EFFECT OF GRAIN SIZE, BED DEPTH AND RETENTION TIME IN INTERMITTENT BIOSAND FILTERS FOR IMPROVEMENT OF SECONDARY SEWAGE EFFLUENT QUALITY

BY

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DECLARATION

Declaration by the candidate

This thesis is my original work and has not been presented for a degree in any other University. No part of this thesis may be reproduced without the prior written permission of the author and/or University of Eldoret.

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Declaration by supervisors

This thesis has been submitted for examination with our approval as University Supervisors.

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To my husband, Semeon, and my lovely daughters; June, Mercy and Babra and son, Emmanuel.

ABSTRACT

Biosand filtration (BsF) is a simple technique used for pathogen and particle removal in drinking water purification. It may be adapted for wastewater stabilization but only a few studies have been done on improvement of wastewater quality using BsF. Application of Biosand filters (BsFs) for wastewater treatment has spread rapidly across the globe in recent years, creating a need for sound scientific understanding of mechanisms and factors controlling BsF microbial removal. The primary aim of this study was to evaluate the effects of sand grain size, bed depth and retention time in BsFs on secondary sewage effluent quality before disposal into waterways. This was achieved by comparing effluent quality data from filtered and unfiltered effluent. The BSFs were assembled with varying sand bed depths and sand grain sizes. The study was a three factorial design consisting of 27 treatments with three levels each of sand bed depth, sand grain size and retention time. The samples of filtered and unfiltered effluents were collected on a monthly basis for six consecutive months and analyzed for efficiency of the various designs of BsFs. Removal efficiency was determined in terms of selected parameters such as total coliforms (TC), faecal coliforms (FC), faecal *Streptococcus* (FS), total bacterial counts (TBC) and physicochemical parameters such as biochemical oxygen demand (BOD), conductivity, pH, Total Suspended Solids (TSS) and nutrients as well as percentage increase in dissolved oxygen (DO). ANOVA was used to evaluate performance of the various BSFs and Duncan's Multiple Range Test to separate means if the differences were significant. Results of this study indicated that there was a significant number of bacteria present in the raw or unfiltered effluent compared to water treated by filtration through various filters. In addition, there were significant differences in the removal efficiency of the different filters. Sand bed depth, retention time and sand grain size influenced filter performance for all the dependent variables under investigation. The physicochemical parameters decreased significantly in effluents from fine sand grain size (0.1- 0.5) mm filters while the bacteriological parameters decreased significantly in effluents from large sand (1.1-2.0) mm filters. Sand bed depth affected removal of most physicochemical parameters (except PO_4^{3-}) and removal was better for the longest depth (0.7 m). However, removal of bacteria in effluents was not significantly affected by sand bed depth. The highest removal of both physicochemical and bacteriological parameters under study was achieved for the longest period of retention (72 hours) in the filters. The study recommends that for efficiency of BsFs in removal of bacteria, retention time should be extended to 72 hours, sand grain size should be between 1.1-2.0 mm and sand bed depth need not be longer than 0.5 m. However, if the removal of physicochemical parameters is targeted, sand bed depth should be increased to 1.0 m, sand grain size should be fine (between 0.1-0.5 mm) and the retention time should also be extended to 72 hours.

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LIST OF ABBREVIATIONS AND ACRONYMS

BA	Bile Aesculin agar
BOD	Biochemical Oxygen Demand
BOD_5	Biochemical Oxygen Demand (5 day-period)
BsF	Biosand Filtration
BsFs	Biosand Filters
CFUs	Colony Forming Units
CV^+	Crystal Violet ions
CV-I	Crystal Violet- Iodine complex
DMAB	Dimethylaminobenzaldehyde
DO	Dissolved Oxygen
FAO	Food and Agriculture Organization (of the United States)
FC	Faecal Coliforms
FS	Faecal *Streptococcus*/ci
GIT	Gastro Intestinal Tract
HPC	Heterotrophic Plate counts
IMViC	Indole, Methyl Red, Voges Proskauer, Citrate
KOH	Potassium Hydroxide
MR	Methyl Red
MR-VP broth	Methyl Red-Voges Proskauer broth
NA	Nutrient Agar
NaCl	Sodium Chloride
NHMRC-ARMCANZ	National Health and Medical Research Council and Agriculture and Resource Management Council of Australia and New Zealand
NRC	National Research Council

PVC	PolyVinyl Chloride
TBC	Total Bacterial Counts
TC	Total Coliforms
TN	Total Nitrates
TP	Total Phosphates
TPC	Total Plate Counts
TSS	Total Suspended Solids
UoE	University of Eldoret
US EPA	United States Environmental Protection Agency
USDOD	United States Department of Defense
VP	Voges Proskauer
WHO	World Health Organization

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CHAPTER ONE

INTRODUCTION

1.1 Background information

The world is faced with problems related to management of wastewater due to extensive industrialization, increasing population density and urbanization of societies (McCasland *et al*., 2008). Wastewater treatment plants and especially sewage effluent treatment plants are aimed at reducing the pollutant load on the environment but in most cases the effluent that is released into water ways is still high in BOD_5, nutrients (N and P) and bacterial load thus posing danger to the receiving environment (Morrison *et al*., 2001; USDOD, 2004). Effluent generated from domestic and industrial activities constitutes the major source of natural water pollution load (Amir *et al*., 2004). Many infectious diseases are associated with faecally contaminated water and are a major cause of mortality worldwide (Leclerc *et al*., 2001; Theoron & Cloete, 2002).

Intermittent or continually operated BsF is a simple process that can be used to reduce the pollutant load of secondary wastewater (Logsdon, 2002). Filtration of secondary wastewater is necessary in order to eliminate the bacteria and other pollutants that exist in these wastewaters before they can be discharged into natural waterways. Biosand filtration is a simple technology for purification of surface water and an effective particle and pathogen filter that combines biological and physical mechanisms (Logsdon, 2002). It is one of the earliest forms of water treatment and an efficient process of reducing water contamination (Rooklidge *et al*., 2009). However, little work has been done on the application of BsFs in wastewater quality improvement (Adin, 2003). The process is passive and the effectiveness of the filters

is dependent upon the development of a biofilm attached to sand grains called the hypogeal. The hypogeal is a biologically active mat that develops on the filter surface that functions to remove the pathogens and organic wastes from water (Weber-Shirk & Chan, 2006).

Use of intermittent and continually operated BsFs in wastewater treatment has recently gained popularity, especially in the context of wastewater use (Sadiq *et al.*, 2003). In this study, the effluent from University of Eldoret sewage treatment plant was sampled and analysed using standard methods to determine its characteristics and intermittent BsFs were used to improve its quality thereafter. The objective of using the intermittent BsF in this study was to reduce the pollutant load of effluent in the receiving water bodies.

1.2 Statement of the problem

Health problems and specifically waterborne diseases are often caused by discharging untreated or inadequately treated effluent into waterways. The biggest risk to health is the presence of disease-causing pathogens that are present in untreated or partially treated wastewater (EPA, 2000; Choukr-Allah & Hamdy, 2001; Sharma *et al*., 2010). Bacteria are the most common microbial pathogens found in wastewater (Rusin *et al*., 2000). Disease-causing microbes in these wastewaters can lead to diarrhea, cramps, nausea, and headaches (EPA, 2000; Meays *et al*., 2004). These pathogens pose severe health risks to infants and people with compromised immune systems (EPA, 2000).

WHO (2003) reported that in developing countries, including Kenya, 90% of the wastewater was discharged without treatment thereby harming both the environment

and drinking water sources and that of the 1.8 million deaths that were caused by diarrheal diseases every year, 88% were due to unsafe water supplies. It was also estimated that 5.7% of all diseases and 4% of all deaths worldwide were caused by inadequate water, sanitation and hygiene (Pruss *et al*., 2002). The release of raw or partially treated effluent into water bodies may also lead to other problems such as fish kills and algal blooms resulting from high organic contents in the wastewater (EPA, 2000; Eynard *et al*., 2000).

1.3 Justification of the study

With increased volumes of treated wastewater being targeted for reuse, there is need to develop reliable methods to mitigate the health risks that can be caused by microorganisms in wastewater. Biosand filtration is a simple process used for pathogen and particle removal in potable water purification and may be adapted for tertiary treatment of sewage effluent. However, only a few studies have been conducted on its use in treatment of wastewater and the filtration of effluent from wastewater treatment processes is a relatively new technology (Caliskaner & Tchobanoglous, 2000; Metcalf & Eddy, 2003). Increasing the knowledge about the dynamics of BsFs is highly important as this will aid in optimizing filtration systems for improved and stable operation efficiency. This includes understanding of how performance is affected by variations in design, construction materials, sand characteristics, and household operation and maintenance practices. Such knowledge would provide a rational basis to inform development of design standards, quality control measures, and guidelines for local construction and operation to maximize BsF performance in local settings. The purpose of this study was therefore to evaluate the effects of sand grain size, bed depth and retention time in intermittent BsFs at

laboratory scale for improvement of secondary effluent quality from University of Eldoret sewage treatment plant.

1.4 Objectives of the study

1.4.1 Broad objective

To evaluate the effects of sand grain size, bed depth and retention time in intermittent BsFs on secondary effluent quality.

1.4.2 Specific objectives

i) To determine selected indicator bacterial counts in unfiltered and filtered effluents.
ii) To determine the levels of selected physicochemical parameters in unfiltered and filtered effluent.
iii) To determine the effects of sand grain size, sand bed depth and retention time in intermittent BsFs on secondary effluent quality.

1.5 Research questions

i) Do the numbers of selected indicator bacteria and total bacterial counts differ in unfiltered and filtered effluents?
ii) Do the levels of selected physicochemical parameters differ in unfiltered and filtered effluents?
iii) Do sand grain size, sand bed depth and retention time have effects in BsFs on secondary effluent quality?

CHAPTER TWO

LITERATURE REVIEW

2.1 Bacteriological indicators of water quality

Sewage water contains disease-causing microorganisms (EPA, 2000; Rusin *et al*., 2000; Girones *et al*., 2010). These microorganisms cause water-borne diseases such as typhoid, dysentery and cholera (EPA, 2000). The number of different types of pathogens present in water as a result of pollution with human or animal excreta is very large and it is not possible to test water samples for each specific pathogen (Gerba, 2000; Steven, 2003). The presence of pathogens is therefore normally indirectly detected by studying indicator organisms (Paillard *et al*., 2005) the presence of which simply indicates that pathogens are expected to be present (Warrington, 2001; Fresno County Department of Public Health, 2009). The most common indicator organisms include heterotrophic plate counts, total coliform bacteria, faecal coliform bacteria, *Escherichia coli*, faecal streptococci, *Clostridium perfringens* and bacteriophages (Gerba, 2000; WHO, 2003).

Microbial indicators of water quality enter water at the same time as faeces and are easier to identify than the full range of pathogens. There are several qualities that are desirable for water quality indicators (NHMRC-ARMCANZ, 2001; NRC, 2001; WHO, 2003; Bitton, 2005):

• They must be universally present in the faeces of humans and warm-blooded animals in large numbers

• They should be readily detected by simple methods

• They should not grow in natural waters or the general environment

• They should be persistent in water and the extent to which they are removed by water treatment should be similar to those of waterborne pathogens.

2. 1.1 Coliforms

Coliform is a term first used in the 1880s to describe rod-shaped bacteria isolated from human faeces. The coliform group of bacteria belong to the family Enterobacteriaceae (Kilb *et al*., 2003). They comprise many genera and species that ferment lactose (Leclerc *et al*., 2001) and include total coliforms and faecal coliforms. These genera are *Escherichia, Klebsiella*, *Citrobacter* and *Enterobacter* among others (Gerba, 2000: Leclerc *et al*., 2001).

Total coliforms have been defined by APHA *et al*., (2005) and Madigan and Martinko (2008) as all facultatively anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose with gas and acid formation within 48 hours at 35°C. They exist naturally in the intestines of warm-blooded animals and so are present in faeces in very large numbers (approximately 108-1010 per gram). However, they can also be found in unpolluted environments so their presence in water does not necessarily indicate that water is faecally polluted (Madigan & Martinko, 2008). Of the total coliforms present in the human gut, *E. coli* represents a majority of the population (Edberg *et al*., 2000).

The faecal coliforms are a subgroup of coliforms that differ from the total coliform group by being able to grow at higher temperatures 44 ± 0.5^{o}C (APHA *et al*., 2005; Doyle and Erickson, 2006) and are found only in the faecal matter of warm-blooded animals (Leclerc *et al*., 2001; Rompre *et al*., 2002). They are more specific and are

better indicators of faecal pollution than total coliforms (Maier *et al*., 2000; Rompre *et al*., 2002; Doyle and Erickson, 2006). The use of total coliforms is common but thermo-tolerant faecal coliforms are only used as co-indicators of water pollution (Edberg *et al*., 2000). The division of TC members into faecal and non-faecal groups is shown in Table 1.

Table 1: Division of the total coliforms into faecal and non-faecal groups

Bacteria	Faecal	Non-faecal
Citrobacter	+	+
Enterobacter	+	+
Escherichia	+	-
Klebsiella	+	+

Source: Leclerc et al., 2001.

+ and - mean present and absent respectively.

The concept of coliforms as bacterial indicators of microbial water quality is based on the fact that coliforms are present in high numbers in the faeces of humans and other warm-blooded animals and if faecal pollution is present, these bacteria would be present (Figueras *et al*., 2000; Edberg *et al*., 2000; Leclerc *et al*., 2001). With few exceptions, coliforms themselves are not considered to be a health risk, but their presence indicates that faecal pollution may have occurred and pathogens might be present as a result (NRC, 2001).

Escherichia coli is a facultatively anaerobic Gram-negative, rod-shaped and non-sporulating bacterium that is commonly found in the lower intestines of warm-blooded organisms (Edberg *et al*., 2000). Most *E. coli* strains are harmless, but some

serotypes can cause serious food poisoning in humans (Hudault *et al*., 2001). The harmless strains form part of the normal flora of the gut and benefit their hosts by producing vitamin K2 and by preventing the establishment of pathogenic bacteria within the intestine (Hudault *et al.*, 2001). *Escherichia coli* constitute about 0.1% of gut flora (Eckburg *et al.*, 2005) and faecal-oral transmission is the major route through which pathogenic strains of the bacterium cause disease. Cells are able to survive outside the body for a limited period which makes them ideal indicator organisms to test environmental samples for faecal contamination (Feng *et al.*, 2002). The genus belongs to the coliform group of bacteria and is a member of the Enterobacteriaceae family (George, 2005). Optimal growth of *E. coli* occurs at 37°C but some laboratory strains can multiply at temperatures of up to 49°C (Furtado *et al.*, 2005). *Escherichia coli* is the only member of the total coliform group of bacteria that is found exclusively in the faeces of humans and other animals (Edberg *et al*., 2000; Doyle and Erickson, 2006) and its presence indicates not only recent faecal contamination but also the possible presence of disease-causing bacteria, viruses, and protozoa (Edberg, 2000).

Escherichia coli is therefore regarded as the most sensitive indicator of faecal pollution (Edberg *et al*., 2000). The large numbers of *E. coli* present in the gut of humans and other warm-blooded animals and the fact that they are not generally present in other environments support their continued use as the most sensitive indicator of faecal pollution available (Edberg *et al*., 2000; Meays *et al*., 2004). However, because *E. coli* is not as resistant to disinfection as intestinal viruses and protozoa, its absence does not necessarily indicate that intestinal viruses and protozoa are also absent.

Enterobacter aerogenes is also a Gram-negative rod-shaped bacterium belonging to the Enterobacteriaceae family. It forms part of the endogenous human gastrointestinal microflora. It also resides in soil, water and dairy products (Cabral, 2010). It has frequently been implicated in urinary tract, respiratory, GIT and skin infections (Cabral, 2010).

Klebsiella species are non-motile, rod-shaped, aerobic and Gram negative bacteria that possess a prominent polysaccharide capsule composed of 63% capsular polysaccharide, 30% lipopolysaccharide and 7% protein (Ryan and Ray, 2004). They can be found in natural environments such as soil, vegetation or surface waters where their presence is not necessarily related to faecal contamination (Leclerc *et al*., 2001).

Citrobacter is a genus of Gram-negative coliform bacteria in the Enterobacteriaceae family (Leclerc *et al*., 2001). The species uses solely citrate as a carbon source and this characteristic is commonly used to differentiate them from other genera of the Enterobacteriaceae family (Harley, 2005). It is also differentiated by their ability to convert tryptophan to indole, ferment lactose, and use malonate (Winn *et al.*, 2006). These bacteria can be found in soil, human GIT, water and wastewater (Leclerc *et al*., 2001).

2.1.2 Faecal streptococci (FS)

Faecal streptococci are bacteria that have been used as indicators of faecal pollution although some can occasionally originate from other habitats (Leclerc *et al*., 2001; Borrego *et al*., 2002). They are Gram-positive cocci that often occur in pairs or short

chains (Gilmore, 2002). They are facultative anaerobic organisms (Maier *et al*., 2000) that are not capable of forming spores and are tolerant of a wide range of environmental conditions such as extreme temperature (10 - 45°C), pH (4.5 - 10) and high sodium chloride concentrations (Maier *et al*., 2000; Giraffa, 2003; Fisher and Phillips, 2009). Faecal Streptococci can be distinguished from other Gram positive, catalase negative cocci by their ability to grow in the presence of 40% bile and sodium azide (Domig *et al*., 2003). The WHO (2003) recommends the use of FS as an additional indicator of faecal pollution and when combined with the measurement of *E. coli*, the result is increased confidence in the absence or presence of faecal pollution.

Faecal Streptococcus is a Gram-positive spherical bacterium inhabiting the GIT of humans and other mammals (Ryan and Ray, 2004). It is a facultatively anaerobic microbe that usually occurs in pairs or short chains, ferments glucose without gas production and catalase negative (Franz *et al*., 2003). This microbe uses peroxidase which is an enzyme that does not evolve oxygen to detoxify hydrogen peroxide. Isolation of FS in water is used in South Australia as an additional indicator when TC are present in the absence of *E. coli* (Cunliffe, 2000), while in Sydney, FS are used to confirm faecal contamination if either TC or *E. coli* are detected (Cunliffe, 2000).

2.2 Effects of raw or partially treated wastewater on natural water bodies

Surface water bodies in developing countries are under serious threats as a result of indiscriminate discharge of polluted effluent (Kambole, 2003*)*. Raw or partially treated sewage effluent fertilizes water bodies with nitrogen (N) and phosphorus (P) leading to eutrophication and degradation of water quality (EPA, 2000; CDC, 2002).

Eutrophication has become an environmental problem in recent years (Oke *et al*., 2006; McCasland *et al*., 2008). This is because N and P are essential components of structural proteins (EPA, 2000; Eynard *et al*., 2000) and their nutrient enrichment of waters promotes algal growth (Feng *et al*., 2002; Owili, 2003). Algal biomass form aggregates that sink and fuel bacterial growth in bottom waters and sediments leading to depletion of oxygen in the bottom waters (USDOD, 2004) creating conditions that are lethal for invertebrates and fish (Feng *et al.*, 2002). Algal bloom also shades and submerges aquatic vegetation reducing photosynthesis and productivity (Kurosu, 2001; Alm *et al.*, 2003; Mbewele, 2006; McCasland *et al.*, 2008).

Organic pollution occurs when large quantities of organic compounds which act as substrates for microorganisms are released into watercourses. During the decomposition process the dissolved oxygen in the receiving water is used up at a greater rate than it can be replenished causing oxygen depletion and having severe consequences for the stream biota (USDOD, 2004). Organic effluent also frequently contains large quantities of suspended solids which reduce the light available to photosynthetic organisms and after settling, they alter the characteristics of the river bed rendering it an unsuitable habitat for many invertebrates (Feng *et al*., 2002).

