THE EFFICACY OF USING THE MICROALGAE *CHLORELLA* SP. FOR THE TREATMENT OF HAZARDOUS LANDFILL LEACHATE

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ABSTRACT

The expansion of urbanisation and industrialisation, particularly in developing nations, has driven a concomitant increase in the generation of solid waste. Currently, the only feasible manner to manage and dispose of solid waste is through landfilling. However, landfilling can form wastewater, termed leachate, via the percolation of water through the landfill. Leachate composition tends to be highly toxic and variable with discharge into the natural environment potentially leading to detrimental ecological impacts. Therefore, the treatment of leachate prior to discharge is an imperative practice. A variety of treatment techniques are available but the use of biological treatment is prevalent due to its reliability and cost-effectiveness. The primary aim of the research undertaken was to determine the efficacy of utilising the microalgae *Chlorella* sp. as the primary and secondary treatment of hazardous landfill leachate. Primary treatment was defined as treating leachate recently derived from landfill, thereby possessing high ammoniacal-nitrogen (NH_3-N) and Chemical Oxygen Demand (COD) concentrations. Secondary treatment was defined as treating pre-treated leachate that possessed a high nitrate (NO_3^-) and lower COD concentration. The amelioration of leachate was temporally monitored via the abatement of NH_3-N, NO_3^-, COD and 5-day Biochemical Oxygen Demand (BOD_5). Chlorophyll-*a* (chl-*a*) was measured to determine *Chlorella* biomass dynamics. Chemical analyses were undertaken for 10%, 25%, 50% and 85% diluted treatment and controls. Toxicity tests were conducted subsequent to secondary treatment batch tests. Both treatments effectively abated their respective nitrogenous compounds to below discharge limits with significant correlations between the nitrogenous compounds and chl-*a*. The efficacy of abating organic compounds demonstrated substantial variability between dilution treatments and treatment types. COD experiment termination concentration (ETC) for the primary treatment did not decline to below discharge limits, with COD levels increasing in the 10% and 50% treatments possibly due to extracellular polysaccharide expulsion by the microalgae. However, the 25% and 85% secondary treatments demonstrated ETCs below discharge limits. Toxicity tests revealed no significant differences between controls and treatments. In conclusion, only secondary treatment by *Chlorella* sp. is effective in treating leachate in terms of nitrogenous and organic compounds and further research should focus on multi-species treatment.

Keywords: Landfill, leachate, *Chlorella*, treatment

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1. INTRODUCTION

Solid waste production is inevitable as a result of current human activity (Vergara, 2012). Apart from the expected increase in Municipal Solid Waste (MSW) production due to urbanization (Renou *et al.*, 2008), the composition is becoming extraordinarily complex owing to plastics and electronics use spreading (Vergara, 2012). More than 1 billion tons of MSW are discarded globally with an expected increase to over 2 billion tons by the year 2025 (Vergara, 2012). Waste characteristics tend to vary between cities, with industrialized cities discarding waste in the form of recyclable products and electronics and cities in developing countries discarding biodegradable waste (Vergara, 2012). Industrialized cities also tend to produce more waste. Composition of waste does not only vary between cities but also within a city over time. Within a relatively short temporal scale, waste properties tends to differ seasonally over the span of a year but over a longer time scale changes in waste depend on cultural trends and technological advancement (Vergara, 2012).

The main imperatives for solid waste management are to protect human health and the natural environment. Incompetent waste management could lead to public health issues such as attracting disease vectors (eg. rodents and mosquitoes) and production of toxic chemicals (Vergara, 2012). Solid waste management is a form of land use and thereby causes habitat destruction. However, management is vital to prevent pollution and therefore toxic chemicals from spreading into more pristine areas. There are several waste management techniques that could be utilized but all methods employed ultimately require a final disposal system (Vergara, 2012). Currently waste complexity is the reason for reusing and recycling complicatedness.

The solid waste can be discarded in an open dump, placed in a landfill or incenerated. The global trend for the management of solid waste is landfilling (Warith, 2003; Renou *et al.*, 2008). This is due to controlled landfilling currently possessing the most economical feasibility as well as being environmentally sensitive (Renou *et al.*, 2008; Umar *et al.*, 2010). This is because the technique allows waste to decompose under controlled conditions to a relatively inert substance (Renou *et al.*, 2008).

Landfill leachate is created by the penetration of water, either as precipitation, runoff or groundwater, into the landfill (Kjeldsen *et al.*, 2002). Products or by-products of physico-chemical and biological processes occurring within the waste dissolve into the water creating a toxic solution (Baderna, 2011). The constituents of the leachate can

vary between sites depending on the waste contained within the landfill, the age of the landfill and the technology employed (Baderna, 2011; Kjeldsen *et al.*, 2002). Leachate composition may vary within a single landfill due to varying stages of waste degradation present (Kjeldsen *et al.*, 2002). There are a range of toxic compounds present within the leachate including inorganic salts, xenobiotics, dissolved organic matter and heavy metals (Baderna, 2011). Hence, if leachate is able to enter ground or surface water, there could be negative consequences for human and ecological health. The toxic compounds of the leachate will adversely affect the health of aquatic organisms; particularly fish (Baderna, 2011). As fish are at the higher trophic levels of aquatic food webs there may be serious intoxication to organisms that consume fish including humans.

In order to protect the natural environment, possess re-usable water and protect human health it is critical to treat landfill leachate. There are several methods employed to treat landfill leachate and the method used is dependent on its composition (Renou *et al.*, 2008; Abbas *et al.*, 2009). Biological treatment is generally widespread as it has proven to be one of the most successful treatments in abating pollutants and is cost-effective (Renou *et al.*, 2008).

Biological treatment that involves the utilisation of micro-organisms to ameliorate wastewater streams has become a necessary practice. Currently, there is much focus on the use of microalgae for the phycoremediation of effluent due to their metabolism (Vilchez *et al.*, 1997). Microalgae are able to assimilate inorganic nitrogen compounds and would be vital in removal of these compounds in wastewater. They would also assist in removing phosphorous and decreasing dissolved organic matter (Tam & Wong, 1996). Advantages of using microalgae over macrophytes in phycoremediation are a drastic decrease of surface area required, economic feasibility, minimal sludge formation, sequestration of greenhouse gases, low energy requirements and the production of potentially useful biomass (Packer, 2009).

Microalgae are regularly used as a tertiary treatment for wastewater (Tam & Wong, 1996). A limiting issue for the use of microalgae for the secondary treatment of effluent is the high concentration of ammonia and urea present (Tam & Wong, 1996). Abeliovich and Azov (1976) have demonstrated that high concentrations of ammonia are toxic to photosynthetic organisms. The microalgae that were researched by Abeliovich and Azov (1976) were *Scenedesmus obliquus, Anacystis nidulans, Chlorella pyrenoidosa* and *Plectonema boryanum*. These species are typical of oxidation ponds

and it was demonstrated that high levels of un-ionized ammonia inhibited photosynthesis. It may inhibit photosynthesis either because of an absence of an electron acceptor or the penetrating ammonia increases the pH of the cell to an inhibitory level (Abeliovich & Azov, 1976). They had proposed that it was probably the latter as the pH maximum limit for undisturbed photosynthesis was 7.9 and that ammonium is known not to be able to penetrate microalgal cells.

Most species of *Chlorella* Beijerinck 1890 are somewhat tolerant to pollution and will rapidly colonise an aquatic body rich in nitrogen, phosphorous and organic matter (Tam & Wong, 1996). *Chlorella* belong to the class Trebouxiophyceae with a morphology of spherical, subspherical or ellipsoid (Bock, 2011). They can be found singly or in colonies with a maximum of 64 individuals with mucilage present or absent (Bock, 2011). The chloroplast is single, parietal with pyrenoid present and surrounded by starch grains (Bock, 2011). Reproduction is by autospores with zoospores not being produced (Bock, 2011). Autospores are released through disruption of mother cell wall (Bock, 2011). The daughter cell can attach to remnants of mother cell and form colonies with mucilage envelopes (Bock, 2011). They can have planktonic, edaphic or endosymbiotic lifestyles (Bock, 2011).

Tam & Wong (1996) have indicated that *Chlorella* growth patterns grown under different nitrogen sources are similar and therefore *Chlorella* able to utilise both nitrate and NH_3-N as a nutrient source. *Chlorella* growth is possible in high concentration NH_3-N but it was observed that maximal cell density was much lower than low NH_3-N concentration Tam & Wong (1996). A more recent study by Termini *et al.* (2011) demonstrated that there was continuous removal of NH_3-N in both indoor and outdoor photo-bioreactors. There was an ammonium removal of 90% with the outdoor configuration and 99.9% in the indoor one (Termini *et al.*, 2011). An advantage of using microalgae for the treatment of wastewater is the coupled biomass production for energy (Sturm & Lamer, 2011). Contrasting with other biofuel stocks, microalgae do not threaten food security, can grow non-arable land and do not necessitate vast quantities of water (Sturm & Lamer, 2011). For this technology to be applied on a large scale a total energy surplus must be obtained (Sturm & Lamer, 2011). Biofuel production has proven to be energetically favourable for open pond reactors exploiting wastewater as a source of nutrients (Sturm & Lamer, 2011). If lipid yields of microalgae are low, direct combustion of the dry algal biomass can be used as a source of energy.

Reports on the utilisation of algae for the treatment of municipal wastewater are available (Craggs *et al.*, 1997; Olguin, 2003; Zimmo *et al.*, 2004; Rawat *et al.*, 2011). However, knowledge and development on the use of microalgae for the treatment of landfill leachate is largely lacking. Therefore, the comprehensive aim of this study was to determine the efficacy of using the microalgae *Chlorella* sp. in the phycoremediation of hazardous landfill leachate.

This was achieved through three objectives:

a) Determining the efficacy of utilising *Chlorella* sp. in the primary treatment of hazardous landfill leachate.
b) Determining the efficacy of utilising *Chlorella* sp. in the secondary treatment of hazardous landfill leachate. In this scenario the leachate underwent primary treatment in an SBR and therefore possessed no NH_3-N but possessed a relatively large concentration of NO_3^-.
c) Using these two experiments to conclude the feasibility of using *Chlorella* sp. for the treatment of landfill leachate.

2. LITERATURE REVIEW

2.1 Solid Waste and Landfilling

Solid waste is defined as material that is discarded from residential, commercial and industrial sources that ceased to have value to the possessor (Williams, 2005; McDougall, *et al.*, 2001). However, the definition of waste may change in the way that it is treated. It holds no value for refuse workers hauling it to be disposed of but it holds great value for waste pickers (Assaad, 1996). Currently the global solid waste generation is approximately 1 billion tonnes per annum (Themelis and Zhang, 2010; Vergara, 2012). This is probably expected to increase due to the continuance of industrial and urban growth globally (Renou *et al.*, 2008). Most urbanisation growth is taking place in small and medium sized cities in low-income countries (Cohen, 2004). According to Myers and Kent (2003) these nations host approximately a billion new consumers expending on cars, electricity, meat and other consumable goods. There are two major consequences from this amplified consumption viz. the increase of utilising natural resources and more waste being produced (Vergara, 2012)

Poor waste management can have detrimental effects on the environment at varying scales (Vergara, 2012). The open dumping of wastes can pollute nearby aquatic ecosystems and can adversely affect human health by attracting disease vectors and exposing people to any deleterious waste products (McDougall *et al.*, 2001). Solid waste also affects the air by emitting poisonous gases as well as greenhouse gases (GHG's) (Vergara, 2012). Waste management only contributes a small degree of GHG emissions nevertheless it is capable of acting as either a source or a sink (Bogner *et al.*, 2007). Waste tends to affect poor people more than the middle and higher income group as they are likely to reside closer to waste and they are more probable to be waste-workers (Vergara, 2012).

The management of solid waste requires land-use change and therefore potentially destroys natural habitat but the emission of toxic chemical by-products will have a heightened effect on fauna and flora (Vergara, 2012). This is especially so if they dumped openly or burnt. This can be avoided by implementing environmentally sensitive methods (Read *et al.*, 1997). These techniques involve minimization, recycling, composting and waste to energy (Read *et al.*, 1997). The global trend for the management of industrial and urban solid waste is landfilling (Warith, 2003; Renou *et al.*, 2008). This is due to controlled landfilling currently possessing the most economical feasibility as well as being environmentally sensitive (Renou *et al.*, 2008;

Umar *et al.*, 2010). This is because the technique allows waste to decompose under controlled conditions to a relatively inert substance (Renou *et al.*, 2008).

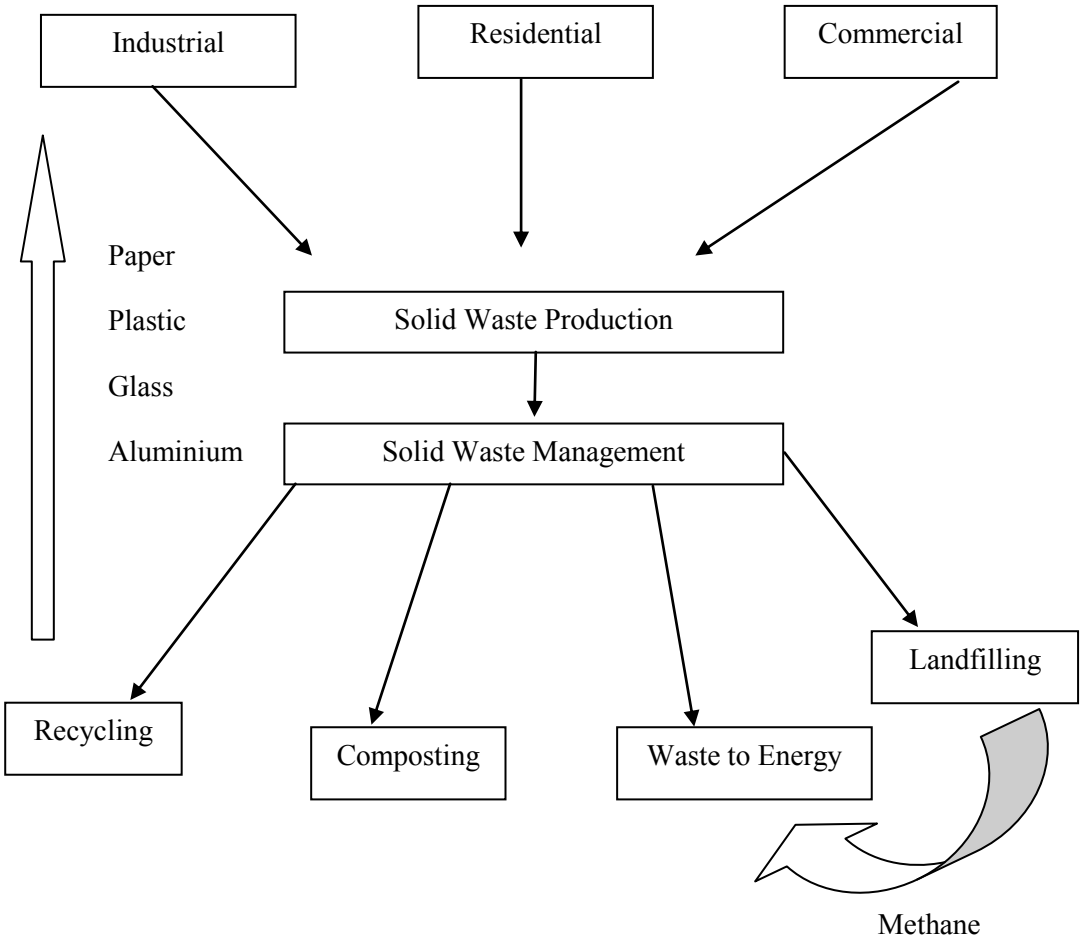

Figure 2.1 Solid Waste Pathway (adapted from Centre for Ecological Sciences, 2015)

2.2 Landfill as a Bioreactor

"The traditional MSW landfill has undergone a transformation from a basic contained dump site, to highly engineered facilities with sophisticated containment systems, environmental monitoring, improved operational practices, and increased regulation" (Reinhart *et al.*, 2002).

Landfills are the foremost disposal technique for municipal solid waste and are the most commonly employed waste management system on a global scale (Warith, 2003). According to Warith (2003) landfills have functioned as the final waste recipients for "municipal refuse, industrial or agricultural residues, wastewater sludge, incinerator ash, recycle discards, and/or treated hazardous wastes". In a conventional landfill the waste is spread out, compacted into a cell and covered with a thin layer of soil (Warith, 2003). Once the maximum height is achieved the waste is enclosed with a layer of clay (Warith, 2003). The problem with running a conventional landfill is that the waste takes several decades to fully decompose and liner failure is possible in the future causing groundwater contamination (Rosenberg, 2000; Warith, 2003).

The possible release of pollutants has transformed the methods of waste management by operating landfills as bioreactors. An engineered bioreactor landfill exploits microbiological processes to stabilize the waste in a landfill within 5 to 10 years (Warith, 2003). This stabilization ensures that potential pollutant parameters are not subjected to dramatic increases due to partial confinement failures (Warith, 2003). The major difference between a traditional MSW landfill and a bioreactor landfill is the addition of moisture either in the form of water (Reinhart *et al.*, 2002) or the recirculation of leachate (Warith, 2003). This addition of moisture stimulates the biodegradation of waste and enhances the rate of waste breakdown when compared to a traditional landfill. This method allows for a rapid stabilisation period i.e. from decades to 2-3 years (Reinhart *et al.*, 2002) thus reducing chances as a potential source of pollution. Managing a landfill in this manner also allows for the optimisation of landfill gas capture, increased landfill capacity as well as opportunities for alternate leachate treatment (Reinhart *et al.*, 2002).

2.2.1 Aerobic Bioreactor

Aerobic respiration has a higher energy yield than anaerobic respiration therefore aerobes have a higher growth rate than anaerobes (Warith, 2003). Aerobe activity thus rapidly accelerates the degradation of waste and full degradation can be achieved at around 2 years. Environmental conditions in the landfill are optimized for the growth

of aerobes in order that they breakdown waste and this achieved by injecting air into the landfill (Warith, 2003).

2.2.2 Anaerobic Bioreactor

The anaerobic landfill reactor utilizes anaerobic micro-organisms to degrade the waste (Warith, 2003). In order for optimum anaerobic activity to be achieved moisture content in the waste mass must be 35-40% (Warith, 2003). However the typical moisture content of a landfill is 10-20% but this can be compensated by the addition of moisture into the landfill. Anaerobic activity consequently produces methane and carbon dioxide that can be collected (Warith, 2003).

2.2.3 Aerobic-Anaerobic Bioreactor

The landfill is engineered and operated such that it possesses attributes of both the aerobic and anaerobic bioreactors. The uppermost portion of waste is aerated while the bottom portion receives the liquid, both being transported by horizontal wells (Warith, 2003). The intention of this design is to cause rapid breakdown in readily degradable waste in the aerobic stage and to reduce organic acids in the anaerobic stage (Warith, 2003).

2.2.4 Facultative Bioreactor

The facultative bioreactor utilizes anaerobic micro-organisms and it also possesses a mechanism that treats the high level of ammonia produced when liquids are added to the landfill (Warith, 2003). Ammonia (NH_3) is converted to Nitrate (NO_3^-) by the process of nitrification and the NO_3^- converted to Nitrogen (N_2) in the absence of oxygen. Liquid has to be added in order for the landfill to contain an elevated moisture content and consequently function at an optimum level (Warith, 2003). The process described above is a brief summary of the Nitrogen cycle which is discussed in further detail below.

2.3 Nitrogen Cycle

The Nitrogen Cycle is defined as the environmental flow of Nitrogen and its inter-conversion with its compounds (Figure 2.2). Nitrogen gas (N_2) is the most stable form and is a reservoir from which N-compounds are produced (Galloway, 2003). Nitrogen fixation is a process wherein N_2 is reduced to Ammonia (NH_3) or Ammonium (NH_4^+) and is performed by biological activity (Galloway, 2003). Ammonia and NH_4^+ are two species that can be grouped as inorganic reduced nitrogen and collectively termed ammoniacal nitrogen (NH_3-N). Ammonia is the main species emitted into the

atmosphere and is caused by the decomposition of organic matter by heterotrophic micro-organisms (Galloway, 2003). This process is termed Ammonification and is the conversion of reduced organic nitrogen to reduced inorganic nitrogen (Galloway, 2003, Figure 2.2). The two inorganic reduced nitrogen species can be consumed by biological organisms and incorporated into their biomass. This process is termed Ammonia assimilation (Galloway, 2003, Figure 2.2).

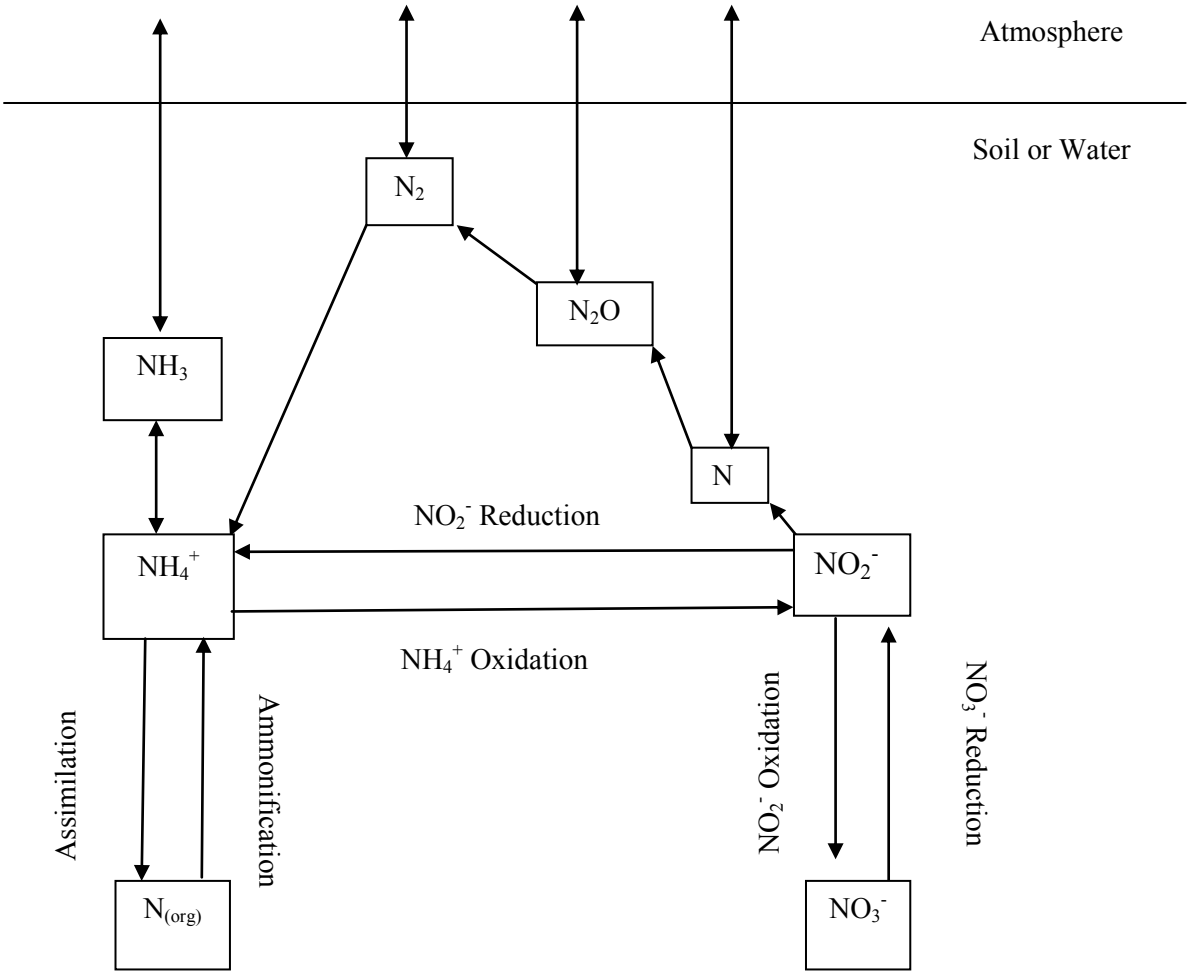

Figure 2.2 A summary of the Nitrogen Cycle (adapted from Galloway, 2003)

Specialised micro-organisms (chemoautotrophs) are able to obtain energy from $NH4^+$ oxidation (Galloway, 2003). This process called Nitrification causes NH_4^+ to be converted to NO_3^- by a series of chemical reactions. There are two groups of micro-organisms that are involved in this aerobic process (Galloway, 2003). The first group oxidizes NH_4^+ to NO_2^- (e.g. *Nitrosomonas*), thereafter another group oxidizes NO_2^- to NO_3^- (e.g. *Nitrobacter*) (Galloway, 2003). The $NO3^-$ end-product of nitrification is an important source of nitrogen for many organisms. This uptake of $NO3^-$ and incorporation into biomass to form organic nitrogen compounds is termed Assimilatory nitrate reduction (Galloway, 2003).

