approximately 3 g). The flasks, shaken at 25 rpm, were incubated at 22°C at night and between 25°C and 29°C during daytime. A total of four cycles was performed with liquid inoculation (i.e. media and inoculum) changed after each cycle. Samples were taken during each cycle at 0, 4, and 48 hrs. Serial dilutions (100 μL of two dilutions) were spread-plated in triplicate on nutrient agar (Oxoid Nutrient Broth CM001 and Oxoid Agar bacteriological no.1 LP011) and incubated at 22°C. The colony forming units (CFU) was counted after 48 hrs, 72 hrs, 1 week and 2 weeks incubation. In addition, pH was recorded for each flask at the time of the sampling.

9.4.3. Results & Discussion
Control liquor pH was stable (~7) during the four cycles (Figure 9.2). Raw Bauxsol™ liquor pH was also ~7 and showed a slight increase (~1 pH unit) towards the end of each cycle. Bauxsol™ pellets liquor pH started at ~7 but increased quickly to pH 10 during the first 4 hrs of the first two cycles. During cycles II and IV, the Bauxsol™ pellets liquor pH increased from 6.8 to 8.5 after 48 hrs (Figure 9.2). These results demonstrated that one of the ingredient used during the pellets production (most likely the unreacted calcium oxide from the cement binder; Chapter 3) induced the pH spike. However, this laboratory experiment has also demonstrated that the spike was short-lived and after a few washes, the pH came back to an appropriate level.
CFU counts showed similar curves for both control and raw Bauxsol™ flasks (Figures 9.3 & 9.4), suggesting that raw Bauxsol™ had no effect on bacteria growth. A decrease of CFU was observed after 4 hrs in the control flask at cycle III and in raw Bauxsol™ flasks at cycle III and IV (Figures 9.3 & 9.4; Table 9.9). These findings may be explained by a predator effect and/or a change in bacterial population (i.e. uncultured bacteria).

Bauxsol™ pellet flasks recorded a substantial drop of CFU during cycle I and II between T = 0 and T = 4h. However, after 48 hrs, the growth increased again, although very slowly during cycle I (Figures 9.3 & 9.4; Table 9.9). These findings suggest that bacteria have been knocked out by the high pH prevailing in Bauxsol™ pellet flasks (i.e. bactericide effect) and therefore, needed some time to recover.
Figure 9.3: Colony forming unit count (cfu/mL) after 48 hrs incubation for Control (C), Bauxsol™ pellets (Bp), and raw Bauxsol™ (rB) at different sampling time (hr) and cycle number.

Figure 9.4: Colony forming unit count (cfu/mL) after 2 weeks incubation for Control (C), Bauxsol™ pellets (Bp), and raw Bauxsol™ (rB) at different sampling time [h] and cycle number.
Table 9.9: Percentage increase in cfu/mL between 48 hrs and 2 weeks incubation for Control (C), Bauxsol™ pellets (Bp), and raw Bauxsol™ (rB) at different sampling time (hr) and cycle number.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Time (hr)</th>
<th>C</th>
<th>Bp</th>
<th>rB</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0</td>
<td>83</td>
<td>73</td>
<td>467</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>19</td>
<td>1726</td>
<td>527</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>10</td>
<td>229</td>
<td>20</td>
</tr>
<tr>
<td>II</td>
<td>0</td>
<td>7</td>
<td>174</td>
<td>n/a</td>
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<tr>
<td></td>
<td>4</td>
<td>n/a</td>
<td>95</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>59</td>
<td>29</td>
<td>21</td>
</tr>
<tr>
<td>III</td>
<td>0</td>
<td>30</td>
<td>12</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6</td>
<td>n/a</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>65</td>
<td>14</td>
<td>142</td>
</tr>
<tr>
<td>IV</td>
<td>0</td>
<td>64</td>
<td>73</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7</td>
<td>128</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>52</td>
<td>51</td>
<td>278</td>
</tr>
</tbody>
</table>

In conclusion, raw Bauxsol™ powder has no effect on bacteria growth. However, Bauxsol™ pellets produced with cement induced a pH spike that temporally knock-out bacteria and therefore slowed down their growth. But this effect is short-lived and once the pH returns to the optimum range, bacteria growth becomes normal.
9.5. Additional information on Bauxsol™ pellet production

The subsequent section presents additional information (Figure 9.5: example of Bauxsol™ pellet material in a curing bath and after the drying process) related to the Bauxsol™ pellet production (Chapter 3).

![Figure 9.5: Example of Bauxsol™ pellet materials after drying process (left) and in a curing bath (right) – Trial 2 (100 g batch).](image)

On the basis of the data from the Bauxsol™ pellets production experiment (Chapter 3), larger quantities of pellets have been produced with a Bauxsol™ to cement ratio of 2.8:1. The general recipe for each batch (25 batches in total) consisted of: 5,568 g of Bauxsol™ powder (<125 µm; poorly buffered reaction of pH 12), 1,992 g of Ordinary Portland Cement (<63 µm), 400 g of hydrophilic fumed silica (0.014 µm; 200 ±25 m²/g), 40 g of aluminium powder (AG8, A. Van Lerberghe), and approximately 7 L of Carbopol 940 (viscosity modifier) and Milli-Q water mix (~0.5% w:v). All dry ingredients except the aluminium powder were mixed with water in a 20 L bucket until a smooth thick paste was achieved. Then the aluminium powder was quickly added and mixed. The mix was left to dry for at least a month in the bucket; some batches dried an extra month outside the bucket. The dried blocks were then broken in smaller pieces and were plunged in a brine curing bath for 7 days: 141 g/L of magnesium chloride hexahydrate (MgCl₂·6H₂O) and 42 g/L of calcium chloride dihydrate (CaCl₂·2H₂O). After the curing process, the small blocks were washed with tap water for approximately 15 mins and then dried in an oven at 45°C for 48 to 72
hrs; silica beads were placed in the oven to absorb humidity. The small blocks were then crushed by hand using a sieve to obtain pellets of 5–10 mm diameter. Because of some friability of the material, only 250 L of Bauxsol™ pellets were produced (loss of about 50% of the original material).
9.6. Additional information on microcosm columns field experiment

The subsequent section presents additional information related to the microcosm columns field experiment (Chapter 4). Figure 9.6 shows the cleaning process of the system; Table 9.10 shows the date of the experiment and sampling time–bed volumes conversion; Table 7.11 presents the electrical conductivity data for each liquid sample; Table 9.12 presents the alkalinity speciation data for each liquid sample; Table 9.13 shows the total phosphorus, orthophosphate, total nitrogen, ammonia, nitrite, and nitrate concentrations for each liquid sample; Table 9.14 shows the concentrations of total carbon, total nitrogen, phosphate, ammonia, and nitrate on solids (sand particles; sand pellets; Bauxsol™ pellets) pre- and post-experiment; Figures 9.7 & 9.8 display the colony forming units and the biomass estimation, respectively, in influent and column’s effluents over the one month trial; Table 9.15 display the colony forming units and biomass estimation on solids (sand particles; sand pellets; Bauxsol™ pellets) post-experiment; Figures 9.9–9.13 present the DGGE profiles of 16S rDNA products from liquid samples; Figure 9.14 displays the non-metric multi-dimensional scaling (MDS) of bacterial communities generated by the analysis of solids DGGE 16S rDNA patterns.
Figure 9.6: Microcosm columns experiment before cleaning (left) and after cleaning (right), featuring the inlet bucket, the peristaltic pump, and the peristaltic tubes and cassettes.
Table 9.10: Date of the microcosm columns field experiment with conversion of sampling time and bed volumes.

