

**INVESTIGATING THE EFFICACY OF HYDROGEN PEROXIDE AGAINST
ENVIRONMENTAL *ESCHERICHIA COLI* STRAINS**

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DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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ABSTRACT

Surface water used for irrigation is often highly contaminated on a microbial level. Using contaminated surface water for the irrigation of fresh produce can lead to foodborne disease outbreaks and *Escherichia coli* has been a major cause of foodborne outbreaks associated with fresh produce over the past few years. There are many possible on-farm treatment options available to decrease the high microbial loads present in surface water, one of these is H₂O₂ and various factors can influence its use. The aim of this study was to determine the efficacy of H₂O₂ on different *E. coli* strains.

Water from the Plankenburg River was sampled and treated with (250, 300 and 350 mg.L⁻¹) H₂O₂ and the impact at 0, 30, 60, 90 and 120 min was then evaluated. It was found that the log reductions differed between samples. Log reductions ranged between 1.60 – 2.63 for Aerobic colony counts (ACC), total coliforms and *Escherichia coli*. The water was not considered safe for irrigation use although it had been treated with H₂O₂.

Reference (ATCC) and environmental *E. coli* strains were individually treated with H₂O₂ (250, 300 and 350 mg.L⁻¹) at 0, 30, 60, 90 and 120 min. Log reductions for the ATCC strains ranged between 2.13 – 5.48. This indicated a variation in H₂O₂ resistance between the different reference strains tested. Log reductions for the environmental *E. coli* strains ranged between 2.17 – 3.93. *Escherichia coli* M53 and MJ56 were the most resistant and most sensitive environmental strains to the H₂O₂ treatment, respectively. Once again it was observed that variations existed between the log reductions achieved for different strains. Overall, it was observed that the ATCC *E. coli* strains were more sensitive to the H₂O₂ treatments when compared the environmental strains. This indicates that ATCC strains should not be used for H₂O₂ treatment optimisation.

Certain factors can influence the efficacy of H₂O₂ such as concentration and organic matter (chemical oxygen demand) present in the water. Different H₂O₂ concentrations were evaluated (50, 350, 700 and 1 000 mg.L⁻¹) on two *E. coli* strains (M53 and W1371). Results indicated that 50 mg.L⁻¹ was not effective as less than 1 log reduction was achieved after 120 min. When 350 and 700 mg.L⁻¹ were used similar log reductions were achieved (1.78 – 2.27), which was not expected. Using 1 000 mg.L⁻¹ was considered an effective concentration that resulted in no growth present after 120 min. *Escherichia coli* strain W1371 carried EPEC virulence factors (potential pathogen). This was included in the study in order to determine how a strain carrying virulence factors would react to H₂O₂. *Escherichia coli* W1371 was considered resistant to the H₂O₂ treatment and log reductions were similar to that achieved for M53.

The catalase activity of the *E. coli* strains was studied to determine if a link existed between catalase activity and H₂O₂ resistance. Although a trend was observed between heat-stable catalase activity and H₂O₂ resistance, there were exceptions. It was concluded that high catalase activity does not always coincide with H₂O₂ resistance and that other mechanisms might also contribute to *E. coli* survival.

Overall, it was observed that there are certain factors that influence the efficacy of H₂O₂ as a treatment option. It can be concluded that environmental *E. coli* strains are generally more resistant to the H₂O₂ treatment compared to ATCC *E. coli* strains, this needs to be considered when using H₂O₂ or other chemical disinfectants as a treatment option.

UITTREKSEL

Oppervlakwater wat gebruik word vir besproeiing is dikwels op 'n mikrobiële vlak hoogs gekontamineer. Die gebruik van oppervlakwater vir die besproeiing van vars produkte kan tot die uitbraak van voedselgedraagde siektes lei. *Escherichia coli* was een van die hoofoorsake van voedselgedraagde uitbrake geassosieer met vars produkte gedurende die laaste paar jaar. Daar is verskeie moontlike behandelingsmetodes op plaasvlak beskikbaar om die hoë mikrobiële las in oppervlakwater te verlaag. Een hiervan is waterstofperoksied (H_2O_2) en verskeie faktore kan die gebruik hiervan beïnvloed. Die doel van hierdie studie was om die doeltreffendheid van H_2O_2 op verskillende *E. coli* isolate te bepaal.

Watermonsters uit die Plankenburg Rivier is behandel met drie konsentrasies H_2O_2 (250, 300 en 350 $mg.L^{-1}$) en die impak is na 0, 30, 60, 90 en 120 minute geëvalueer. Daar is gevind dat die log reduksies tussen monsters verskil het. Log reduksies het gewissel tussen 1.60 en 2.63 vir aerobiese kolonietellings (AKT), totale kolivorme en *E. coli*. Selfs na H_2O_2 behandeling, is die water nie as veilig vir besproeiing beskou nie.

Verwysingsisolate (ATCC) en omgewingsisolate van *E. coli* is afsonderlik met H_2O_2 behandel (250, 300 en 350 $mg.L^{-1}$) vir 0, 30, 60, 90 en 120 minute. Log reduksies vir die ATCC isolate het gewissel tussen 2.13 en 5.48. Hierdie verskille dui op die variasies wat tussen die getoetste verwysingsisolate voorkom. Log reduksies vir die omgewingsisolate het gewissel tussen 2.17 en 3.93. *Escherichia coli* M53 en MJ56 was onderskeidelik die mees weerstandbiedende en mees sensitiewe verwysingsisolate wat getoets is. Verskille in log reduksies het daarop gedui dat isolaat variasies voorkom. In geheel is dit gevind dat die ATCC *E. coli* isolate meer sensitief was vir die H_2O_2 behandelings vergeleke met die omgewingsisolate. Dit toon dat die ATCC isolate nie gebruik moet word vir H_2O_2 behandeling optimering nie.