Under anaerobic conditions where there is organic matter and NO_3^- available, the process of Denitrification can occur. The denitrification process is defined as the reduction of nitrates (NO_3^-) to nitrogen gas (N_2), via the intermediates nitrite (NO_2^-), nitric oxide (NO) and nitrous oxide (N_2O) (Trois *et al.*, 2010).Micro-organisms use the NO_3^- as an oxidant to obtain energy from the organic matter (Galloway, 2003). Nitrogen is used as an electron acceptor in the place of oxygen. Dissimalatory nitrate reduction is a respiratory process whereby nitrates (instead of oxygen as in aerobic respiration) serve as the terminal electron acceptor.

2.4 Landfill Design

Incorporated into the design of the landfill is an impermeable barrier to prevent contamination of groundwater (Reinhart *et al.*, 2002). These liners prevent waste from entering the surrounding environment but prevent degradation of the waste (Reinhart and Al-Yousfi, 1996; Reinhart *et al.*, 2002). Liners used are a clay layer, a geo-membrane or both (Katsumi *et al.*, 2001). The liners have a limited lifespan and once worn away the waste is then exposed to the environment. This waste that is not yet degraded and was dormant is now as hazardous to the environment as it was when initially landfilled (Reinhart *et al.*, 2002).

The two principal channels for the escape of leachate are leakage through holes and molecular diffusion through the membrane (Katsumi *et al.*, 2001). Holes in the geo-membrane layer are caused by faults in the seams, punctures by sharp objects, tension caused by the mass of the waste and material failure (Katsumi *et al.*, 2001). Clay liners have low hydraulic conductivity and are generally unsaturated as long as they are placed above the water table (Katsumi *et al.*, 2001). A composite liner i.e. liner that incorporates a geo-membrane and a clay layer, typically possesses leachate leakage appreciably less than a single layer. This is because the geo-membrane reduces the area

through which leakage can occur and the clay liner below minimises leakage due to geo-membrane defects (Katsumi *et al.*, 2001).

Once the landfill has reached its maximum disposal capacity it is imperative that it is capped in order to enclose the waste. The crucial purpose of the cap is to prevent or restrain precipitation from entering the landfill thereby controlling leachate generation (Simon and Müller, 2004). The cap also prevents the emission of landfill gas into the atmosphere and wind transport of waste and odour (Simon and Müller, 2004). There are several capping systems available but the standard capping method is a plastic geo-membrane in contact with a clay layer (Simon and Müller, 2004). The geo-membrane comprises of both, a hydrophobic and hydrophilic material, and any faults in it are sealed with the clay layer (Simon and Müller, 2004).

2.5 Formation of Leachate

One of the key issues associated with landfilling of waste is the release of wastewater referred to as leachate. According to Renou *et al.* (2008) leachate is defined as "the aqueous effluent generated as a consequence of rainwater percolation through wastes, biochemical processes in waste's cells and the inherent water content of wastes themselves." Leachate contains heavy metals, xenobiotics, organic and inorganic compounds and can cause major environmental predicaments such as ground- and surface water contamination (Kjeldsen *et al.*, 2002).

Comparisons of different landfills have indicated a wide variation in leachate composition (Kulikowska and Klimiuk, 2008). A contributing factor to this occurrence is the age of the landfill (Kurniawan *et al.*, 2006; Renou *et al.*, 2008), because the age of the landfill influences the degradation state of the waste (Baderna *et al.*, 2011). The older a landfill is the greater the stability of the waste (Renou *et al.*, 2008). Therefore the leachate composition and characteristics can vary within an individual landfill itself, depending on the differences in the waste age (Baderna *et al.*, 2011; Table 2.1). Climate is a dynamic phenomenon that also considerably effects leachate production, as it affects volume through precipitation and losses through evaporation (Renou *et al.*, 2008).

Table 2.1 Landfill leachate composition (Qasim and Chiang, 1994)

Constituent	Unit	New Landfill (younger than 2 years)	Old Landfill (older than 2 years)
5-day Biochemical Oxygen Demand	mg/L	2 – 30 000	100 – 200
Total Organic Carbon	mg/L	1500 – 20 000	80 – 160
Chemical Oxygen Demand	mg/L	3000 – 60 000	100 – 500
Total Suspended Solids	mg/L	200 – 2000	100 – 400
Organic Nitrogen	mg/L	10 – 800	80 – 120
Ammonia	mg/L	10 – 800	20 – 40
Nitrate	mg/L	5 – 40	5 – 10
Total Phosphorus	mg/L	5 – 100	5 – 10
Ortho-Phosphates	mg/L	4 – 80	4 – 8
Alkalinity	mg/L	1000 – 10 000	2 – 1000
pH	pH units	4.5 – 7.5	6.6 – 7.5
Total hardness as $CaCO_3$	mg/L	300 – 10 000	200 – 500
Calcium	mg/L	200 – 3000	100 – 400
Magnesium	mg/L	50 – 1500	50 – 200
Potassium	mg/L	200 – 1000	50 – 400
Sodium	mg/L	200 – 2500	100 – 200
Chloride	mg/L	200 – 3000	100 – 400
Sulfate	mg/L	50 – 1000	20 – 50
Total Iron	mg/L	50 – 1200	20 – 200

2.6 Leachate Toxicity and Impact on Natural Ecosystems

2.6.1 Background

Landfill leachate is a water-based solution that contains a diversity of pollutants including heavy metals, inorganic ions and xenobiotics (Table 2.1). Therefore, it may be harmful to living organisms including humans (Baderna *et al.*, 2011). Leachate can induce cognitive and behavioural abnormalities, neurotoxicity and DNA damage. Exposure to leachate causes oxidative damage to particular organs via lipid peroxidation and changes in antioxidant status (Baderna *et al.*, 2011). Studies on the effect of mice have indicated that leachate causes increased frequency in micronuclei, chromosomal aberrations and alteration in sperm morphology (Baderna *et al.*, 2011).

Talorete *et al.* (2008) tested the *in vitro* effects of raw leachate on MCF-7, a human breast cancer cell. The leachate instigates oxidative stress on the cells causing DNA damage. The normal sequence of cell cycles, including mitosis, is blocked and if the cells are unable to undergo DNA repair, cell death eventually occurs.

Baderna *et al.* (2011) conducted a study wherein industrial waste landfill leachate characteristics were monitored over a period of 11 years and *in vitro* assays conducted to test the hepatotoxicity of leachate. It was observed that raw leachate significantly inhibited cell multiplying at small doses (greater than 2.5% v/v). The study also determined that it was the hydrophilic compounds in the leachate were responsible for the inhibition and not the organic components. Cell viability was also monitored and it was observed that within 24 hours no significant inhibition occurs, however after 72 hours cell viability had declined with concentrations greater than 5% v/v.

An important compound detected at high concentrations is Bisphenol A (BPA). BPA is classified as a high production volume compound that is used for the production of polycarbonate plastics and epoxy resins (Baderna, 2011). It is known to cause adverse health effects in animals and humans particularly during early development periods. Furthermore, exposure to levels lower than those required for acute toxicity have proven to result in endocrine-related affects in an array of aquatic ectotherms (Baderna *et al.*, 2011).

Although the physico-chemical properties and pollutants present in leachate have the potential to be toxic, there are specific physico-chemical properties and compounds that are of primary concern as they:

- Are present in relatively large concentrations;
- Negatively affect the physiological functioning of living organisms; and
- Negatively influence physico-chemical characteristics of natural ecosystems, thereby negatively impacting ecosystem functioning.

These leachate constituents are discussed in further detail below.

2.6.2 Nitrogenous Compounds

NH_3-N is an important reducing agent in landfill leachate and partakes in complex redox reactions (Baderna *et al.*, 2011). The two aqueous forms of NH_3-N are the ammonium ion (NH_4^+) and ammonia (NH_3) and their relative abundance depends on temperature and pH (Figure 2.3).

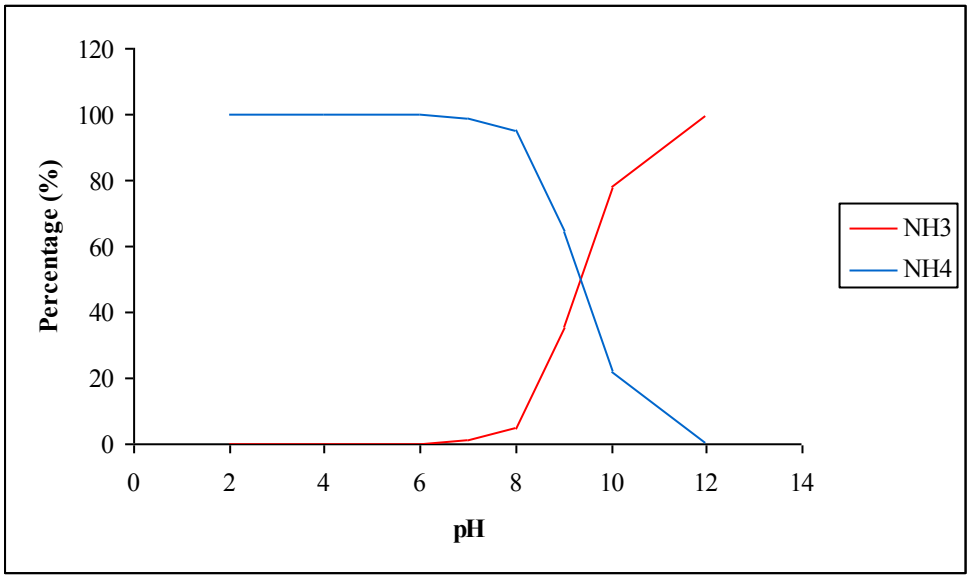

Figure 2.3 **The effect of pH on ammoniacal-nitrogen relative concentration (Huckstedt, 1973)**

When equilibrium is shifted to the right at low temperatures and pH < 7, NH_4^+ dominates and conversely when equilibrium is shifted to the left at high temperatures and pH > 7 NH_3 dominates.

Due to its long-term persistence NH_3-N plays a significant role in human and ecological toxicity and is regarded as the main cause of acute toxicity from landfill leachate exposure (Baderna *et al.*, 2011). Depending on other constituents and physico-chemical properties of the leachate, NH_3-N toxicity could be altered causing increased or

decreased toxicity of the leachate as a whole (Byrne *et al.*, 2008). These parameters include total dissolved solids, heavy metals, dissolved oxygen, and carbon dioxide (DWS, 1996). Unionized ammonia (NH_3) is more toxic to organisms and inhibits cell metabolism and decreases O_2 permeability through cell membranes (DWS, 1996). The acute toxic effects of NH_3 to fish include "loss of equilibrium, hyper-excitability, an increased breathing rate, an increased cardiac output and oxygen intake, and in extreme cases convulsions, coma and death" (DWS, 1996). "Chronic effects include a reduction in hatching success, reduction in growth rate and morphological development, and pathological changes in tissue of gills, liver and kidneys" (DWS, 1996).

NH_3-N is eventually converted to nitrate under aerobic conditions. Nitrate is highly soluble, chemically stable and persists in polluted waters (Pisano, 2007). The symptoms of excessive nitrate intake include abdominal pains, diarrhea, vomiting, hypertension, increased infant mortality, central nervous system birth defects, diabetes, spontaneous abortions, respiratory tract infections, and changes to the immune system (Lohumi *et al.*, 2004). The toxic activity of nitrate itself is termed primary toxicity.

Secondary toxicity is the noxious action of nitrite that was formed from the reduction of nitrate by intestinal bacteria. Methemoglobin (MetHb) is formed when nitrite oxidizes the ferrous iron in haemoglobin (Hb) to the ferric form. MetHb cannot bind to oxygen and ultimately leads to a condition called methemoglobinemia (Kross *et al.*, 1992). Methemoglobinemia is characterised by cerebral anoxia, cyanosis and stupor (Samatya *et al.*., 2006). Symptoms include an unusual greyish skin color and irritability (Samatya *et al.*., 2006). Furthermore, excessive crying in children with moderate MetHb levels and drowsiness and lethargy at higher levels has been recorded (Samatya *et al.*., 2006).

Reactions between nitrite and secondary or tertiary amines in an acidic medium may lead to the formation of N-nitroso compounds, several of which are carcinogenic, mutagenic and teratogenic (Pisano, 2007). This is termed tertiary toxicity.

In addition, excessive nitrate loads may potentially alter ecosystem characteristics and functioning. The excessive presence of nitrates in surface waters drive eutrophication, wherein primary producers occur in nuisance abundances (DWS, 1996). These blooms may consist of species that are toxic to man and other biota (DWS, 1996).

2.6.3 Organic Carbon Compounds

The oxidisable organic matter present within leachate can possibly negatively impact any receiving aquatic ecosystem (DWS, 1996). When the organic matter is discharged

into the receiving water body, it serves as a source of nutrients for microbes and results in the rapid increase in microbial metabolism. Consequently, there is a rapid decline in the concentration of Dissolved Oxygen (DO) (DWS, 1996). Therefore, untreated or improperly treated leachate discharged into the natural environment will have negative consequences for biota as DO is required for respiration.

2.7 Treatment of Landfill Leachate

The Department of Water and Sanitation (DWS), previously known as the Department of Water Affairs (DWA), is regarded as the custodian of South Africa's water resources and their policy dictates that aquatic ecosystems remain ecologically healthy and are utilised in a sustainable manner. To ensure the ecological health of aquatic ecosystems is maintained, they need to possess an array of specific ecological properties including optimum water quality. The term *water quality* as defined by DWS (1996) is the "physical, chemical, biological and aesthetic properties of water that determine its fitness for a variety of uses and for the protection of the health and integrity of aquatic ecosystems."

According to the DWS policy "pollutants which pose the greatest threat to the environment, because of their toxicity, extent of bio-accumulation and persistence, a precautionary approach aimed at minimizing or preventing inputs to the water environment should be adopted." Consequently, effluent including landfill leachate must be pre-treated before it is discharged into water courses.

One of the management objectives of DWS is ensuring that no adverse effects are brought about by the introduction of pollutants into the aquatic system and is achieved by the Target Water Quality Range (TWQR). It is derived from a range of quantitative and qualitative criteria. It is set group of concentrations and levels that will not impair the health of ecosystems and will form a guideline to what degree water quality may be altered (DWS, 1996). The Government Gazette No. 20526 8 October 1999 indicates the established limits for several parameters pertaining to the discharge of effluent into a water resource through a conduit. The concentration of constituents permitted for discharge into natural water systems are indicated in Table 2.2 below.

Table 2.2 Established General Limit and Special Values for the discharge of wastewater (DWS, 1999)

Parameter	Unit	General Limit	Special Limit
Faecal Coliforms	Per 100 mL	1000	0
Chemical Oxygen Demand	mg/L	75	30
Ammoniacal nitrogen	mg/L	3	2
Nitrate/Nitrite	mg/L	15	1.5
Free Chlorine	mg/L	0.25	0
Suspended Solids	mg/L	25	10
Conductivity	mS/m	70 – 150 above intake	50 – 100 above intake
Ortho-Phosphate	mg/L	10	1 – 2.5
Fluoride	mg/L	1	1
Soap, oil or grease	mg/L	2.5	0
Dissolved Arsenic	mg/L	0.02	0.01
Dissolved Cadmium	mg/L	0.005	0.001
Dissolved Chromium	mg/L	0.05	0.02
Dissolved Copper	mg/L	0.01	0.002
Dissolved Cyanide	mg/L	0.02	0.01
Dissolved Iron	mg/L	0.3	0.3
Dissolved Lead	mg/L	0.01	0.006
Dissolved Manganese	mg/L	0.1	0.1
Dissolved Selenium	mg/L	0.02	0.02
Dissolved Zinc	mg/L	0.1	0.04
Mercury and Mercury Compounds	mg/L	0.005	0.001
Boron	mg/L	1	0.5

The complexity of leachate makes it problematical to make general recommendations for its treatment (Renou *et al.*, 2008; Abbas *et al.*, 2009). Due to this wide variation in leachate characteristics treatment methods employed must be flexible (Abbas *et al.*, 2009). There are several methods employed to treat landfill leachate and the method used is dependent on the composition of the leachate (Renou *et al.*, 2008; Abbas *et al.*, 2009).

2.7.1 Leachate Transfer

2.7.1.1 Combined Treatment

A common practice was to combine landfill leachate with municipal sewage and treat the combined wastewater in the municipal sewage treatment plant (Renou *et al.*, 2008). The leachate is pumped out from the bottom of the landfill and stored in basins where it is later transported to the treatment plant (Warith, 2003). This method has received wide criticism due the presence of heavy metals and organic inhibitory compounds that may reduce treatment efficiency thus increasing pollutant concentration in the effluent (Cecen and Aktas, 2004). In a study by Diamodopoulos (1997) it was demonstrated that the ratio of 9:1 for sewage and leachate respectively yielded nearly 95% Biochemical Oxygen Demand (BOD) and 50% Nitrogen removals at the end of the daily cycle.

2.7.1.2 Recycling

Recycling the leachate back through the landfill has been widely used as it is currently the most economically feasible (Renou *et al.*, 2008). Recirculating the leachate increases the moisture content of the landfill and supplies assorted nutrients thus promoting microbial activity (Warith, 2003). Chugh *et al.* (1998) demonstrated that there was significant lowering of COD and methane production using this method. This was observed when the recycled volume was 30% of the initial waste volume. An immense advantage is that the stabilisation process time is reduced from decades to 2-3 years (Reinhart and Al-Yousfi, 1996). This method also assists in the removal of sulphides and hydroxides thus decreasing the concentration on heavy metals in the leachate (Reinhart and Al-Yousfi, 1996). The passage of the recycled leachate back through the solid waste augments contact between micro-organisms and the leachate thus optimising microbial treatment of the leachate (Reinhart and Al-Yousfi, 1996).

There are several methods that are employed in order to facilitate the recirculation of leachate. The leachate can be applied directly to the waste as it is being landfilled. The disadvantages of this technique are odour problems, health risk due to contact with the

leachate and off-site migration (Warith, 2003). The leachate can be spray irrigated onto the surface of the landfill to ensure that the leachate comes into contact with more of the solid waste than with direct application (Warith, 2003). Another advantage is that some of the leachate volume is lost due evaporation (Warith, 2003). Surface application of leachate involves either ponding or spreading the leachate (Warith, 2003). A larger amount of land is required for this and ponds have to be monitored for any leaks (Warith, 2003). In order to avoid these problems, subsurface methods can be applied. This is achieved by constructing vertical recharge wells and horizontal drains within the solid waste (Warith, 2003). This method reduces the risk of atmospheric exposure.

2.7.2 Physical/Chemical Treatment

"When treating stabilised (fewer biodegradables) leachate, physico-chemical treatments have been found to be suitable as a refining step for biologically treated leachate, in order to remove organic refractory substances" (Renou *et al.*, 2008).

2.7.2.1 Coagulation and Flocculation

Coagulation and flocculation can be used to treat aged and therefore stabilised landfill leachate (Silva *et al.*, 2004). These coagulants destabilize any colloidal particles within a solution thus facilitating the settling of the particles out of solution. Flocculation follows the coagulation step and serves to increase the particle size, thus further assisting the settling of particles (Renou *et al.*., 2008). Aluminium sulfate, ferrous sulfate, ferric chloride and ferric chloro-sulfate are commonly used as coagulants (Amokrane *et al.*, 2009). This method is commonly employed as either a pre-treatment and/ or final polishing step in order to remove non-biodegradable matter (Amokrane *et al.*, 2009). Addition of flocculants together with coagulants may enhance the floc-settling rate (Amokrane *et al.*, 2009). There are disadvantages to this method these include consistent production of sludge volume and an increase in the concentration of aluminium or iron in the liquid phase (Silva *et al.*, 2004).

2.7.2.2 Chemical Precipitation

Landfill leachate typically has a high concentration of ammonia present as NH_4^+ (Renou *et al.*, 2008). Li *et al.* (1999) indicated that increasing NH_4^+ significantly affected a conventional activated sludge process. The Chemical Oxygen Demand (COD) removal declined from 95 to 79%, when the concentration of NH_4^+ increased from 50 to 800 mg/L (Li *et al.*, 1999). Li *et al.* (1999) precipitated ammonium ions as Magnesium Ammonium Phosphate (MAP) with the addition of $MgCl_2.6H_2O$ and $Na_2HPO_4 \cdot 12H_2O$ with an $Mg/NH_4^+/PO4^{3-}$ ratio of 1/1/1 at a pH of 8.5–9. Chemical

treatment is used in leachate treatment to remove high concentrations of NH_4^+ (Renou *et al.*, 2008).

2.7.2.3 Adsorption

The use of activated carbon columns for the adsorption of pollutants from leachate is more efficient in removing COD from landfill leachate than the chemical precipitation method (Morawa *et al.*, 1995; Fettig *et al.*, 1996). The combined use of activated carbon with biological treatment effectively treats landfill leachate (Morawa *et al.*, 1995). Employing this technique ensures that the leachate is suitable for biological treatment by lowering non-biodegradable organics, inert COD and colour (Renou *et al.*, 2008).

2.7.2.4 Air Stripping

This is currently the most common method utilised to remove excess levels of ammonia (Renou *et al.*, 2008). For efficient processing there must be a high pH (Gotvajn *et al.*, 2009) and the contaminated gas phase must be treated with either H_2SO_4 or HCl (Renou *et al.*, 2008). Marttinen *et al.* (2002) reported an 89% ammonia decline at pH 11 and 20°C within a 24 hour retention time. Ammonia stripping is a first-order reaction therefore the mass transfer rate from liquid to gas depends on the initial concentration of ammonia (Marttinen *et al.*, 2002). A foremost concern with this process is the release of ammonia into the atmosphere if the ammonia cannot be absorbed by H_2SO_4 or HCl (Renou *et al.*, 2008). According to Ozturk *et al.* (2003) air stripping is the most economically viable alternative for high ammonium removal.

2.7.3 Membrane Processes

The main membrane processes used in landfill leachate treatment are reverse osmosis (RO), ultrafiltration (UF), nanofiltration (NF) and microfiltration (MF) (Renau *et al.*, 2008). The key issue with using pressure-driven membrane processes for leachate treatment is membrane fouling (Renou *et al.*, 2008). This requires large-scale pre-treatment and intensive cleaning of the membranes, resulting in a shortened life span of the membrane itself and thus impedes treatment efficiency (Renou *et al.*, 2008). Another drawback is the production of a large volume of concentrate that has to be further treated and discharged.

2.7.4 Biological Treatment

According to Vilchez *et al.* (1997) "bioremoval is defined as the accumulation and concentration of pollutants from aqueous solutions by the use of biological materials,

thus allowing the recovery and/or environmentally acceptable disposal of the pollutants". Biological treatment is prevalent due to the technique being simple, reliable and cost-effective (Renou *et al.*, 2008). Micro-organisms degrade organic compounds into sludge and CO_2 or CO_2 and CH_4, under aerobic or anaerobic conditions, respectively (Lema *et al.*, 1988). Biological treatment is especially effective in eradicating organic and nitrogenous matter from young leachate where the COD/BOD ratio is high (Renou *et al.*, 2008). Biological treatment has been found to be ideal for the treatment of young leachate with regards to the elimination or diminution of NH_3-N, COD and heavy metals (Renou *et al.*, 2008).

2.7.4.1 Aerobic Treatment

Aerobic Treatment allows for the partial abatement of organic pollutants and for the ammonium nitrogen nitrification (Renou *et al.*, 2008). Aerated lagoons have been identified as an effective and economically feasible treatment for the removal of pathogens and pollutants from wastewater (Renou *et al.*, 2008). They are generally used in wastewater treatment particularly in developing countries (Renou *et al.*, 2008). Maehlum (1995) indicated that over 70% of N, P, and Fe was removed from leachate using anaerobic-aerobic lagoons and constructed wetlands. However lagooning may not be an effective method for the treatment of leachate. This is due to the dependence on the temperature on microbial activity and with strict requirements for leachate discarding it may not be effectual (Zaloum and Abbott, 1997).

Activated sludge processes are used for the treatment for municipal wastewater and could be used for the co-treatment of wastewater and landfill leachate. However this method has proven to be ineffective due to various reasons (Renou *et al.*, 2008). A longer aeration time is required for treatment and there is meagre sludge settling (Loukidou and Zouboulis, 2001). The method requires substantial energy and there is unwarranted sludge formation (Hoilijoki, 2000).

The sequencing batch reactor (SBR) system is ideal for the nitrification-denitrification process. It allows for concurrent nitrification and organic carbon oxidation (Diamadopoulos *et al.*, 1997). Process techniques demonstrated by Diamadopoulos *et al.* (1997) has resulted in the wide application of a SBR system for the treatment of leachate. This system has proved to remove up to 75% COD and Lo (1996) has reported a removal of 99% NH_4^+ during the aerobic treatment of domestic leachates in a SBR with a 20–40 days residence time. An advantage of the SBR system is that it has

an outsized process flexibility which is critical for the treatment of landfill leachate, which itself is highly variable (Renou *et al.*, 2008).