<table>
<thead>
<tr>
<th>Date</th>
<th>Sampling time</th>
<th>Bed volumes</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.01.2009</td>
<td>T = 0</td>
<td>1</td>
</tr>
<tr>
<td>13.01.2009</td>
<td>T = 4h</td>
<td>16</td>
</tr>
<tr>
<td>14.01.2009</td>
<td>T = 1d</td>
<td>96</td>
</tr>
<tr>
<td>16.01.2009</td>
<td>T = 3d</td>
<td>288</td>
</tr>
<tr>
<td>20.01.2009</td>
<td>T = 7d</td>
<td>672</td>
</tr>
<tr>
<td>23.01.2009</td>
<td>T = 10d</td>
<td>952</td>
</tr>
<tr>
<td>27.01.2009</td>
<td>T = 14d</td>
<td>1336</td>
</tr>
<tr>
<td>30.01.2009</td>
<td>T = 17d</td>
<td>1616</td>
</tr>
<tr>
<td>04.02.2009</td>
<td>T = 22d</td>
<td>2024</td>
</tr>
<tr>
<td>06.02.2009</td>
<td>T = 24d</td>
<td>2216</td>
</tr>
<tr>
<td>10.02.2009</td>
<td>T = 28d</td>
<td>2600</td>
</tr>
</tbody>
</table>

Table 9.11: Electrical conductivity in influent and column’s effluents (sand control; sand pellets; Bauxsol™ pellets) at predetermined bed volumes; ± standard error for sand pellets (2 columns) and Bauxsol™ pellets (3 columns). All data are in μS.

<table>
<thead>
<tr>
<th>Bed volumes</th>
<th>Influent</th>
<th>Sand Control</th>
<th>Sand pellets</th>
<th>Bauxsol™ pellets</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>942</td>
<td>980</td>
<td>10055 ± 555</td>
<td>7513 ± 252</td>
</tr>
<tr>
<td>16</td>
<td>784</td>
<td>828</td>
<td>1074 ± 103</td>
<td>1272 ± 34</td>
</tr>
<tr>
<td>96</td>
<td>905</td>
<td>886</td>
<td>915 ± 10</td>
<td>933 ± 8</td>
</tr>
<tr>
<td>288</td>
<td>824</td>
<td>798</td>
<td>850 ± 16</td>
<td>848 ± 8</td>
</tr>
<tr>
<td>672</td>
<td>790</td>
<td>811</td>
<td>839 ± 9</td>
<td>821 ± 12</td>
</tr>
<tr>
<td>952</td>
<td>876</td>
<td>857</td>
<td>907 ± 21</td>
<td>902 ± 5</td>
</tr>
<tr>
<td>1336</td>
<td>599</td>
<td>596</td>
<td>645 ± 3</td>
<td>634 ± 4</td>
</tr>
<tr>
<td>1616</td>
<td>688</td>
<td>663</td>
<td>721 ± 1</td>
<td>690 ± 8</td>
</tr>
<tr>
<td>2024</td>
<td>676</td>
<td>760</td>
<td>787 ± 13</td>
<td>785 ± 27</td>
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<tr>
<td>2216</td>
<td>813</td>
<td>786</td>
<td>818 ± 0</td>
<td>804 ± 4</td>
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<td>2600</td>
<td>928</td>
<td>910</td>
<td>907 ± 6</td>
<td>900 ± 5</td>
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Table 9.1: Alkalinity speciation (CaCO$_3$; OH$^-$; CO$_3^{2-}$; HCO$_3^-$) of influent and column’s effluents (sand control; sand pellets; Bauxsol™ pellets) at predetermined bed volumes; ± standard error for sand pellets (2 columns) and Bauxsol™ pellets (3 columns). All data are in mg/L.

<table>
<thead>
<tr>
<th>Bed volumes</th>
<th>Liquid sample</th>
<th>Total alkalinity as CaCO$_3$</th>
<th>Alkalinity as OH$^-$</th>
<th>Alkalinity as CO$_3^{2-}$</th>
<th>Alkalinity as HCO$_3^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Influent</td>
<td>266.5</td>
<td>0.0</td>
<td>0.2</td>
<td>324.4</td>
</tr>
<tr>
<td></td>
<td>Sand control effluent</td>
<td>298.6</td>
<td>0.0</td>
<td>1.2</td>
<td>361.7</td>
</tr>
<tr>
<td></td>
<td>Sand pellets effluent</td>
<td>168.8 ± 6.1</td>
<td>0.0 ± 0.0</td>
<td>3.0 ± 1.4</td>
<td>199.8 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>Bauxsol™ pellets effluent</td>
<td>213.4 ± 5.1</td>
<td>0.0 ± 0.0</td>
<td>3.7 ± 0.1</td>
<td>252.5 ± 6.2</td>
</tr>
<tr>
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<td>Influent</td>
<td>536.9</td>
<td>0.0</td>
<td>4.3</td>
<td>645.8</td>
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<tr>
<td></td>
<td>Sand control effluent</td>
<td>159.8</td>
<td>0.0</td>
<td>0.5</td>
<td>193.7</td>
</tr>
<tr>
<td></td>
<td>Sand pellets effluent</td>
<td>291.6 ± 6.8</td>
<td>0.1 ± 0.0</td>
<td>12.1 ± 1.7</td>
<td>330.7 ± 11.8</td>
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<tr>
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<td>Bauxsol™ pellets effluent</td>
<td>182.6 ± 50.4</td>
<td>0.0 ± 0.0</td>
<td>1.9 ± 1.2</td>
<td>218.5 ± 59.0</td>
</tr>
<tr>
<td>96</td>
<td>Influent</td>
<td>219.3</td>
<td>0.1</td>
<td>10.0</td>
<td>246.8</td>
</tr>
<tr>
<td></td>
<td>Sand control effluent</td>
<td>276.0</td>
<td>0.1</td>
<td>10.1</td>
<td>315.8</td>
</tr>
<tr>
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<td>Sand pellets effluent</td>
<td>187.6 ± 116.8</td>
<td>0.0 ± 0.0</td>
<td>4.0 ± 2.1</td>
<td>220.6 ± 138.3</td>
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<tr>
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<td>Bauxsol™ pellets effluent</td>
<td>209.2 ± 66.4</td>
<td>0.0 ± 0.0</td>
<td>3.1 ± 1.6</td>
<td>248.7 ± 79.3</td>
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<td>Influent</td>
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<td>0.2</td>
<td>167.2</td>
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<td>Sand control effluent</td>
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<td>0.0</td>
<td>2.2</td>
<td>264.1</td>
</tr>
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<td>Sand pellets effluent</td>
<td>226.4 ± 1.7</td>
<td>0.1 ± 0.1</td>
<td>8.0 ± 1.5</td>
<td>259.6 ± 1.0</td>
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<td>Bauxsol™ pellets effluent</td>
<td>238.9 ± 26.3</td>
<td>0.0 ± 0.0</td>
<td>7.0 ± 1.7</td>
<td>276.9 ± 30.1</td>
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<td>672</td>
<td>Influent</td>
<td>234.9</td>
<td>0.0</td>
<td>1.9</td>
<td>282.6</td>
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<td>Sand control effluent</td>
<td>285.1</td>
<td>0.0</td>
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<td>Sand pellets effluent</td>
<td>232.8 ± 72.2</td>
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<td>8.5 ± 3.5</td>
<td>266.5 ± 80.9</td>
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<td>Bauxsol™ pellets effluent</td>
<td>315.4 ± 60.4</td>
<td>0.0 ± 0.0</td>
<td>5.2 ± 1.0</td>
<td>373.8 ± 72.7</td>
</tr>
<tr>
<td>952</td>
<td>Influent</td>
<td>313.7</td>
<td>0.0</td>
<td>2.7</td>
<td>376.9</td>
</tr>
<tr>
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<td>Sand control effluent</td>
<td>215.8</td>
<td>0.0</td>
<td>2.1</td>
<td>258.8</td>
</tr>
<tr>
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<td>Sand pellets effluent</td>
<td>689.0 ± 324.4</td>
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<td>11.2 ± 5.4</td>
<td>817.3 ± 384.7</td>
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<td>Bauxsol™ pellets effluent</td>
<td>283.3 ± 36.2</td>
<td>0.0 ± 0.0</td>
<td>4.4 ± 1.7</td>
<td>336.4 ± 41.1</td>
</tr>
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<td>1336</td>
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<td>0.0</td>
<td>0.4</td>
<td>271.9</td>
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<td>Sand control effluent</td>
<td>300.5</td>
<td>0.0</td>
<td>2.2</td>
<td>362.0</td>
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<tr>
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<td>Sand pellets effluent</td>
<td>303.0 ± 25.1</td>
<td>0.0 ± 0.0</td>
<td>2.3 ± 0.7</td>
<td>364.9 ± 32.0</td>
</tr>
<tr>
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<td>Bauxsol™ pellets effluent</td>
<td>446.9 ± 83.6</td>
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<td>10.5 ± 2.1</td>
<td>523.4 ± 99.8</td>
</tr>
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<td>Influent</td>
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<td>3.4</td>
<td>404.9</td>
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<tr>
<td></td>
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<td>0.0</td>
<td>2.4</td>
<td>349.6</td>
</tr>
<tr>
<td></td>
<td>Sand pellets effluent</td>
<td>449.0 ± 255.2</td>
<td>0.1 ± 0.1</td>
<td>27.8 ± 24.6</td>
<td>490.5 ± 260.9</td>
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<td>Bauxsol™ pellets effluent</td>
<td>781.3 ± 383.1</td>
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<td>927.8 ± 457.0</td>
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<td>2024</td>
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<td>187.8</td>
<td>0.0</td>
<td>4.0</td>
<td>220.8</td>
</tr>
<tr>
<td></td>
<td>Sand control effluent</td>
<td>447.4</td>
<td>0.0</td>
<td>2.8</td>
<td>539.7</td>
</tr>
<tr>
<td></td>
<td>Sand pellets effluent</td>
<td>284.5 ± 0.4</td>
<td>0.0 ± 0.0</td>
<td>5.2 ± 1.9</td>
<td>336.2 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>Bauxsol™ pellets effluent</td>
<td>262.4 ± 117.1</td>
<td>0.0 ± 0.0</td>
<td>6.5 ± 4.4</td>
<td>306.6 ± 134.1</td>
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<td>2216</td>
<td>Influent</td>
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<td>1.3</td>
<td>345.9</td>
</tr>
<tr>
<td></td>
<td>Sand control effluent</td>
<td>197.2</td>
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<td>2.3</td>
<td>235.8</td>
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<td>Sand pellets effluent</td>
<td>258.5 ± 82.4</td>
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<td>7.7 ± 6.2</td>
<td>299.4 ± 87.6</td>
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<tr>
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<td>Bauxsol™ pellets effluent</td>
<td>249.9 ± 13.6</td>
<td>0.1 ± 0.1</td>
<td>14.8 ± 9.6</td>
<td>311.0 ± 24.3</td>
</tr>
<tr>
<td>2600</td>
<td>Influent</td>
<td>255.8</td>
<td>0.0</td>
<td>1.5</td>
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<tr>
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<td>Sand control effluent</td>
<td>237.0</td>
<td>0.0</td>
<td>1.7</td>
<td>285.4</td>
</tr>
<tr>
<td></td>
<td>Sand pellets effluent</td>
<td>236.4 ± 24.5</td>
<td>0.1 ± 0.1</td>
<td>5.8 ± 2.8</td>
<td>276.3 ± 35.8</td>
</tr>
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<td>Bauxsol™ pellets effluent</td>
<td>239.6 ± 18.7</td>
<td>0.0 ± 0.0</td>
<td>5.1 ± 1.5</td>
<td>281.7 ± 24.4</td>
</tr>
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</table>
Table 9.13: Total phosphorus (TP), orthophosphate (PO43–), total nitrogen (TN), ammonia (NH4+),
nitrite (NO2–), nitrate (NO3–) concentrations in influent and column’s effluents (sand control; sand
pellets; Bauxsol pellets) at predetermined bed volumes; ± standard error for sand pellets (2 columns)
and Bauxsol pellets (3 columns). All data are in mg/L.
Bed
volumes

