Determination of the quality of environmental water using GC-MS based faecal sterol analysis

C Swanepoel

13075942

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Supervisor: Prof. CC Bezuidenhout

Assistant supervisor: Mr. JS Hendriks

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"In an age when man has forgotten his origins and is blind even

to his most essential needs for survival,

water along with other resources has become victim of his

indifference"

~ Rachel Carson: Author - Silent Spring ~

ABSTRACT

Faecal indicator bacteria have traditionally been used in the detection of faecal pollution in water, but due to concerns about the lack of reliability of these indicators, alternative methods have been developed. One of which is the detection of sterols present in human and animal excreta via GC-MS analysis of water in this study. The Szűcs method was used to detect six target sterols (coprostanol, cholesterol, dehydrocholesterol, stigmasterol, β-sitosterol, and stigmastanol) in environmental water samples. An initial study was done by analysing raw sewage and effluent (human faecal sterol biomarkers) and water samples were spiked with excreta from cattle, chickens, horses, pigs, and sheep to determine faecal sterol fingerprints. The method was evaluated for quantitation and differences between the water samples from each species. Following liquid-liquid extraction, silylation and derivatization, samples were analysed by GC-MS. Standard curve assays were linear up to 160ng and the limit for quantification was 20ng. The human faecal sterol biomarker was coprostanol, while herbivore profiles were dominated by terrestrial sterol biomarkers (stigmasterol and stigmastanol). Sterol fingerprints and differences in concentrations of sterols between various animals and between animals and humans occurred, providing the opportunity to determine whether faecal pollution was from humans or from animals. The method proved sensitive enough to evaluate faecal contamination in environmental water. Groundwater was collected from bore-holes and surface water samples were collected from the Baberspan Inland Lake. Physico-chemical parameters analysed indicated that pH for surface water samples was above 6.9. The total dissolved solids (TDS) in groundwater indicated that the water was not suitable for human consumption, but could be used for livestock watering. Surface water electrical conductivity (EC) and inorganic nitrates was too high to be used for irrigational purposes. Nitrates in groundwater were too high to be consumed by humans. In groundwater, the total

coliform target water quality range (TWQR) was exceeded at 53% of sites analysed and faecal coliform TWQR were exceeded at 77% sites. Surface water samples complied with TWQR with regards to faecal coliforms for full contact recreational activities and livestock watering. The TWQR for *E. coli*, with regards to full contact recreational activities, was within a safe range for surface water. Faecal streptococci were found in 85% of groundwater sampling sites. And surface water faecal streptococci counts exceeded the TWQR for full contact recreational activities. There is no TWQR for faecal sterols in water, but concentrations of cholesterol and coprostanol was found at three of the groundwater sites analysed. This indicates faecal contamination from possible animal and human origin. Surface water samples analysed showed that the Harts River water is clean and free of faecal sterols, while the water analysed from the inflow, hotel and outflow, cholesterol eluted, which showed faecal contamination, possibly from animals. Faecal sterol markers could be detected in groundwater and surface water, adding an extra dimension to determining the quality of water systems. An optimization and sensitivity study of the method was done on waste water treatment plant (WWTP) raw sewage and effluent. The WWTP sample analysed form Potchefstroom and Carletonville WWTP yielded all six target sterols in the raw sewage water samples, but no sterols eluted in the effluent samples. The raw sewage water sample taken from the Fochville WWTP yielded all six target sterols as well, however, the effluent yielded an unknown compound as well as cholesterol. An alternative study was done where the effluent sample volume was increased. By increasing the volume of water, one can possibly increase the amount ("load") of sterols extracted and analysed, resulting in a higher abundance of target sterols. By using the target qualifier ions of the six target sterols, and the GC-TOF/MS software, the target sterols could still be qualitatively determined. Optimal volume for raw sewage is 300 ml water sample as this is enough to yield all 6 target sterols. For optimum water quality monitoring via faecal sterol analysis of effluent and other

environmental samples, at least 1L sample volume needs to be collected and analysed. The methods described here can be applied to the analysis of environmental water samples. The technical advantages also make it suitable for routine environmental monitoring of faecal pollution.

Keywords: Faecal sterols; Coprostanol; Cholesterol; Faecal pollution; Faecal Streptococci; Faecal coliforms.

This work is dedicated to my parents, Leo and Alta van Vuuren. Thank you for a lifetime of support, motivation and love. For helping me realize that I am capable of anything if I put my mind to it and thank you for pushing me to be more than I ever thought possible.

I am grateful…

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Lastly, and most importantly, I owe my deepest gratitude to my Creator, for presenting me with such wonderful opportunities. Without Him, none of this would have been possible.

Soli Deo Gloria

DECLARATION

I, Chantel Swanepoel, declare that this dissertation is my own work in design and execution. It is being submitted for the degree Master of Science in Environmental Science at the North West University, Potchefstroom Campus. It has not been submitted before for any degree or examination at this or any other university. All material contained herein has been duly acknowledged.

Chantel Swanepoel

Date

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Introduction

GENERAL INTRODUCTION AND PROBLEM STATEMENT

One of the world's main challenges is the prevention of further deterioration of its water quality. Sewage and micro-pollutant inputs from anthropogenic sources contribute to the deterioration of all water bodies (Pratt, 2005). Increasing emphasis has thus been placed on the importance of evaluating and controlling sources of water pollution (Leeming, 1996; Chan *et al.*, 1998; Afzal, 2006; Derrien *et al.*, 2012; Gottschall *et al.*, 2013). There still exist a need to develop methods for collecting and analysing water for the presence of specific classes of compounds that can be correlated quantitatively with major sources of faecal pollution. Sterols offer such a class of compounds because certain sterols are characteristic of wastes from higher forms of life and anthropogenic sources (Leeming, 2006; Derrien *et al*., 2012). Sterol biomarkers can be used as chemical tracers to determine sewage transport and distribution in the environment (Leeming *et al,*. 1996; Derrien *et al*., 2012; Furtula *et al*., 2012). Other chemical tracers also include caffeine and pharmaceuticals from humans, veterinary remnants from animals and pesticides and herbicides from agricultural run-off (Leeming, 2006; Froehner *et al.*, 2010; Jaffrezic *et al.*, 2011).

Faecal indicator bacteria (*E. coli*, *Enterococci* spp. and *Clostridium perifringens*) have been used as a traditional method for detecting and determining faecal contamination in water (Pratt, 2005). Concerns have been raised about the reliability of these bacterial counts as an accurate indicator of faecal pollution. The lack of reliability is mainly due to:

- Extreme variability that exists for the bacteria to survive under various conditions (Pratt, 2005).

- Faecal coliforms may come from a multitude of sources thus lacking source specificity (Cabelli *et al.*, 1983; Jaffrezic *et al.*, 2011).

- There is no correlation between faecal coliforms and the occurrence of pathogens (Rhodes and Kator, 1988; Ferguson *et al*., 1996), heightened by the fact that coliforms are more vulnerable to disinfection, particularly chlorination, than many pathogens and viruses (Dukta, 1973).

- Chlorination of coliforms can cause the degree of faecal pollution to be greatly underestimated (Tabak *et al.*, 1972).

- Faecal coliforms have the ability to grow in water, or they may come from a non-faecal origin (plants or pulp mill effluent) which may lead to false positive readings (Cabelli *et al.*, 1983).

- Survival rates of bacterial indicators can also be affected by toxic compounds present in the water or environment (such as nitrogen, pesticides, antibiotics, etc.) (Tabak *et al.*, 1972)

Sewage tracers can be used to examine a) the presence of current, past, or even historic sewage inputs (Gottschall *et al*., 2013) and b) the distribution and transport of sewage in the environment (Pratt, 2005; Biache and Philp, 2013). In this respect, biomarkers are organic compounds that, once released into the environment, retain their structural integrity for their source to be recognized (Leeming *et al*., 1996). The distribution of sterols found in faeces and hence their source-specificity, is caused by a combination of diet and the animal's ability to synthesize its own sterols as well as the intestinal micro-biota in the digestive tract (Leeming,

2006). These factors determine “the sterol fingerprint”. Field studies (Leeming, 1996; Derrien *et al*., 2012) and a major validation study (Leeming *et al.,* 1998b) highlighted the possibility of using sterols together with bacterial indicator concentrations as faecal pollution indicators in any water column. Although sterols can be degraded in surface water, it rapidly bounds to micro-particulates, which are then deposited and eventually incorporated into the anaerobic sediment where they have limited decomposition (Hatcher and McGillivary, 1979; Gottschall *et al.,* 2013).

Many of the pioneering studies on faecal sterols have been on sediments and not water (McCalley *et al.,* 1980; Pratt, 2005) and were also taken in temperate to cool environments in the United Kingdom (Goodfellow *et al.,* 1977) and the United States (Hatcher and McGillivary, 1979). Additionally, most of these studies relate to large sewage input. Very few studies have been done in warmer climates, relating to small scale sewage inputs.

Therefore, this study examines the use of sterol biomarkers in water as a tool for detecting faecal contamination in various aquatic environments. Because effectiveness is of concern when testing environmental water samples for faecal sterol content, the identification and quantitation of sterols in a single chromatographic run is of importance. This dissertation attempts to fill in the gaps of how sterols can be used as a toll in indicating water quality of environmental water, with regards to faecal pollution, in South Africa

RESEARCH AIM AND OBJECTIVES

The aim of this study was to assess the use of faecal sterol analysis to determine the quality of environmental water by using GC-MS based techniques.

The objectives of this study are to:

i. optimize the GC-MS methods and conditions used for faecal sterol analysis of environmental water samples,
ii. determine the sterol fingerprints of various animal species, that may be common sources of pollution in the North West Province, by using the GC-MS method,
iii. determine the quality of environmental water based on physico-chemical properties, bacteriological parameters and faecal sterols,
iv. determine the efficiency of wastewater treatment plants using the GC-MS method to analyse effluent and other surface water samples.

2

Literature Survey

2.1 CURRENT WATER SITUATION IN SOUTH AFRICA

South Africa is a semi-arid region, with some geographical regions receiving higher rainfall than others. The average rainfall of the country is 500 mm per annum (mm/a), which is far below the global average of 860 mm/a (Basson *et al.*, 1997*;* Karlberg *et al*., 2004; DWAF, 2004a). South Africa's estimated mean annual runoff is 43 500 million m³ per annum (m^3/a), while the total available yield is 13 227 million m³/a. For the year 2000 the total water requirements were 12 871 million m³/a (DWAF, 2004a). The national water resource strategy (2004) estimates that at the current usage, available water resources will be insufficient to meet the demands for 2025. The estimated total water requirements for 2025 will be approximately 17 billion m^3, but the projected reliable yield only 15 billion m^3 (CSIR, 2010). Factors such as global warming, climate change, water pollution and international obligations (South Africa shares water from four of its main rivers, the Inkomati, Pongola, Orange and Limpopo rivers, with neighbouring countries) limit the available water and are placing increasing demands on South Africa's existing water resources (DWAF, 2004a). South Africa's major water requirements are provided by surface water supplies (Stats SA, 2010a). Surface water resources contribute to 77% of the total water needed across the country, while groundwater contributes to 9% and 14% is water which is available from return flows (DNT, 2011).

To meet South Africa's current water needs, surface water resources are well developed to supply the majority of urban, industrial and irrigation water needs. The 550 government dams, have a total capacity of about 37 000 million m³ (Anon[1], 2012). These dams capture about 70% of the total mean runoff. One of the challenges is, however, to get the water to where it is needed. For this South Africa have transfer schemes between the rivers within and between the 19 water management- and catchment areas (CSIR, 2010). There are, however, criticism as to how much more water can be transferred between river basins, and how many more dams can be built to provide water for future uses and needs (CSIR, 2010).

Economic activity and standard of living drives increased water demand. Furthermore the standard of living is also directly connected to economic growth (CSIR, 2010; UNEP, 2011). Unfortunately social, political and economic activities drive environmental change and as South Africa's population increases the day-to-day consumption patterns challenge and compromise the water resources ever more (CSIR, 2010; UNEP, 2011). Poor water quality not only limits its utilization value, but also adds economic stress on society through both primary treatment costs and the secondary impacts on the environment and the economy (CSIR, 2010). The more polluted the water, the higher the treatment costs.

Water use in South Africa (Figure 1) is dominated by irrigation (agricultural activities), which accounts for 62% of all water used in the country. Domestic and urban use accounts for 27% and mining and large industries 8% (CSIR, 2010). Commercial forestry plantations account for about 3% of water use (DWAF, 2004). There is thus particularly a large return flow from urban areas as well as agriculture. This return flow is generally of poor quality (DWAF, 2009a).

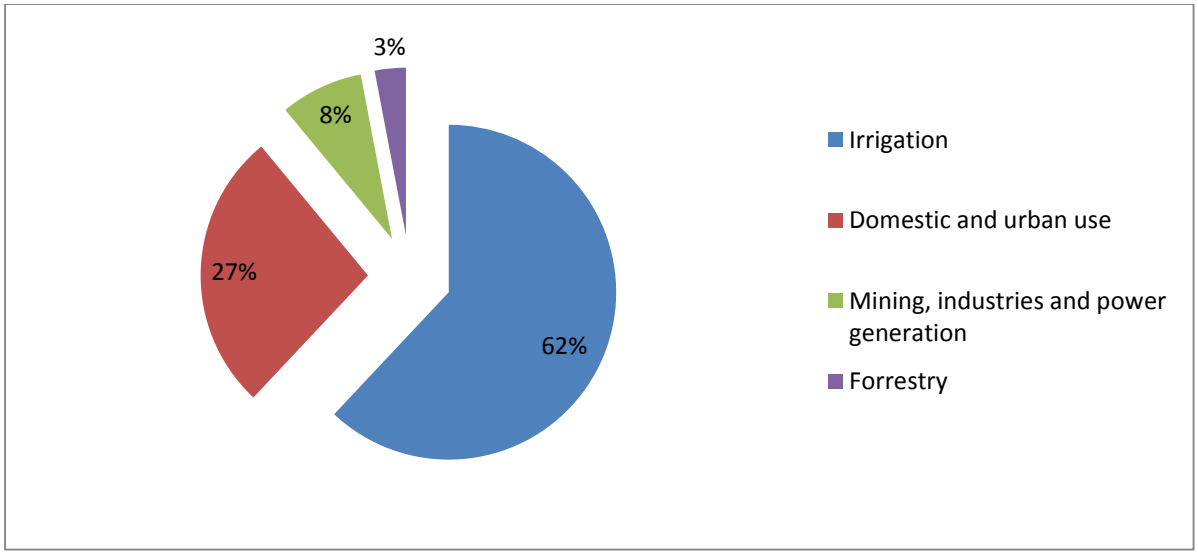


Figure 1: Water use per sector in South Africa.

South Africa's Human Reserve is required to satisfy basic human needs by securing water for people who are, or will be relying upon, taking water from, or being supplied with water from relevant sources (DWAF, 2010). The country's water resources are, however, a social, environmental and economic entity and as such, there is always competition for access to remaining available water once the basic human needs and requirements for a healthy ecosystem have been met (DWAF, 2004b). Moloko Matlala stated during a briefing in parliament in 2011 that SA's river water quality was deteriorating (Water and Sanitation Africa, 2011). He attributed this deterioration to faecal pollution, eutrophication, high salinity and toxicity. This is worrisome, especially considering the existent link between the state of environmental water and the well-being of humans (CSIR, 2010). As a result, measures regarding the protection of South Africa's water quality are imperative.

In the North West Province (NWP) only 8 Local Municipalities, representing nearly a 67% of the waste water treatment plants (WWTPs), have been tested in the Green Drop Certification programme (DWAF, 2012). Most of the municipalities and their WWTPs had relatively low

scores. Areas such as the Rustenburg, Mogwase and Tlokwe WWTPs scored quite high and have earned Green Drop Status. The overall waste water quality in the North West Province was, however, very low (DWAF, 2012). Thus a large proportion of the domestic return flows are of poor quality. All of these users must effectively treat the used water. If this does not happen it will cause severe degradation of the quality of environmental water.

2.2 THE NORTH WEST PROVINCE'S WATER SITUATION

The North West Province (NWP) has all of the above mentioned water resource constraints, (Howard *et al.*, 2003). NWP is a land locked province with Botswana as its western border. It is bordered by the Kalahari Desert in the west, Gauteng province to the east, and the Free State to the south. Known as the Platinum Province, it is extremely rich in underground metals (NWDACE, 2007) A large portion of available surface water contributes to the mining, property, agricultural and industrial sectors (Kalule-Sabiti and Heath, 2008). Mining contributes 23.3% to the North West economy, and makes up more than one fifth of South Africa's mining industry. Ninety-four percent of the platinum of South Africa is produced in the North West Province. This is more than any other single area in the world (NWDACE, 2007). Other mining in the province include gold (a quarter of South Africa's gold is produced in the North West), granite, marble, diamonds and flouspar. The province also contains some of the largest cattle herds in the world (at Stellaland near Vryburg) (NWDACE, 2007). The Marico region is also cattle country, while the land surrounding Brits and Rustenburg are utilized for mixed crop farming. Crops produced include maize and sunflowers (Anon[2], 2012). Water in the NWP and in South Africa also plays an integral part in many cultural ceremonies and religious rights and beliefs (Zenani and Mistri, 2005). Thus,

water from rivers, streams, dams and springs may be utilized for cultural and religious purposes such as baptism or initiation (Zenani and Mistri, 2005).

Rainfall varies from the east to the west of the province. There is a higher rainfall in the east (500-650 ml per annum), compared to the lower rainfall in the west (350 – 500 ml per annum) (Figure 2). The rainfall in the central and eastern part of the province is significantly greater than the western part of the province.

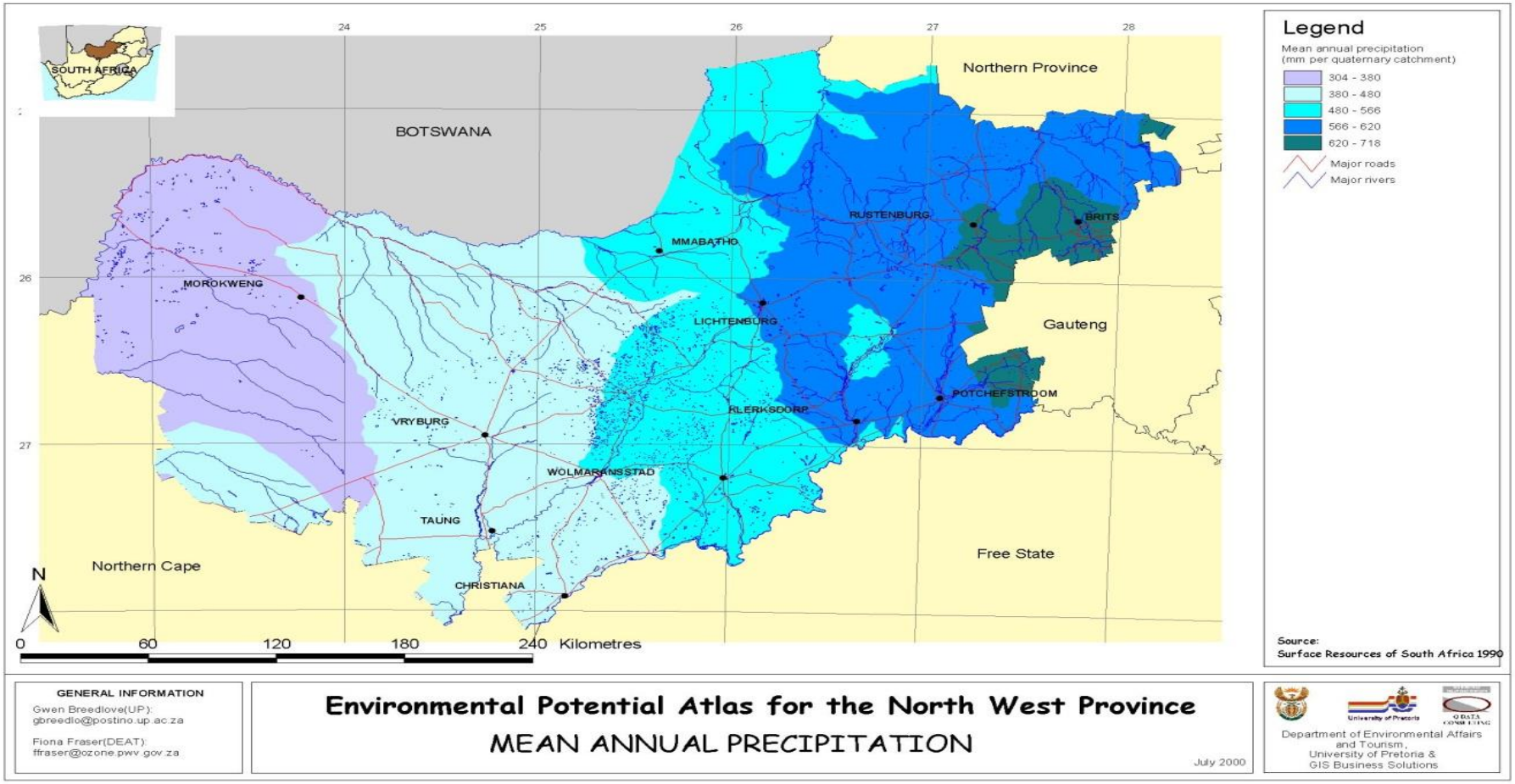


Figure 2: The North West Province's average rainfall per annum.

The NWP surface water comprises rivers, dams, pans, wetlands and dolomitic eyes fed by underground water sources (DWAF, 2004a; NWDACE-SoER, 2008). The province's ground and surface water are integrated and interdependent as dolomitic eyes are the sources of several major rivers that rise within the province's boundaries, such as Groot Marico, Mooi and Molopo Rivers (DWAF, 2004a). Groundwater is of vital importance in the NWP, predominantly in rural and underdeveloped areas. Groundwater is in many instances the only source of water for many rural people, particularly in the arid western region of the Province (DWAF, 2004a). Water quality and quantity issues affecting groundwater also have implications for surface waters (DWAF, 2004a).

General driving forces (natural factors and human activities) exist that has an effect on surface water quality and groundwater quality and quantity, (DWAF, 2004a). These driving forces are:

1.) Climatic conditions - All surface water systems within the NWP are susceptible to rainfall and evaporation patterns. In all catchments the evaporation exceeds rainfall. Rainfall is the most significant driving force with respect to groundwater recharge and the frequency and occurrence of wet and dry periods play a major role in the quantity and quality of the groundwater. Climatic conditions affect other environmental factors such as vegetation and soil cover, which affect the groundwater quality. It also influences permeability of the surface and percolation of surface water into aquifers and other groundwater features.

2.) Increased population growth - Rising population numbers and increasing wealth and standard of living places stress on every aspect of the environment - Higher standards of living and raised expectations of rural communities, means that greater volumes of water are required to assure basic supply. Demands of groundwater may, in many instances, exceed the

supply, because groundwater quantity is not exactly specified, as well as the discharge of used water through sewerage and other effluents. These pressures exerted on the water resources are manifested as:

i.) Changed hydrology - The volumes of water required for domestic use, industry, mining and agriculture have prompted the construction of many dams in rivers. The seasonal and natural cycle of periods of high and low flow has been significantly altered. Many aquatic organisms depend on seasonal floods for survival. The regulation of floods has had an effect on the ecosystem functioning of many rivers. In addition, transfer of water from one area to the next has resulted in altered flow patterns and the translocation of aquatic fauna and flora between catchments. As ground and surface water resources are interdependent, pressures on surface water are felt on groundwater hydrology and *vica versa*. Artificial influence on stream flows has immediate and medium-term impact on groundwater and the surrounding environment.

ii.) Mining - Mining places significant negative pressure on the NWP's water resources because most mines need large volumes of water for production. Mines also dispose of waste products into this used water. This is then discharged into rivers and other surface waters (industrial effluent). Mines dewater aquifers in order to work at depth safely. Many local aquifers have become depleted because of this, and this, in turn, leads to an effect on both surface and subsurface systems.

iii.) Agriculture - An agricultural industry is essential to provide food for the population, and raw materials for industry. The irrigation industry is the biggest single water user in the NWP, with a very high demand to use groundwater for irrigation. Another negative effect of irrigation is the washout of fertiliser and agrochemicals

into the receiving surface water, which can have an effect on the quality of the groundwater as well.

iv.) Industry - Industrial activities largely support the mining and agriculture industries. These industries have an effect through the demand for water, the requirement for people living close to the work place and through discharging a variety of waste products into the environment. Locally the subsurface release of harmful chemicals from the industry may affect groundwater resources, because organic chemicals tend to be dangerous in minute concentrations (e.g. benzene derivates in fuels, industrial solvents).

3.) Policy and legislation is another general driving force. The National Water Act (Act 36 of 1998) is central to the management of water resources from national to water management area level. Legislation therefore influences the pressures, state, impact and responses pertaining to the water resources (Howard *et al*., 2003). South Africa has a policy that all domestic water supplies should be clean and drinkable (Wilson and Trollip, 2009). All drinking water quality has to comply with the South African National Standards (SANS 241: 2011), which is in line with most international drinking quality standards (Wilson and Trollip, 2009). By law, all municipalities have to monitor their drinking water quality but not all municipalities comply due to a lack of skills, funding and management capacity (DWAF, 2010).

2.3 WATER BOURNE DISEASES

In the NWP, more than 80% of the rural population depend on groundwater as their main drinking water source (Bezuidenhout, 2011). The rural population remain susceptible to numerous water-borne diseases because of the quality of groundwater in the province

(Mpenyana-Monyatsi and Momba, 2012). Surface and groundwater remain an integral part in the sosio-economics of the NWP, and requires some closer monitoring and regulation.

The South African National Environmental Management Act (Act 107/1998) stipulates that everyone has the right to an environment that is not harmful to their health and wellbeing. As a result, an understanding of the inter-relationship that exists between water and human health is essential for the sustainable management of water quality, so as to attain optimum human health gains (CSIR, 2010b). In South Africa, water borne diseases are a major concern (DNT, 2011). The National Environmental Health Policy (DoH, 2011), stipulates that South Africa's is surrounded by developing countries that have health challenges such as water-borne diseases, and since disease causing microbes are easily spread in the environment, this posts a worrisome problem. In most of Africa communities are still reliant on easily accessible surface water systems, which place inhabitants in a vulnerable position, especially relating to water borne diseases (DWAF, 2002a).

Transmission of water borne diseases occur mainly through poor water quality, human contact, eating using contaminated utensils, food and soil (DoH, 2011). It has been estimated that as many as 43 000 South Africans may die annually as a result of diarrhoeal diseases from water origin (DWAF, 2002a). An estimated 70% of diarrhoeal disease incidents in South Africa occur in children under the age of five years while 60% are related to people receiving lower than the acceptable basic level of service (DWAF, 2010). The National Environmental Health Policy (DoH, 2011) explains that children of 5 years and younger are the most susceptible to diarrhoea caused by amongst others lack of clean and palatable water. Intestinal infectious diseases are among the leading underlying natural causes of death through all age groups according to the mortality and causes report issued by Statistics South Africa (2010b). Additionally, this report confirmed that the leading cause of infant mortality

(22.4%) in 2008 was attributed to intestinal infectious diseases. While deaths for children aged 1-4 years (27.3%) were due to intestinal infectious diseases.

Although not reported in South Africa, incidents have been found in many parts of the world where epidemics relating to contact water sports have been found (WHO, 2005). Several studies have illustrated that human and animal waste discarded in water used for full body contact activities can have an effect on humans using the water mostly resulting in gastroenteritis, acute respiratory disease, eye, ear and skin infections (Saliba and Helmer, 1990; Dwight *et al*., 2004).

2.4 WATER QUALITY MONITORING

In water resource management, a lot of emphasis revolves around ensuring that users have sufficient quantities of water. Impending threats of water scarcity, as well as increases in the amount of water being used and re-used daily, it is the quality thereof that begins to take a toll (DWAF, 2004a). Water quality thus, become an issue in the front seat in of water resource management. In South Africa water quality is defined on the basis of its physical, chemical, biological and aesthetic characteristics and determines the health and integrity of an aquatic ecosystem (DWAF, 1996a).

According to Lemarchand and co-workers (2004), water quality is taken for granted even though health risks from polluted water remain a major public concern. The lack of access to good quality drinking water and sanitation are the cause of huge health impacts such as diarrhoeal diseases (Rijisberman, 2006). In 2003, the World Health Organisation (WHO) reported that 1.2 billion people in the world lacked access to safe and affordable water for domestic purposes. This validates the statement made by DWAF that water quality is still not

yet taken its rightful place in integrated water resource management (DWAF, 2004a). The main objective for water quality monitoring is to control and minimize the incidence of pollutant-oriented problems, so as to provide good quality water for drinking, irrigation and other purposes (Boyacioglu, 2006).

Water quality monitoring is imperative for the protection of surface and ground water resources. It provides water resource managers and politicians with information they need to make all necessary decisions regarding the equitable access, sustainable, efficient and effective use of water. Much of the information needed will be generated by water quality monitoring programmes (Van Niekerk, 2004).

The Department of Water Affairs and Forestry (DWAF), is responsible for ensuring that the country's water systems are fit for various uses while remaining sustainable (Murray, 1999). A number of physical, chemical and biological constituents are usually assessed as they may possibly have an effect on the suitability of water for a specific use (DWAF, 2004a). As a result, a number of water quality monitoring programmes have been set up and are functioning.

In South Africa there are two monitoring programmes - The National Chemical and National Microbial Monitoring Programmes. The National Chemical Monitoring Programme analyse physical and chemical variables during water quality monitoring. The physical quality of water refers to properties that may be determined by physical methods and mainly affects the aesthetic quality of the water. Parameters include the pH of the water tested, temperature, total dissolved solids (TDS), electrical conductivity (EC). High levels of TDS in the water may indicate pollution in drinking water (Kempster *et al.*, 1997) while pH of the water influences the taste as well as the hardness of the water (Adams, 2001). Chemical quality of the water refers to the nature and concentration of dissolved substances, such as salts, metals

and organic chemicals. Chemical parameters measured are nitrites (NO_2^+) and nitrates (NO_3^{2-}). Nitrates in water have a special significance in measuring the quality of water, as infants who constantly consume water with high levels (20 ng/L and higher) of nitrates have a high risk of developing methaemoglobinaemia (Afzal, 2006).

The National Microbial Monitoring Programme utilizes microbial testing to determine and monitor the quality of water bodies. Microbial analysis of water includes testing for the presence of indicator organisms such as heterotrophic plate count (HPC) bacteria, total coliforms (TC), faecal coliforms (FC), and *Enterococci*, using selective media. The presence of TC and FC bacteria in water may be associated with faecal contamination (Pavlov *et al*., 2004; DWAF, 2006).

2.5 AN OVERVIEW OF WATER QUALITY TESTING AND MONITORING TECHNIQUES

2.5.1 Traditional faecal pollution indicators

Indicator organisms are generally used for assessing microbial content of domestic and recreational water for safety (Sankaranakrishnan and Guo, 2005). Among indicator organisms, heterotrophic organisms, total coliforms (TC), faecal coliforms (FC), and faecal streptococci (FS) bacteria are also used to measure surface water quality (Kim *et al*., 2005; Zheng *et al.*, 2013).

Coliforms are a group of bacteria which consist of Gram negative, non-spore forming, oxidase negative, rod-shaped, facultative anaerobes, which have the ability to ferment lactose (WHO, 2008). These total coliforms (TC) can be from both faecal (human and animal

wastes), or vegetative origin (soil, sediment, etc.). They are primarily used as a practical indicator of the general hygienic quality of water, mainly used in routine monitoring of drinking supplies (Wutor *et al*., 2009). Alone, total coliforms are not a good indicator of faecal pollution as many non-faecal strains are included in this group. These strains can originate from the environment (Bezuidenhout *et al.,* 2002; WHO, 2008).

Faecal coliform are a subset of total coliforms and includes *E.coli.* Faecal coliforms are associated with human and animal waste. These are facultative anaerobic, Gram negative, non-spore forming, rod-shaped bacteria (WHO, 2001). When FC bacteria are present in high numbers in water, it is indicative of faecal matter from one source or another. Faecal coliform bacteria may also indicate the possible presence of pathogenic organisms, which live in the same environment (Noble and Furman, 2001; Pachepsky and Shelton, 2011).

Another group of bacteria that has been used as an index of faecal pollution is called the faecal streptococci. Faecal streptococci are Gram-positive; catalase negative cocci that are not inhibited by bile salts (Lemarchand *et al*., 2004). It is important to identify human enterococci and streptococci, as these genera pose a greater human health risk (Holt *et al.,* 1993; Rincè *et al.,* 2003).

Since the introduction of thermo-tolerant faecal bacteria as indicators (Escherich, 1885), many other methods have been proposed for the detection of sewage pollution, including testing for specific organic and inorganic compounds and also other biological and microbial indicators (Vivian, 1986). Many researchers have repeatedly demonstrated that traditional microbial assays for sewage monitoring have serious short-comings (Evans *et al.,* 1968; Dukta, 1973; McCalley *et al*., 1980 and 1981; Cabelli *et al*., 1983; WHO, 1996; Romprè *et al*,. 2001; WHO, 2008; Boehm *et al.,* 2009).