Jokela *et al.* (2002) investigated the efficiency of using trickle filters for the treatment of landfill leachate. The advantage of using this type of biofilter is the relatively low cost of filter media (Jokela *et al.*, 2002). Jokela *et al.* (2002) had found that there was over 90% nitrification of the leachate under laboratory conditions and *in situ* pilot aerobic crushed brick filters. "MBBR (Moving-bed biofilm) reactor process is based on the use of suspended porous polymeric carriers, kept in continuous movement in the aeration tank, while the active biomass grows as a biofilm on the surfaces of them" (Renou *et al.*, 2008). This method is also termed as a fluidised bed reactor. This method allows for superior microbial biomass growth and there is less sensitivity to toxic chemicals (Loukidou and Zouboulis, 2001). MBBRs also eliminate the need for long sludge-settling periods. Loukidou and Zouboulis (2001) had achieved a maximum of 90% NH_4^+ reduction and 81% COD reduction.

2.7.4.2 Anaerobic Treatment

The anaerobic digestion process allows for the treatment of high strength organic effluents. As opposed to aerobic treatment anaerobic digestion conserves energy and produces very few solids but does so at low reaction rates (Renou *et al.*, 2008). SBRs operated under anaerobic conditions are able to accomplish solid capture and organic lowering in one vessel and thus eliminates the need for a clarifier (Renou *et al.*, 2008). By running the leachate through an aerobic reactor methanogenesis and denitrification occurs, thus enhancing nitrification in a proceeding aerobic reactor (Renou *et al.*, 2008). An aerobic-anaerobic system is recommended to bring down organic and nitrogen matter simultaneously (Renou *et al.*, 2008).

The Up-flow Anaerobic Sludge Blanket (UASB) reactor has prominent treatment efficiency and a short hydraulic retention time (Lin *et al.*, 2000). When a volume of high organic loading rate is introduced into the system, it exhibits superior performances when compared to other anaerobic treatments (Garcia *et al.*, 1996).

The anaerobic filter is a high rate system that maintains biomass as a biofilm on a support (Nedweld and Reynolds, 1996). Henry *et al.* (1987) demonstrated that anaerobic filter could reduce the COD by 90%, at loading rates varying from 1.26 to 1.45 kg COD/m^3/day, and is applicable for different landfill ages. Total biogas production ranged between 400 and 500 L gas per kg COD destroyed with a methane

content between 75 and 85%. The hybrid bed filter involves an up-flow sludge blanket and an anaerobic filter above it (Renou *et al.*, 2008). This type of filter is a gas-solid separator (Renou *et al.*, 2008). It enhances the retention of solids without causing channelling or short-circuiting (Renou *et al.*, 2008). For the treatment of older leachate a carbon-assisted fluidised bed reactor is more effective than the conventional one such as activated sludge and fixed film processes (Imai *et al.*, 1993).

2.7.4.3 Microalgae Treatment

The high concentration of inorganic nitrogen compounds from ever increasing human and animal waste in wastewater streams are of considerable concern as they affect the quality of the water for domestic and industrial use (Vilchez *et al.*, 1997). Therefore the use of micro-organisms to eliminate heavy metals and toxic chemicals from wastewater has become an imperative practice (Vilchez *et al.*, 1997). Currently there is much interest in the biotechnological use of microalgae due to characteristics of their metabolism (Vilchez *et al.*, 1997). The removal of inorganic nutrients from wastewater by microalgae has been widely reported (Craggs *et al.*, 1997; Oswald and Gotaas, 1957; Vilchez *et al.*, 1997 and Zimmo *et al.*, 2004). Microalgae systems are able to efficiently remove nitrogen and phosphorous compounds and thus aid in alleviating eutrophication issues (Vilchez *et al.*, 1997). Microalgae are photoautotrophs meaning that they utilise sunlight energy to manufacture their own nutritive sources through a process called photosynthesis; this is described in further detail below. There are several advantages for using a microalgae treatment system. The energy source required is sunlight so is widely available and highly cost-effective. Due to the ability of microalgae to assimilate inorganic nitrogen compounds into biomass the resultant quantity of biomass can be used for livestock feed and the production of high added-value compounds and fine chemicals (Vilchez *et al.*, 1997). The use of microalgae for the removal or biotransformation of pollutants with simultaneous biomass production is termed phycoremediation (Olguin, 2003).

High Rate Algal Ponds (HRAPs) were developed as an alternative for the removal of pathogens, BOD and suspended solids (Rawat *et al.*, 2011). HRAPs are shallow (30–100 cm) include a large paddle wheel vane pump to create a channel velocity sufficient for gentle mixing. Unlike anaerobic ponds which must be several meters deep, HRAPs have to be shallow to ensure that there is maximum light penetration (Rawat *et al.*, 2011). They are able to operate at a hydraulic retention time of 4 – 10 days (Rawat *et al.*, 2011). The paddle wheel ensures that there is mixing to enable exposure of the

microalgae to sunlight and to keep them in suspension. HRAPs are the most cost effective treatment for liquid waste management (Rawat *et al.*, 2011). Open raceway pond systems used for the treatment of wastewater are economically feasible but biomass concentrations remain low as they are poorly mixed and cannot sustain an optically dark zone (Chisti, 2007) and are not as effective as the cascade design of HRAPs which ensures prolonged mixing and extended retention times.

Photobioreactors (PBR) are able to permit the maintenance of microalgae that produce a large biomass (Chisti, 2007). The most common design is the tubular PBR as it is a continuous system (Chisti, 2007). The tubes can be arranged either vertically or horizontally so as to allow for maximum solar capture (Chisti, 2007). Tubular photobioreactors are not suitable for large scale phycoremediation, however.

A problem with maintaining microalgae in suspension is solids handling and the difficulty in harvesting for biotechnological uses. Cell immobilization techniques have been developed to overcome these tribulations (Moreno-Garrido, 2007). It was reported that *Chlamydomonas reinhardtii* cells that were immobilised were more resistant to nitrite toxicity as well as the ammonium-dependent inhibition of nitrite assimilation and the system is more stable with regards to cell viability (Vilchez *et al.*, 1997). Some microalgae have the tendency to adhere themselves to surfaces and grow on them (Moreno-Garrido, 2007). This characteristic allows for microalgae to become attached to carriers (Moreno-Garrido, 2007). This natural attachment is referred to as passive immobilization (Moreno-Garrido, 2007). Adsorbent materials can be natural or synthetic. Currently efforts have focused on the use of loofa sponges for a carrier material (Moreno-Garrido, 2007). Loofa sponges are the fibrous support of the fruits of the genus *Luffa*. This carrier is noted to be cheap, strong, inert and stable in the long term (Moreno-Garrido, 2007). Synthetic materials such as polyvinyl and polyurethane can also be used for immobilizing microalgae (Moreno-Garrido, 2007).

Active immobilisation can be undertaken by the use of flocculants or gel entrapment. Flocculant agents were initially used for the removal of microalgae from a liquid medium (Moreno-Garrido, 2007). The most widely utilised is chitosan. Chitosan is a linear polysaccharide obtained from the alkaline deacetylation of chitin (Moreno-Garrido, 2007). The most widely used technique for active immobilization is gel entrapment. “Gel entrapment can be performed by the use of synthetic polymers (acrylamide, photocrosslinkable resins, polyurethanes), proteins (gelatine, collagen or egg white) or natural polysaccharides (agars, carrageenans or alginates)” (Moreno-

Garrido, 2007). When the main purpose of the microalgae is for removal of pollutants it is more viable for the microalgae to be adsorbed than entrapped (Moreno-Garrido, 2007). A packed bed is more suitable for this purpose than a fluidized reactor because in the latter collisions of particles bring about the desorption of cells (Moreno-Garrido, 2007). Immobilized microalgae are not efficient at removing low levels of nutrients due to the limitation of diffusion through the carrier matrix (Moreno-Garrido, 2007). Thankur and Kumar (1999) undertook a study on a halotolerant algal species, *Dunaliella salina*. Thankur and Kumar (1999) reported that immobilized *D. salina* always removed more nutrients than free cells. After 36 h, the levels of removed NO_3^-, NH_4^+ and PO_4^{3-} were 62%, 42% and 65% of initial concentrations, respectively (Thankur and Kumar, 1999). This was further supported by Fierro *et al.* (2008) that reported improved rates of nitrate and phosphate removal. Therefore, the immobilization of microalgae may be useful in treatment processes under relatively high nutrient conditions.

Reports on the use of microalgae for the treatment of municipal wastewater are available (Craggs *et al.*, 1997; Olguin, 2003; Zimmo *et al.*, 2004; Rawat *et al.*, 2011). However there are few studies on the use of microalgae for the treatment of MSW landfill leachate. The feasibility of using microalgal phycoremediation for leachate is not clear especially under high NH_3-N conditions. A study by Lin *et al.* (2007) indicated that high-concentration leachate inhibited algal growth and was probably attributed to the high level of NH_3-N. Although the typical nitrogen source of algae is NO_3^- (Vilchez et al, 1997), Lin *et al.* (2007) recorded a positive correlation between algal growth and NH_3-N consumption. Initially nitrate is taken up using energy by a specific permease enzyme followed by reduction of nirate to ammonium (Vilchez et al, 1997). This requires no ATP but 8 electrons and is catalyzed by nitrate reductase and nitrite reductase (Vilchez et al, 1997). Finally the ammonium is incorporated into carbon skeletons such as the α-amino group of L-glutamate. Therefore, it may be possible and energetically favorable to bypass the nitrate uptake step and utilise NH_3-N directy. In addition to the treatment of nitrogenous waste, significant quantities of ortho-phosphate and COD were removed by the microalgae (Lin *et al.*, 2007).

The relative removal rates of these characteristics were higher in the more dilute leachate (Lin *et al.*, 2007). Furthermore, it was concluded that phytotoxicity of the leachate was lowered after algal growth, indicated with a seed germination toxicity test using *Brassica chinensis* seeds (Lin *et al.*, 2007).

2.8 Photosynthesis in Microalgae

This chapter describes the process of photosynthesis and the information, unless otherwise referenced, has been sourced from Chemistry for Biologists (2015).

Photosynthesis can be defined as the coordinated series of biological reactions that converts light energy, via the absorption of photons, and inorganic carbon into stable organic compounds (Rubio *et al*., 2002). Photosynthesis in a microalgal cell occurs only in the photosynthetic unit (PSU), which is a portion of the thylakoidal membrane of the chloroplast (Rubio *et al*., 2002; Figure 2.4). Typically associated with chloroplasts is the pigment termed chlorophyll. Chlorophyll is a complex molecule that consists of a lipid-soluble hydrocarbon tail joined by an ester bond to a flat hydrophilic head with a magnesium centre. There are several modifications of chlorophyll that occur amongst photosynthetic organisms but all possess Chlorophyll *a*. Accessory pigments absorb energy that Chlorophyll *a* does not.

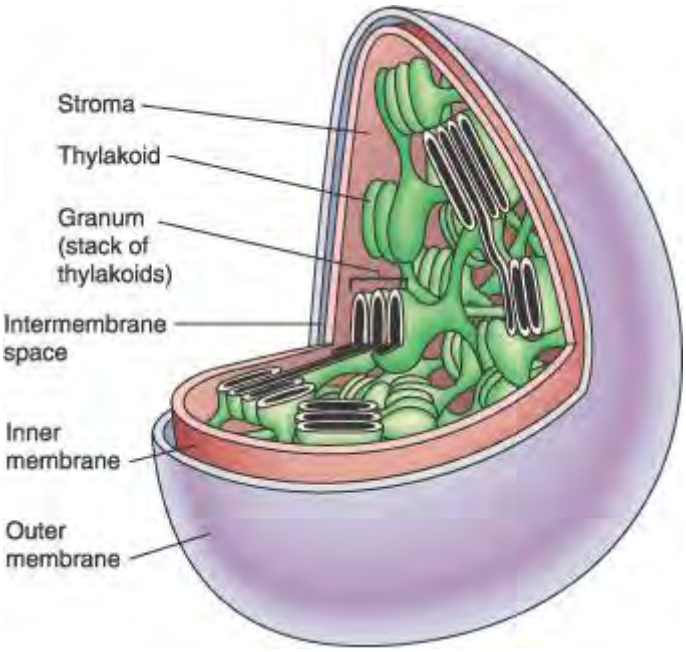

Figure 2.4 The structure of the chloroplast present in photosynthetic organisms (image source – Chemistry for Biologists, 2015)

Photosynthesis is regarded as a two-stage process: i) the light-dependent reactions; and ii) the light-independent reactions.

2.8.1 Light-dependent reactions (Light Phase)

During the Light Phase a resting PSU becomes activated by the non-enzymatic absorption of a photon (Rubio *et al*., 2002). Electrons present within the chlorophyll

gain energy and are transferred to a primary electron receptor. The chlorophyll is therefore oxidised and possesses a positive charge. The positively charged chlorophyll ion then takes a pair of electrons from a neighbouring electron donor such as water.

An electron transfer system carries the two electrons to and fro across the thylakoid membrane. The energy to drive these processes comes from two photosystems:

- Photosystem II (PSII) (P680); and
- Photosystem I (PSI) (P700).

Sufficient energy is released during electron transfer to enable Adenine Tri-Phosphate (ATP) to be made from Adenine Di-Phosphate (ADP) and phosphate. ATP is formed from the electrochemical gradient created by the pumping of Hydrogen ions (H^+) across the thylakoid membrane into the thylakoid compartment, due to the energy provided by the movement of electrons through the transport chain. Diffusion of the H^+ drives production of ATP. The electrons then react with a carrier molecule Nicotinamide Adenine Dinucleotide Phosphate (NADP), changing it to NADPH.

The photoionisation of chlorophyll and the eventual synthesis of ATP and NADPH are termed as Non-cyclic Phosphorylation or the Z-scheme. The components of the Z-scheme are found in the thylakoid membrane of the chloroplast.

2.8.2 Light-independent reactions (Dark Phase)

During the Dark Phase Carbon Dioxide (CO_2) from the water is taken up by the algae and is modified by the addition of H^+ to form carbohydrates in a process termed carbon fixation. The energy required for this process is supplied by the Light Phase of photosynthesis. CO_2 combines with a five-carbon sugar, Ribulose1,5Bi-Phosphate (RuBP) to form a six-carbon carbohydrate which is unstable, and therefore breaks down to form two Glycerate 3-Phosphate (GP) molecules. The GP molecules are phosphorylated by ATP into glycerate di-phosphate molecules. These are subsequently reduced by NADPH into two molecules of Glyceraldehyde 3-phosphate (GALP). One GALP molecule is the initial end product of photosynthesis and the other froms RuBP through a series of chemical reactions.

3. METHODS

3.1 Study Site

The Bulbul Drive landfill is situated near Havenside/Silverglen adjacent to the Doringspruit River, approximately 15 km from the Durban CBD located in the eThekwini Municipal area (GreenEng, 2011). The landfill is a hazardous (H:h) landfill and was constructed in accordance with the Minimum Requirements for Landfill devised by the Department of Water and Sanitation (DWS) previously known as Department of Water Affairs and Forestry (DWS) (GreenEng, 2011).

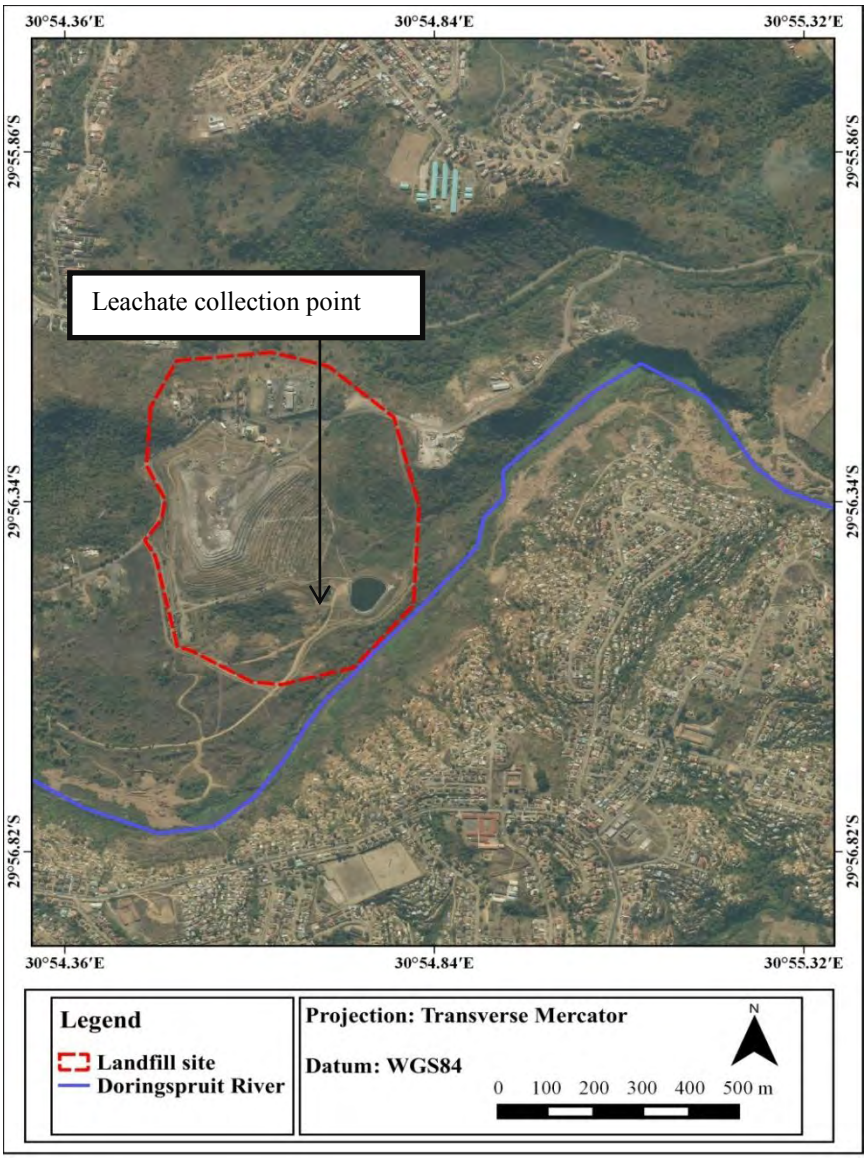

Figure 3.1 Location of the Bulbul Drive landfill

The landfill site was decommissioned from 2011 and had contained approximately 2.5 to 3 million m^3 of waste on the verge of its closure (GreenEng, 2011). Although the

landfill does not receive waste, it currently produces approximately 100 m^3 leachate per day. The leachate is transported off site and disposed of at the eThekwini Municipality's sea outfall. Disposal of the leachate is only allowed with pretreatment using Hydrogen Peroxide (H_2O_2) (GreenEng, 2011).

3.2 *Chlorella* sp. Cultures

Chlorella sp. cultures were obtained from the Centre for Algal Biotechnology at the Mangosuthu University of Technology, South Africa. *Chlorella* cultures were maintained on the BG-11 artificial medium.

Table 3.1 BG-11 algal growth medium chemical ingredients and their respective concentration

Chemical Ingredient	Concentration (mg/L)
$NaNO_3$	1500
$K_2HPO_4.3H_2O$	40
$MgSO_4.7H_2O$	75
$CaCl_2.2H_2O$	36
$C_6H_8O_7$	6
$C_6H_8O_7.xFe.xNH_3$	6
$C_{10}H_{16}N_2O_8$	1
Na_2CO_3	20
Trace Metal Solution	1 ml/L

Table 3.2 BG-11 algal growth medium trace metal ingredients and their respective concentration

Chemical Ingredient	Concentration (g/L)
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.222
NaMoO4.$5H_2O$	0.390
$CuSO_4.5H_2O$	0.079
$Co(NO_3)_2.6H_2O$	0.0494

Chlorella cultures obtained from the Centre for Algal Biotechnology were up-scaled to a 10 L glass reactor vessel. The reactor was provided with a light: dark ratio of 16:8 hours daily. Lighting consisted of two "Dual Pro T5 High Output (HO) Fluorescent Light" manufactured by ODYSSEA®. Each unit possesses two 24W HO fluorescent lights with a single reflector behind both lights. One light is a 10 000 K daylight T5 and the other is an Actinic T5. In one of the ODYSSEA® units the Actinic T5 was replaced with a Sylvania Aquastar 10 000 K T5 fluorescent light. The reactor was continuously aerated with a SONIC® 108 air pump with an attached diffuser.

Figure 3.2 ***Chlorella* sp. seed culture maintained in a 10 L reaction vessel with BG-11 as the nutrient source**

3.3 Batch Tests

The batch tests were the *in vivo* experiments that were undertaken to determine the efficacy of using the microalgae *Chlorella* sp. as either a primary or secondary treatment for hazardous landfill leachate.

3.3.1 Primary Treatment Batch Test

This batch test was undertaken to determine the feasibility of utilising *Chlorella* sp for the primary treatment of hazardous landfill leachate. The basis of this experiment was to determine if *Chlorella* would utilise the NH_3-N and organic carbon as a nutrient source thereby treating the leachate.

Batch tests were conducted using 1 L conical flasks (Figure 3.3). The flasks were acid washed with 1 M Nitric Acid (HNO_3) and autoclaved prior to use. All treatment cultures and controls were performed in triplicate. Treatment cultures were continually

shaken at 110 rpm (Eaton, 2005) and irradiated at a light: dark ratio of 16:8 hours (Figure 3.3). Lighting consisted of two "Dual Pro T5 High Output (HO) Fluorescent Light" manufactured by ODYSSEA® placed on either side of the shaker. Each unit possesses two 24W HO fluorescent lights with a single reflector behind both lights (Figure 3.3). The Actinic T5 was replaced with a Sylvania Aquastar 10 000 K T5 fluorescent light in each of the units.

Figure 3.3 Conical flask batch test on a shaker with T5 lights off (left) and on (right)

The batch test treatments were undertaken at leachate dilutions of 10%, 25%, 50% and 85%. The dilution was made using distilled water (dH_2O) and 150 mL *Chlorella* seed culture. Refer to Table 3.3 for constituent volumes used. In conjunction, controls containing identical dilutions of raw leachate containing no microalgae were maintained and tested in order to determine any significant differences in the physico-chemical parameters (Table 3.3).

Table 3.3 Summary of constituent volumes for primary treatment batch tests

	Dilution	Leachate Volume (mL)	dH_2O (mL)	Algae Inoculation (mL)
Treatment	10%	100	750	150
	25%	250	600	150
	50%	500	350	150
	85%	850	-	150
Control	10%	100	900	-
	25%	250	750	-
	50%	500	500	-
	85%	850	150	-

In order to undertake physico-chemical analysis, a volume of 20 mL was extracted from each test culture and filtered using a cellulose nitrate filter paper with a particle retention size 0.45 µm (GEMA MEDICAL) to remove algae. This was to ensure no erroneous values when testing the leachate. The batch test was carried out until the NH_3/NH_4^+ value had reached 0 mg/L or the *Chlorella* population in the leachate culture had been decimated.

3.3.2 Secondary Treatment Batch Tests

This experiment was used to determine the efficacy of *Chlorella* in the secondary treatment of hazardous landfill leachate. The basis of this experiment was to determine if *Chlorella* would consume NO_x and thereby "polishing" the treated leachate.

Batch tests were conducted using 1 L conical flasks. The flasks were acid washed with 1 M Nitric acid (HNO_3) and autoclaved prior to use. The batch test was operated at a series dilution of 10%, 25%, 50% and 85%. The dilution was made using distilled water (dH_2O) and 150 mL *Chlorella* seed culture. In conjunction, controls containing identical dilutions of raw leachate containing no microalgae were maintained and tested in order to determine any significant differences in the physico-chemical parameters.

Table 3.4 Summary of constituent volumes for secondary treatment batch tests

	Dilution	Leachate Volume (mL)	dH_2O (mL)	Algae Inoculation (mL)
Treatment	10%	100	750	150
	25%	250	600	150
	50%	500	350	150
	85%	850	-	150
Control	10%	100	900	-
	25%	250	750	-
	50%	500	500	-
	85%	850	150	-

All treatment cultures and controls were performed in triplicate. Treatment cultures were continually shaken at 110 rpm (Eaton, 2005) and irradiated at a light: dark ratio of 16:8 hours (Figure 3.3). Lighting consisted of two "Dual Pro T5 High Output (HO) Fluorescent Light" manufactured by ODYSSEA® placed on either side of the shaker.

Each unit possesses two 24W HO fluorescent lights with a single reflector behind both lights (Figure 3.3). The Actinic T5 was replaced with a Sylvania Aquastar 10 000 K T5 fluorescent light in each of the units.

In order to undertake physico-chemical analysis, a volume of 20 mL was extracted from each test culture and filtered using a cellulose nitrate filter paper with a particle retention size 0.45 µm (GEMA MEDICAL) to remove algae. This was to ensure no erroneous values when testing the leachate. The batch test was carried out until the NO_3^- value had reached 0 mg/L or the *Chlorella* population in the leachate culture had been decimated.

3.4 Characterisation Analyses

Although hazardous landfill leachate possesses an array of pollutants, 4 were of primary concern given their high potential for human toxicity and driving environmental degradation. In addition, three of these parameters are listed in the established DWS discharge limits (Table 2.2). Accordingly, the principle parameters analysed for the batch tests consisted of:

- Ammoniacal nitrogen (NH_3-N);
- Nitrate (NO_3^-);
- Chemical Oxygen Demand (COD); and
- 5-day Biochemical Oxygen Demand (BOD_5).