Sekere faktore, soos die konsentrasie en organiese materiaal (chemiese suurstof vereiste) in die water, kan die doeltreffendheid van H_2O_2 behandeling beïnvloed. Verskillende H_2O_2 konsentrasies is geëvalueer (50, 350, 700 en 1000 $mg.L^{-1}$) op twee *E. coli* isolate (M53 en W1371). Resultate dui daarop dat 50 $mg.L^{-1}$ nie effektief was nie omdat minder as 1 log reduksie behaal is na 120 minute. Toe 350 en 700 $mg.L^{-1}$ gebruik is, is soortgelyke log reduksies (1.78 – 2.27) teen verwagting in behaal. Die gebruik van 1000 $mg.L^{-1}$ is as 'n effektiewe behandeling beskou aangesien daar geen groei na 120 minute teenwoordig was nie. *Escherichia coli* isolaat W1371 besit EPEC virulensie faktore (potensiële patogeen). Dit is in die studie ingesluit ten einde te bepaal hoe 'n isolaat met virulensie faktore sou reageer op H_2O_2 . *Escherichia coli* W1371 is as weerstandbiedend

teen die H₂O₂ behandeling beskou en log reduksies was soortgelyk aan die van M53 .

Die katalase aktiwiteit van die *E. coli* isolate is bestudeer om te bepaal of 'n skakel bestaan tussen katalase aktiwiteit en H₂O₂ weerstandbiedendheid. Alhoewel 'n tendens waargeneem is tussen hitte-stabiele katalase aktiwiteit en H₂O₂ weerstandbiedendheid, was daar uitsonderings. Die gevolgtrekking was dat hoë katalase aktiwiteit nie altyd saamval met H₂O₂ weerstandbiedendheid nie en dat ander meganismes ook mag bydra tot *E. coli* oorlewing.

In geheel is dit waargeneem dat daar sekere faktore is wat die doeltreffendheid van H₂O₂ as 'n behandelingsmetode beïnvloed. Daar is gevind dat omgewingsisolate van *E. coli* in die algemeen meer weerstandbiedend is teenoor H₂O₂ behandeling in vergelyking met ATCC *E. coli* isolate. Dit moet in ag geneem word wanneer H₂O₂ of ander chemiese ontsmettingsmiddels oorweeg word as 'n behandelingsopsie.

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CONTENTS

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Language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

ABBREVIATIONS

ACC:	Aerobic colony count
ATCC	American Type Culture Collection
CDC:	Centers for Disease Control and Prevention
COD:	Chemical Oxygen Demand
DAEC:	Diffusely Adhering <i>Escherichia coli</i>
DBP:	Disinfectant-by-products
DNA:	Deoxyribonucleic acid
DWA:	Department of Water Affairs
DWAF:	Department of Water Affairs and Forestry
<i>E. coli</i> :	<i>Escherichia coli</i>
EAEC:	Enteraggregative <i>Escherichia coli</i>
EHEC:	Enterohemorrhagic <i>Escherichia coli</i>
EIEC:	Enteroinvasive <i>Escherichia coli</i>
EPEC:	Enteropathogenic <i>Escherichia coli</i>
ETEC:	Enterotoxigenic <i>Escherichia coli</i>
ExPEC:	Extraintestinal pathogenic <i>Escherichia coli</i>
GDP:	Gross Domestic Product
HPA:	Health Protection Agency
HPI:	Hydroperoxidase I
HPII:	Hydroperoxidase II
HPP:	Hydrogen Peroxide Plus
HUS:	Haemolytic uremic syndrome
Hydroxyl radical:	$\cdot\text{OH}$
InPEC:	Intestinal pathogenic <i>Escherichia coli</i>
L-EMB	Levine Eosin Methylene-Blue Lactose Sucrose Agar
m-PCR:	Multiplex PCR
MH:	Mueller Hinton
MIC:	Minimum inhibitory concentration
NA:	Nutrient Agar
NaOCl:	Sodium hypochlorite
NB:	Nutrient Broth
NESID:	National Epidemiological Surveillance of Infectious Disease
NHLS:	National Health Laboratory Service

NICD:	National Health for Communicable Diseases
NNDSS:	National Notifiable Disease Surveillance System
NTU:	Nephelometric Turbidity Units
PCR:	Polymerase Chain Reaction
PSS:	Physiological saline solution
RNA:	Ribonucleic acid
ROS:	Reactive oxidative species
t-PCR:	Triplex PCR
TSA:	Tryptone Soy Agar
USEPA:	US Environmental Protection Agency
UTI:	Urinary Tract Infection
UV:	Ultraviolet
VRBA:	Violet Red Bile Agar
WHO:	World Health Organization
WWTW:	Waste water treatment works

CHAPTER 1

INTRODUCTION

Due to continuous population growth and economic development the demand for water has increased, therefore fresh water resources are limited (Guo *et al.*, 2009; Norton-Brandão *et al.*, 2013). Although water scarcity is considered a growing problem worldwide, South Africa's water resources are extremely limited (NWRS, 2004; Norton-Brandão *et al.*, 2013). The agricultural sector relies heavily on water, and dominates the use of water, as it is responsible for using 62% of the water available in South Africa (DWAF, 2009; Basson, 2011). South Africa depends mainly on surface water for its irrigation requirements (NWRS, 2004). In a country like South Africa where water scarcity exists, the quality of natural water resources is of utmost importance (Le Roux *et al.*, 2012). Many rivers in South Africa are considered unsuitable for irrigation of fresh produce, due to high levels of faecal contamination (Britz *et al.*, 2012). However, many farmers are forced to use water of marginal quality for agricultural use and using water of a compromised quality may have negative implications on agricultural sustainability as well as posing a threat to human health (Srinivasan & Reddy, 2009).