2.5.2 A review of the applications, in water, of sterol analysis as faecal pollution indicators

Measurement of faecal pollution and sewage water inputs by means of sterol analysis is relatively recent compared to faecal bacterial methods (McCalley *et al*., 1980; Nichols *et al*., 1996; Isobe *et al*., 2002; Leeming, 2006; Pratt *et al.,* 2007). Faecal pollution detection through sterol analysis has, however, become ever increasing popular (Mudge and Bebianno, 1997; Devane *et al.,* 2006; Sullivan *et al.,* 2010) and coprostanol has been successfully used as sewage tracer in a wide variety of environments (Writer *et al.,* 1995; Pratt, 2005; Sankaramakridhnan and Guo, 2005; Pratt *et al.,* 2007; Shah *et al.,* 2007). The acceptance of coprostanol as a sanitary indicator has not been considered as it is not a direct health risk (Leeming, 1996).

Sterols can provide an extra tool for sewage pollution and source identification when used in conjunction with bacterial analysis. Leeming (1996) noted that a broad correlation is found between coprostanol and *Enterococci* sp., but the relationship is dependent on the type of environment. Leeming and Nichols (1996) conducted a study in a range of environments in cold temperatures (sub-Anarctic Australia) in the water column at the Derwent Estuary, Tasmania, and mimicked the results of Leeming (1996) in which a correlation was found between coprostanol and *Enterococci* sp. Isobe *et al.* (2002) investigated the relationship between coprostanol, faecal streptococci and *E.coli* in tropical waters in Western Malaysia and the Mekong Delta, Vietnam. They concluded that among the three bacterial indicators measured, *E.coli* showed the strongest correlation with coprostanol in both Malaysia and Vietnam. This suggests that temperature and probably other physico-chemical factors have different effects on both coprostanol and bacteria, and possibly also viruses (Leeming and Nichols, 1996).

Churchland *et al.,* (1982) measured variations in faecal pollution indicators in the Frasier River estuary, during tidal cycles. Faecal coliforms were measured using the membrane filter technique, while coprostanol and cholesterol were extracted using hexane and analysed by GC-MS. The faecal coliform counts correlated with coprostanol and cholesterol levels. In receiving waters it was found that faecal coliform counts, but not sterol concentrations were reduced by chlorination of sewage treatment plant effluents. Nichols *et al.* (1996), have applied sterol analysis for measuring faecal-derived sterols in storm water and sea surface micro-layers. The sterol composition from a variety of sample types were determined by GC-MS. Coprostanol concentrations in the samples readily provided an estimate of human faecal contamination. The technique was successfully used for storm water, the sea-microlayer, beach sand and greases, and in regional studies of coastal sediments. Nichols *et al.,* (1996) concluded that sterol profiles can be used to distinguish between human and non-human sewage pollution and algal blooms. Mudge and Duce (2005) utilized sterols in identifying the source, transport path, and sinks of sewage derived organic matter in the Ria Formosa Lagoon (Algarve, Portugal). Ratios between key biomarkers were able to identify the sewage sources and effected deposition sites (Mudge and Duce, 2005).

Shah *et al.,* (2007) took water samples at sites potentially impacted by septic tanks, cattle, sewage treatment plants and natural forests, and analysed them for FC and faecal sterols. Faecal sterol ratios were used to assign human and/or herbivore contamination. Sullivan *et al.,* (2010) used faecal sterols to identify human faecal pollution in a non-sewered catchment in Southeast Queensland, Australia. Stanols concentration increased with increased catchment runoff and high coprostanol levels were found in water indicating human faecal pollution due to defective septic systems. Sterol profiles were also able to point to

cattle farm pollution during modest catchment runoff. In that study Sullivan *et al.,* (2010) were able to use faecal sterols in identifying human and animal faecal pollution in water.

Adnan *et al.,* (2012) was able to use sterol ratios to identify source, occurrence and positioning of faecal matter in sediments from the Langat River, Malaysia. Sterol ratios demonstrated that sewage contamination was occurring at most of the sampling sites along the Langat River. These sewage contaminations were in the low to mid-range level, while others contained significant levels of contamination.

Most of the earlier studies using sterols as indicators of faecal contamination focused on the distribution of coprostanol only (Boehm *et al.,* 1984; Eganhouse, 1986; Gardner *et al.,* 2008) or coprostanol, cholesterol and some of its major reduction products (Jeng and Han, 1994; Writer *et al.,* 1995). Later work sought to investigate the use of sterols as biomarkers to other source contributions such as algae and terrestrial sterols for non-anthropogenic inputs (Fernandes *et al.,* 1999; Mudge and Duce, 2005).

Sterol analyses from previous studies have mostly been done in cooler areas, and/or in the Northern hemisphere. There is certainly a lack of literature that has dealt with sterols in warmer climates. Nevertheless, sterol analysis still appears to be the more accurate way in detecting faecal contamination in water and sediment. Even though results in literature highlight major differences between coprostanol and bacterial indicators, all findings do confirm that coprostanol is overall much more stable and resistant to stresses in the environment that FC bacteria (McCalley *et al.,* 1981; Leeming and Nichols, 1996; Isobe *et al.,* 2002). Therefore, coprostanol may be a more reliable indicator of faecal pollution in water.

2.5.3. Gas Chromatography as a method for analysing faecal sterols in water

For determining water quality via sterol analysis, gas chromatograph-mass spectrometry (GC-MS) and gas chromatograph time-of flight mass spectrometry (GC-TOF/MS) techniques could be used. The gas chromatograph-mass spectrometer is a combined analyser that has superior ability in analysing organic compounds qualitatively and quantitatively. Gas chromatography exploits the differences in the partition coefficients between a stationary liquid phase and a mobile gas phase of the volatilised analytes (Wilson and Walker, 2005). The sample is injected into a tubular column (chromatography column), usually made of a high boiling point liquid material. In this case silicone grease, that is supported on a granular solid, which is packed into the column (Wilson and Walker, 2005; Douglas, 2010). The analytes are carried through the column by helium gas. Individual chemical characteristic of an analyte determines how long it will take to go through the chromatography column. The time it takes for any given analyte to travel the length of the column is referred to as its retention time (RT). The RT for a given chemical is an identifying characteristic (Douglas, 2010).

Very high resolutions are obtained during this technique and it is used for a variety of qualitative and quantitative analysis of a large number of compounds. It also has a high sensitivity, reproducibility and speed of resolution (Wilson and Walker, 2005). Mass spectrometry detector is used in series to the GC. As an analyte exits the end of the GC column it is fragmented by ionization (e.g. electron spray ionization) and the fragments are sorted by mass to form a fragmentation pattern (Wilson and Walker, 2005). The fragmentation pattern for a given analyte is unique and therefore is an identifying characteristic of the analyte. Thus, the GC uses chemical property differences between

molecules to separate them, while the MS detects ionized fragments using their mass to charge ratio (Douglas, 2010).

The GC-TOF/MS has a time-of-flight (TOF) in series to the GC and MS. The TOF/MS determines an ion's mass-to-charge ratio as a measurement of time (Agilent, 2012). Ions are accelerated by an electrical field in the TOF. This acceleration causes the ion to have the same kinetic energy as other ions with the same charge (Agilent, 2012; Barden, 2012). Velocity of the ion and the time it will take for it to reach the detector (MS) all depends on the mass-to-charge ratio of the particle. Heavier compounds move slower than lighter ones. The TOF thus separate the time of compounds due to their velocities, while the MS detects these compounds using their mass to charge ratios (Agilent, 2012). TOF/MS brings together the best of the SIM and full-scan modes, providing data across a full mass range while retaining good sensitivity and minimizing ion wastage (Barden, 2012).

2.5.4 Sterols used in present study to identify faecal pollution in water

Cholesterol (CHL; 5-cholesten-3β-ol; Figure 3) is a lipid steroid found in the cell membranes and is transported in the blood plasma of all animals. It is an important precursor molecule for the biosynthesis of bile acids, steroid hormones and several fat-soluble vitamins (Wedro and Kulick, 2011). Cholesterol is the main sterol synthesised in the body of animals, and is completely absent among prokaryotes (bacteria) and plants. It is not completely absorbed by the body and is also excreted as a component of bile (Anon[3], 2007).

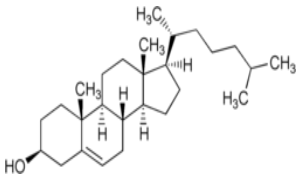


Figure 3: The structure of Cholesterol (5-cholesten-3β-ol).

The structure of cholesterol (Figure 3) contributes to the decreased fluidity of the cell membrane since the molecule is in a trans structural conformation state, it makes the side chain of cholesterol rigid and planar (Ohvo-Rekilä *et al.*, 2002). In this structural role, cholesterol changes the permeability of the cell membrane to neutral solutes, protons and Na – ions (Haines, 2001). Within cells cholesterol is the precursor molecule in several biochemical pathways (Figure 4). Figure 4 shows one such biotransformation pathway, in which cholesterol undergoes microbial reduction to form 5β-stanols. These are the major sterols found in humans and animals (Leeming *et al.*, 1996).

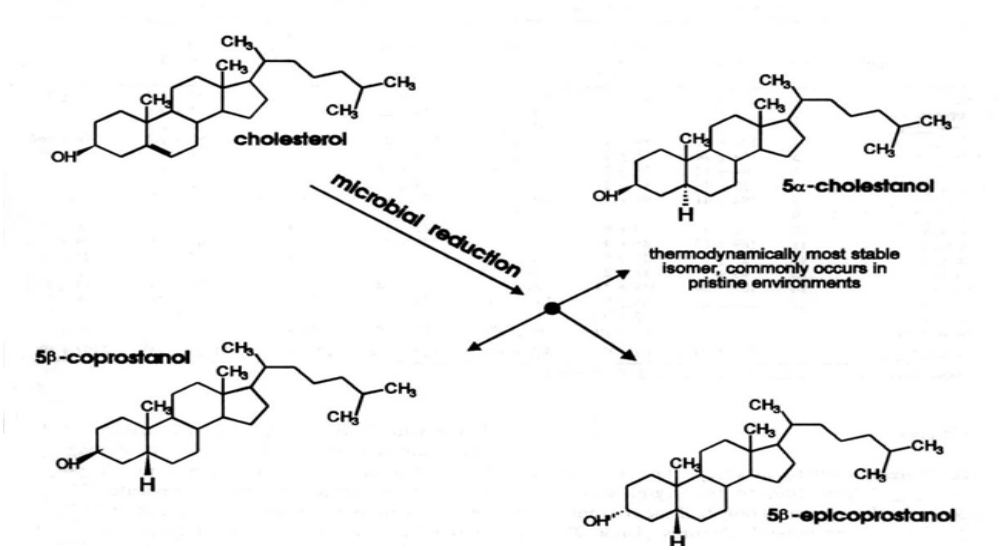


Figure 4: Sterol structure, biotransformation pathways and indication of the major sterols in human and animal faeces.

Reduction of cholesterol, in the body of higher animals, by enteric microbial activity (Figure 4) is the main source of coprostanol (Sherwin, 1993). Animals that cannot convert cholesterol

to 5β-stanols include; dolphins, penguins, birds and dogs (Leeming *et al.,* 1994; Pratt, 2005). Humans, sea lions, pigs and cats are able to convert cholesterol into coprostanol (Figure 4) (Pratt, 2005). The principle faecal sterol excreted by herbivores is 5β-epi-coprostanol (Figure 4).

The principal human faecal sterol, coprostanol (COP; 5β(H)-cholestan-3β-ol; Figure 5), constitutes about 60% of the total sterols found in human faeces. Coprostanol has been used as a sewage pollution indicator in a wide range of environments (Nichols *et al.,* 1996). Coprostanol itself is not a contaminant of concern, but it provides the opportunity to assess other particulate bound contaminants such as viruses, PCB's, bacteria, hydrocarbons and endocrine disrupters (Writer *et al.,* 1995). Literature highlights major differences in bacterial indicator concentrations and coprostanol (mainly due to temperature) (Nishimura and Koyama, 1977; Pratt, 2005). Findings confirmed that coprostanol is much more stable and resistant to environmental changes than faecal coliform bacteria (Nishimura and Koyama, 1977; Leeming *et al.,* 1996; Pratt, 2005). This faecal sterol may be a reliable indicator of sewage pollution and could be used to support faecal bacterial data (Leeming and Nichols, 1996).

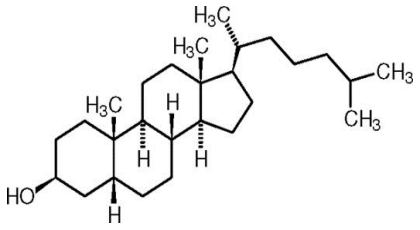


Figure 5: The structure of Coprostanol (5β(H)-cholestan-3β-ol).

Coprostanol is associated with organic solid particles in the water column (Brown and Wade, 1984) and because of that can be incorporated into the sediment. If the sediment remains anoxic, the coprostanol will persist (Bartlett, 1987). Experiments done on the decay and

degradation of coprostanol and dehydrocholesterol in lake sediments (Nishimura and Koyama, 1977; Bachtiar *et al.*, 1996) found that there was little degradation observed. Other studies showed that coprostanol in anoxic mud was very persistent, and that coprostanol levels can even reflect sewage input into water for over 160 years (Muller *et al*., 1979). Coprostanol overcomes many of the shortcomings of microbial sewage pollution, such as die-off and lack of correlation with pathogens, as it is non-ionic and non-polar (Figure 5), (Nichols and Leeming, 1991). Due to high concentrations coprostanol in human faeces and its specificity to faecal origin, coprostanol has been used in many studies as a human faecal pollution biomarker (Hatcher and McGillivary, 1979; Nichols *et al*,. 1991a and 1991b; Leeming and Nichols, 1992a and 1992b; Sullivan *et al*., 2010).

The naturally occurring stanol in unspoiled environments is 5α-cholestanol (dehydrocholesterol; DCHL; Figure 6), because it is the most thermodynamically stable isomer (Figure 6) (Nishimura, 1982). This sterol is more commonly found in pristine environments, because of its thermo-dynamical stability. By comparing ratios of coprostanol to 5α-cholestanol (Leeming and Nichols, 1995), one can determine if coprostanol found in organic–rich, or anaerobic sediments are of faecal origin. 5α-cholestanol is found in trace amounts (relative to coprostanol) in human faeces, but increases in relative proportions in digested sewage sludges (Leeming, 2006). Increased amounts of 5α-cholestanol relative to coprostanol indicate older human faecal contamination (such as faecal sludges from sewer pipes). Measurement of these sterols from a non-faecal origin in the water column indicates that organic matter has been re-suspended from highly anaerobic sediments (Leeming, 2006).

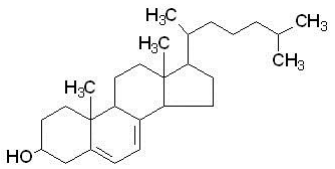


Figure 6: The structure of Dehydrocholesterol (5α-cholestanol).

In the body, dehydrocholesterol is located extracellular and functions in cell signalling and fuel and energy source and storage. As a component of the cell membrane, it assists in membrane integrity or stability (Anon[4], 2005).

The C_{29} homologue of coprostanol is 24-ethylcoprostanol (24-ethyl-5β(H)-cholestan-3β-ol; Figure 7) which is the main faecal sterol excreted by herbivores (Leeming *et al.*, 1996). Both coprostanol and 24-ethylcoprostanol are present in different ratios in both human and animal faeces. It is possible to determine the relative contributions by calculating the ratio of coprostanol to 24-ethylcoprostanol in human and herbivore (e.g. sheep, cow, and possum) faeces (Leeming *et al.*, 1996). 24-ethylcoprostanol is a 29 carbon stanol (Figure 7), formed from the bio-hydrogenation of β-sitosterol (which is a phytosterol found in plants) in the gastrointestinal tract of most higher animals, especially herbivores.

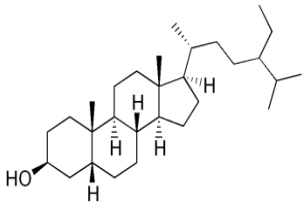


Figure 7: The structure of 24-ethylcoprostanol (24-ethyl-5β(H)-cholestan-3β-ol).

Stigmasterol (SROL; 3β-hydroxy-24-ethyl-5,22-cholestadiene; Figure 8a) is an un-saturated plant sterol occurring in plant fats and oils. It is chemically similar to cholesterol, and soluble

in most organic solvents. Stigmasterol is found in various species of vegetables, nuts, seeds, and un-pasteurized milk (Baxter, *et al.,* 1999). Stigmastanol (β-sitostanol; SNOL; Figure 8b) is an algae sterol and is found in reducing environments. Stigmastanol's presence in water indicates algae present and does not signify human or animal waste (Pratt *et al*., 2007). Stigmastanol has the same structure as stigmasterol, but without the double bonds (Figure 8). Sterols that are fully saturated (no double bonds) are called stanols.

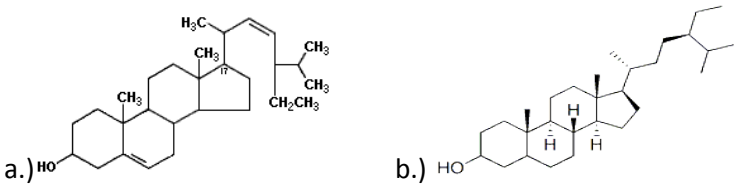


Figure 8: The structure of sterols a.) Stigmasterol (3β-hydroxy-24-ethyl-5,22-cholestadiene) and b.) Stigmastanol (β-sitostanol).

β-sitosterol (β-SIT; 24β-ethylcholesterol; Figure 10) is a phytosterol, which come from a multitude of sources including, wheat germ, peanuts, soybeans, pumpkin seeds, and corn oil. The presence β-sitosterol in water thus indicates sterols originating from plants and not humans or animals (Lam, 2009).

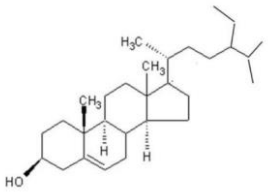


Figure 9: The structure of the phytosterol, β-sitosterol (24β-ethylcholesterol).

The significance of phytosterols is that they resemble cholesterol in structure (Figure 9). They are used in medicine to lower cholesterol in the body, as they inhibit cholesterol from being re-absorbed in the intestinal tract (Anon[5], 2012).

3

Sterol fingerprints of animals and humans by using the Szűcs Method

A pilot study was conducted for analysis of sterols in the water column. The pilot study was done, to provide insight into sterol concentrations of different species of animals and humans from the North West Province. The principle of the Szűcs method used is to have a simple and rapid GC-MS method for the simultaneous identification and quantitation of six target sterols in environmental water. The GC-MS analysis was evaluated for linearity, precision, extraction efficiency, limit of detection (LOD), limit of quantitation (LOQ), and stability according to the guidelines and regulations of Good Laboratory Practice specified for chromatographic techniques (Szűcs *et al.*, 2006). The cost- and time-effectiveness, technical advantages, and good precision and recovery make this GC-MS analysis method suitable for routine environmental monitoring of faecal pollution in aquatic systems (Szűcs *et al.*, 2006). To date, the method has not as yet been used in field studies.

Because significant differences occur in individual composition of human and animal faeces due to alterations in dietary intake, sterol patterns in water can provide information to distinguish and determine faecal pollution in water (Leeming *et al.*, 1996). High concentrations of coprostanol could be associated with anthropogenic faecal contamination in water, while abundances in cholesterol and β-sitosterol are typical sterols from dogs or birds (Szűcs *et al.*, 2006). In accordance, herbivore profiles have terrestrial sterols and phytosterol abundances. Sterols, according to the Szücs method, are extracted and isolated from water samples by liquid-liquid extraction using dichloromethane. Coprostanol, however, has an

a-polar character, which slightly reduces its solubility in dichloromethane, making it more difficult to recover. A surrogate solution is added (perylene) in order to test the recovery of the sterols in the water samples, and an internal standard (perylene-d12) is added to quantitate the sterols that eluted (Szűcs *et al.,* 2006).

Leeming *et al.,* (1996) conducted a study using faecal sterols from humans and animals to distinguish faecal pollution in receiving waters. The major human faecal sterol was coprostanol (60% of the total sterols found in human faeces), while the principal faecal biomarkers of herbivores were stigmasterol, β-sitosterol, and stigmastanol. The sterol content of bird faeces was extremely variable and largely dependent on the animal's diet. Cats and pigs were the only animals that had similar faecal sterol profiles to humans. However, the concentration of coprostanol was more abundant in human's sterol profiles than in those of cats and pigs. Leeming *et al.,* (1996) concluded that the "sterol fingerprints" of the faeces of humans and animals are sufficiently distinctive to be of diagnostic value in determining whether faecal pollution in water samples are of human or animal origin.

3.1 AIM AND OBJECTIVES

The aim of this study was to use the Szűcs method (Szűcs *et al.,* 2006) to determine faecal sterol fingerprints for various animal species and humans (as well as the quantitation of these sterol profiles and the differences between each of the profiles).

The objectives of this study were to;

- determine the faecal sterol profiles of treated effluent and raw sewage of a waste water treatment plant (WWTP) (Potchefstroom)

- determine the faecal sterol profiles of water containing faeces from cattle, sheep, horses, pigs and chickens
- compare faecal sterol profiles of the different water samples tested

3.2 MATERIALS AND METHODS

The method used was based on that from Szűcs *et al.* (2006). The method was used to determine faecal sterol fingerprints of humans and various species of animals in water using GC-MS. Six target sterols (COP, CHL, DCHL, SROL, SNOL, β-SITO) were analysed using this method.

3.2.1 Materials

Dichloromethane (HPLC grade), acetonitrile (HPLC grade), sodium hydroxide, and standards of faecal sterols, including coprostanol (COP; 5β-cholestan-3β-ol), cholesterol (CHL; 5-cholesten-3β-ol), dehydrocholesterol (DCHL; 3β-hydroxy-5α-cholestane), stigmasterol (SROL; 3β-hydroxy-24-ethyl-5,22-cholestadiene), β-sitosterol (β-SIT; 24β-ethylcholesterol), and stigmastanol (SNOL; 24α-ethyl-5α-cholestan-3β-ol)as well as the surrogate standard solution (Perylene), and the Internal standard (Perylene-d12) were all provided by Sigma-Aldrich (Stenheim, Germany). N,O-bis-trimethylsilyl-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was from Supelco (Germany).

3.2.2 Preparation of standard solutions

For the optimization of GC-MS conditions, a standard mixture of COP, CHL, DCHL, SROL, β-SIT, SNOL, and perylene (surrogate standard) was prepared in dichloromethane at a concentration of 1.0 mg/ml for each component. For quantitative determination, perylene-d12

was used as an internal standard (IS) and dissolved in dichloromethane to obtain a stock solution of 1.0 mg/ml. In order to determine the extraction efficiency (recovery) of the faecal sterols in unknown water samples, a 0.25 mg/ml perylene surrogate standard solution was prepared in acetonitrile.

3.2.3 Derivatization procedure

GC-MS analysis of target sterols was carried out after derivatization with N,O-bis-trimethylsilyl-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS). A 25 µl aliquot of the standard mixture and 20 µl of IS solution were pipetted into 1.5 ml auto sampler vial (Supelco, Germany) and dried completely under nitrogen flow. The residue was reconstituted in 125 µl of dichloromethane and mixed with 125 µl of BTSFA-TMCS. The vial was closed with a Teflon-lined screw cap, and the sample was silylated at 70˚C for 30 min. Following derivatization, 1 µl of the mixture was injected into the GC-MS.

3.2.4 Instrumentation and GC-MS conditions

The samples were analysed on a Hewlett-Packard (Palo Alto, CA) GC-MS system consisting of an HP 6890 GC with an HP 5973 mass selective detector (MSD) and an Agilent 7683 automatic liquid sampler (Agilent Technologies, Palo Alto, CA). Separations were accomplished using an Supelco Equity-5 fused silica capillary column (Hewlett-Packard) coated with phenylmethylsiloxane (30 m x 0.25 mm x 0.25 µm film thickness) using the Environmental Protection Agency (EPA) Method 8270 with minor modifications.

Briefly, the GC-MS parameters were as follow: Carrier gas: helium 1.4 ml/min, constant flow; injection: splitless, 1 µl sample; inlet temperature: 280˚C; oven ramps, 150˚C for 0.5 min, 150˚C- 300˚C at 20˚C/min, and 300˚C-310˚C at 25˚C/min; GC-MS interface

temperature: 280˚C; MSD ion source temperature: 230˚C; MSD quadrupole temperature: 150˚C; ionization energy: 70 eV; and solvent delay: 7.0 min. System control, data acquisition, and analysis were performed with the HP G1701AA MSD Productivity ChemStation software, Rev. A.03.01 (Agilent) on a HP (Hewlett-Packard) computer. Data was acquired in the full scan mode between ions of m/z 50 and 550.

3.2.5 Preparation of calibrators and calibration curves

Target sterols and surrogate standard were dissolved in dichloromethane to prepare the standards as 1.0 mg/ml stock solutions. Calibrators were prepared by measuring 20 µl of IS and 5-, 10-, 15-, 20-, 25-, 30-, 35-, and 40 µl aliquots of the stock solution into auto sampler vials and dried under nitrogen flow. Residues were constituted in 125 µl of dichloromethane, mixed with 125 µl of BSTFA-TMCS, and derivatized as described previously. Final mass concentrations of calibration standards were 20-, 40-, 60-, 80-, 100-, 120-, 140-, and 160 ng for each of the components and that of IS was 80 ng in 1 µl injected volume. Calibration curves were obtained by plotting the target compound: IS response ratios of characteristic ions selected from the mass spectra as a function of the respective concentrations of the individual components. Target (T) and qualifier ions of m/z (Q1 and Q2) applied for the GC-MS quantitation are shown in Table 1.

Table 1: Target (T) and qualifier (Q1 and Q2) ions of m/z applied for GC-MS quantitation of faecal sterols.

Target Compound	T (m/z)	Q1 (m/z)	Q2 (m/z)
Perylene	252	253	250
Perylene-d12	264	260	132
Coprostanol	370	215	355
Cholesterol	329	368	353
Dehydrocholesterol	215	445	355
Stigmasterol	255	394	484
ß-sitosterol	357	396	381
Stigmastanol	215	473	383

Identity of the target sterols was examined by comparing the obtained mass spectra with those of the corresponding reference substances using the HP 5973 MSD reference collection, NIST MS search program, and Spectrald database collection, Version 1.5 (1996) (Hewlett-Packard).

3.2.6 Recovery studies

Extraction efficiency (recovery) was determined at three different concentrations, adjusted by adding known amounts of COP, CHL, DCHL, SROL, β-SIT, STOL, and perylene (surrogate standard) to surface water samples. Standards were dissolved in dichloromethane to obtain a 160 µg/ml stock solution, and 62.5-, 125.0-, and 250.0 µl aliquots of this were mixed with 250 ml of surface water and 10 g of NaOH (three replicates at each concentration). Spiked water samples were shaken in an incubator at 250 rpm/min at 60˚C for 1 h. After transferring to a separatory funnel, the samples were vigorously shaken with 4 x 50 ml of dichloromethane. Extractions were left overnight in order to separate and settle. The dichloromethane extracts were completely dried in a Rota-vapour (Büchi, Flawil,

Switzerland). Any water Residue present in the sample after evaporation was azeotroped with methanol and evaporated again. Residues were washed into auto sampler vials with 4 x 0.5 ml of dichloromethane and mixed with 20 µl of IS. Mixtures were then evaporated to dryness under nitrogen flow and reconstituted in 125 µl of dichloromethane, mixed with 125 µl of BSTFA-TMCS, derivatized, silylated, and analysed by GC-MS as described previously.

3.2.7 Preparation of water samples for GC-MS analysis

A raw domestic wastewater sample (influent) as well as a treated wastewater sample (effluent) was obtained from an urban sewage treatment plant (Potchefstroom WWTP). Surface water samples were also spiked with faeces from animals. These were cattle, chickens, horses, pigs and sheep. Samples were processed for extraction within 24-36 h. Water samples (300 ml) were mixed with 200 µl of 0.125 mg/ml surrogate standard solution and 10 g NaOH. They were then saponified in a shaking incubator at 60˚C for 1 h. Following saponification, the spiked water samples and the effluent were extracted (Section 3.2.6), derivatized (Section 3.2.3), and analysed by GC-MS (Section 3.2.4). The untreated wastewater sample (influent) was prepared for GC-MS in the same manner, but it was mixed with 50 ml of dichloromethane and ultra-sonicated with 10 µm amplitude for 10 min before extraction. Concentrations of target sterols and surrogate were calculated on the basis of calibration curves by the data analysis software (HP ChemStation).

3.3 RESULTS

3.3.1 Standard curves

Standard curves (Figure 10 a - f) were constructed by dividing the area of the peak for perylene-d12 (IS), by the area of the peak for each of the target sterols (COP, CHL, DCHL,

SROL, SNOL, β-SIT). This was done for each concentration of the standard solution. The ratios were determined and plotted on a concentration/response graph.

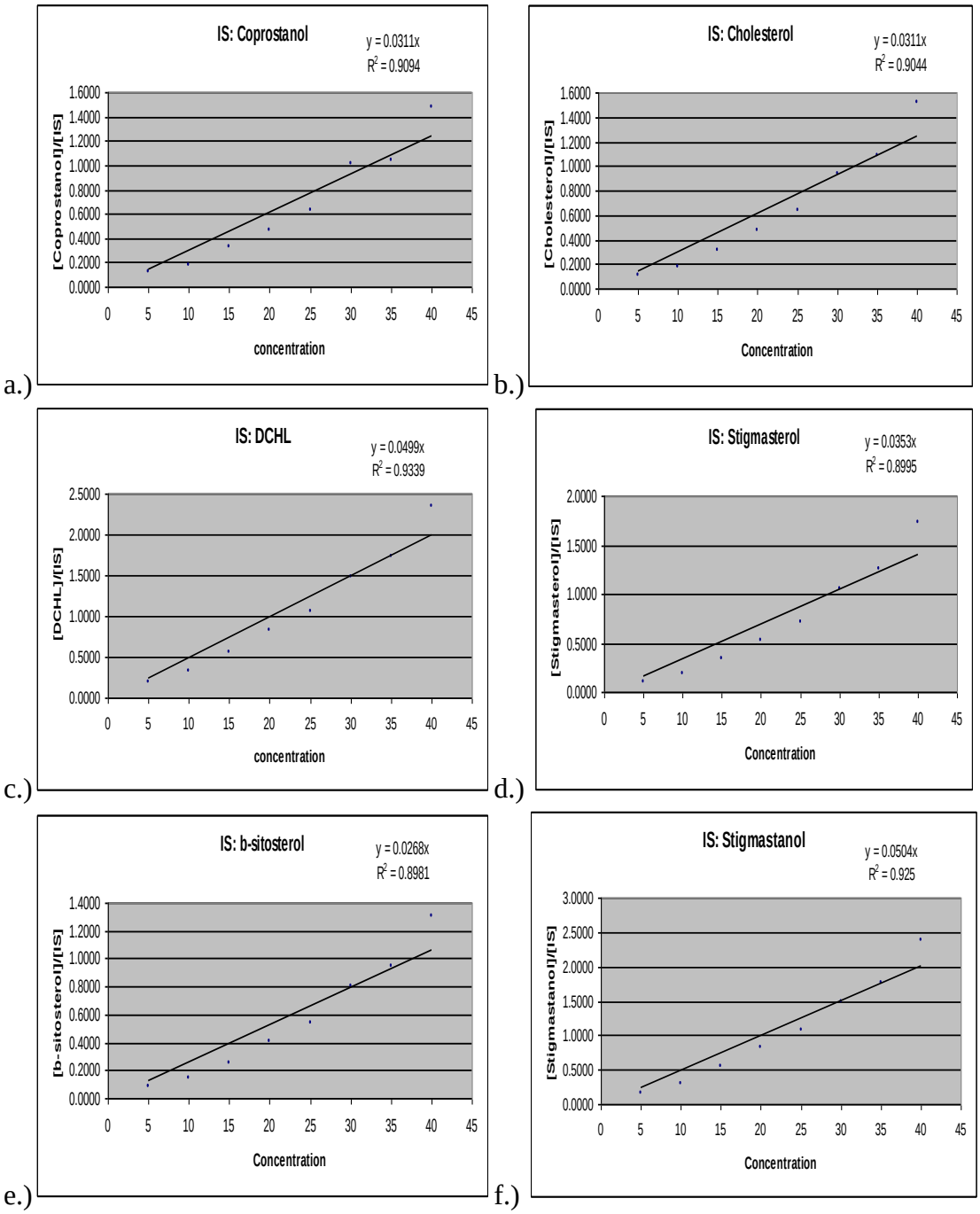


Figure 10: Standard curves of a.) IS: coprostanol (humans); b.) IS: cholesterol (humans); c.) IS: dehydrocholesterol (pristine environments); d.) IS: stigmasterol (plants); e.) IS: β-sitosterol (plants) and f.) IS: stigmastanol (algae).

The calibration curves for COP, CHL, DCHL, SROL, β-SITO and SNOL were obtained from three different series. The target compound-IS response ratios were linear up to 160 ng for each target sterol molecule. The lines of best fit obtained by linear regression were described by:

COP: $y = 0.0311x - 0.1999$, where $r^2 = 0.91$;

CHL: $y = 0.0311x - 0.2285$, where $r^2 = 0.90$;

DCHL: $y = 0.0499x - 0.2802$, where $r^2 = 0.93$;

SROL: $y = 0.0353x - 0.2711$, where $r^2 = 0.90$;

β-SITO: $y = 0.0268x - 0.2131$, where $r^2 = 0.90$;

SNOL: $y = 0.0504x - 0.3302$, where $r^2 = 0.93$.

These equations were used in the determination of concentrations of the various sterols.