2.3 Biosand filtration

Biosand filtration is a water purification process in which water is passed through a porous bed of sand that contain a biological film that traps and metabolizes the organic compounds in water (Rooklidge *et al*., 2009). The process percolates untreated water slowly through the bed of sand, with the influent water being introduced over the surface of the filter and then draining from the bottom. The filters

can reduce the level of nutrients and other pollutants entering waterways to considerable levels (Logsdon, 2002; Rooklidge *et al*., 2009).

Intermittent BsFs are sand filters just like slow and rapid sand filters but they differ from these two in that the flow of water or filtration through them is demand driven and therefore not continuas (Buzunis, 1995).

Biosand filters are comprised of the supernatant that is the water above the SSF, the filter bed and the under-drain medium consisting of graded gravel (Foppen *et al*., 2006). The supernatant provides a head of water that creates a detention time of several hours for the treatment of the raw water (Logsdon, 2002). Depth of a filter bed ranges between 1.0 and 1.4 meters (Logsdon, 2002).

The under-drain system provides unobstructed passage for the collection of treated water and it supports the bed of filter medium (Logsdon, 2002). The under drain gravel is placed so that the finest gravel is directly underneath the sand and the coarsest gravel is surrounding the under drain pipes or covering the under drain block to prevents the filter grains from being carried into the treated water system (Dizer *et al*., 2004).

In a mature sand bed, a thin gelatinous layer called the hypogeal or *schmutzdecke* forms in the upper layer that consists of biologically active microorganisms such as bacteria, fungi and protozoa (Powelson and Mills, 2001; Campos *et al*., 2002; Auset and Keller, 2006; Foppen *et al*., 2006; Morales *et al*., 2007). These microorganisms break down the organic matter in the water and provide effective purification of water

with the underlying sand providing support for the biological treatment layer (Hendel *et al*., 2001; Dizer *et al*., 2004; Unger and Collins, 2006).

As water passes through the h*ypogeal*, particles are trapped in the mucilaginous matrix and dissolved organic material is adsorbed and metabolized by the microorganisms. Biological removal mechanisms in the BsF include predation, scavenging, natural death, inactivation and metabolic breakdown (Powelson and Mills, 2001; Auset and Keller, 2006; Foppen *et al*., 2006; Morales *et al*., 2007). It has also been proposed that another biologically-mediated particle removal mechanism is by attachment to biofilms (Bomo *et al*., 2004; Weber-Shirk and Chan, 2006) and that microorganisms also produce exocellular polymers that stick to raw-water particulates and enhance their removal (Jellison *et al*., 2000).

The organic impurities are biologically converted to water, carbon dioxide and harmless salts. Hydrophilic materials such as carbohydrates, aldehydes and simple organic acids are removed by biodegradation activities of microorganisms in the biofilm (Logsdon, 2002). In addition to the biological filtration mechanisms of the filter bed, physical processes such as adsorption and chemical oxidation also contribute to purification of water (Logsdon, 2002).

2.4 History of Biosand filtration

For over 150 years, BsF has been an effective means of treating water for the control of microbiological contaminants (Li *et al*., 1996). Biosand filters were built to serve communities in North America before 1900s but the advent of effective coagulation, sedimentation and rapid filtration resulted in declining interest in BsF. The situation

changed during the latter part of the 20th century when BsF was evaluated for the removal of viruses and *Giardia* cysts which were not known as pathogens before. A driving force for the reevaluation of BsFs in the U.S. was the need for simple yet effective water treatment processes for small water systems in rural areas.

According to Li *et al*., (1996), BsF was used in China in 1980s for rural areas for small water treatment facilities. Along with the renewed interest in BsFs, some different approaches to design and operation have come about and operators have developed approaches for filter cleaning that depart from the use of manual labor as was initially practiced (Hijnen *et al*., 2007).

Recent improvements in BsF capabilities now enable this technology to be applied more widely than would have been possible three decades ago. In recent years, resurgence in BsF application has occurred particularly because of its efficiency in removing bacteria, viruses and cysts of *Cryptosporidium* which are usually quite persistent (Hijnen *et al*., 2007). Biosand filtration has also been shown to be effective for the removal of nitrates in drinking water (Aslan and Cakici, 2007). High removal rates of antimicrobial contaminants have also been reported (Rooklidge *et al*., 2009).

Although BsF has often been replaced by faster and more high-rate filtration methods such as rapid sand filtration, its low cost, ease of operation, minimal maintenance requirements, and success in removing pathogenic microorganisms make BsF an attractive option for use in developing nations and rural communities (Logsdon, 2002). Biosand filtration of surface water is one of the oldest and most successful drinking water treatment techniques available for rural regions (Rooklidge *et al*., 2009).

2.5 Tests for identification and enumeration of bacteria in water

2.5.1 Viable plate counts for total coliforms, faecal coliforms and faecal streptococci

The viable plate count method is an indirect measurement of cell density and reveals information related only to live bacteria. The number of bacteria in a given sample is usually too great to be counted directly. Due to this reason, samples are normally serially diluted and plated out on an agar surfaces and the suspension spread over the surface of the growth medium before incubation at suitable temperature conditions. Multiplication of a single bacterium on the solid media results in the formation of a macroscopic colony visible to naked eye so each colony is assumed to arise from an individual viable cell. Plates with 30-300 colonies are chosen for enumeration as this range is considered statistically significant (Tomasiewicz *et al*., 1980). The total number of colonies is counted and multiplied by the dilution factor of the plate counted to determine the number of colony forming units (CFUs) per millilitre of sample.

2.5.2 Gram staining technique

Gram staining is a biological technique of differentiating bacterial species into two large groups (Gram-positive and Gram-negative) based on the chemical and physical properties of their cell walls (Ryan and Ray, 2004). The test detects peptidoglycan which is present in a thick layer in Gram positive bacteria but a thin layer in Gram negative bacteria (Bergey *et al.*, 2001; Madigan and Martinko, 2008). Gram-positive bacteria have thick cell walls made of peptidoglycan (50-90% of cell envelope) which are stained purple by crystal violet, whereas Gram-negative bacteria have a thinner

layer (10% of cell envelope) which are stained pink by the counter-stain (Madigan & Martinko, 2008).

The Gram staining technique involves four basic steps:

i) Crystal violet (CV) dissociates in aqueous solutions into CV^+ and chloride (Cl^-) ions that penetrate the cell wall and membrane of both Gram-positive and Gram-negative cells. The CV^+ ion interacts with negatively charged components of bacterial cells and stains the cells purple.

ii) Iodine ions interacts with CV^+ and forms large complexes of crystal violet and iodine (CV–I) within the inner and outer layers of the cell. Iodine is a mordant that acts as a trapping agent to prevent the removal of the CV–I complexes from the cells (Beveridge, 2001).

iii) A decolorizer (alcohol/acetone) is added and interacts with the lipids of the cell membrane. Gram-negative cells lose the outer lipopolysaccharide membrane and the inner peptidoglycan layer left exposed as the CV–I complexes are washed from the cells along with the outer membrane (George, 2005). In contrast, Gram-positive cells become dehydrated from the ethanol treatment and the large CV–I complexes become trapped within them due to the thick nature of its peptidoglycan (George, 2005).

iv) Counterstaining with a secondary stain (safranin/basic fuchsin) follows decolorization and the Gram-positive cells remain purple or retain the primary stain while the Gram-negative cell lose the purple color of the primary stain

and take up the red colour of the secondary stain (Beveridge, 2001; George, 2005).

2.5.3 IMViC tests

This is a group of tests group of tests that are used to distinguish between members of the Enterobacteriaceae family based on their metabolic by-products (Harley, 2005).

a) Indole test

The indole test is used as a classic test to distinguish indole-positive bacteria such as *E. coli* and *Citrobacter spp*. from indole-negative *Enterobacter* (Winn *et al.*, 2006). The indole test screens the ability of an organism to degrade the amino acid tryptophan and produce indole. Tryptophan is an amino acid that can undergo deamination and hydrolysis by bacteria that express tryptophanase enzyme. The pre-requirement for culturing an organism prior to performing the indole test is that the medium contains a sufficient quantity of tryptophan (MacFaddin, 2000). The presence of indole when a microbe is grown in a tryptophan-rich medium shows that the organism is capable of degrading tryptophan. Detection of indole relies upon the chemical reaction between indole and p-dimethylaminobenzaldehyde (DMAB) that is a constituent of the Kovac's reagent under acidic conditions to produce the red dye rosindole (MacFaddin, 2000; Winn *et al.*, 2006).

b) Methyl red test

Escherichia coli ferments sugars resulting in a low ratio of CO_2 to H_2 gas produced by fermentation and the acidic products cause a significant decrease in the pH of the

culture medium (Madigan & Martinko, 2008). In contrast, *E. aerogenes* produces a high ratio of CO_2 to H_2 from the fermentation of glucose via the butanediol fermentation pathway that results into a lower degree of acidification of the culture medium (Madigan & Martinko, 2008). When the culture medium turns red after addition of MR because of acidic pH from the fermentation of glucose, the culture has a positive result for the MR test. A negative MR test is indicated by formation of a yellow color in the culture medium which occurs when less acid is produced from the fermentation of glucose (Madigan & Martinko, 2008).

c) Voges-Proskauer Test

Voges Proskauer test together with the MR test are used to distinguish between members of the family Enterobacteriaceae (Schumann *et al*., 2003). Bacteria fermenting sugars via the butanediol pathway produce acetoin as an intermediate. In the presence of potassium hydroxide (KOH), acetoin is oxidized to diacetyl, a reaction which is catalyzed by α-naphthol (MacFaddin, 2000). Diacetyl reacts with the guanidine group associated with molecules contributed by peptone in the medium, to form a pinkish-red-colored product that is indicative of a positive VP reaction.

d) Citrate test

The citrate test screens a bacterial isolate for the ability to utilize citrate as its sole carbon and energy source (Harley, 2005). A positive diagnostic test rests on the generation of alkaline by-products of citrate metabolism and the subsequent increase in the pH of the medium is demonstrated by the color change of a pH indicator. Citrate is the sole source of carbon in the Simmon's citrate medium while inorganic

ammonium salt ($NH_4H_2PO_4$) is the sole fixed nitrogen source (Harley, 2005; MacFaddin, 2000; Reddy, 2007). When citrate is used as a carbon and energy source, alkaline carbonates and bicarbonates are produced (MacFaddin, 2000). The visible presence of growth on the medium and the change in pH indicator color due to the increased pH are the signs that an organism can use citrate as a sole source of carbon. Citrate is a Krebs cycle intermediate and is generated by many bacteria, however utilization of exogenous citrate requires the presence of citrate transport proteins (MacFaddin, 2000). Upon uptake by the cell, citrate is cleaved by citrate lyase to oxaloacetate and acetate and the former is metabolized to pyruvate and CO_2. The CO_2 is released and subsequently reacts with water and the sodium ion in the medium to produce sodium carbonate, an alkaline compound that raises the pH. In addition, ammonium hydroxide is produced when the ammonium salts in the medium are used as the sole nitrogen source. Bromothymol blue pH indicator is part of the citrate medium and is deep green at neutral pH and increase in medium pH causes bromothymol blue to change from green to blue (MacFaddin, 2000). The probable identification of coliforms based on the IMViC test results is shown in Table 2.

Table 2: IMViC test results and probable identification of coliforms

Test/Bacteria	*E. coli*	*Klebsiella spp.*	*E. aerogenes*	*Citrobacter spp.*
Indole	+	_	_	+
Methyl Red	+	_	_	_
Voges Proskauer	_	+	+	_
Citrate	_	_	+	+

Source: Bergey (2001).

+- Positive reaction; - - Negative reaction.

2.6 Isolation and identification of faecal s*treptococcus*

Faecal s*treptococcus* can easily be distinguished from other Gram-positive, catalase-negative, cocci like lactococci by their ability to grow between 10 and 45℃, between 5 and 10% Sodium chloride (NaCl), in the presence of 40% bile and sodium azide (Borrego *et al.*, 2002; Franz *et al*., 2003). Streptococci are catalase negative in that they don't produce the catalase enzyme in order to counter the effects of harmful oxygen metabolites but instead produce peroxidase that allow them to repair the oxidative damage of H_2O_2 (Wheelis, 2008). The catalase test is a reaction that is evidenced by rapid formation of bubbles in reaction tubes (MacFaddin, 2000) upon addition of H_2O_2. The test facilitates detection of catalase enzyme in bacteria and is essential for differentiating catalase-positive Micrococcaceae such as *Staphylococcus aureus* and *Micrococci spp.* from catalase-negative Streptococcaceae such as and *Enterococcus faecalis* (Chelikani *et al*., 2004). Bile aesculin (BA) agar is among the recommended media for enterococcal isolation and enumeration with incubation at 35-37℃ (Domig *et al*., 2003). The FS can hydrolyze aesculin to form aesculetin and dextrose and it is the aesculetin that reacts with ferric citrate in the medium to form a dark brown or black complex which is indicative of a positive result. The bile salts in the medium inhibit Gram-positive organisms other than Enterococci and the result is positive for bile salt tolerance and aesculin hydrolysis if blackening of the medium occurs (Franz *et al*., 2003).

The ratio of Faecal coliforms to Faecal streptococci (FC: FS) is sometimes used as an indicator of human faecal pollution/contamination. If the ratio is high, then pollution is concluded to be from human faeces. If it is less than 1, the pollution is assumed to be from domestic animals (Mara, 1974).

2.7 Heterotrophic plate counts /Total bacterial counts

Heterotrophic plate counts or total bacterial counts are tests used to estimate the total number of all types of bacteria in an environmental sample (WHO, 2003). It is a procedure that is used to estimate the number of live heterotrophic bacteria. Heterotrophs are those microorganisms that use organic compounds for all of their carbon requirements. A heterotrophic plate count is a microbial method that uses colony formation on culture media to approximate the levels of heterotrophic flora. The results obtained using HPC test are not an accurate assessment of total heterotrophic concentrations but are indications of culturable organisms present as some bacteria may be present in viable but non-culturable state (Bartram *et al*., 2003). The test does not also detect the types of bacteria present in the water (WHO, 2003). The density of HPC is influenced by quality of the water entering the system, temperature, residence time and the availability of nutrients for growth. Methods for performing the HPC include spread plate, pour plate and membrane filtration methods (Bartram *et al*., 2003) with either Yeast extract agar, Plate count agar or R2A agar and incubation at room temperature (25^{o}C) for 5 days or 35-37^{o}C for 48 hours (Lillis & Bissonnette, 2001). Total plate count methods rely on bacteria forming a colony on a nutrient medium so that the colony becomes visible and the number of colonies can be counted. To ensure that an appropriate number of colonies are generated, several dilutions of samples are cultured (WHO, 2003). Heterotrophic plate counts alone cannot indicate a health risk and additional studies on the presence of *E. coli* and other indicators are needed to establish the potential harm or risk of the water analyzed (WHO, 2003).

2.8 Physicochemical parameters of water

The physicochemical characteristics of wastewater of special concern are pH, dissolved oxygen, oxygen demand, suspended and dissolved solids, nitrogen and phosphate (Larsdotter, 2006).

2.8.1 Dissolved Oxygen

Dissolved oxygen is a measure of the amount of oxygen that is dissolved in a given medium. The quantity of oxygen that can be present in a solution is governed by the temperature, partial pressure of the atmosphere and the concentration of impurities such as salts and suspended solids in the water (Metcalf & Eddy, 2003). The presence of sufficient oxygen promotes the aerobic biological decomposition of organic wastes (Metcalf & Eddy, 2003). The bacterial breakdown of organic solids present in wastewater and the oxidation of chemicals can consume much of the dissolved oxygen in the receiving water bodies (USDOD, 2004).

2.8.2 Biochemical oxygen demand

Biochemical oxygen demand is the amount of dissolved oxygen needed by aerobic biological organisms in a body of water to break down organic material present in a given water sample at certain temperature over a specific time period (Gray, 2002; FAO, 2007). This test is widely used as an indication of the organic quality of water (Gray, 2002; FAO, 2007). The BOD_5 value is expressed in milligrams of oxygen consumed per liter of sample during 5 days of incubation at 20°C and is often used as a true representation of the degree of organic pollution of water. The BOD_5 value can be used as a gauge of the effectiveness of wastewater treatment plants.

Microorganisms living in oxygenated waters use DO to convert the organic compounds into energy for growth and reproduction (USDOD, 2004). Populations of these microorganisms tend to increase in proportion to the amount of food available and microbial metabolism creates an oxygen demand proportional to the amount of organic compounds useful to them as food (Gray, 2002; FAO, 2007). Biochemical Oxygen Demand is therefore the amount of oxygen required for microbial metabolism of organic compounds in water.

Oxidation of reduced forms of nitrogen such as ammonia and organic nitrogen are also mediated by microorganisms and exert nitrogenous demand which has been considered as interference in the determination of BOD_5. The interference from nitrogenous demand is prevented by an inhibitory chemical. If an inhibiting chemical is not used, the oxygen demand measured is the sum of carbonaceous and nitrogenous demand (FAO, 2007).

2.8.3 Nitrites and phosphates

Nitrogen is a nutrient necessary for growth of all living organisms and is as an essential component of life and is recycled continually by plants and animals (Jenkins *et al*., 2009). Inorganic nitrogen may exist in the free-state as di-nitrogen gas (N_2), as ammonia when combined with hydrogen, or as nitrite or nitrate when combined with oxygen (Kurosu, 2001). Nitrites and nitrates are produced naturally as part of the nitrogen cycle when bacteria break down toxic ammonia wastes into nitrites and then into nitrates (Kurosu, 2001). Nitrogen in the form of ammonia is toxic to fish and exerts an oxygen demand on receiving water because they get oxidized, creating oxygen demand (CDC, 2002; Amir *et al*., 2004). Nitrites are relatively short-lived and

not found in high concentrations in water because they're quickly converted to nitrates by bacteria (EPA, 2000). Nitrite is therefore present in samples from biological processes such as nitrification, denitrification or biological nutrient removal. Nitrite concentration and trend in samples can be used as an indicator of biological process efficiency.

Phosphates are salts of phosphoric acid that can condense to form pyrophosphates at elevated temperatures. In biological systems, phosphorus is found as a free phosphate ion in solution and is called inorganic phosphate to distinguish it from phosphates bound in various phosphate esters. Inorganic phosphate is often a limiting nutrient in environments and its availability may govern the rate of growth of organisms. In the context of pollution, phosphates are a component of total dissolved solids which is a major indicator of water quality (Hogan, 2010).

Increased levels of phosphates and nitrates often indirectly harm the environment by causing bacterial growth and huge algae blooms (Yanamadala, 2005). When an algal bloom resulting from phosphate and nitrate addition ends, the resulting decay of the algae may lead to the growth of disease-causing bacteria (Ansar & Khad, 2005). Eutrophication by high amounts of phosphates and nitrates causes destruction of water bodies around the world (Ansar & Khad, 2005). Studies have shown that the levels of phosphates and nitrates heavily impact the overall health of the water and its inhabitants (Yanamadala, 2005)

2.8.4 pH

pH is a measure of the acidity or basicity of an aqueous solution (Gray, 2002). The balance of positive hydrogen ions (H^+) and negative hydroxide ions (OH^-) in water determines how acidic or basic the water is. Water's pH is affected by its age and the chemicals discharged by communities and industries. As organic substances decay, carbon dioxide forms and combines with water to produce a weak acid, carbonic acid, which lowers water's pH (Gray, 2002).