The number of foodborne disease outbreaks linked to fresh produce has increased over the years (Velázquez *et al.*, 2009). Irrigation water quality has been linked to outbreaks associated with fresh produce and therefore has been identified as an important potential source of contamination (Gelting & Baloch, 2013). Microbial pathogens that may be present in the surface water can cause serious health problems. Infants, elderly persons and people with compromised immune systems are considered at greater risk when exposed to these pathogens (Britz *et al.*, 2012). Therefore, in South Africa where a large proportion of the population is suffering from compromised immune systems, due to malnutrition or disease such as HIV/AIDS, this is considered a serious problem (Britz *et al.*, 2012). From previous studies it is clear that farmers need to treat surface water by implementing on-farm treatment systems to decrease the high microbial loads, prior to irrigation.

Faecal coliforms are important indicator organisms as many waterborne outbreaks are caused by faecal pollution (Yan & Sadowsky, 2005). *Escherichia coli* is also a common indicator organism for detecting faecal contamination of water, and is often used as part of water guidelines and regulations (Campos, 2008). Faecal coliforms and *E. coli* are seen as good indicator organisms as they are present in the faeces of humans and animals, they can be detected by simple methods, and they are not naturally present in

water (Campos, 2008). According to the WHO and DWA, faecal coliforms must be ≤ 1000 per 100 mL for wastewater irrigation or irrigation of any land or property, consequently the regulations regarding irrigation water are based on faecal coliforms (WHO, 1989; DWAF, 1996; DWA, 2013). For the above-mentioned reasons, *E. coli* was selected as the organism of choice for this study.

On-farm treatment options such as hydrogen peroxide (H_2O_2) are often being used to decrease the microbial loads of surface water used for irrigation. Hydrogen peroxide acts as a disinfectant due to the formation of strong oxidant chemical species including; singlet oxygen, superoxide radicals and hydroxyl radicals. These oxidising agents are reactive and can disinfect water by damaging lipids, proteins and DNA of microorganisms - exposed sulfhydryl groups and double bonds are easily targeted (McDonnell & Russell, 1999). Hydrogen peroxide has been used as a disinfectant for many years due to its advantages. The main advantage of H_2O_2 compared to other chemical treatment options is the lack of environmental toxicity, as H_2O_2 biodegrades to environmentally friendly by-products (hydrogen and water) without the formation of disinfectant-by-products (DBP's) (Linley *et al.*, 2012). This is an important factor to consider when selecting an on-farm treatment option.

Usually, well-characterised reference *E. coli* strains are used to determine the efficiency of treatment options (Koivunen & Heinonen – Tanski, 2005; Labas *et al.*, 2008). This is not considered the most accurate method to test the efficiency of treatment options as environmental *E. coli* strains may have developed resistance mechanisms that enable them to withstand higher concentrations of a specific chemical disinfectant (Maillard, 2007; Ortega Morente *et al.*, 2013).

Antibiotic resistant *E. coli* strains have been isolated from rivers in the Boland region (Lamprecht *et al.*, 2014). *Escherichia coli* strains showing antibiotic resistance are considered a serious health risk, as, if they do result in illness they are more difficult to treat due to their resistance. It is important that surface water treatment options are implemented in order to prevent carry-over of these strains from irrigation water to fresh produce. Many similarities and differences exist between the mechanisms by which *E. coli* can protect themselves against biocides and antibiotics (Russell, 2003). If these strains are able to resist biocide treatment there is an increased risk of contamination of fresh produce.

In summary, certain factors may influence the efficacy of H_2O_2 treatment. Environmental *E. coli* strains can be better adapted to adverse environmental conditions than reference *E. coli* strains. It is, therefore, important to determine if differences

between strains can also influence the way the strains react to biocides such as H₂O₂. *Escherichia coli* strains carrying virulence factors as well as antibiotic resistance have been isolated from rivers in the Boland region and a possibility exists that there is a link between virulence factors, antibiotic resistance and chemical resistance. Vargas *et al.* (2013) reported that the biocidal effect of H₂O₂ is decreased in water containing pollutants, as these pollutants are oxidised by the H₂O₂. The amount of organic material present in river water has been shown to vary (Britz *et al.*, 2013) and this could influence the efficacy of H₂O₂. *Escherichia coli* strains have shown to synthesise catalase enzymes, which degrade H₂O₂ into hydrogen and water consequently rendering it non-toxic. This could be possible mechanism for some *E. coli* strains to protect themselves against H₂O₂.

The overall aim of this study was to determine the efficacy of H₂O₂ on both environmental and reference *E. coli* strains. The study had the following objectives: determine the effect of H₂O₂ on river water samples; determine whether environmental *E. coli* strains are more resistant to the H₂O₂ treatment compared to ATCC strains; determine if a link exists between antibiotic and H₂O₂ resistance; determine the effect that different H₂O₂ concentrations had on the *E. coli* strains; establish how a pathogenic *E. coli* strain would react to the H₂O₂ treatment; determine if the organic matter (COD content) would influence the efficacy of the H₂O₂ treatment; and to determine if a link was observed between H₂O₂ resistance and increased catalase activity.

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CHAPTER 2

LITERATURE REVIEW

A. WATER SITUATION IN SOUTH AFRICA

South Africa is a water scarce country where the demand exceeds the natural water available (UNEPFI, 2009). Water scarcity is a critical issue especially in developing countries, as it can lead to a decrease in food production (FAO, 2007). A clean water supply is of utmost importance as almost every industry relies heavily on water to function productively (Tarver, 2008). The low rainfall, high evaporation rates, growing economy as well as the growing population, demands the availability of large amounts of water (CSIR, 2010).