3.3.2 Chromatograms

The following chromatograms (Figures 11 - 13) indicate results in determining the sterol fingerprint for the six species examined. Elution of perylene (surrogate) indicates that the recovery of the sterols was successful. Although sterol profiles may look similar and peaks elute at the same retention times, it may not be the same sterol. The identification of the target sterols are done by the obtained mass spectra for each eluted compound and not by retention times only. Some compound may overlap when eluting. Target and qualifier ions (Table 1), obtained by the MS detector, were used to identify target sterols of all species. Establishing sterol fingerprints is necessary because it forms the baseline for the faecal sterol analysis and helps with understanding the origin of faecal pollution in a water body (Leeming *et al.*, 1996). Figure 11 shows the sterol fingerprint for water samples spiked with faeces from chickens.

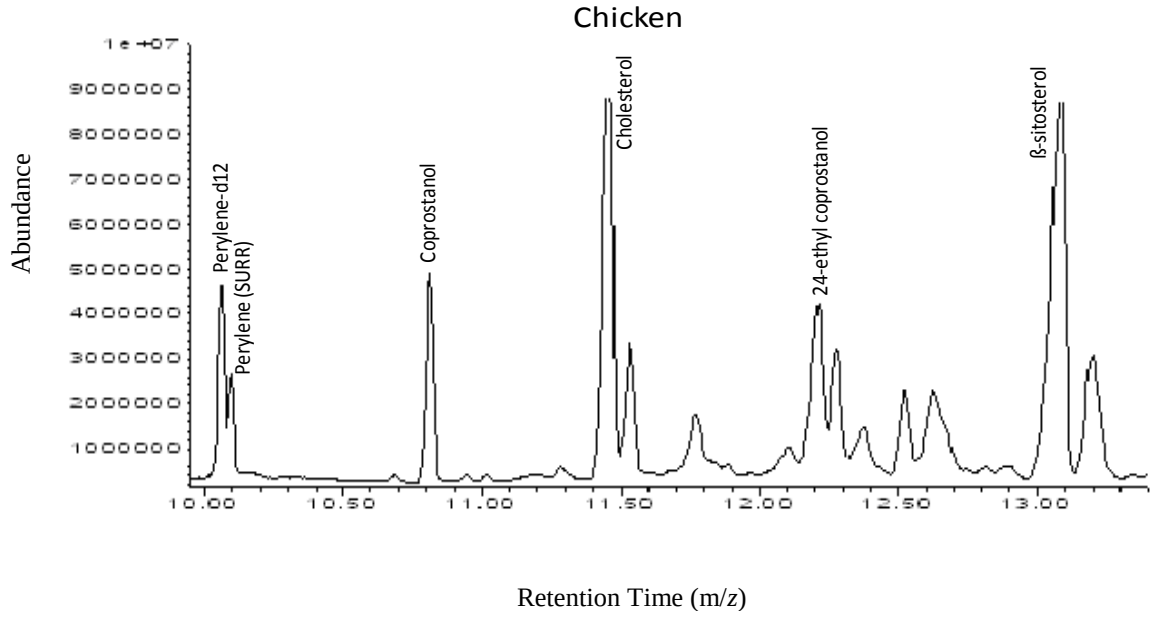


Figure 11: Sterol profile for the water sample spiked with chicken faeces.

In Figure 11, a higher abundance of cholesterol and β-sitosterol was observed compared to levels of coprostanol. This is in agreement with Leeming *et al.*, (1996), which suggested such results indicate faecal pollution from birds. While other sterols also eluted, the most important concentrations would be that of cholesterol and β-sitosterol as they are also the most abundant. This phenomenon is also demonstrated in Table 2.

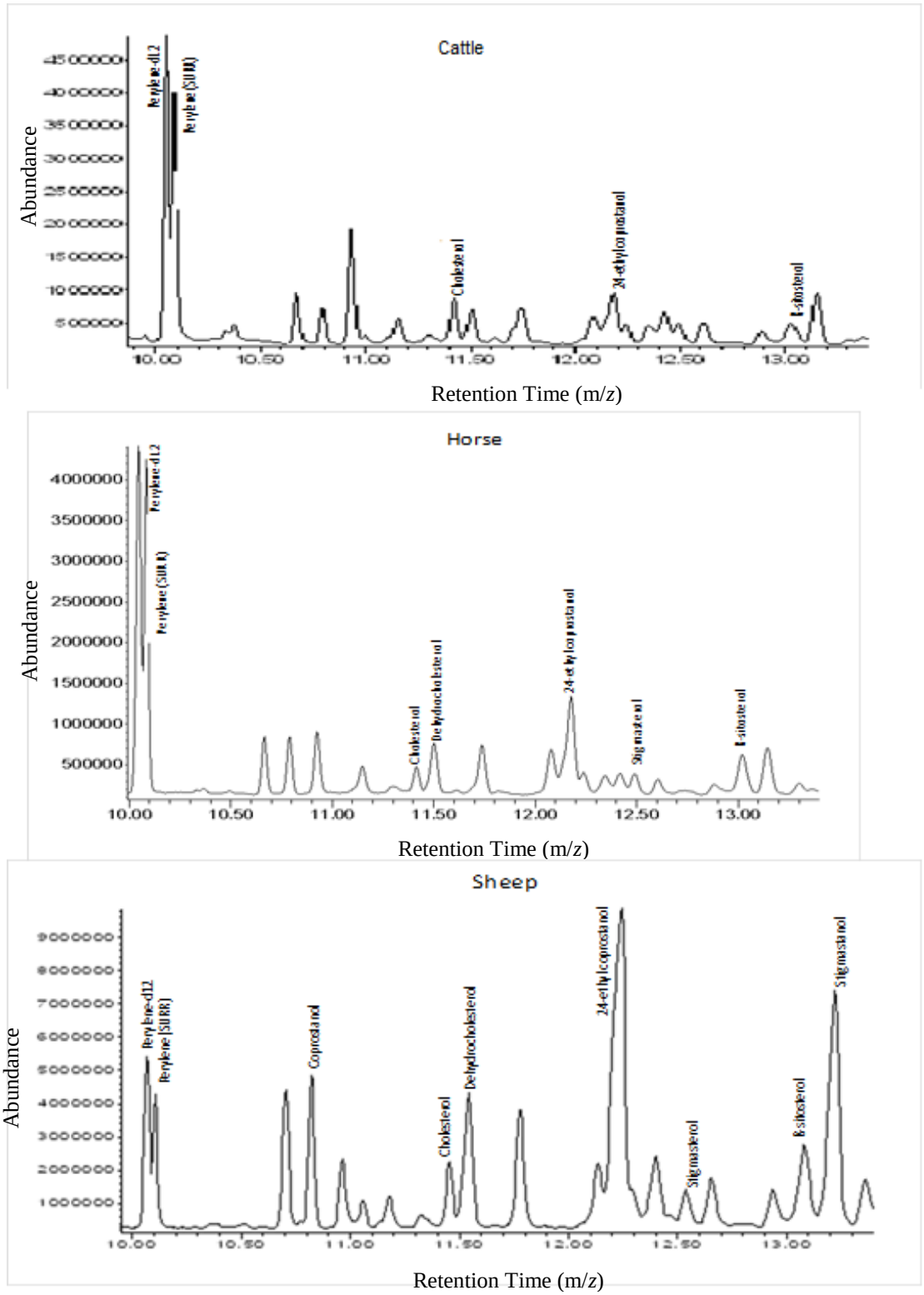


Figure 12: The sterol profiles of the herbivore species; a.) cattle, b.) horse and c.) sheep.

Elution of cholesterol and the phytosterol, β-sitosterol, occurs in all sterol profiles. Leeming *et al.*, (1996), indicated that sterol profiles of herbivores were dominated by C_{29} sterols.

Horse sterol profile (Figure 12b) also eluted dehydrocholesterol and stigmasterol that was not found in the sterol profile of cattle faeces, but was detected in sheep sterol profile (Figure 12c). The dehydrocholesterol (horse and sheep sterol profile) is a natural occurring sterol which is usually found in pristine environments, its absence in cattle suggests that cattle faeces were freshly collected at a non-pristine environment. Usually dehydrocholesterol is found in abundance in sewage sludge (Leeming, 2006) and by comparing concentrations of dehydrocholesterol to coprostanol, one can determine if faecal contamination is of fresh human faecal origin. In Figure 12 (a and b), no coprostanol eluted, which is in accordance to literature (Leeming *et al.*,1996 and Szűcs *et al.*, 2006), which states that coprostanol is found in human faeces, but in Figure 12c (sheep profile) coprostanol did elute, although the abundance of it was lower than that if the β-stanols. High abundance of 24-ethylcoprostanol eluted for all sterol profiles (Figure 12 a-c), since 24-ethylcoprostanol is the main faecal sterol excreted by herbivores (Leeming *et al.*, 1996), this was to be expected. The elution of β-sitosterol in all profiles is because of the diet of cattle, horses and sheep (herbivores) and β-sitosterol is a phytosterol found in plants.

Sterol profiles of human raw sewage, and effluent were also determined (Figure 13 b and c, respectively) and compared to one another. Because the food sources of humans and pigs are more or less the same, pig sterol profile (Figure 13 a) was also compared to the human profiles in order to determine significant and diagnostic differences between the two species sterol profiles.

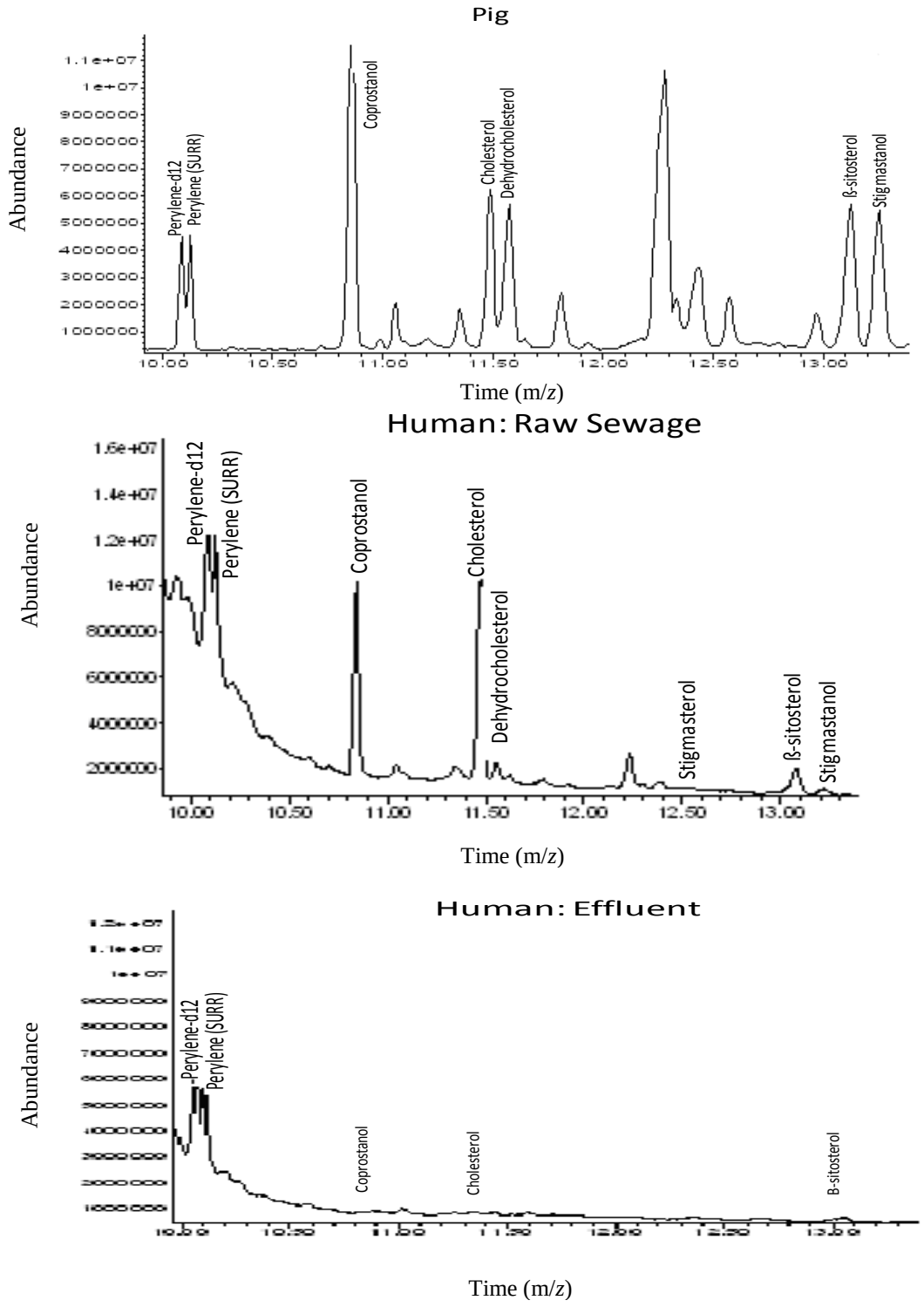


Figure 13: Sterol profiles of a.) pig, b.) human raw sewage (untreated sewage influent), and c.) treated sewage effluent.

Sterol profile of the pig excreta (Figure 13a) showed very high abundances of coprostanol, cholesterol, β-sitosterol, and stigmastanol. In untreated wastewater (Figure 13b), cholesterol and coprostanol was found. Coprostanol is the main human faecal sterol biomarker, indicating fresh human faecal contamination (Leeming, 1996; Szűcs *et al.*, 2006). All other five sterols were found in varying abundances in the WWTP influent. Coprostanol, cholesterol and β-sitosterol was found in extremely low abundances in treated effluent (Figure 13c). Comparing the sterol profiles of pigs (Figure 13a) and sewage (Figure 13b), a distinct difference between the two profiles can be seen. All target sterols eluted in the sewage sterol profile (Figure 13b), but β-sitosterol and the terrestrial sterols (stigmastanol and stigmasterol) were all of low abundance, while coprostanol and cholesterol were of higher abundance. In comparison, the pig sterol profile (Figure 13a), had high coprostanol abundance but low cholesterol abundance, while the abundance of dehydrocholesterol and stigmastanol were much higher than that of human raw sewage (Figure 13b). The differences that exists between the sterol profiles of pigs and humans are sufficient enough to depict that through sterol ratios, one would be able to distinguish between human and pig faecal contamination of a water source. Comparing the untreated wastewater sterol profile (Figure 13b) with that of the treated water (Figure 13c), it can be deducted that the target sterols tested for in this study has been radically decreased or completely removed. There were also fewer peaks in the treated water demonstrating that the wastewater treatment works was removing sterols from wastewater. This result implies that one could evaluate the efficiency of wastewater treatment plants using GC-MS analysis of faecal sterols.

In the samples that contained faecal matter that is of human origin (Figure 13b), the coprostanol and cholesterol concentrations were found to be of higher abundance than the other sterols. Whereas with cattle, horse, sheep (Figure 12) and chicken (Figure 11) faecal samples, the concentration of coprostanol was lower than that of other sterols. This

observation forms the basis for differentiating human from non-human sources of faecal contamination in environmental water samples (Leeming *et al.,* 1996).

Concentrations of the various sterols that eluted is important because from the concentrations, ratios can be established (Leeming, 2006), determining the origin of faecal pollution. The concentrations of the target sterols were calculated by integrating the peaks that eluted for each species and determining the area of each peak. Using the standard curve's y-values and r^2-values, the concentrations of each sterol that eluted for each of the various species could be calculated by the straight line equation: y = mx + c. The values are depicted in Table 2.

Table 2: Concentrations of all marker sterols that eluted for the seven samples analysed**.**

	COP (ppm)	**CHL (ppm)**	**DCHL (ppm)**	**SROL (ppm)**	**ß-SITO (ppm)**	**SNOL (ppm)**
Chicken	29.998	81.126	<LOQ	<LOQ	119.828	<LOQ
Cattle	<LOQ	5.859	<LOQ	<LOQ	4.875	<LOQ
Horse	<LOQ	3.785	4.740	3.078	8.024	<LOQ
Sheep	26.995	12.698	21.227	8.442	28.126	74.828
Pig	111.008	49.280	34.957	<LOQ	14.070	68.336
Sewage Influent	14.297	15.012	1.685	0.636	3.513	1.012
Sewage Effluent	1.368	2.004	<LOQ	<LOQ	2.887	<LOQ

<LOQ – Below limit of quantitation

Table 2 indicates that the treated effluent had very low concentrations of sterol to be of any consequence. All species had cholesterol and β-sitosterol in their profiles Cholesterol is synthesized in the body of all of the species samples and tested, and since all of the cholesterol synthesized and ingested is not re-absorbed by the body, it is excreted. Sheep, horse, pig and raw sewage samples had dehydrocholesterol and are consistent with Leeming *et al.,* (1996). The raw sewage sample, horse and sheep samples were the only profiles that

had stigmasterol, a plant sterol found in plant fats and oils (Baxter *et al.*, 1999). Large amounts of β-sitosterol were found among horse and sheep profiles, but less in cattle faeces. This is contradictory since they are all herbivores. All the herbivore species showed large amounts of stigmastanol and stigmasterol compared to the other species. This is because of their diet, as stigmasterol and stigmastanol are both terrestrial sterol biomarkers (Leeming, 2006).

3.4 DISCUSSION AND CONCLUSION

The aim of this study was to use the Szűcs method (Szűcs *et al.,* 2006) to determine faecal sterol fingerprints for various animal species and humans, as well as the quantitation of these sterol profiles and the differences between each of the profiles. In order to achieve this, determination of the faecal sterol profiles of treated effluent and raw sewage of a WWTP (Potchefstroom), as well as faecal sterol profiles of surface water containing faeces from cattle, sheep, horses, pigs and chickens, had to be accomplished. Results presented showed that the faecal sterol concentration was measured in five spiked water samples and two sewage water samples (influent and effluent) using GC-MS. Their chemical identity was confirmed by spectral analysis, and the mass spectra of the target compounds showed a higher than 80% identity with those of reference substances (Table 1).

Comparison of the faecal sterol profiles also had to be made in order to assess significant differences between the sterol concentrations of species. This in turn, forms the basis of which faecal origins can be determined. The major human faecal sterols were coprostanol and cholesterol. Leeming *et al.,* (1996) determined that coprostanol was the main human faecal sterol, and that it constituted about 60% of the total sterols found in human faeces. This was not the case in our study as cholesterol (15.012 ppm) was more abundant than coprostanol

(14.297 ppm). Coprostanol and cholesterol concentrations were, however, far above that of dehydrocholesterol (DCHL), stigmasterol (SROL), stigmastanol (SNOL) and β-sitosterol (β-SITO), which also eluted in human sterol profile. The high abundance of cholesterol may be because a raw sewage sample was taken to be analysed. This sample may contain other organic matter (eg. plant rests), which could possibly have contributed to the amount of cholesterol in the sample. Leeming *et al.,* (1996), also determined that the main faecal biomarker for herbivores was 24-ethylcoprostanol. Although 24-ethylcoprostanol was not used as a target sterol in this method (Szűcs *et al.*, 2006), it did elute in the sterol profiles of herbivores as determined by Leeming *et al.,* (1996), and was thus shown on the chromatograms of the sterols (Figure 12 a-c). The concentration could however, not be determined as no standard was acquired for 24-ethylcoprostanol. Herbivore sterol profiles all had traces of terrestrial sterol biomarkers, because of the animals' diet. In the sterol profile of bird faeces, both 5β and 5α stanols were low compared to β-sitosterol. Birds possess an inability (due to the lack of specific bifidobacteria) to convert cholesterol to 5β-stanols (Leeming *et al.,* 1996), which explains why the cholesterol concentration was higher than all other sterol concentrations. Pigs and sheep were found to have sterol profiles similar to that of humans, although the concentrations of sterols found varied immensely (Table 2).

The main conclusion from this part of the study is that faecal sterol biomarkers have unique source specificity dependent on a combination of sterol intake and the metabolic production of sterols produced by microbial biota in the digestive tracts of various animal species. It can therefore be concluded that the 'sterol fingerprints' of human and animals are sufficiently distinctive to be of value in determining the quality of environmental water and the origin of faecal pollution in water samples.

4

Applications in using sterol analysis in the determination of groundwater and surface water quality

Groundwater resources occur in openings in the rock material in the subsurface (DWAF, 2004a). These openings are fracture zones in hard rocks and dissolved fissures in dolomites (in the North West Province) (Colvin, 2007), these specific openings are mostly recharged by rainfall and stream infiltration (DWAF, 2004a). Groundwater resources act as reservoirs and slowly release water back to rivers and wetlands. Ecosystems connected to these groundwater replenishment zones are dependent on the water they provide all year round (Colvin, 2007).

Pollution of groundwater in the North West and Northern Cape Provinces are a main challenge as rural communities depend almost exclusively on these water resources for drinking purposes (Kalule-Sabiti and Heath, 2008; Woodford *et al.,* 2009). These communities obtain their drinking water directly from uncovered or covered boreholes and wells (Kalule-Sabiti and Heath, 2008). There are four main routes by which groundwater can be contaminated, 1.) infiltration; 2.) direct migration; 3.) inter-aquifer exchange; and, 4.) recharge from surface water (Barcelona *et al.,* 1988). Thus, microbiological pollution of groundwater is most probably caused by human and animal activities, which include on-site sanitation, cemeteries, waste disposal, feedlots and un-sewered settlements (Engelbrecht and Tredoux, 2000). In certain areas, the groundwater does not comply with the DWAF and SANS 241 limits set for physico-chemical properties, microbial indicators or trace elements (Engelbrecht and Tredoux, 2000). Recently, Mpenyana-Monyatsi and Momba, (2012),

conducted a study of groundwater in rural areas of the NWP and concluded that 86% of the boreholes tested did not comply with the limits set by the national guidelines (SANS 241 and DWAF) in terms of faecal (FC) (0 cfu/100 ml) and total coliforms (TC) (0 to 5 cfu/100 ml).

In most urban areas in South Africa, surface water are utilized more readily that groundwater. Surface water systems in the NWP consist of dams, rivers, wetlands, and pans (NWDACE-SoER, 2008). A large portion of available surface water in the North West and Northern Cape Provinces contributes to the mining, agricultural and industrial sectors (Kalule-sabiti and Heath, 2008). Surface water quality in the North West and Northern Cape Provinces are impacted by a number of point and non-point source factors; these include, acid mine drainage, domestic and industrial sewage effluents, and agricultural and storm run-off (NWDACE-SoER, 2008). Additionally, raw municipal waste water is sometimes discharged into the rivers and dams in these Provinces, introducing a wide range of potentially infectious agents into the rivers (Awofolu *et al*., 2007). Surface water in the NWP is critical for socio-economic growth and poverty reduction, and plays a central role in providing water recreational, cultural and religious beliefs of the people in communities (Molale, 2012).

Groundwater in this study was collected from boreholes and water storage tanks in townships across the North West and Northern Cape Provinces, South Africa. These groundwater samples were of significant bad quality (Results, 4.3A). As previously mentioned, groundwater is of vital importance in the North-West and Northern Cape Provinces as it is in many instances the only source of water for many rural people (Mpenyana-Monyatsi and Momba, 2012). We know from previous studies done on boreholes in the NWP that a large number of groundwater sources in the Province are contaminated (Momba *et al.*, 2006; Mpenyana-Monyatsi and Momba, 2012).

Surface water collected and analysed in this study was gathered from the Harts River and Baberspan Nature reserve situated between Delareyville and Sannieshof in the NWP, South Africa. The Harts River falls under the Lower Vaal WMA (Water Management Area) and is regulated by the Taung and Spitskop dams and the Little Harts River and the Great Hearts River (DWAF, 2009c). The Lower Vaal WMA is located on the north-western region of South Africa, and borders Botswana in the North (DWAF, 2009). Intensive irrigation practices happen in this area and they include the planting and harvesting of maize, wheat and cotton (Ferreira, 2008), as well as extensive livestock farming of beef, dairy, goats and sheep (DWAF, 2004a). According to Ellington (2003) the water quality of the Harts River can be impacted by irrigation return flows and the Vaal Harts irrigation scheme, which contributes fertilizer and salts to the Harts River from the Upper and Middel Vaal. The Harts River is connected with an off channel-pan, Baberspan, by a channel (DWAF, 2004a). The Baberspan bird sanctuary is one of the largest waterfowl sanctuaries in South Africa (Anon[6], 1997; NWP-SoeR, 2002). It consists of 2000 ha body of water, which is of international importance for migratory birds (Anon[6], 1997; DWAF, 2002). It lies between Sannieshof and Delareyville in the NWP of South Africa (DWAF, 2004a). Baberspan is the largest of a series of pans that lie in the fossil bed of the Harts River. More than 12 000 birds and over 365 bird species have been recorded here (Swart and Cowan, 1994; Anon[6], 1997). The pan is state controlled and is used for angling and bird watching, while land use surrounding the pan include cattle and maize farming (Swart and Cowan, 1994).

4.1 AIM AND OBJECTIVES

The aim of this study was to determine if the Szűcs method can be used to detect faecal contamination in groundwater and surface water samples, which were taken from various sites across the North West Province, South Africa.

The objectives included;

For groundwater samples;

- determining the physico-chemical properties of groundwater sampled from boreholes, water storage tanks and water storage facilities in rural areas of the North West Province, South Africa.
- determining the microbial counts of groundwater samples.
- measuring target faecal sterol concentrations in groundwater samples collected from boreholes and water storage tanks from rural communities across the North West Province. This also included water storage facilities in townships.

For surface water samples;

- determining the physico-chemical properties of surface water collected from the Baberspan bird sanctuary
- determine the microbial content of the surface water samples collected.
- analysing faecal sterols of surface water collected from the Baberspan inland lake in the North West Province.

4.2 MATERIALS AND METHODS

4.2.1 Sample design and collection

Sampling was done according to the guidelines provided in the sampling guide of the Water Research Commission (WRC, 2000). Ground water samples were collected from boreholes and water storage tanks all over the North West and Northern Cape Provinces (Figure 14) and analysed for their physico-chemical properties, their microbial content and for faecal sterols by using the Szűcs *et al.* (2006) method. Since sterols are known to have a high binding capacity with organic-rich, fine grained sediment, (Bilibro *et al.*, 2011), which is ubiquitous in ground water, little to no sterol abundances was expected.

Surface water was collected at four pre-marked sites from the Harts River and Baberspan bird Sanctuary in the NWP, South Africa (Figure 15) and analysed for its physico-chemical properties and microbial concentrations. The Szűcs method was then used in determining faecal sterol levels in surface water.

All groundwater and surface water samples were collected in 1L Schott bottles and transported to the laboratory in cooler boxes for microbial analysis, sterol analysis and for the determination of certain chemical properties (chemical oxygen demand (COD), NO_2 and NO_3). Analysis was done within 12-24 hours from collection due to the distance travelled. Water was analysed on site for physical parameters such as temperature, pH, total dissolved solids (TDS), dissolved oxygen (DO) (not all samples), salinity and conductivity, using a transportable multi-probe meter (Multi-parameter PCSTestr 35, Eutech Tehnologies). Sites where groundwater was collected are shown in Figure 14 and where surface water was collected, Figure 15.

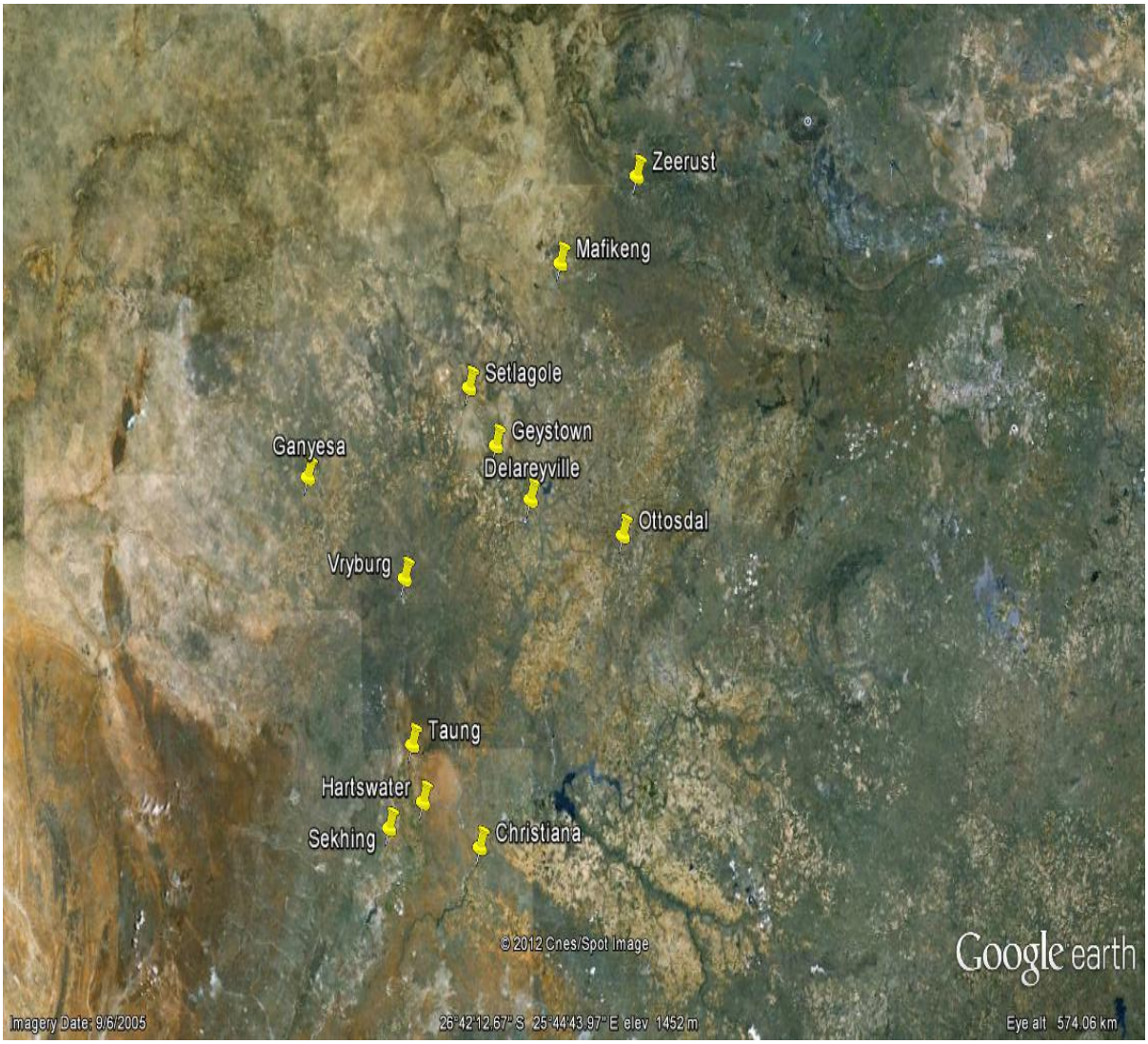


Figure 14: A map of towns were groundwater samples were taken, taking into account that most of the samples were taken on farms with boreholes near to these towns, or from water storage tanks and water storage facilities in informal settlements near these towns.

Two samples were taken from the Christiana and Ganyesa area, while one water sample each was collected from Geystown, Hartswater, Mafikeng, Sekhing, Setlagole, Taung, Vryburg, Zeerust, and a rural sample between towns Delareyville and Ottosdal (Delarey-Otto). All GPS coordinates of sampling sites are listed in Appendix A

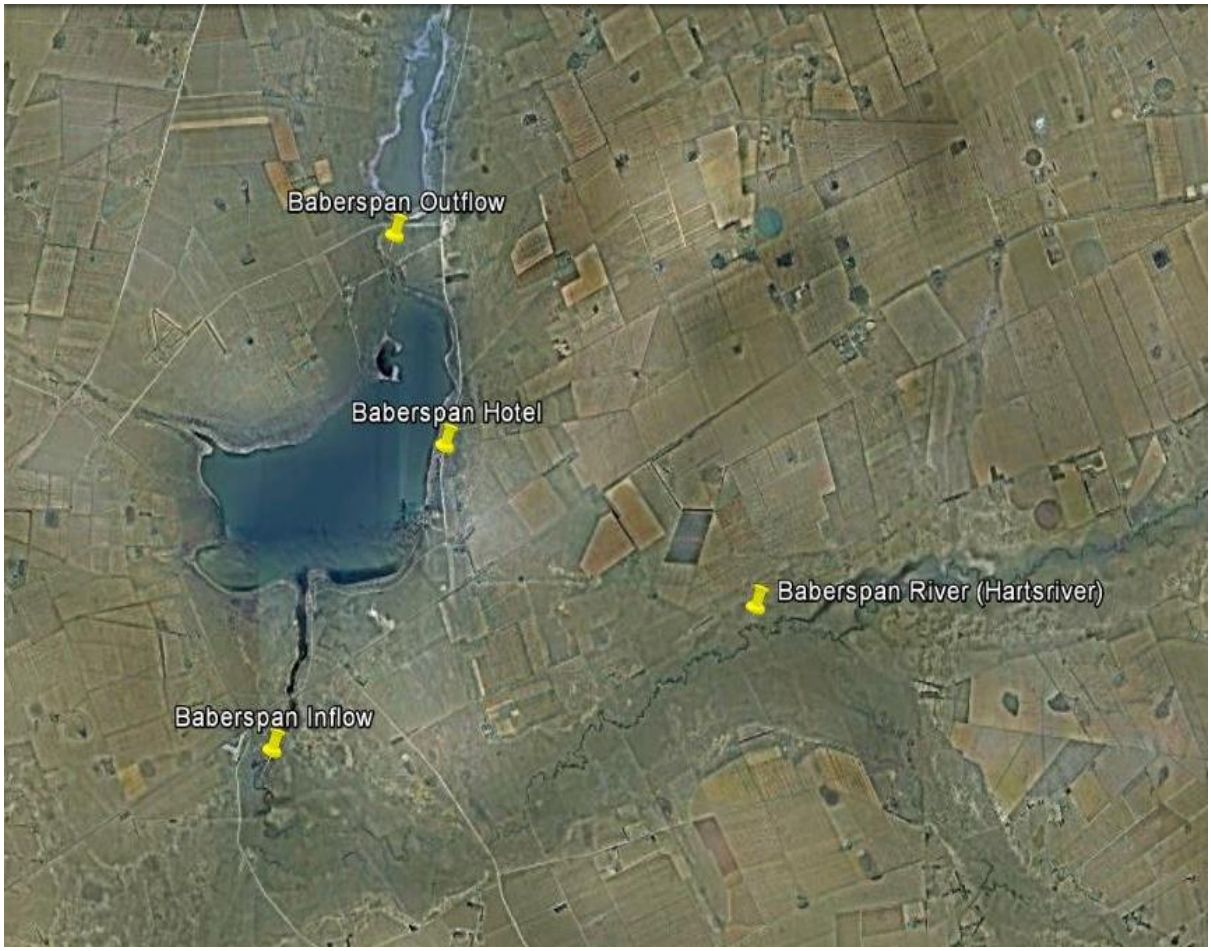


Figure 15: Sampling sites of surface water, taken from the Baberspan bird sanctuary and Harts River, in the North West Province of South Africa.