2.8.5 Conductivity

Conductivity is the ability of water to carry an electrical current and it indicates the physical presence of dissolved chemicals in the water. The specific conductance test measures the ability of water to pass an electrical current. Conductivity in water is affected by the presence of inorganic dissolved solids such as chloride, sulfate, sodium and calcium. Industrial effluents have high conductance readings and after extended dry periods and low flow conditions, high specific conductance readings can also be achieved. Temperature also affects conductivity; warm water has a higher conductivity. Specific conductance is measured in microsiemens per centimeter (µs / cm) (Weiner, 2013).

2.8.6 Total Suspended Solids

Total suspended solids is a measure of the suspended and dissolved solids in water. Suspended solids are those that can be retained on a water filter and are capable of settling out of the water column onto the stream bottom when stream velocities are low. They include silt, clay, plankton, organic wastes, and inorganic precipitates such as those from acid mine drainage (AWWA, 2005). The TSS of a water sample is

determined by pouring a carefully measured volume of water through a pre-weighed filter of a specified pore size, then weighing the filter again after drying to remove all water. Filters for TSS measurements are typically composed of glass fibers (AWWA, 2005). The gain in weight is a dry weight measure of the particulates present in the water sample expressed in units derived or calculated from the volume of water filtered (mg/l) (AWWA, 2005).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

The study was carried out at the University of Eldoret sewage treatment plant, Uasin Gishu County, lying between longitude 35° 18' 13'' East and latitude 0° 34' 35'' North at an altitude 2180 meters above sea level.

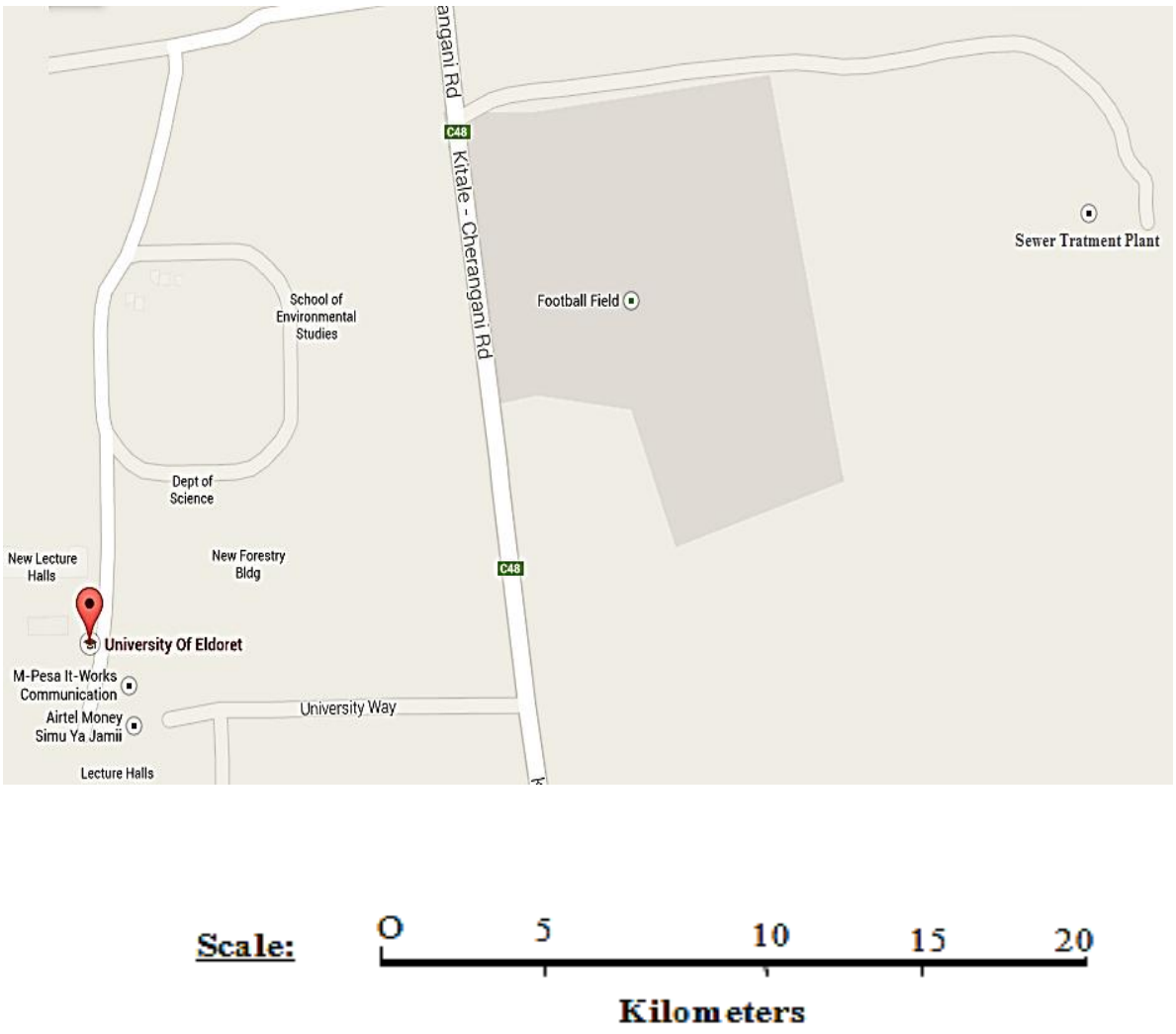

Figure 1: Map of the study area (Source: Google Maps, 2014)

The sewage treatment plant serves the university which has a population of approximately 15,000 students and staff. The area receives a mean annual rainfall of between 900 mm and 1600 mm occurring between the months of March and September with two distinct peaks in May and August. It has a mean annual temperature of 24^{o}C.

3.2 Study site

Sampling of sewage effluent for microbiological and physicochemical analyses was carried out at UoE sewage treatment plant. The treatment plant consists of four oxidation ponds that receive sewage water from the entire university community. Sewage water is channelled to the first pond that drains into the 3 consecutive ponds before it is released into River Marura. Sewage effluent was sampled from the fourth oxidation pond.

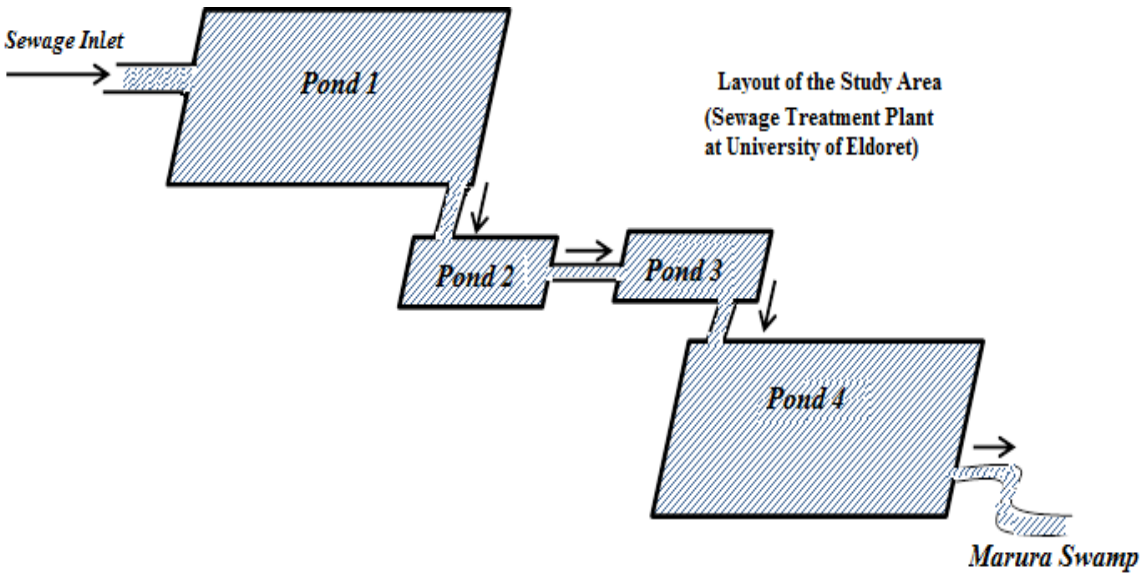

Figure 2: Illustration of the University of Eldoret Sewage treatment plant showing the arrangement of the oxidation ponds (Source: Author, 2014)

3.3 Design and operation of Biosand filters

Nine experimental BsFs were designed and assembled at the Biotechnology laboratory at UoE using plastic (PVC) pipes sealed at the bottom and fitted with stainless steel valves to regulate the flow of filtered effluents (Plate 1). Coarse gravel was used to make the under drain medium of about 0.5 m in each of the filters to hold the filter medium. Three sand grain sizes were prepared and tested for their suitability to remove bacteria and other contaminants from the effluent collected from UoE sewage treatment plant. The different sand grain sizes were prepared by sieving sand using standard sieves of different pore sizes. The sand grain sizes were (1.1 - 2.0) mm in the first set of three filters, (0.6 - 1.0) mm in the next set of three filters and (0.1 - 0.5) mm in the last set of three filters. The gravel and sand for the filters were washed and sterilized in the oven at 105^{o}C overnight before placing into the plastic pipes. The sand bed depth was 1 m for the first filter in each of the three sets of filters, 0.7 m in the second filter in each of the sets and 0.5 m in the third filter in each set of filters. Five liters of sampled effluent were introduced into each of the BsFs and samples were collected from each of the filters after 24, 48 and 72 hours for bacteriological and physicochemical analyses.

A summary of the dimensions of the filters is provided in Table 3 and the details of the design of BsFs based on various grain sizes, sand bed depths and retention times are provided in Table 4. The filters (Figure 1) were operated for six consecutive months to investigate the effects of the three control variables (sand grain size, bed depth and retention time) on effluent quality.

Table 3: Summary of the dimensions of the assembled Biosand filters

Particular		Dimensions/Details of measurements
Inner diameter	-	20 cm
Height of pipes	-	1.8 m
Gravel layer/under drain	-	0.5 m
Headspace	-	varying according to sand bed depth
Filter bed depth	-	varying per filter in each set of filters
Sand grain sizes	-	varying per set of filters
Influent temperature	-	$20^{o}C – 23^{o}C$

Table 4: Design of the Biosand Filters on various grain sizes, sand bed depths and retention times

		Sand grain size		
		(0.1 - 0.5) mm	(0.6 - 1.0) mm	(1.1 – 2.0) mm
Sand bed depth	1.0 m	Time 1	Time 1	Time 1
		Time 2	Time 2	Time 2
		Time 3	Time 3	Time 3
	0.7 m	Time 1	Time 1	Time 2
		Time 2	Time 2	Time 2
		Time 3	Time 3	Time 3
	0.5 m	Time 1	Time 1	Time 1
		Time 2	Time 2	Time 2
		Time 3	Time 3	Time 3

Key: Time 1 = 24 hours, Time 2 = 48 hours and Time 3 = 72 hours.

The quality of unfiltered effluent was determined by analyzing three replicate samples for bacteriological indicators and the physicochemical parameters. The effluents were passed through the various columns filled with sand for the varying periods of time. After filtration, the filtered effluent underwent bacteriological and physicochemical

analyses again to determine the reduction efficiency of various response parameters by the various sand columns.

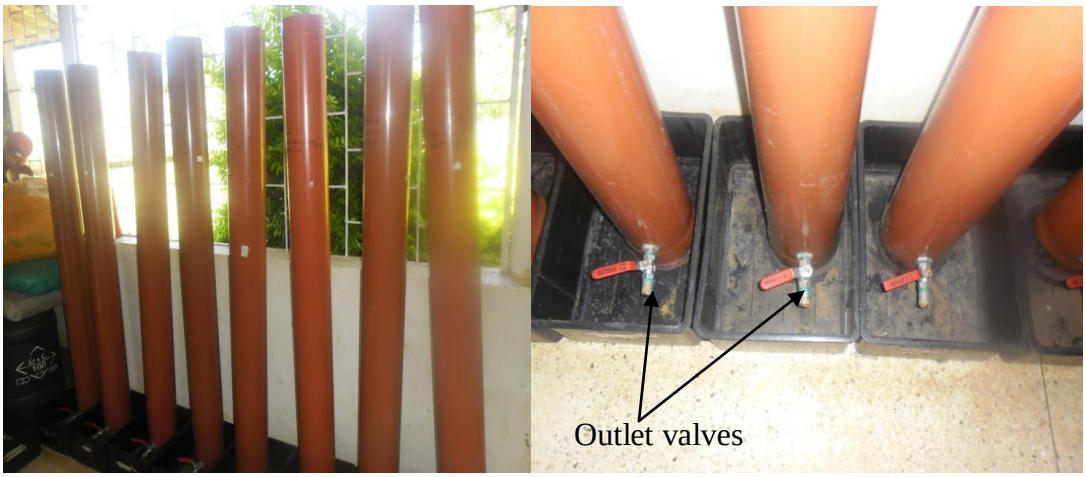

Figure 3: Assembled Biosand filters showing the outlet valves (Source: Author, 2012)

3.4 Sampling and laboratory analyses

Samples of filtered and unfiltered effluents were collected on a monthly basis for six consecutive months (June to December 2012).

3.4.1 Sampling for bacteriological analyses

Samples for bacteriological analyses were collected in one litre pre-sterilized sampling bottles from the study site and transported to the laboratory in iced cooler boxes. Nine 5-litre pre-sterilized plastic containers were used to collect samples from the same site and transported to the laboratory where they were introduced into the assembled sand filters.

Proper labeling of the sampling bottles was done before transporting them to the laboratory. In the laboratory, the 1-liter samples were stored under refrigeration ($4^{o}C$) to minimize changes in populations of bacteria. The samples were handled aseptically to ensure that they were representative of the effluents being examined. Analyses were done within 24 hours of collection to avoid any changes in the bacterial flora of the samples.

3.4.2 Bacteriological laboratory procedures

Isolation and enumeration of various bacterial groups in filtered and unfiltered effluent samples were done in order to monitor the effects of sand grain size, bed depth and retention time on filter performance. Effluent samples were analysed following standard plating techniques for enumeration of three bacterial types and for total bacterial counts. The bacterial types that were identified and enumerated included total coliforms (TC) and faecal coliforms (FC) that are members of family Enterobacteriaceae, and Faecal *Streptococcus* (FS) of Streptococcaceae family. Positive isolates from effluents were selected based on different morphologies and identified using Gram staining properties and biochemical tests. Isolation of the different groups of bacteria was done using selective media at suitable temperatures of incubation while identification was done by means of Gram stain reactions, microscopy for determination of morphology and biochemical tests.

a) Isolation and enumeration of bacteria

Standard plating techniques (Booth, 2006) were used to isolate and enumerate bacteria. For every effluent sample, 3 dilution tubes, each containing 9 ml of sterile

distilled water were used to serially dilute the effluent samples by dispensing 1 ml of the samples to the first dilution tube. Using the same procedure, 1 ml of sample from the first dilution tube was drawn and dispensed into the next dilution tube. This procedure was repeated up to a dilution of 10^{-3}. Dilution was necessary so that the bacteria in samples could be reduced to countable numbers upon culturing. One milliliter of sample from each of the three dilution tubes was transferred onto the surface of petri-plates containing MacConkey (MAC) agar, Bile Aesculin (BA) agar and Plate Count (PC) agar and the plates incubated at 37°C for 48 hours for enumeration of TC, FS and TBC on MAC, BA and PC agar plates respectively. A second lot of MAC agar plates were incubated at 44.5^{o}C for 48 hours for enumeration of FC. The plates were kept in the refrigerator at 4^{o}C after incubation for 48 hours in order to arrest growth of bacteria. The average composition of BA and MAC agars are given in Appendices 3 and 6 respectively.

Plates with 30-300 colonies were chosen for enumeration and identification of the bacterial types using appropriate biochemical tests. The pink or red colonies growing on MAC agar plates were tested for identification of members of the family Enterobacteriaceae. The colonies growing on the BA agar plates were used for identification of FS. The colonies on the BA and MAC agar plates were sub-cultured on Nutrient Agar (NA) and Nutrient Broth media and incubated at 37^{o}C for 48 hours to get pure cultures for further identification procedures.

b) Gram staining procedure

Gram staining was done to group the pure cultures into Gram positive and Gram negative bacteria before performing biochemical tests for identification of the

bacteria. The Gram staining procedure was done according to Bergey *et al.*, (2001) and Madigan and Martinko, (2008).

A thin smear of bacterial cell sample was prepared on a glass slide and heat fixed by passing over flame. The purpose of heat fixing was to affix the bacterial cells to the slide to prevent them from rinsing out during the staining procedure. The prepared smear was flooded with crystal violet stain for 1 minute. The sample on the slide was rinsed with a gentle stream of water for 5 seconds to remove the unbound crystal violet. Gram's iodine was added to bind to the primary stain and trap it in the cell. Rapid decolorization with alcohol followed after which the slide was rinsed with a gentle stream of water and counterstained with safranin and left on the slide for 1 minute. The slide was rinsed gently with a stream of water for 5 seconds.

c) Microscopy

After the Gram staining procedure, the stained slides were viewed under the microscope (oil immersion; ×100) to determine the morphological characteristics of the bacteria. The morphological characteristics together with the preceding biochemical tests were used for identification of the bacteria. Gram negative rods gave the initial identification FC and TC bacteria while Gram positive cocci that appeared in chains gave the initial identification of FS.

d) Biochemical tests

The biochemical tests for identification of TC and FC were carried out after microscopy on bacterial isolates that were lactose fermenters and Gram negative rods to get the probable identification of the genera and/or species of the isolated bacteria.

i) Indole test procedure

A tube of tryptone broth was inoculated with a loopfull of a pure culture to be tested and incubated at 35°C for 24 to 48 hours. To test for indole production, about 5 drops of Kovac's reagent were added to the tube (MacFaddin, 2000; Harley, 2005).

ii) Methyl Red and Voges Proskauer test procedures

The Methyl Red (MR) and Voges Proskauer (VP) tests were done as described by MacFaddin (2000**).** One tube of 5 ml MR-VP broth was inoculated with test culture to be identified. The test cultures were incubated at 35°C for 48 hours.

For the methyl red test, approximately 2.5 ml of culture from MR-VP broth was transferred into a new sterile culture tube and 5 drops of the MR reagent added. For the VP test, the remaining 2.5 ml of culture grown in MR-VP broth was mixed with 12 drops of Barritt's reagent A (5% α-naphthol) and 4 drops of Barritt's reagent B (40% KOH). The tube was shaken for a period of between 30 seconds to 1 minute, to expose the medium to atmospheric oxygen for oxidation of acetoin to obtain a color reaction, and allowed to stand for 30 minutes.

iii) Citrate test procedure

The Citrate test was performed according to MacFaddin, (2000). Pure cultures of the test bacteria were used as inoculation sources into Simmon's citrate medium. The inoculated culture tubes were incubated at 35^{o}C for 48 hours. Cultures were then observed for growth or lack of it to determine whether isolates were capable of citrate hydrolysis or not.

e) Testing for the presence of Faecal *Streptococcus*

Gram positive cocci bacteria isolated previously on BA agar were subjected to the catalase test to identify the FS. The catalase test was performed using the tube method (South Bend Medical Foundation, 2010). Four to five drops of 3% H_2O_2 were placed into a sterile test tube and a small amount of the pure culture of the test organism added using a sterile wooden applicator stick. The test tube was placed against a dark background and observed for immediate effervescence at the end of the wooden applicator stick (Wheelis, 2008).

f) Total bacterial counts in effluent samples

The standard spread plate technique was used to enumerate bacteria in effluent samples (Booth, 2006). This procedure involved making serial dilutions of the effluent sample (1:10, 1:100 and 1:1000) in sterile distilled water and cultivating 1 ml of each dilution on Plate Count Agar (PCA) plates that were sealed and incubated. The inoculum was transferred and spread onto the agar surface using an inoculation rod and allowed to be absorbed by the medium. Replica plates were inverted and incubated at 35+0.5^{o}C for 48 hours after which the number of colonies per plate were

counted using a Quebec colony counter (Gallenkamp; England) and reported as total viable counts. The CFU/ml of effluent samples was calculated by multiplying the counted number of colonies by the dilution used and dividing by total volume plated.

$$\frac{\text{CFU}}{\text{ml}} = \frac{\text{No. of colonies} \times \text{dilution factor}}{\text{Total volume plated (ml)}}$$

3.5 Physicochemical analyses

3.5.1 Measurement of conductivity, temperature and pH

Conductivity, temperature and pH of effluent samples were measured *in situ* using a JENWAY 3405 Electrochemical analyser (Figure 2 A) and a HACH thermometer and pH meter (Figure 2 B) respectively.