1

TN

NH4+

NO2

NO3

Influent
Sand control effluent

6.35
1.31
0.60
±0.21
0.96
±0.10
5.80
5.37
1.59
±0.38
1.70
±0.21
6.81
6.91
3.09
±0.24
4.20
±0.67
7.02
6.95
4.19
±1.20
5.07
±0.92
7.21
7.27
6.54
±0.06
6.59
±0.14
7.35
7.15
6.65
±0.02
6.63
±0.07
4.62
4.64
4.53
±0.06
4.15
±0.03
4.79
4.93
4.48
±0.02
4.27
±0.03
5.19
9.28
5.37
±0.13
5.42
±0.12
5.80
5.84
5.31
±0.02
5.15
±0.03
7.29
7.22
6.47
±0.03
6.26
±0.03

6.26
1.46
0.32
±0.12
0.59
±0.09
5.09
4.97
0.75
±0.09
1.57
±0.31
5.84
6.11
2.76
±0.06
3.74
±0.52
6.14
6.36
3.86
±1.17
4.43
±0.90
6.50
6.83
5.94
±0.04
5.91
±0.12
6.66
6.62
6.11
±0.08
6.07
±0.05
4.32
4.27
4.26
±0.06
3.82
±0.03
4.35
4.40
4.15
±0.01
4.00
±0.01
4.74
8.46
4.94
±0.28
4.98
±0.07
5.37
5.49
4.97
±0.00
4.77
±0.04
6.56
6.68
6.11
±0.08
5.70
±0.04

39.02
84.59
31.02
±0.53
34.70
±0.47
33.56
35.32
32.72
±0.04
32.72
±0.14
54.76
54.70
54.64
±2.08
50.57
±3.27
41.27
37.61
36.66
±0.30
37.96
±0.34
42.22
39.16
40.65
±0.42
39.90
±0.71
40.41
36.93
40.25
±0.34
38.94
±0.24
24.34
21.49
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±0.32
23.20
±0.08
31.22
27.04
27.51
±0.21
27.75
±0.11
29.99
45.00
28.86
±0.77
25.01
±0.91
28.12
22.98
23.20
±0.23
22.93
±0.26
46.83
41.17
41.41
±0.24
41.41
±0.07

8.01
5.73
7.87
±0.16
7.93
±0.07
5.48
6.02
6.16
±0.11
6.37
±0.15
9.65
10.35
10.51
±0.06
10.09
±0.48
8.76
8.93
8.38
±0.05
8.44
±0.16
7.07
8.65
8.84
±0.02
8.57
±0.09
8.07
8.65
10.03
±0.02
9.70
±0.08
3.41
3.40
3.66
±0.03
3.71
±0.09
5.10
5.51
5.29
±0.08
5.37
±0.09
5.59
19.97
8.19
±0.37
9.01
±0.73
6.28
6.24
5.79
±0.21
5.82
±0.06
9.76
10.11
9.65
±0.34
9.84
±0.02

0.23
0.25
0.23
±0.00
0.25
±0.01
0.25
0.27
0.27
±0.01
0.28
±0.01
0.35
0.41
0.56
±0.01
0.46
±0.02
0.41
0.05
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±0.01
0.15
±0.01
0.40
0.06
0.33
±0.04
0.30
±0.01
0.27
0.18
0.26
±0.02
0.19
±0.01
0.23
0.19
0.57
±0.07
0.46
±0.06
0.23
0.05
0.37
±0.11
0.27
±0.05
0.22
0.10
0.21
±0.08
0.16
±0.04
0.50
0.10
0.47
±0.13
0.23
±0.04
0.43
0.12
0.63
±0.24
0.36
±0.03