The availability of water across South Africa varies due to the uneven distribution of the rainfall, therefore most inland areas are prone to drought (Van Heerden *et al.*, 2008). Due to the hot climate in South Africa, a large amount of precipitation is lost due to high evaporation rates (Van Heerden *et al.*, 2008; UNEPFI, 2009). As little as 9% of the rainfall in South Africa is converted to river runoff, which is then available as surface water (CSIR, 2013). South Africa stores the majority of its water in dams and water abstraction schemes, therefore allowing water to be accessible for agriculture, domestic and industry use (Paulse *et al.*, 2009). Surface water, ground water and return flows are the three sources, which are responsible for providing water in South Africa. Surface water is the most important source of water as 77% of the country's water comes from this source. Only 14% of the country's water comes from return flows and even less from ground water (9%) (DWAF, 2008). Surface water is the main source of water used for irrigation and industrial purposes in South Africa, unfortunately, surface water is susceptible to contamination and can lead to health problems (NWRS, 2004; Won *et al.*, 2013). Ground water is not easily available in South Africa as it is limited by the hard rock geology (CSIR, 2013). In the Western Cape, the harvest potential for ground water is 3.2 million m³ per annum. The Western Cape has more available ground water than other provinces in South Africa (Anon, 2005; UNEPFI, 2009). Ground water is more available in the southern areas of the Western Cape, and is often overexploited (Anon, 2005; UNEPFI, 2009).

Water is used in many different sectors, and the volume of water needed varies between these sectors (Table 1). In South Africa, the agricultural sector demands the most water compared to the other sectors (FAO, 2007). The irrigation sector in South Africa is responsible for 62% of the water withdrawals (Table 1) (DWAF, 2009). Large

volumes of water are not only needed for irrigation in South Africa, as 70% of the world's water withdrawals are for the agricultural sector (FAO, 2013).

Irrigation water of a suitable quality is one of the most important limiting factors for increasing food production in South Africa, as water scarcity can lead to decreased food production therefore negatively impacting food security (Hanjra & Qureshi, 2010). The availability of a clean water supply is not only critical to alleviate hunger in South Africa but it is also critical for the cultivation of fruit and vegetables. These are also important for South Africa's economy (DWAF, 2009).

Table 1 Water resource allocations per water user group (DWAF, 2009)

Water sector	Proportion of allocation (%)
Agriculture	62
Urban (domestic)	23
Rural (domestic)	4
Industrial	3.5
Afforestation	3
Mining	2.5
Power generation	2

Changes in the spatial distribution of rainfall and the availability of rainfall due to climate changes can impact the agricultural market, as less water is available for irrigation (Hanjra & Qureshi, 2010). The demand for water has increased worldwide but fresh water resources are declining, and therefore water scarcity is becoming a major problem in the agricultural sector (Hanjra & Qureshi, 2010; Norton-Brandão *et al.*, 2013).

Due to the increasing water scarcity problem that the world is facing, many arid and semi-arid countries such as South Africa, are forced to use low quality water for agricultural purposes. Using questionable quality water for irrigation may affect human health as well as influencing the sustainability of agriculture (Srinivasan & Reddy, 2009).

Another problem associated with irrigation water contamination, is the resistance of microorganism. Antibiotic and biocide resistance is increasing and becoming a major cause for concern.

B. AGRICULTURE IN SOUTH AFRICA

The fresh produce industry is an important, continuously growing sector in the global market (Joshi *et al.*, 2013). The demand for fresh fruit and vegetables has increased over the years, as consumers are becoming more aware of the health benefits (Matthews *et al.*, 2014). A variety of fruits and vegetables can be grown in South Africa due to the diverse weather conditions, the winter rainfall in the Boland region and the all year round rainfall of the Southern Cape (PDC, 2005; Ntombela, 2012). Fruit production has been increasing over the past few years, with 2.7 million tons being produced in 1981, which increased to 5.5 million tons by 2011 (Ntombela, 2012). Primary agriculture is of utmost importance to the South African economy. It contributes about 4% to the gross domestic product (GDP), which is relatively small, but the agro-processing industry contributes 20% to the GDP and is indirectly linked to agriculture as it relies on irrigation water (DWAF, 2009). Exports of agricultural products increased from 5% in 1988 to 46% in 2009 (Van Niekerk, 2012). The agricultural sector contributes about 7% to formal employment in South Africa. Employment increases in the summer months, as farmers need more workers during the harvesting period (DAFF, 2012; Van Niekerk, 2012). About 8.5 million people are dependent on agriculture either directly or indirectly for their employment, therefore indicating the importance of agriculture in South Africa (Van Niekerk, 2012). The fruit industry is one of the most important export markets in South Africa (Nieuwoudt *et al.*, 2004). Most of the fruit grown in South Africa is exported to Europe but this has extended to the Far East, USA as well as Africa. The fruit industry is one of the most important export industries as it contributes 20% of the total agricultural market (WESGRO, 2006). Vegetables in South Africa are mainly sold in the domestic market, between 2006 and 2011 the domestic market sales increased significantly. This was due to consumers becoming more aware of the health benefits of consuming fresh fruits and vegetables (Ntombela, 2012).

The gross farming income (GFI) increased from R27.8 billion (first quarter of 2011) to R30.8 billion (first quarter 2012) for total agricultural products. In the first quarter of 2012 the gross income from horticulture products was R9.2 billion and for the first quarter of 2011 it was R8.7 billion, therefore a 5.2% increase was achieved (DAFF, 2012). Between 2011 and 2012 income from fruit and vegetables increased by 11.5% and 6.7%, respectively (DAFF, 2012).