One surface water sample was collected from the Harts River that runs past, and into the inland lake. Another water samples was taken from the inflow of the water into the lake. Another was taken at the Baberspan Hotel, which is situated on the banks of the lake, and the last sample was taken at the outflow of the water from the lake. All samples were tested for pH and temperature of water, EC and TDS on site, while nitrate-nitrogen analysis was conducted in the laboratory. Determination of microbial counts and sterol analysis were then also done on all of these samples. Samples were processed for extraction within 24-36 h.

4.2.2 Physico-chemical analysis of groundwater and surface water samples

The physical properties (pH, temperature, total dissolved solids and electrical conductivity) of the surface water systems were determined on site using a calibrated Oakton PCStestrTM 35 waterproof field multi-parameter probe (Thermo Fisher scientific, US). The probe was rinsed with distilled water prior and after use. For measurement of the physical parameters, 20 ml of sample water was dispensed in a sterile glass beaker, the multi-probe was then submerged in the water and the parameters (temperature, pH, salts, EC and TDS) measured.

The chemical properties of the surface water systems nitrites (NO2-), (CAT No: 21071-69); and nitrates (NO3-), (CAT No: 21061-69) were measured in the laboratory as milligrams per litre (mg/l) using a HACH DR 2800TM (HACH, US). All physico-chemical values obtained were compared to the South African Target Water Quality Range (TWQR) for full and intermediate recreational contact, livestock watering and irrigation (DWAF, 1996d). All the applicable TWQR as set by the Department of Water Affairs and Forestry are summarised in Appendix B.

4.2.3 Microbial analysis of groundwater and surface water

Water samples were transported to the laboratory in cooler boxes on ice, for standard microbial analysis. Analysis was done within 12-24 hours of sampling, due to distance travelled. All bacteria was analysed following the standard membrane filtration technique were 100 ml of water sample were aseptically filtered through a 0.45 µm pore size Whatman© filter using a vacuum. The Whatman filters, which now contain the bacterial cells, were placed aseptically on selective media: m-FC agar (Merck, Germany) was used, for the cultivation of total coliforms, MLG agar (Oxoid, UK), and for the cultivation of faecal

Streps, KF-Streptococcus agar containing 1ml of 2,3,5-Triphenyltetrazolium chloride (TTC) per 100ml (Sigma-Aldrich, South Africa) was used. The filter funnel apparatus was treated with 70% Ethanol (ETOH) and washed thoroughly with sterile distilled water when analysing 2 different samples. The culture media was pre-selected due to their selective ability; which means that it is a culture medium that is enriched with a particular substance to allow the growth of particular organisms. All samples were done in triplicate.

All m-FC agar plates were incubated at 45°C for 24 hours while all MLG agar plates were incubated at 37°C for 24 hours. The KF-Streptoccus agar plates were incubated at 37°C for 48 hours. Colonies of interest on the surface of the filter membranes (blue and yellow colonies on m-FC agar as total coliforms, blue colonies on m-FC agar as faecal coliforms, green colonies on MLG agar as *E. coli* and pink colonies on KF-Streptococcus agar as presumptive faecal streptococci) were observed, counted and recorded (DWAF, 1996). Results were converted and expressed as colony forming units (CFU) per 100 ml.

For identification of bacteria gram staining was performed on all colony samples taken from the cultured agar and results and cell morphology were noted under a microscope at x 1000 magnification with immersion oil. Faecal indicator organisms are small rod shaped organisms and gram negative, all organisms that were gram positive and didn't have the specific morphology were discarded, whereas gram negative, small rod shaped organisms were subjected to further identification tests. For further identification, the triple sugar iron (TSI) test, and API 20E strips were used.

4.2.4 Isolation and purification of presumptive E. coli and faecal streptococci

For isolation and purification purposes, selected pink colonies on KF-Streptococcus and green colonies on MLG agars were aseptically sub-cultured at least 3 times on nutrient agar using the streak plate technique and incubated for 24 hours at 37ºC.

4.2.5 Identification and confirmation of presumptive E. coli and faecal streptococci

In this study, Gram staining was performed in order to confirm that all presumptive *E. coli* and faecal streptococci isolates was Gram negative and Gram positive, respectively, while ensuring they were pure and not mixed cultures. Briefly, a bacterial smear was heat fixed and stained with a drop of crystal violet for 1 minute and rinsed with distilled water. A drop of Grams iodine was placed for 1 minute and rinsed with distilled water. Thereafter the smear was destained with ethanol for 10 seconds and washed with distilled water again. Lastly, the smear was counterstained with safranin for 1 minute and rinsed with distilled water once more. This staining procedure was done according to the method prescribed in Pandolfi and Pons (2004). Schleifer and Kilpper-Bälz (1984) described the observation of a faecal streptococci isolate under a microscope as Gram positive, elongated, ovoid shaped cells in pairs or short chains. Expected results for all presumptive faecal streptococci were indicated by a bacterial mat composed of deep purple stained cocci arranged in chains. *E. coli* is a gram negative, rod-shaped bacterium (Anon[7], 2005). Expected results for presumptive *E. coli* were indicated by a bacterial mat composed of red or pink stained rod shaped bacteria.

The TSI test was then used for confirming all presumptive *E. coli* isolates The triple sugar iron (TSI) agar (Merck, Germany) test used for the further confirmation of presumptive *E. coli* isolates was performed according to (Fankhauser, 2001). Briefly, purified presumptive *E. coli* isolates were inoculated into a TSI agar slant using a zig-zag streak pattern and stabbed

using an inoculation needle. These slants were incubated at 37°C for 18 hours. A positive TSI agar reaction test was indicated by a change in the colour of the agar from red to yellow as well the development of an air bubble in the test tube and the absence of a black precipitate indicative of production of Hydrogen sulphide (H_2S).

4.2.6 Faecal sterol analysis of groundwater and surface water via GC-MS

All groundwater sampling sites and surface water sampling sites were analysed for faecal sterols with the Szűcs method (Szűcs *et al.*, 2006). The Szűcs method was designed to rapidly measure faecal pollution in water by GC-MS determination of faecal sterols present in human and animal faeces. Sterol biomarkers tested for were; cholesterol, coprostanol, β-sitosterol, stigmastanol, stigmasterol and dehydrocholesterol (Szűcs *et al.*, 2006). The materials used in this method, as well as the GC-MS instrumentation and conditions can be viewed in Chapter 3 (Sections 3.2.1 and 3.2.4, respectively). Recovery studies of sterols (Section 3.2.6) and the derivatization procedure (Section 3.2.3) for sterol analysis in groundwater and surface water were the same as that of sterol fingerprint determination (chapter 3).

4.2.6.1 Preparation of water samples for GC-MS analysis

Water samples (300 ml) were mixed with 200 µl of 0.125 mg/ml surrogate standard solution and 10 g NaOH. They were then saponified in a shaking incubator at 60˚C for 1 h. Following saponification, the water samples were extracted (Section 3.2.6), derivatized (Section 3.2.3), and analysed by GC-MS (Section 3.2.4). Concentrations of target sterols and surrogate were calculated on the basis of calibration curves (Section 3.2.5 and Section 3.3.1) and by the data analysis software (HP ChemStation).

4.2.6.2 Statistical analysis

Where appropriate; averages, standards and standard deviations were calculated with Microsoft Excel 2010. Sterol quantitation and analysis were done by the data analysis software, HP ChemStation.

4.3A RESULTS: GROUNDWATER

This section includes the measured results from the groundwater samples that were analysed. physico-chemical results are given in section 4.3A.1. Microbial results are given in section 4.3.A.2. This entails the bacterial counts enumerated on culture media. Section 4.3A.3 will give the GC-MS profiles of the groundwater samples that indicated faecal pollution through elution of certain sterols.

4.3A.1 Determination of the physico-chemical properties of groundwater

Sampling for the groundwater study was done only once between January 2010 – November 2010, with the aim to detect any amount of faecal pollution in groundwater in the North West Province of South Africa. Thirteen sites were sampled and the physico-chemical results depicted. Some of the physical properties were measured on site (temperature and pH of water, $NaCl_2$, TDS and EC), while other physico-chemical properties (NO_3-N) were tested for in the laboratory. Results for the physico-chemical properties are shown in Table 3.

Table 3: Physico-chemical properties of the groundwater samples taken at the various sites across the North West Province.

Site	pH	Temp (°C)	EC (mS/m)	TDS (mg/L)	Nitrate-nitrogen (mg/L NO_3-N)
Mafikeng	7.1	19.3	142	997	8.2
Zeerust	7.8	20.1	62	450	9.5
Delarey-Ottosdal	7.2	20.5	133	948	23.9
Setlagole	7.0	19.5	253	1790	18.4
Geystown	7.8	18.1	81	583	15.2
Taung	7.7	24.4	92	650	16.4
Hartswater	7.6	20.2	286	2003	22.3
Sekhing	7.3	24.0	143	1007	1.6
Christiana	7.5	19.4	144	1002	23.2
Christiana 2	6.9	22.2	86	614	11.6
Vryburg	7.2	16.0	125	886	28.9
Ganyesa	7.5	17.3	126	895	28.6
Ganyesa 2	7.1	21.2	84	595	15.2

The physico-chemical properties of the water sample taken may have an effect on the bacterial coliform counts and it is therefore a necessity when taken the water sample. Groundwater samples taken in Table 3 were taken from all over the North West Province of South Africa and analysed.

From Table 3 the following was observed: The pH of all the sites sampled complied with the DWAF TWQR (Target Water Quality Range) (6.0 – 9.0) for domestic and irrigational purposes and ranged from 6.9 – 7.8 (Table 3). The groundwater temperature of sample areas ranged from 16.0 ˚C – 24.4˚C (Table 3). The EC and TDS (average 135.15 mS/m and 955.38 mg/L, respectively) were generally high compared to SANS 241 (2011), which indicates levels of 0 - 70 mS/m and 0 - 450 mg/L, respectively (DWAF, 1996). It is to be expected as this is groundwater samples and particulate matter with high salt content because of the surrounding rocks in the aquifers is, most likely, the cause (Anon[8], 2007). Slightly elevated TDS may cause water to taste salty, but no health effects are likely, on the other hand, high TDS values such those found at Hartswater (2003 mg/L) causes the water to taste extremely salty and may cause corrosion of pipes and appliances (Oram, 2012). Short term consumption may be tolerated, but may cause a disturbance in the body's salt balance. TDS is tested as a secondary drinking water standard, and is therefore more of an aesthetic hazard (Oram, 2012). The high EC counts are related to the high TDS counts, but the EC counts need to exceed 300 mS/m to be of any major health concern (DWAF, 2001).

The nitrite-nitrogen is very high (average 17.15 mg/L NO_3-N) in some areas which water samples were taken compared (Table 3) to the 6 mg/L NO_3-N TWQR for domestic use. High nitrite values pose a serious health risk; such water should not be ingested. Upon digestion, nitrite combines with haemoglobin (oxygen carrying red blood pigment) to form methaemoglobin, which is not capable to carry oxygen; this condition is then called *methaemoglobinemia* (DWAF, 1996). *Methaemoglobinemia* is particularly hazardous in infants, as adults possess the enzymes needed to reverse the methaemoglobin to haemoglobin reaction and infants do not. *Methaemoglobinemia* then occurs. The DWAF (1996) target water quality range for Nitrate/Nitrite is 0 – 6 mg/L. It is rather worrying then that all but one (Sekhing) of the groundwater sample sites showed values far exceeding that of the TWQR.

Some sites even reaching levels of 28 mg/L (Vryburg; 28.9 mg/L and Ganyesa; 28.6 mg/L) Table 4 indicates the effects the different nitrate/nitrite values have on human health.

Table 4: Effects of nitrate/nitrite values on human health (taken from DWAF, 1996).

Nitrate/Nitrite range (mg/L)	Effects
0 - 6	No adverse health effects
6 - 10	Rare instances of methaemoglobinemia in infants No effects in adults
10 - 20	Concentrations in this range are generally we tolerated methaemoglobinemia may occur in infants No effects in adults
>20	*methaemoglobinemia* occurs in infants Occurrence of membrane irritation in adults

Of all the groundwater sites tested (n=13), 5 sites were above 20 mg/L, 5 sites were in the 10-20 mg/L range, 2 sites were in the 6-10 mg/L range, and only 1 site was within the target water quality range for domestic use (0-6 mg/L) (Table 3). Because of the health effect associated with groundwater nitrate/nitrite values, it is of major importance that groundwater and especially boreholes used as drinking water source, is tested and monitored on a regular basis.

4.3A.2 Microbial analysis of groundwater

All groundwater samples were analysed for TC, FC, *E. coli* and FS (Faecal Streps) as depicted in Section 4.2.3 and. the results are shown in Table 5 below. All microbial parameters were extracted with the membrane filter technique, as previously mentioned, and grown on selective agar. The identified colonies were counted and depicted as CFU/100 ml (colony forming units per 100 ml). Table 5 also contains the concentrations of sterols that

were found at the different groundwater sampling sites, although their TIC (total ion chromatograph) and concentrations will only be shown and interpreted in Section 4.3A.3.

Table 5: Coliform and bacterial counts for groundwater samples.

Site	TC (CFU/100 ml)	FC (CFU/100 ml)	E.coli (CFU/100 ml)	FS (CFU/100 ml)	Cholesterol (ppm)	Coprostanol (ppm)
Mafikeng	TFTC	TFTC	TFTC	TFTC	<LOQ	<LOQ
Zeerust	TFTC	TFTC	TFTC	TFTC	<LOQ	<LOQ
Delarey-Ottosdal	TFTC	TFTC	TFTC	TFTC	<LOQ	<LOQ
Setlagole	>300	>300	34	TFTC	<LOQ	<LOQ
Geystown	TFTC	TFTC	TFTC	TFTC	6.135	5.485
Taung	TFTC	TFTC	TFTC	TFTC	6.135	5.538
Hartswater	>300	>300	TFTC	180	<LOQ	<LOQ
Sekhing	TFTC	TFTC	TFTC	TFTC	<LOQ	<LOQ
Christiana	233	65	TFTC	32	6.696	6.037
Christiana 2	TFTC	TFTC	TFTC	TFTC	<LOQ	<LOQ
Vryburg	TFTC	TFTC	TFTC	TFTC	<LOQ	<LOQ
Ganyesa	300	220	TFTC	TFTC	<LOQ	<LOQ
Ganyesa 2	TFTC	TFTC	TFTC	TFTC	<LOQ	<LOQ

TFTC – CFU were less than 30 CFU/100ml; <LOQ – Below the limit of quantitation

The groundwater samples in Table 5 were taken at various sites in the North West Province, South Africa. These water samples were then analysed and the total coliform counts (TC), the faecal coliform counts (FC), *E. coli* counts and the Faecal Streps (FS) were determined.

Total coliforms (TC) give an indication of the general sanitary quality of water, since this group may contain bacteria from faecal and non-faecal origin (DWAF, 1996). The DWAF (1996) (SANS 241; 2011) TWQR for water that is used for human consumption is a count of 0-5 CFU/100 ml for TC. There are 6 sites (Mafikeng, Zeerust, Geystown, Sekhing, Vryburg and Ganyesa 2) that fall within this target water quality range, which means the 54 % of the groundwater sample sites did not comply with the DWAF (1996) TC standards. Two sites (Setlagole and Hartswater) showed a bacterial CFU/100 ml count of above 300 CFU/100 ml (To many to count (TMTC)). Christiana (233 CFU/100 ml) and Ganyesa (300 CFU/100 ml) also showed extremely high TC counts. These extremely high TC counts significantly increases the risk of infectious disease transmission. The remaining groundwater sampling sites (Delarey-Ottosdal, Taung and Christiana 2) were all in the 5-100 CFU/100 ml range. Continuous use of groundwater at these sites increases the risk of infectious disease transmission and, there exists a slight risk with occasional exposure.

Faecal Coliforms (FC) are indicators of possible faecal pollution in water and are used to evaluate the quality of waste water, river water, raw water for drinking supply, recreational water and water used for irrigation, livestock watering and aquaculture (DWAF, 1996). The DWAF (1996) (SANS 241; 2006) target water quality range for FC is 0 CFU/100 ml. Three of the 13 groundwater sites sampled had a FC value of 0 CFU/100 ml (Mafikeng, Vryburg and Ganyesa 2). This means that 77% of the groundwater sampling sites did not comply with DWAF (1996) target water quality standards for faecal coliforms. Setlagole and Hartswater again had values exceeding 300 CFU/100 ml (TMTC), while Christiana (65 CFU/100 ml) and Ganyesa (220 CFU/100 ml), also showed extremely high FC counts. DWAF (1996) states that any water sample that exceed 20 CFU/100 ml pose a significant and high risk of infectious disease transmission. The remaining six groundwater sample sites all fall within the 0-10 CFU/100 ml category of the DWAF (1996) standards. This means that with

prolonged or continuous use of the groundwater there exists a slight risk of microbial infection, while occasional or short-term exposure hold negligible health effects.

E.coli is used to evaluate drinking water for the possible faecal origin (DWAF, 1996). The presence of *E.coli* can be used to monitor more harmful microbes (Giardia and Shigella) (Lewis, 2006). From the 13 groundwater sites sampled, 8 sites (Mafikeng, Geystown, Taung, Hartswater, Christiana 2, Vryburg, Ganyesa and Ganyesa 2) had no *E.coli*, three sites (Zeerust, Delarey-Ottosdal and Sekhing) showed 2 CFU/100 ml and one site (Christiana) had 3 CFU/100 ml and only Setlagole showed a high amount of *E.coli* in the water. Heavy precipitation may have caused *E.coli* count in the Setlagole groundwater to be high. If this water is consumed without prior treatment, it may result in illness. Faecal streptococci (FS) are used in the evaluation of treatment processes and recreational water. Faecal streptococci are more stable than coliform bacteria and often appear in human and animal faeces (DWAF, 1996). There is no DWAF (1996) target water quality range for FS in drinking water, only for recreational water (0-30 CFU/100 ml). Hartswater groundwater quality exceeds even this range with an amount of 180 CFU/100 ml found in the groundwater sample. The rest of the groundwater sample values varied from 0 CFU/100 ml (Geystown and Ganyesa 2) to 32 CFU/100 ml (Christiana). Because faecal streptococci are more stable in the environment, it may provide a better estimate of possible viruses in contaminated water than coliforms (Cohen and Shuval, 1972).

4.3A.3 GC-MS analysis of groundwater

Twelve groundwater samples (Ganyesa 2 was not analysed) were then analysed by GC-MS for the target faecal sterols described by Szűcs *et al.,* (2006). The instrumentation and GC-MS conditions are explained in Section 3.2.4. Because particles form a constituent part of

groundwater and faecal sterols tend to attach themselves to particles in water, no sterols were expected to elute from any of the groundwater samples.

The TIC (Total Ion Chromatographs) for the faecal sterol analysis of groundwater from Christiana 2, Delarey-Ottosdal, Ganyesa, Hartswater, Mafikeng, Sekhing, Setlagole, Vryburg and Zeerust sampling sites yielded none of the target sterols, only perylene (SURR) and perylene-d12 (IS) eluted (the chromatograms for these can be found in Appendix C). Perylene, of known concentration, is added as a surrogate in order to check the efficiency of the recovery of the sterols in the water. Perylene-d12 is added in order to quantitate the amount of sterols found in the water. In the groundwater samples of the sites mentioned previously, perylene and perylene-d12 were the only sterols that eluted, indicating no pollution of the water. This is contradictory to the microbial results, as extremely high TC and FC counts were found for at least Setlagole, Hartswater, Christiana and Ganyesa. According to literature (Leeming, 1996; Isobe *et al.*, 2002) a broad correlation can be found between coprostanol and specific species of microbes, but the relationship is dependent on the type of environment (Leeming and Nichols, 1996; Isobe *et al.*, 2002). There is, however, a lack of literature regarding sterol and bacterial correlation in groundwater.

Some groundwater sites were found to contain faecal sterols. Since the physico-chemical data has shown (Table 3) a very high amount of TDS in the water, one could argue that it might be the reason sterols were found in the groundwater samples (Writer *et al.*, 1995). The sterol analysis of groundwater sampling sites; Christiana, Geystown and Taung showed the elution of Coprostanol and Cholesterol, these are shown in Figure 16 to 18.

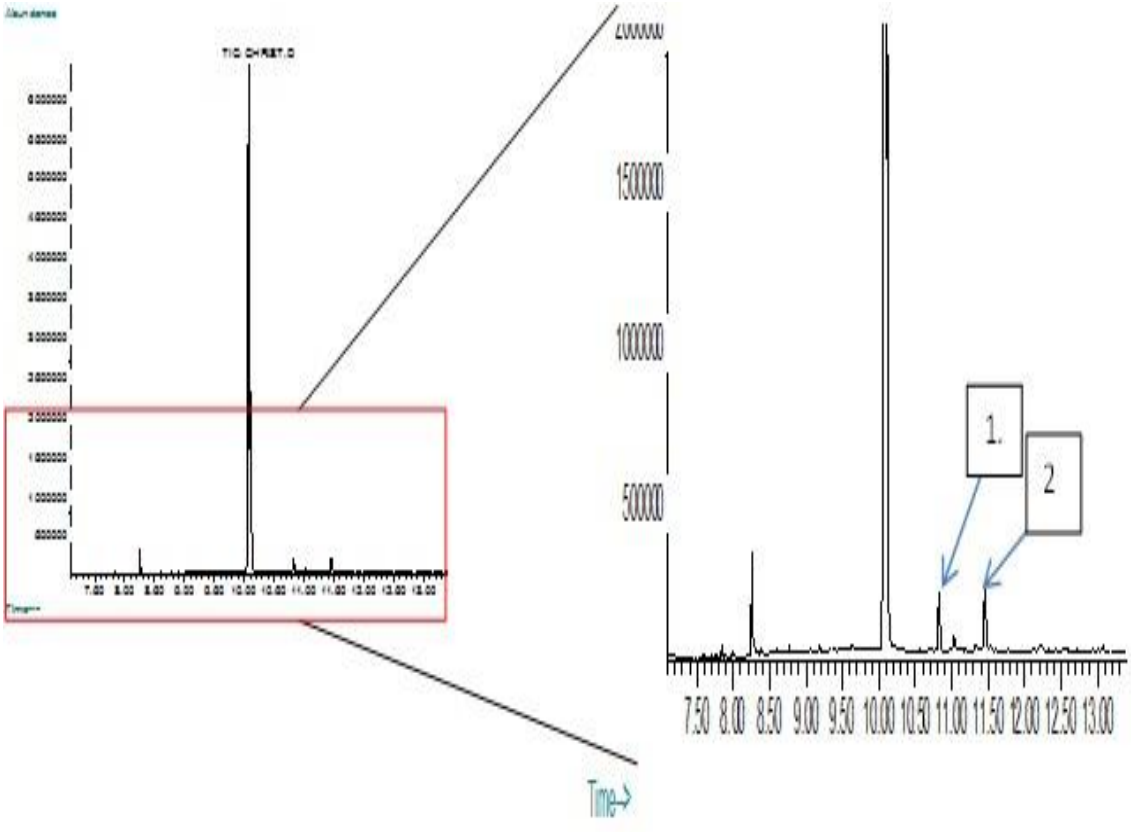


Figure 16: The Christiana groundwater sample that was analysed showed abundances of (1) coprostanol (6.037 ppm) and (2) cholesterol (6.696 ppm) in the groundwater. Coprostanol indicates human faecal pollution, while cholesterol is found in the faeces of most animals (Pratt, 2005; Szűcs *et al*., 2006).

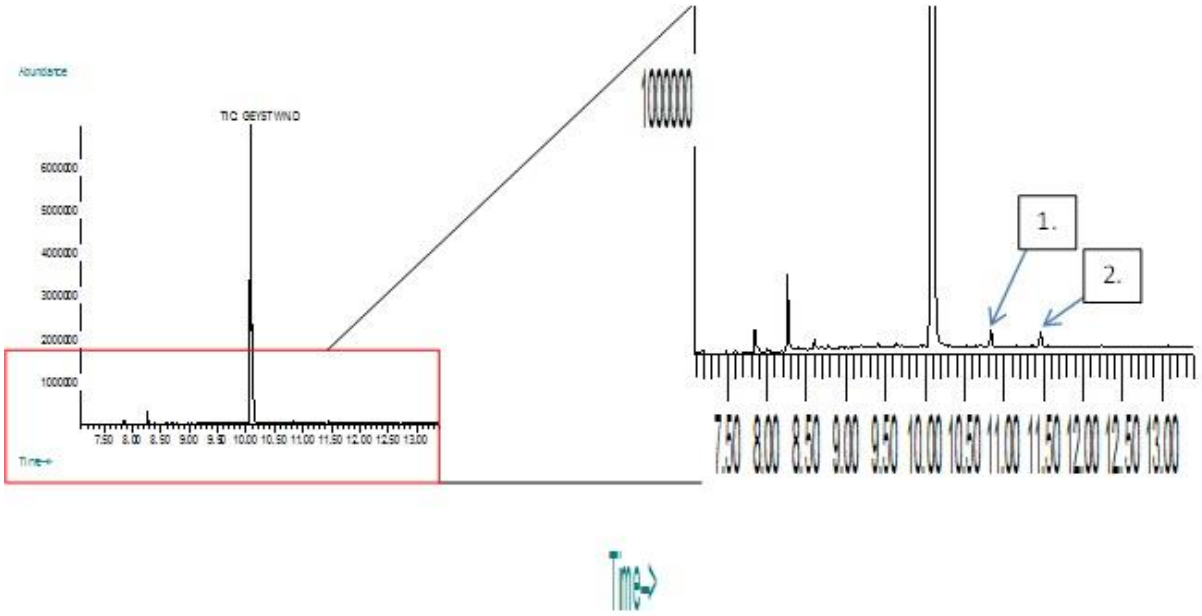


Figure 17: In the Geystown groundwater sample elution of (1) coprostanol (5.485 ppm), which may indicate human faecal contamination (Leeming *et al.*, 1996; Pratt, 2005), and (2) cholesterol (6.135 ppm), which may indicate animal faecal contamination was found (Leeming *et al*,. 1996).

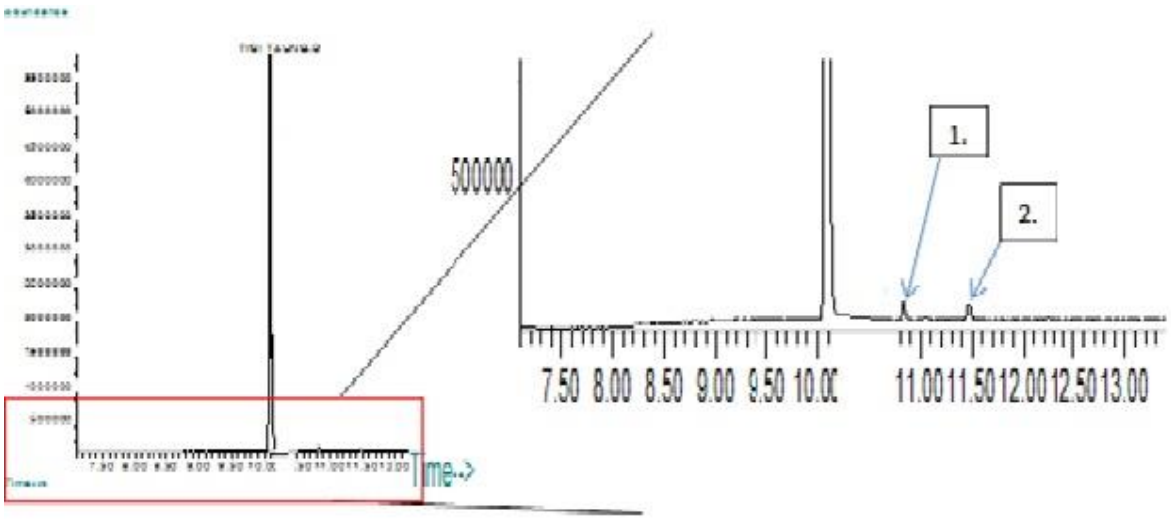


Figure 18: The Taung groundwater sample showed abundances of (1) coprostanol (5.538 ppm) and (2) cholesterol (6.135 ppm) eluding, which may indicate human and animal faecal pollution respectively.

The results in Figure 16, 17 and 18 show the elution of coprostanol and cholesterol, which may indicate human and animal faecal contamination of the water (Leeming *et al.,* 1996). Concentrations of target sterols were calculated on the basis of calibration curves (Section 3.2.5 and Section 3.3.1) and by the data analysis software (HP ChemStation). Concentration results for the sterols that eluted are shown in Table 5. Since there exist no standard for faecal sterols found in water in South Africa, these concentrations could not be compared to any standards, but the fact that they were found even in trace amounts in these groundwater samples are worrying. Qualitatively, cholesterol concentrations were higher than that of coprostanol in all of the samples. Since Christiana, Geystown and Taung are all agricultural and livestock areas one could suggest that the cause of cholesterol in the water may be because of seepage through the ground and into the groundwater table (Barcelona *et al.,* 1988).

4.3B. RESULTS: SURFACE WATER

Four sites from the Baberspan bird sanctuary were sampled, once in 2010 and their surface water were analysed. All samples were analysed for physico-chemical properties (Section 4.3.B.1), microbial counts (section 4.3.B.2) and faecal sterol analysis (section 4.3.B.3) and the results depicted in the sections below.

4.3B.1 Determination of the Physico-chemical properties of surface water

The physico-chemical properties of the water can have an effect on the bacterial coliform counts and is thus important when sampling. Samples and measurements were taken from four different areas near the Inland Lake (Bird Sanctuary); in the river (Harts River) that flows to the Inland Lake (Bird Sanctuary), the inflow, where water enters the Inland Lake, near the hotel, which is situated on the edge of the Inland Lake within the bird sanctuary, and the outflow, where water leaves the lake and exits in a small stream. Surface water samples collected from the Baberspan was analysed for its physico-chemical properties (pH, temperature, EC, and TDS of water) on site (Section 4.2.2).

Table 6: Physico-Chemical properties for the water samples taken in the Inland Lake area.

Site		**pH**	**Temp (°C)**	**EC (mS/m)**	**TDS (mg/L)**	**NO_2^- (mg/L)**	**NO_3^- (mg/L)**
TWQR		**6.5-8.5**	**No TWQR exists for recreational use of water**				
Inland	**River**	8.61	12.0	147	956	1.20	4.00
Lake	**Inflow**	9.23	14.7	135	961	0	0
	Hotel	8.58	15.2	115	820	0	4.00
	Outflow	8.62	13.7	121	862	0.70	3.00

The pH of the water samples is above average, with the pH of all of the samples exceeding the DWAF (1996) standard for recreational use (6.5 – 8.5). The TDS and EC of all the water samples are also very high, but there exist no DWAF (1996) standard for TDS and EC for

recreational use. As mentioned previously, TDS is tested as a secondary drinking water standard, and is therefore more of an aesthetic hazard (Oram, 2012). The TDS is higher in the river and inflow of water into the pan; this makes sense as the TDS will settle out of suspension once in the pan itself, and form part of the sediment. There also exists no DWAF (1996) standard for nitrate-nitrogen ratio for recreational water, although the values found are low (Section 4.3B.1.)

4.3B.2 Microbial analysis of surface water

Surface water samples were transported to the laboratory and then analysed for their microbial content. All surface water samples were analysed for TC, FC, *E. coli*, and FS as described in Section 4.2.3 and the results for microbial counts are depicted in Table 7 below. Table 7 also contain the concentrations of cholesterol that was found at the different sampling sites. This will be further discussed in Section 4.3B.3.

Table 7: Bacterial coliform counts for the surface water samples taken in the Inland Lake area.

Site		**TC (cfu/100ml)**	**FC (cfu/100ml)**	***E. coli* (cfu/100ml)**	**FS (cfu/100ml)**	**Cholesterol (ppm)**
			2010			
Inland	**River**	TFTC	TFTC	TFTC	61.30	<LOQ
Lake	**Inflow**	TFTC	TFTC	TFTC	50.00	6.212
	Hotel	TFTC	TFTC	TFTC	36.00	6.008
	Outflow	270.30	130.00	33.60	127.30	7.258

TFTC – cfu were less than 30 cfu/100ml; <LOQ – Below the limit of quantitation

There exists no DWAF (1996) standard for TC in recreational waters use. The DWAF (1996) standards for recreational use for *E. coli* is 0-130 cfu/100 ml and for FC, 0-150 cfu/100 ml. None of the four surface water samples exceeds these standards for coliforms. Thus, indicating that there exists no risk of infectious disease or microbial infection (DWAF, 1996; SANS 241, 2006). The DWAF (1996) standard for recreational use for FS is however, 0-30

cfu/100 ml, and the river water sample (127 CFU/100 ml), hotel water sample (50 CFU/100 ml), and outflow water sample (61 CFU/100 ml) all exceed this standard, indicating the high FS counts at these sites. Faecal streptococci (FS) is, however, used in describing water quality as it appears to behave similarly to actual faecal derived pathogens (Prüss, 1998), thus giving us a better estimate of pathogens in water.