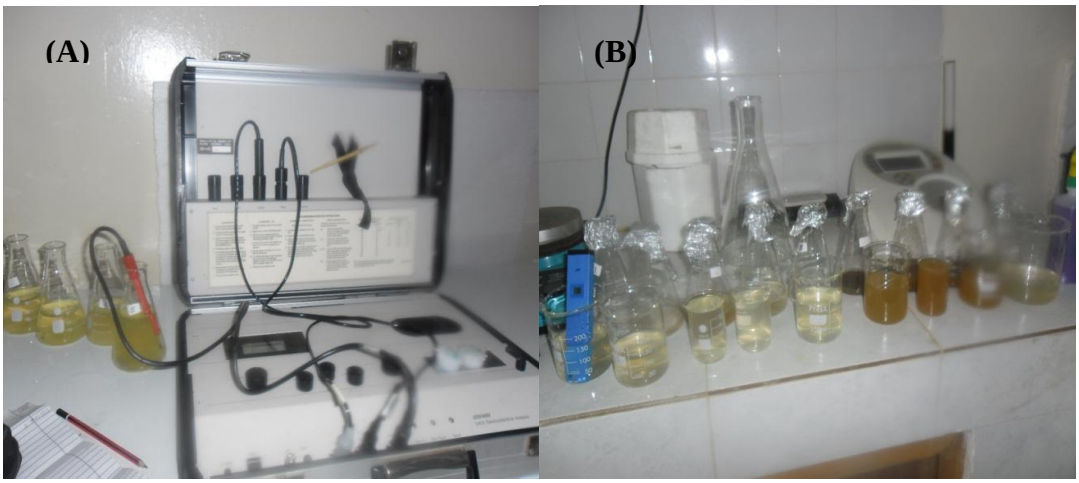

Figure 4: Measurement of (A): Conductivity and (B): pH of effluent samples. Source (Author, 2012)

3.5.2 Measurement of total suspended solids

To determine the TSS of effluent samples, well-mixed samples were filtered through pre- weighed glass fibre filter pads. The residues retained on the filter pads were dried overnight at 105°C in the oven to remove any remaining water. The pads were placed

in a desiccator to cool to room temperature before weighing on balance (Kern & Sohn GMBH, D-72336, Balingen, Germany). The increase in weight of the filter pads represented the TSS of the effluent samples in mg/l, (APHA, 2005).

3.5.3 Determination of dissolved oxygen, BOD_5, phosphates and nitrogen compounds.

Triplicate 500 ml effluent samples were collected in plastic bottles from the fourth pond at the study site. The samples were transported to the laboratory for analysis of DO, BOD_5, phosphates and nitrogen compounds. Effluent samples were kept below 4°C and analysis done within 6 hours of collection. Chilled samples were first warmed to 20 ± 3°C in a microwave before analyses.

a) Determination of phosphates

Amino acid method was used to measure phosphates using the HACH colorimeter (DR/820) (Figure 3). Two 25-ml sample cells were filled with 25 ml of sample each. To one of the cells, 1 ml of Molybdate reagent followed by 1 ml of Amino Acid reagent was added with calibrated droppers. The cell was capped and inverted several times to mix the sample well and the reaction allowed to run for 10 minutes. A blue color (Figure 3; Tube 1) formed in the cell with an intensity depicting the amount of phosphates in the sample. The blank (sample cell with no reagent) (Figure 3; Tube 2) was placed into the cell holder and used to zero the machine after which the prepared sample cell was put in the cell holder and the concentration of phosphates obtained in mg/l PO_4^{3-}.

Figure 5: The HACH colorimeter (DR/820) and HACH phosphate reagents for determination of phosphates in samples (Source: Author, 2012)

b) Determination of nitrites in samples

The Diazotization method was used to measure nitrites in samples using the HACH colorimeter (DR/820) (Figure 4). Two 10-ml sample cells were filled with 10 ml of sample each. To one of the cells, the contents of one Nitriver 3 Nitrite Reagent Powder Pillow (NRPP) was added, capped and shaken well to mix. A pink color (Figure 4; Tube 1) formed in the cell with an intensity depicting the amount of nitrites in the sample. A 15-minutes reaction was allowed to take place after which the blank (sample cell with no reagent) (Figure 4; Tube 2) was placed into the cell holder and used to zero the machine. The prepared sample cell was put in the cell holder and the concentration of nitrites in the sample obtained in mg/l NO_2^-N.

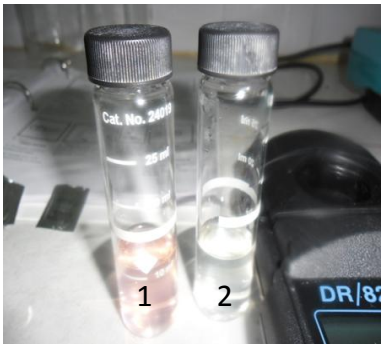

Figure 6: The HACH colorimeter (DR/820) and HACH nitrite reagents for determination of nitrites in samples (Source: Author, 2012)

c) Determination of nitrates in samples

The Cadmium reduction method was used to determine the concentration of nitrates in effluent samples using the HACH colorimeter (DR/820) (Figure 5). Two 10-ml sample cells were filled with 10 ml of sample each. To one of the cells, the contents of one Nitra ver 5 Nitrate Reagent Powder Pillow was added and shaken well for one minute after which the reaction was left to take place for 5 minutes. An amber colour (Figure 5; Tube 1) developed in the sample cell with an intensity depicting the quantity of nitrates in the sample. After 5 minutes, the blank (sample cell with no reagent) (Figure 5: Tube 2) was placed into the cell holder and used to zero the machine and the prepared sample cell was put in the cell holder in order to obtain the concentration of nitrates in mg/l NO_3^-N.

Figure 7: The HACH colorimeter (DR/820) and HACH nitrate reagents for determination of nitrates in samples (Source: Author, 2012)

d) Determination of dissolved oxygen in samples

Measurement of DO was carried out using a HANNA DO meter (HI 9143) (Figure 6). Initial DO measurements were carried out *in situ* to minimize errors of added oxygen during transportation. Calibration of the machine was done by exposing the probe to air until the machine displayed 100% concentration of oxygen in air. The tip of the probe was immersed into the sample and the machine allowed to stabilize before obtaining the concentration of oxygen in ppm which is equivalent to mg/l.

Figure 8: HANNA DO meter (HI 9143) for determination of DO in samples. (Source: Author, 2012)

e) Determination of Biochemical Oxygen Demand (BOD_5) in samples

Determination of BOD_5 was done by getting the difference in DO between day 1 of sampling and day 5 (FAO, 2007). After the initial DO measurements, the samples were stored in dark bottles to prevent the algae in the effluent samples from adding to the oxygen quantity from photosynthetic processes. The samples were kept in an incubator at 20^oC and on the fifth day of incubation, the DO was measured again. The difference in the two DO readings represented the BOD_5 which is the amount of oxygen consumed after five days to degrade the organic content in the samples.

3.6 Data analysis

Statistical analyses were performed using Statistical Package for Social Sciences (SPSS 13.1). Differences in removal efficiencies for physicochemical and bacteriological parameters among the filters were assessed by ANOVA. This was done in order to determine if there were significant differences between influent and effluent parameters. The replications were treated as the blocks and sand bed depth, sand grain size and retention time were the different treatments. The response parameters were the bacteriological and the physicochemical parameters of the filtered and unfiltered effluent samples. Duncan's Multiple Range Test (DMRT) was used to separate means that were significantly different. All statistical analyses were done at 95% level of confidence.

CHAPTER FOUR

RESULTS

4.1 Bacteriological analyses

4.1.1 Isolation and identification of total coliforms, faecal coliforms and faecal streptococci in effluents

The first objective of this study was to isolate, identify and enumerate indicator bacteria in addition to enumeration of total bacterial counts in unfiltered and filtered effluents. A summary of the isolation media, incubation temperature, Gram stain reaction and morphology of isolated bacteria as seen under the microscope is shown in Table 5. The initial isolation of the three major bacterial groups under study was done on BA and MAC agars.

Table 5: A summary of isolation media, incubation temperature, Gram stain reaction and morphology of isolated bacterial groups

Medium	Incubation temperature (^{o}C)	Gram-stain reaction	Morphology	Bacterial group
MAC	37	_	Rods	TC
MAC	44.5	_	Rods	FC
BA	37	+	Cocci	FS

MAC= MacConkey agar, BA= Bile Aesculin agar, TC = Total Coliforms, FC = Faecal Coliforms, FS = Faecal Streptococci.

Gram positive bacteria appeared red under the microscope while Gram negative bacteria appeared red under the microscope. Gram negative rods growing on MAC agar (37^{o}C) after 48 hours were identified as the TC while Gram negative rods

growing on MAC agar (44.5^{o}C) after 48 hours were identified as the FC (Table 5). Gram positive culture isolates on BA agar (37^{o}C) after 48 hours were identified as the FS (Table 5).

The colonies of TC and FC that were isolated on MAC agar plates were pinkish and reddish with smooth edges (Figure 7 A) while the colonies of FS that were isolated on BA agar appeared dark or black (Figure 7 B).

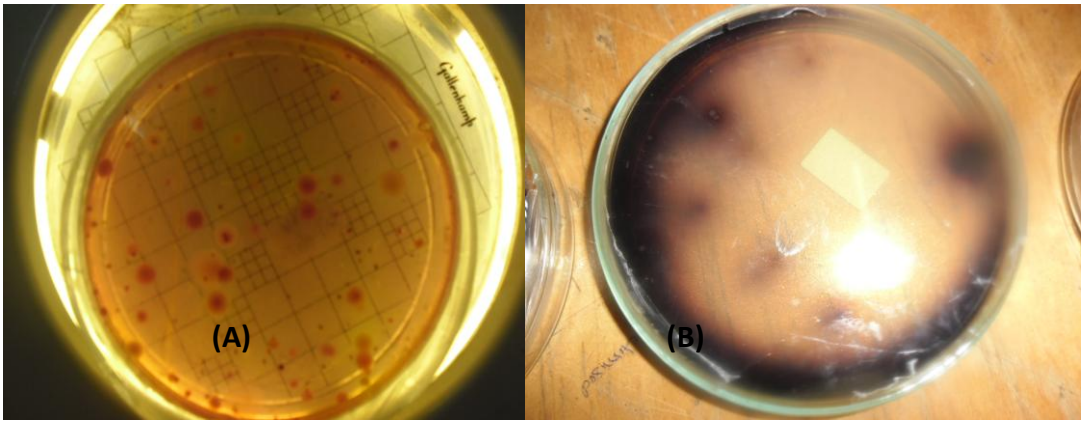

Figure 9: (A): Total coliform colonies on MacConkey agar plate and (B): Faecal Streptococci colonies on Bile Aesculin agar plates (Source: Author, 2012)

Results on catalase test were used to determine whether BA isolates were FS or non-FS bacteria. Isolates that produced bubbles upon addition of H_2O_2 were identified as catalase positive and were indicative of non-FS (Figure 8 A). Isolates that did not produce bubbles upon addition of H_2O_2 (Figure 8 B, Tube 1) were identified as catalase negative organisms and since the Gram stain reactions and microscopy had been done prior to the catalase test, isolates were identified as FS or non-FS bacteria.

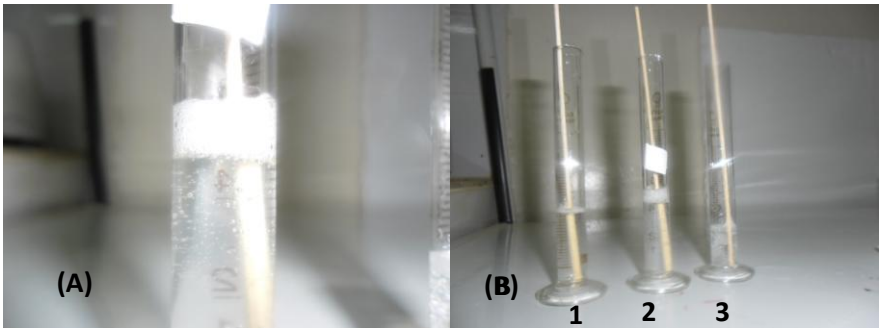

Figure 10: Reaction tubes for catalase test on isolates (Source: Author, 2012)

The results on the tests performed during isolation and identification of the FS bacteria from filtered and unfiltered effluents are shown in Table 6. The FS bacteria were found to be catalase negative, Gram positive cocci capable of aesculin hydrolysis, bile tolerance and citrate hydrolysis, the last three qualities being observed in their ability to grow on the BA agar.

Table 6: Results on tests performed during isolation and identification of faecal streptococci bacteria in filtered and unfiltered effluents

Identification/Test	**FS reaction/appearance**
Catalase test	–
Morphology	Cocci
Gram stain	+
Aesculin hydrolysis	+
Bile tolerance	+
Citrate hydrolysis	+

+ and – mean positive and negative reactions respectively

4.1.2 Identification and differentiation of the total coliform bacteria isolated from unfiltered and filtered effluents.

Total Coliform isolates from filtered and unfiltered effluents included *E. coli*, *Klebsiella spp.*, *Enterobacter spp.* and *Citrobacter spp*. A summary of the biochemical test reactions for TC that were isolated and identified are shown in Table 7. Results for Indole, MR and VP biochemical tests are shown in Figure 9 A, B and C respectively.

Table 7: Biochemical reactions for isolated and identified total coliform bacteria

Test	Results		Identification			
	Positive	**Negative**	***E. coli***	***Enterobacter***	***Citrobacter***	***Klebsiella***
Indole	Red colour	No colour change	+	_	+	_
MR	Pinkish colour	No colour change	+	_	_	_
VP	Pinkish colour	No colour change	_	+	_	+
Citrate	Blue colour	Green colour	_	+	+	_

MR = Methyl Red, VP= Voges Proskauer
+ and – mean positive and negative reactions respectively

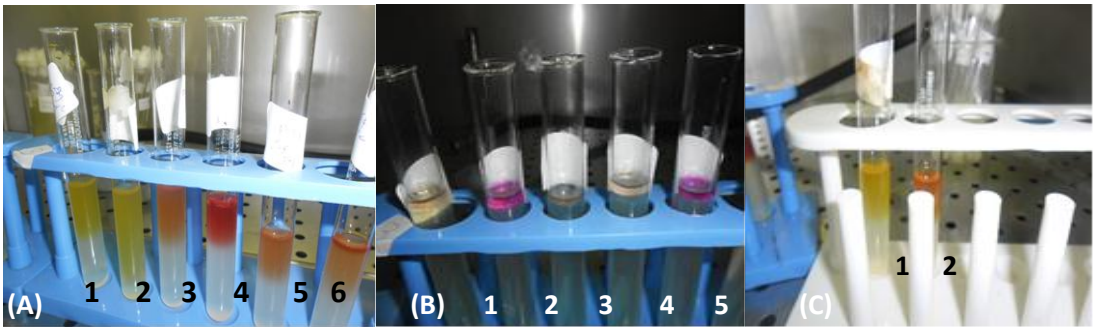

Figure 11: Tubes for (A): Indole, (B): Methyl Red and (C): Voges Proskauer test reactions (Source: Author, 2012)

The Indole positive results (Figure 9 A; Tubes 3, 4, 5 and 6) indicated the presence of either *E. coli* or *Citrobacter spp.* while Indole negative results (Figure 9 A; Tubes 1 and 2) indicated the presence of *Enterobacter spp.* or *Klebsiella spp*.

The MR^+ results (Figure 9 B; Tubes 2 and 5) confirmed the presence of *E. coli* while the MR^- results (Figure 9 B; Tubes 1, 3 and 4) confirmed the presence of *Enterobacter spp*., *Klebsiella spp. or Citrobacter spp*.

The VP^+ results (Figure 9 C; Tube 2) confirmed the presence of *Enterobacter spp.* or *Klebsiella spp.* and VP^- results (Figure 9 C; Tube 1) confirmed the presence of *E. coli* or *Citrobacter spp*.

The citrate test performed on TC isolates (Figure 10) gave positive results (Figure 10 A) for citrate positive isolates like *Enterobacter spp.* and *Citrobacter spp*. and negative results (Figure 10 B) for citrate negative isolates like *E. coli* and *Klebsiella spp*.

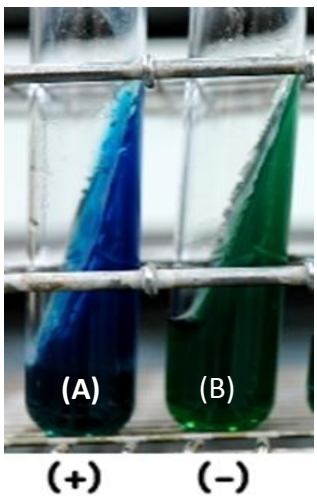

Figure 12: Simon's Citrate agar slants showing (A): Positive and (B): Negative reactions for citrate test on bacterial isolates (Source: Author, 2012)

4.1.3 Enumeration of total coliforms, faecal coliforms, faecal streptococci and total bacterial counts in effluents.

The enumeration of TC, FC, FS and TBC was done in both filtered and unfiltered effluents as part of the first objective of this study. This was done in order to assess the effectiveness of the various designs of the SSFs in reduction of the numbers of these bacterial groups in the filtered effluents. The number of CFUs was determined by multiplying the number of colonies in a plate by the dilution factor of the counted plate.

Table 8: Means of CFUs /ml data of bacteriological parameters in unfiltered and filtered effluents

	FS	**TBC**	**FC**	**TC**
Filtered	185[a]	3060[a]	355[a]	164[a]
Unfiltered	5258[b]	24840[b]	1924[b]	5258[b]
% removal	96.48	87.68	81.55	96.88
F – value	10.092	2.455	4.736	2.117
P – value	0.000	0.001	0.000	0.003
CV%	10.1	32.8	10.7	48.4
S.E	370.4	3192.9	5232	3325.3
Effect	*	*	*	*

**Significant at p < 0.05. TC = Total Coliforms, FC = Faecal Coliforms, FS = Faecal Streptococci, TBC = Total Bacterial counts.*

Means with different letter superscripts in same column are significantly different at p<0.05.

Results on the enumeration of the three bacterial groups and the TBC under study were found to differ significantly ($p < 0.05$) in filtered and unfiltered effluents

showing that filtration reduced the TC, FC, FS and TBC significantly (Table 8). The results on bacterial numbers in filtered and unfiltered effluents based on the different experimental parameters are further shown in Figures 1 - 6. The highest per cent reductions were observed for FS and TC bacteria (96.48% and 96.88% respectively) while the least per cent reductions were observed for TBC and FC (87.68% and 81.55% respectively).

4.2 Physicochemical analyses

The second objective of the study was to determine the levels of selected physicochemical parameters in filtered and unfiltered effluents.