27.06
71.93
17.56
±1.55
16.32
±0.27
15.29
15.63
14.97
±0.10
16.21
±0.66
24.25
24.87
26.77
±1.49
24.85
±1.48
18.15
16.61
17.37
±0.35
17.30
±0.28
21.46
18.39
19.22
±0.10
19.43
±0.16
20.22
17.99
18.58
±0.15
19.66
±0.48
17.37
15.35
17.19
±0.34
17.94
±0.25
23.59
20.24
21.71
±0.68
21.08
±0.60
20.56
13.87
15.99
±0.52
10.72
±1.93
18.30
14.99
15.31
±0.19
15.71
±0.29
30.75
27.64
29.00
±0.00
28.37
±0.42

Sand pellets effluent

Sand pellets effluent
Bauxsol pellets effluent
Influent
Sand control effluent
Sand pellets effluent
Bauxsol pellets effluent
Influent
Sand control effluent

288

Sand pellets effluent
Bauxsol pellets effluent
Influent
Sand control effluent

672

Sand pellets effluent
Bauxsol pellets effluent
Influent
Sand control effluent

952

Sand pellets effluent
Bauxsol pellets effluent
Influent
Sand control effluent

1336

Sand pellets effluent
Bauxsol pellets effluent
Influent
Sand control effluent

1616

Sand pellets effluent
Bauxsol pellets effluent
Influent
Sand control effluent

2024

Sand pellets effluent
Bauxsol pellets effluent
Influent
Sand control effluent

2216

Sand pellets effluent
Bauxsol pellets effluent
Influent
Sand control effluent

2600

–

PO43

Influent
Sand control effluent

96

–

TP

Bauxsol pellets effluent

16

–

Liquid sample

Sand pellets effluent
Bauxsol pellets effluent

237


Table 9.1: Total carbon (TC; %), total nitrogen (TN; %), phosphate (PO$_4^{3-}$; mg/kg), ammonia (NH$_4^+$; mg/kg) and nitrate (NO$_3^-$; mg/kg) on solids (sand particles; sand pellets; Bauxsol™ pellets) pre- and post-experiment.

<table>
<thead>
<tr>
<th>Time</th>
<th>Material</th>
<th>TC</th>
<th>TN</th>
<th>PO$_4^{3-}$</th>
<th>NH$_4^+$</th>
<th>NO$_3^-$</th>
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</thead>
<tbody>
<tr>
<td>Pre-experiment</td>
<td>Sand particles</td>
<td>0.08</td>
<td>0.01</td>
<td>4.78</td>
<td>4.53</td>
<td>61.32</td>
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<td>Sand pellets</td>
<td>0.88</td>
<td>0.01</td>
<td>0.07</td>
<td>2.92</td>
<td>5.35</td>
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<tr>
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<td>Bauxsol™ pellets</td>
<td>1.57</td>
<td>0.02</td>
<td>0.05</td>
<td>3.06</td>
<td>7.02</td>
</tr>
<tr>
<td>Post-experiment</td>
<td>Sand particles</td>
<td>0.25</td>
<td>0.04</td>
<td>13.76</td>
<td>13.57</td>
<td>1.49</td>
</tr>
<tr>
<td></td>
<td>Sand pellets</td>
<td>± 0.01</td>
<td>± 0.01</td>
<td>± 0.25</td>
<td>± 0.74</td>
<td>± 0.86</td>
</tr>
<tr>
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<td>Bauxsol™ pellets</td>
<td>± 0.09</td>
<td>± 0.02</td>
<td>± 3.58</td>
<td>± 17.29</td>
<td>± 9.69</td>
</tr>
</tbody>
</table>

Figure 9.1: Colony forming units (cfu/mL) in influent and column’s effluents (sand control; sand pellets; Bauxsol™ pellets) at predetermined bed volumes; thick grey error bar = ± standard error for sand pellets (2 columns); thin black error bar = ± standard error for Bauxsol™ pellets (3 columns).

Figure 9.8: Biomass estimation (µg of protein per mL) in influent and column’s effluents (sand control; sand pellets; Bauxsol™ pellets) at predetermined bed volumes; thick grey error bar = ± standard error for sand pellets (2 columns); thin black error bar = ± standard error for Bauxsol™ pellets (3 columns).
Table 9.15: Colony forming units (cfu/mL) and biomass estimation (µg of protein per mL) on solids (sand particles; sand pellets; Bauxsol™ pellets) post-experiment.

<table>
<thead>
<tr>
<th>Material</th>
<th>CFU</th>
<th>Biomass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sand particles</td>
<td>$6.03 \times 10^6$</td>
<td>444.82</td>
</tr>
<tr>
<td>Sand pellets</td>
<td>$3.68 \times 10^6 \pm 1.25 \times 10^5$</td>
<td>$340.21 \pm 89.99$</td>
</tr>
<tr>
<td>Bauxsol™ pellets</td>
<td>$1.43 \times 10^6 \pm 4.21 \times 10^5$</td>
<td>$200.96 \pm 44.84$</td>
</tr>
</tbody>
</table>

Figure 9.9: DGGE analysis of 16S rDNA gene products amplified from total DNA. Gel image of liquid samples collected at $T = 0$: lane 1 – influent, lane 2 – Sand control effluent, lane 3 – Sand pellet effluent I, lane 4 – Sand pellet effluent II, lane 5 – Bauxsol™ pellets effluent I, lane 6 – Bauxsol™ pellets effluent II, lane 7 – Bauxsol™ pellets effluent III; at $T = 4h$: lane 8 – influent, lane 9 – Sand control effluent, lane 10 – Sand pellet effluent I, lane 11 – Sand pellet effluent II, lane 12 – Bauxsol™ pellets effluent I, lane 13 – Bauxsol™ pellets effluent II, lane 14 – Bauxsol™ pellets effluent III. St = DNA marker standard from wastewater isolate colony.
Figure 9.14: Non-metric multi-dimensional scaling (MDS) of bacterial communities generated by the analysis of solids DGGE 16S rDNA patterns. Fourth roots transform and the Bray-Curtis measure used. Samples displayed according to the materials: sand particles (SC); sand pellets (Sp) from duplicate columns (I and II); Bauxsol™ pellets (Bp) from triplicate columns (I, II, and III). Similarity between samples is represented in: continuous line (75%), dash line (80%), and point line (90%).
9.7. Additional information on mesocosm columns field experiment

The subsequent section presents additional information related to the mesocosm columns field experiment (Chapters 5 & 6). Figure 9.15 presents the mesocosm columns experiment setting; Table 9.16 shows the date of the experiment and sampling time–bed volumes conversion; Figure 9.16 presents the electrical conductivity data for column inlet and outlet waters samples; Table 9.17 presents the alkalinity speciation data for inlet and column’s outlets over the six months trial; Table 9.18 presents the average concentration of total phosphorus and orthophosphates in the inlet, Bauxsol™ half-way and outlet and gravel half-way and outlet between 0–1st month, 1st–3rd month, 3rd–6th month of the mesocosm columns experiment; Table 9.19 shows the pre-experiment geochemistry of Bauxsol™ pellet and gravel; Figures 9.17–9.24 present the DGGE profiles of 16S rDNA products from liquid samples; Figure 9.25 displays the photo of gel electrophoresis of functional PCR amplification (bacterial amoA genes, archaeal amoA genes, hzo genes, nosZ genes) products run through a 2% agarose gel.

Figure 9.15: Mesocosm columns experiment setting (3 Bauxsol™ pellets packed columns and 1 Gravel packed column) at the Sewage Treatment Plant, South Lismore (Australia). The effluent is flowing in an updraft direction at 15 mL/min (regulated by peristaltic pump system). Samples ports at one-quarter-way, half-way and three-quarter-way along the columns length are shown on the left image.
Table 9.16: Date of the experiment with conversion of sampling time and bed volumes.