3.4.1 Ammoniacal Nitrogen

Ammoniacal nitrogen was analysed daily utilising the Titrimetric method as described in Eaton (2005). The following reagents were prepared for the analysis as follows:

a) Mixed Indicator Solution – 200 mg methyl red indicator was dissolved in 100 mL 95% ethanol. 100 mg methylene blue was dissolved in 50 mL 95% ethanol. The solutions were then combined.
b) Boric Acid (H_3BO_3) Indicator – 20 g H_3BO_3 was dissolved in distilled water and 10 mL mixed indicator solution was added. This mixture was subsequently diluted to 1 L.
c) Hydrochloric Acid (HCl) Titrant – A 0.1 N HCl solution was prepared using the ampoules manufactured by Merck (Art. No. SAAR3063170YA).

Prior to analysis samples were distilled by means of a behr distillation unit from United Scientific (Pty) Ltd (Figure 3.4). In the distillation apparatus a borosilicate glass flask

was attached to a vertical condenser into which the sample was placed and the outlet tip submerged into the receiving solution (Eaton, 2005). In order to ensure that NH_3-N completely distills out of the sample solution 6N Sodium Hydroxide (NaOH) is automatically added by the distiller unit. The distillate was collected in a 250 mL Erlenmeyer flask containing 50 mL H_3BO_3 indicator solution. At least 200 mL of distillate was collected. The indicator solution including distillate was titrated with 0.1 N HCl on a magnetic stirrer until the color of the solution turned pale lavender (Eaton, 2005, Figures 3.4 – 3.5). The acid was titrated with a Jencons Scientific digitrate.

The quantity of NH_3-N was determined by using the following equation;

$$CNH3\text{-}N = 14 * NHCl * VHCl \qquad \textbf{(Equation 3-1)}$$

This equated to mass (mg) per sample volume. This was then converted to mg/L.

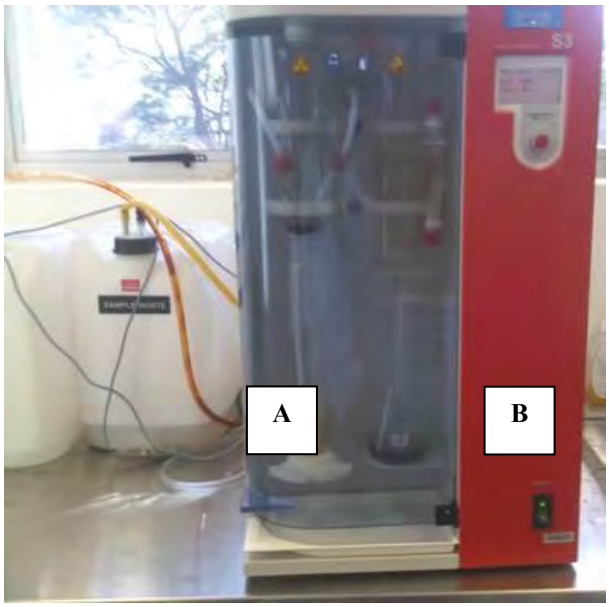

Figure 3.4 **The behr distillation unit with the borosilicate flask into which the sample was placed (A) and the Erlenmeyer flask containing the H_3BO_3 indicator solution (B)**

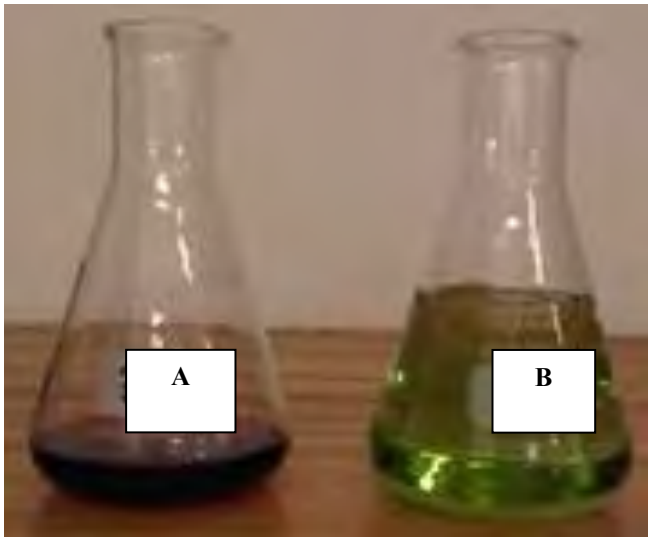

Figure 3.5 Boric Acid indicator solution pre-distillation (A) and post-distillation (B)

Figure 3.6 Jencons digitrate used to titrate 0.1 N HCl with magnetic stirrer at bottom

3.4.2 Nitrate

The procedure for analysing NO_3^- was the same as described for NH_3-N with the exception that 50 mg Magnesium Oxide (MgO) and 100 mg Devarda's alloy was added to the sample prior to distillation.

3.4.3 Chemical Oxygen Demand

Chemical Oxygen Demand (COD) is a frequently used measurement of pollutants in water or wastewater samples (Eaton, 2005). "COD is defined as the amount of a specified oxidant that reacts with the sample under controlled conditions" (Eaton, 2005). The quantity of oxygen demanded is equivalent to the quantity of oxidant consumed (Eaton, 2005).

In order to determine COD of samples the Closed Reflux Colorimetric Method was used as Closed Reflux methods are necessary for the analysis of samples with COD values greater than 50 mg O_2/L (Eaton, 2005). In addition this method is more economical in terms of reagents used and generates minimal quantities of hazardous waste (Eaton, 2005). In this method the dichromate ($Cr_2O_7^{2-}$) ion is used as the oxidant.

The following reagents were prepared as described in Eaton (2005):

a) Digestion solution – 10.216 g $K_2Cr_2O_7$ was dried for 2 hours at 150°C and was added to 167 mL concentrated sulfuric acid (H_2SO_4) and 33.3 g mercuric sulfate ($HgSO_4$). This was dissolved in 500 mL distilled water, cooled to room temperature and then diluted to 1L.
b) Sulfuric acid reagent – 25.3 g silver sulfate (Ag_2SO_4) was dissolved into 2.5 L H_2SO_4 for 24 hours.
c) Potassium Hydrogen Phthalate (KHP) solution – A mass of KHP was crushed and dried at 110°C. 425 mg was dissolved into distilled water and a total volume of 1 L was prepared.

Suitable volumes of sample, digestion solution and H_2SO_4 reagent were measured into COD tubes manufactured by HACH. Samples were diluted with distilled water in the event of excessive COD levels. The KHP solution was used as the standard (Eaton, 2005). COD tubes were then placed in a HACH COD reactor for 2 hours (Figure 3.7). After digestion samples were cooled and absorbance was read at 600 nm (Eaton, 2005) using a HACH DR/2000 spectrophotometer (Figure 3.8). A digested blank was used to determine the blank for the COD and to ensure good quality analytical reagents. A calibration curve was prepared and values of sample COD were consequently determined. COD analysis of cultures was performed weekly.

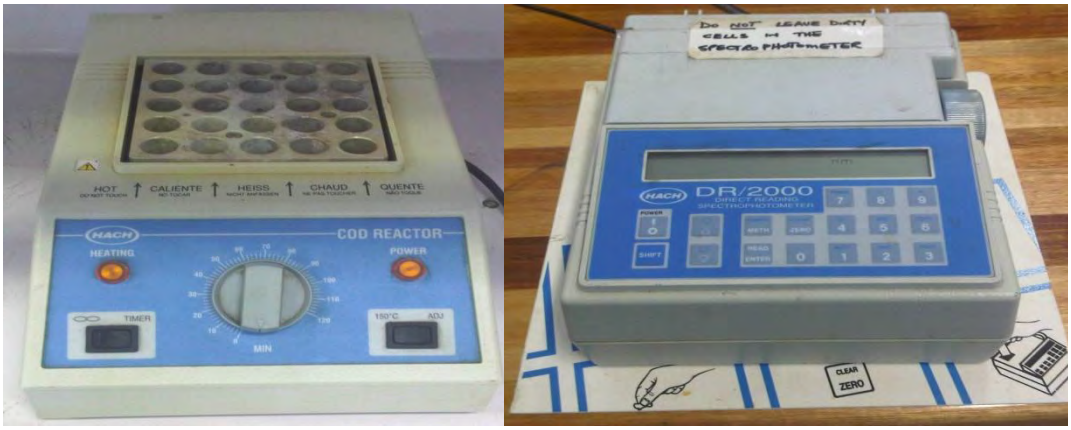

Figure 3.7 The HACH COD reactor (left) and the HACH DR/2000 spectrophotometer (right)

3.4.4 Biochemical Oxygen Demand

Biochemical Oxygen Demand (BOD) is an index of biodegradable organics present in water and wastewater (Eaton, 2005). The technique measures the oxygen (O_2) consumed during a finite incubation period for the breakdown of organic matter (Eaton, 2005). The manometric method as described by Robertz (2006) was utilised for this study. The system was purchased from AQUALYTIC.

According to Robertz (2006) this method is very reliable for routine analysis and there are several advantages when compared to the dilution method. Samples do not have to be diluted, there is a much wider measurement range and there is less work involved (Robertz, 2006).

The principle of the measurement as explicated by Robertz (2006) is as follows. The O_2 in the water or wastewater sample is converted into carbon dioxide (CO_2) which is subsequently removed by potassium hydroxide (KOH). Therefore a drop in pressure occurs in the BOD reaction flask. This pressure change is measured by electronic pressure sensors and this drop in pressure is proportional to the amount of oxygen consumed.

The BOD_n test is widely used to determine the efficiency of treatment processes (Eaton, 2005) and therefore, BOD_5 (i.e. measured over 5 days) was utilised to evaluate the effectiveness of using *Chlorella* as an organics remover.

Samples were filtered with 0.45 µm filter paper in order to prevent contamination and errors from *Chlorella*. A sample volume of 56 ml was placed in the BOD flask, as this

volume is recommended for the range of 0-2000 mg/L BOD (Robertz, 2006). In order to prevent nitrification which also consumes O_2 thereby causing erroneous results, 3 drops of N-allylthiourea (ATH) were added to samples. In order to ensure thorough gas exchange a magnetic stirring rod which would agitate the sample, was added into the flask. A dry grease free gasket was placed into the neck of the flask and filled with 5 drops of KOH solution (Robertz, 2006). The vessel was subsequently sealed with a BOD-sensor (Robertz, 2006). The sample was then placed in an incubator maintained at 20°C (incubation temperature) for 5 days. The temperature had to remain at the incubation temperature within the range of $\pm$ 1°C in order to prevent errors of up to 10% per 1°C (Robertz, 2006). BOD_5 analysis was performed on samples prior to and after inoculation with *Chlorella*.

Figure 3.8 **The BOD-sensor, gasket and flask (left) and samples in BOD flasks placed in incubator (right).**

3.5 Ancillary Data

3.5.1 pH

Although pH is not listed in the DWS discharge limits (Table 2.2) it was monitored daily because the pH of a solution influences the activity of other chemical compounds within a given solution (DWS, 1996). In addition, the monitoring of pH was vital as fluctuations can occur due to photosynthesis by the microalgae (Taub, 2009). The fluctuations of pH are due to the uptake of CO_2 leading to a reduced level of carbonic acid and thereby increasing the pH (Lin *et al.*, 2007).

Furthermore, the assimilation of NO_3^- increases alkalinity and pH via the production of hydroxyl (OH^-) production (Goldman and Brewer, 1980).

Therefore, pH was analysed to enable comprehension of the monitored parameter dynamics as well as to serve as an indirect indicator of NO_3^-uptake. The pH of treatment cultures were analysed daily using a Thermo Scientific pH meter.

3.6 Chlorophyll-*a* Assay

In order to assess *Chlorella* survivability and growth rate during the batch tests, the chlorophyll *a* (chl-*a*) content of treatments were examined daily. Chl-*a* can be used as a proxy for biomass as there is a direct relationship between them (Henriques *et al.*, 2007). A volume of 10 mL was drawn from a culture and placed in a 50 mL centrifuge tube. The tubes containing the cultures were centrifuged at 3000 rpm for 15 minutes (Henriques *et al.*, 2007; Parvin *et al.*, 2007). The supernatant was discarded and the pellet was re-suspended in 5 mL of 95% ethanol. Ethanol was used as the solvent due to its efficiency and superiority when compared to other solvents (Downes *et al.*, 1993; Lan *et al.*, 2011). The suspension was then boiled for 15 minutes (Downes *et al.*, 1993). After boiling, the suspension was centrifuged at 4000 rpm for 5 minutes (Henriques *et al.*, 2007). The supernatant was removed and its absorbance was read at 650 nm and 665 nm using a HACH DR/2000 spectrophotometer. Absorbance readings were applied to the equation below to obtain readings in µg/mL (Arai *et al.*, 2008):

$$\text{Chlorophyll-}a = (16.5 \times A665) - (8.3 \times A650) \quad \textbf{(Equation 3-2)}$$

The constants 16.5 and 8.3 refer to the specific absorption coefficients for the absorbance of Chl-*a* at 665 nm and 650 nm respectively. The specific absorption coefficient refers to the factor that measures the absorbance of light per unit of path length and per unit of mass concentration.

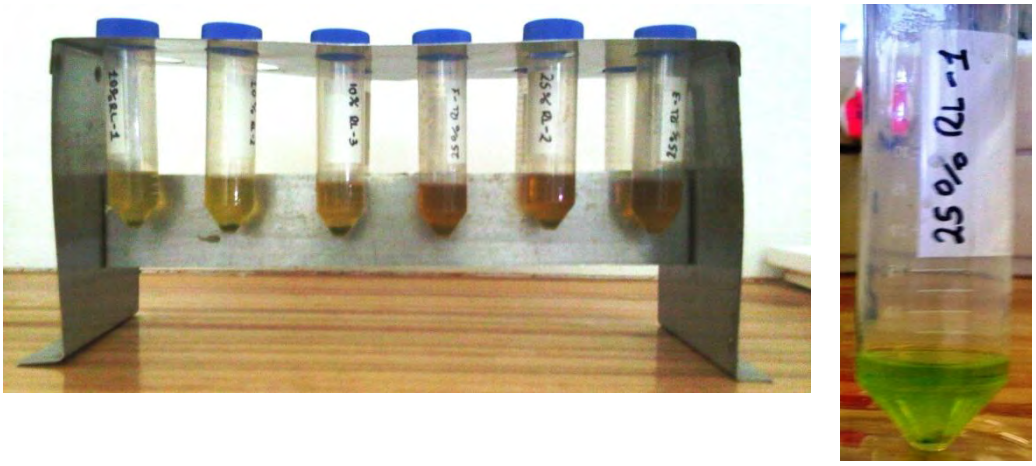

Figure 3.9 **Centrifuge tubes containing microalgae treatment cultures (left) and centrifuge with chlorophyll-*a* supernatant obtained after dissolving with boiling ethanol and centrifuging at 4000 rpm for 5 minutes (right)**

3.7 Toxicity Tests

Phytotoxicity tests were undertaken for the secondary treatment batch test to determine if *Chlorella* is effective in reducing leachate toxicity. Radish (*Raphanus sativus*) seeds (STARKE AYRES brand) were used, as it is a commonly used species for phytotoxicity tests (Lin, 2007). In order to ensure viability of the seeds, a pre-study using distilled water was performed. A percentage germination of 100% indicates seed viability for use in the tests. In addition, this test was used to determine the maximum number of days to operate the toxicity tests, by virtue of the number of days required for all the seeds to germinate.

Three replicates of 15 seeds were placed in a 90 mm diameter, 15 mm high petri dish lined with two filter papers (Whatman® Cat No. 1001110). Before the seeds were placed on the filter paper, 5 mL of the leachate to be tested was added (Mosse, 2010). The number of seeds germinated was recorded daily for 7 days as indicated by the pre-test. Germination was defined as the presence of a radicle $\geq$ 5 mm in length (Mosse, 2010). The total percentage germinated and mean time to germinate (MTG) was determined. MTG was calculated as indicated in Brenchley (1998):

$$MTG = \Sigma (n \times d)/ N \qquad \textbf{(Equation 3-3)}$$

n = the number of seeds germinated between scoring intervals.

d = the incubation period in days at that time point.

N = the total number of seeds germinated in the treatment.

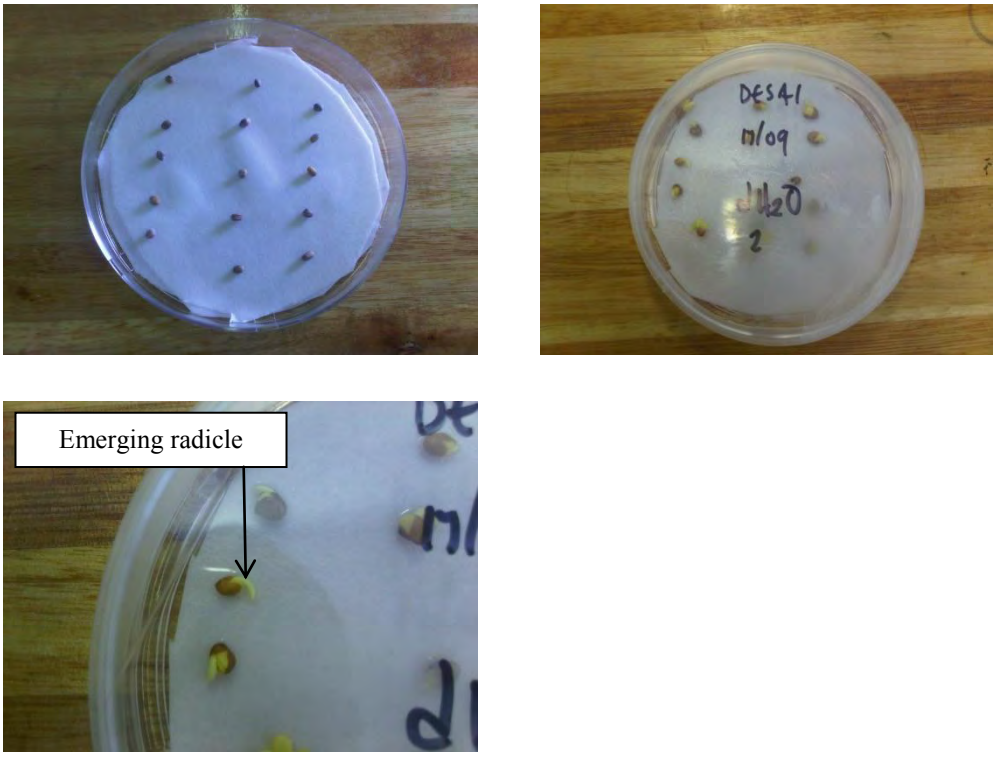

Figure 3.10 **Clockwise from top – Radish (*Raphanus sativus*) seeds placed in petri dish prior to incubation; *R. sativus* seeds in petri dish during incubation and germinated R. sativus seeds showing emerging radicle**

3.8 Statistical Analysis

Statistical analysis was undertaken using the software R™ Version 3.1.2. A range of univariate analyses were utilised to comprehend and confirm results and these are discussed in further detail in the following sections. Prior to all analyses a Shapiro-Wilcox test was undertaken to determine if the data were parametric or non-parametric.

3.8.1 Primary Treatment Statistical Analysis

To determine if there were significant differences between the treatment and control COD concentration upon experiment termination, i.e. Experiment Termination Concentration (ETC), Mann-Whitney *U* tests were undertaken. In addition, independent-samples t-tests were undertaken to determine if there were significant differences between BOD_5 concentrations and pH between the treatment and control at the termination of the batch test.

However, statistical analysis could not be applied to testing for significant differences between NH_3-N concentrations due to no variance in the treatments at termination of the experiment.

A Spearman Rank Correlation was utilised to determine if there was a significant relationship between the physico-chemical parameters and chl-*a* recorded. This particular correlation was used as data proved to be non-parametric even after transformations. It is important to note that unlike regression analysis, correlations do not indicate a cause and effect relationship but rather existence of a relationship between two variables. Therefore, the analysis informed on the presence and strength of a relationship as well as percentage of parameter variability explained by one variable on the other.

Subsequently, a slope analysis was undertaken to determine the rate of parameter change between microalgal treatments and controls. To achieve this, parameter values of the treatment and control plotted against time. Where it was required to achieve linearity, data was $\log_{10}$ transformed. This was undertaken only for principle parameters that were both, determined to be significantly different between control and treatment at the experiment termination and were significantly correlated to chl-*a*.

3.8.2 Secondary Treatment Statistical Analysis

Independent-samples t-tests were undertaken to determine if there were significant differences between the physico-chemical parameters of the treatment and control upon termination of the batch test.

A Spearman Rank Correlation was utilised to determine if there was a significant relationship between the physico-chemical parameters recorded and chl-*a*. This particular correlation was used as data proved to be non-parametric even after transformations.

A slope analysis was undertaken to determine and compare the rate of parameter change between microalgal treatments and controls. To achieve this, parameter values of the treatment and control plotted against time. Where it was required to achieve linearity, data was $\log_{10}$ transformed. This was undertaken only for principle parameters that were both, determined to be significantly different between control and treatment at the experiment termination and were significantly correlated to chl-*a*.

Statistical analysis undertaken for the toxicity tests to compare the percentage of seeds germinated and the MTG initially comprised of a Mann-Whitney *U* test to determine if there were significant differences between the treatments and controls. Subsequently, a Kruskal Wallis test was undertaken to determine if there were significant differences between treatments, with a Tukey post-hoc test to indicate between which two samples the significant difference occurs.

4. RESULTS

This section reports on the results obtained from the Primary Treatment Batch Test and Secondary Treatment Batch Test. Accordingly, these are separated into two sections; the Primary Treatment Batch Test and Secondary Treatment Batch Test. In addition, a third section will compare the Primary and Secondary Treatment batch test.

4.1 Primary Treatment Batch Test

4.1.1 Ammoniacal Nitrogen

The batch test indicated a simultaneous decrease in NH_3-N concentrations between the treatment and control flasks. NH_3-N (Figure 4.1). All treatments had reduced NH_3-N concentration to 0 mg/L after 20 days except the 10% treatment, wherein the concentration was reduced to 0 mg/L after 15 days (Figure 4.1). However, none of the controls had their NH_3-N concentrations reduced to 0 mg/L (Figure 4.1). The 85% control possessed the highest ETC of NH_3-N, with an average of 42.0 ± 6.4 mg/L, whereas the 10% control possessed the lowest concentration after 20 days with an average of 4.2 ± 0.6 mg/L.

The Spearman Rank correlation indicated that there was a significant ($p < 0.05$) relationship between NH_3-N and chl-*a* concentrations for all treatments, and that the relationship was inversely proportional (Table 4.1). Accordingly, when chl-*a* concentration increases NH_3-N levels decrease (Figure 4.2). The highest co-efficient of variations (R^2) was the 25% treatment at 0.92. This denotes that one measurable accounted for approximately 92% of the variability in the other, respectively. However, the lowest co-efficient of variation was 0.14 in the 50% treatment. Figure 4.2 illustrates the inconstant relationship between the two variables for the 50% and 85% treatment.