Table 9: Means of physicochemical parameters in unfiltered and filtered effluents

	BOD_5	**Conduct.**	**DO**	**NO_3^-**	**NO_2^-**	**pH**	**TSS**	**PO_4^{3-}**
Filtered	0.36^a	319.2^a	7.22^a	2.78^a	0.0279^a	7.5^a	0.12^a	1.55^a
Unfiltered	4.98^b	439.6^b	6.02^b	4.18^b	0.1126^b	8.1^b	0.39^b	16.0^b
% removal	92.77	27.39	-19.33	33.49	75.22	7.41	69.23	90.31
F – value	39.16	1.917	0.779	0.780	4.313	3.269	39.67	3.528
P – value	0.000	0.000	0.009	0.008	0.012	0.00	0.000	0.000
CV%	29.6	11.3	12.9	20.3	15.4	3.5	22.6	29.1
S.E	0.1557	36.45	0.924	1.705	0.0264	0.262	1.578	0.117
Effect	*	*	*	*	*	*	*	*

**Significant at $p < 0.05$. DO = Dissolved Oxygen, NO_2^- = Nitrites, NO_3^- = Nitrates, TSS = Total Suspended Solids, Conduct. = Conductivity.*

Means with different letter superscripts in same column are significantly different at $p<0.05$.

All the physicochemical parameters under investigation were found to differ significantly ($p < 0.05$) in filtered and unfiltered effluents (Table 9). The pH, BOD_5, NO_2^-, NO_3^-, Conductivity and TSS were reduced significantly upon filtration using the assembled sand columns while DO was increased significantly in the filtered effluents. The highest reduction was observed for BOD_5 followed by PO_4^{3-} (92.77% and 90.31% respectively) while the least reduction was observed, for pH (7.41%). The negative percent reduction for DO (-19.93%) showed that DO rather than reducing, increased in the filtered effluents.

4.3 Effects of sand bed depth, sand grain size and retention time in Biosand filters on secondary effluents

Results showed that all filters reduced bacterial load and levels of physicochemical parameters significantly except for DO that increased. However, there were statistical differences in the removal efficiency of the filters as a result of the differences in sand grain sizes, sand bed depths and retention times as outlined in the following subsections.

4.3.1 Effect of sand bed depth in Biosand filters

Sand bed depth had a significant effect on pH, DO, BOD_5, NO_2^-, NO_3^-, PO_4^{3-}, conductivity and TSS (Figure 11; Appendix 1). The pH of unfiltered effluents was significantly higher ($p < 0.05$) than that of filtered effluents. Although pH of the effluents was lowered significantly with increasing sand bed depth, there was no significant difference in filtered effluent pH at depths 0.5 m and 0.7 m. However, the

pH at these two depths (0.5 m and 0.7 m) differed significantly from that obtained at depth 1.0 m which showed the lowest pH of 7.35 (Fig. 11 A).

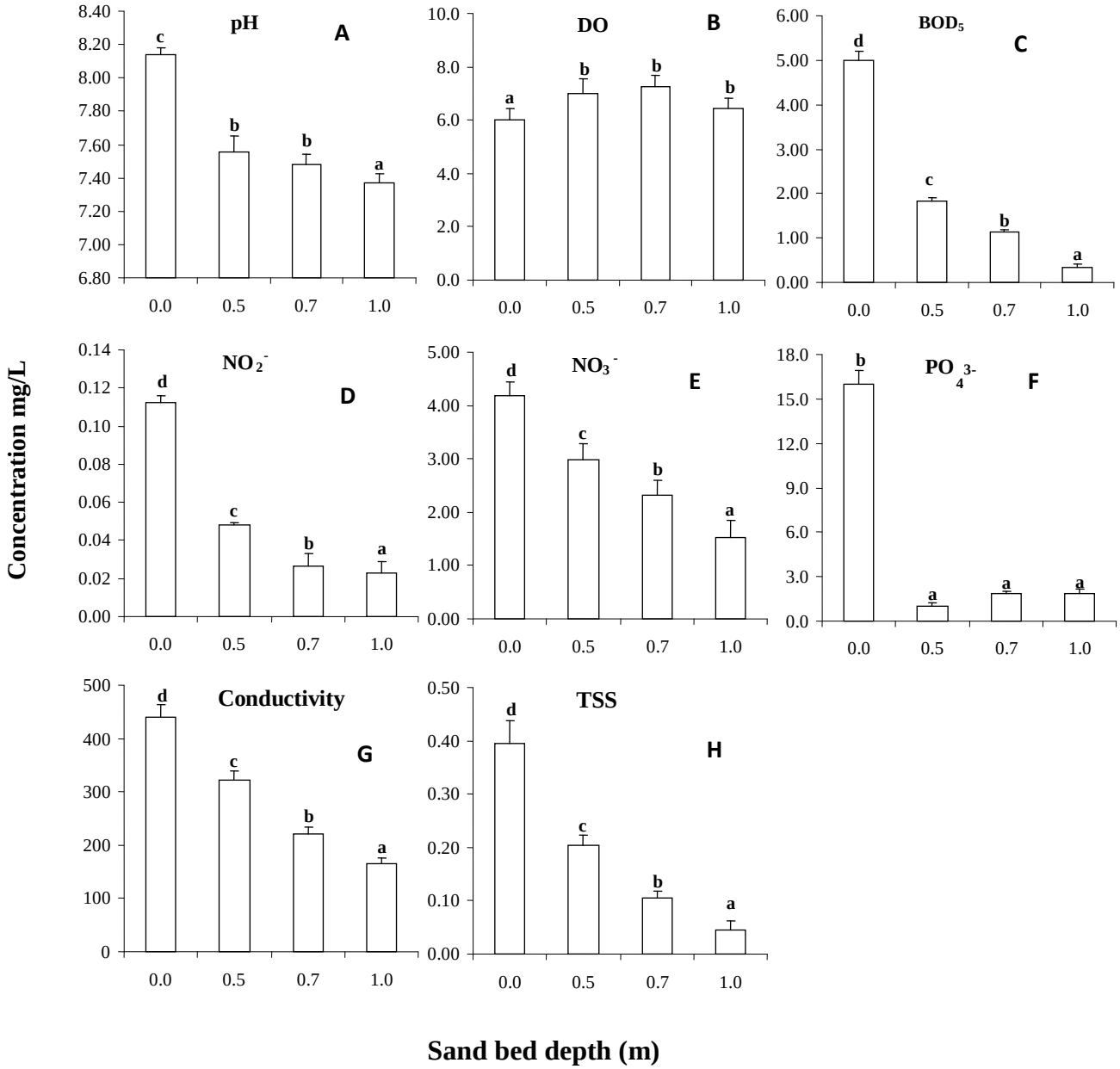

Figure 13: Levels of physicochemical parameters (A): pH, (B): DO, (C): BOD_5, (D): NO_2^-, (E): NO_3^-, (F): PO_4^{3-}, (G): Conductivity and (H): TSS in the effluents at various sand bed depths.

Sand bed depth of 0.0 m depth means filtration not done/raw sewage effluent before filtration

Bars with the same letter superscript on the same graph are not significantly different at $p < 0.05$

The DO of effluents increased significantly ($p < 0.05$) in filtered effluents as compared with the unfiltered effluents. Although, there were no significant differences in DO increment at the three levels of depth, increase in DO was found to be highest (7.60 mg/l) at depth 0.7 m and lowest (6.50 mg/l) at depth 1.0 m (Fig. 11 B).

The BOD_5 decreased significantly ($p < 0.05$) with an increase in sand bed depth. The BOD_5 was found to be highest in unfiltered effluents but lower at all the three levels of experimental depths. Significant differences in BOD_5 were also observed among the three experimental depths (0.5 m, 0.7 m and 1.0 m) with the highest reduction being observed at 1.0 m depth and lowest reduction at 0.5 m depth (Fig 11 C).

Results on effects of sand bed depth on NO_2^- reduction showed that sand bed depth had significant effect on NO_2^- reduction. NO_2^- reduced significantly with an increase in sand bed depth. The mean concentration of NO_2^- in unfiltered effluents was found to be 0.15 mg/l, decreasing significantly ($p < 0.05$) at 0.5 m depth (0.012 mg/l), and further decreasing to 0.02 mg/l at 1.0 m depth (Fig. 11 D). A similar trend was observed in NO_3^- reduction in effluents with the concentration of NO_3^- being highest in unfiltered effluents at 4.05 mg/l, reducing significantly ($p < 0.05$) at 0.5 m depth to 2.75 mg/l and further reducing to 1.5 mg/l at the highest experimental depth (1.0 m) (Fig. 11 E).

Although mean PO_4^{3-} concentration in effluents reduced significantly upon filtration, there were no significant differences in mean PO_4^{3-} concentration at 0.5 m, 0.7 m and 1.0 m (Fig. 11 F).

Reduction of mean conductivity and TSS levels were similar to those observed for BOD_5, nitrates and nitrites where the highest conductivity and TSS values were recorded in unfiltered effluents (0 m depth) and the three experimental depths (0.5 m, 0.7 m and 1.0 m) recorded lower values of these parameters (Figs. 11 G and H). These two parameters increased significantly with increase in sand bed depth.

Results of bacterial counts in the filtered effluents at various sand bed depths showed that sand bed depth had no significant effect on reduction of bacterial counts (Fig. 12). Although the numbers of FC, TC, FS and TBC were significantly ($p < 0.05$) different in filtered and unfiltered effluents, there was no significant difference in their counts at the various depths except for FC (Fig. 12).

Total coliform counts were found to be 6800 CFUs/ml in unfiltered effluents and reduced significantly ($p < 0.05$) to below 1000 CFUs/ml after filtration in all the three levels of experimental depths (Fig. 12 A). However, there was no significant difference in reduction of TC counts at the three levels of experimental depths and the average reduction of TC was 96.29 % (Fig. 12 A; Table 10).

Faecal coliform counts were found to be 1900 CFUs/ml in unfiltered effluents and reduced significantly ($p < 0.05$) to below 500 CFUs/ml after filtration in all three experimental depths. There was no significant difference observed in FC reduction at depths 0.5 m (93%) and 0.7 m (94%) (Fig. 12 B; Table 10). Faecal coliform removal at 1.0 m sand bed depth was found to be 82.81% which was significantly different from removal at 0.5 m and 0.7 m depths (Table 10). Faecal streptococci had a similar

trend to that of TC counts at the various sand bed depths, with an average removal of 98.12% (Fig. 12 C).

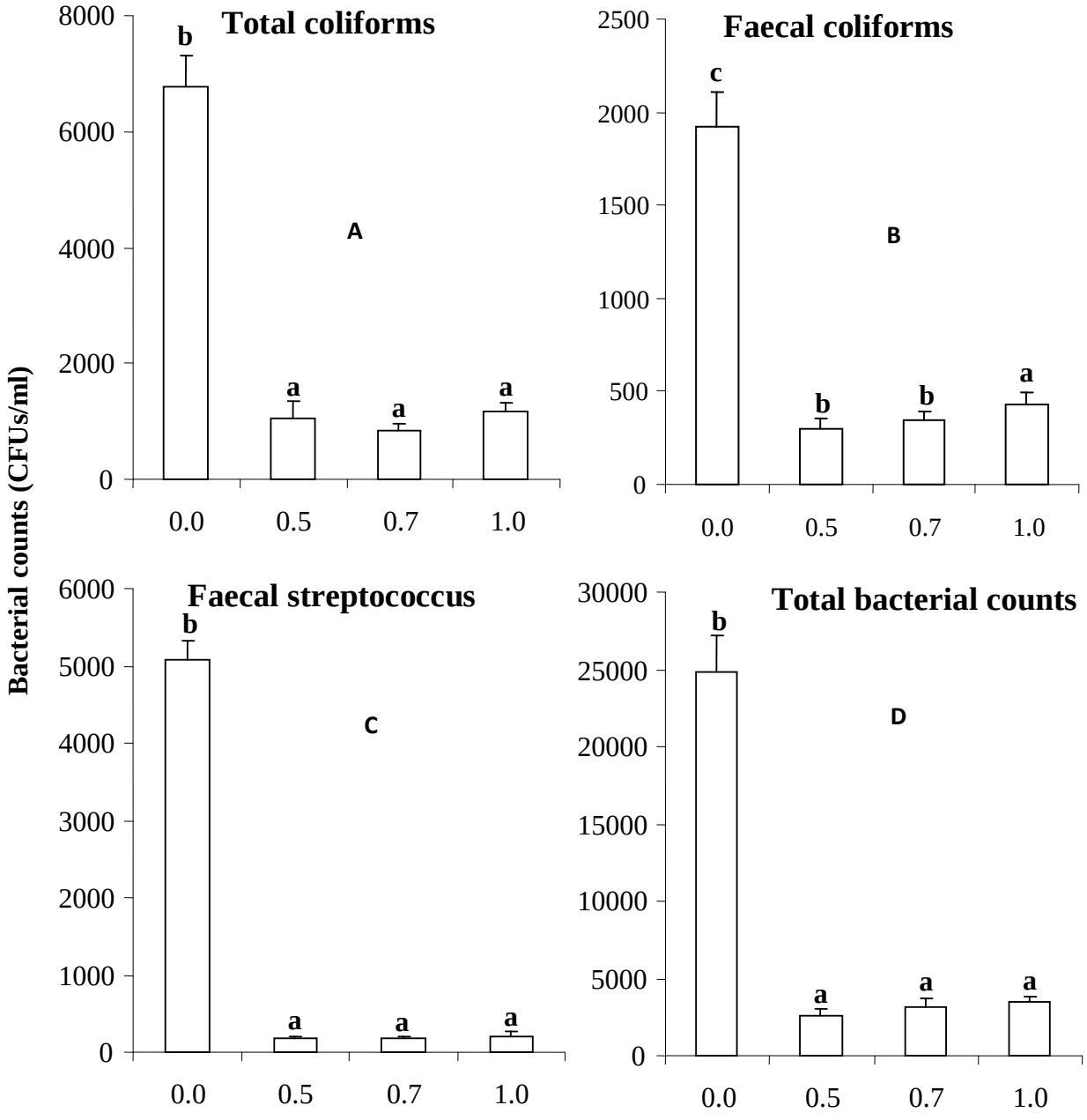

Figure 14: Bacteriological quality parameters (A): TC, (B): FC, (C): FS and (D): TBCs in the effluents at various sand bed depths.

Bars with the same letter superscript in the same graph are not significantly different at $p < 0.05$

Sand bed depth of 0.0m means filtration not done/raw sewage effluent before filtration

Total bacterial counts were found to be 25000 CFUs/ml in unfiltered effluents and reduced significantly ($p < 0.05$) to below 5000 CFUs/ml after filtration in all the three experimental depths. However, just like for TC and FS counts, TBC reduction was not significantly different at the three levels of depth (Fig. 12 D). The average percent removal of TBC after filtration was 91.59% (Table 10).

Table 10: Percentage removal of bacteria in filters of different depths

Sand bed depth (m)	Average % removal			
	TC	FC	FS	TBC
0.5	98.02^{a}	93.42^{b}	99.34^{a}	93.48^{a}
0.7	95.53^{a}	94.02^{b}	99.02^{a}	91.32^{a}
1.0	95.32^{a}	82.81^{a}	96.02^{a}	89.99^{a}

Percentage values with the same letter superscript in the same column are not significantly different at $p < 0.05$

4.3.2 Effect of sand grain size in Biosand filters

Sand grain size had a significant ($p < 0.05$) effect on reduction of pH, BOD_5, NO_2^-, NO_3^-, PO_4^{3-}, conductivity and TSS and increase in DO of effluents (Fig. 13; Appendix 4). The pH, BOD_5, NO_2^-, NO_3^-, conductivity and TSS of effluents were significantly ($p < 0.05$) highest in the unfiltered effluents and decreased significantly ($p < 0.05$) after filtration.

The pH in unfiltered effluents was found to be 8.1 and reduced significantly ($p < 0.05$) upon filtration with reduction efficiency increasing significantly ($p < 0.05$) with decreasing sand grain size. The small grain sized filters (0.1 - 0.5) mm recorded the least mean pH of 7.35 followed by medium grain sized filters (0.6 -1.0) mm which gave a mean pH of 7.45 and the least reduction of pH (7.5) was observed in the large grain sized filters (1.1 - 2.0) mm (Fig. 13 A).

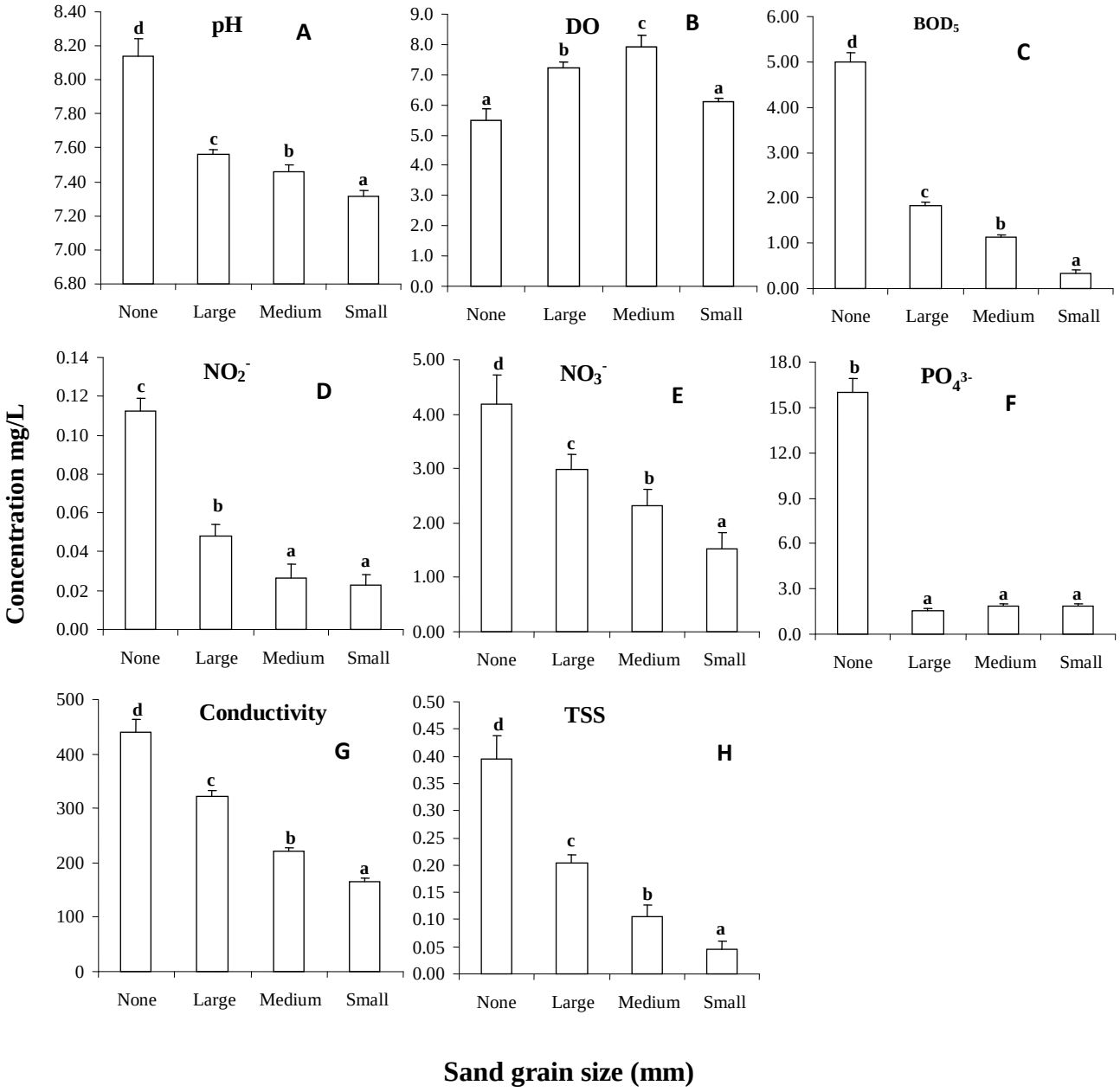

Figure 15: Levels of physicochemical parameters (A): pH, (B): DO, (C): BOD_5, (D): NO_2^-, (E): NO_3^-, (F): PO_4^{3-}, (G): Conductivity and (H): TSS in effluents at different sand grain sizes.