Agriculture is important for the economy of South Africa, but the microbiological quality of the fruits and vegetables being exported is of great importance as an outbreak of foodborne illness could result in a ban on the exporting of fresh produce to the European

market (Britz *et al.*, 2012). Therefore, it is important to ensure that the irrigation water quality in South Africa is of a safe microbiological standard to prevent food and waterborne outbreaks.

The poor quality of the rivers in South Africa negatively influences the economy, as polluted water needs to be treated to decrease the microbial level to an acceptable standard. The higher the microbial level the higher the treatment costs therefore costing the country more money (CSIR, 2010). It is important that the microbial quality of irrigation water and in turn fresh fruits and vegetables, be monitored as contamination can result in economic loss for the country (Chaidez *et al.*, 2005).

The agricultural sector relies on 62% of the countries water for irrigation, and is the most important limiting factor with regards to agriculture (DWAF, 2009). Therefore, it is of utmost importance that there is enough water of good quality in order to sustain the agricultural sector, to prevent negative impacts on the economy of the country.

C. IRRIGATION IN SOUTH AFRICA

Irrigation has been used for many years to increase the productivity of agriculture, especially in arid and semi-arid regions (Hanjra & Qureshi, 2010; Goldblatt, 2013). The use of irrigation stabilises food production and in turn stabilises food prices (Hanjra & Qureshi, 2010). Lack of water for irrigation is often the limiting factor when it comes to food production, especially in a water scarce country such as South Africa (Won *et al.*, 2013). Rivers, dams, ponds and streams are often utilised by farmers for irrigation, often these water sources do not meet the required standards for irrigation due to contamination, but farmers have no other choice but to use these water sources due to water scarcity (Ijabadeniyi *et al.*, 2011).

Modes of irrigation

As agriculture is the largest user of water it is important that effort be made to reduce the amount of irrigation water needed. If the amount of water needed for irrigation can be reduced more water will be available for the domestic sector (Reinders, 2011). The method of irrigation can influence the risk of microbial contamination, as different irrigation systems are available. Certain factors need to be considered before an irrigation system can be chosen (Bihn & Gravani, 2006). These factors include: capital cost to install the irrigation system; as well as the maintenance costs; energy availability and costs; labour skills needed; availability of water; quality of water; source of water; and the type of product being irrigated (Ross & Hardy, 1997). The method chosen should result in using

water effectively and therefore optimising the available water (Ross & Hardy, 1997). There are three main irrigation classifications, which include: flood irrigation; mobile irrigation; and static systems (Reinders, 2011).

Flood irrigation includes systems, which flood the surface in order to provide water. Flood irrigation systems have low capital costs as well as low energy requirements. The disadvantage of using flood irrigation is that water will be wasted if the system is not well maintained or designed incorrectly (Koegelenberg, 2007). Flood irrigation systems include furrow, short furrow, basin and border-based systems (Reinders, 2011).

Mobile irrigation systems are able to move as they apply water as they move by their own power generation. Examples of mobile irrigation systems include linear, travelling gun-spray and centre-pivot systems (Koegelenberg, 2007; Reinders, 2011). This type of irrigation is expensive but has low labour costs (Koegelenberg, 2007).

Static systems are systems that do not move while they are applying water. Static systems can be further divided into two types, which include sprinkler and micro-systems. Sprinkler systems apply water to the ground from a sprayer or sprinkler system, micro-systems include micro irrigation systems and drip irrigation (Koegelenberg, 2007). The different irrigation systems have different efficiencies (Table 2), which is an important factor especially when water scarcity is a problem (ARC-ILI, 2003).

Table 2 Different irrigation modes and the corresponding efficiencies (ARC-ILI, 2003)

Type of irrigation system	Efficiency (%)
Flood	
Furrows	60 - 80
Basins	60 - 80
Border	60 - 80
Mobile	
Linear	80
Centre-pivot	80
Static	
Micro-sprays	80

Drip irrigation has been known as an effective method to reduce microbial contamination risks by minimising water contact with the surface of the crop being irrigated. Mini-sprinklers and drip irrigation are the most effective irrigation methods to prevent contamination compared to other methods, where the water comes into contact with the crop (Jones & Shortt, 2010). Farmers should use drip irrigation for vegetables that are consumed raw as it reduces the risk of bacterial contamination when compared to spray irrigation (Ijabadeniyi & Buys, 2012). However, a study done by the Water Research Commission (WRC) confirmed that drip irrigation can be ineffective against preventing contamination if it is not maintained properly or if water quality is compromised (Reinders *et al.*, 2005).

South African irrigation water standards and regulations

The quality of irrigation water significantly affects the microbiological safety of the fresh produce being irrigated (Bihn & Gravani, 2006). In many instances, farmers often have no choice but to use water of a compromised quality or even wastewater (Qadir *et al.*, 2010). Some farmers also use wastewater deliberately without considering the health implications as wastewater contains nutrients that can be beneficial for farmers by decreasing fertiliser costs (Gemmell & Schmidt, 2012). Irrigation water quality standards are therefore very important in order to ensure that fresh produce is safe to consume, as health problems can be associated with using contaminated surface water to irrigate fresh produce (Ijabadeniyi & Buys, 2012).