4.3B.3 GC-MS analysis of surface water

Surface water samples collected at the Inland Lake (Baberspan Bird Sanctuary) were then analysed for the target faecal sterols by GC-MS (the Szűcs method), (Szűcs *et. al*., 2006). Results are depicted in Figures 19 to 21 below, but also in Table 7 which shows the concentrations of cholesterol that eluted. Concentrations of target sterols were calculated on the basis of calibration curves (Section 3.2.5 and Section 3.3.1) and by the data analysis software (HP ChemStation). The River analysed yielded no faecal sterols, This TIC can be found in Appendix C.

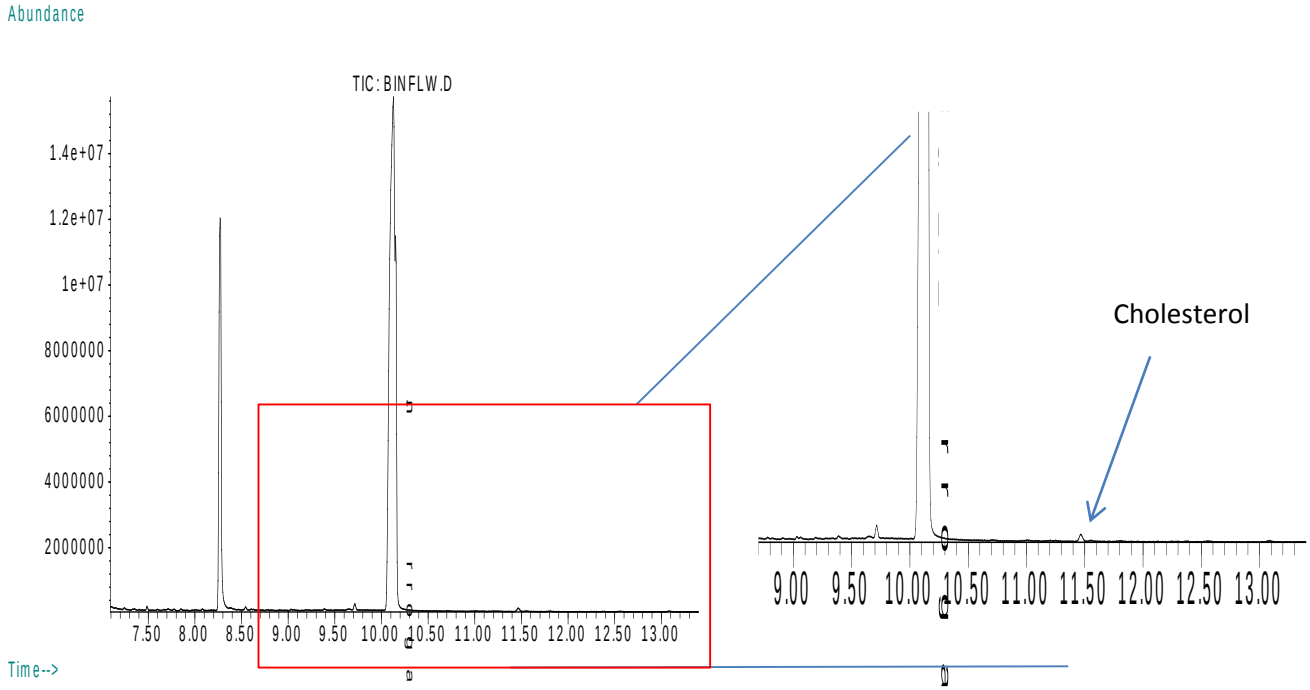


Figure 19: The faecal sterol profile of water collected from the inflow of water into the pan, showed the elution of cholesterol (6.212 ppm).

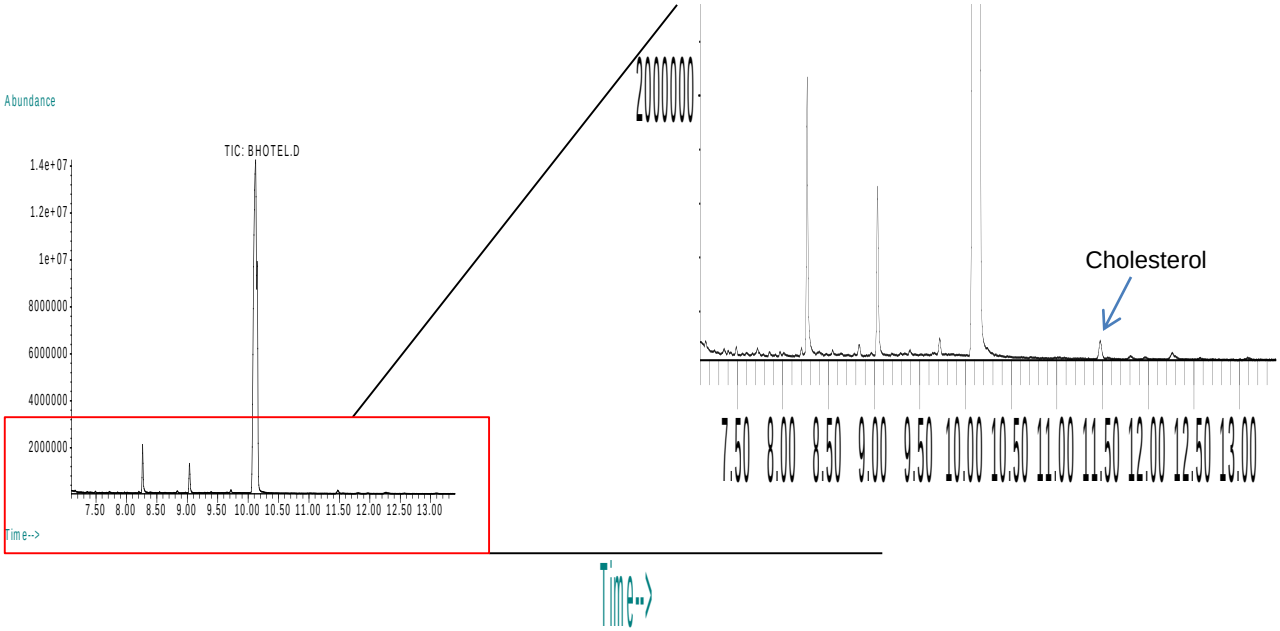


Figure 20: The faecal sterol analysis of the surface water collected from the Baberspan Hotel, showed the elution of cholesterol (6.008 ppm), which could indicate animal faecal contamination (Leeming *et al.*,1996).

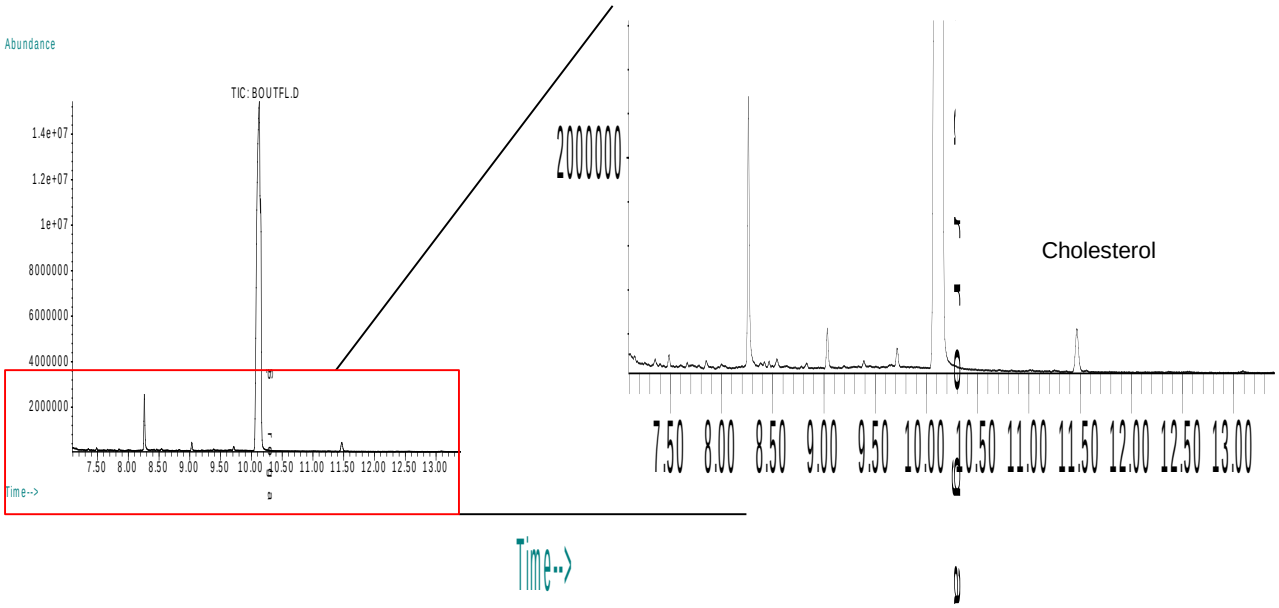


Figure 21: The water sample taken at the outflow of the pan, showed the presence of cholesterol (7.258 ppm) in the water.

Figures 19, 20 and 21, showed the elution of Cholesterol in all three water samples. Cholesterol concentrations found were illustrated in Table 7 (Section 4.3.2.2). Previous pilot studies and literature cited (Leeming *et al.*, 1996) specify that this is indicative of faecal pollution from birds. Birds do not have the ability to convert cholesterol into 5β-stanols and thus show a high cholesterol concentration in their sterol fingerprints (Figure 11) (Leeming *et al.*, 1996).

4.4 DISCUSSION AND CONCLUSION: GROUNDWATER AND SURFACE WATER

The Szűcs method (Szűcs *et al.*, 2006) was used to detect faecal contamination in groundwater and surface water samples, which were taken from various sites across the North West Province, South Africa.

4.4.1 Physico-chemical parameters analysed

The pH of a water system can be a good indicator of water quality (Nola *et al.*, 2002) because anthropogenic activities can have an influence on a water system's pH. These anthropogenic activities could include; runoff from agricultural fields and mines, as well as infiltration of untreated wastewater into surface water and groundwater (Monwq, 2007). Anthropogenic effects on an aquatic ecosystem are usually evident by pH values that are not in the TWQR (Dallas and Day, 2004).

The pH of the groundwater samples taken from boreholes were complaint with the TWQR (Table 3). The pH of the 4 surface water sampling sites was slightly alkaline. Based on the values from the sampling sites at Baberspan, and the TWQR for full contact recreational activities (6.5-8.5) and irrigation (6.5 -8.4), all surface water sampling sites for Baberspan exceeded the TWQR.

Humans directly exposed to water sites exceeding the TWQR for recreational activities may suffer from mild effects of irritation of the eyes, skin, ears, nose, mouth and throat mucous membranes (DWAF, 1996c; WHO, 2003b). Water found at sites with elevated pH levels that are used for irrigation, will have an effect on crop yield, while the erosion of irrigation pipes may also occur (DWAF, 1996b; Valdez-Aguilar *et al.*, 2009).

The TDS TWQR for livestock watering is 0-2000 mg/L for horses and cattle and 0-3000 mg/L for sheep. All of the groundwater at the various sites sampled complies with these TWQR. The TWQR for domestic use of water is 0-450 mg/L TDS. Only the groundwater sampled at Zeerust complied (Table 3). All other groundwater samples were above the TWQR for domestic use (DWAF, 1996b) (Table 3). Groundwater regularly has elevated levels of TDS and EC. High levels of TDS and EC cause water to be aesthetically unfavourable. High TDS and EC levels could indicate hydrocarbon contamination that is

undergoing microbial biodegradation (Atekwana *et al.,* 2004).Water that contain TDS an EC values exceeding the TWQR may cause a salt balance disturbance in humans and animals.

All four surface water samples from Baberspan exceeded the EC TWQR for irrigation (< 40 mS/m) (DWAF, 1996d).Surrounding farms use the Baberspan for irrigation. Water with a high EC value, which indicates a high ion concentration, can have profound effects on crop production (Grattan, 2002).

The permissible TWQR for domestic use of nitrates in water is 6 mg/L NO_3-N. All groundwater samples analysed exceeded the TWQR for nitrate concentration, except Sekhing, which had a low nitrate concentration. Groundwater nitrate id expected to increase in relation to land use practises that require nitrogen application (e.g. fertilizer). Remnant fertilizer after plant uptake plays a major role in contributing to the high nitrate levels in groundwater (Van der Voet *et al.,* 1996). Furthermore, trees and plants store massive quantities of nutrients temporarily during the growing season, and release it into the aquifers during the following non-growing season (Walker, 1973). The water quality guidelines for irrigation by DWAF (1996d) refer to nitrogen as all inorganic forms of nitrogen present in water. The irrigation TWQR of inorganic nitrogen is < 0.05 mg/L. Surface water samples collected at Baberspan hotel and outflow of the water from the Baberspan, exceed this TWQR (Table 6).

4.4.2. Microbial water quality analysis of surface and groundwater.

The presence of faecal indicator bacteria in groundwater is very alarming, as it indicates the presence of possible disease causing pathogens (Jagals *et al.,* 1995; Grabow, 1996; Barrel *et al.,* 2000; Ashbolt *et al.,* 2001).The TWQR for domestic use, is 5 cfu/100ml for total coliforms and 0 cfu/100ml for faecal coliforms (DWAF, 1996b).The groundwater samples

analysed showed 6 groundwater sites that complied with the domestic TWQR for total coliforms (Table 5). Seven groundwater sites analysed exceeded the domestic TWQR, varying from 10 cfu/100ml to over 300 cfu/100ml (too many to count).Ten groundwater sites exceeded TWQR for faecal sterols (Table 5), while only 3 sites showed no faecal coliforms (comply with TWQR). Exposure to water with an elevated concentration of faecal indicator bacteria (total and faecal coliforms, *E. coli,* and enterococci) increases the risk of contracting gastrointestinal and respiratory illnesses (Haile *et al.*, 1999).

The present study assessed surface water that is predominately used for recreational activities, livestock watering and irrigation. TWQR for full contact recreational activities are 0-150 cfu/100ml, for livestock watering, 0-200 cfu/100ml, and for irrigation, 0-1 cfu/100ml. The TWQR for livestock watering for faecal coliforms primarily focuses on the toxicological effects associated with the ingestion of water borne pathogens that are present in the water (DWAF, 1996e). All the surface water samples collected and analysed from the Baberspan Lake complied with the faecal coliform TWQR for full contact recreational activities and livestock watering. However, the Baberspan hotel and outflow water samples exceeded the TWQR for irrigation. A wide range of problems can exist if the quality of water used for irrigational purposes does not meet acceptable standards (Gemmell and Schmidt, 2010) Contaminated irrigation water acts as a vehicle and facilitates the movement of viral and bacterial pathogens to fresh produce and processed produce (Steele and Odumeru, 2004; Heaton and Jones, 2007).

4.4.2.1 E. coli detection

The detection of *E. coli* is a good indicator of recent faecal contamination, as it forms part of the intestinal micro-flora of warm blooded animals (Edhberg *et al.,* 2000;

Kaper, 2005). However, Ishii and Sadowsky (2008) highlighted in a mini-review article, the survival and potential replication of *E. coli* in water, algae and soils. Therefore, *E. coli* detected in groundwater may have been *E. coli* strains that are attenuated in the soil and infiltrated into the groundwater. Groundwater sites analysed had very low *E.coli* values, although 5 sites did show the presence of *E.coli* in the water.

The *E. coli* TWQR for full contact recreational activities is 0-130 cfu/100ml. All sampling sites at Baberspan were within this range. Water resources used for recreation may contain a combination of pathogenic and non-pathogenic organisms from various anthropogenic sources (WHO, 2003b). Pathogenic organisms present in water can cause infections in the intestine when ingested, upper respiratory tract, ears and eyes infections, upon full human body contact (WHO, 2003b).

4.4.2.2 Faecal streptococci

Four key points makes faecal streptococci a favourable indicator organism of faecal contamination (Ashbolt *et al.,* 2001): 1) relatively high numbers occur in the faeces of humans and other warm blooded animals; 2) their presence in wastewaters polluted waters sources; 3) their absence from pure waters, virgin soils and environments free from human and animal contact and 4) their persistence in the environment without multiplication.

A TWQR for faecal streptococci is only available for recreational activities. However, faecal streptococci are an indicator of faecal matter in water; thus, its presence in water used for domestic purposes is undesirable. Groundwater sites analysed only two sites had a 0 cfu/100ml faecal streptococci count. Eleven groundwater sites showed

various values of faecal streptococci, with the highest number found in Hartswater (180 cfu/100ml).

The surface water sites analysed at Baberspan all exceeded the TWQR for full recreational activities (0-30 cfu/100ml), but complied with the TWQR for intermediate contact (0-230 cfu/100ml) (DWAF, 1996c). According to Given *et al.* (2006), the contamination of recreational water by sewage or runoff can lead to an increase in swimmer illness from bodily exposure to water borne pathogens. Exposure to water with high concentrations of *E.coli* and faecal streptococci is associated with increased risk of contracting gastro-intestinal and respiratory infections (Kay *et al.,* 1994; Haile *et al.,* 1999). Although swimming in microbiologically polluted water is not usually life threatening, it can have serious effects on children and immune-compromised individuals (Clark *et al.,* 2003).

4.4.3 Faecal sterol analysis of water samples analysed

The faecal analysis for the groundwater samples showed three sites that may contain human and animal faecal contamination. Since sterols are particle bound (Roser *et al.,* 2003), it was thought that most of the sterols would cling to the particles in the ground, and not be found in the groundwater. Gottshall and colleagues (2013) also proved that sterols can be found in groundwater. There are no TWQR for faecal sterols in water, but concentrations of cholesterol and coprostanol was found at 3 of the groundwater sites analysed. Since a combination of cholesterol and coprostanol was found, this indicates faecal contamination of the groundwater from possible animal and human origin (Leeming, 2006). Faecal sterol concentrations in water is not a direct health risk, but sterol concentrations indicate faecal contamination of water, and it has been well documented that exposure to water contaminated

with faecal matter almost certainly result in serious health effects (Kay *et al*., 1994; Haile *et al.,* 1999; Dwight *et al.,* 2004; WHO, 2005; DNT, 2011).

The water samples analysed for Baberspan showed that the Harts River of water into the Baberspan are clean and free of faecal sterols, while the water collected at the inflow, hotel and outflow of Baberspan showed faecal contamination, possibly from animals, since only cholesterol eluted at these sites (Leeming, 2006). Since Baberspan is a bird sanctuary, and a multitude of bird species can be found there in great numbers, it seems as though the birds may be the ones that are responsible for the contamination, as only cholesterol eluted, indicating possible faecal pollution from birds (Leeming *et al.*, 1996; Leeming, 2006; Szùcs *et al*., 2006).

The conclusion that can be drawn from this part of the study is that faecal sterol markers can be detected in groundwater and surface water. Thus, adding an extra dimension to determining the quality of water systems. Faecal sterols can be used as a tool in faecal source tracking as it has unique source specificity (Chapter 3), with regards to a combination of sterol intake and metabolic production of sterols by various animal species (Leeming *et al.,* 1996).Faecal sterol analysis did, however, not correspond with faecal indicator bacteria found. This is contradictory to what Gottshall and co-workers (2013) found in groundwater and Derrien *et al.* (2012) found in river water.

5

Applications in using sterol analysis in waste water treatment plants (WWTP) effluent water quality

In determining if the Szűcs (2006) method would be ideal for the determination of faecal contamination in water by GC-TOF/MS, an optimization and sensitivity study of the method was also done on WWTP effluent in the North West and Gauteng provinces of South Africa in the year 2011.

The objective of WWTPs worldwide is to produce disposable effluent without causing harm to the surrounding environment and thus prevent faecal pollution (DWAF, 2009). Sewage treatment is described as the process of removing physical, chemical and biological contaminants from waste water originating in households and industrial and agricultural processes. Treated water (effluent) is then deposited into the environment and treated sludge is used as farm fertilizer (Anon[9], 2013). Sewage collection and treatment are usually subjected to local, municipal, provincial, and national regulations and standards.

Sewage treatment generally involves 3 stages:

i) Preliminary treatment - Preliminary treatment consists of screening out large objects from the incoming wastewater (SA Water, 2010). Sand and grit are removed by allowing those to settle. The grit is removed from the bottom of the tank, dewatered, deposited into large dumpsters (Anon[9], 2013).

ii) Primary treatment - The incoming wastewater is then slowed to allow the remaining solids in the raw wastewater to sink to the bottom of a large tank, these are to be

removed for further processing and fats and oils are skimmed off the top. The only solids remaining in the water are either suspended or dissolved (SA Water, 2010; Anon[9], 2013). This water is then called "primary effluent".

iii) Secondary treatment - The primary effluent is then mixed with activated sludge (biological solids) and flows into the aeration tanks. The activated sludge contains a wide variety of bacteria that ingest the suspended and dissolved solids in the wastewater (Anon[9], 2013). Aeration tanks are configured to select certain bacteria to do the work. The first stage of the aeration tank (anoxic selector) has very little free oxygen. Bacteria capable of utilizing bound oxygen in the form of nitrite, nitrate or phosphate for respiration dominate in this stage (SA Water, 2010). During the remaining stages of the aeration tank (aerobic selector**)**, fine bubble aeration is used to mix and provide free dissolved oxygen to the bacteria for respiration (SA Water, 2010; Anon[9], 2013).

The recycling and saving of water are an advantage of water purification, and because wastewater is treated before entering the environment, groundwater and surface water, in theory, are "protected" from pollution. Treated water is also used for vegetation and creating an artificial wetland, resulting in habitat for various species of fauna and flo

The operations and maintenance (OandM) of many of the WWTP are poor and poor quality effluents are discharged (DWAF, 2009). In many cases, the WWTP are not able to handle the hydraulic or the organic loads. As a result, the installed treatment technology is not always working to specification (DWAF, 2009). An audit of the WWTPs, is required to determine the works that are not working to specification and develop a programme to retrofit and upgrade these works. It is essential to address the issue of insufficient OandM resources in this process (DWAF, 2009; DWAF Green Drop Report, 2012).

5.1. AIM AND OBJECTIVES

The aim of this study was to determine if the Szűcs (2006) method, ran on a GC-TOF/MS would be able to detect the six target sterols in raw sewage and effluent of the Potchefstroom WWTP situated in the North West Province and the Carletonville and Fochville WWTPs situated in the Gauteng Provinces, South Africa.

Objectives of this study included;

- sampling of raw sewage and effluent from WWTPs situated in the North West and Gauteng Provinces, South Africa.
- optimization of the Szűcs (2006) method with the GC-TOF/MS.
- Detection of target faecal sterols in raw sewage water and effluent of the WWTPs sampled.

5.2. MATERIALS AND METHODS

5.2.1 Sample design and collection

Potchefstroom is located in the south eastern part of the North West Province. It is an academic town which plays host to the North West University. The town is situated on the banks of the Mooi River, which then also passes through the town itself. Carletonville is a gold mining town located in the western end of the Gauteng province. It is one of the richest gold producing areas in the world, with Western Deep Levels gold mine (Anglo Gold (Pty) ltd) being one of the world's deepest mines at 3 777 m deep (Britannica, 2012). Fochville is farming and mining town in the western Gauteng province, and it was first established as an agricultural town in 1920. Each of these towns has their own WWTP. A raw sewage water

sample and effluent water sample were collected for sterol analysis by GC-TOF/MS. Sites were the water samples were collected is shown in Figure 22 below.

Figure 22: Sampling sites of the WWTP sensitive study. Sampling sites were all WWTP's across the North West and Gauteng province of South Africa. At each sampling site, a raw sewage water sample and effluent sample was collected.

Sampling for the WWTP sensitivity study was only done once in 2011. In all cases water samples were collected in sterile 2 L glass Schott bottles. Water samples were tested for some physico-chemical parameters on site (pH, temperature, EC and TDS) as previously described in Section .4.2.2. Water samples were then transported to the laboratory and analysis was done within 36 hours of sample collection.

Not all physico-chemical properties were tested for, and there were no microbial counts recorded. Microbial counts were unnecessary for this study as only the GC-TOF/MS analytical method was tried and tested – to detect all six target sterols in raw sewage water samples, and none in the effluent water samples. Because no microbial data was recorded, only some of the physico-chemical properties were monitored, as physico-chemical

properties have an effect on the microbial population in a water body, but have little or no effect on the sterol composition in the water.

5.2.2 GC-TOF/MS analysis of water samples collected from WWTPs

The raw sewage and effluent of all three WWTP were extracted and run in accordance to the Szűcs method (Szűcs *et* al., 2006). As done with all previous pilot and environmental studies. Materials (Section 3.2.1), derivatization procedure (Section 3.2.3) and recovery studies (Section 3.2.6) all remain the same.

5.2.2.1 Instrumentation and GC-TOF/MS conditions

The samples were analysed on an Agilent Technologies (Palo Alto, CA) 7890A GC system with a LECO TruTOF HT High Troughput TOF/MS and an LEAP Technologies, CTC Analytics, PAL system autosampler (Agilent Technologies, Palo Alto, CA). Separations were accomplished using an Supelco Equity-5 fused silica capillary column (Hewlett-Packard) coated with phenylmethylsiloxane (30m x 0.25mm x 0.25µm film thickness) using the Environmental Protection Agency (EPA) Method 8270 with minor modifications.

The GC-MS parameters were as follow: Carrier gas: helium 1.4 ml/min, constant flow; injection: splitless, 1µl sample; inlet temperature: 280˚C; oven ramps, 150˚C for 0.5 min, 150˚C- 300˚C at 20˚C/min, and 300˚C-310˚C at 25˚C/min; GC-MS interface temperature: 280˚C; MSD ion source temperature: 230˚C; MSD quadrupole temperature: 150˚C; ionization energy: 70 eV; and solvent delay: 7.0 min. System control, data acquisition, and analysis were performed with the TruTOF software,

(LECO) on a HP (Hewlett-Packard) computer. Data was acquired in the full scan mode between ions of m/z 50 and 550.

5.2.2.2 Preparation of water samples for GC-TOF/MS analysis

A 300 ml raw domestic wastewater sample and the treated effluent, also 300ml, were obtained from the Potchefstroom WWTP (North West Province, South Africa), Carletonville WWTP (Gauteng Provinces, South Africa) and the Fochville WWTP (Gauteng Provinces, South Africa). Samples were processed for extraction within 24-36 h. Water samples (300 ml) were mixed with 200 µl of 0.125 mg/ml surrogate standard solution and 10 g NaOH. They were then saponified in a shaking incubator at 60˚C for 1 h. Following saponification, the water samples and the effluent were extracted (Section 3.2.6), derivatized (Section 3.2.3), and analysed by GC-TOF/MS (Section 4.2.2.1). The untreated wastewater samples (raw sewage) were prepared for GC-TOF/MS in the same manner, but it was mixed with 50 ml of dichloromethane and ultra-sonicated with 10 µm amplitude for 10 min before extraction. Concentrations of target sterols and surrogate were calculated on the basis of calibration curves by the data analysis software (TruTOF, LECO Software).

5.3. RESULTS

5.3.1 Determination of the physico-chemical properties

As previously mentioned, not all physico-chemical properties were measured for in this study. Physical properties were done on site; these include the pH and temperature of the water. The EC and TDS were not measured for water samples collected from Carletonville WWTP and Fochville WWTP as these sites were not measured with the multi-probe, but with

a thermometer and pH strips. Previous results (chapter 4) indicate however, that the EC and TDS of the water do not have an effect on the results for the choromatographic analysis of sterols in the water. Wastewater treatment plant samples of Potchefstroom WWTP were, however, measured with the Multi-probe (Eutech instruments; Multi-parameter PCSTestr 35) and EC and TDS was measured. Results of measured physico-chemical parameters are depicted in Table 8 below.

Table 8: Physico-chemical properties of the water samples collected from the WWTPs.

Site		**pH**	**Temperature (˚C)**	**EC (mS/m)**	**TDS (mg/L)**
TWQR		**5.5-9.5**	**<25 ˚C for effluent**	**<250mS/m for effluent**	**No TWQR**
Carletonville WWTP	Raw sewage	8.1	16.0	N/M	N/M
	Effluent	7.7	14.0	N/M	N/M
Fochville WWTP	Raw sewage	8.0	13.0	N/M	N/M
	Effluent	7.0	12.0	N/M	N/M
Potchefstroom WWTP	Raw sewage	7.4	18.9	118.0	767
	Effluent	7.5	17.3	110.6	719

N/M – not measured.

Table 8 shows the physico-chemical properties of the raw sewage and effluent collected at the WWTPs. The effluent water samples of all sites were taken in the effluent dams, before the water is deposited into the reed banks.

The DWAF (1996) standards for pH are 5.5-9.5. The pH in the effluent sampled ranged from 7.0-7.7, so all effluent complies with DWAF standards. Temperature of all samples collected ranged from 12-23.1 ˚C. The DWAF (1996) standards for effluent are <25 ˚C, so all samples (even raw sewage samples) comply. The DWAF (1996) specific standard for EC in effluent is <250 mS/m. Electrical conductivity (EC) for Potchefstroom effluent was 110.6 mS/m and

is below the required standard. The TDS and EC are high for raw sewage sample; however, this is completely normal. There exist no specific DWAF TDS values for effluent, although DWAF (1996) states that effluent may not contain any substance in a concentration capable of producing colour, odour or taste. This was not the case with Potchefstroom WWTP effluent. The water did not have any colour, or odour. Taste was not tested for.

5.3.2 GC-TOF/MS analysis of WWTP water samples

The faecal sterol analyses of all three sites were done on the GC-TOF/MS and the results illustrated in Figures 23 to 25. The TOF/MS was used in this study as it is non-comparable in analysing samples in full spectrum. The software technology that is incorporated into the GC-TOF/MS is also of a higher calibre then that of the GC-MS, allowing the raw sewage and effluent peaks of the samples to be compared on one TIC (total ion chromatograph) profile.

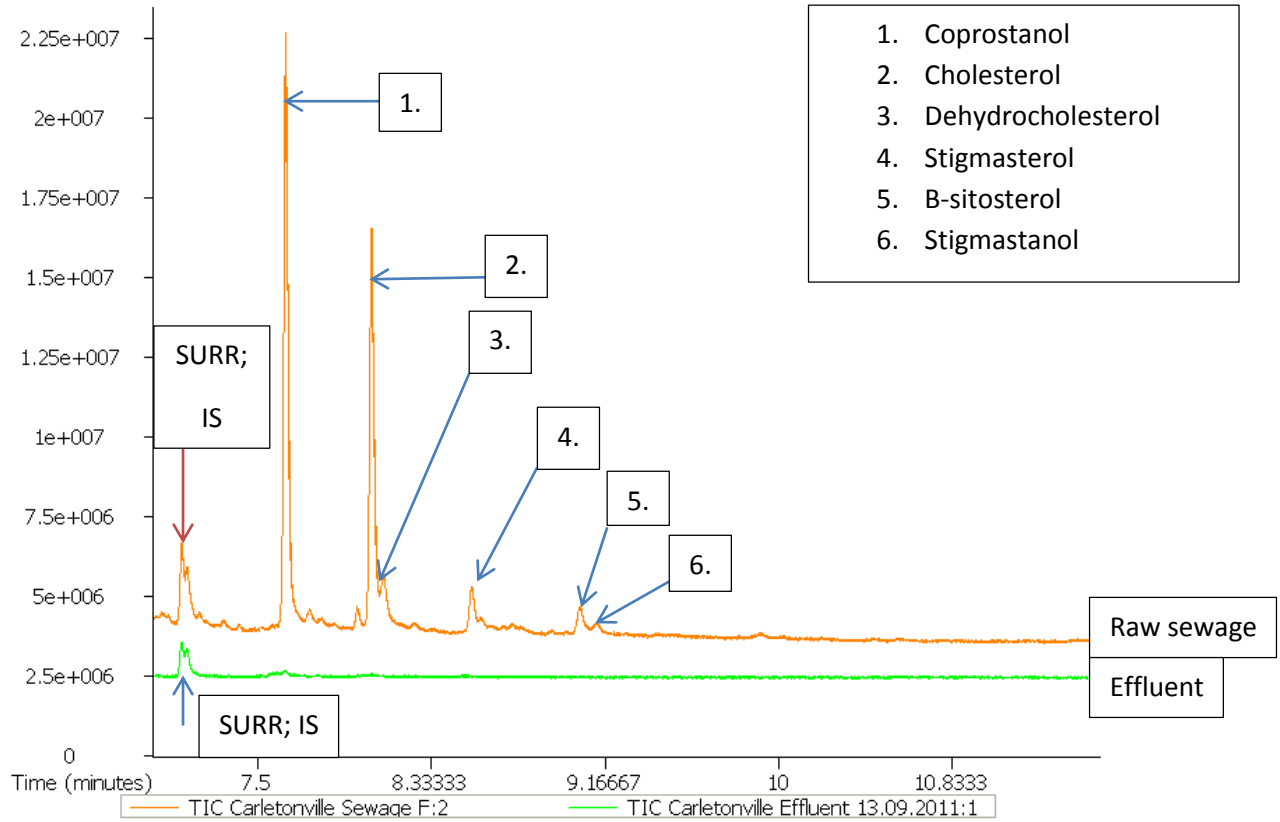


Figure 23: Sterol profile for raw sewage (orange line) and effluent (green line), of water samples collected from Carletonville WWTP.