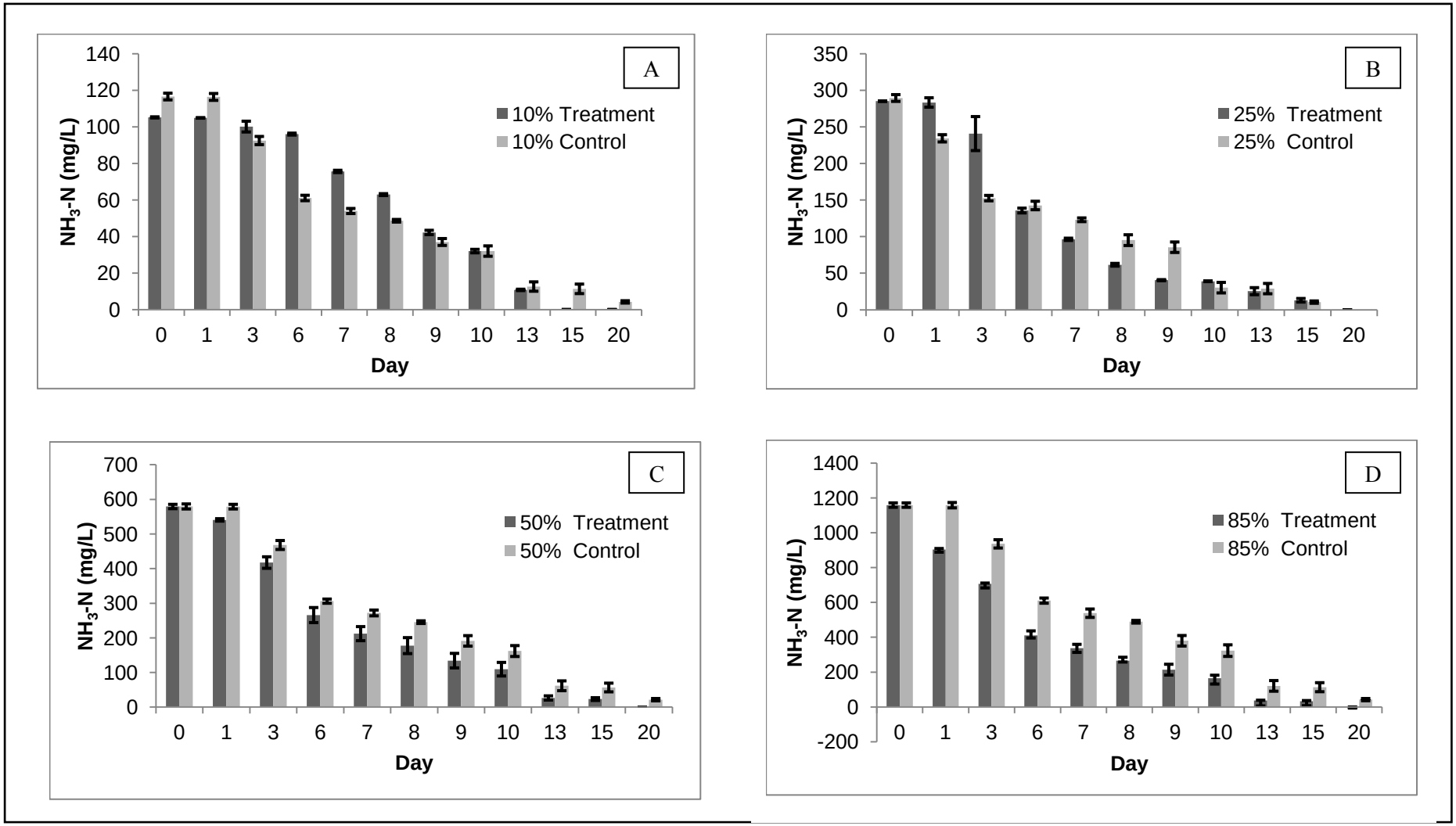

Figure 4.1 **Temporal dynamics of ammoniacal nitrogen concentration (mg/L) for treatments and controls during the primary treatment batch test. The vertical lines indicate standard deviation**

Table 4.1 **Statistical summary of Spearman Rank Correlation analysis between ammoniacal nitrogen and chlorophyll-*a* during the primary treatment batch test**

	Treatment			
Statistic	**10%**	**25%**	**50%**	**85%**
p	0.001	0.001	0.03	0.02
r	-0.93	-0.96	-0.38	-0.40
R^2	0.86	0.92	0.14	0.16

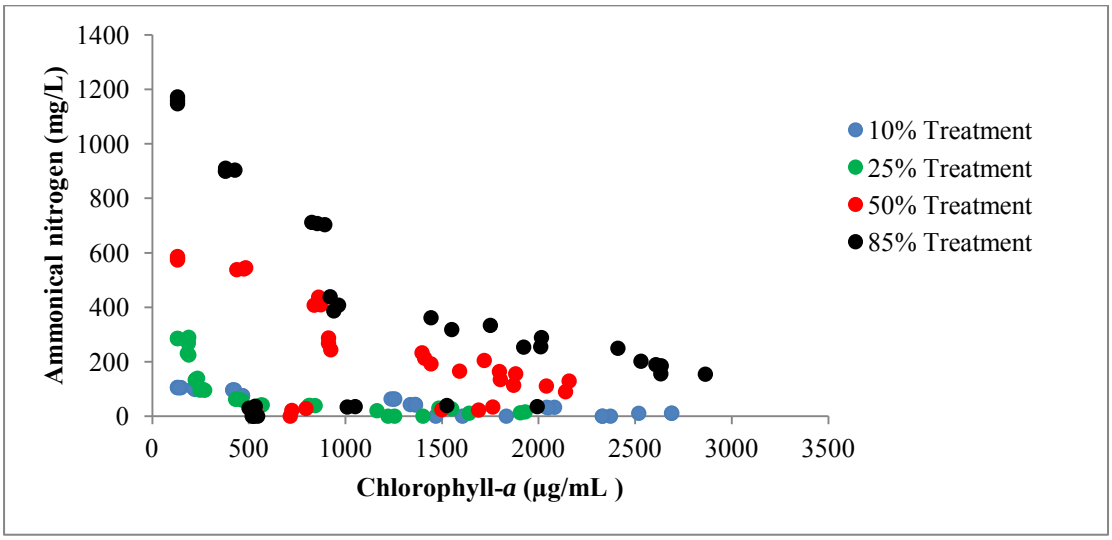

Figure 4.2 **Scatterplot to illustrate the relationship between chlorophyll-*a* (µg/mL) and ammoniacal nitrogen (mg/L) for all treatments during the primary treatment batch test**

The slope analysis revealed that the gradients (m) were consistently higher in the treatment than in the control (Figure 4.3). In terms of the treatments, the highest gradient recorded was for the 85% treatment with -0.1389, while the lowest gradient recorded was for the 25% treatment with -0.1153 (Figure 4.3). Regarding the controls, the 85% control had possessed the highest gradient of -0.0753 and the 10% control the lowest with -0.0717 (Figure 4.3).

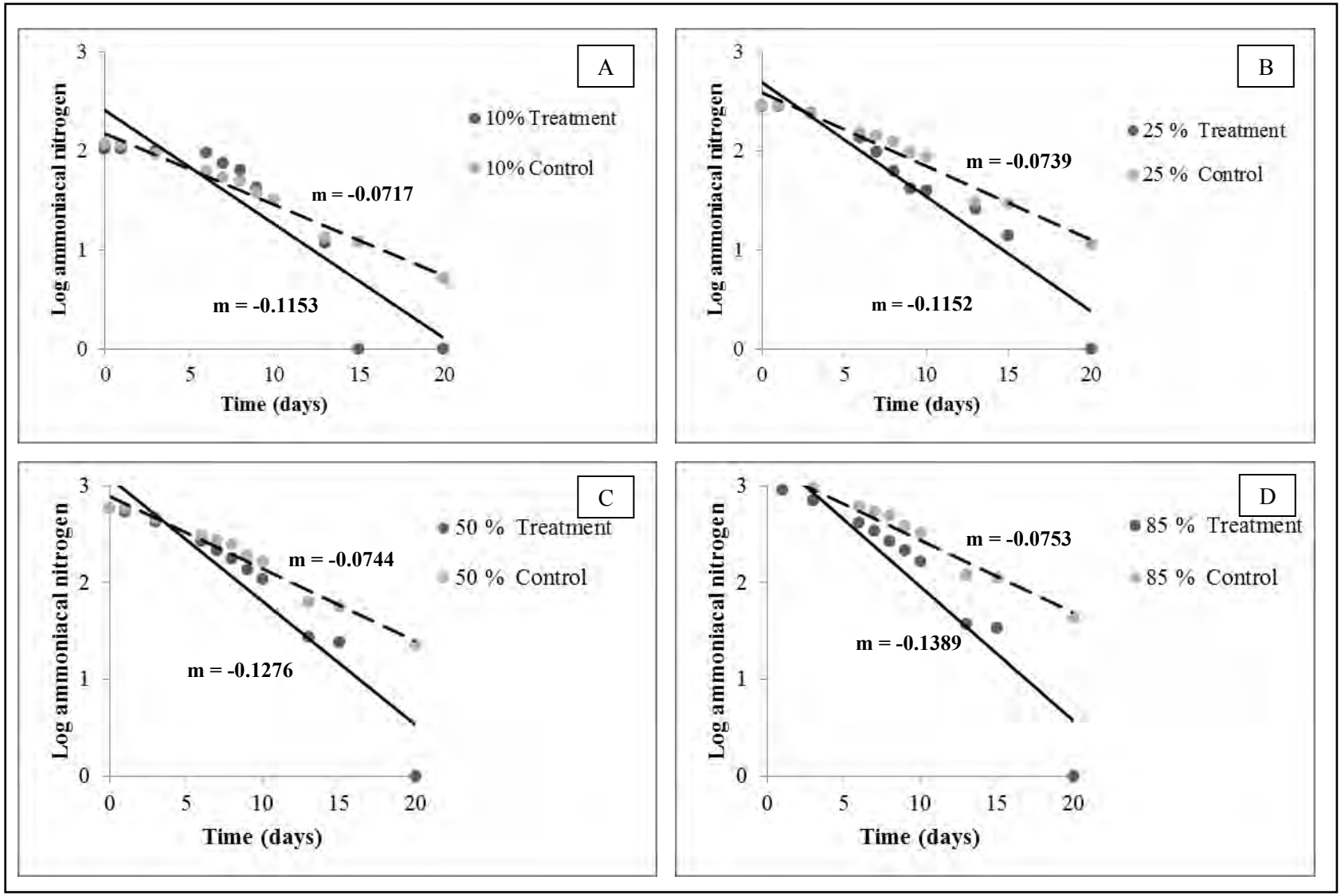

Figure 4.3 **Comparison of average $\log_{10}$ ammoniacal nitrogen trendlines of treatments and controls during the primary treatment batch test. The solid and dash line illustrate the trend in data for the treatment and control respectively**

4.1.2 Chemical Oxygen Demand

The temporal dynamics of COD was demonstrated to be variable within and amongst the treatments and controls (Figure 4.4). The 25% and 85% treatments had exhibited COD ETCs lower than the control (Figure 4.4). Conversely, the 10% and 50% treatments exhibited COD values higher than the control at experiment termination (Figure 4.4). Furthermore, for all treatments and controls there was an increase in the COD level after day 7 (Figure 4.4).

The highest ETC difference between control and treatment was the 25% treatment, which possessed an average COD concentration of 288.22 ± 89.33 mg O_2/L. This was a 447.9 mg O_2/L difference between itself and the control (Figure 4.4). However, the difference between the medians of all treatments and controls proved to be insignificant ($p > 0.05$) indicating that there was no significant difference between the COD levels of the control and treatment on day 20 (Table 4.2).

The correlation analysis indicated that only the 85% treatment exhibited a significant ($p < 0.05$) relationship between COD and chl-*a* (Table 4.3). It was demonstrated that an increase in chl-*a* resulted in a concomitant decrease in COD, with a co-efficient of variation of 0.69 (Table 4.3; Figure 4.5).

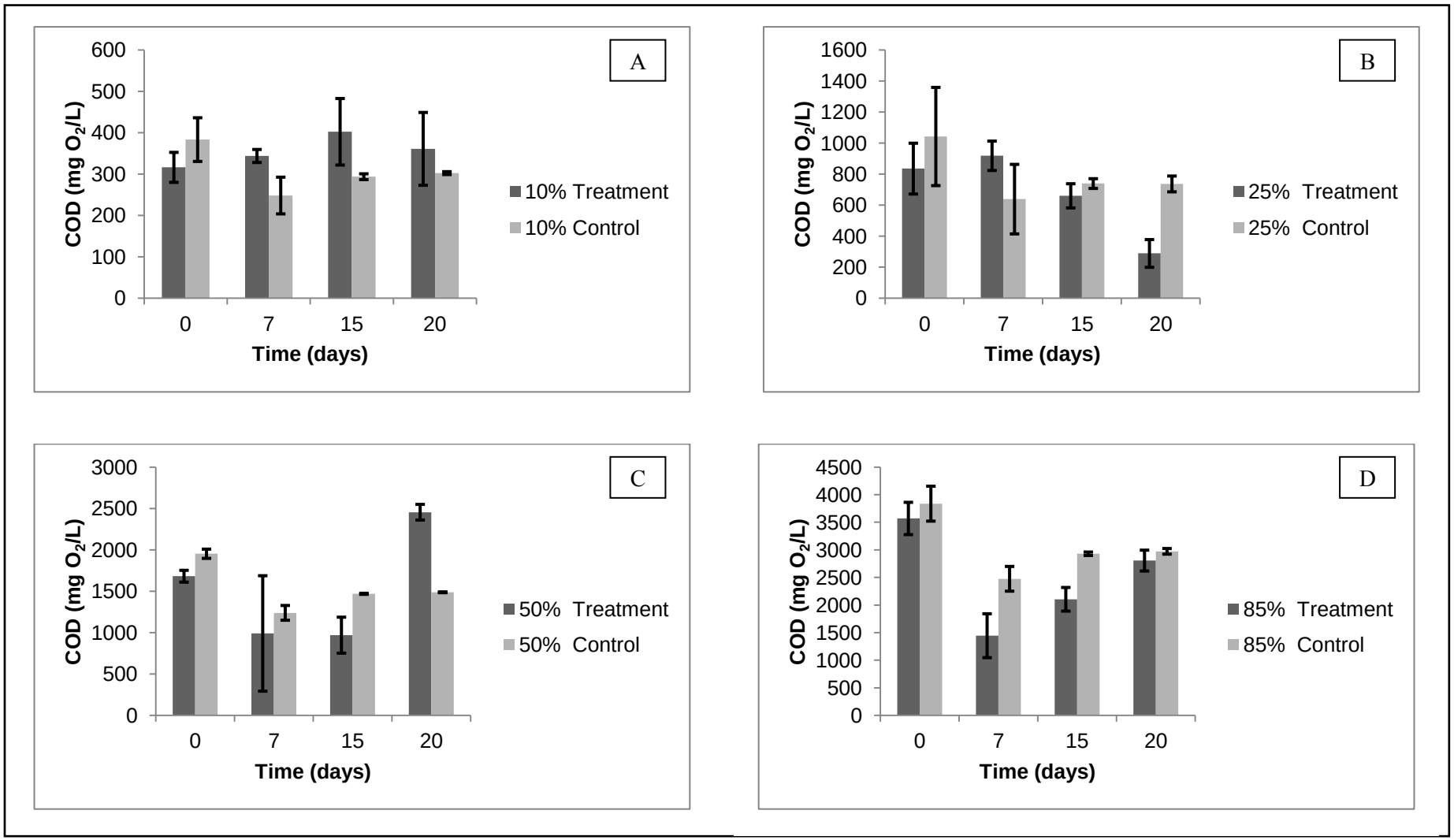

Figure 4.4 **Temporal dynamics of average COD concentration (mg O_2/L) for treatments and controls during the primary treatment batch test. Vertical bars indicate standard deviation from the mean**

Table 4.2 **Summary of probability (p) values obtained from Mann-Whitney *U* analysis between COD concentrations of the treatment and control at the termination of the primary treatment batch test**

		Treatment			
		10%	**25%**	**50%**	**85%**
Control	**10%**	0.06			
	25%		0.07		
	50%			0.08	
	85%				0.38

Table 4.3 **Statistical summary of Spearman Rank Correlation analysis between COD and chlorophyll-*a* during the primary treatment batch test**

	Treatment			
Statistic	**10%**	**25%**	**50%**	**85%**
p	0.05	0.06	0.26	0.001
r	0.57	-0.56	-0.35	-0.83
R^2	0.32	0.31	0.12	0.69

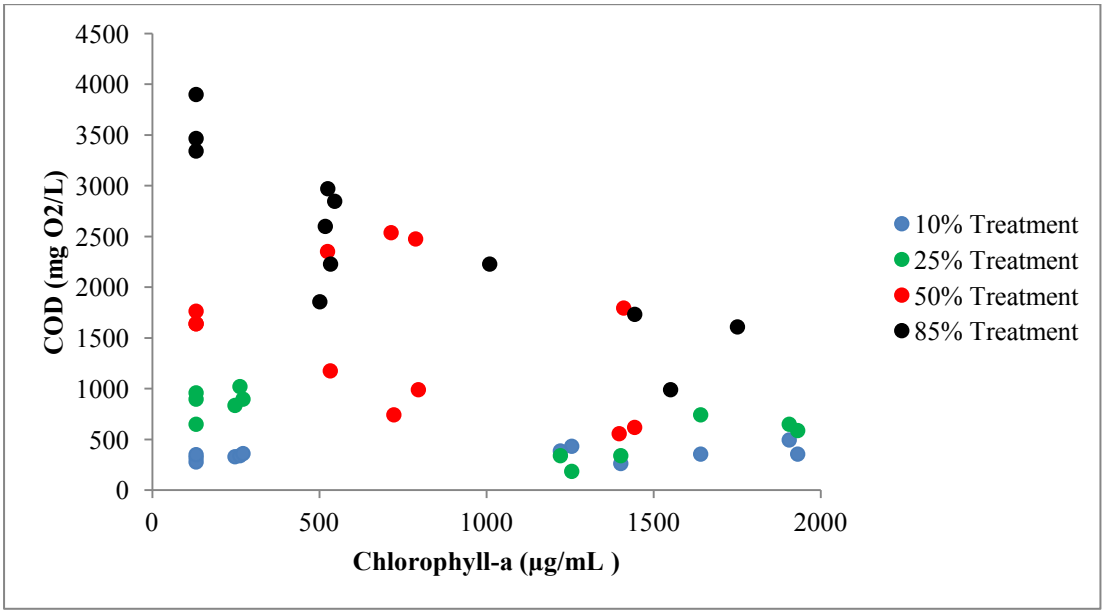

Figure 4.5 **Scatterplot to illustrate the relationship between chlorophyll-*a* (µg/mL) and COD (mg O_2/L) for all treatments during the primary treatment batch test**

4.1.3 Biochemical Oxygen Demand

During the primary treatment batch test BOD_5 levels had decreased in all treatments and controls (Figure 4.6). In terms of the treatments, the lowest recorded ETC was exhibited by the 10% treatment with 77±1 mg O_2/L, whereas the highest recorded BOD_5 ETC was the 85% treatment, possessing a BOD_5 value of 191±11 mg O_2/L (Figure 4.6).

In terms of the control, a similar result was recorded with the 10% and 85% treatment possessing the lowest and highest recorded BOD_5 levels, respectively. The values recorded for the 10% and 85% controls were 155±6 and 781±11 mg O_2/L respectively (Figure 4.6).

The independent t-test analysis indicated significant differences ($p < 0.05$) in BOD_5 levels between each set of treatment and control at experiment termination on day 20 (Table 4.4). The control BOD_5 levels were therefore significantly higher than the treatment levels (Figure 4.6).

The spearman rank correlation demonstrated that there were significant relationships between BOD_5 levels and chl-*a* for the 10%, 25% and 50% treatments (Table 4.5). Furthermore, the highest recorded co-efficient of variation was 0.79 for the 10% treatment (Table 4.5). Figure 4.7 illustrates the relationship between BOD_5 and chl-*a*.

The slope analysis revealed that the gradients of the trendlines were consistently higher for the treatment than the control. The highest gradient recorded was the 50% treatment with a value of -9.7167 (Figure 4.8). The lowest treatment gradient recorded was the 10% treatment with a value of -3.8167. Overall in terms of the BOD_5 analysis, the lowest gradient recorded was the 25% control at -1.2167 (Figure 4.8).

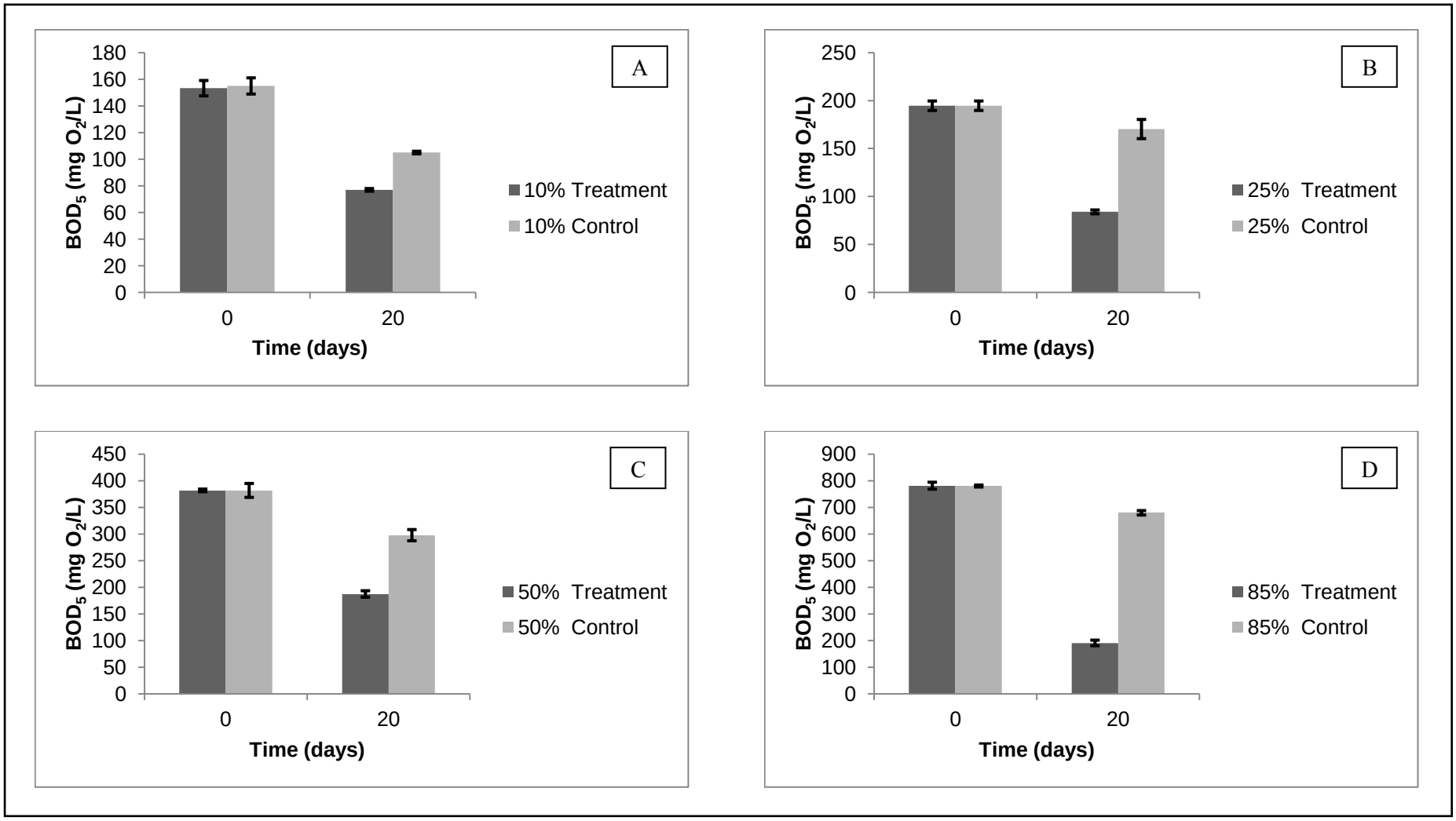

Figure 4.6 **Temporal dynamics of average BOD_5 (mg O_2/L) concentrations for treatments and controls during the primary treatment batch test. Vertical bars indicate standard deviation from the mean**

Table 4.4 **Summary of probability (p) values obtained from the independent samples t-test between BOD_5 of the treatment and control at the termination of the primary batch test**

		Treatment			
		10%	**25%**	**50%**	**85%**
Control	**10%**	0.001			
	25%		0.001		
	50%			0.001	
	85%				0.001

Table 4.5 **Statistical summary of Spearman Rank Correlation analysis between BOD_5 and chlorophyll-*a* during the primary treatment batch test**

	Treatment			
Statistic	**10%**	**25%**	**50%**	**85%**
p	0.02	0.02	0.02	0.08
R	-0.89	-0.88	-0.88	-0.76
R^2	0.79	0.77	0.77	0.58

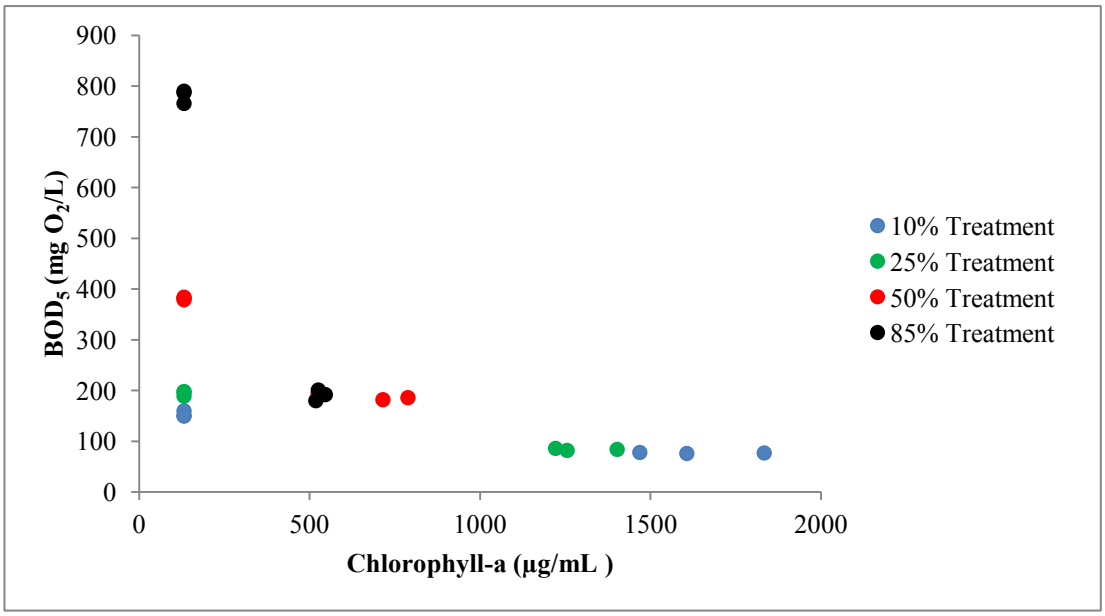

Figure 4.7 **Scatterplot to illustrate the relationship between chlorophyll-*a* (µg/mL) and BOD_5 (mg O_2 /L) for all treatments during the primary treatment batch test**