Faecal coliforms are naturally found in the intestines of warm-blooded animals, and are used as an indication of sewage waste and faeces present in the water. Although faecal coliforms are not pathogenic, presence of faecal coliforms indicates that pathogenic viruses and bacteria may be present in the water (McCaffrey, 2011). According to the World Health Organization (WHO), wastewater used for irrigation of crops intended on being eaten raw should contain ≤ 1 intestinal nematode and ≤ 1000 faecal coliforms per 100 mL (WHO, 1989). According to the Department of Water Affairs (DWA) if using 2000 cubic meters of wastewater a day for irrigation the faecal coliforms cannot exceed 1000 faecal coliforms per 100 mL (DWA, 2013). The DWA also has water quality guidelines for irrigation water of fresh produce stating that faecal coliforms should not exceed 1 000 faecal coliforms per 100 mL (DWA, 1996). Only the WHO includes a standard for nematodes, but WHO and DWA do not include viruses or protozoan limits in the standards. *Cryptosporidium* and *Giardia* are protozoans that have been associated with

surface water used for irrigation but these protozoans are not often monitored as there are no regulations or limits to adhere to (Bihn & Gravani, 2006; Rad *et al.*, 2007).

Other important chemical standards include pH, chemical oxygen demand (COD), suspended solids, turbidity and electrical conductivity. The pH is considered an important variable when considering water quality, as pH is the measure of the concentration of hydrogen ions in the water. The pH must not be less than 5.5 or higher than 9.5 when wastewater is intended for irrigation use. However, according to the water quality guidelines the pH should be between 6.5-8.4 (DWA, 1996). Suspended solids refer to inorganic and organic matter, and these suspended particles are usually between 10 nm and 0.1 mm in size (Gray, 2010). Suspended solids content must not exceed 25 mg.L⁻¹ if the water is to be used for irrigation (DWA, 2013). Suspended solids have an influence on the turbidity of water. Turbidity indicates the cloudiness of the water, as it is a measure of the ability of light to pass through water. As with suspended solids, the turbidity reading is affected by both organic and inorganic components in the water (GHD, 2005; McCaffrey, 2011). Turbidity is often used to measure the suspended solids of the water in industry, and is measured in Nephelometric Turbidity Units (NTU) (GHD, 2005). Salinity refers to the measure of dissolved salt in the water, while electrical conductivity indicates the property of a substance to conduct electricity. According to DWA, electrical conductivity of wastewater intended for irrigation may range between 70 and 150 milliSiemens per m (mS.m⁻¹) (DWA, 2013). The COD has been used to measure the oxygen equivalent of the organic matter in water, by using a strong oxidant such as dichromate. The higher the COD value the more polluted the water is (Sincero & Sincero, 2003). There is no regulation with regards to COD levels for irrigation water. However, there is a regulation that states wastewater intended for irrigation must not exceed 75 mg.L⁻¹ (DWA, 2013).

Surface water quality in South Africa

Due to the increasing concerns regarding the deteriorating quality of surface water in South Africa, studies have been performed in order to achieve a better understanding of the problem (Paulse *et al.*, 2009; Ackermann, 2010; Gemmell & Schmidt, 2010; Huisamen, 2012; Le Roux *et al.*, 2012). Irrigation water quality has been linked to pathogens associated with fresh produce, which in turn leads to disease outbreaks (Gelting & Baloch, 2013).

Le Roux *et al.* (2012) investigated the microbial quality of the water in the upper Olifants River catchment, located in the Mpumalanga Province. Sampling took place at various sites along the river and *Escherichia coli* (*E. coli*) was found in varying numbers

along the different sites. Counts ranged from 34 to 44 110 MPN (most probable number).100 mL⁻¹. These results indicated microbial contamination of the river, with the highest counts of *E. coli* (13 632 to 44 110 MPN.100 mL⁻¹) occurring at sites located downstream from a wastewater treatment works (WWTW). This therefore, resulted in elevated *E. coli* levels due to faecal contamination. *Escherichia coli* levels were also elevated at a site located downstream from a cattle feedlot, which therefore led to faecal contamination from the animals. *Cryptosporidium* and *Giardia* were both detected at these sites. This was expected since protozoans are often associated with faecal contamination from both domestic sources and livestock animals (Traub, 2008; Castro-Hermida *et al.*, 2009; Le Roux *et al.*, 2012).

A study done by Gemmell & Schmidt (2012) on the Baynespruit River in Pietermaritzburg indicated high levels of contamination for samples taken in January and April. Total coliforms and faecal coliforms were monitored. Results indicated high levels of contamination with total coliforms and faecal coliform counts of > 160 000 and 35 000 MPN.100 mL⁻¹ and 33 000 and 7 000 MPN.100mL⁻¹, respectively. The river water was used directly to irrigate small-scale gardens, therefore the water had not been treated in order to decrease the microbial loads. This can lead to serious health problems (Gemmell & Schmidt, 2012). This faecal contamination was said to be caused from sewage entering the river, illegal dumping of faecal matter or contamination from animal feedlots.

Huisamen (2012) investigated the microbial levels in the Plankenburg and Eerste Rivers, which are both located in the Stellenbosch region. Results indicated that the Plankenburg River was highly contaminated with faecal matter. Faecal coliform counts up to 7 000 000 cfu.100 mL⁻¹ were found therefore indicating extreme faecal contamination of the river. The Eerste River had total coliform counts ranging from 330 to 35 000 cfu.100 mL⁻¹ and faecal coliforms ranging from 230 to 3 300 cfu.100 mL⁻¹ (Huisamen, 2012). Kayamandi is an informal settlement located near these rivers, and is one of the main factors leading to the excessive contamination. This informal settlement is under-serviced causing faecal contamination of the rivers (Nleya & Jonker, 2005). Ackermann (2010) also investigated the microbial loads of the Plankenburg River and found faecal coliforms ranging from 490 to 160 000 cfu.100 mL⁻¹, therefore, indicating faecal contamination of the river. Paulse *et al.* (2009) performed a study on the microbial contamination at various sites along the Plankenburg River. Results showed that the (most probable number) MPN of the, faecal coliforms and *E. coli* on average were very high and above regulations. Site B was the most contaminated with regards to MPN, faecal contamination and *E. coli* counts, this was expected, as it was the site located closest to the informal settlement.