All six target sterols eluted from the raw sewage (orange line) water sample collected. The peaks were not distorted in any way and eluted completely, with 1. being Coprostanol; 2.

Cholesterol; 3. Dehydrocholesterol; 4. Stigmasterol; 5. β-sitosterol and 6. Stigmastanol. In the effluent water sample only the SURR and IS eluted, which is what is expected of a well-run and optimal operating WWTP.

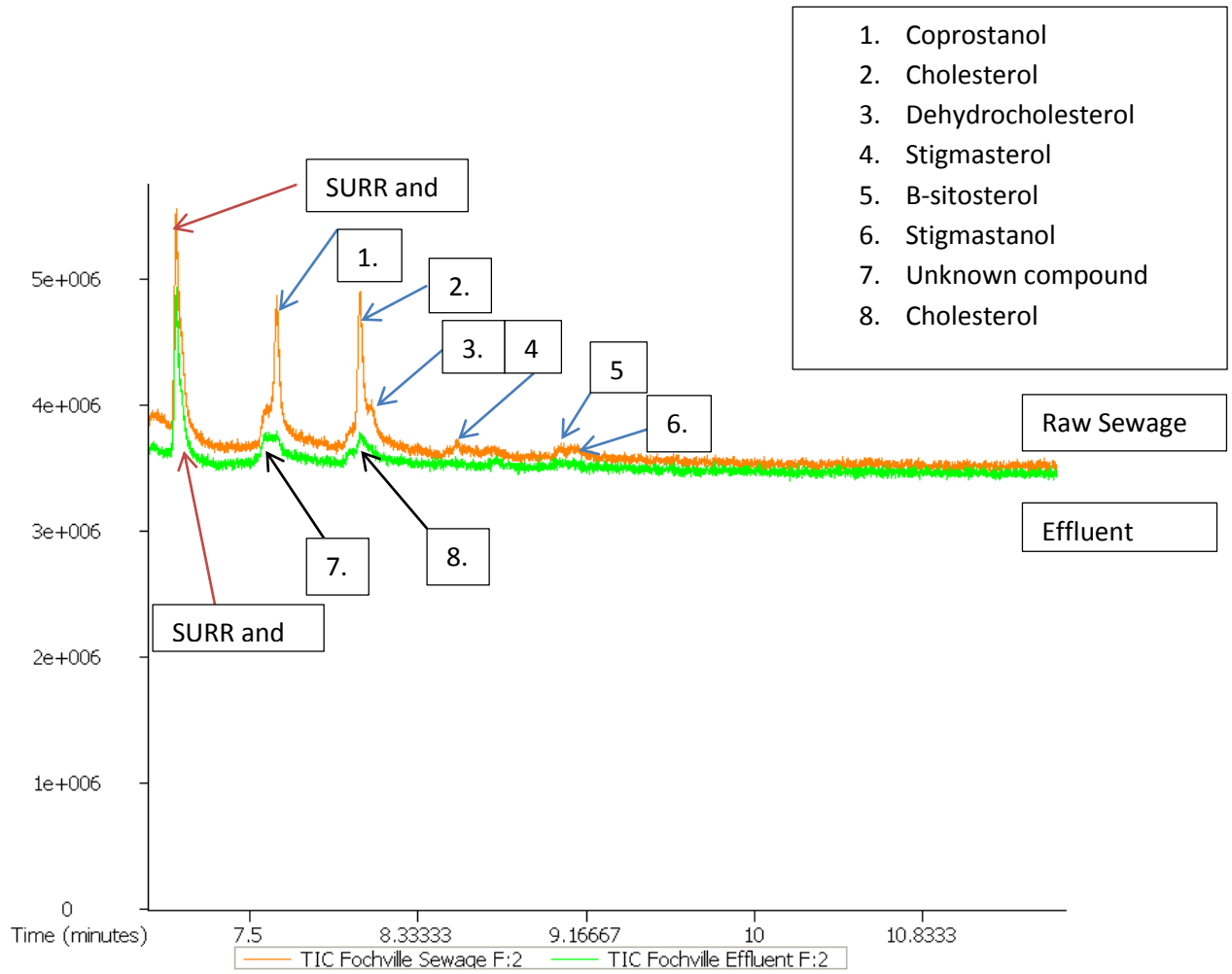


Figure 24: The sterol profile of Fochville WWTP, with the raw sewage sample being the orange line and the effluent the green line.

All six target sterols eluted from the raw sewage water. The peaks were a little distorted, but could still be clearly identified. The raw sewage sample is represented by the orange line in Figure 24, where, 1. Coprostanol; 2. Cholesterol; 3. Dehydrocholesterol; 4. Stigmasterol; 5. β-sitosterol and 6. Stigmastanol. The effluent water sample however, showed the elution of two other compounds (7. and 8). 7.) was an unknown compound, while 8.) was identified as

cholesterol. No compounds or sterols are expected in the effluent sample as all should be removed during the water treatment process.

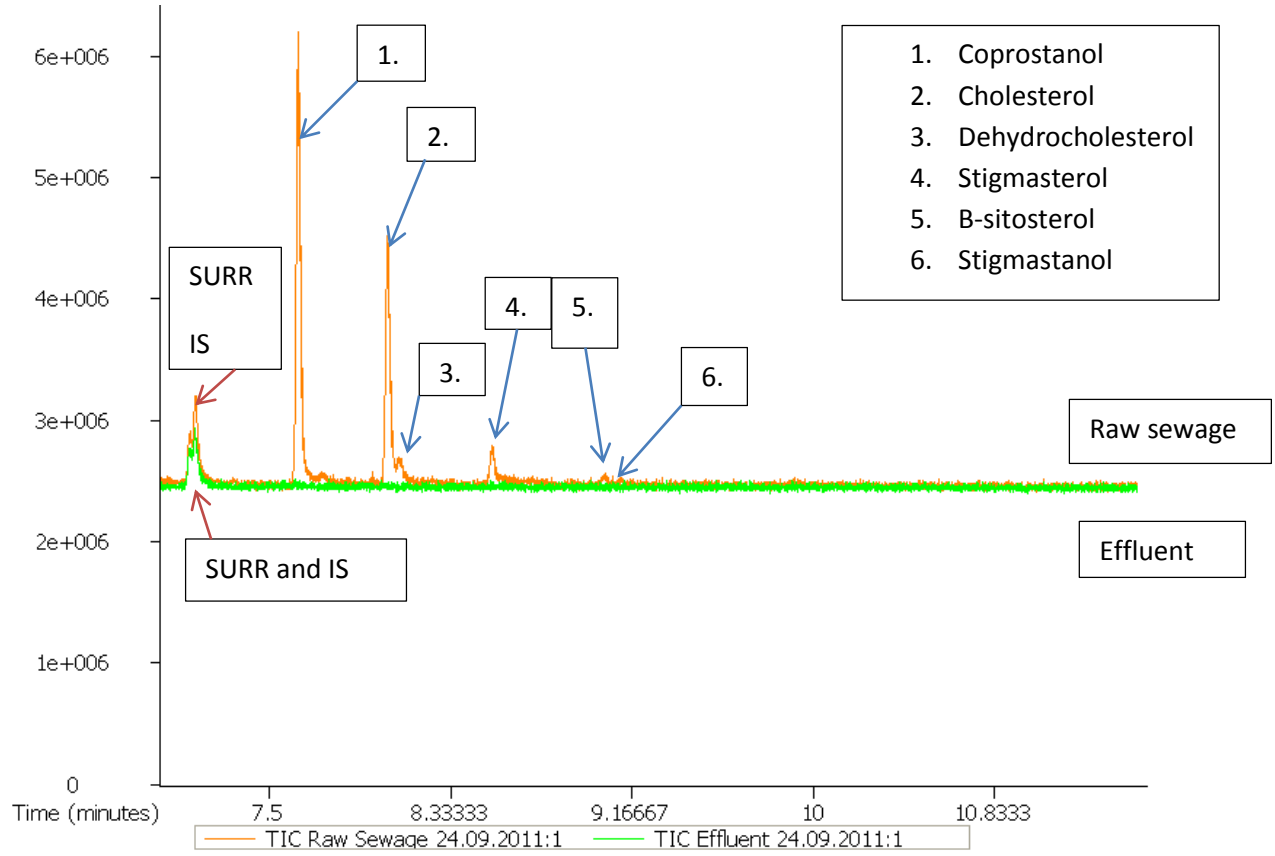


Figure 25: The sterol profile of water sampled from the Potchefstroom WWTP, where the orange line is the raw sewage water sample, and the green line is the effluent water sample.

All six sterols that were tested for in this study eluted in the raw sewage water sample. The peaks were precise and complete, without distortion. The six sterols that eluted were 1. Coprostanol; 2. Cholesterol; 3. Dehydrocholesterol; 4. Stigmasterol; 5. β-sitosterol and 6. Stigmastanol. No compounds could be found eluted in the effluent water sample. Again, this is the mark of a WWTP that is running optimally (DWAF, 2004a).

5.4 DISCUSSION AND CONCLUSION

The Szűcs method (Szűcs *et al.,* 2006), was used to analyse raw sewage and effluent of the Potchefstroom WWTP (North West Province, South Africa), the Carletonville and Fochville WWTPs (Gauteng Province, South Africa) on a GC-TOF/MS. Six target sterols (coprostanol, cholesterol, dehydrocholesterol, stigmasterol, β-sitosterol and stigmastanol) were detected in all raw sewage samples from Potchefstroom, Carletonville and Fochville WWTP. These six target sterols are always present in raw sewage samples, as it represents all aspects of faecal pollution in water, which is found in raw sewage. In order for a WWTP to function optimum, these target sterols, reflecting faecal pollution, should be removed by the water treatment process during water purification.

The WWTP sample collected from the Potchefstroom and Carletonville WWTP indicated just such a result. Where all six target sterols were found in the raw sewage water samples, but no sterols eluted in the effluent samples analysed (Figure 23 and 25). The raw sewage water sample taken from the Fochville WWTP yielded all six target sterols as well (Figure 24). However, the effluent yielded two extra peaks that indicate sterols found in the water and thus, possible faecal contaminants in the effluent (Figure 24). One was determined to be an unknown particle (had a 42% matching percentage, instead of the 80% matching percentage used in identifying compounds when using the GC-TOF/MS compound libraries), while the other was identified as cholesterol. A WWTP that is working optimally would not show these peaks in their effluent. The Fochville WWTP is, however, still a relatively new plant, build in 2010 (sampled in 2011). Their treatment process of the water may not yet be fully optimized and established (DWAF, 2011).

The conclusion from this part of the study is that the Szűcs method (Szűcs *et al*., 2006) proved to be more than adequate in analysing WWTP samples. It was time saving and very

accurate in detecting the sterols in the raw sewage sample and the lack thereof in the effluent samples. There is, however, apprehension that effluent samples analysed with the GC-TOF/MS from the WWTPs (Figure 23 to 25) are not very exact, as literature (DWAF, 2009; DWAF, 2011) suggests that WWTP are not working adequately and as a result, more organic pollutants and sterols are to be found in the WWTP effluent than were detected. Due to this fact, more water was collected and analysed to possibly increase the "load" of organic pollutants, this is depicted in the following chapter (Chapter 6).

6

Applications in using sterol analysis in waste water treatment plants (WWTP) effluent water quality – Further analysis

There existed a concern in this study in that effluent samples analysed with the GC-TOF/MS from the WWTPs (Figure 23 to 5.4) were not optimal. Literature (DWAF, 2009; DWAF, 2011) suggests that WWTP are not working adequately and as a result, more organic pollutants and sterols are to be found in the WWTP effluent than were found in chapter 5 of this study. In order to try and rectify this, an alternative study was done where the sample volume was increased. By increasing the volume of water, one can possibly increase the amount ("load") of sterols extracted and analysed, resulting in possible higher abundance of target sterols.

6.1 AIM AND OBJECTIVES

The aim of this study was to detect faecal sterols, using the Szűcs *et al.,* (2006) method in WWTP effluent samples, with larger volumes of water samples collected and in doing so, possibly increase the "load" of faecal sterols to be found in the effluent.

Objectives of the study include;

- qualitative determination of faecal sterols in WWTP effluent, using larger sample volumes.

6.2 MATERIALS AND METHODS

6.2.1 Sampling design and collection

Waste water treatment plant effluent was collected at the Potchefstroom WWTP. Raw sewage water sample was not collected and analysed as the 300 ml sample used in the previous study (Chapter 5) was adequate to get good elution of all the target sterols in high abundances (Section 5.3.2, Figure 25). It can thus be concluded that 300 ml sample volume is required for raw sewage water, when conducting faecal analysis of WWTPs.

Three other sample points were chosen; the point where effluent water flows from the effluent tanks to the environmental reed beds (Figure 27 and 28, Effluent), the point where water flows from the reed bed into the Mooi River (Figure 29 and 30, Reed/River Barrier), and a point in the Mooi River, a few kilometres from the WWTP (Figure 31 and 32, Mooi River). All these sampling points are shown Figure 26 below.

Figure 26: Map of the points where WWTP effluent samples were taken.

Sample volumes that were collected at each site to be analysed via GC-TOF/MS, for faecal sterols are depicted in Table 9. All water samples were collected in 2L Schott bottles and transported to the laboratory for sterol analysis. Analysis was done within 6 hours from collection.

Table 9: Volumes of samples collected at the WWTP effluent, Reed/River barrier and the Mooi River.

Sampling Site	Sample volumes	
	Reference sample volume	**Analysis sample volume**
Effluent	300 ml (control sample)	1L
Reed/River barrier	1L	2L
Mooi river	1L	2L

6.2.2 GC- TOF/MS analysis

All samples collected were extracted and run in accordance to the Szùcs method (Szùcs *et* al., 2006), as done with all previous pilot (Chapter 3), environmental (Chapter 4) and WWTP (Chapter 5) studies. Materials (Section 3.2.1), derivatization procedure (Section 3.2.3) and recovery studies (Section 3.2.6) all remain the same. Instrumentation and GC-TOF/MS conditions are unchanged from that from Section 5.2.2. Preparation of water samples are referred to in the next paragraph.

6.2.2.1. Preparation of water samples for GC-TOF/MS analysis

Three samples were collected at, and in the vicinity of the Potchefstroom WWTP. The samples were from the effluent from the WWTP, in the reed bed following the effluent discharge from the WWTP, and from the Mooi River downstream from the WWTP. Samples were processed for extraction within 6 h. Water samples, of various volumes (Table 9), were mixed with 200 µl of 0.125 mg/ml surrogate standard solution and 10 g NaOH. They were then saponified in a shaking incubator at 60˚C for 1 h. Following saponification, the water samples and the effluent were extracted (Section 3.2.6), derivatized (Section 3.2.3), and analysed by GC-TOF/MS (Section B.4.2.2.1).

6.3 RESULTS

The TICs of the water samples are shown in the figures below (Figures 27 - 32). Within these TICs the targeted sterols are shown when eluted. Many other organic compounds eluted as well as a list of all these compounds together with the TICs can be found in Appendix D.

6.3.1 GC-TOF/MS analysis of environmental water samples

Figure 27: The 300ml effluent reference sample used, showing the elution and of only perylene-d12, the IS.

With the GC-QTOF/MS analysis of the 300ml reference sample volume collected from the effluent tank at the Potchefstroom WWTP. Only Perylene-d12, the internal standard was detected, and also with an abundance of only 800. Other organic compounds such as Heptacosane, were found in very low abundances, but are not looked at specifically in this study, and are thus irrelevant.

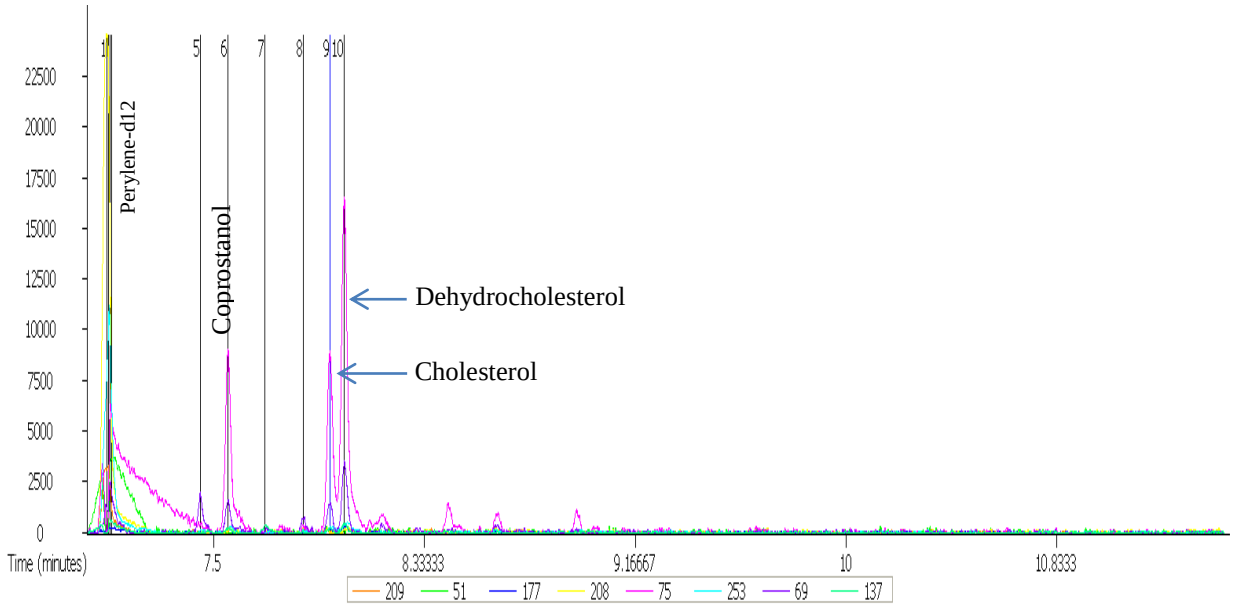


Figure 28: The TIC of the 1L effluent water sample, taken from the effluent tank of the Potchefstroom WWTP.

The TIC of the 1L effluent sample, however, showed that there are faecal sterols present in water that is entering the natural reed bed. Coprostanol indicates human faecal pollution (Leeming, 2006). Dehydrocholesterol is found in sludge, used during the treatment process, and cholesterol is found in most animal faeces, but also in human defecate, although not in as high a concentration as coprostanol (Leeming *et al.*, 1996).

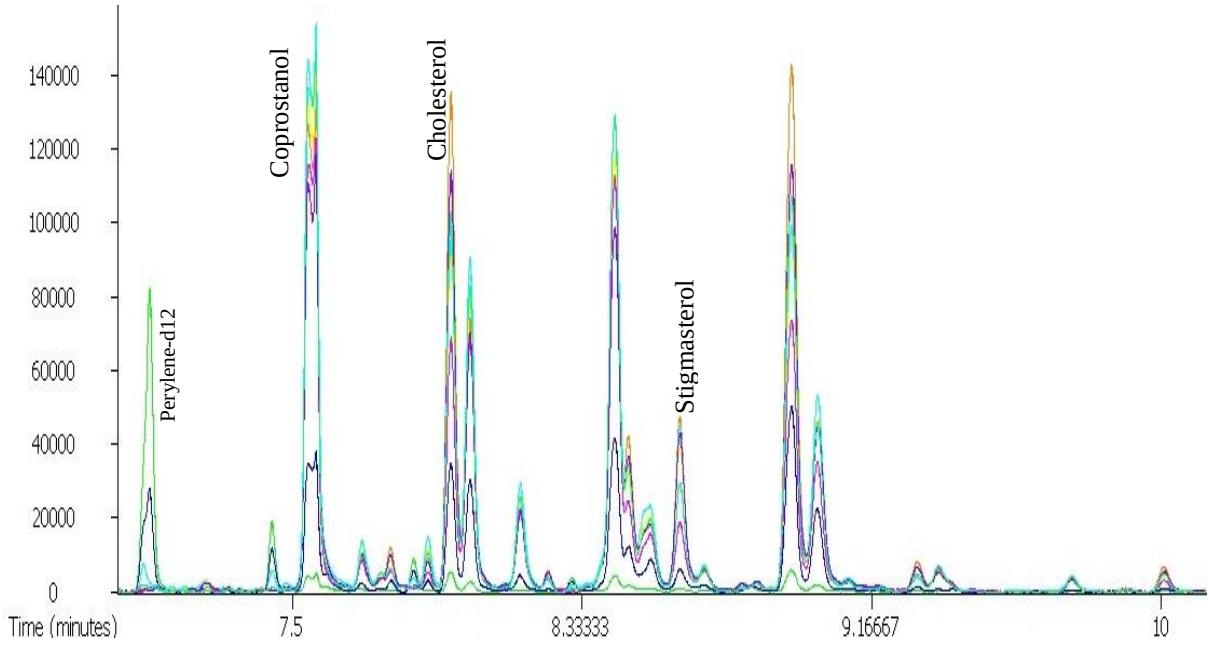


Figure 29: The 1L water sample taken from the reed-river barrier, where the effluent exits the natural reed bed, and flows into the Mooi River.

The TIC of the 1L reed-river barrier water sample that was analysed indicated the presence of Coprostanol, which indicates human faecal contamination (Leeming *et al.*,, 1996; Leeming, 2006). The abundance of cholesterol that eluted from the 1L effluent water sample is also high, this is most probably faecal pollution from birds (Leeming *et al.,* 1996), as the reed barrier is situated in a the bird sanctuary of Potchefstroom (OMP Prozesky Bird Sactuary), which is home to over 200 species of birds (Jenkins, 1971) Stigmasterol is a terrestrial sterol and is an indication of plant sterols. It is therefore expected to be found in environmental surface waters where plant growth usually flourishes (Leeming, 2006).

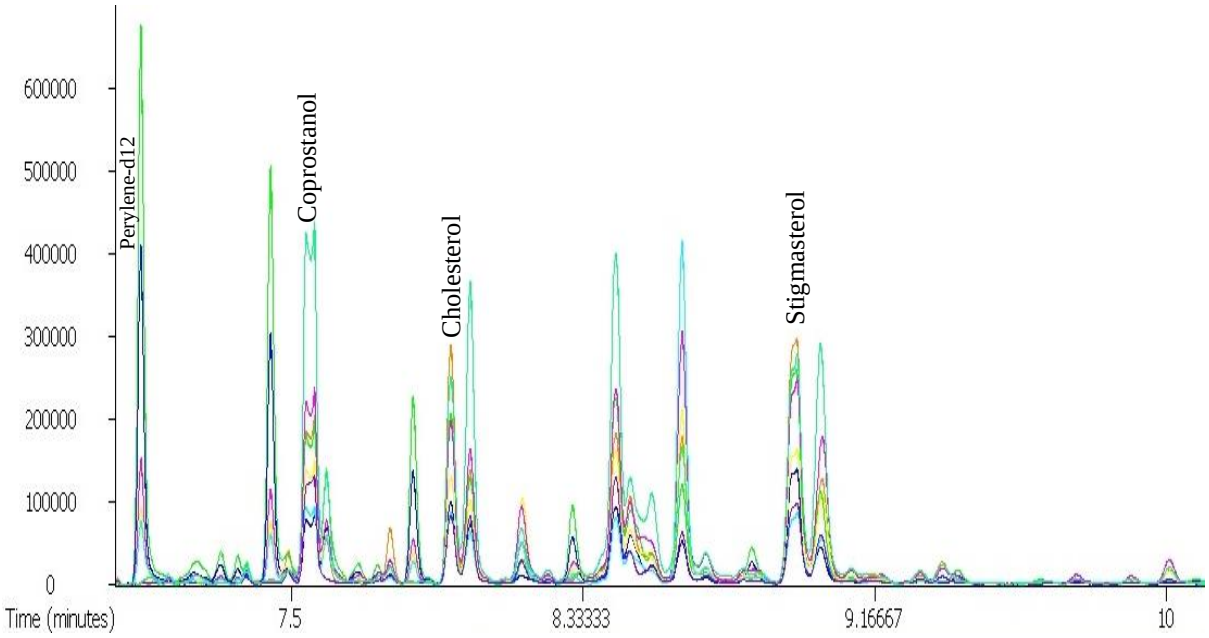


Figure 30: The TIC of the 2L water sample collected at the reed-river barrier, where the effluent flows from the natural reed bed, into the Mooi River.

The 2L reed-river barrier sample eluted the same sterols (along with other organic compounds, see Appendix D). All of which were at a higher abundance than the faecal sterols that was eluted from the 1L sample (Figure 29). This is most probably because the "load" of sterols increased with the increased volume of water sample.

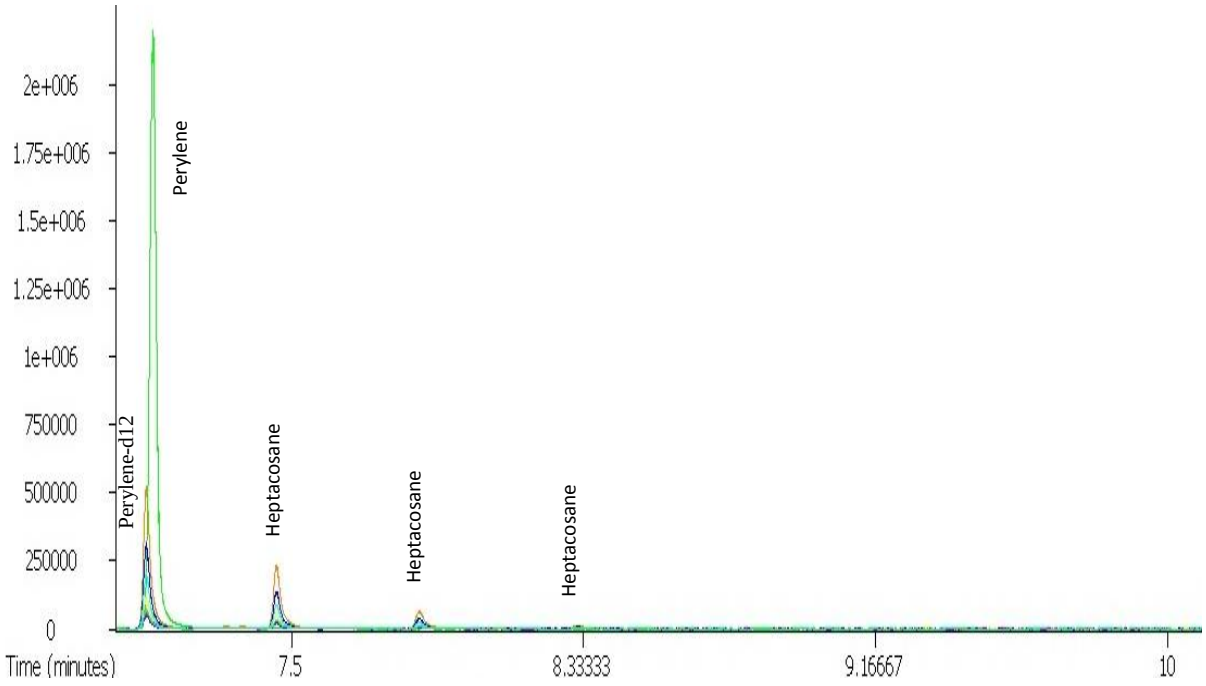


Figure 31: The 1L river water sample, collected from the Mooi River, downstream from the WWTP.

The TIC of the 1L Mooi River water sample, taken a few kilometers downstream from the WWTP, indicates no presence of faecal sterols. Other organic compounds were howerver found. Heptacosane being one of the more predominent compounds. The perylene-d12 and perylene dit elute hat high abundace, which indicates that the extraction and analysis procedure were not flawed.

A 2L Mooi River water sample was also analysed in order to try to increase the "load"of sterols in the water.This was done to determine a possible adequate volume of water that should be sampled for river surface water samples.

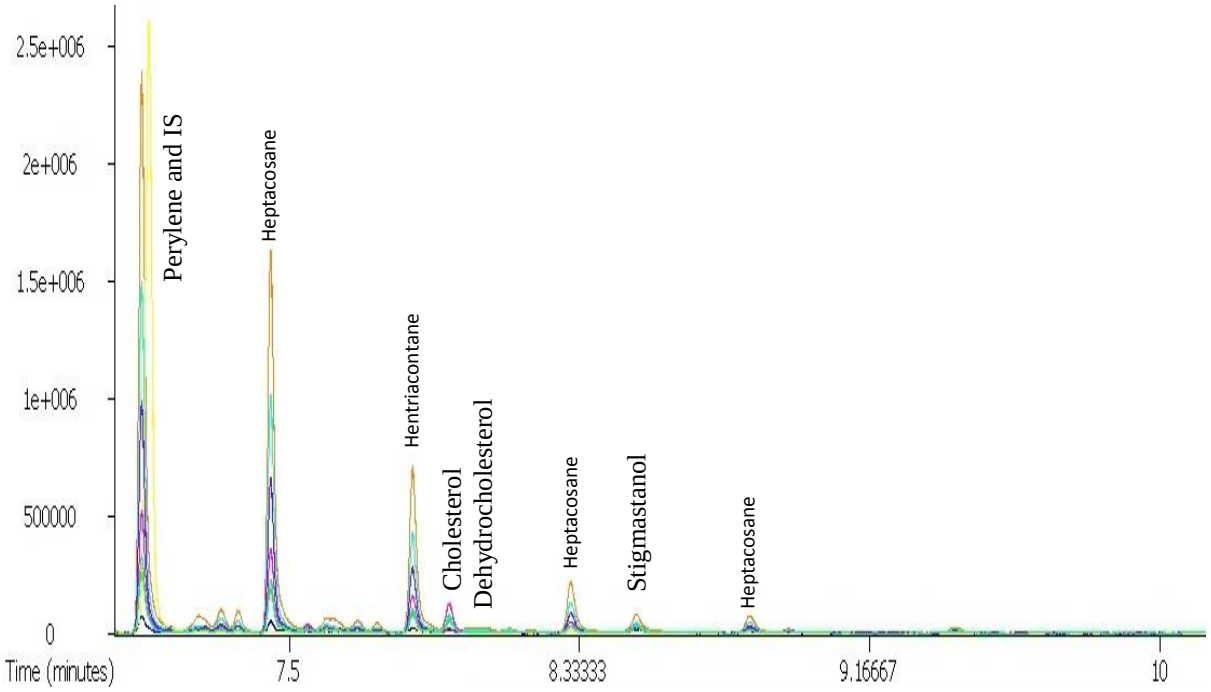


Figure 32: The 2L surface water sample collected from the Mooi River, downstream from the WWTP.

When the sample volume of the Mooi River surface water sample was increased (from 1L to 2L) cholesterol, dehydrocholesterol and stigmastanol eluted, Cholesterol indicating faecal pollution from birds or other animals (Leeming *et al.*, 1996). The abundance of cholesterol also increased from that of the reed-river water sample. The cholesterol load in the water has been increased as the river flows through the rural parts of the Potchefstroom area. A very small amount of dehydrocholesterol was also present; this could indicate older human faecal contamination in that fraction of the river at the specific time of sampling. Remembering that dehydrocholesterol is found in sludge (Szucs *et al.*, 2006) and that sludge can be used on farm lands (Heathcote, 2013). Stigmastanol is another terrestrial sterol that is usually found in algae (Leeming *et al.*, 1996). It is expected to be found in water bodies where algae are to be present.

6.4 DISCUSSION AND CONCLUSION

In this alternative study for the detection and qualitative determination of faecal sterols, using the Szűcs method, in WWTP effluent, (from the Potchefstroom WWTP) the sample volumes were increased. By increasing the volume of water, an increase in the "load" of sterols extracted and analysed, could be seen, resulting in a higher abundance of target sterols, which could then be detected in the effluent and other water samples.

A disadvantage of increasing the sample volume and faecal sterol load, is that all other compound loads get increased as well, resulting in a lot of "noise" (unwanted elution peaks at different abundances) appearing in the TIC (Appendix D). But by using the target qualifier ions of the six target sterols (Table 1), and the GC-TOF/MS software, the target sterols could still be qualitatively determined.

Though this study it can be concluded that 300 ml water sample collected from raw sewage it an optimal volume for the detection of all 6 target sterols. However, for optimum water quality monitoring via faecal sterol analysis of effluent and other environmental samples, at least 1L sample volume needs to be collected and analysed in order to accurately determine water quality.

7

Conclusion and Recommendations

The aim of this study was to assess the use of faecal sterol analysis to determine the quality of environmental water by using GC-MS and GC-TOF/MS techniques. These techniques were optimized (Chapter 3) for faecal sterol analysis of groundwater (Chapter 4 A), surface water (Chapter 4 B), and water from WWTPs (Chapter 5 and 6).

Monitoring water quality is essential in the efficient managing of South Africa's water resources (CSIR, 2010). Poverty and poor water quality are paralleled in developing countries. Poor people cannot contribute to water infrastructure, and are thus deprived of good water quality (DWAF, 2004). Lack of good water quality, or the depravation thereof could regrettably force people to drink from inferior, or worse, contaminated water sources (WHO, 2005). The current study was focused on a new and improved method of faecal detection in environmental water. The analytical detection method is faster, less time consuming and less expensive in the long run.

7.1 THE PILOT AND SENSITUVITY STUDIES

The method used in the determination of faecal sterols in water, was designed to detect six target sterols in water (Szùcs *et al.*, 2006). It is a time saving, less expensive and more accurate way of analysing water (Leeming, 2006).