<table>
<thead>
<tr>
<th>Date</th>
<th>Sampling time</th>
<th>Bed volumes</th>
</tr>
</thead>
<tbody>
<tr>
<td>16/09/2009</td>
<td>T = 0</td>
<td>1</td>
</tr>
<tr>
<td>17/09/2009</td>
<td>T = 1d</td>
<td>2.1</td>
</tr>
<tr>
<td>19/09/2009</td>
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</tr>
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<td>23/09/2009</td>
<td>T = 7d</td>
<td>15</td>
</tr>
<tr>
<td>30/09/2009</td>
<td>T = 2w</td>
<td>30</td>
</tr>
<tr>
<td>07/10/2009</td>
<td>T = 3w</td>
<td>45</td>
</tr>
<tr>
<td>14/10/2009</td>
<td>T = 4w</td>
<td>60</td>
</tr>
<tr>
<td>27/10/2009</td>
<td>T = 6w</td>
<td>90</td>
</tr>
<tr>
<td>10/11/2009</td>
<td>T = 8w</td>
<td>120</td>
</tr>
<tr>
<td>24/11/2009</td>
<td>T = 10w</td>
<td>150</td>
</tr>
<tr>
<td>08/12/2009</td>
<td>T = 12w</td>
<td>180</td>
</tr>
<tr>
<td>22/12/2009</td>
<td>T = 14w</td>
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</tr>
<tr>
<td>05/01/2010</td>
<td>T = 16w</td>
<td>240</td>
</tr>
<tr>
<td>19/01/2010</td>
<td>T = 18w</td>
<td>270</td>
</tr>
<tr>
<td>02/02/2010</td>
<td>T = 20w</td>
<td>300</td>
</tr>
<tr>
<td>16/02/2010</td>
<td>T = 22w</td>
<td>330</td>
</tr>
<tr>
<td>01/03/2010</td>
<td>T = 24w</td>
<td>360</td>
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</tbody>
</table>

Figure 9.16: Electrical conductivity (µS) of column inlet and outlet waters samples (Bauxsol™ pellets and Gravel) - at predetermined bed volumes; ± standard error for Bauxsol™ pellets (3 columns).

Table 9.17: Alkalinity speciation (CaCO₃; OH⁻; CO₃²⁻; HCO₃⁻) of inlet and column’s outlets (Bauxsol™ pellets and gravel) at predetermined bed volumes; ± standard error for Bauxsol™ pellets (3 columns). All data are in mg/L.

<table>
<thead>
<tr>
<th>Bed volume</th>
<th>Liquid sample</th>
<th>Total alkalinity as CaCO₃</th>
<th>Alkalinity as OH⁻</th>
<th>Alkalinity as CO₃²⁻</th>
<th>Alkalinity as HCO₃⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inlet</td>
<td>145.1</td>
<td>0</td>
<td>0.1</td>
<td>176.7</td>
</tr>
<tr>
<td></td>
<td>Gravel outlet</td>
<td>158.9</td>
<td>0</td>
<td>1.6</td>
<td>190.5</td>
</tr>
<tr>
<td></td>
<td>Bauxsol™ outlet</td>
<td>119.4 ± 15.2</td>
<td>6.2 ± 1.0</td>
<td>54.2 ± 8.9</td>
<td>13.2 ± 2.8</td>
</tr>
<tr>
<td>Time (min)</td>
<td>Inlet</td>
<td>Gravel outlet</td>
<td>Bauxsol outlet</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>------</td>
<td>--------------</td>
<td>---------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.1</td>
<td>150.4</td>
<td>152.4</td>
<td>166.8 ± 5.9</td>
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<td></td>
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<td>9.2 ± 0.8</td>
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<td>75.3 ± 2.4</td>
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<td>17.4 ± 0.5</td>
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<tr>
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<td>3.4 ± 1.2</td>
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<td>179.5 ± 2.7</td>
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<td>114.8</td>
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<td>1.6 ± 0.3</td>
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<td>1.3 ± 0.1</td>
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<td>1.3 ± 0.1</td>
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<td>204.1 ± 1.9</td>
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Table 9.1: Average concentration of total phosphorus (TP) and orthophosphates (PO$_4^{3-}$) in the inlet, Bauxsol™ half-way and outlet and gravel half-way and outlet between 0–1$^{\text{st}}$ month, 1$^{\text{st}}$–3$^{\text{rd}}$ month, 3$^{\text{rd}}$–6$^{\text{th}}$ month of the mesocosm columns experiment. ± Standard error. All data are in mg/L.

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<tr>
<th>Time</th>
<th>Treatment</th>
<th>Distance</th>
<th>TP</th>
<th>PO$_4^{3-}$</th>
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</thead>
<tbody>
<tr>
<td>0 to 1$^{\text{st}}$ month</td>
<td>nil</td>
<td>inlet</td>
<td>8.05 ±0.31</td>
<td>7.13 ±0.45</td>
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<tr>
<td></td>
<td>Bauxsol™</td>
<td>half</td>
<td>1.89 ±0.22</td>
<td>0.48 ±0.19</td>
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<tr>
<td></td>
<td></td>
<td>outlet</td>
<td>1.63 ±0.36</td>
<td>0.23 ±0.07</td>
</tr>
<tr>
<td></td>
<td>Gravel</td>
<td>half</td>
<td>6.84 ±0.33</td>
<td>6.46 ±0.36</td>
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<tr>
<td></td>
<td></td>
<td>outlet</td>
<td>7.09 ±0.30</td>
<td>6.62 ±0.35</td>
</tr>
<tr>
<td>1$^{\text{st}}$ to 3$^{\text{rd}}$ month</td>
<td>nil</td>
<td>inlet</td>
<td>6.51 ±0.75</td>
<td>6.03 ±0.75</td>
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<td>Bauxsol™</td>
<td>half</td>
<td>2.23 ±0.15</td>
<td>1.93 ±0.17</td>
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<tr>
<td></td>
<td></td>
<td>outlet</td>
<td>1.67 ±0.12</td>
<td>1.42 ±0.09</td>
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<tr>
<td></td>
<td>Gravel</td>
<td>half</td>
<td>6.31 ±0.63</td>
<td>5.84 ±0.57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>outlet</td>
<td>6.34 ±0.71</td>
<td>5.75 ±0.91</td>
</tr>
<tr>
<td>3$^{\text{rd}}$ to 6$^{\text{th}}$ month</td>
<td>nil</td>
<td>inlet</td>
<td>4.85 ±0.50</td>
<td>3.74 ±0.24</td>
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<tr>
<td></td>
<td>Bauxsol™</td>
<td>half</td>
<td>1.70 ±0.11</td>
<td>1.50 ±0.09</td>
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<tr>
<td></td>
<td></td>
<td>outlet</td>
<td>1.17 ±0.09</td>
<td>1.00 ±0.09</td>
</tr>
<tr>
<td></td>
<td>Gravel</td>
<td>half</td>
<td>4.30 ±0.37</td>
<td>3.78 ±0.26</td>
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<tr>
<td></td>
<td></td>
<td>outlet</td>
<td>4.33 ±0.41</td>
<td>3.86 ±0.35</td>
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Table 9.19: Pre-experiment geochemistry of Bauxsol™ pellet and gravel. All data are mg/kg except iron and aluminium which are in weight %.