Key: None-Unfiltered/control; Small – (0.1-0.5) mm; Medium – (0.6-1.0) mm; Large – (1.1 - 2.0) mm. Filters with the same letter superscript are not significantly different at $p < 0.05$.

Sand grain size of none- filtration not done/raw sewage effluent before filtration

The DO of effluents displayed different trends and was lowest in unfiltered effluents (5.5 mg/l), increasing significantly ($p < 0.05$) in filtered effluents except for effluents from filters with the small grain size (0.1 - 0.5) mm where there was no significant increase in DO observed after filtration.

The greatest reduction of NO_2^- and NO_3^- measurements were observed in filters with small grain sizes (0.1 - 0.5) mm while the least reduction of the two parameters were observed in filters with large grain sizes (1.1 - 2.0) mm (Figs. 13 D and E).

The PO_4^{3-} concentration was highest (16 mg/l) in unfiltered effluents and decreased to the lowest value (< 2.0 mg/l) at the least experimental sand grain size (0.1 - 0.5) mm. However, this was not significantly ($p < 0.05$) different to the values for medium (0.6 - 1.0) mm and large grain sizes (1.1 - 2.0) mm (Fig. 13 F).

Results for conductivity and TSS measurements in filtered and unfiltered effluents followed the same trend as pH and BOD_5 measurements. Conductivity and TSS were found to be at 450 and 0.40 mg/l respectively in unfiltered effluents, reducing significantly ($p < 0.05$) after filtration with the greatest reduction for the two parameters occurring in filters with small grain sizes (0.1 - 0.5) mm and the least reduction of the two occurring in filters with large grain sizes (1.1 - 2.0) mm (Figs. 13 G and H).

Sand grain size was found to affect bacterial removal significantly (Figure 14; Appendix 7). The TBC, FC and TC were significantly ($p < 0.05$) high in unfiltered effluents and decreased with increasing sand grain sizes (1.1-2.0) mm (Fig. 14 A, B

and D). The average percentage removal of TC, FC, FS and TBC means at the three levels of sand bed depth under study are shown in Table 11.

The FS counts displayed a slightly different trend and were highest in unfiltered effluents and decreased by 99.35% at small sand grain size (0.1- 0.5) mm. A percentage decrease of 96.33% was recorded at medium sand grain size (0.6 – 1.0) mm. This removal was found to be significantly different from removal at the two other experimental grain sizes. At the largest sand grain size (1.1 - 2.0) mm, percentage FS removal was found to be lower (99.03%), though not significantly different from removal at small grain size (0.1 - 0.5) mm (Fig. 14 C; Table 11).

Percent removal of TC was found to increase significantly ($p < 0.05$) with an increase in sand grain size. The experimental grain size of (0.1 - 0.5) mm showed the greatest removal of TC (99.48%) followed by 96.26% at medium grain size (0.6 - 1.1) mm and 95.92% at smallest grain size (0.1 - 0.5) mm (Table 11).

Removal efficiency of TBC followed the same trend as TC removal and was found to increase significantly with an increase in sand grain size. The experimental grain size of (0.1 - 0.5) mm showed the greatest removal of TBC (93.15%) followed by 90.88% at medium grain size (0.6 - 1.1) mm and 90.76% at smallest grain size (0.1 - 0.5) mm (Table 11).

Removal of FC followed the same trend as that of TC with significant increase in percent removal with increasing sand grain sizes. Interestingly, the removal efficiency at medium grain size (0.6 - 1.0) mm was found not to be significantly different from

removal at the smallest grain size (0.1 - 0.5) mm and the largest gain size (1.1 - 2.0) mm (Table 11).

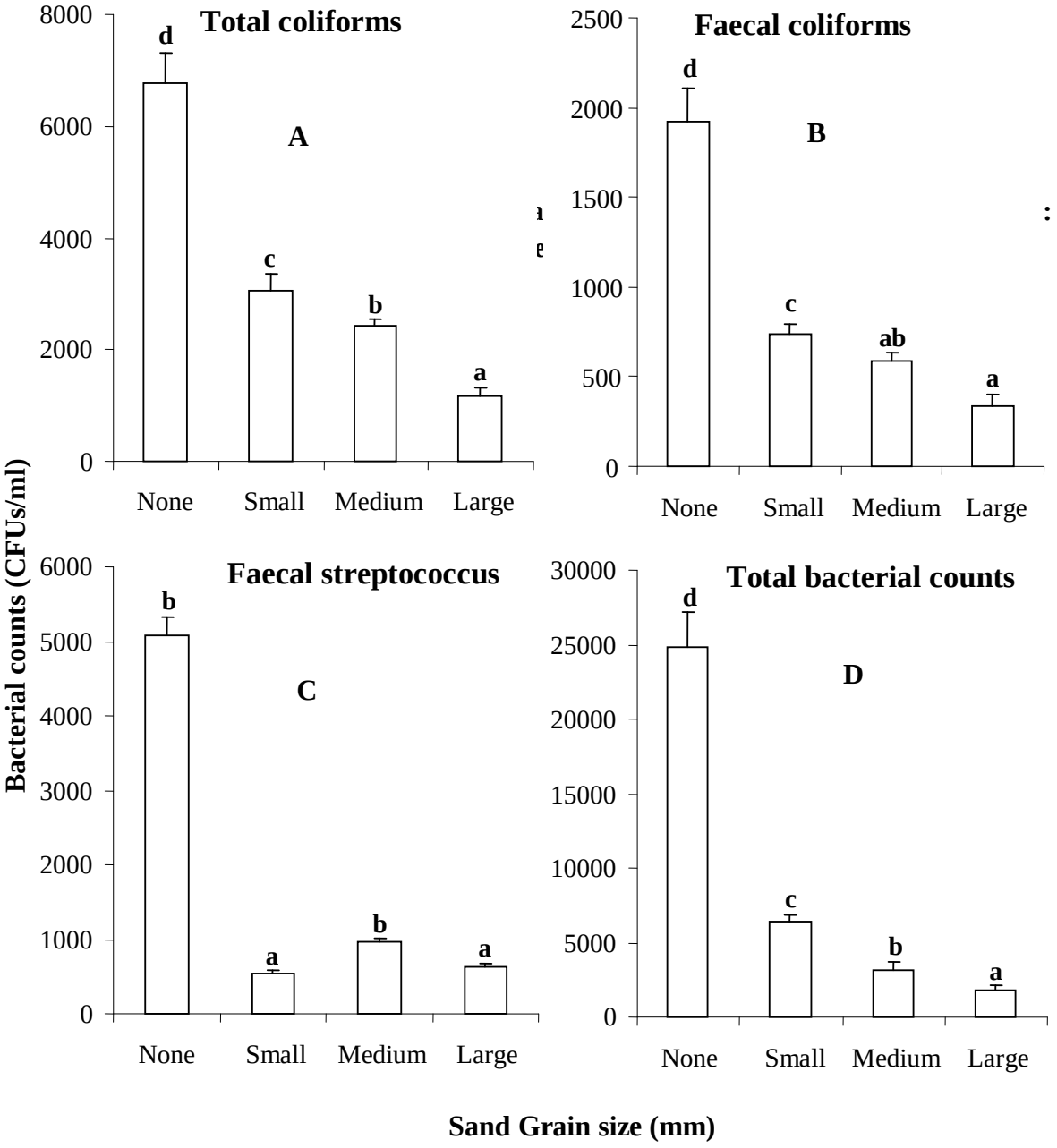

Figure 17: Bacteriological quality parameters (A): TC, (B): FC, (C): FS and (D): TBC in effluents in the filters of different sand grain sizes.

Key: None- unfiltered/control; Small- (0.1-0.5)mm; Medium- (0.6-1.0)mm; Large-(1.1-2.0)mm. Bars with the same letter superscript on the same graph are not significantly different at $p < 0.05$.

Sand grain size of none- filtration not done/raw sewage effluent before filtration

Table 11: Percentage removal of bacteria in Biosand filters at different sand grain sizes

Sand grain size (mm)	Average % removal			
	TC	FC	FS	TBC
0.1-0.5	95.92^{c}	85.45^{c}	99.35^{a}	90.76^{c}
0.6-1.0	96.46^{b}	87.38^{ab}	96.03^{b}	90.88^{b}
1.1-2.0	96.48^{a}	91.22^{a}	99.33^{a}	93.15^{a}

Values with the same letter superscript in the same column are not significantly different at $p < 0.05$

Means with different letter superscripts in same column are significantly different from each other.

4.3.3 Effects of retention time in Biosand filters

The physicochemical parameters in the filters at various levels of retention time in the filtered effluents are shown in Figure 15. Further analysis of the differences in various parameters was also done using ANOVA as provided in Appendix 5. All the physicochemical parameters except PO_4^{3-} were significantly different at various levels of retention time ($p < 0.05$).

The pH of effluents was found to differ significantly ($p < 0.05$) among the different retention periods and decreased significantly with increased retention period of effluents in filters. Mean pH level was 7.63, 7.55 and 7.52 at 24, 48 and 72 hours respectively (Fig. 15 A)

The DO of effluents also differed significantly ($p < 0.05$) among the different levels of retention time and was found to increase significantly ($p < 0.05$) with increased retention time. Mean DO levels in filtered effluents were 6.85 mg/l, 7.1 mg/l and 7.3 mg/l at 24, 48 and 72 hours respectively (Fig. 15 B).

The BOD_5 in filtered effluents at 24 hours was significantly ($p < 0.05$) different from that at 48 and 72 hours of retention in filters. However, mean BOD_5 levels at 48 and 72 hours was not significantly different, both being 0.70 mg/l (Fig. 15 C).

Mean NO_2^- reduction in filtered effluents followed the same trend as BOD_5 reduction. Mean NO_2^- level at 24 hours was significantly ($p < 0.05$) different from that at 48 and 72 hours of retention in filters. However, NO_2^- levels at 48 and 72 hours was not significantly different, both being 0.03 mg/l (Fig. 15 D).

Mean NO_3^- reduction in filtered effluents followed the same trend as mean pH reduction and differed significantly among the different levels of retention periods. The mean NO_3^- levels decreased significantly with increased retention time and was 3.50 mg/l, 3.01 mg/l and 2.01 mg/l at 24, 48 and 72 hours respectively (Fig. 15 E).

Although mean PO_4^{3-} level at 24 hours was higher than that at 48 and 72 hours by 0.9 mg/l, mean PO_4^{3-} reduction did not differ significantly at the different levels of retention time (Fig. 5 F). Trends in conductivity and TSS reduction in filtered effluents were similar to reduction of pH and NO_3^-. Reduction of these two parameters was significantly affected by retention time with increased reduction being observed at increased hours of retention in the filters. Mean conductivity level in effluents was 345 µs/cm, 330 µs/cm and 320 µs/cm at 24, 48 and 72 hours respectively while mean TSS level in filtered effluents was 0.20 mg/l, 0.14 mg/l and 0.11 mg/l at 24, 48 and 72 hours respectively (Fig. 15 G and H).

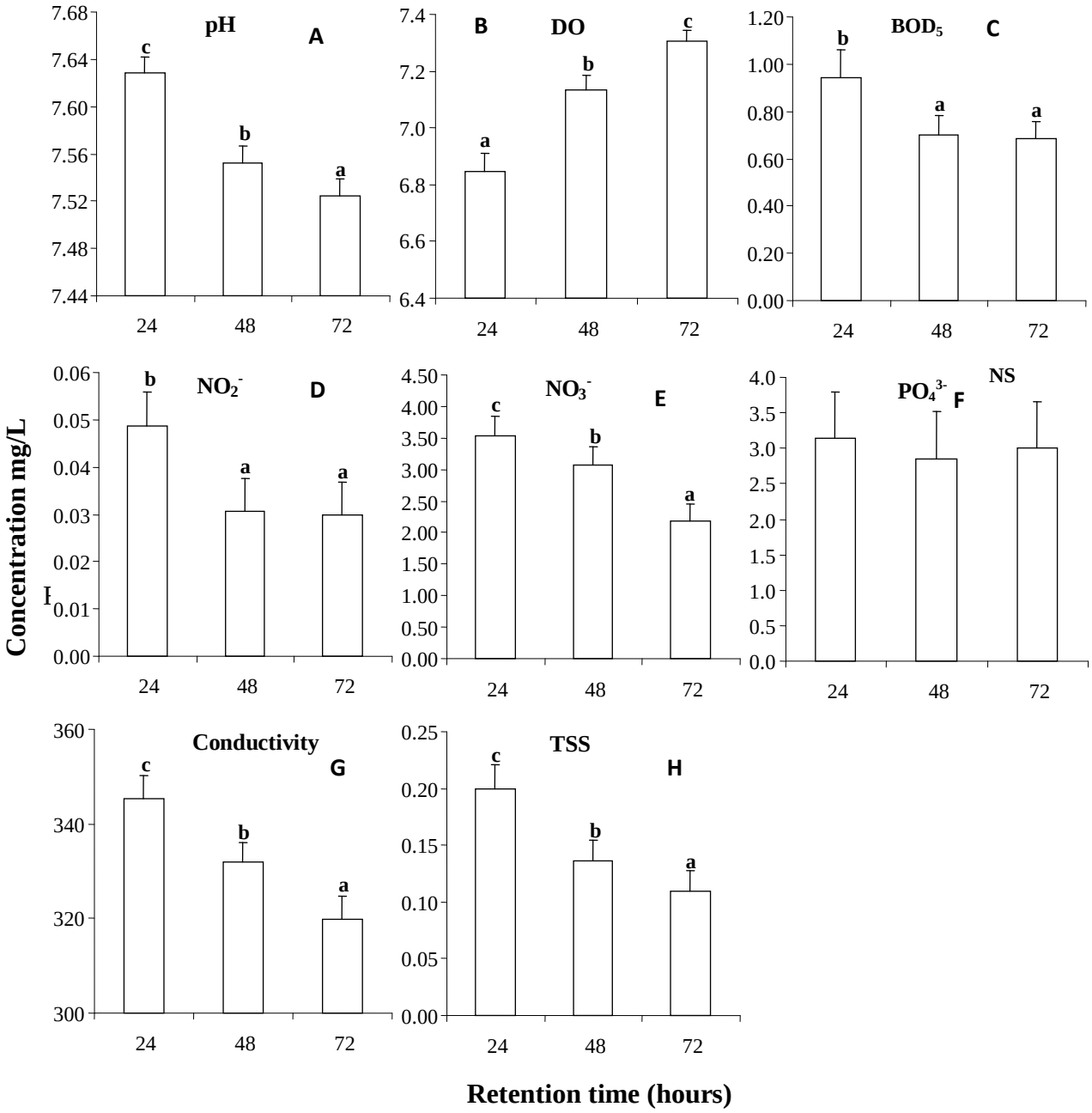

Figure 18: Levels of the physicochemical parameters (A): pH, (B): D), (C): BOD_5, (D): NO_2^-, (E): NO_3^-, (F):PO_4^{3-}, (G): Conductivity and (H): TSS in the filters at different retention times.

NS – Not Significant. Bars with the same letter superscript on the same graph are not significantly different at $p < 0.05$.

Results on the quantitative counts of bacteria in the filtered effluents at various retention times in the sand bed showed that there were significant ($p < 0.05$) differences in mean bacterial counts relative to the different levels of retention time (Fig. 16; Appendix 8).

Reduction of TC and FC counts in filtered effluents followed a similar trend. Although mean TC and FC counts were higher at 24 hours than at 48 hours of retention in the filters, reduction of these two coliform bacteria at these two retention times was not significantly different. However, the means of these two bacterial groups at 72 hours were found to differ significantly ($p < 0.05$) from those at 24 and 48 hours (Fig. 16 A and B).

The means for FS and TBC also followed a similar trend with the values at the three different levels of retention time being significantly ($p < 0.05$) different (Fig. 16 C and D). This showed that the longer the effluents were retained in the filter columns, the more the numbers of bacteria in the filtered effluents were reduced.

A summary of the effects of sand bed depth, sand grain size and retention time on the physicochemical parameters in the effluents is shown in Appendix x while a summary of the effects of sand bed depth, sand grain size and retention time on the bacteriological parameters in the effluents is shown in Appendix ix.

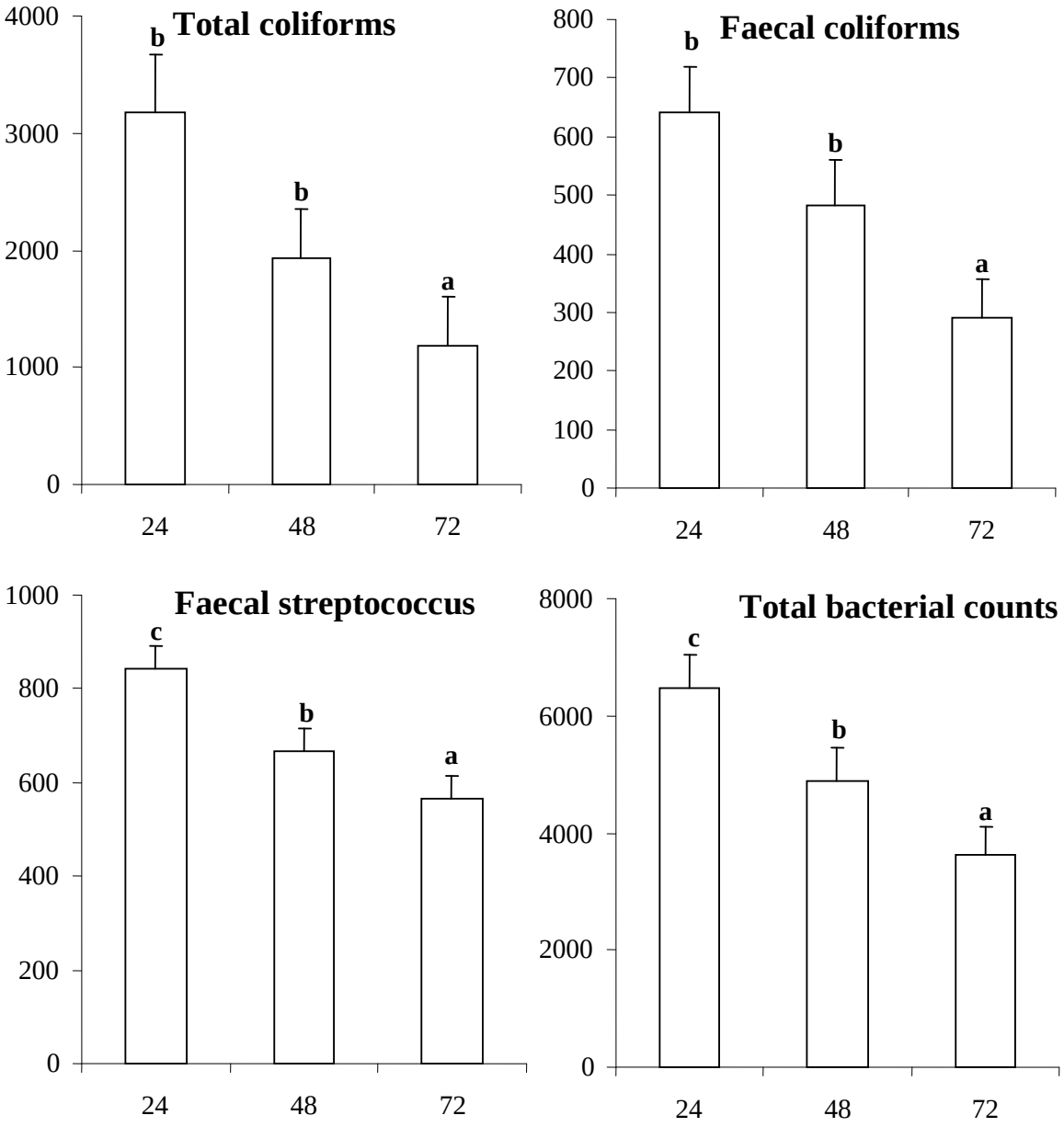

Figure 19: Bacteriological quality parameters (A): TC, (B): FC, (C): FS and (D): TBCs in the filtered effluents at different levels of retention time.