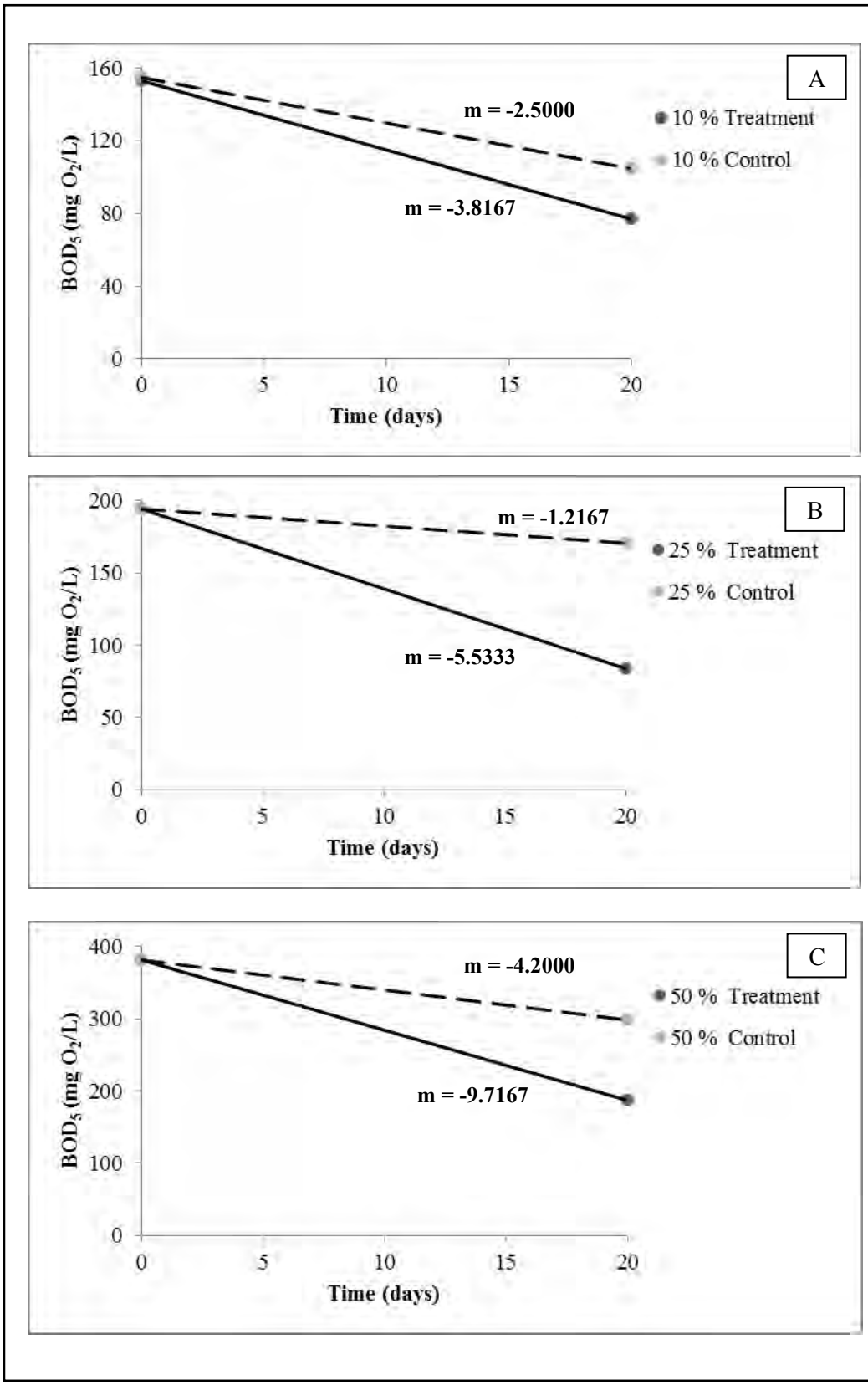

Figure 4.8 **Comparison of average BOD_5 trendlines of treatments and controls during the primary treatment batch test. The solid and dash line illustrate the trend in data for the treatment and control respectively**

4.1.4 pH

The pH levels recorded for the treatments and control during the batch test demonstrated a simultaneous increase from the onset of the experiment to the termination (Figure 4.9). However, it was recorded that there was a general decline in the first seven days of the experiment (Figure 4.9). Furthermore, the 10% treatment exhibited a considerable decline in pH on day 1 with a recorded pH of 6.41 ± 0.34. The highest recorded pH was the 85% treatment at the onset of the experiment at a pH of 9.95 ± 0.06 (Figure 4.9). In terms of the treatments, the lowest recorded pH at the termination point was the 25% treatment with a level of 9.08 ± 0.12 (Figure 4.9).

Although the treatments and controls exhibited simultaneous increases, the independent samples t-test indicated that the 25% control possessed a significantly higher pH than its associated treatment at experiment termination (Table 4.6).

It was determined that pH was significantly correlated to chl-*a* concentration for the 10% and 25% treatment (Table 4.7). The positive *rho* (*R*) values indicate that when chl-*a* concentration increased, there was a concomitant increase in pH (Table 4.7; Figure 4.10).

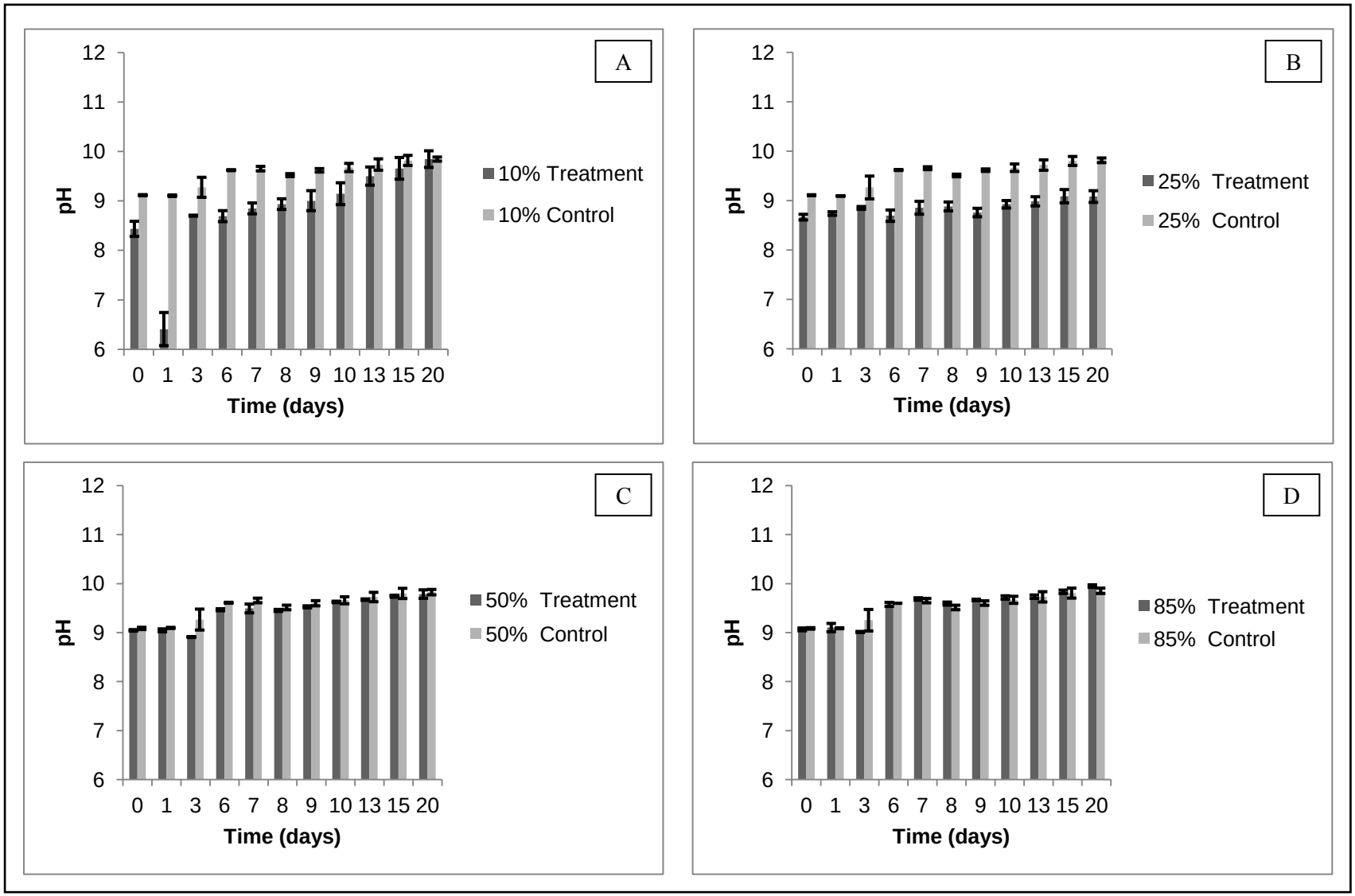

Figure 4.9 **Temporal dynamics of average pH for treatments and controls during the primary treatment batch test. Vertical bars indicate standard deviation from the mean**

Table 4.6 **Summary of probability (p) values obtained from the independent samples t-test between pH of the treatment and control at the termination of the primary treatment batch test**

		Treatment			
		10%	**25%**	**50%**	**85%**
Control	**10%**	1			
	25%		0.03		
	50%			0.54	
	85%				0.07

Table 4.7 **Statistical summary of Spearman Rank Correlation analysis between pH and chlorophyll-*a* during the primary treatment batch test**

	Treatment			
Statistic	**10%**	**25%**	**50%**	**85%**
p	0.001	0.001	0.14	0.10
R	0.88	0.74	0.25	0.29
R^2	0.77	0.55	0.06	0.08

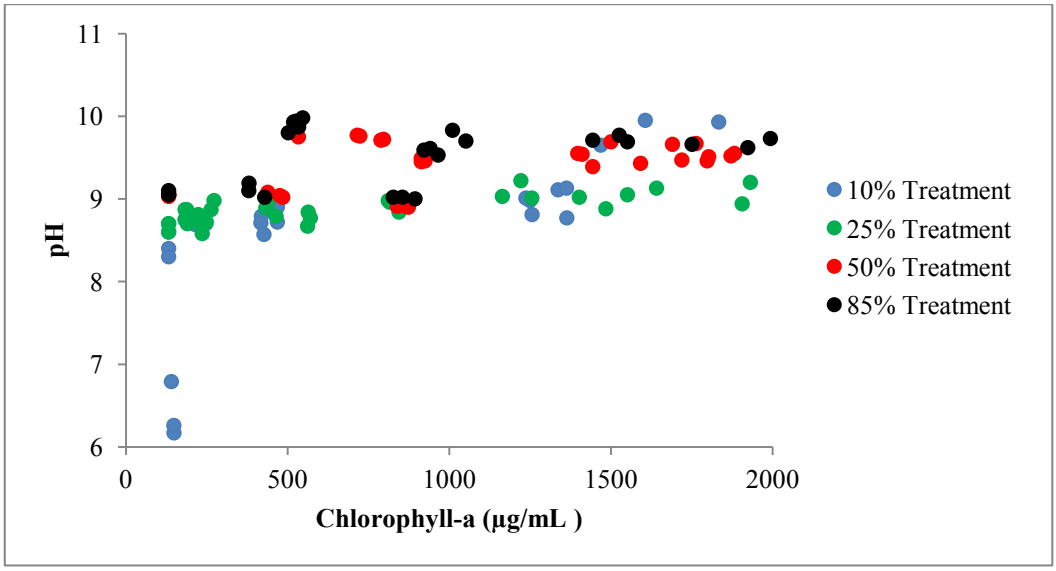

Figure 4.10 **Scatterplot to illustrate the relationship between chlorophyll-*a* (µg/mL) and pH for all treatments during the primary treatment batch test**

4.2 Secondary Treatment Batch Test

4.2.1 Nitrate

The batch test demonstrated a decline in the NO_3^- concentration for all treatments (Figure 4.11). Contrariwise, the controls exhibited no discernible decline in NO_3^- concentrations (Figure 4.11). The lowest recorded NO_3^- ETC was exhibited by the 10% treatment at 9.4 ±0.1 (Figure 4.11). Furthermore, the treatment that exhibited the highest ETC was the 25% treatment that possessed a concentration of 10.0 mg/L (Figure 4.11).

Nitrate concentrations were determined to be significantly different ($p < 0.05$) between treatments and controls at the termination of the batch test (Table 4.8). Therefore, final nitrate concentrations for all treatments were significantly lower than their respective controls (Table 4.8).

Spearman rank correlations indicated a significant relationship between NO_3^- and chl-*a* for all treatments (Table 4.9). In addition, the two variables are inversely related denoting that when one parameter increases, there is an associated decrease in the other (Table 4.9). The highest co-efficient of variation was possessed by the 50% treatment (R^2 = 0.89) (Table 4.9). Figure 4.12 illustrates the relationship between chl-*a* concentration and NO_3^- concentration for all treatments. In addition, it can be noted from Figure 4.12 that the 85% treatment possessed the highest concentration of chl-*a*, while the 10% treatment possessed the lowest concentration of chl-*a*.

The slope analysis indicated that there was great variability in the rate of NO_3^- concentration decline (Figure 4.13). The highest gradient and therefore rate of change, was exhibited by the 85% treatment with a gradient of -121.69 (Figure 4.13). In contrast, the lowest rate of change amongst the treatments was exhibited by the 10% treatment with a gradient of -11.135 (Figure 4.13). Furthermore, all treatment trendlines possessed substantially higher gradients than their respective controls.

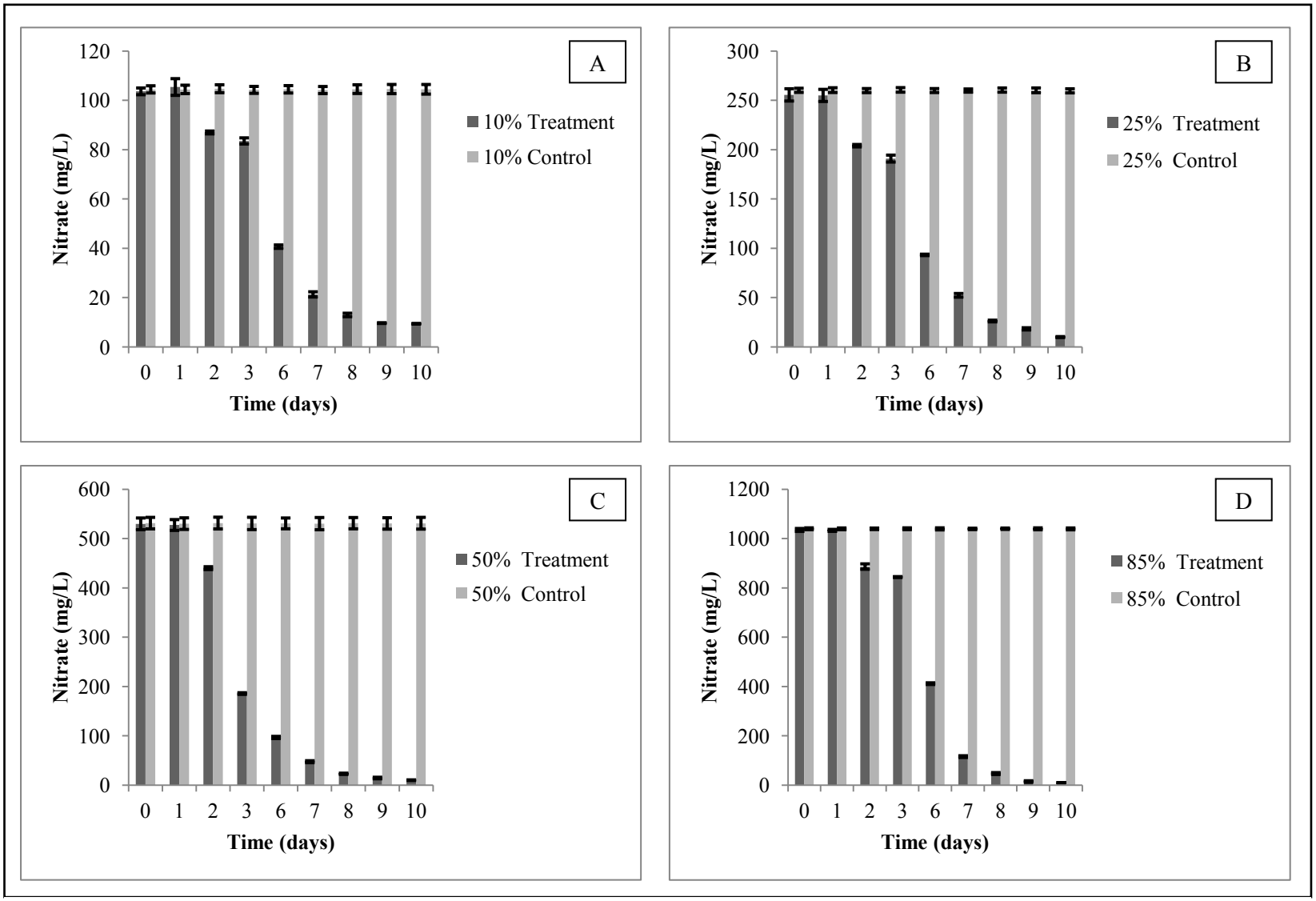

Figure 4.11 **Temporal dynamics of nitrate concentrations (mg/L) for treatment and controls during the secondary treatment batch test. Vertical lines indicate standard deviation**

Table 4.8 **Summary of probability (p) values obtained from independent samples t-test analysis between nitrate concentrations of the treatment and control at the termination of the secondary treatment batch test**

		Treatment			
		10%	**25%**	**50%**	**85%**
Control	**10%**	0.001			
	25%		0.001		
	50%			0.001	
	85%				0.001

Table 4.9 **Statistical summary of Spearman Rank Correlation analysis between nitrate and chlorophyll-*a* during the secondary treatment batch test**

	Treatment			
Statistic	**10%**	**25%**	**50%**	**85%**
p	0.001	0.001	0.001	0.001
R	-0.87	-0.91	-0.93	-0.80
R^2	0.76	0.83	0.86	0.64

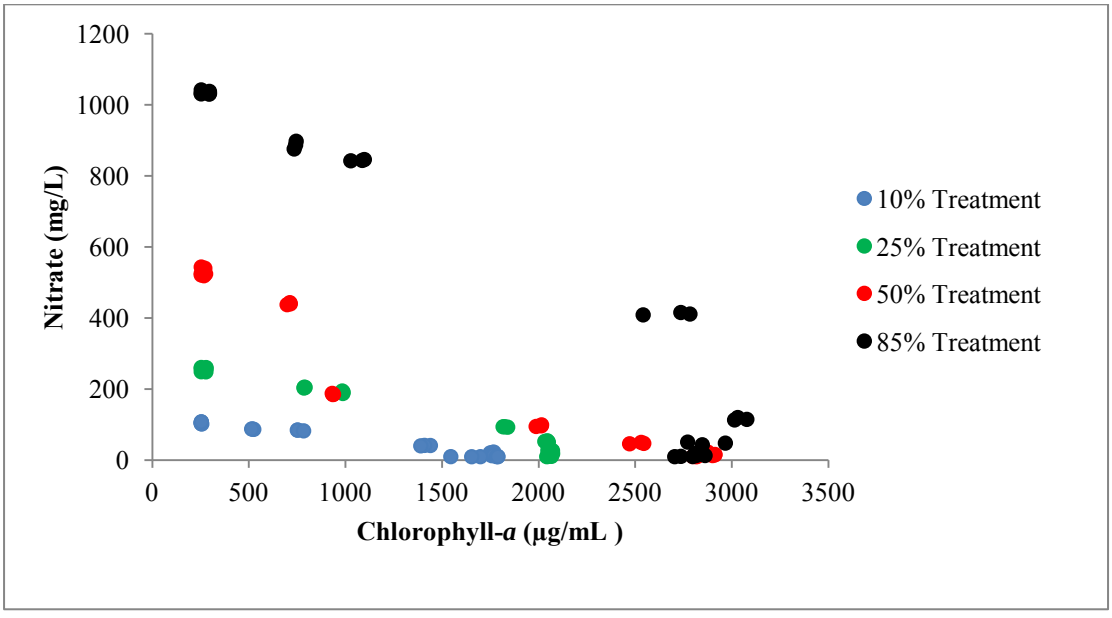

Figure 4.12 **Scatterplot to illustrate the relationship between chlorophyll-*a* (µg/mL) and nitrate (mg/L) for all treatments during the secondary treatment batch test**

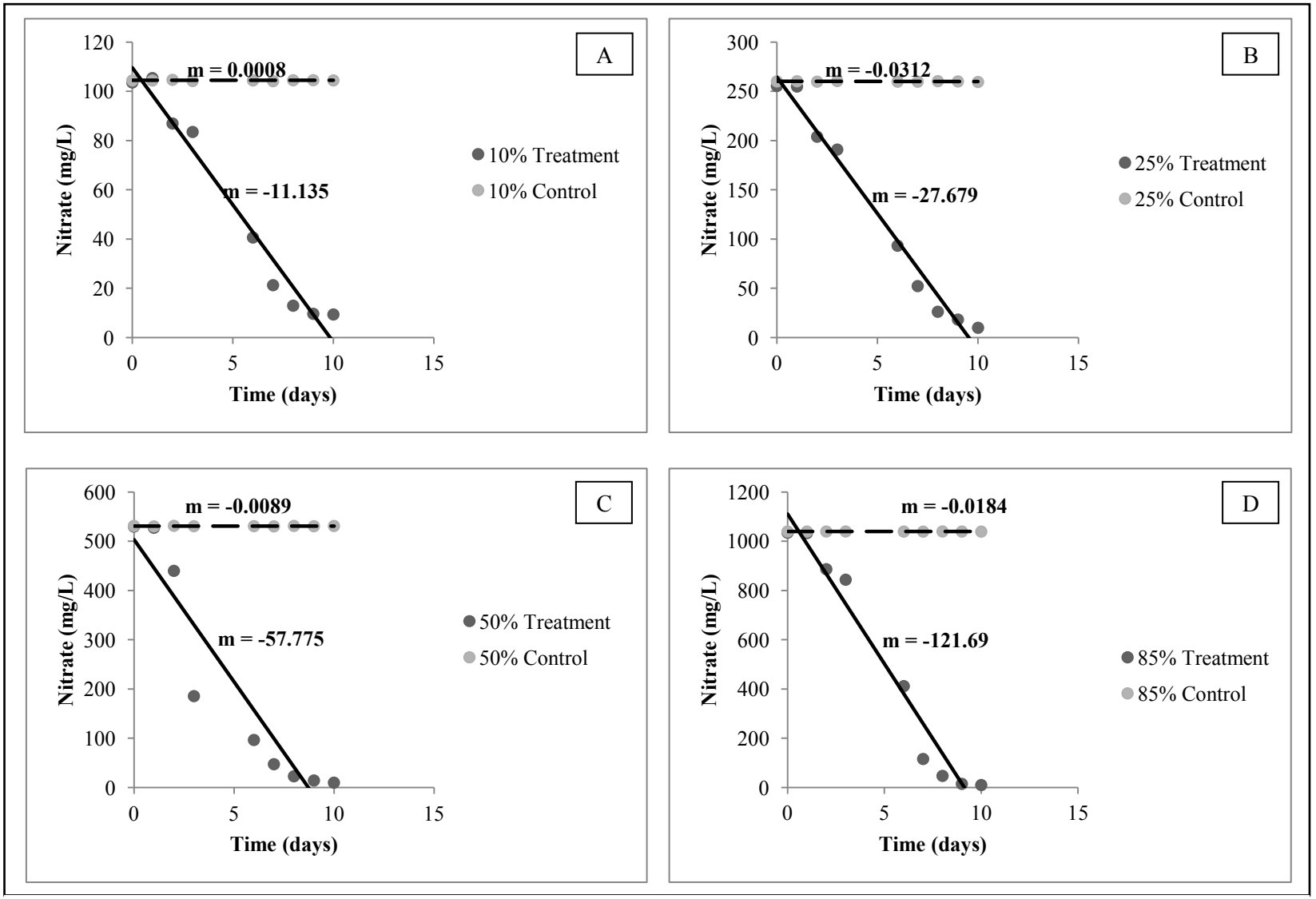

Figure 4.13 **Comparison of average nitrate trendlines of treatments and controls during the secondary treatment batch test. The solid and dash line illustrate the trend in data for the treatment and control respectively**

4.2.2 Chemical Oxygen Demand

COD concentrations for the secondary treatment batch test demonstrated temporal declines for both the treatments and controls (Figure 4.14). The lowest recorded average COD ETC was exhibited by the 10% treatment at 18.95 ±0.34 mg O_2/L (Figure 4.14). In terms of the treatments, the 50% treatment possessed the highest COD level at 105.26 ±2.61 mg O_2/L (Figure 4.14).