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<tr>
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<th>Bauxsol™ pellet</th>
<th>Gravel</th>
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<tr>
<td>Total phosphorus</td>
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<td>3687.24</td>
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<td>Colwell Phosphorus</td>
<td>47.89</td>
<td>9.84</td>
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<td>Phosphate (KCl extract)</td>
<td>0.12</td>
<td>1.10</td>
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<tr>
<td>Ammonia</td>
<td>1.38</td>
<td>0.19</td>
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<tr>
<td>Nitrate</td>
<td>14.38</td>
<td>0.79</td>
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<tr>
<td>Silver</td>
<td>0.32</td>
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<tr>
<td>Arsenic</td>
<td>17.20</td>
<td>0.30</td>
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<td>Lead</td>
<td>45.86</td>
<td>2.37</td>
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<td>Cadmium</td>
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<td>Chromium</td>
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<td>Copper</td>
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<td>Manganese</td>
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<td>Nickel</td>
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<td>Selenium</td>
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<td>Zinc</td>
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<td>Mercury</td>
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<tr>
<td>Iron</td>
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<td>7.49</td>
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<tr>
<td>Aluminium</td>
<td>3.26</td>
<td>1.67</td>
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Figure 9.17: DGGE analysis of 16S rDNA gene products amplified from total DNA. Gel image of liquid samples collected at $T = 0$: lane 1 – inlet, lane 2 – gravel outlet, lane 3 – gravel half-way, lane 4 – Bauxsol™ pellets outlet III; at $T = 1$ d: lane 5 - inlet, lane 6 – gravel outlet, lane 7 – gravel half-way, lane 8 – Bauxsol™ pellets outlet I, lane 9 – Bauxsol™ pellets half-way I, lane 10 – Bauxsol™ pellets outlet II, lane 11 – Bauxsol™ pellets half-way II, lane 12 – Bauxsol™ pellets outlet III, lane 13 – Bauxsol™ pellets half-way III. St = DNA marker standard from wastewater isolate colony.
Figure 9.18: DGGE analysis of 16S rDNA gene products amplified from total DNA. Gel image of liquid samples collected at $T = 1w$: lane 1 – inlet, lane 2 – gravel outlet, lane 3 – gravel half-way, lane 4 – Bauxsol™ pellets outlet I, lane 5 – Bauxsol™ pellets outlet II, lane 6 – Bauxsol™ pellets outlet III, lane 7 – Bauxsol™ pellets half-way III; at $T = 3w$: lane 8 – inlet, lane 9 – gravel outlet, lane 10 – Bauxsol™ pellets outlet I, lane 11 – Bauxsol™ pellets half-way I, lane 12 – Bauxsol™ pellets outlet II, lane 13 – Bauxsol™ pellets half-way II, lane 14 – Bauxsol™ pellets outlet III, lane 15 – Bauxsol™ pellets half-way III. St = DNA marker standard from wastewater isolate colony.
Figure 9.19: DGGE analysis of 16S rDNA gene products amplified from total DNA. Gel image of liquid samples collected at $T = 2w$: lane 1 – inlet, lane 2 – gravel outlet, lane 3 – gravel half-way, lane 4 – Bauxsol™ pellets outlet I, lane 5 – Bauxsol™ pellets half-way I, lane 6 – Bauxsol™ pellets outlet II, lane 7 – Bauxsol™ pellets half-way II, lane 8 – Bauxsol™ pellets outlet III, lane 9 – Bauxsol™ pellets half-way III; at $T = 6w$: lane 10 – inlet, lane 11 – Bauxsol™ pellets half-way I, lane 12 – Bauxsol™ pellets outlet II, lane 13 – Bauxsol™ pellets half-way II, lane 14 – Bauxsol™ pellets half-way III. St = DNA marker standard from wastewater isolate colony.
Figure 9.20: DGGE analysis of 16S rDNA gene products amplified from total DNA. Gel image of liquid samples collected at $T = 4\text{w}$: lane 1 – inlet, lane 2 – gravel outlet, lane 3 – gravel half-way, lane 4 – Bauxsol™ pellets outlet I, lane 5 – Bauxsol™ pellets half-way I, lane 6 – Bauxsol™ pellets outlet II, lane 7 – Bauxsol™ pellets half-way II, lane 8 – Bauxsol™ pellets outlet III, lane 9 – Bauxsol™ pellets half-way III; at $T = 14\text{w}$: lane 10 – inlet, lane 11 – Bauxsol™ pellets outlet II. St = DNA marker standard from wastewater isolate colony.
Figure 9.21: DGGE analysis of 16S rDNA gene products amplified from total DNA. Gel image of liquid samples collected at $T = 12\text{w}$: lane 1 – inlet, lane 2 – gravel outlet, lane 3 – gravel half-way, lane 4 – Bauxsol™ pellets outlet I, lane 5 – Bauxsol™ pellets half-way I, lane 6 – Bauxsol™ pellets outlet II, lane 7 – Bauxsol™ pellets half-way II, lane 8 – Bauxsol™ pellets outlet III, lane 9 – Bauxsol™ pellets half-way III; at $T = 16\text{w}$: lane 10 – inlet. St = DNA marker standard from wastewater isolate colony.
Figure 9.22: DGGE analysis of 16S rDNA gene products amplified from total DNA. Gel image of liquid samples collected at $T = 10\text{w}$: lane 1 – inlet, lane 2 – gravel half-way, lane 3 – Bauxsol™ pellets outlet I, lane 4 – Bauxsol™ pellets half-way I, lane 5 – Bauxsol™ pellets outlet II, lane 6 – Bauxsol™ pellets half-way II, lane 7 – Bauxsol™ pellets outlet III, lane 8 – Bauxsol™ pellets half-way III; at $T = 20\text{w}$: lane 9 – inlet, lane 10 – gravel outlet, lane 11 – Bauxsol™ pellets outlet I, lane 12 – Bauxsol™ pellets outlet II, lane 13 – Bauxsol™ pellets half-way II, lane 14 – Bauxsol™ pellets outlet III, lane 15 – Bauxsol™ pellets half-way III. St = DNA marker standard from wastewater isolate colony.
Figure 9.23: DGGE analysis of 16S rDNA gene products amplified from total DNA. Gel image of liquid samples collected at $T = 22w$: lane 1 – inlet, lane 2 – gravel outlet, lane 3 – gravel half-way, lane 4 – Bauxsol™ pellets outlet I, lane 5 – Bauxsol™ pellets half-way I, lane 6 – Bauxsol™ pellets outlet II, lane 7 – Bauxsol™ pellets half-way II, lane 8 – Bauxsol™ pellets outlet III, lane 9 – Bauxsol™ pellets half-way III. St = DNA marker standard from wastewater isolate colony.
Figure 9.24: DGGE analysis of 16S rDNA gene products amplified from total DNA. Gel image of liquid samples collected at $T = 18w$: lane 1 – inlet, lane 2 – gravel outlet, lane 3 – gravel half-way, lane 4 – Bauxsol™ pellets outlet I, lane 5 – Bauxsol™ pellets half-way I, lane 6 – Bauxsol™ pellets outlet II, lane 7 – Bauxsol™ pellets half-way II, lane 8 – Bauxsol™ pellets outlet III, lane 9 – Bauxsol™ pellets half-way III; at $T = 24w$: lane 10 – inlet. St = DNA marker standard from wastewater isolate colony.
Figure 9.25: Photo of gel electrophoresis of functional PCR amplification products run through a 2% agarose gel. Panel A corresponds to bacterial *amoA* genes (AOB); panel B of archaeal *amoA* genes (AOA); panel C of *hzo* genes; and panel D of *nosZ* genes. Lane 1 – Bauxsol™ pellets at 0 cm, lane 2 – Bauxsol™ pellets at 75 cm, lane 3 – Bauxsol™ pellets at 130 cm, lane 4 – Gravel at 0 cm, lane 5 – Gravel at 75 cm, lane 6 – Gravel at 130 cm, lane 7 – Inlet T = 1d, lane 8 – Inlet T = 22w; Sta = standard 1 Kb Plus DNA ladder™ (Life Technologies), Stb = standard HyperladderIV™ (Bioline).
9.8. Additional information on constructed wetland canal field experiment

The subsequent section presents additional information related to the constructed wetland canal field experiment (Chapter 7). Table 9.20 shows the date of the experiment and sampling time–bed volumes conversion; Table 9.21 shows the pre-experiment geochemistry of Bauxsol™ pellet, gravel and soil particle; Figures 9.26–9.33 present the DGGE profiles of 16S rDNA products from liquid samples; Figures 9.34–9.45 display the DGGE profiles of 16S rDNA products from solid samples; Figure 9.46 displays the photo of gel electrophoresis of functional PCR amplification (bacterial amoA genes, archaeal amoA genes, hzo genes, nosZ genes) products run through a 2% agarose gel; Figure 9.47 shows the X-ray diffraction of Bauxsol™ pellets pre-experiment, post-experiment, and the difference between the two; Figure 9.48 displays a picture of the scanning electron analysis undertaken on Bauxsol™ pellets pre-experiment; Figures 9.49–9.51 display a picture of the scanning electron analysis undertaken on Bauxsol™ pellets post-experiment; Figure 9.52 shows a picture of the micro-CT scan of Bauxsol™ pellets post-experiment.