Bars with the same letter superscript on the same graph are not significantly different at $p < 0.05$.

CHAPTER FIVE

DISCUSSION

5.1 Bacteriological analyses

Bacteriological analyses in this study involved isolation, identification and enumeration of selected bacterial groups in filtered and unfiltered effluents. The bacterial counts of TC, FC, FS and TBC were found to differ significantly ($p < 0.05$) in filtered and unfiltered effluents. The numbers of the bacterial groups under study reduced significantly upon filtration using the assembled sand columns. The average removal efficiency of these bacterial groups was approximately 96%, 97%, 82% and 89% for FS, TC, FC and TBC respectively. These findings are consistent with previous findings done on laboratory efficiencies of BsFs by Stauber *et al.*, (2006). These researchers did an 8-month study in Ghana and found out that BsFs operated intermittently achieved a mean reduction of 97% for FS bacteria.

In the present study, results showed an average removal of 82% for FC in effluent samples which agree with those of Elliot *et al.*, (2008). The authors reported that the average *E. coli* reduction in laboratory studies was 80%. Other researchers have reported even higher removals of FC bacteria in laboratory trials of BsFs. For instance, Lee, (2001) and Earwaker, (2007) reported average FC removals of 90 - 99% and 87.9% respectively. However, it is important to note that performance of the investigated filters varied largely, and while some filters provided 100% removal, others provided none or even negative removals of FC (Lee, 2001; Earwaker, 2007).

The findings in this study are also consistent with those obtained from field samples of two and three-year old filters collected during studies on intermittently operated BsFs by Burt (2012) in Afghanistan which also showed an average removal efficiency of 91.7% for *E. coli* bacteria. Another evaluation carried out by Kaiser *et al.,* (2002) on 577 intermittent SSFs located in 6 countries showed an average of 93% FC removal in the effluent samples. Earlier studies by Ellis (1985) on laboratory scale SSFs also revealed that the SSFs gave consistent coliform removals greater than 95% and recommended BsFs as a tertiary treatment process for municipal sewage waters.

5.2 Physicochemical analyses

The filters were found to remove the PO_4^{3-}, NO_2^- and NO_3^- to significant levels in filtered effluents as compared to the levels in unfiltered effluents. This is probably due to the fact that the nutrients were filtered out mechanically by adsorption or by the organisms present in the biofilm that break them down or metabolize them to other intermediate products (Logsdon, 2002). The BsFs was also found to reduce possibly because some of the organic elements were being metabolized by the biofilm organisms (IWA, 2000).

Unlike the other physicochemical parameters under investigation in this study, DO was found to increase significantly ($p < 0.05$) upon filtration of the effluents. This could be due to the reason that most if not all of the organic compounds that require oxygen to degrade become eliminated from the effluents during the filtration process (Gray, 2002). Elimination of organic matter from effluents meant that the oxygen that got dissolved from aeration could be retained thereby increasing the levels of DO in

the filter effluents. Increase in DO was not entire because some of the organic matter could have been in dissolved form and had not been eliminated. As the DO increased in the filtered effluents, the BOD_5 was found to decrease. This can be hypothesized to be so because the organic components of the effluents were being eliminated in the filter column by the action of the biofilm bacteria. In fact, Gray (2002) argued that BOD_5 concentration in effluents can be used as a gauge of the effectiveness of wastewater treatment plants. Gray (2002) also noted the relationship between the removal of TSS and BOD_5 in sand bed filter systems stating that the removal of BOD_5 occur rapidly as a result of filtering and settling of solids with which much of the BOD_5 is associated (IWA, 2000).

5.3 Effect of sand bed depth in Biosand filters

The vertical height of the sand bed in BsFs that the water has to pass through is important in terms of filtration efficiency. The reasons for this are the existence of biological activity in a sand filter which is known to occur at depths of up to 0.5 m within a sand bed and the available surface area for mechanical filtration (Vigneswaran *et al*., 2009).

Literature on the effects of sand depth on the performance of BsF on wastewater is limited and most of the results available in the literature have been obtained based on experimental observations in laboratory scale models (Stevik *et al*., 2004). Removal rates of bacteriological and physicochemical parameters observed during this particular study are consistent with those of other studies using intermittent sand filters in the treatment of wastewater (Stevik *et al.,* 2004).

Elliot *et al.,* (2008), using laboratory scale model showed that longer depths of BsFs can be applied effectively as tertiary treatment for secondary effluents. The author reported that most of the removal of suspended solids and BOD_5 occurred at the lower sand layer. The results in this study also showed that BOD_5, NO_2^-, NO_3^-, conductivity and TSS were significantly higher ($p < 0.05$) in unfiltered effluents but decreased significantly with increasing depth and the values for these parameters were found to be lowest at depths of 1.0 m. This showed that sand bed depth affected the reduction of the physicochemical parameters under study. These findings also agree with those of Bellamy *et al.,* (1985) who reported an average percent removal of pH at 97%, BOD_5 at 87%, NO_3^- at 56%, and conductivity at 62% using sand bed depth of 1 m.

The findings in this study showed that PO_4^{3-} reduction was not significantly different at the different levels of sand depth. The low reduction capacity of PO_4^{3-} suggests that biomass communities within the sand bed removed negligible phosphorus from the wastewater (Arias *et al*., 2001). Removal of phosphorus within sand beds is predominantly achieved through adsorption onto substratum and precipitation/fixation reactions (Arias *et al*, 2001). Consequently, the surface area for chemistry can substantially govern the rate of phosphorus removal (Arias *et al*., 2001; Fisher and Reedy, 2001; Pant *et al*., 2001). Although no chemical analysis of the substrate composition was undertaken, it can be assumed that it would be composed primarily of somewhat inert polymer chains of carbon and hydrogen that would have limited potential to bind phosphorus. Burgoon *et al.,* (1991) reported a maximum TP load removal rate of 44% in sand beds utilizing plastic tricking filter medium to treat

primary treated, noting substrate adsorption and sedimentation as the major mechanism for the removal of TP.

The present study also sought to find out the role of sand bed depth on removal of TC, FC, FS and TBC and established that removal efficiency of these bacterial groups was not entirely pegged on sand bed depth. Results from this study showed that although maximum removal of the coliform organisms (98%) occurred at a depth of 0.5 m, there was no significant difference in bacterial removal in effluents at the different levels of sand bed depth. These results are consistent with those of Bellamy *et al*., (1985) who reported a 97% removal of coliform bacteria at a sand depth of 0.9 m.

In a follow up study, Bellamy *et al*., (1985) also reported 88% - 91% removal of standard plate counts at a depth of 1.0 m. These findings concur with those of our study that showed 90% removal of TBCs at the same sand bed depth. In addition, the present study established that percent removal of FC, TC and TBCs were not significantly different at 0.5 m, 0.7 m and 1.0 m depths. This confirms that bacterial removal in SSFs is not sensitive to sand bed depth. Likewise, Bellamy *et al*., (1985) also found that removal of standard plate counts still ranged from 88% to 91 % when the sand depth was increased from 0.35 to 1.0 m suggesting that sand depth could be reduced to 0.48 m and still produce satisfactory bacteriological removal efficiency.

It can be hypothesized that sand bed depth is less significant in removal of bacteria. This is because most of the biomass and biological treatment occurs in the upper portion of the sand bed and the increasing depth would therefore have little effect on the filtered effluent quality in terms of bacterial removal (Arias *et al*., 2001). For

instance, Williams (1987) found that all bacterial reduction occurs in the top 0.2 m of the filter bed. Research by ASCE, (1991) also confirmed that majority of biological processes occur in the top 0.4 m of the sand bed. Bellamy *et al.,* (1985) and Muhammad *et al*., (1996) reported that bacteriological treatment was not highly sensitive to sand bed depth and thereby increased surface area. However, while this is generally true, bacteriological treatment efficiency becomes sensitive to bed depth with larger sand sizes. This is because the total surface area within the filter is reduced in a sand bed with larger grains and higher flow rates also occur, potentially increasing percolation rates (Jenkins *et al*., 2009).

Wheelis (2008) revealed interesting trends in bacterial removal in BsFs. This author reported 98%, 74% and 85% FS, FC and total plate count removals respectively at sand bed depths of 0.5 m. These results showed that FS removals were highest in BsFs followed by total plate count removals and the least removal was observed for FC. These findings coincide with those in the present study that revealed approximately 99%, 93%, 93% reductions of FS, FC and TBC respectively at the same sand bed depth. All these results portray satisfactory bacterial removals at a very small sand bed depth of 0.5 m showing that shallow bed depth probably allows more oxygen to diffuse to the microbes and the biologically active zone can grow deeper within the sand bed.

5.4 Effect of sand grain size in Biosand filters

In the current study, levels of the physicochemical parameters and bacteriological indicators in the filtered effluents were also affected by sand grain size. Among the physicochemical parameters, the percent removals of pH, BOD_5, NO_2^-, NO_3^-,

conductivity and TSS increased with decreased sand grain size. The reduction in levels of these parameters at varying sand grain sizes can be explained by adsorption sites and microbial communities within the BsFs becoming saturated, with numerous authors reporting a decrease in removal with an increase in filter size (EPA, 2000).

The pattern of BOD_5 removal during the study is comparable to that observed by Mitchell and McNevin, (2001). Farooq *et al*., (1993) conducted a comprehensive study on the effect of sand size on treatment efficiency of BsFs using 0.31 mm and 0.56 mm grain sizes. These authors reported that removals of BOD_5, nitrates and phosphates varied from 79 - 92%, 17 - 30% and 83 - 84% respectively at the two sand grain sizes investigated and concluded that percent removals of the various parameters decreased by increasing sand grain size. The DO of effluents were lowest in filters with small sand grain size (0.1 - 0.5) mm and highest in filters with medium sand grain size (0.6 - 1.0) mm. This trend was different from that observed for other parameters which increased with decreased sand grain size. This could be attributed to the large pore sizes that allowed more oxygen to diffuse through the filter medium.

Reduction of TP in the present study did not differ significantly for the different sand grain sizes. Drizo *et al.,* (2000) reported a similar trend when they investigated treatment of synthetic sewage using sand filters suggesting that reduction of TP may be controlled by other factors. Few studies have tracked the concentration of TP at various sand grain sizes of SSF treating domestic wastewater. Arias *et al.,* (2001) investigated the influence of abiotic factors such as pH, redox potential, DO and certain ions in controlling phosphorus removal mechanisms in SSFs. These factors are important as they affect the chemical precipitation and adsorption of phosphorus

onto the sand filter substrate (IWA, 2000). Reddy and D'Angelo (1997) reported that reducing conditions (low redox potentials) decrease phosphorus precipitation rates and can cause the release of phosphorus from pre formed precipitates. Arias *et al.*, (2001) also reported an increase in phosphorus adsorption rates with an increase in redox potential conditions.

Except for the FS bacteria, least bacterial counts were recorded for filters with large sand grain sizes (1.1-2.0) mm. These findings agree with those of Van der Hook *et al.*, (1996) who found that the use of small grain size (0.19 mm) did not result in better filtrate quality than a larger sand grain size (0.25 mm). The reason for this may be hypothesized to be because large sand grain size translates into large pore sizes that contribute to penetration of biofilm into the sand thereby allowing biological removal mechanisms for bacteria to continue throughout the filters. Goitom (1990) revealed that filters with large sand grain sizes had higher filtration rates while those with smaller grain sizes had lower rates of filtration. It can be assumed that the slow filtration rates in filters with small sand grain sizes lead to premature clogging of the filters thus the reduced filtration efficiency observed in them.

The FS counts decreased with decreasing sand grain size probably because they are physiologically different from the coliform bacteria, and the smaller sand sizes have larger surface area available for supporting the biofilm necessary for their removal (Leclerc, 2001). Nam *et al.*, (2000) demonstrated that finer sand beds had close to three times the biofilm compared to coarse sand. Therefore filters with larger sand sizes have larger interstices between sand grains, smaller surface area and higher flow rates which give rise to less total surface area for biofilm to grow on.

Fine sand grain size has been reported to produce filtrates of better bacteriological quality by many researchers. However, most of the research has been carried out on continually-operated sand filter systems. In contrast, research carried out on intermittently-operated filters, as in the present study, does not seem to indicate that fine sand size is important in improving efficiency. The results from this study indicated that using coarse sand (1.1 - 2.0) mm rather than fine sand (0.1 - 0.5) mm yielded a significant and meaningful efficiency of SSF removal of bacteria independent of retention time and sand bed depth. The findings concur with those of Bellamy *et al*., (1985) who reported that an increase in the effective sand grain size did not necessarily result in poor filter performance. These researchers established that an increase in effective diameter of sand grains from 0.128 mm to 0.615 mm resulted only in a small decrease in bacterial removals from 99.4% to 96%. Muhammad *et al.,* (1996) also observed that treatment efficiency in terms of removal of bacteria was not sensitive to sand sizes up to 0.45 mm, although a slight increase in treatment efficiency was observed with decreasing sand size. They concluded that in terms of removal efficiency of bacteria, the argument for using very fine sand is not strong. It seems that coarse sand provides acceptable filtration results in continually-operated systems (Barrett, 1989).

5.5 Effect of retention time in Biosand filters on improvement of secondary effluent quality

The removal efficiency of most physicochemical parameters in the filtered effluents was also influenced by the period of retaining the effluents in the filters. Effluents

retained for the longest period in the filters (72 hours) had the lowest levels of the physicochemical parameters under study except for DO that was found to increase. These results agree with those observed by Madigan and Martinko (2008) and Rooklidge *et al*. (2009) who recorded greater removal of nitrogen, phosphates and BOD_5 under long retention periods. This was attributed to microbial communities within the system that acted on the organics to break them down into simpler substances. It therefore follows that short retention time does not give the microbial communities in the biological layer enough time to remove particles and contaminants present in the effluents.

The removal of bacteria in BsFs is a biological process and is therefore affected by the time available for reactions to take place in the filter bed. The removal efficiency of the various bacterial groups improved with increased retention period of effluents in the filters. Most removal occurs when water is in contact with the biofilm and so the pause or retention period allows the microorganisms in the biofilm to consume the bacteria and nutrients in water (Dizer *et al*., 2004). For satisfactory removal of bacteria, sufficient time must therefore be allowed to maintain a long contact time with the sand bed.

Studies by Elliot *et al.,* (2008) Jenkins *et al*., (2009) showed that greater reduction of bacteria can be attained with greater retention time within the filters. These researchers found that microbial reductions tended to improve as retention time increased and thus the last parcels of water to leave the filters had much better quality than the initial parcels of water withdrawn from the filters. The results from these studies are consistent with those from the present study which also showed improved

bacterial removal under long retention periods confirming that the longer the effluents are retained in the filter columns, the more the numbers of bacteria in the filtered effluents are reduced.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

From this study, it can be concluded that filtration produced effluents of better quality in terms of reduction of bacterial counts and reduction/increase of physicochemical parameters. Although bacterial numbers reduced significantly after filtration of effluents, TC and FS were removed the most (approximately 97% and 96% respectively) and FC and TBC were removed the least (approximately 82% and 87% respectively).

The levels of all physicochemical parameters except DO also improved significantly after filtration of effluents. Maximum removals were observed for BOD_5 and PO_4^{3-} (93% and 90% respectively). Minimum removals were observed for pH, conductivity, NO_3^-, TSS and NO_2^- (approximately 7%, 27%, 33%, 69% and 75% respectively). The DO in filtered effluents increased (unlike the other physicochemical parameters) by approximately 19%.

It can also be concluded that sand bed depth, retention time and sand grain size influence filter performance for all the dependent variables under investigation. Filtration using large sand grains (1.1 - 2.0) mm improve bacterial removal than filtration using fine sand grains (0.1 - 0.5) mm and medium sand grains (0.6 – 1.1) mm. However, physicochemical parameters such as pH, BOD_5, NO_3^-, NO_2^-, conductivity and TSS decreased significantly in effluents from fine sand grains.

Physicochemical parameters such as pH, BOD_5, NO_3^-, NO_2^-, conductivity and TSS decreased significantly when sand bed depth increased. However, reduction of PO_4^{3-} in filtered effluents was not significantly affected by sand bed depth. Similarly, removal of bacterial groups and TBC was not significantly influenced by sand bed depth. The removal of different response parameters both physicochemical and bacteriological increased with increased retention time showing that longer retention of effluents in the filters improved filtration efficiency irrespective of sand size or bed depth.

6.2 Recommendations

- The study recommends that for efficiency of BsFs in eliminating of bacteria, the retention period be extended, the grain size should be large but the bed depth needs not be high.
- If removal of physicochemical parameters such as pH, BOD_5, NO_3^-, NO_2^-, conductivity and TSS is targeted, the bed depth should be increased to 1 m while sand grain size should be fine to improve filtration efficiency.
- Further studies on the indicator to pathogen ratios in filtered and unfiltered effluents in order to correlate the work done on the indicator microorganisms to the actual risk of microbial infection.
- There is also need for more research to understand the mechanisms that are responsible for microbial reduction in the BsFs so as to optimize its design and operation in terms of reduction of harmful bacteria in secondary effluents before they can be discharged into waterways.

- Field trials on BsFs based on different bed depths, retention times and sand grain sizes are needed in order to determine whether similar results as those obtained from laboratory studies would be achieved.