The independent samples t-test indicated that there were significant differences between treatments and controls at experiment termination (Table 4.10). This denotes that treatments possessed significantly lower COD levels than the controls (Table 4.10).

The spearman rank correlation analysis indicated that there was only a significant ($p < 0.05$) relationship between chl-*a* and COD for the 50% treatment (Table 4.11). The correlation denotes that for the treatment the two variables were inversely related (Table 4.11). Furthermore, any changes in either variable are accounted for approximately 88% by the other (Table 4.11).

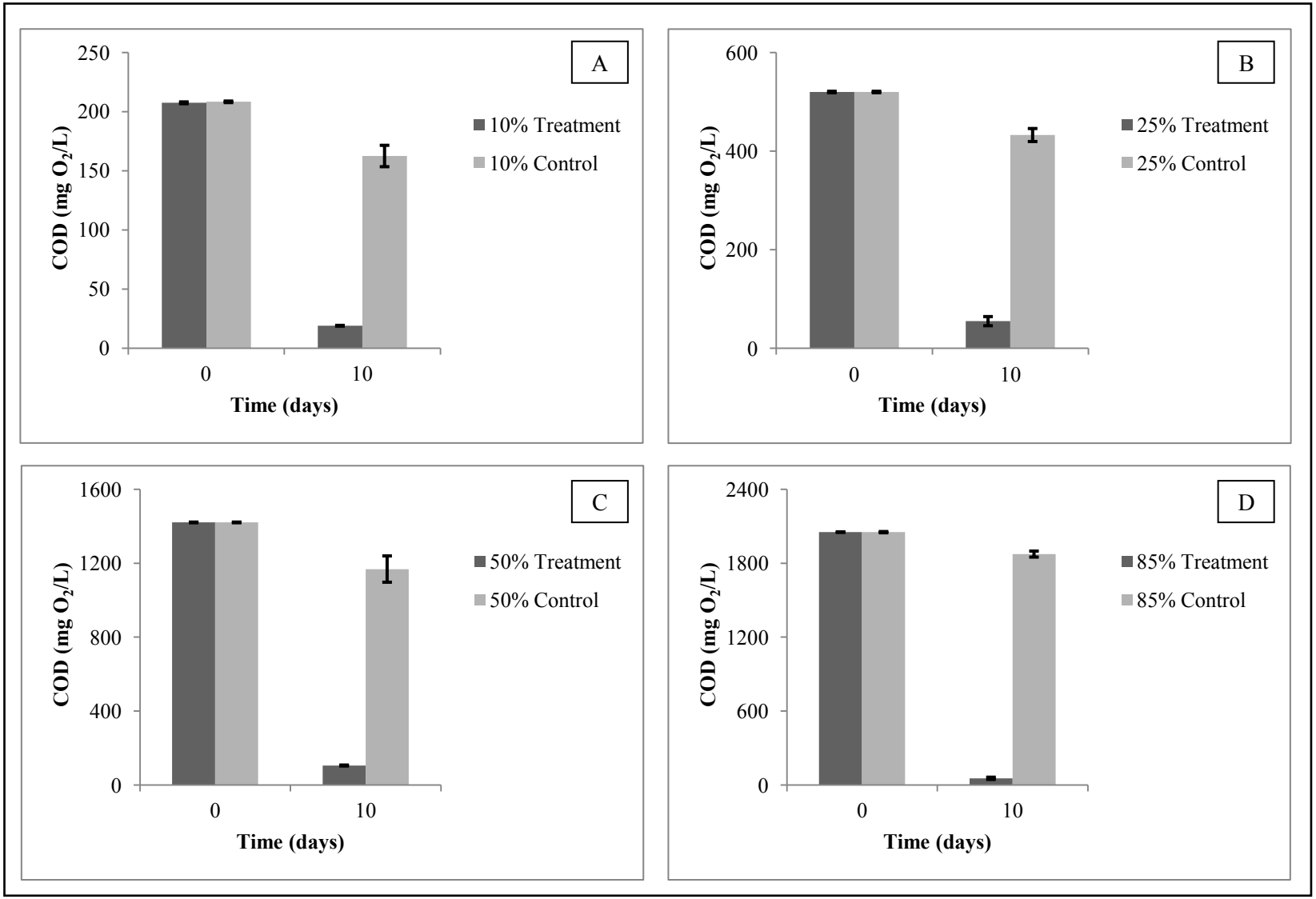

Figure 4.14 **Temporal dynamics of COD concentrations (mg O_2 /L) for treatment and controls during the secondary treatment batch test. Vertical lines indicate standard deviation**

Table 4.10 **Summary of probability (p) values obtained from independent samples t-test analysis between COD concentrations of the treatment and control at the termination of the secondary treatment batch test**

		Treatment			
		10%	**25%**	**50%**	**85%**
Control	**10%**	0.001			
	25%		0.001		
	50%			0.001	
	85%				0.001

Table 4.11 **Statistical summary of Spearman Rank Correlation analysis between COD and chlorophyll-*a* during the secondary treatment batch test**

	Treatment			
Statistic	**10%**	**25%**	**50%**	**85%**
p	0.1228	0.08	0.005	0.08
R	-0.70	-0.76	-0.94	-0.76
R^2	0.49	0.58	0.88	0.58

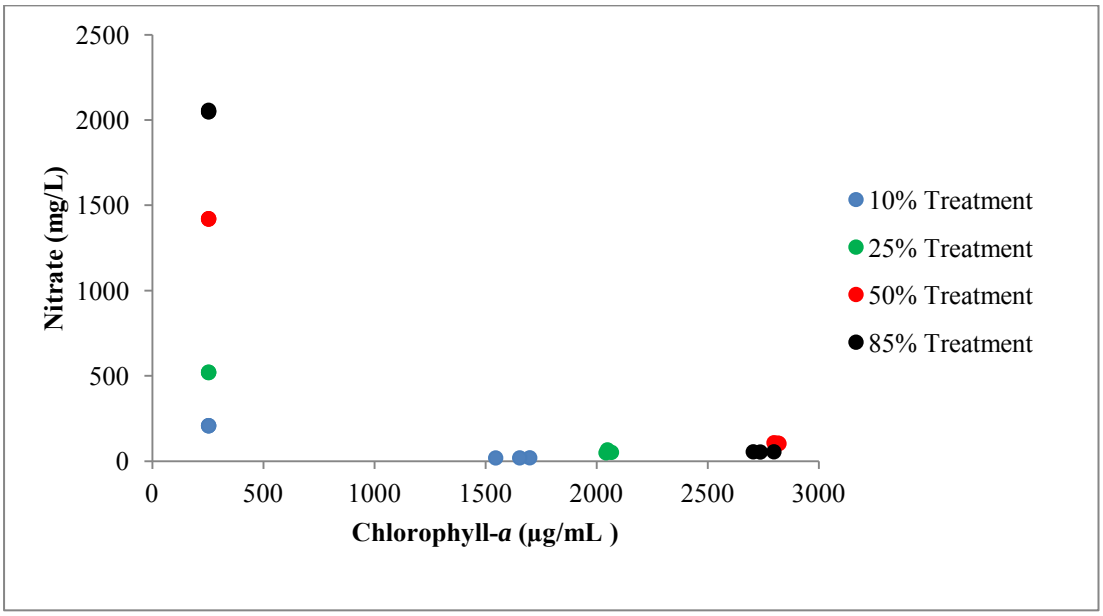

Figure 4.15 **Scatterplot to illustrate the relationship between chlorophyll-*a* (µg/mL) and COD (mg O_2 /L) for all treatments during the secondary treatment batch test**

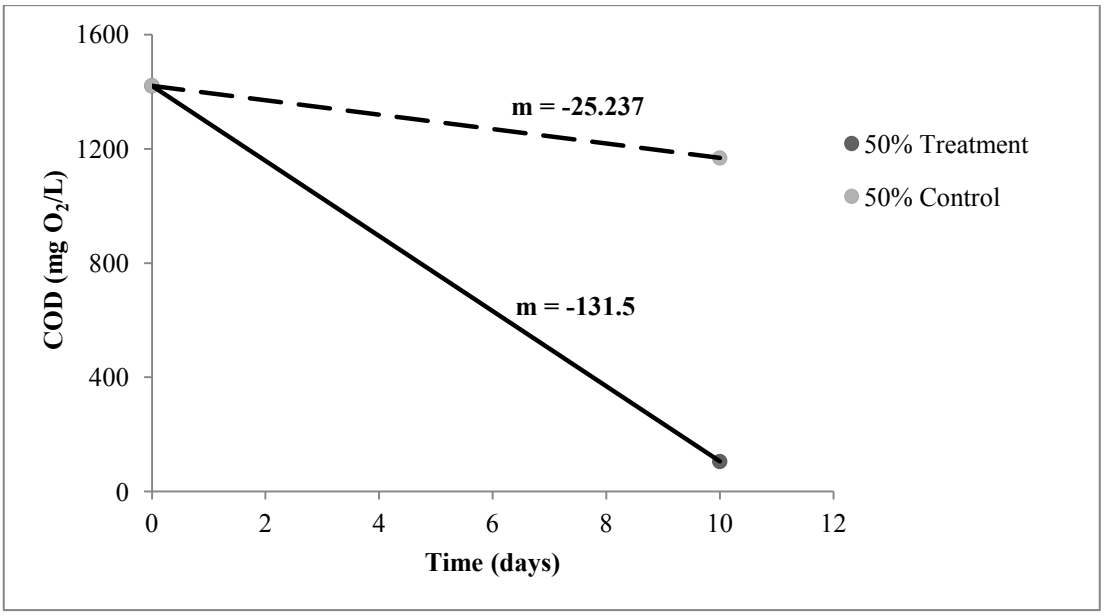

Figure 4.16 **Comparison of average COD trendline of the 50% treatment and control during the secondary treatment batch test. The solid and dash line illustrate the trend in data for the treatment and control respectively**

4.2.3 Biochemical Oxygen Demand

BOD_5 levels for the secondary treatment batch tests demonstrated temporal declines (Figure 4.17). Upon experiment termination the lowest and highest recorded BOD_5 level was exhibited by the 10% and 85% treatments, respectively (Figure 4.17). The 10% and 85% treatments possessed an average ETC of 17 ±1 and 232 ±6 mg O_2/L, respectively (Figure 4.17). In addition, the independent samples t-test indicated significant differences between control and treatments ETC (Table 4.12).

Spearman rank correlations indicated significant ($p < 0.05$) a relationship between BOD_5 and chl-*a* for all treatments except the 25% treatment (Table 4.13). The highest co-efficient of variation was displayed by the 10% treatment ($R^2 = 0.88$) (Table 4.13). Figure 4.18 illustrates the relationship between chl-*a* and BOD_5.

The slope analysis indicates that from amongst the treatments the highest gradient was exhibited by the 85% treatment (-19.493), whereas in contrast the 10% treatment possessed the lowest gradient (-1.9967) (Figure 4.19). However, overall the 85% control possessed the lowest gradient (-0.4467) (Figure 4.19).

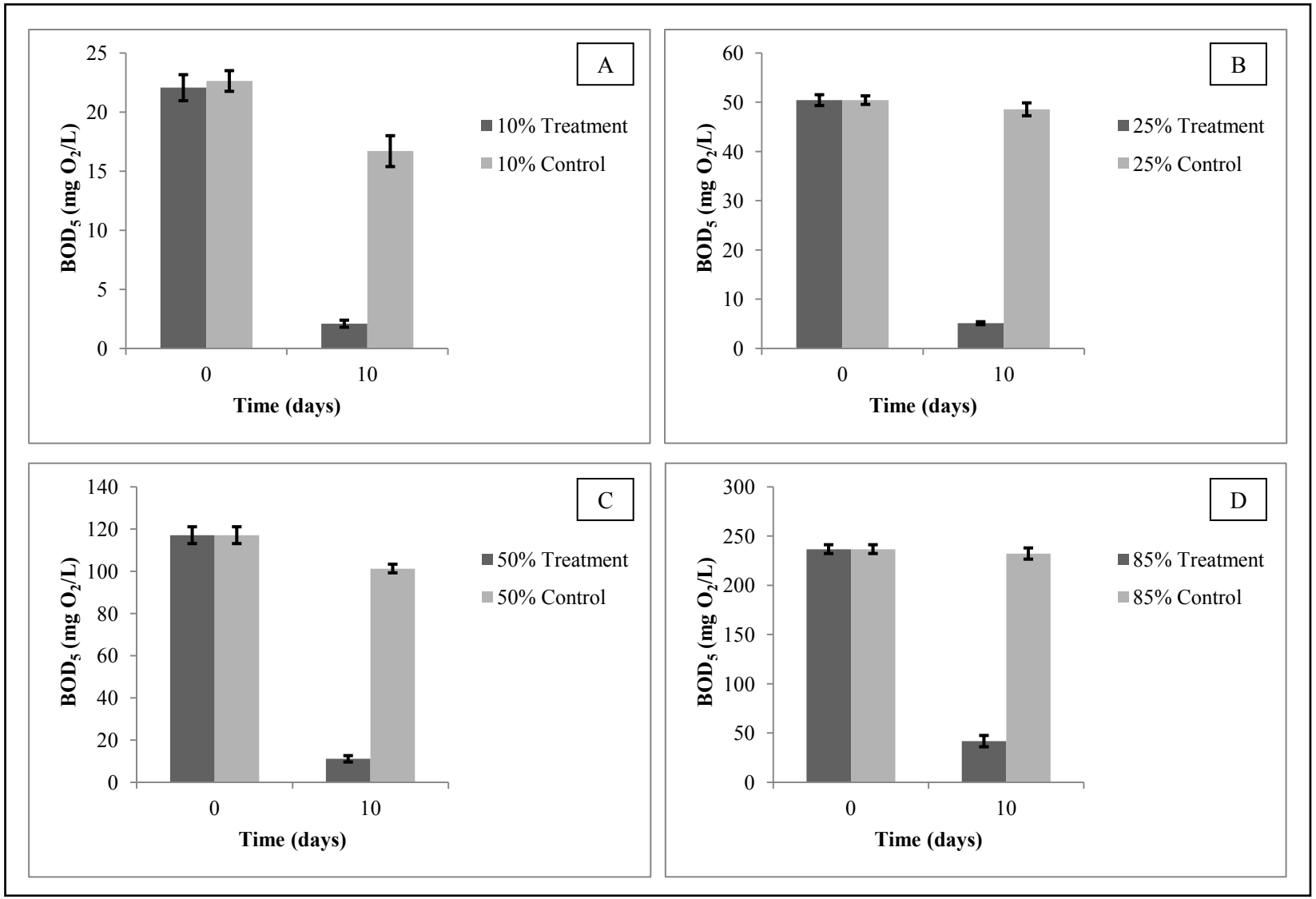

Figure 4.17 **Temporal dynamics of BOD_5 concentrations (mg O_2 /L) for treatment and controls during the secondary treatment batch test. Vertical lines indicate standard deviation**

Table 4.12 **Summary of probability (p) values obtained from independent samples t-test analysis between BOD_5 concentrations of the treatment and control at the termination of the secondary treatment batch test**

		Treatment			
		10%	**25%**	**50%**	**85%**
Control	**10%**	0.002			
	25%		0.001		
	50%			0.001	
	85%				0.001

Table 4.13 **Statistical summary of Spearman Rank Correlation analysis between BOD_5 and chlorophyll-*a* during the secondary treatment batch test**

	Treatment			
Statistic	**10%**	**25%**	**50%**	**85%**
p	0.005	0.08	0.02	0.02
R	-0.94	-0.76	-0.88	-0.88
R^2	0.88	0.58	0.77	0.77

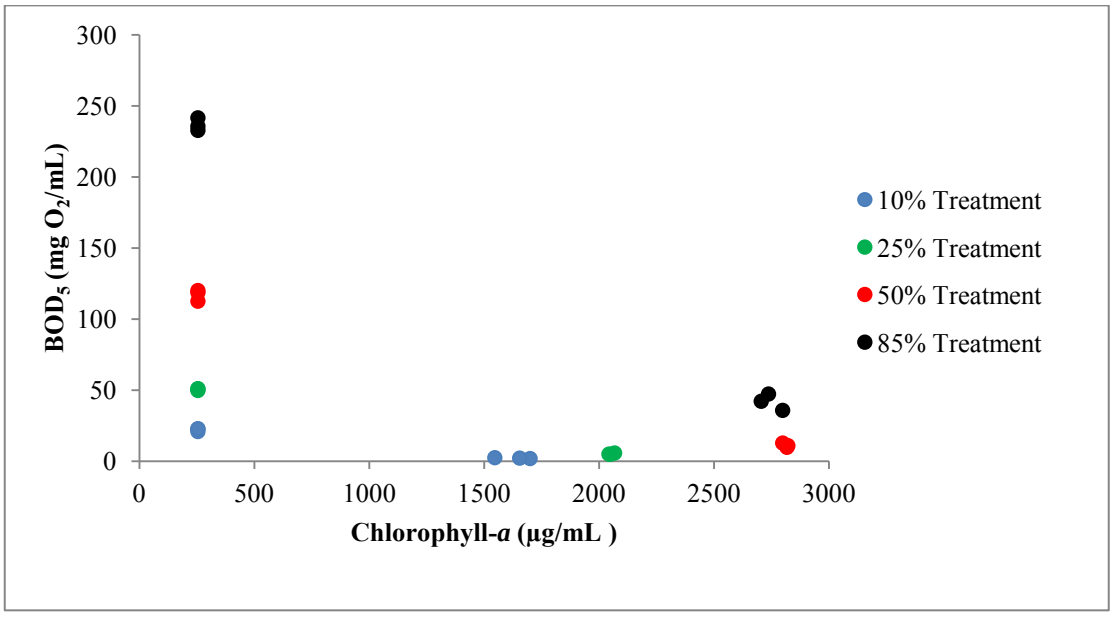

Figure 4.18 **Scatterplot to illustrate the relationship between chlorophyll-*a* (µg/mL) and BOD_5 (mg O_2 /L) for all treatments during the secondary treatment batch test**

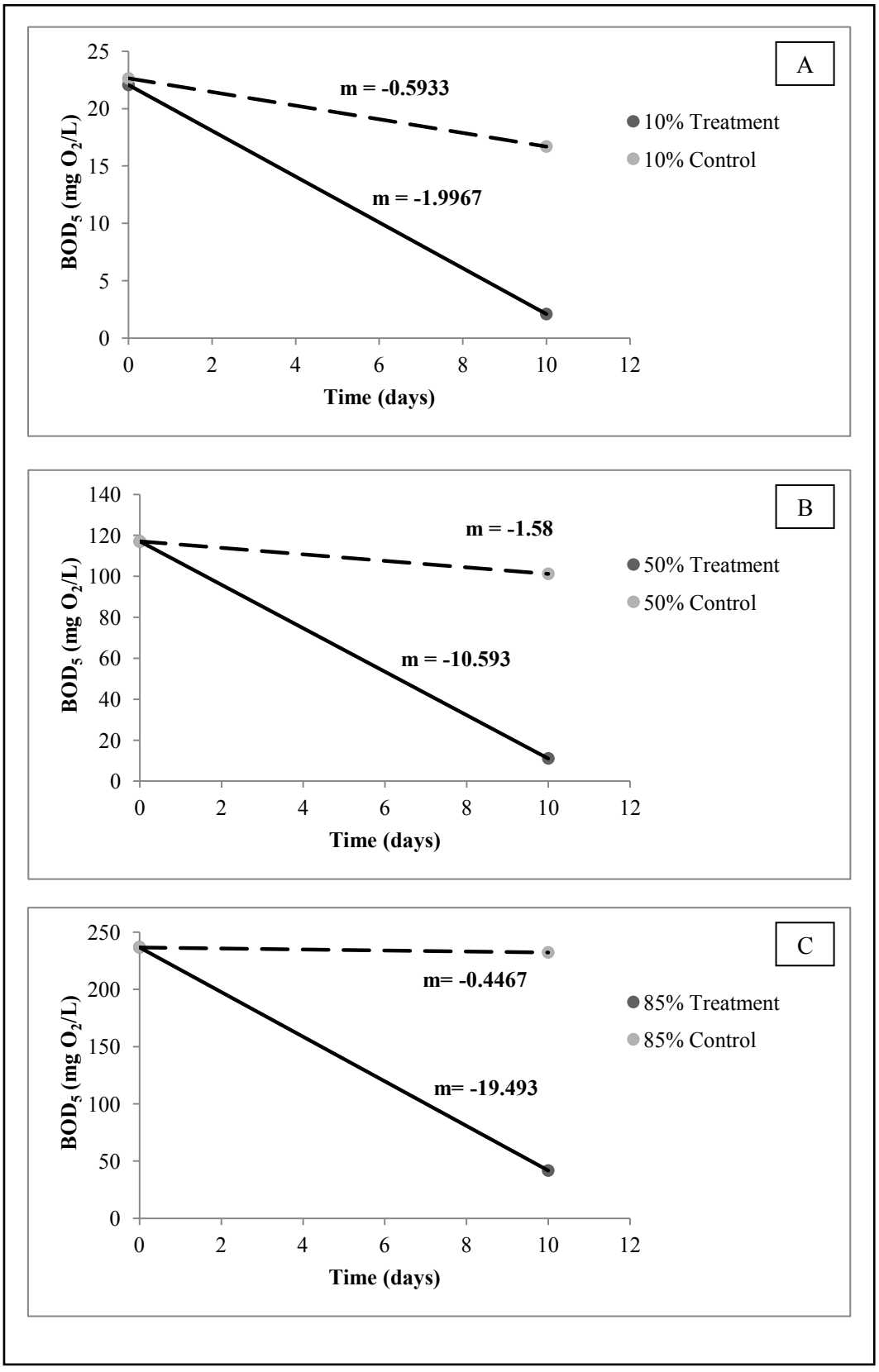

Figure 4.19 **Comparison of average BOD_5 trendlines of treatments and controls during the secondary treatment batch test. The solid and dash line illustrate the trend in data for the treatment and control respectively**

4.2.4 pH

pH levels for the secondary treatment batch test demonstrated substantial temporal variability (Figure 4.20). Furthermore, there was a general increase for both treatments and controls for the duration of the experiment (Figure 4.20). However, there was a recorded decrease in average pH for the controls on day 2 with a subsequent average increase on day 3 (Figure 4.20).

The independent samples t-test indicated that there were only significant differences ($p < 0.05$) between the 85% treatment and control upon termination of the experiment (Table 4.14). The treatment possessed an average pH of 10.81 ±0.14, while the control possessed an average pH of 9.75 ±0.16 (Figure 4.20).

The spearman rank correlation indicated that there were significant relationships between pH and chl-*a* concentration for all treatments apart from the 85% treatment (Table 4.15). The highest co-efficient of variation was exhibited by the 10% treatment ($R^2 = 0.90$) whereas the lowest was possessed by the 25% treatment (($R^2 = 0.90$) (Table 4.15). Figure 4.21 illustrates the relationship between pH and chl-*a*.