The Szùcs method (Szùcs *et al.*, 2006), was successfully used in producing "sterol fingerprints" for humans and five other animal species. The sterol fingerprints provide us with a "control" to which we can measure all other unknown water samples. The spiked chicken faeces had the highest abundance of cholesterol, which is in accordance with Szùcs *et al*., (2006), which indicates that such results are indicative of faecal pollution from birds. The sterol 24-ethylcoprostanol, was the most abundant and notable faecal sterol found among the herbivore *spp*., this is also supported by Leeming *et al*., (1998a). In samples that contained mainly human faecal matter, the coprostanol and cholesterol were found to be in higher abundances then any of the other sterols that eluted, while the concentration of coprostanol was less abundant at any of the other species of animals (Table 3) – this then forms the bases for discriminating between human faecal and non-human faecal contamination.

7.2 WATER ANALYSIS

7.2.1 Ground Water

Groundwater of the North West Province is vulnerable to contamination, and restoration and replenishment of contaminated groundwater could take several decades (Reasoner, 1990). It is vital to manage the groundwater resources of the North West Province in a sustainable manner due to the large population size depending on groundwater for their day-to-day water needs.

Groundwater analysis was done on sites across the North West Province. Samples were collected from boreholes, water storage tanks, and water storage facilities. The physico-chemical quality of these groundwater sources showed that the pH of the groundwater sampled complied with the national TWQR. The TDS and EC of these groundwater samples

were high, but still at an accepted level. The nitrate contamination in some of these samples was shockingly high. Nitrate contamination was found to be a major contaminant in the groundwater of the North West Province. Agricultural or faecal contamination could have contributed to the high levels of nitrate in the groundwater (Ferreira, 2011).

Microbiological results showed that groundwater of the North West Province is vulnerable to faecal contamination as faecal indicator bacteria were identified in a high levels of the groundwater analysed. Thus, the microbiological quality of groundwater in the North West Province was not satisfactory.

The sterol analysis of the groundwater samples yielded both coprostanol and cholesterol in three sites where groundwater was sampled and analysed. This indicates possible mixed faecal pollution sources from humans and animals.

Compared to the target water quality range set by the DWAF, groundwater is not safe to use for domestic purposes. Groundwater of the North West Province is subjected to various contaminants, and should therefore be used with caution. The low compliance of the groundwater tested to national standards indicates that there is numerous health risks involved if these water is used for domestic purposes without any prior treatment. Exposed communities are mainly poor communities that have no alternatives. Therefore there exists a need to uplift these communities by providing them with safe water.

7.2.2 Surface Water

The quality of Baberspan inland lake situated in the North West Province was successfully determined by the use of physico-chemical parameters, microbial parameters and faecal sterol analysis. Physico-chemical and microbial parameters were compared to the TWQR for full

and intermediate contact recreational activities, livestock watering and irrigation. These comparisons tell us if the water is suitable for all of the above activities. Surface water samples analysed from the Baberspan area showed high pH levels, while microbial results for TC, FC and *E. coli*, but the FS counts were high, exceeding the DWAF (1996) standards. The high levels of FS is cause for concern, since their presence in surface water systems used for recreational, cultural and agricultural activities could have serious human and animal health implications.

Faecal sterol analysis showed the elution of cholesterol at three of the four sampling sites. No sterols were found in the Harts River, indicating that the water is free of faecal pollution in the river. The inflow of the water into the Baberspan did however yield cholesterol (6.212 ppm), indication faecal contamination from birds (Szùcs *et al.,* 2006). The surface water sample collected and analysed from the hotel at Baberspan also yielded cholesterol (6.008 ppm), and the outflow of the water out of the Baberspan yielded the highest concentration of cholesterol (7.258 ppm). All of these results at each site indicates that the Baberspan area's faecal contamination is as a direct result of the birds (Szùcs *et al.,* 2006), as Baberspan is one of the largest bird sanctuaries in South Africa, with over 12 000 birds recorded.

7.3 STEROL ANALYSIS OF WWTPs

The aim of this study was to determine if the Szűcs (2006) method, ran on a GC-TOF/MS, which is more sensitive than a GC-MS, would be able to detect the six target sterols in raw sewage and effluent of the Potchefstroom WWTP situated in the North West Province and the Carletonville and Fochville WWTPs situated in the Gauteng Provinces, South Africa. Therefore, also determining effluent water quality, and the efficiency of the various WWTPs.

The Szùcs method (Szùcs *et al.*, 2006) proved to be accurate and successful. At all the WWTPs two water samples was collected, one from the raw sewage entering the plant and the other from the effluent of the WWTP. In all of the raw sewage samples, all six target sterols (coprostanol, cholesterol, dehydrocholesterol, stigmasterol, β-sitosterol, stigmastanol) were found. The WWTP of Potchefstroom and Carletonville did not illustrate any sterols in the effluent. The effluent sample collected from the Fochville WWTP differed in that the effluent yielded two extra peaks. One was determined to be an unknown particle, while the other was identified as cholesterol.

There was, however, concern about the results for effluent, and so an alternative sub-study was done in order to try and rectify this, the sampling volumes were increased from 300ml to 1L and 2L sampling volumes. By increasing the volume of water, the amount ("load") of sterols extracted and analysed were increased, resulting in a higher abundance of target sterols. Three samples were analysed, each with different volumes of water. Coprostanol, dehydrocholesterol, and cholesterol were found in effluent when 1L of water sample was analysed. Coprostanol, cholesterol and stigmasterol was found in the reed bed sample taken, when 1L and 2L water samples were analysed. Cholesterol, dehydrocholesterol and stigmastanol were found in the river sample, kilometres downstream from the WWTP when a 2L water sample was analysed.

Knowing the amount of coprostanol expected, given a certain amount of human sewage pollution, provided a measure against which managers could quantitatively assess faecal pollution as a whole and relate that assessment to variables with which they are more familiar with. Sterols can provide an extra tool along with bacterial analysis for sewage pollution measurements in effluent.

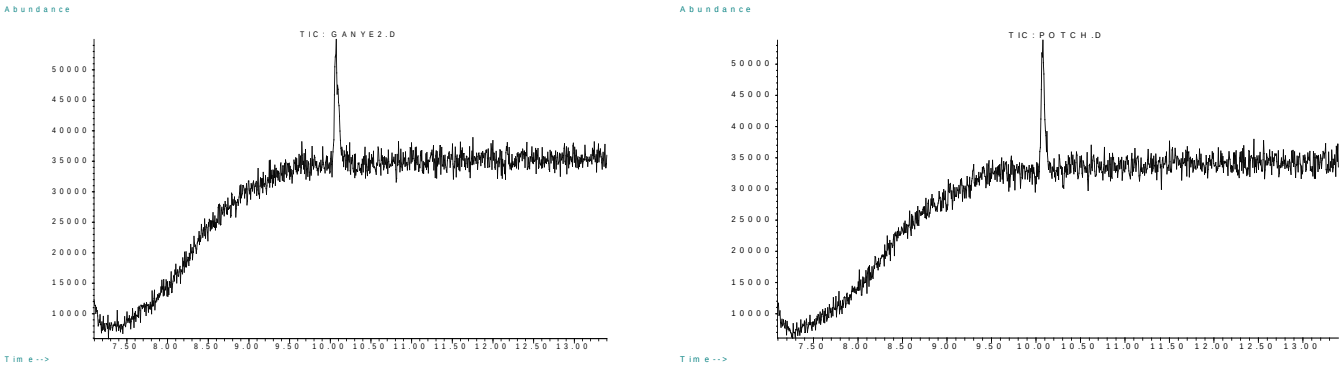
7.4 PROBLEMS ASSOCIATED WITH THE SZùCS METHOD

Some shortcomings obtaining to the Szűcs method .Using the Szűcs method (Szűcs *et al.*, 2006) it was difficult to recover sufficient amounts of sterols in the water samples. The liquid-liquid extraction procedure is time consuming and may not always be successful causing distortion of the gas chromatic profile or no elution of peaks, rendering the results inconclusive. Cost is also a drawback as some reagents and standards used are expensive and not always available. Although the Szűcs Method (Szűcs *et al*., 2006) is accurate in detecting faecal contamination in water, it does not possess the necessary protocols to detect the origin of the faecal pollution accurately, therefore other methods need to be incorporated into the faecal sterol study of environmental water in order to overcome the problems found with the Szűcs method (Leeming, 2006). The Szűcs method could be used in evaluating the efficiency of wastewater treatment plants since it is a relatively quick method and small amounts of water need be analysed. This could prove to be very beneficial since South Africa is a country of inherent wastewater treatment plant failures (DWAF, 2009). Sterol analysis can aid in the water quality determination of both ground and surface water. It can be used to determine the point source or non-point source of pollution and determine faecal contamination in all water bodies, whether from human or animal sources.

The method that is currently being used (Szűcs *et al.*, 2006) has some drawbacks which include:

- Extracts may sometimes not be pure enough, and result in inconclusive results (see Figure 1 a and b).
- Coprostanol is non-polar, and therefore more difficult to extract from the water samples.
- Cost. Some reagents and standards used are expensive and not always available.

- Differentiation between human and animal faecal sterols is difficult because not all the sterols needed for ratio determination is tested for in this method.



a.) b.)

Figure 33: When extraction of sterols is not successful, distortion of the gas chromatic profile occurs, and no elution picks can be observed, rendering the results inconclusive and useless.

Figure 33 shows the sterol profiles of analysis done on groundwater from a.) Ganyesa and b.) Potchefstroom, in which the extraction process was unsuccessful. Because of the drawbacks that the Szűcs *et al.* (2006) method had, a few adjustments were made to the original method to try and overcome its drawbacks. These adjustments included:

- Running the extraction over 2 days, thus leaving the extractions overnight to separate and settle.
- Increasing the volume of the sample. Extracting 300ml water samples instead of the normal 250ml.
- If water was present in the sample after evaporation by the Rota-vapor (Büchi, Flawil, Switzerland), methanol was added to the sample and evaporated again, thus removing the water.

- GC-MS was run on SIM (Selective Ion Monitoring) instead of Scan, in order to get better elution of sterols at their various retention times.
- When raw sewage samples were extracted, the bottom half (containing the dichloromethane and sterols) was removed from the separatory flask and placed in a 'new' separatory flask. These were then extracted again with 3 x 50 ml dichloromethane. The top half was discarded.

Even though these modifications helped in the extraction and analysis of the sterols from groundwater and surface water, some results were still determined inconclusive.

Cutting edge biomarker methods such as sterols, fatty acids and isotope ratios can possibly be used in conjunction for the detection of faecal pollution in environmental water sources. For local authorities to establish and incorporate

7.5 RECOMMENDATIONS

With reference to this study, the following recommendations are suggested:

1) Parameters such as Chemical Oxygen Demand (COD) and Biological Oxygen Demand should be monitored. COD and BOD are indicators of organic pollution measured during water quality studies because they provide information that indicates the toxic conditions of a water system while reporting on the potential presence of biologically resistant organic substances (Venkatesharaju *et al*., 2010).

2) *E. coli* species confirmed by biochemical techniques (such as API, Entropleuri tests) and the identification should be further confirmed by molecular methods. A

polymerase chain reaction (PCR) amplification procedure that uses *E. coli* housekeeping genes could be used for this purpose (Bej *et al*., 1991).

3) Biochemical techniques used in this study for the identification of faecal streptococci proved to be limiting. Sequencing data can be more confirmatory and should always be used for the identification of DNA.

4) Groundwater sites should be identified that may be vulnerable to a certain contamination source. These boreholes should be sampled and analysed on a regular basis. During every sample event, the surrounding area, human and environmental influences as well as seasonal variables should be recorded. This will allow for a deeper insight into the factors promoting the contamination of groundwater.

5) It is vital that communities are aware of and understand the risks involved with using untreated groundwater and surface water as a source for domestic purposes. By having an understanding of the water quality they use, communities will be encouraged to treat the water at household level (boiling of water, point-of-use chlorination, UV treatment and safe storage practices). These practises will aid in the prevention of water borne diseases.

6) Policies, guidelines and programs (such as well-head protection, source-control, minimum allowable distances of contaminant sources from groundwater and surface water sources, etc.) should be developed and enforced by local government and relevant stakeholders to ensure the protection of water sources.

Finally, this study presented some baseline data on the relationship between physico-chemical water quality, microbiological parameters and faecal sterol concentrations of

surface water and groundwater systems in the North West Province. In addition, attention was drawn to the faecal pollution trends in the surface water and groundwater systems as well as their potential health risk when water is used for full and intermediate contact recreational activities, livestock watering and irrigation, and domestic use.

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APPENDICES

Appendix A

Table 10: GPS co-ordinates of all sampling sites.

River System	Sampling Site	GPS Co-ordinates	
		Longitude (S)	**Latitude €**
Surface water samples			
Baberspan	Town - Harts River	S 26° 38' 37.2"	E 25° 36' 58.8"
	Inflow	S 26° 37' 06.6"	E 25° 34' 40.0"
	Hotel	S 26° 35' 26.1"	E 25° 36' 10.9"
	Outflow	S 26° 33' 00.8"	E 25° 35' 48.1"
Groundwater samples			
Zeerust		S 25° 32' 11.05"	E 26° 04' 33.68"
Mafikeng		S 25° 52' 33.85"	E 25° 36' 05.27"
Delareyville/ Ottosdal		S 26° 44' 39.59"	E 25° 44' 14.94"
Setlagole		S 26° 17' 08.13"	E 25° 06' 47.86"
Geystown		S 26° 32' 13.21"	E 25° 18' 34.22"
Taung		S 27° 33' 10.10"	E 24° 45' 57.51"
Hartswater		S 27° 45' 08.64"	E 24° 48' 53.07"
Sekhing		S 27° 50' 52.13"	E 24° 37' 30.27"
Christiana		S 27° 54' 58.98"	E 25° 09' 22.17"
Vryburg		S 26° 57' 15.59"	E 24° 44' 00.13"
Ganyesa		S 26° 35' 42.43"	E 24° 09' 59.00"
Waste water treatment pland samples			
Carletonville WWTP		S 26° 21' 08.75"	E 27° 23' 32.45"
Fochville WWTP		S 26° 29' 49.32"	E 27° 29' 28.44"
Potchefstroom WWTP		S 26° 43' 45.45"	E 27° 06' 00.65"
Potchefstroom WWTP samples			
Effluent		S 26° 45' 05.14"	E 27° 05' 39.92"
Reed/ River barrier		S 26° 45' 01.06"	E 27° 05' 59.36"
Mooi River		S 26° 46' 41.98"	E 27° 05' 09.25"

Appendix B

Table 11: Target water quality variables and ranges (DWAF, 1996b)

Variable		Target Water Quality Range (TWQR)				
		Domestic	Recreation		Livestock watering	Irrigation
			Full contact	Intermediate contact		
pH (pH units)		6-9	6.5-8.5	NA	NA	6.5-8.4
TDS (ppm)		0-450	NA	NA	<1000[a]	260
					<2000[b]	
					<3000[c]	
Electrical conductivity (mS/m)		0-70	NA	NA	NA	0-40
Nitrite (mg/L)		0-6	NA	NA	0-10	0-0.5
Faecal streptococci (cfu/100 ml)		NA	0-30	0-230	NA	NA
Coliforms (cfu/100 ml)	**Total Coliforms**	0-5	NA	NA	NA	NA
	Faecal Coliforms	0	0-150	0-1000	0-200	<1
	E. coli	NA	0-130	NA	NA	NA

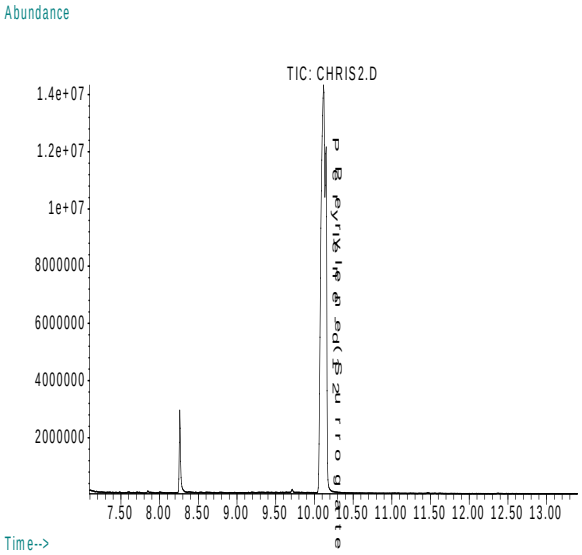
NA – Not applicable

[a]Dairy, pigs and poultry; [b]Cattle and horses; [c]Sheep.

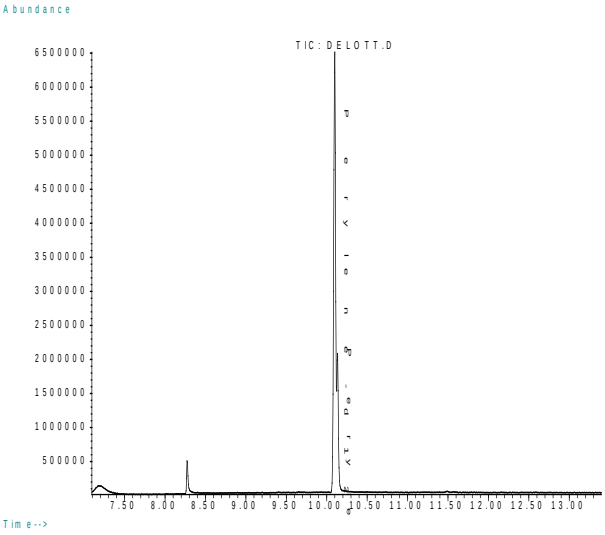
Appendix C

GC-MS Analysis of Groundwater:

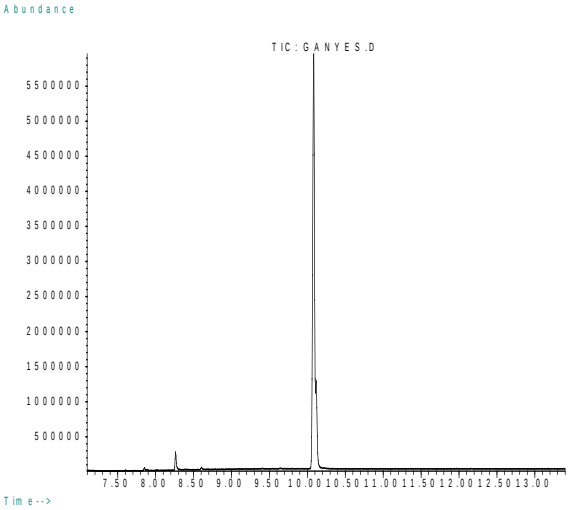
Groundwater samples collected and analysed for the presence of faecal sterols by GC-MS. The water sample TIC below indicated no presence or levels of faecal sterols in the water (Figure 34 a – i).



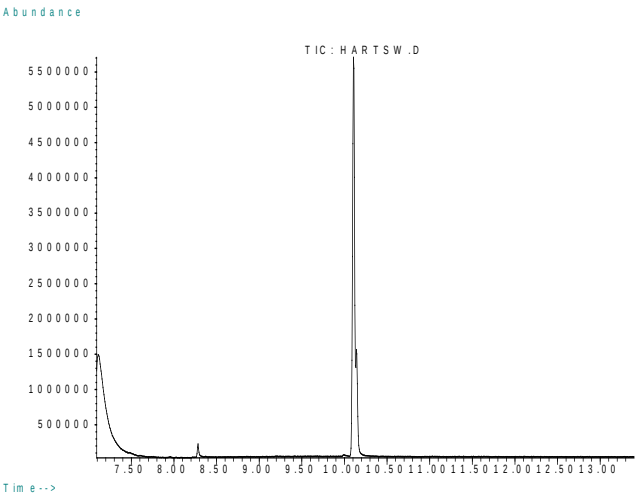
a.) Christiana 2 groundwater.



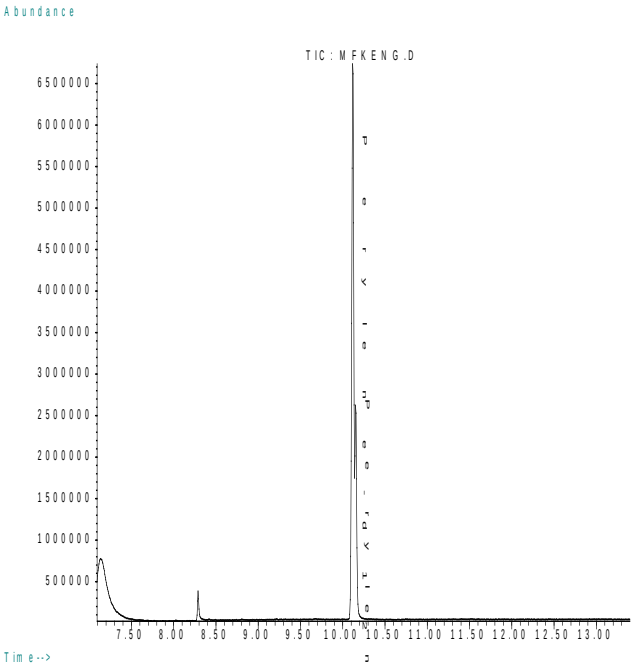
b.) Delarey-Ottosdal groundwater.



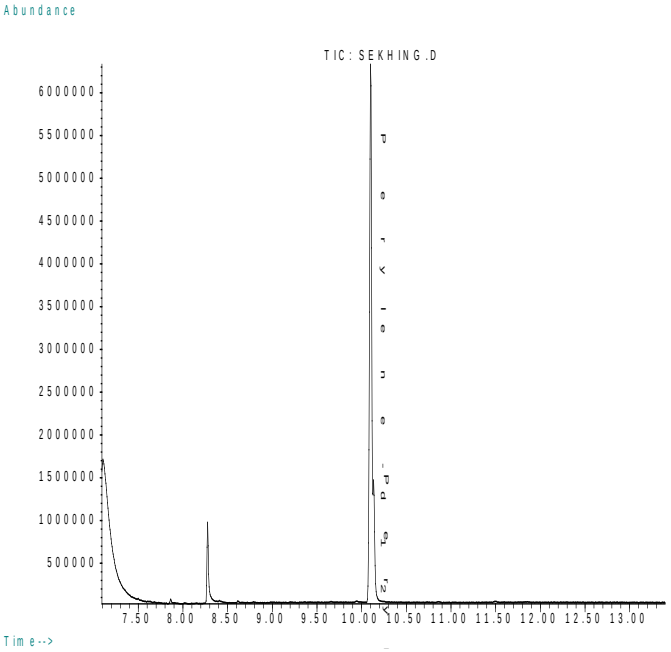
c.) Ganyessa groundwater.



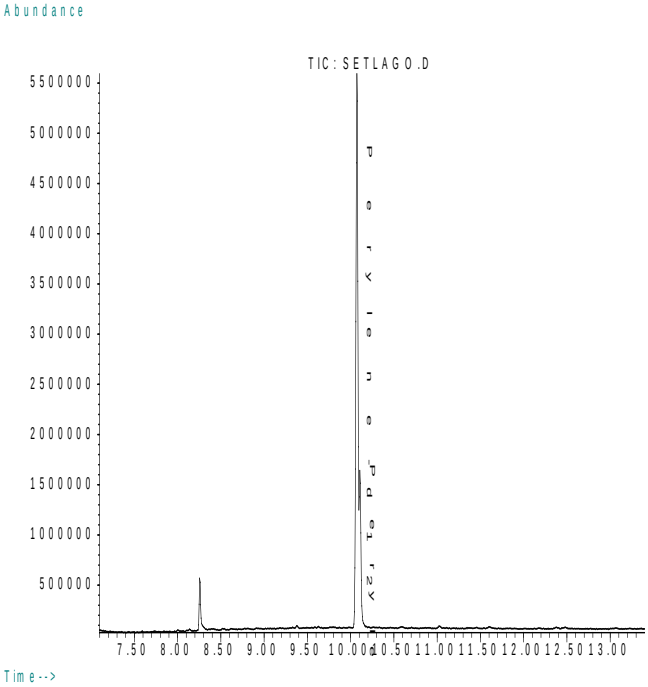
d.) Hartswater groundwater.



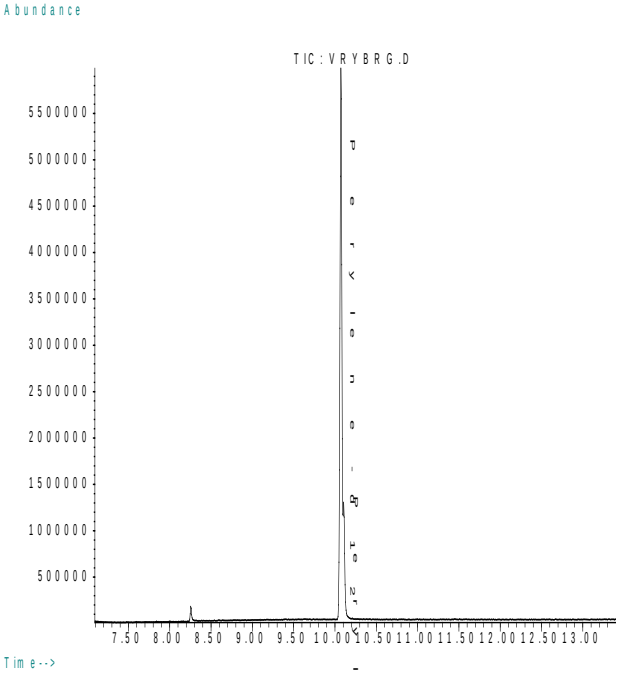
e.)Mafikeng groundwater.



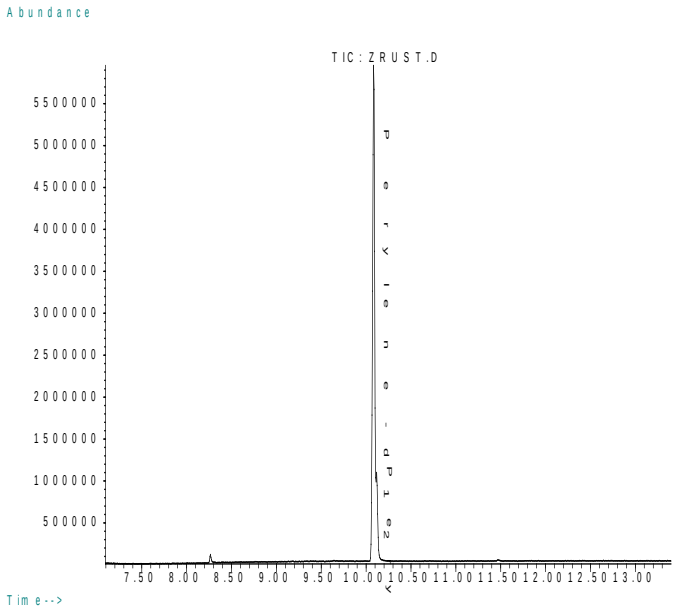
f.) Sekhing groundwater.



g.) Setlagole groundwater.



h.) Vryburg groundwater.



i.) Zeerust groundwater.

Figure 34 a. – i**.:** Groundwater samples that were taken at various sites all over the North West Province of South Africa and analyzed by GC-MS (Szűcs method) for faecal sterols in order to determine faecal contamination in groundwater. At the specific sampling sites, no sterols were found that were of any consequence. Only the SURR (perylene) and IS (perylene-d12) eluted.

GC-MS analysis of surface water

Surface water samples collected at the Inland Lake (Bird Sanctuary) were then analysed for faecal sterols by GC-MS (the Szűcs method), (Szűcs *et. al*., 2006). Results are depicted in the figures below, but also in table 5.5

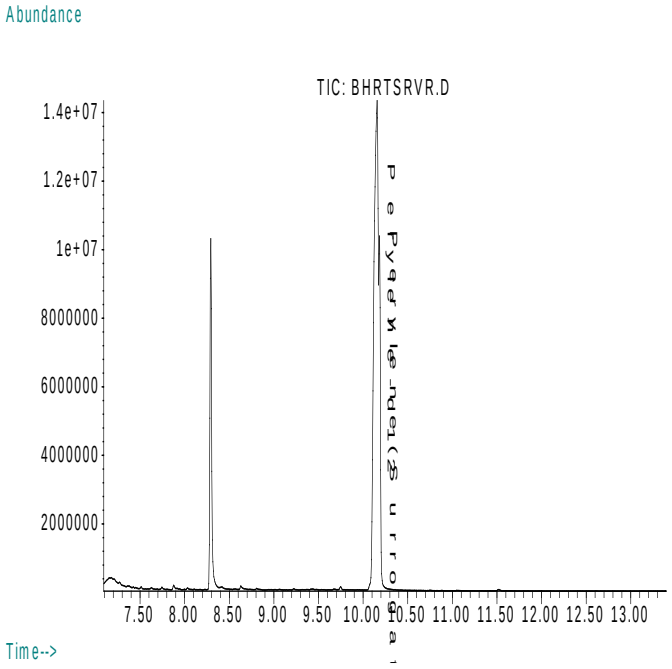


Figure 35: Faecal sterol analysis done on the water of the Harts River that flows into the Baberspan Inland Lake showed no sterols of any consequence that eluted.

Appendix D

Table 12: Effluent (1L) from the Potchefstroom WWTP.

PEAK #	NAME	EXACT MASS	R.T (MIN)	AREA	HEIGHT	UNIQUE MASS
1	Perylene-d12	264.1692	7.07583			264
2	1,12 Dicarbododecarborane	262.2244	7.0825	18083589	546140	262
3	Isophthalic acid	390.1643	7.09	297120	11903	207
4	Benzo[e]pyrene	252.0939	7.09583	117640	2334.6	250
5	Heptarosane	380.4382	7.445	408712	14501	57
6	**5b-Cholestane-3a, 3-(ethylthio)**	**432.3790**	**7.55417**	**57607**	**1916.3**	**215**
7	Pentacene, 6,13-dihydro	280.1252	7.70167	19415	546.57	278
8	Tetratriacontane	478.5478	7.85167	148099	4950.2	57
9	**Cholesterol TMS**	**458.3944**	**7.9575**	**71038**	**2566.5**	**129**
10	**5a-cholestan-3a-ol**	**460.4100**	**8.015**	**134372**	**4818.3**	**215**
	Total			18083578	589676.5	

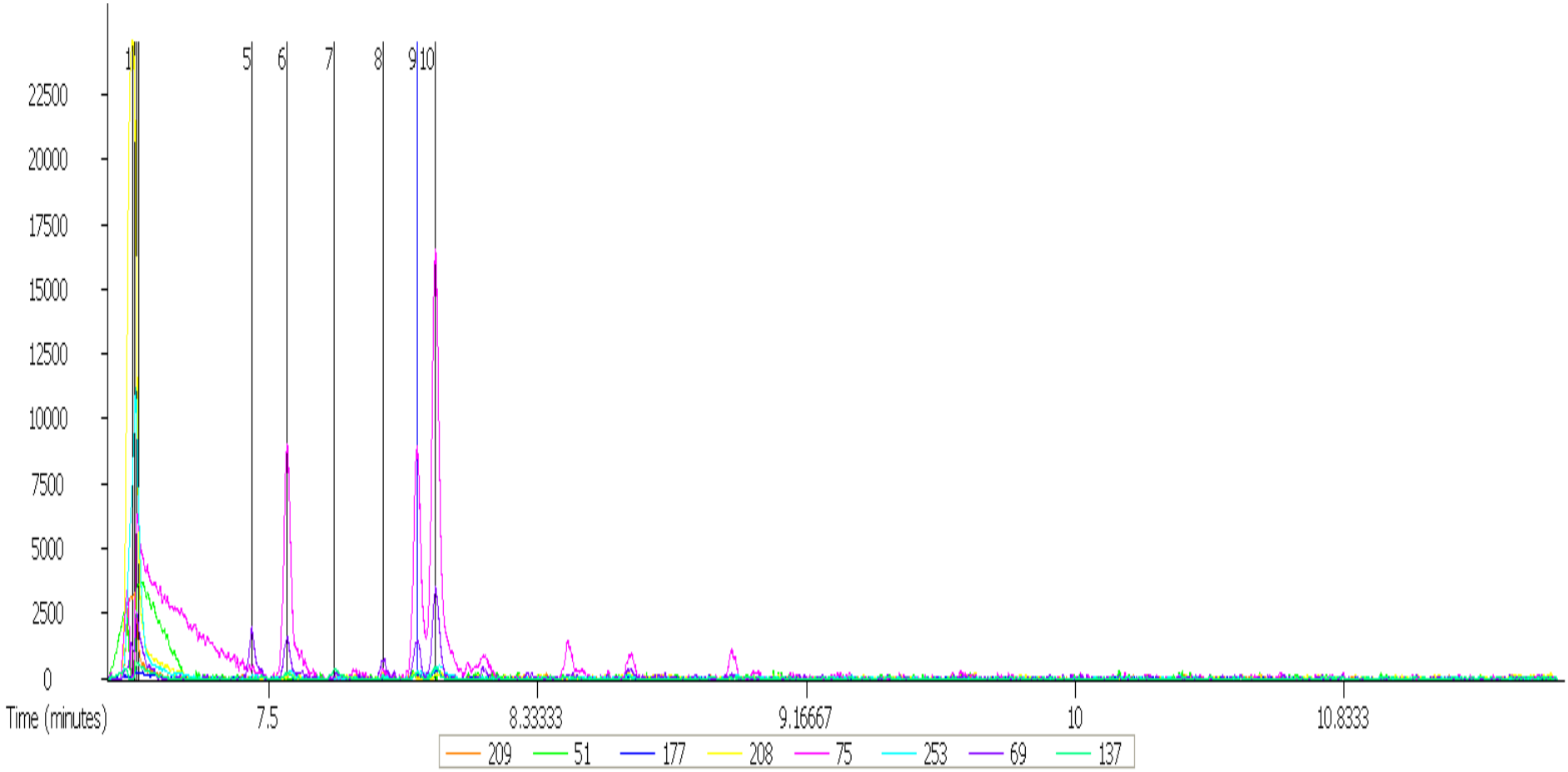


Figure 36: TIC of the Effluent (1L) water sample from the Potchefstroom WWTP.