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APPENDICES

Appendix i: Effects of sand bed depth on the physicochemical parameters in effluents

Physicochemical parameter		Sum of Squares	Df	Mean Square	F	P-value
pH	Between Groups	5.518	3	1.839	29.23	0.000
	Within Groups	9.188	146	0.063		
	Total	14.706	149			
DO	Between Groups	22.625	3	7.542	2.433	0.074
	Within Groups	452.578	146	3.1		
	Total	475.203	149			
BOD_5	Between Groups	288.722	3	96.241	410.534	0.000
	Within Groups	34.227	146	0.234		
	Total	322.949	149			
Nitrites (NO_2^-)	Between Groups	0.134	3	0.045	10.639	0.000
	Within Groups	0.612	146	0.004		
	Total	0.746	149			
Nitrates (NO_3^-)	Between Groups	37.63	3	12.543	2.936	0.035
	Within Groups	623.672	146	4.272		
	Total	661.302	149			
Phosphates	Between Groups	2847.617	3	949.206	439.29	0.060
	Within Groups	315.473	146	2.161		
	Total	3163.09	149			
Conductivity	Between Groups	202977.411	3	67659.137	18.201	0.000
	Within Groups	542718.089	146	3717.247		
	Total	745695.5	149			
TSS	Between Groups	1.026	3	0.342	24.534	0.000
	Within Groups	2.035	146	0.014		
	Total	3.061	149			

Appendix ii: Effects of sand bed depth on the bacteriological quality parameters in the effluents

Bacterial quality parameters		Sum of Squares	Df	Mean Square	F	P-value
TC	Between Groups	4.51E+08	3	1.501E+08	20.851	0.060
	Within Groups	1.05E+09	146	7201334.46		
	Total	1.50E+09	149			
FC	Between Groups	3.35E+07	3	1.121E+07	44.112	0.000
	Within Groups	3.70E+07	146	253138.904		
	Total	7.05E+07	149			
FS	Between Groups	3.48E+08	3	1.16E+08	12.72	0.075
	Within Groups	1.33E+09	146	9105906.575		
	Total	1.68E+09	149			
TBC	Between Groups	6.42E+09	3	2.14E+09	16.088	0.087
	Within Groups	1.94E+10	146	1.33E+08		
	Total	2.59E+10	149			

Appendix iii: Composition of Bile Aesculin Agar: Approximate formula per liter of purified water

Pancreatic Digest of Gelatin.....5.0 g

BeefExtract .....3.0 g

Oxgall/Oxbile.....20.0 g

Ferric Citrate .....0.5 g

Aesculin .....1.0 g

Agar .....14.0 g

Appendix iv: Effects of sand grain sizes on the physicochemical parameters in the effluents

		Sum of Squares	Df	Mean Square	F	P-value
pH	Between Groups	5.686	3	1.895	30.681	0.000
	Within Groups	9.02	146	0.062		
	Total	14.706	149			
DO	Between Groups	31.645	3	10.548	3.472	0.018
	Within Groups	443.557	146	3.038		
	Total	475.203	149			
BOD_5	Between Groups	288.851	3	96.284	412.263	0.000
	Within Groups	34.098	146	0.234		
	Total	322.949	149			
Nitrites	Between Groups	0.099	3	0.033	7.405	0.000
	Within Groups	0.648	146	0.004		
	Total	0.746	149			
Nitrates	Between Groups	28.446	3	9.482	2.188	0.009
	Within Groups	632.856	146	4.335		
	Total	661.302	149			
Phosphates	Between Groups	2829.965	3	943.322	413.433	0.000
	Within Groups	333.125	146	2.282		
	Total	3163.09	149			
Conductivity	Between Groups	193226.3	3	64408.767	17.021	0.000
	Within Groups	552469.2	146	3784.036		
	Total	745695.5	149			
TSS	Between Groups	1.018	3	0.339	24.255	0.000
	Within Groups	2.043	146	0.014		
	Total	3.061	149			

Appendix v: Effects of retention times on the physicochemical parameters in the filtered effluents

		Sum of Squares	df	Mean Square	F	Sig.
pH	Between Groups	0.29	2	0.145	1.476	0.032
	Within Groups	14.417	147	0.098		
	Total	14.706	149			
DO	Between Groups	5.42	2	2.71	0.848	0.030
	Within Groups	469.783	147	3.196		
	Total	475.203	149			
BOD_5	Between Groups	1.445	2	0.722	0.33	0.019
	Within Groups	321.504	147	2.187		
	Total	322.949	149			
Nitrites	Between Groups	0.011	2	0.006	1.132	0.025
	Within Groups	0.735	147	0.005		
	Total	0.746	149			
Nitrates	Between Groups	45.917	2	22.959	5.484	0.005
	Within Groups	615.385	147	4.186		
	Total	661.302	149			
Phosphates	Between Groups	2.22	2	1.11	0.052	0.950
	Within Groups	3160.87	147	21.503		
	Total	3163.09	149			
Conductivity	Between Groups	16344.84	2	8172.42	1.647	0.016
	Within Groups	729350.66	147	4961.569		
	Total	745695.5	149			
TSS	Between Groups	0.216	2	0.108	5.584	0.005
	Within Groups	2.845	147	0.019		
	Total	3.061	149			

Appendix vi: Composition of MacConkey Agar

Component	***Quantity in Grams***
Peptone (Difco) or Gelysate (BBL)	17.0 g
Proteose peptone (Difco) or Polypeptone (BBL)	3.0 g
Lactose	10.0 g
NaCl	5.0 g
Crystal Violet	1.0 mg
Neutral Red	30.0 mg
Bile Salts	1.5 g
Agar	13.5 g

Appendix vii: Effects of sand grain sizes on the bacteriological parameters in the effluents

		Sum of Squares	df	Mean Square	F	P-value
TC	Between Groups	4.52E+08	3	1.51E+08	20.93	0.000
	Within Groups	1.05E+09	146	7193192.694		
	Total	1.50E+09	149			
FC	Between Groups	3.40E+07	3	1.14E+07	45.493	0.000
	Within Groups	3.64E+07	146	249427.489		
	Total	7.05E+07	149			
FS	Between Groups	3.48E+08	3	1.16E+08	12.719	0.000
	Within Groups	1.33E+09	146	9106048.311		
	Total	1.68E+09	149			
TBC	Between Groups	6.44E+09	3	2.15E+09	16.143	0.000
	Within Groups	1.94E+10	146	1.33E+08		
	Total	2.59E+10	149			

Appendix viii: Effects of retention times on the bacteriological quality parameters in the filtered effluents

		Sum of Squares	Df	Mean Square	F	P-value
TC	Between Groups	2.28E+07	2	1.14E+07	4.73	0.035
	Within Groups	1.48E+09	147	1.01E+07		
	Total	1.50E+09	149			
FC	Between Groups	1269337.333	2	634668.667	3.35	0.026
	Within Groups	6.92E+07	147	470669.361		
	Total	7.05E+07	149			
FS	Between Groups	1943004	2	971502	10.09	0.000
	Within Groups	1.68E+09	147	1.14E+07		
	Total	1.68E+09	149			
TBC	Between Groups	1.20E+08	2	6.01E+07	5.34	0.010
	Within Groups	2.57E+10	147	1.75E+08		
	Total	2.59E+10	149			

Appendix ix: Effects of sand bed depth, sand grain size and retention time on the bacteriological parameters in effluents

Depth (m)	Grain size (mm)	Retention time (hours)	TC	FC	FS	TBC
0.0	None	24	6780 ± 3634^{a}	1924 ± 5612^{a}	5258 ± 563^{a}	26842 ± 174^{a}
		48	6180 ± 3634^{a}	1224 ± 521^{a}	5258 ± 436^{a}	24140 ± 123^{a}
		72	6082 ± 3521^{a}	924 ± 412^{b}	5258 ± 312^{a}	22840 ± 102^{a}
0.5	Small	24	3194 ± 256^{b}	448 ± 214^{c}	318 ± 178^{b}	5570 ± 3045^{b}
		48	790 ± 323^{c}	382 ± 216^{c}	82 ± 36^{c}	3360 ± 1772^{bc}
		72	646 ± 260^{c}	372 ± 209^{c}	48 ± 20^{c}	2756 ± 1211^{bc}
	Medium	24	1064 ± 274^{b}	576 ± 194^{c}	458 ± 167^{b}	2300 ± 417^{bc}
		48	894 ± 304^{c}	356 ± 130^{c}	88 ± 33^{c}	1504 ± 473^{c}
		72	638 ± 256^{c}	288 ± 143^{c}	62 ± 26^{c}	2652 ± 117^{bc}
	Large	24	680 ± 348^{c}	130 ± 46^{cd}	270 ± 128^{b}	1978 ± 326^{c}
		48	826 ± 449^{c}	84 ± 38^{d}	182 ± 155^{bc}	1448 ± 481^{c}
		72	646 ± 369^{c}	66 ± 25^{d}	40 ± 24^{c}	1380 ± 434^{c}
0.7	Small	24	1094 ± 188^{b}	582 ± 146^{c}	386 ± 160^{b}	5172 ± 506^{b}
		48	720 ± 242^{c}	312 ± 123^{c}	92 ± 31^{c}	3380 ± 912^{bc}
		72	372 ± 224^{c}	208 ± 120^{c}	38 ± 19^{c}	1740 ± 506^{c}
	Medium	24	960 ± 210^{c}	344 ± 190^{c}	364 ± 141^{b}	7696 ± 2110^{b}
		48	678 ± 235^{c}	218 ± 129^{c}	114 ± 59^{cb}	3340 ± 1012^{bc}
		72	526 ± 201^{c}	216 ± 119^{c}	52 ± 38^{c}	2040 ± 623^{bc}
	Large	24	1634 ± 554^{b}	500 ± 175^{c}	280 ± 72^{b}	2384 ± 216^{bc}
		48	932 ± 292^{c}	408 ± 190^{c}	202 ± 137^{b}	1340 ± 370^{c}
		72	764 ± 320^{b}	304 ± 166^{c}	20 ± 4^{c}	1324 ± 344^{c}
1.0	Small	24	2132 ± 438^{b}	772 ± 292^{c}	488 ± 89^{b}	4676 ± 370^{b}
		48	1244 ± 294^{b}	350 ± 130^{c}	218 ± 145^{b}	2708 ± 501^{bc}
		72	634 ± 107^{b}	316 ± 133^{c}	54 ± 19^{c}	2216 ± 541^{bc}
	Medium	24	2688 ± 1107^{b}	718 ± 204^{c}	352 ± 112^{b}	4836 ± 1021^{b}
		48	1362 ± 329^{b}	630 ± 213^{c}	228 ± 123^{b}	3460 ± 1254^{bc}
		72	432 ± 196^{c}	418 ± 173^{c}	54 ± 9^{c}	2272 ± 742^{bc}
	Large	24	940 ± 299^{c}	406 ± 129^{c}	250 ± 113^{b}	5232 ± 1526^{b}
		48	590 ± 208^{c}	158 ± 63^{cd}	208 ± 140^{b}	3512 ± 871^{bc}
		72	378 ± 184^{c}	106 ± 54^{cd}	44 ± 12^{c}	2396 ± 711^{bc}

Key: Small-0.5mm and below; Medium- 0.6mm-1.0mm; Large- 1.1mm-2.0mm.TC=Total Coliforms, FC= Faecal Coliforms, FS= Faecal Streptococci, TBC = Total Bacterial Counts. Means with the same letter superscript in the same column are not significantly different by Duncan's Multiple Range Test at $p < 0.05$

Appendix x: Effects of sand bed depth, sand grain size and retention time on the physicochemical parameters of the effluents.
Key: Small-0.5mm and below; Medium- 0.6mm-1.0mm; Large- 1.1mm-2.0mm

Depth (m)	Grain size (mm)	Retention time (hrs)	Ph	DO	BOD_5	Nitrites	Nitrates	Phosphates	Conductivity	TSS
0	None	24	8.14 ± 0.19^{a}	6.02 ± 0.75^{a}	4.98 ± 0.43^{a}	0.112 ± 0.062^{a}	4.19 ± 1.21^{a}	18.01 ± 1.64^{a}	469.6 ± 41.2^{a}	0.43 ± 0.08^{a}
		48	8.10 ± 0.14^{a}	6.09 ± 0.19^{a}	4.98 ± 0.41^{a}	$0.12\ 3\pm 0.052^{a}$	4.12 ± 1.12^{a}	16.09 ± 1.44^{a}	439.3 ± 40.1^{a}	0.39 ± 0.07^{a}
		72	8.07 ± 0.12^{a}	6.11 ± 0.74^{a}	4.98 ± 0.37^{a}	0.114 ± 0.041^{a}	4.02 ± 0.09^{a}	11.03 ± 1.21^{a}	412.5 ± 32.5^{a}	0.37 ± 0.06^{a}
0.5	Small	24	7.52 ± 0.07^{c}	6.46 ± 0.32^{ab}	0.31 ± 0.16^{c}	0.001 ± 0.001^{c}	3.62 ± 0.91^{b}	1.92 ± 0.64^{bc}	341.6 ± 26.5^{b}	0.13 ± 0.04^{bc}
		48	7.52 ± 0.08^{c}	6.99 ± 0.21^{ab}	0.21 ± 0.06^{bc}	0.004 ± 0.001^{c}	2.62 ± 0.52^{b}	1.12 ± 0.51^{c}	318.4 ± 26.4^{bc}	0.08 ± 0.03^{c}
		72	7.51 ± 0.06^{c}	7.21 ± 0.21^{b}	0.45 ± 0.17^{bc}	0.002 ± 0.002^{c}	1.34 ± 0.21^{c}	1.51 ± 0.23^{c}	300.2 ± 21.3^{bc}	0.07 ± 0.04^{c}
	Medium	24	7.58 ± 0.07^{c}	6.87 ± 0.51^{ab}	0.42 ± 0.08^{bc}	0.016 ± 0.006^{c}	3.42 ± 0.02^{b}	0.78 ± 0.51^{d}	339.8 ± 44.2^{b}	0.29 ± 0.11^{b}
		48	7.52 ± 0.08^{c}	7.27 ± 0.41^{b}	0.20 ± 0.06^{c}	0.011 ± 0.003^{c}	2.78 ± 0.24^{b}	0.52 ± 0.45^{d}	332.8 ± 32.1^{b}	0.12 ± 0.06^{bc}
		72	7.46 ± 0.07^{c}	7.03 ± 0.32^{b}	0.33 ± 0.19^{c}	0.013 ± 0.003^{c}	1.26 ± 0.41^{c}	1.02 ± 0.41^{c}	312.2 ± 31.4^{bc}	0.11 ± 0.05^{bc}
	Large	24	7.50 ± 0.11^{c}	6.89 ± 0.52^{ab}	0.83 ± 0.37^{b}	0.017 ± 0.009^{c}	3.26 ± 0.81^{b}	0.78 ± 0.51^{d}	354.1 ± 29.7^{b}	0.16 ± 0.05^{bc}
		48	7.48 ± 0.12^{c}	7.07 ± 0.48^{c}	0.21 ± 0.11^{c}	0.006 ± 0.003^{c}	2.18 ± 0.42^{b}	0.44 ± 0.39^{d}	324.6 ± 31.1^{b}	0.08 ± 0.02^{c}
		72	7.44 ± 0.12^{c}	7.23 ± 0.21^{b}	0.27 ± 0.22^{c}	0.002 ± 0.002^{c}	1.54 ± 0.44^{c}	0.78 ± 0.47^{d}	281.0 ± 23.6^{c}	0.05 ± 0.02^{c}
0.7	Small	24	7.70 ± 0.07^{b}	7.44 ± 0.82^{b}	0.61 ± 0.33^{b}	0.016 ± 0.008^{c}	4.01 ± 1.12^{ab}	2.02 ± 0.15^{b}	321.8 ± 11.2^{b}	0.13 ± 0.05^{bc}
		48	7.62 ± 0.07^{b}	7.90 ± 0.82^{b}	0.42 ± 0.24^{bc}	0.012 ± 0.006^{c}	3.42 ± 0.62^{b}	1.86 ± 0.20^{bc}	304.6 ± 17.2^{bc}	0.07 ± 0.03^{c}
		72	7.58 ± 0.05^{c}	8.24 ± 0.97^{c}	0.30 ± 0.19^{c}	0.022 ± 0.018^{bc}	2.26 ± 0.56^{b}	1.76 ± 0.41^{c}	299.4 ± 16.2^{c}	0.06 ± 0.03^{c}
	Medium	24	7.58 ± 0.12^{c}	6.61 ± 0.37^{ab}	0.34 ± 0.08^{c}	0.083 ± 0.047^{b}	3.46 ± 0.45^{b}	2.22 ± 0.21^{b}	318.6 ± 10.9^{bc}	0.17 ± 0.04^{bc}
		48	7.46 ± 0.13^{c}	6.96 ± 0.28^{ab}	0.15 ± 0.05^{c}	0.015 ± 0.005^{b}	3.08 ± 0.65^{b}	1.98 ± 0.22^{bc}	300.0 ± 21.7^{bc}	0.13 ± 0.03^{bc}
		72	7.42 ± 0.12^{c}	7.56 ± 0.36^{b}	0.41 ± 0.23^{bc}	0.012 ± 0.004^{b}	2.36 ± 0.36^{b}	1.68 ± 0.43^{c}	296.6 ± 20.6^{c}	0.07 ± 0.03^{c}
	Large	24	7.58 ± 0.11^{c}	6.56 ± 0.31^{a}	0.51 ± 0.13^{b}	0.048 ± 0.016^{bc}	3.92 ± 0.15^{b}	1.82 ± 0.32^{c}	319.8 ± 16.1^{b}	0.15 ± 0.04^{bc}
		48	7.44 ± 0.09^{c}	6.85 ± 0.22^{ab}	0.29 ± 0.10^{c}	0.015 ± 0.006^{c}	3.32 ± 0.47^{b}	1.48 ± 0.41^{c}	316.2 ± 16.4^{bc}	0.12 ± 0.05^{bc}
		72	7.42 ± 0.08^{c}	7.14 ± 0.25^{b}	0.38 ± 0.18^{c}	0.012 ± 0.005^{c}	2.64 ± 0.23^{b}	1.58 ± 0.43^{c}	297.6 ± 15.6^{c}	0.06 ± 0.03^{c}
1	Small	24	7.54 ± 0.11^{c}	6.77 ± 0.33^{ab}	0.59 ± 0.19^{b}	0.088 ± 0.048^{b}	2.66 ± 0.56^{b}	1.92 ± 0.22^{bc}	358.6 ± 19.9^{b}	0.21 ± 0.05^{b}
		48	7.54 ± 0.17^{c}	6.96 ± 0.39^{ab}	0.16 ± 0.06^{c}	0.068 ± 0.052^{bc}	2.42 ± 0.82^{b}	1.68 ± 0.12^{c}	343.1 ± 11.2^{b}	0.12 ± 0.05^{bc}
		72	7.54 ± 0.11^{c}	7.05 ± 0.39^{b}	0.54 ± 0.34^{b}	0.072 ± 0.048^{bc}	2.08 ± 0.61^{b}	2.46 ± 0.23^{b}	335.4 ± 15.6^{b}	0.09 ± 0.04^{c}
	Medium	24	7.46 ± 0.15^{c}	8.53 ± 0.35^{c}	0.51 ± 0.32^{b}	0.053 ± 0.036^{bc}	2.92 ± 0.51^{b}	1.74 ± 0.11^{c}	324.4 ± 13.3^{b}	0.16 ± 0.06^{bc}
		48	7.34 ± 0.15^{c}	8.63 ± 1.22^{c}	0.17 ± 0.02^{c}	0.028 ± 0.017^{c}	2.98 ± 0.36^{b}	1.44 ± 0.21^{c}	315.8 ± 14.6^{bc}	0.11 ± 0.04^{bc}
		72	7.32 ± 0.14^{c}	8.72 ± 2.11^{c}	0.29 ± 0.21^{c}	0.022 ± 0.014^{c}	1.86 ± 0.56^{c}	1.28 ± 0.14^{c}	316.8 ± 16.2^{bc}	0.03 ± 0.01^{c}
	Large	24	7.68 ± 0.14^{b}	6.28 ± 0.51^{b}	0.32 ± 0.08^{c}	0.044 ± 0.032^{c}	3.82 ± 0.35^{b}	2.28 ± 0.15^{b}	335.0 ± 17.2^{b}	0.19 ± 0.05^{bc}
		48	7.48 ± 0.08^{c}	6.64 ± 0.44^{ab}	0.22 ± 0.12^{c}	0.034 ± 0.024^{c}	3.71 ± 0.25^{b}	1.98 ± 0.22^{bc}	324.1 ± 14.3^{b}	0.15 ± 0.04^{bc}
		72	7.42 ± 0.09^{c}	6.86 ± 0.46^{ab}	0.24 ± 0.11^{c}	0.031 ± 0.023^{c}	2.42 ± 0.33^{b}	1.92 ± 0.24^{bc}	318.8 ± 17.2^{bc}	0.14 ± 0.05^{bc}