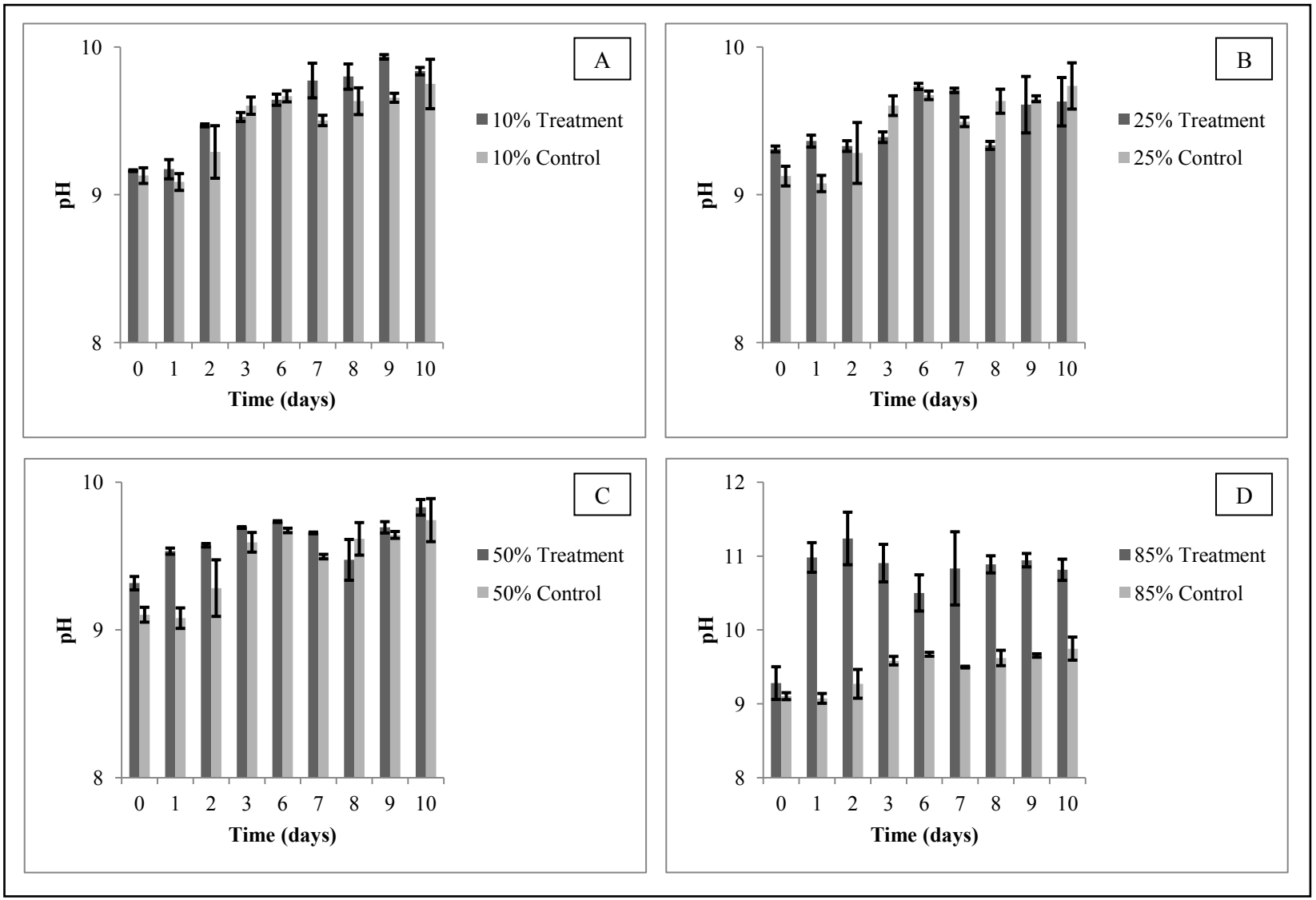

Figure 4.20 **Temporal dynamics of pH for treatment and controls during the secondary treatment batch test. Vertical lines indicate standard deviation**

Table 4.14 **Summary of probability (p) values obtained from independent samples t-test analysis between pH of the treatment and control at the termination of the secondary treatment batch test**

		Treatment			
		10%	**25%**	**50%**	**85%**
Control	**10%**	0.46			
	25%		0.46		
	50%			0.42	
	85%				0.001

Table 4.15 **Statistical summary of Spearman Rank Correlation analysis between pH and chlorophyll-*a* during the secondary treatment batch test**

	Treatment			
Statistic	**10%**	**25%**	**50%**	**85%**
p	0.001	0.01	0.01	0.49
R	0.95	0.47	0.48	0.14
R^2	0.90	0.22	0.23	0.02

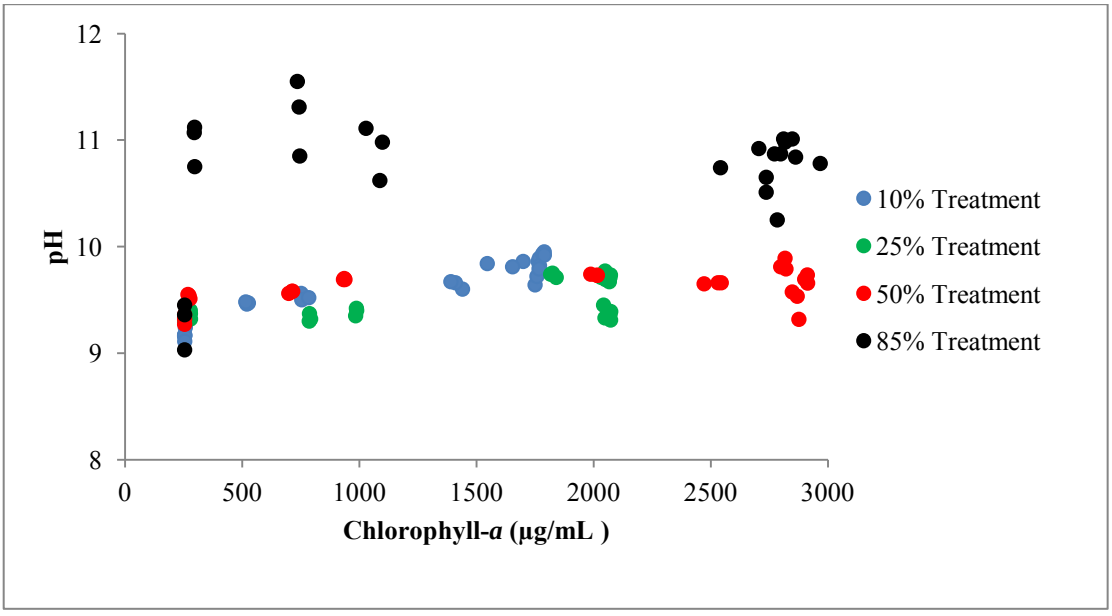

Figure 4.21 **Scatterplot to illustrate the relationship between chlorophyll-*a* (µg/mL) and pH for all treatments during the secondary treatment batch test**

4.2.5 Toxicity Test

Toxicity tests demonstrated substantial variability in the percentage of seeds germinated for treatments (Table 4.16). In terms of the treatments, the highest percentage of seeds germinated was recorded for the 10% treatment at an average of 96±4% while the lowest was recorded for the 85% treatment (Table 4.16). Overall, the lowest percentage of seeds germinated was recorded for the 85% control at an average of 18 ±3%. In addition, there were notable differences in the percentage of seeds germinated between the treatments and their respective controls (Table 4.16).

The Mann Whitney *U* test indicated that there were no significant differences between the median values of percentage of seeds germinated for treatments and controls (Table 4.17). However, the Kruskal Wallis analysis indicated a significant difference ($p < 0.05$) between median values of the percentage of seeds germinated for the 10% and 85% treatments (Table 4.18).

Table 4.16 **Summary of the percentage of Radish (*Raphanus sativus*) seeds germinated in toxicity tests for the treatments and controls**

10%		25%		50%		85%	
Control	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**
25±5	96±4	22±3	44±8	18±3	58±4	18±3	29±4

Table 4.17 **Summary of probability (p) values obtained from the Mann Whitney *U* test between treatments and controls for the percentage of Radish (*Raphanus sativus*) seeds germinated in toxicity tests**

		Treatment			
		10%	**25%**	**50%**	**85%**
Control	**10%**	0.08			
	25%		0.07		
	50%			0.07	
	85%				0.07

Table 4.18 **Summary of probability (p) values obtained from the Kruskal Wallis Test test between the percentage of Radish (*Raphanus sativus*) seeds germinated in toxicity tests for the treatments**

	Treatment		
	10%	**25%**	**50%**
25%	0.20		
50%	0.70		
85%	0.01	0.70	0.20

With regards to the treatments, the lowest MTG was recorded for the 10% treatment at at an average of 2.11 ±0.77 days, whereas the highest recorded MTG was the 85% treatment at an average of 4.15 ±0.86 days (Table 4.19). Overall the highest average MTG recorded was exhibited by the 85% control at an average of 4.44±1.26 days (Table 4.19).

However, the Mann Whitney *U* test indicated that there were no significant differences between the median values of MTG for treatments and controls (Table 4.20). Furthermore, the Kruskal Wallis analysis indicated that there were no significant differences between the treatment MTG (Table 4.21).

Table 4.19 **Summary of the mean germination time in days for Radish (*Raphanus sativus*) seeds in toxicity tests for the treatments and controls**

10%		**25%**		**50%**		**85%**	
Control	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**
4.22±0.51	2.11±0.77	4.29±0.40	3.51±0.46	4.29±0.36	4.13±0.67	4.44±1.26	4.15±0.86

Table 4.20 **Summary of probability (p) values obtained from the Mann Whitney *U* test between treatments and controls for the mean germination time of Radish (*Raphanus sativus*) seeds germinated in toxicity tests**

		Treatment			
		10%	**25%**	**50%**	**85%**
Control	**10%**	0.10			
	25%		0.12		
	50%			1	
	85%				1

Table 4.21 **Summary of probability (p) values obtained from the Kruskal Wallis Test test between treatments for the mean germination time of Radish (*Raphanus sativus*) seeds germinated in toxicity tests**

	Treatment		
	10%	**25%**	**50%**
25%	0.32		
50%	0.81	0.91	
85%	0.17	0.99	0.99

4.3 Comparison between Primary Treatment and Secondary Treatment

The primary and secondary treatment batch tests had reduced their respective nitrogenous pollutants to below the discharge limits (Table 4.22). All primary treatments reduced NH_3-N by 100% as the Experiment Termination Concentration (ETC) were 0 mg/L (Table 4.22). Based on average values this was achieved in 15 days for the 10% treatment and 20 days for the 25%, 50% and 85% treatment.

Based on average values, nitrate levels below discharge limits was achieved in 8 days for the 10% treatment, 10 days for the 25% treatment and 9 days for the 50% and 85% treatment. The highest recorded reduction in NO_3^- concentration was achieved by the 85% secondary treatment with a 99% reduction (Table 4.22). The lowest reduction in NO_3^- concentration was exhibited by the 10% treatment (Table 4.22).

Pertaining to COD, only the 25% and 85% secondary treatments achieved final concentrations below the discharge limits (Table 4.22). The highest percentage reduction in COD level was achieved by the 85% secondary treatment, whereas the lowest percentage reduction was demonstrated by the 85% primary treatment (Table 4.22). Furthermore, the 85% primary treatment and the 85% secondary treatment exhibited the highest and lowest COD ETC, respectively (Table 4.22). Conversely, the 50% primary treatment demonstrated the highest increase in COD, with a 46% increase. The 10% primary treatment had also exhibited an increase in COD (Table 4.22).

All treatments demonstrated a reduction in BOD_5 levels upon termination of the respective experiments (Table 4.22). The lowest BOD_5 ETC was possessed by the 10% secondary treatment, whilst the highest was possessed by the 85% primary treatment (Table 4.22). However, the former exhibited the lowest reduction in BOD_5 levels. Furthermore, the largest reduction was achieved by the 50% secondary treatment with a 91% reduction (Table 4.22).

Table 4.22 **Summary of principle parameter records for the primary treatment and secondary treatment batch tests. Average values of Experiment Termination Concentration (ETC) and the Percentage Change (PC) from the initial concentration are indicated. Positive PC values indicate an increase in parameter concentration and negative PC values indicate a decrease in parameter concentration.**

Parameter	**Primary Treatment**								**Secondary Treatment**							
	10%		25%		50%		85%		10%		25%		50%		85%	
(General value discharge limits)	ETC	PC	ETC	PC	ETC	PC	ETC	PC	ETC	PC	ETC	PC	ETC	PC	ETC	PC
NH3-N (mg/L) **(3 mg/L)**	0.0	-100	0.0	-100	0.0	-100	0.0	-100	-	-	-	-	-	-	-	-
NO_3^- (mg/L) **(15 mg/L)**	-	-	-	-	-	-	-	-	9.4	-91	10	-96	9.9	-98	9.8	-99
COD (mg O_2/L) **(75 mg O_2/L)**	361.03	14	288.82	-35	2454.97	46	2805.68	-21	207.44	-91	55.19	-89	105.26	-93	54.02	-97
BOD_5 (mg O_2/L) **(N/A)**	77.0	-50	84.0	-57	187.0	-51	191.0	-76	2.0	-90	5.0	-90	11.0	-91	42	-82

5. DISCUSSION AND CONCLUSION

5.1 Primary Treatment Batch Test

Ammoniacal-nitrogen concentrations for the treatments and controls declined throughout the batch test. The primary cause of decline in NH_3-N in the control was possibly ammonia oxidation by microbes. However, the decline and eventual absence of NH_3-N in the treatments was possibly due to the combination of ammonia oxidation by microbes and ammonia assimilation by *Chlorella*. This was indicated by the significance of the correlations that indicated a decline of NH_3-N was associated with an increase in *Chlorella*. In addition, the higher gradient of NH_3-N for all the treatments, when compared to their respective controls, alludes to assimilation by *Chlorella* as a driver of NH_3-N abatement in the treatments. This is further supported by the relatively high co-efficient of variations for the 10% and 25% treatments.

In order to confidently determine if *Chlorella* were the principle cause of NH_3-N decline, an experiment wherein the leachate would be sterilised through autoclaving and the experiment conducted in an enclosed photo-bioreactor would have to been undertaken. However, this does not mimic the actual nature of leachate where constituents include a microbial population.

The duration of NH_3-N abatement varied amongst the treatments with the 10% treatment exhibiting the lowest time. The longer duration of the higher concentration treatments are likely due to the toxicity of high levels of ammonia to *Chlorella* (Lin *et al.,* 2000). Once the microalgae are inoculated into the leachate with a relatively high concentration of ammonia from the seed culture, *Chlorella* would require an acclimatisation period, hence requiring a longer duration for the assimilation of NH_3-N into metabolic pathways (Lin *et al.,* 2000).

The rates of NH_3-N abatement varied between treatments with the 85% treatment possessing the highest rate of decline. This was possibly attributed to the presence of microalgae within the treatment assimilating the NH_3-N. However, the co-efficient of variation indicates that the relatively high rate of NH_3-N decline is more likely attributed to other factors including microbial metabolic activity within the leachate.

The rate of decline in the other treatments, while higher than their respective controls, may be lower than the 85% treatment due to their diluted nature. The dilution procedure may reduce microbe populations within the diluted treatments and therefore not achieve

the rate of NH_3-N removal. However, the dilution reduces the NH_3-N concentration to levels that the algae can tolerate and thus the algae are possibly the primary removers of NH_3-N. The 10% possessed the lowest concentration of NH_3-N at the onset of the batch test and is possibly the reason that NH_3-N was not recorded on day 15, whilst all other treatments possessed NH_3-N.

Regarding COD, none of the treatments had achieved an ETC level below discharge limits. Furthermore, statistical analysis revealed that there were no significant differences ($p > 0.05$) between the treatment and controls. This suggests that primary treatment by *Chlorella* sp. is not effective in abating COD levels to discharge limits. However, the spearman rank correlation analysis indicated a significant relationship between *Chlorella* and COD for the 85% treatment. The possible reason is that as water quality improved, indicated by the decrease in COD, *Chlorella* sp. are able to tolerate the leachate conditions and thus grow.

In the cases where the treatments COD concentration had exceeded the control, this may have resulted from the excretion of organic compounds from the algae themselves. According to Helebust (1965) although microalgae tend to excrete 3%-6% of their assimilated carbon during the logarithmic growth phase, approximately 17%-38% is exuded at the end of a bloom. Furthermore, higher rates of carbon compound excretion occur during periods of stress such as nutrient depletion (Hulatt and Thomas, 2011). Therefore, there was the possibility that after growth phase in the 10% and 50% treatments there was excrement of carbon, thereby increasing COD levels. In addition, cell lysis and Programmed Cell Death (PCD) may account for the increase in COD levels. These phenomena may have caused the increase in COD levels recorded after 7 days. Consequently, the utilisation of microalgae for the abatement of carbon compounds in the primary treatment of leachate is ineffective. However, this does not indicate that COD should not be measured when microalgae are used as the primary treatment, as it is an important leachate quality determinand considering the potential impact on the receiving environment, but it suggests that removal of algae prior to a population crash due to PCD is required.

BOD_5 levels had decreased in all treatments and controls with the treatments possessing significantly ($p < 0.05$) lower concentrations. Furthermore, significant relationships were present between *Chlorella* sp. and BOD_5 levels for the 10%, 25% and 50% treatment. This denotes that *Chlorella* were possibly effective in improving the BOD_5 of the leachate when diluted to these levels. This was expected as many species in the

genus *Chlorella* are able to shift between CO_2 and organic compounds as a carbon source due to their ability to undergo heterotrophic growth (Samejima and Myers, 1958; Perez-Garcia, 2011). Under conditions favourable for photosynthesis, *Chlorella* undergo autotrophic growth wherein CO_2 is converted to carbohydrates and O_2 produced. All organisms including microalgae utilise the same metabolic pathways for respiration, with the consumption of oxygen and the production of carbon dioxide (Perez-Garcia, 2011). In microalgae that are able to undergo heterotrophic growth, including *Chlorella*, dark respiration occurs during periods with no available light for photosynthesis and the carbon source is other available organic compounds rather than CO_2 (Samejima and Myers, 1958; Perez-Garcia, 2011). Consequently, the growth of *Chlorella* sp. would have abated organic compounds during heterotrophic growth.

The 50% treatment possessed the highest rate of BOD_5 reduction with the spearman rank correlation suggesting that *Chlorella* accounting for approximately 77% of the variability in BOD_5. However, similar co-efficients of variation were recorded for the 10% and 25% treatments that possessed lower rates of BOD_5 decline. This suggests that the extent of assimilation by *Chlorella* is similar for all treatments, but the lower dilution in the 50% treatment implies that it possessed additional microbes that aided in decreasing BOD_5 levels at a higher rate.

pH levels increased from the baseline value during the duration of the batch test. This was possibly attributed to the uptake of CO_2 leading to a reduced formation of carbonic acid thereby increasing pH (Lin *et al.*, 2007). However, correlation analysis indicated a significant relationship between pH and chl-*a* for the 10% and 25% treatments. Therefore in these treatments microalgae growth are the principle influencers of pH. The pH was possibly further influenced by the consumption of acidic organic compounds such as carboxylic acids by micro-organisms (Kjeldsen *et al.* 2002). Furthermore, this was possibly the reason for the increase in pH for the controls and 50% and 85% treatment.

The decline in pH for the initial seven days of the batch test may be attributed to the uptake of ammonium (Shi *et al.*, 2000). Considering that pH plays a vital role in biochemical processes within organisms (DWS, 1996), the reduction in pH possibly had toxic affects to the microalgae, particularly in the 10% treatment (Shi *et al.*, 2000). This further explains the increase in COD after the initial 7 days. The rapid decline in the 10% treatment was possibly due to a lack of buffering capacity of the diluted leachate.

The shifting of the leachate from an acidic medium to a basic medium has important implications for the treatment of landfill leachate. Acidic mediums tend to increase the toxicity and bioavailability of metals including silver, aluminium, cadmium, cobalt copper, mercury, manganese, lead and zinc (DWS, 1996). Therefore, increasing the pH to a basic medium will reduce the toxicity of the leachate. Conversely, high pH values increase the toxicity of NH_3-N as unionised NH_3 is the dominant form (DWS, 1996).

5.2 Secondary Treatment Batch Test

The secondary treatment batch test exhibited declines in NO_3^- for all treatments in contrast to the control that exhibited no discernible decrease. Furthermore, all treatments recorded ETC of NO_3^- below the established DWS discharge limits. ETC for NO_3^- in treatments were significantly ($p < 0.05$) lower than ETC for controls. Taking into consideration that NO_3^- is the primary source of nitrogen for microalgae (Vilchez *et al.*, 1997), the results indicate that *Chlorella* sp. accounted for the decline in NO_3^- in the treatments. This is further supported by significance and co-efficient of variations determined by the spearman rank correlation.

The highest and lowest rate of NO_3^- decline was recorded in the 85% and 10% treatments, respectively. This may be attributed to the biomass of *Chlorella* sp. within the treatments. In general, when microalgae are exposed to nutrient limited conditions, they demonstrate reduced photosynthetic activity and biomass growth (Hao *et al.*, 2012). Conversely, higher nutrient concentrations are able to support relatively higher algal abundances (Shi *et al.*, 2000). Therefore, the higher concentration of NO_3^- in the 85% treatment denotes that it had the potential to support a higher abundance of *Chlorella* sp. when compared to the 10% treatment. Hence, the higher abundance of *Chlorella* was able to assimilate the NO_3^- at a relatively higher rate. Contrariwise, the relatively low NO_3^- concentration in the 10% treatment signifies that it was unable to support an algal abundance on par with the 85% treatment. Accordingly, the relatively lower abundance of *Chlorella* assimilates NO_3^- at a relatively lower rate when compared to the 85% rate.

Secondary treatments exhibited temporal declines in COD levels with treatments possessing significantly lower levels than the controls. However, there was only a significant relationship between COD and chl-*a* concentrations for the 50% treatment. Therefore, the decline in COD may be accounted for by the growing population of *Chlorella* sp. assimilating the carbon compounds that were present in the leachate.

However, the treatment was ineffective in reducing levels to below established discharge limits.

The lack of a significant relationship between *Chlorella* and COD for the other treatments, allude to the possibility of amelioration by microbial metabolism as the principal driver in COD abatement. The possible cause for the disparity between treatments may be attributed to a factor not analysed that demonstrates the variability between the treatments.

The BOD_5 levels for the secondary treatment declined during the batch test period, with significant differences recorded in ETC between experiments and their respective controls. Furthermore, significant negative correlations were recorded between BOD_5 and chl-*a*. This postulates that *Chlorella* sp. assimilated organic compounds significantly reducing BOD_5 levels. This was congruent with the primary treatment and indicates the uptake of organic carbon compounds by *Chlorella* sp. (Samejima and Myers, 1958).

The highest co-efficient of variation was demonstrated by the 10% treatment. This postulates that the algae primarily accounted for the decline in BOD_5 for the 10% treatment and may have been a result of the dilution process. Dilution of the leachate may have resulted in a diminution of microbial populations, thereby reducing competition for organic compound assimilation. In addition, the highest rate of decline recorded was for the 85% treatment and was possibly because the treatment possessed the highest recorded biomass of *Chlorella*. The relatively high biomass of *Chlorella* and possible microbial population present suggests the potential high rate of amelioration.

The pH of the secondary treatments increased during the period of the batch test. This is possibly caused by the uptake of carbon dioxide by *Chlorella* sp. (Hullard and Thomas, 2011). In addition, the removal of nitrate increases pH due to the production of hydroxyl ions (Goldman and Brewer, 1980), postulating from the relatively high rate of NO_3^- and CO_2 uptake by *Chlorella*. The 85% treatment possessed the highest levels of pH and was determined to be significantly different from the control, but was not significantly correlated to chl-*a*. The increase in pH was possibly influenced by the utilisation of acidic organic compounds by microbes (Kjeldsen *et al.* 2002). The shift from an acidic to a basic medium has further implications for the treatment of landfill leachate as discussed above.

Toxicity tests revealed substantial variability between the toxicity of the leachate subsequent to secondary treatment with *Chlorella* sp. The 10% treatment possessed the highest percentage of seeds germinated and the lowest mean time to germinate indicating that it was the least toxic from all treatments and controls. Statistical analysis indicated that the percentage of seeds germinated for the 10% treatment was significantly lower than the 85% treatment. This was possibly caused by the dilution of the 10% treatment possessing lower concentrations of potential toxins than the 85% treatment.

However, the lack of significant differences between treatments and controls allude to the presence of a toxin or toxins that were not abated through *Chlorella* sp. metabolic pathways, particularly in consideration of the variability of leachate toxin composition (Kjeldsen *et al.*, 2010).

5.3 Conclusion

The discharge of landfill leachate to the natural environment is one of the foremost ecological impacts pertaining to the disposal of solid waste (Kjeldsen *et al.*, 2010). This is especially considering that possible negative physiological effects that may be observed in aquatic organisms even under a 1:1 000 dilution scenario (Baderna *et al*., 2011). Therefore, the treatment of landfill leachate prior to release is an imperative practice. Accordingly, the experiments undertaken, tested the utilisation of the microalgae *Chlorella* sp. as the primary and secondary treatment of landfill leachate, particularly focusing on the amelioration of nitrogenous compounds.

The results from batch tests indicated that *Chlorella*, although effective at reducing NH_3-N in the primary treatment process, was temporally inefficient when compared to other treatment options. In addition, *Chlorella* sp. was not effective in lowering COD to below discharge limits when utilised as the primary treatment and has the potential to exacerbate COD levels of the leachate through the release of carbon compounds from stressed microalgae as well as PCD. Furthermore, Lin *et al.* (2000) has reported on the toxicity of the relatively high NH_3-N concentrations associated with leachate on microalgae. Therefore, the use of *Chlorella* sp. as the primary treatment of leachate is considered unfeasible.

However, the use of *Chlorella* sp. as the secondary treatment of leachate demonstrates positive results. *Chlorella* sp. is able to effectively reduce NO_3^- to below discharge limits and may assist in the decrease of organic chemical compounds. In addition, the

shifting of leachate to a basic medium lowers the bioavailability of certain heavy metals. However, it was noted that toxicity of leachate was not significantly reduced although an improvement in the quality of the leachate was recorded.

In conclusion, it is recommended that *Chlorella* sp. should not be utilised as the primary treatment of landfill leachate and that further studies should focus on utilising microalgae species as the secondary or tertiary treatment options. Furthermore, studies should not focus on monocultures of species but rather using an array of taxa as particular taxonomic groups may favour the growth of other species that are able to improve leachate quality (de-Bashan *et al.,* 2004). de-Bashan *et al.* (2004) demonstrated that a co-immobilised treatment system with microalgae and microalgae-growth promoting bacteria enhanced the uptake of nitrogen and phosphorus from wastewater. In addition, further leachate phycoremediation studies should also include testing the efficacy of a treatment system possessing a diversity of functional growth forms. This is suggested as different taxonomic groups possess different metabolic pathways and nutrient requirements and uptake capabilities (Vilchez *et al.*, 1997; Wallentinus, 1984). Therefore, a hetero-cultural treatment system may enable the amelioration of leachate through the metabolism of the suite of compounds present in the leachate.

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