Table 9.20: Date of the experiment with conversion of sampling time and bed volumes.

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<td>12.07.2010</td>
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<td>12.10.2010</td>
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<td>29.11.2010</td>
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Table 9.2: Pre-experiment geochemistry of Bauxsol™ pellet, gravel and soil particle. All data are mg/kg except iron and aluminium which are in weight %.

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<th>Soil</th>
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<td>0.1</td>
<td>0.01</td>
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<td><strong>Aluminium</strong></td>
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<td>1.4</td>
<td>4.9</td>
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Figure 9.26: DGGE analysis (triplicate) of 16S rDNA gene products amplified from total DNA extracted from liquid samples. Lane 1 – inlet T = 0, lane 2 – inlet T = 2w, lane 3 – inlet T = 4w, lane 4 – inlet T = 8w, lane 5 – inlet T = 12w. St = DNA marker standard from wastewater isolate colony.
Figure 9.27: DGGE analysis (triplicate) of 16S rDNA gene products amplified from total DNA extracted from liquid samples. Lane 1 – inlet T = 16w, lane 2 – experimental effluent from Bauxsol™ layer at canal’s mid way point T = 16w, lane 3 – experimental effluent from soil layer at canal’s mid way point T = 16w, lane 4 – experimental effluent from Bauxsol™ layer at canal’s distal end T = 16w, lane 5 – experimental effluent from soil layer at canal’s distal end T = 16w. St = DNA marker standard from wastewater isolate colony.
Figure 9.28: DGGE analysis (triplicate) of 16S rDNA gene products amplified from total DNA extracted from liquid samples. Lane 1 – control effluent from gravel layer at canal’s mid way point T = 16w, lane 2 – control effluent from soil layer at canal’s mid way point T = 16w, lane 3 – control effluent from gravel layer at canal’s distal end T = 16w, lane 4 – control effluent from soil layer at canal’s distal end T = 16w, lane 5 – gravel outlet from control canal T = 16w. St = DNA marker standard from wastewater isolate colony.
Figure 9.29: DGGE analysis (triplicate) of 16S rDNA gene products amplified from total DNA extracted from liquid samples. Lane 1 – inlet T = 20w, lane 2 – experimental effluent from Bauxsol™ layer at canal’s mid way point T = 20w, lane 3 – experimental effluent from soil layer at canal’s mid way point T = 20w, lane 4 – experimental effluent from Bauxsol™ layer at canal’s distal end T = 20w, lane 5 – experimental effluent from soil layer at canal’s distal end T = 20w. St = DNA marker standard from wastewater isolate colony.
Figure 9.30: DGGE analysis (triplicate) of 16S rDNA gene products amplified from total DNA extracted from liquid samples. Lane 1 – Bauxsol™ outlet from experimental canal T = 20w, lane 2 – control effluent from gravel layer at canal’s mid way point T = 20w, lane 3 – control effluent from soil layer at canal’s mid way point T = 20w, lane 4 – control effluent from gravel layer at canal’s distal end T = 20w, lane 5 – control effluent from soil layer at canal’s distal end T = 20w. St = DNA marker standard from wastewater isolate colony.
Figure 9.31: DGGE analysis (triplicate) of 16S rDNA gene products amplified from total DNA extracted from liquid samples. Lane 1 – gravel outlet T = 20w, lane 2 – inlet T = 24w, lane 3 – experimental effluent from Bauxsol™ layer at canal’s mid way point T = 24w, lane 4 – experimental effluent from soil layer at canal’s mid way point T = 24w, lane 5 – experimental effluent from Bauxsol™ layer at canal’s distal end T = 24w. St = DNA marker standard from wastewater isolate colony.
Figure 9.32: DGGE analysis (triplicate) of 16S rDNA gene products amplified from total DNA extracted from liquid samples. Lane 1 – experimental effluent from soil layer at canal’s mid way point $T = 24w$, lane 2 – Bauxsol™ outlet from experimental canal $T = 24w$, lane 3 – control effluent from gravel layer at canal’s mid way point $T = 24w$, lane 4 – control effluent from soil layer at canal’s mid way point $T = 24w$, lane 5 – control effluent from gravel layer at canal’s distal end $T = 24w$. St = DNA marker standard from wastewater isolate colony.
**Figure 9.33**: DGGE analysis (triplicate) of 16S rDNA gene products amplified from total DNA extracted from liquid samples. Lane 1 – control effluent from soil layer at canal’s distal end T = 24w. St = DNA marker standard from wastewater isolate colony.
Figure 9.34: DGGE analysis (triplicate) of 16S rDNA gene products amplified from total DNA extracted from solid samples. Lane 1 – Bauxsol™ in experimental canal at 0.6 m (length; proximal end) T = 16w, lane 2 – soil in experimental canal at 0.6 m (length; proximal end) T = 16w, lane 3 – Bauxsol™ in experimental canal at 1.3 m (length) T = 16w, lane 4 – soil in experimental canal at 1.3 m (length) T = 16w, lane 5 – Bauxsol™ in experimental canal at 1.8 m (length) T = 16w. St = DNA marker standard from wastewater isolate colony.
Figure 9.35: DGGE analysis (triplicate) of 16S rDNA gene products amplified from total DNA extracted from solid samples. Lane 1 – interface Bauxsol™/soil layers in experimental canal at 1.8 m (length) T = 16w, lane 2 – soil in experimental canal at 1.8 m (length) T = 16w, lane 3 – Bauxsol™ in experimental canal at 2.5 m (length; distal end) T = 16w, lane 4 – gravel in control canal at 0.6 m (length; proximal end) T = 16w, lane 5 – interface gravel/soil layers in control canal at 0.6 m (length; proximal end) T = 16w. St = DNA marker standard from wastewater isolate colony.
Figure 9.36: DGGE analysis (triplicate) of 16S rDNA gene products amplified from total DNA extracted from solid samples. Lane 1 – soil in control canal at 0.6 m (length; proximal end) T = 16w, lane 2 – gravel in control canal at 1.3 m (length) T = 16w, lane 3 – interface gravel/soil layers in control canal at 1.3 m (length) T = 16w, lane 4 – soil in control canal at 1.3 m (length) T = 16w, lane 5 – interface gravel/soil layers in control canal at 1.8 m (length) T = 16w. St = DNA marker standard from wastewater isolate colony.
Figure 9.37: DGGE analysis (triplicate) of 16S rDNA gene products amplified from total DNA extracted from solid samples. Lane 1 – soil in control canal at 1.8 m (length) T = 16w, lane 2 – interface gravel/soil layers in control canal at 2.5 m (length; distal end) T = 16w, lane 3 – soil in control canal at 2.5 m (length; distal end) T = 16w, lane 4 – Bauxsol™ in experimental canal at 0.6 m (length; proximal end) T = 20w, lane 5 – interface Bauxsol™/soil layers in experimental canal at 0.6 m (length; proximal end) T = 20w. St = DNA marker standard from wastewater isolate colony.