The highest MPN of the faecal coliforms and *E. coli* were 9.2×10^6 , 3.5×10^6 and 3.5×10^6 , respectively.

In the Gauteng Province, the Jukskei River showed 13 million *E. coli* organisms per 100 mL, and the Umgeni River had counts of 1 100 000 cfu.100 mL⁻¹ (Ijabadeniyi & Buys, 2012). These are a few examples of contaminated rivers in South Africa. These results indicate high levels of contamination, which are above the DWA and WHO regulations. Therefore, the overall quality of the surface water in South Africa is seen as a cause for concern (WHO, 1989; Ijabadeniyi & Buys, 2012; DWA, 2013).

Factors affecting surface water quality

The demand for high quality water has increased in South Africa due to population growth, urbanisation and industrialisation. Many of the rivers in South Africa are highly contaminated and this is seen as a major problem as these rivers are often used for irrigation, such as the Eerste and Plankenburg Rivers in Stellenbosch (Britz *et al.*, 2013). Informal settlements, agricultural run-off, urban pollution and untreated wastewater are some of the contributing factors to the decreasing quality of surface water in South Africa (Anon, 2005).

A large proportion of the South African population lives in informal settlements or low cost housing (Gemmell & Schmidt, 2010). Dense informal settlements are situated along many rivers in the Western Cape and these informal settlements do not have adequate sanitation or waste disposal facilities. The lack of adequate facilities has led to high faecal contamination of nearby rivers (Barnes & Taylor, 2004). Human faeces are considered a higher risk than animal faeces as they contain human-specific enteric pathogens. However, animal faeces are still considered harmful (Chaidez *et al.*, 2005). Using highly contaminated surface water for irrigation can be dangerous and cause serious health problems (Barnes & Taylor, 2004). Contamination of irrigation water has been linked to pollution from informal settlements and, runoff from informal settlements is one of the major causes of pollution in the Western Cape (DWAF, 2005; UNEPFI, 2009). The rainy seasons often result in high faecal contamination in surface water due to faecal matter being washed into the rivers (Gadgil, 1998).

Population growth in South Africa has made managing wastewater a challenge, as the infrastructure is very out-dated and wastewater treatment works are not well maintained (Kühn *et al.*, 2000; Qadir *et al.*, 2010). Treatment plants are overloaded and need to be upgraded in order to effectively treat wastewater (CSIR, 2010). In many African countries wastewater infrastructure is considered a serious problem, as population

growth has exceeded the infrastructure and sanitation conditions have not improved (Qadir *et al.*, 2010). Mismanagement of treatment plants is a problem in South Africa and often leads to untreated wastewater being leaked out due to broken or incomplete systems (CSIR, 2010). Untreated wastewater could contain bacteria, protozoa, viruses as well as helminthes and therefore cannot be discharged into rivers without causing serious health problems (WHO, 2006). Wastewater that is insufficiently treated is often discharged into water systems, which is then used for irrigation (Qadir *et al.*, 2010).

Monitoring of water pollution was done in the Western Cape by the City of Cape Town, and certain incidences were thought to have increased the pollution (DWAf, 2005). In 2004, 78 888 sewage blockages occurred with six of these being serious as sewage overflowed into storm water systems. Forty-three sewer incidences occurred as a result of pump stations failing due to mechanical or electrical problems in the treatment plants (DWAf, 2005). The development of properly working wastewater treatment plants is seen as a long-term problem in developing countries due to financial management and technical constraints (Qadir *et al.*, 2010).

Faecal contamination problems from animal faeces are also a contributing factor to contaminated rivers (FDA, 1998; Goss & Richards, 2008). Some farmers use animal faeces as manure therefore increasing the risk of faecal contamination (Panigrahy *et al.*, 2011). All these factors are considered a major problem especially in rural areas as people rely on water that is usually untreated (Kühn *et al.*, 2000).

D. WATER AND FOODBORNE MICROBES ASSOCIATED WITH IRRIGATION WATER

Indicator organisms

Indicator organisms have been used for many years during water quality monitoring, due to the fact that testing for individual pathogens can be technically difficult, time consuming and expensive (Jamieson *et al.*, 2002). Bacteria that indicate the presence of other harmful pathogenic organisms are tested for, and these bacteria are then referred to as indicator organisms. Indicator organisms do not represent the concentration, presence or source of pathogens present, but rather give an indication of what is likely to be present.

Important factors need to be considered when selecting an indicator organism. These factors include: the indicator organisms should be present in the intestines of warm-blooded animals; needs to be present in larger numbers than the pathogen of interest; must be present when the pathogen is present; should be able to multiply in the

environment and be equally resistant to the environment as the pathogen; should be easily identified; and should be non-pathogenic (Bitton, 1999).

Coliforms are the most common indicator organisms used for determining water quality, as they can survive for long periods of time in water and they are easily detected. The U.S. Public Health Service first identified the use of the coliform group as an indication of faecal contamination of drinking water in 1914 (Bitton, 1999). Coliforms do not necessarily indicate faecal contamination, but further tests can be performed in order to detect faecal coliforms, which in turn would confirm the presence of faecal contamination (Jamieson *et al.*, 2002). Faecal coliforms are important indicator organisms as many waterborne outbreaks are the result of faecal pollution (Yan & Sadowsky, 2005). *Escherichia coli* is also a common indicator organism for detecting faecal contamination of water (Campos, 2008). Faecal coliforms and *E. coli* are seen as good indicators as they are present in faeces of humans and animals and they can be detected by simple methods (Campos, 2008). Some *E. coli* are however capable of replicating and surviving in natural environments, such as surface waters and soils (Ishii & Sadowsky, 2008). This may influence water quality monitoring (Goto & Yan, 2011). Thus, the presence of *E. coli* alone may not indicate faecal contamination but rather indicate the persistence of specific strains in the environment (Ishii & Sadowsky, 2008; Janezic *et al.*, 2013). However, the presence of *E. coli* in surface waters is still a cause for concern, in terms of public safety (Janezic *et al.*, 2013).