Table 13: The water sample taken from the Reed/River barrier where effluent from the Potchefstroom WWTP exits and flows onto the natural reed bed, before flowing into the Mooi River. (References Sample of 1L) (47compounds found). Compounds found included Coprostanol, Cholesterol and Stigmasterol.

PEAK #	NAME	EXACT MASS	R.T (MIN)	AREA	HEIGHT	UNIQUE MASS
1	Perylene-D12	264.1692	7.06333	18604112	622685	264
2	Octacosane	394.4539	7.07083	7883480	309745	57
3	Perylene	252.0939	7.09083	26833087	974245	252
4	Mono-TMS of (pyridoxine-H2O)	223.1029	7.185	11325	488.21	224
5	2-Methyl-1-undecanol	186.1984	7.2325	733021	10462	57
6	Silane, [(3,7,11,15-tetramethyl-2-hexadecenyl)oxy]trimethyl-	368.3474	7.26083	159642	6643.9	143
7	Heptacosane	380.4382	7.3	401700	13439	57
8	Sulfurous acid, buryl decyl ester	278.1916	7.34833	337737	12178	57
9	Heptacosane	380.4382	7.44167	5594260	213287	57
10	2,6,10-Dodecatrien-1-ol,3,7,11-trimethyl-	222.1984	7.4825	187133	6643.8	69
11	**5b-Cholestan-3b-ol, trimethylsilyl ether**	**432.3790**	**7.54167**	**802286**	**31222**	**77**
12	**5b-Cholestan-3b-ol, trimethylsilyl ether**	**432.3790**	**7.56583**	**1157948**	**47132**	**143**
13	Unknown 1	191.1310	7.57083	1030627	36659	77
14	Indolo[2,3-b]quinoxaline, 1-fluoro-	237.0702	7.6625	56918	2212.7	237
15	Sulfurous acid, buryl heptadecyl ester	376.3011	7.69583	304328	8482.7	71

16	Lithocholic acid 3-acetate	418.3083	7.7	228369	7887.5	215
17	2-Piperidinone, N-[4-bromo-n-buryl]-	233.0415	7.7475	116102	4261.1	71
18	Cholesterol 3-O-[[2-acetoxy]ethyl]-	472.3916	7.7825	1002543	36946	237
19	Heptacosane	380.4382	7.84917	1753854	61658	71
20	5-a-Cholan-24-oic acid, 3-oxo-	374.2821	7.89	244979	8381.2	231
21	**Cholesterol trimethylsilyl ether**	**458.3944**	**7.95667**	**12179628**	**391704**	**129**
22	Silane, trimethyl[[(3a,5a)-stigmastan-3-yl]oxy]-	488.4413	8.01167	4586244	137671	215
23	Neopregnenolone	298.1933	8.11167	76350	2506.5	195
24	9,19-Cycloergost-24(28)-en-3-ol, 4,14-dimethyl-, acetate, (3a,4a,5a)-	468.3967	8.15583	1744810	47116	83
25	10,12-Tricosadiynoic acid, trimethylsilyl ester	418.3267	8.16	249777	7402.8	125
26	5.Xi.-Ergost-7-ene, 3a-(trimethylsiloxy)-	472.4100	8.23583	125632	4255.4	213
27	Heptacosane	380.4382	8.30333	1482016	45672	57
28	Neoergosterol, acetate	422.3185	8.32	78754	2341.1	195
29	5a,-Ergost-8(14)-ene	384.3756	8.4275	7816536	185333	215
30	**Cholesterol trimethylsilyl ether**	**458.3944**	**8.4675**	**101860**	**3084.1**	**343**
31	9,19-Cyclolanostan-3-ol, acetate, (3a)-	470.4124	8.50917	151197	4542.8	283
32	Silane, trimethyl[[(3a,5a)-stigmastan-3-yl]oxy]-	488.4413	8.53083	1174637	29495	215
33	**Stigmasterol trimethylsilyl ether**	**484.4100**	**8.61417**	**7055188**	**204108**	**83**
34	9,19-Cyclolanostan-3-ol, acetate, (3a)-	470.4124	8.68417	163932	4520.9	123
35	Dodecane, 2,6,10-trimethyl-	212.2504	8.81583	549451	12659	71
36	2-Pentanone, 3-[(acetyloxy)methyl]-3,4-dimethyl-,(.+-.)-	186.1256	8.82167	437038	8335.8	69

37	10,12-Tricosadiynoic acid, trimethylsilyl ester	418.3267	8.83667	93973	2322.7	105
38	**Cholesterol trimethylsilyl ether**	**458.3944**	**8.93583**	**16047668**	**401037**	**129**
39	Dimethyl(bis{[2E,6E)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl]oxy})silane	500.4050	8.94083	22245	536.84	296
40	Pregna-5,8-diene-3a,11a-diol-20-one diacetate	414.2406	8.955	100211	2644.0	343
41	Silane, trimethyl[[(3a,5a)-stigmastan-3-yl]oxy]-	488.4413	9.00833	3015814	78165	215
42	Retinol, acetate	328.2402	9.02167	213961	6396.9	296
43	9,19-Cyclolanostan-3-ol, acetate, (3a)-	470.4124	9.09917	101563	2813.3	81
44	5.Xi.-Ergost-7-ene, 3a-(trimethylsiloxy)-	472.4100	9.2975	237948	6299.7	213
45	9,19-Cyclolanost-24-en-3-ol, acetate, (3a)-	468.3967	9.35833	908630	15747	69
46	Heptacosane	380.4382	9.40167	136191	3455.7	85
47	9,19-Cycloergost-24(28)-en-3-ol, 4,14-dimethyl-, acetate, (3a,4a,5a)-	468.3967	9.74083	179691	3834.3	135
	Total			126474397	4028660	

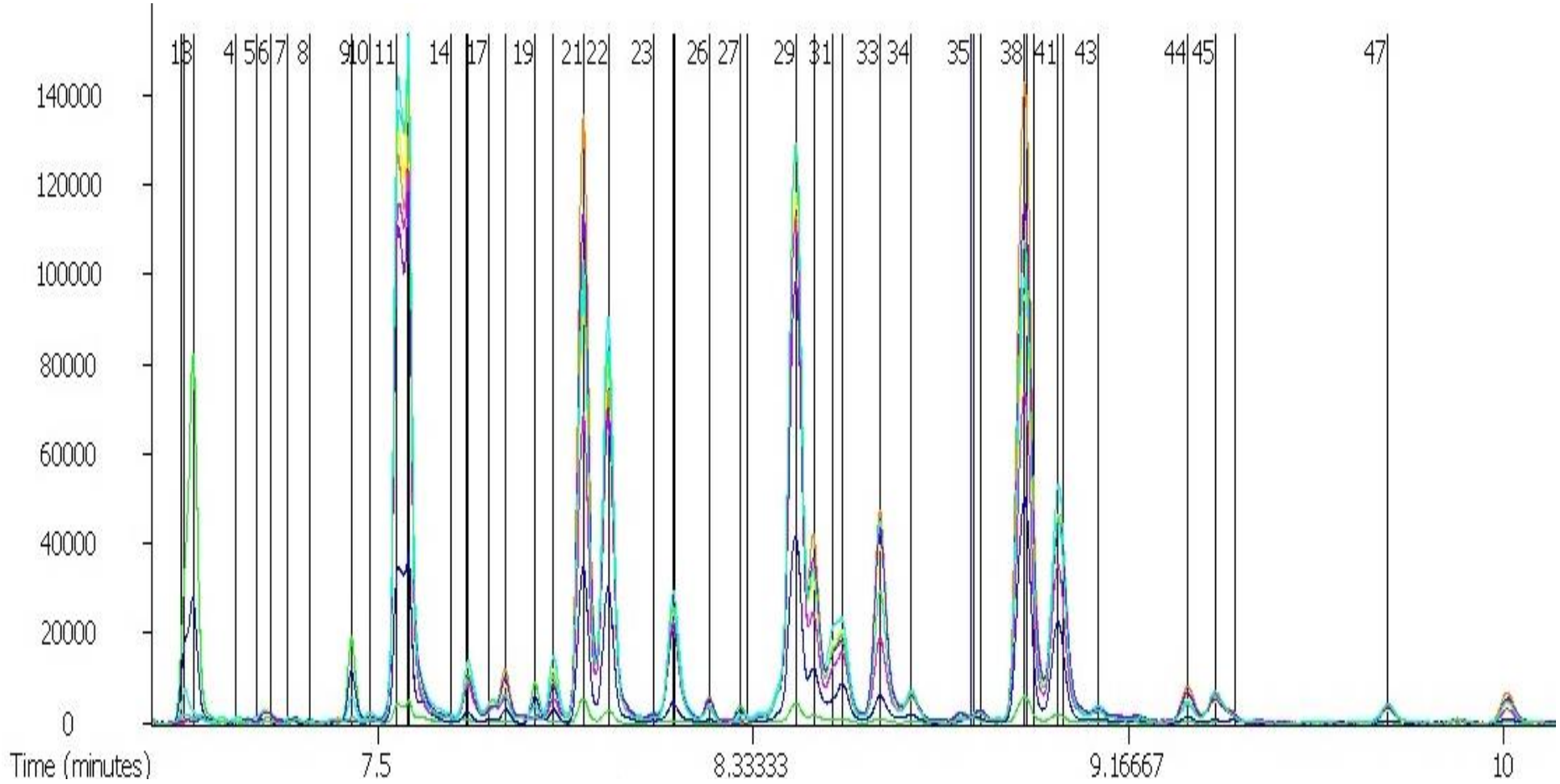


Figure 37: TIC of the 1 L reference water sample taken from the Reed / River barrier, in which 47 compounds were, identified (Table 13)

Table 14: The 2L water sample taken from the Reed/River barrier where effluent from the Potchefstroom WWTP exits and flows into the natural reed bed yielded 64 different compounds including, Coprostanol, Cholesterol and Stigmasterol.

PEAK #	NAME	EXACT MASS	R.T (MIN)	AREA	HEIGHT	UNIQUE MASS
1	Perylene-D12	264.1692	7.06333	17810211	596962	264
2	1-Iodo-2-methylundecane	296.1001	7.07083	17084310	676226	57
3	Perylene	252.0939	7.09083	33713367	1218957	252
4	Silane, [[(3a,5a,20S)-pregnane-3,20-diyl]bis(oxy)]bis[trimethyl-	464.3506	7.10833	130701	5066.3	117
5	n-Heptadecylcyclohexane	322.3600	7.15	217962	9447.8	82
6	Mono-TMS of (pyridoxine-H20)	223.1029	7.185	152654	6499.2	223
7	tert-Hexadecanethiol	258.2381	7.22167	765537	12744	71
8	Pentafluoropropionic acid, octadecyl ester	416.2714	7.2325	272982	4811.3	56
9	Octane, 4,5-diethyl-	170.2035	7.25333	240667	8044.9	85
10	Pentadecanoic acid, 3-methylbutyl ester	242.2246	7.28917	201961	6152.4	70
11	Sulfurous acid, butyl dodecyl ester	306.2229	7.3	965075	34680	57
12	Sulfurous acid, butyl dodecyl ester	306.2229	7.3475	313484	10381	56
13	1,12-Bis(trimethylsiloxy)dodecane	346.2723	7.3725	636311	27474	149
14	Hentriacontane	436.5008	7.44083	7537488	300260	71
15	6-Azabicyclo[3.2.1]octane	111.1048	7.47583	206400	6744.3	68
16	9-Octadecenoic acid, 2-[(trimethylsilyl)oxy]-1-	500.3717	7.48167	70397	2810.9	219

	[[(trimethylsilyl)oxy]methyl]ethyl ester					
17	1,11-Bis(trimethylsiloxy)undecane	332.2567	7.49167	47278	2085.2	440
18	5b-Cholestan-3b-ol, trimethylsilyl ether	432.3790	7.54	135968	5442.5	78
19	5b-Cholestan-3b-ol, trimethylsilyl ether	432.3790	7.56667	2374723	48925	143
20	2-Hexadecanol	242.2610	7.56917	828308	39413	77
21	Octacosanol trimethylsilyl ether	482.4883	7.60083	357572	15514	468
22	Cyclooctacosane	392.4382	7.60833	130768	3747.6	157
23	Indolo[2,3-b]quinoxaline, 1-fluoro-	237.0702	7.66167	123348	4832.2	237
24	1-Octadecanesulphonyl chloride	352.2203	7.6925	653326	19781	57
25	Sulfurous acid, butyl heptadecyl ester	376.3011	7.74583	57871	2039.7	99
26	Dimethyl(bis{[(2E,6E)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl]oxy})silane	500.4050	7.7525	125684	4393.2	109
27	1-(1-Adamantyl)-2-trimethylsilyloxyethane	252.1909	7.7825	1140036	42276	237
28	9,12,15-Octadecatrienoic acid, 2-[(trimethylsilyl)oxy]-1-[[(trimethylsilyl)oxy]methyl]ethyl ester, (Z,Z,Z)-	496.3404	7.78583	144409	4720.6	103
29	Hentriacontane	436.5008	7.84833	6509976	226833	57
30	Cholesterol trimethylsilyl ether	458.3944	7.95583	13758385	440679	129
31	2-Cyclohexene-1-carboxylic acid, 2-(7-hydroxy-3-methyl-1,3-octadienyl)-1,3-dimethyl-4-oxo-, methyl ester	320.1988	8.00833	16645	399.66	343
32	Silane, trimethyl[[(3a,5a)-stigmastan-3-yl]oxy]-	488.4413	8.01167	4591729	142134	215
33	Neopregnenolone	298.1933	8.11083	118862	4010.2	195
34	9,19-Cyclolanostan-3-ol, acetate, (3a)-	470.4124	8.155	20529	660.53	487

35	Stigmasterol trimethylsilyl ether	484.4100	8.16	4036703	102606	69
36	3a-Hydroxy-11-cholenic acid methyl ester	388.2977	8.235	123040	4016.4	213
37	Heptacosane	380.4382	8.30333	2990403	92098	57
38	Isoneoergosterol acetate	422.3185	8.32167	337821	10215	237
39	3a,5-Cyclo-5a-ergosta-6,8(14).22t-triene	378.3287	8.37917	139453	3780.6	253
40	9,19-Cyclolanostan-3-ol, acetate, (3a)-	470.4124	8.42667	7766034	186400	215
41	Unknown 1	342.3287	8.43	17850	419.88	389
42	Cholesterol trimethylsilyl ether	458.3944	8.4675	596206	16062	213
43	3,7,11,15-Tetramethyl-hexadecanol, trimethylsilyl ether	370.3631	8.49	392282	12534	496
44	Methyl 12-acetyl- 7-desoxycholate	448.3189	8.50833	125286	3689.4	283
45	Methyl 2-[3a-acetoxy-5,7,9-estratrien-17-yl]propionate	384.2301	8.51333	93991	2827.4	324
46	Silane, trimethyl[[(3a,5a)-stigmastan-3-yl]oxy]-	488.4413	8.53	115084	2830.4	306
47	Stigmasterol trimethylsilyl ether	484.4100	8.61583	14326850	412680	83
48	9,19-Cyclolanostan-3-ol, acetate, (3a)-	470.4124	8.685	269380	7148.2	123
49	Androst-5-en-17-one, 3-(acetyloxy)-19-hydroxy-, (3a)-	346.2144	8.78833	136820	3595.0	237
50	Sulfurous acid, butyl dodecyl ester	306.2229	8.8175	816597	18625	85
51	Retinol, acetate	328.2402	8.83833	279024	7167.3	129
52	Pregn-5-en-20-one, 3-[(trimethylsilyl)oxy]-, (3a)-	388.2798	8.93917	7691222	152162	145
53	1, 12-Bis(trimethylsi loxy)dodecane	346.2723	8.9425	4612452	85330	83
54	Silane, trimethyl[[(3a)-stigmasta-7,24(28)-dien-3-yl]oxy]-	484.4100	8.9575	148529	4254.3	343
55	Silane, trimethyl[[(3a,5a)-stigmastan-3-yl]oxy]-	488.4413	9.01	3801918	98141	215

56	Retinol, acetate	328.2402	9.0225	401741	11935	296
57	9,19-Cycloergost-24(28)-en-3-ol, 4,14-dimethyl-, acetate, (3a,4a,5a)-	468.3967	9.09917	55690	1296.6	216
58	Silane, trimethyl(1-methylethoxy)-	432.0970	9.155	56089	1179.0	118
59	Cholesterol 3-O-[[2-acetoxy]ethyl]-	472.3916	9.18417	406393	8404.2	55
60	5.Xi.-Ergost-7-ene, 3a-(trimethylsiloxy)-	472.4100	9.29667	212672	5520.6	213
61	Unknown 2	372.2485	9.355	107659	2659.2	255
62	Dimethyl(bis{[(2E,6E)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl]oxy})silane	500.4050	9.36	528709	10658	109
63	Sulfurous acid, butyl dodecyl ester	306.2229	9.4025	426280	9119.0	71
64	9,19-Cyclolanostan-3-ol, acetate, (3a)-	470.4124	9.74083	323764	7244.6	95
	Total			162790865	5219743	

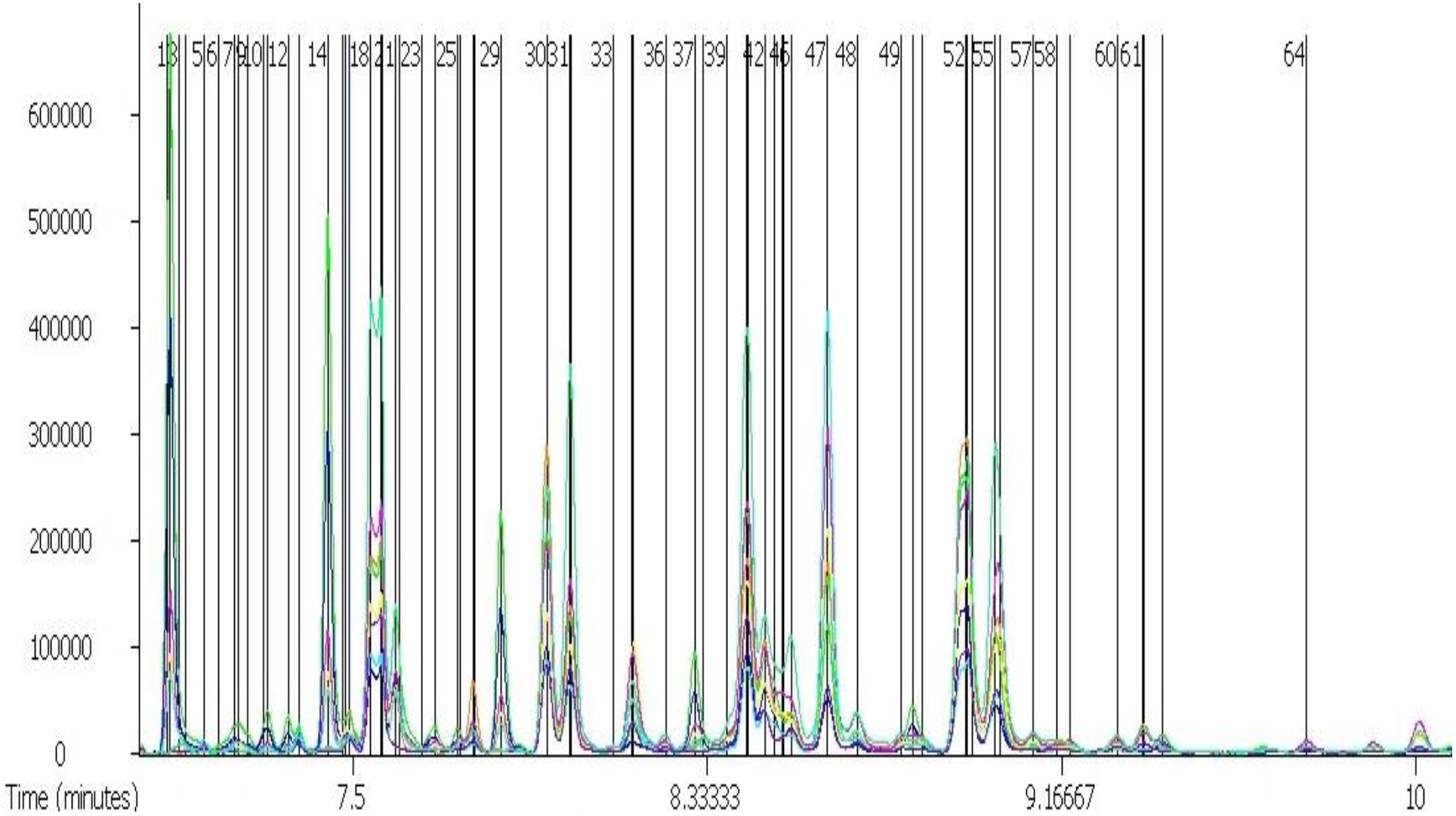


Figure 38: TIC of the 2L water sample taken from the Reed / River barrier, in which 64 compounds were, identified (Table 14)

Table 15: The 1L reference river water sample taken from the Mooi River downstream from the WWTP, yielded 11 different compounds, none of which were identified as faecal sterols.

PEAK #	NAME	EXACT MASS	R.T (MIN)	AREA	HEIGHT	UNIQUE MASS
1	Perylene-D12	264.1692	7.07833	11430645	358228	264
2	Hentriacontane	436.5008	7.085	15186717	523558	57
3	Perylene	252.0939	7.10417	61361445	2206271	252
4	Pentafluoropropionic acid, octadecyl ester	416.2714	7.24667	286891	5642.4	57
5	Heptacosane	380.4382	7.3125	350591	9240	57
6	Heptacosane	380.4382	7.36083	326561	9295.1	57
7	Heptacosane	380.4382	7.45667	7574871	230498	57
8	Dodecane, 1-fluoro-	188.1940	7.615	126811	2200.9	55
9	Heptacosane	380.4382	7.70333	91538	2153.6	71
10	Heptacosane	380.4382	7.865	2479044	63816	57
11	Heptacosane	380.4382	8.31833			57
	Total			99215115	3410904	

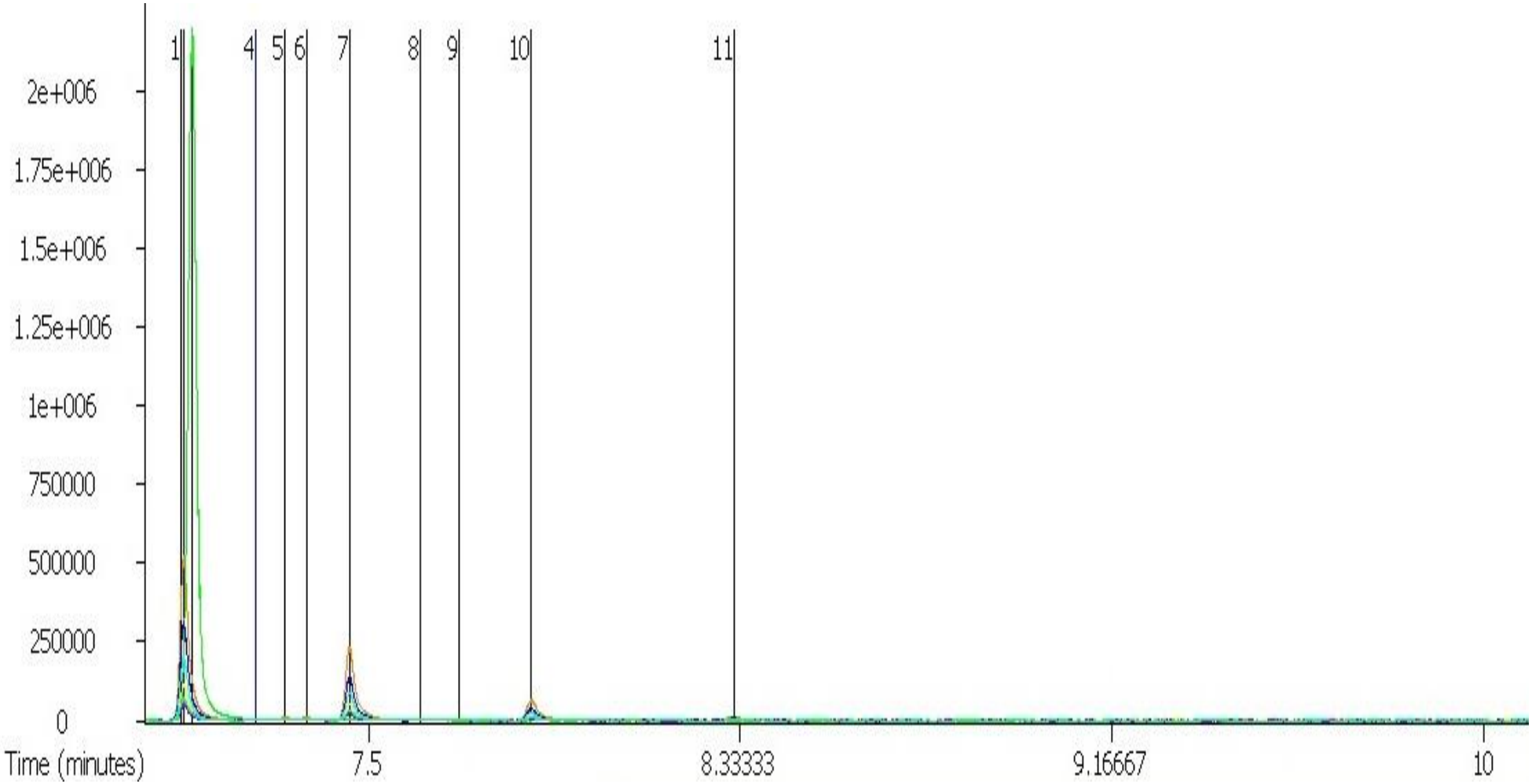


Figure 39: TIC of the 1L reference river water sample taken from the Mooi River, in which 11 compounds were, identified (Table 15)

Table 16: The 2L river water sample taken from the Mooi River downstream from the WWTP, yielded 35 different compounds including, Cholesterol, Dehydrocholesterol and Stigmastanol.

PEAK #	NAME	EXACT MASS	R.T (MIN)	AREA	HEIGHT	UNIQUE MASS
1	Perylene-D12	264.1692	7.06833	19070450	637390	264
2	Hentriacontane	436.5008	7.07583	63825316	2385879	57
3	Perylene	252.0939	7.09667	71717903	2608095	252
4	Dodecane, 2-cyclohexyl-	252.2817	7.15583	604780	21761	82
5	1-Octacosanol	410.4488	7.22917	122967	2847.6	112
6	Silane, trichlorodocosyl-	442.2356	7.24083	445988	11503	82
7	Decane, 2,4,6-trimethyl-	184.2191	7.26	511292	11328	84
8	1-Heptene, 4-methyl-	112.1252	7.2975	658769	16593	70
9	Heptacosane	380.4382	7.30417	298153	8825.5	113
10	Dotriacontane	450.5165	7.3525	849548	27992	56
11	Hentriacontane	436.5008	7.44667	44327043	1624993	57
12	Cyclopentane, heneicosyl-	364.4069	7.47833	633274	19053	68
13	Dodecane, 4-cyclohexyl-	252.2817	7.55	581520	19545	82
14	Lithocholic acid 3-acetate	418.3083	7.55417	404351	14649	215
15	Sulfurous acid, butyl octadecyl ester	390.3168	7.60583	26208	1077.1	468
16	Octadecyl trifluoroacetate	366.2746	7.6275	256113	5199.8	98
17	Sulfurous acid, hexyl pentadecyl ester	376.3011	7.64667	296999	6340.2	84

18	Ethanone, 1-(2,2-dimethylcyclopentyl)-	140.1201	7.6875	379614	8986.0	70
19	Heptacosane	380.4382	7.69667	141750	3794.1	113
20	Sulfurous acid, butyl heptadecyl ester	376.3011	7.7525	373415	11509	56
21	Hentriacontane	436.5008	7.85417	21252993	705475	57
22	Cyclopentane, heneicosyl-	364.4069	7.9	268030	7674.2	68
23	Cholesterol trimethylsilyl ether	458.3944	7.95917	4473253	167146	129
24	Cholestan-3-ol, acetate, (3a)-	430.3811	8.015	118071	3546.4	215
25	Oxalic acid, allyl tridecyl ester	312.2301	8.07417	158869	4067.7	85
26	Heptacosane	380.4382	8.13167	181756	4460.7	85
27	Sulfurous acid, butyl heptadecyl ester	376.3011	8.195	452428	12369	57
28	Hentriacontane	436.5008	8.30833	7870797	214268	57
29	9,19-Cyclolanostan-3-ol, acetate, (3a)-	470.4124	8.43167	137099	3659.8	107
30	10,12-Tricosadiynoic acid, trimethylsilyl ester	418.3267	8.4725	185135	3643.4	129
31	Oxalic acid, allyl octadecyl ester	382.3083	8.49667	510459	16309	496
32	Stigmasta-5,22-dien-3-ol, acetate, (3a)-	454.3811	8.62	71044	1977.2	97
33	Heptacosane	380.4382	8.82333	3161932	73399	57
34	Cholest-5-en-3-ol (3a)-, carbonochloridate	448.3108	8.93417	430468	12809	129
35	Sulfurous acid, butyl dodecyl ester	306.2229	9.41167			57
	Total			244797787	8678166	

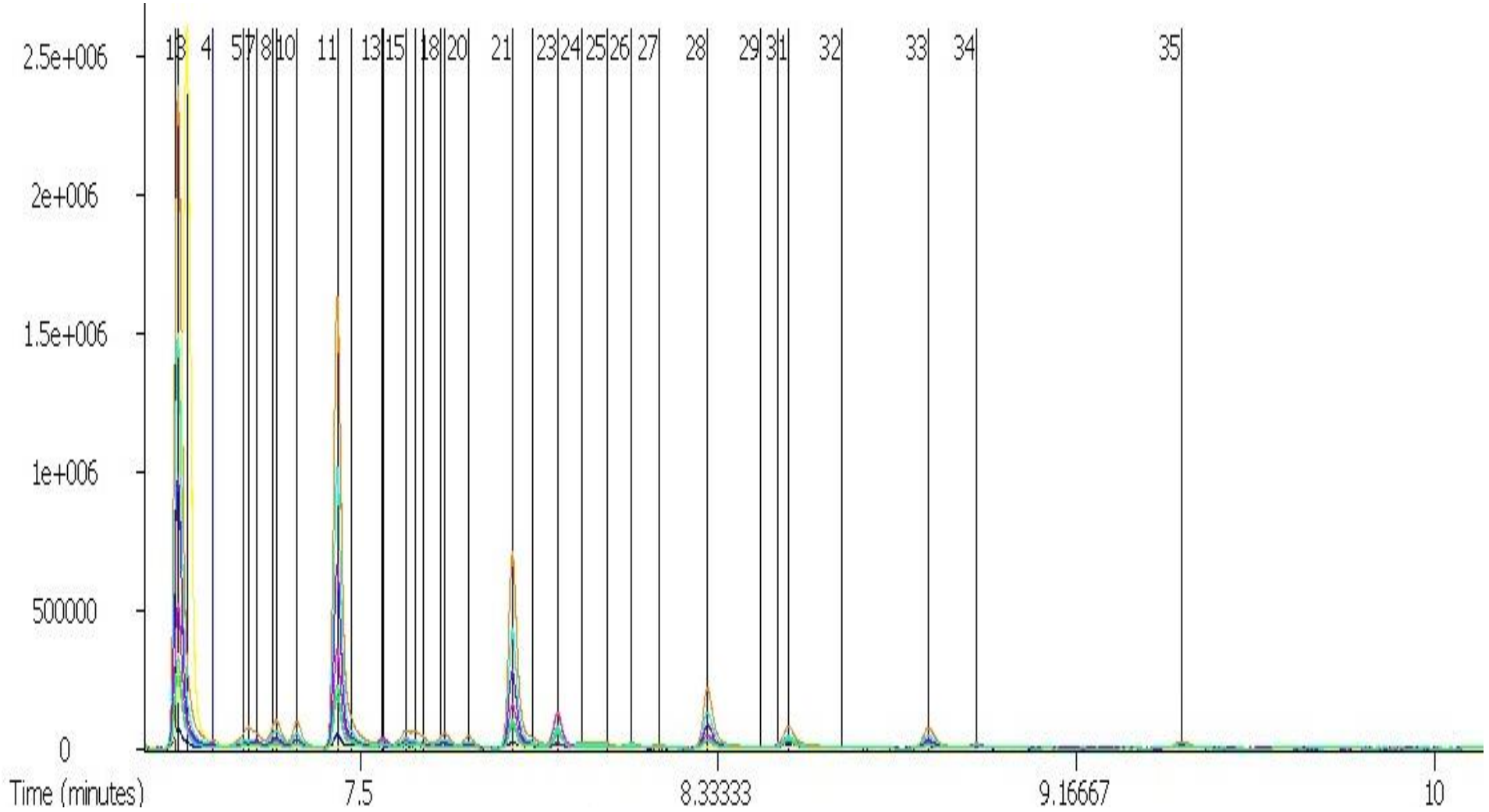


Figure 40: TIC of the 2L river water sample taken from the Mooi River. Thirty five compounds were identified (Table 16).