Figure 9.38: DGGE analysis (triplicate) of 16S rDNA gene products amplified from total DNA extracted from solid samples. Lane 1 – soil in experimental canal at 0.6 m (length; proximal end) T = 20w, lane 2 – Bauxsol™ in experimental canal at 1.3 m (length) T = 20w, lane 3 – soil in experimental canal at 1.3 m (length) T = 20w, lane 4 – Bauxsol™ in experimental canal at 1.8 m (length) T = 20w, lane 5 – interface Bauxsol™/soil layers in experimental canal at 1.8 m (length) T = 20w. St = DNA marker standard from wastewater isolate colony.
Figure 9.39: DGGE analysis (triplicate) of 16S rDNA gene products amplified from total DNA extracted from solid samples. Lane 1 – soil in experimental canal at 1.8 m (length) T = 20w, lane 2 – interface Bauxsol™/soil layers in experimental canal at 2.5 m (length; distal end) T = 20w, lane 3 – soil in experimental canal at 2.5 m (length; distal end) T = 20w, lane 4 – gravel in control canal at 0.6 m (length; proximal end) T = 20w, lane 5 – interface gravel/soil layers in control canal at 0.6 m (length; proximal end) T = 20w. St = DNA marker standard from wastewater isolate colony.
Figure 9.40: DGGE analysis (triplicate) of 16S rDNA gene products amplified from total DNA extracted from solid samples. Lane 1 – soil in control canal at 0.6 m (length; proximal end) T = 20w, lane 2 – interface gravel/soil layers in control canal at 1.3 m (length) T = 20w, lane 3 – soil in control canal at 1.3 m (length) T = 20w, lane 4 – gravel in control canal at 1.8 m (length) T = 20w. St = DNA marker standard from wastewater isolate colony.
Figure 9.41: DGGE analysis (triplicate) of 16S rDNA gene products amplified from total DNA extracted from solid samples. Lane 1 – interface gravel/soil layers in control canal at 1.8 m (length) T = 20w, lane 2 – soil in control canal at 1.8 m (length) T = 20w, lane 3 – gravel in control canal at 2.5 m (length; distal end) T = 20w, lane 4 – interface gravel/soil layers in control canal at 2.5 m (length; distal end) T = 20w, lane 5 – soil in control canal at 2.5 m (length; distal end) T = 20w. St = DNA marker standard from wastewater isolate colony.
Figure 9.42: DGGE analysis (triplicate) of 16S rDNA gene products amplified from total DNA extracted from solid samples. Lane 1 – Bauxsol™ in experimental canal at 0.6 m (length; proximal end) T = 24w, lane 2 – interface Bauxsol™/soil layers in experimental canal at 0.6 m (length; proximal end) T = 24w, lane 3 – soil in experimental canal at 0.6 m (length; proximal end) T = 24w, lane 4 – Bauxsol™ in experimental canal at 1.3 m (length) T = 24w, lane 5 – Bauxsol™ in experimental canal at 1.8 m (length) T = 24w. St = DNA marker standard from wastewater isolate colony.
Figure 9.43: DGGE analysis (triplicate) of 16S rDNA gene products amplified from total DNA extracted from solid samples. Lane 1 – soil in experimental canal at 1.8 m (length) T = 24w, lane 2 – Bauxsol™ in experimental canal at 2.5 m (length; distal end) T = 24w, lane 3 – soil in experimental canal at 2.5 m (length; distal end) T = 24w, lane 4 – gravel in control canal at 0.6 m (length; proximal end) T = 24w, lane 5 – interface gravel/soil layers in control canal at 0.6 m (length; proximal end) T = 24w. St = DNA marker standard from wastewater isolate colony.
Figure 9.44: DGGE analysis (triplicate) of 16S rDNA gene products amplified from total DNA extracted from solid samples. Lane 1 – gravel in control canal at 1.3 m (length) T = 24w, lane 2 – interface gravel/soil layers in control canal at 1.3 m (length) T = 24w, lane 3 – soil in control canal at 1.3 m (length) T = 24w, lane 4 – gravel in control canal at 1.8 m (length) T = 24w, lane 5 – interface gravel/soil layers in control canal at 1.8 m (length) T = 24w. St = DNA marker standard from wastewater isolate colony.
Figure 9.45: DGGE analysis (triplicate) of 16S rDNA gene products amplified from total DNA extracted from solid samples. Lane 1 – gravel in control canal at 2.5 m (length; distal end) $T = 24w$, lane 2 – interface gravel/soil layers in control canal at 2.5 m (length; distal end) $T = 24w$, lane 3 – soil in control canal at 2.5 m (length; distal end) $T = 24w$, lane 4 – soil before the experiment. St = DNA marker standard from wastewater isolate colony.
Figure 9.46: Photo of gel electrophoresis of functional PCR amplification products run through a 2% agarose gel. Panel A corresponds to bacterial amoA genes (AOB); panel B of archaeal amoA genes (AOA); panel C of hzo genes; and panel D of nosZ genes. Lane 1 – Inlet 0 month, lane 2 – Inlet 5th month, lane 3 – Inlet 6th month, lane 4 – Soil pre-experiment, lane 5 – Bauxsol™ pellet (experimental canal) at 0.6 m 5th month, lane 6 – Soil particles (experimental canal) at 0.6 m 5th month, lane 7 – Gravel (control canal) at 0.6 m 5th month, lane 8 – Soil particles (control canal) at 0.6 m 5th month, lane 9 – Bauxsol™ pellet (experimental canal) at 2.5 m 6th month, lane 10 – Soil particles (experimental canal) at 2.5 m 6th month, lane 11 – Gravel (control canal) at 2.5 m 6th month, lane 12 – Soil particles (control canal) at 2.5 m 6th month; St1 = standard 1 Kb Plus DNA ladder™ (Life Technologies), Stb = standard HyperladderIV™ (Bioline).
Figure 9.47: X-ray diffraction of Bauxsol™ pellets pre-experiment (black bottom line) and post-experiment (blue middle line). Pink top line represents pre-experiment minus post-experiment: gain of calcite (CaCO₃) and quartz (SiO₂) — positive peaks on pink line; loss of hydrocalumite [Ca₂Al(OH)ₓ[Clₓ-(OH)ₓ]•3(H₂O)] and halite (NaCl) — negative peaks on pink line.
Figure 9.48: Scanning electron microscopy of Bauxsol™ pellet pre-experiment.

Figure 9.49: Scanning electron microscopy of Bauxsol™ pellet post-experiment, rod-shaped bacteria.
Figure 9.50: Scanning electron microscopy of Bauxsol™ pellet post-experiment, diatoms.

Figure 9.51: Scanning electron microscopy of Bauxsol™ pellet post-experiment (interior), rod-shaped bacteria and diatoms.
Figure 9.52: Micro-CT scan of Bauxsol™ pellets post-experiment. Black area = porosity (all the way through), bright white area = high atomic number compounds (i.e. iron oxides), pale gray area = low atomic number compounds (i.e. fumed silica and cement).

9.9. Sugar cane fly-ash composition

X-ray fluorescence analysis of sugar cane fly ashes undertaken for study on “Development of Value-added Products from Sugar Cane Boiler Ash” is presented in Table 9.22 (Clark et al. 2012). These data are important for further key research on Bauxsol™ pellet production.
Table 9.22: X-ray fluorescence analysis on fly ash samples from Harwood, Condong and Broadwater mills using different type of fuel for major elements Fe, SiO₂, Al₂O₃, MnO, TiO₂, CaO, MgO, K₂O, P₂O₅, Na₂O, and for loss on ignition (LOI); all results are in %. (Source Clark et al. 2012).

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9.10. References


CHAPTER 10: REFERENCES


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