Total coliforms, faecal coliforms and *E. coli* are the most common indicator organisms and they are still used as part of most water quality guidelines and regulations. These indicator organisms usually indicate the presence of the following human pathogens; *Shigella*, Enterotoxigenic *E. coli*, *Campylobacter* and *Vibrio cholerae* (Campos, 2008). Although coliforms and *E. coli* are used as indicator organisms in most regulations it is important to note that coliform indicators do not indicate whether oocysts or cysts are present in the water, therefore the presence of protozoans such as *Cryptosporidium* and *Giardia* need to be tested for before the water is considered safe (Smith & Nichols, 2010). The survival pattern of faecal coliforms differs from protozoan cysts and viruses and therefore is not a reliable indication of these organisms (Bitton, 1999). On the negative side, the absence of indicator organisms in a water system does not indicate the absence of parasites, viruses or protozoan cysts (Gadgil, 1998).

Coliforms

Coliforms are part of the *Enterobacteriaceae* family and are found in the faeces of warm-blooded animals, soil and decaying organic matter (Jamieson *et al.*, 2002; Prescott *et al.*, 2002). Coliforms make up about 10% of the intestinal microorganisms of humans. These bacteria are facultative anaerobes, rod shaped, Gram-negative and oxidase negative organisms that do not produce spores. Coliforms are lactose-fermenting bacteria that produce gas during fermentation (within 48 h at 37°C) (Prescott *et al.*, 2002). *Enterobacter aerogenes*, *Klebsiella pneumonia* and *E. coli* are examples of coliform bacteria. Coliforms are divided into two groups that include total coliforms and faecal coliforms, where total coliforms refers to the entire group of coliforms whereas faecal coliforms refers only to coliforms originating from the intestines of warm-blooded animals (Jamieson *et al.*, 2002). Faecal coliforms differ from coliforms, as they are able to ferment lactose with the production of gas and acid at elevated temperatures (at 44.5°C within 24 h) (Jamieson *et al.*, 2002).

Escherichia coli

A German bacteriologist, Theodor Escherich in 1885, first reported the isolation of *E. coli* from a faecal sample. In 1888, the organism was named *Escherichia coli* (Bhunia, 2008). *Escherichia coli* forms part of the *Enterobacteriaceae* family and is found in the gut of healthy warm-blooded animals. Most *E. coli* are considered harmless and are essential organisms in the gastrointestinal tract of animals and humans (Ingerson-Mahar & Reid, 2011). Certain *E. coli* strains are considered beneficial to mammals as they aid in digestion and protect the body against other microbes, which may be harmful (Ingerson-Mahar & Reid, 2011).

Pathogenic *E. coli* strains are becoming a cause for concern due to many foodborne outbreaks being associated with these strains (Bhunia, 2008). *Escherichia coli* O157:H7 is a common pathogenic strain of *E. coli*, and is responsible for many water and foodborne illnesses. This strain of *E. coli* produces a toxin, which can lead to haemolytic uremic syndrome (HUS) (Jamieson, 2002; Rangel *et al.*, 2005; Chigor *et al.*, 2010).

Categorisation of E. coli strains

Escherichia coli strains are categorised into three main groups, based on genetic and clinical differences. Genetic differences between *E. coli* strains determine whether they can cause disease and whether they can cause intestinal or extraintestinal disease (Russo & Johnson, 2003). These three groups include; commensal strains, intestinal pathogenic

strains (InPEC) and extraintestinal pathogenic strains (ExPEC) (Table 3) (Russo & Johnson, 2000).

Commensal *E. coli* are found in the gut of healthy humans and animals, and are not generally considered harmful to the host. The virulence genes that are present in both intestinal and extraintestinal *E. coli* strains are not usually present in commensal *E. coli* strains and therefore they do not result in disease (Russo & Johnson, 2000). However, commensal *E. coli* are capable of causing extraintestinal infections in certain circumstances. This can be in situations where the host is immunocompromised, as well as a result of a foreign body (due to the presence of an urinary catheter) (Russo & Johnson, 2003).

Pathogenic *E. coli* strains are responsible for many diseases including: urinary tract infections (UTI); HUS; septicaemia; pneumonia; meningitis; and gastroenteritis (Bhunia, 2008). Pathogenic *E. coli* strains are capable of expressing different virulence factors, which are located on the chromosomes, plasmids or genomes of pathogenic *E. coli* (Mühldorfer & Hacker, 1994). Only certain combinations of virulence factors are capable of causing disease in healthy humans. These combinations of successful virulence factors are referred to as pathotypes (Kaper *et al.*, 2004). Intestinal pathogenic *E. coli* are categorised into six pathotype groups including: enterotoxigenic *E. coli* (ETEC); enteropathogenic *E. coli* (EPEC); enterohemorrhagic *E. coli* (EHEC); enteroinvasive *E. coli* (EIEC); enteroaggregative *E. coli* (EAEC); and diffusely adhering *E. coli* (DAEC) (Bhunia, 2008). *Escherichia coli* O157:H7 is the most well-known *E. coli* strain in the EHEC grouping and has been recognised as a food pathogen since 1982 (Schlundt, 2002). Due to the increase in *E. coli* outbreaks it has been suggested that an increase in vertical and horizontal gene transfer of pathogenic genes between *E. coli* and other bacterial species is occurring (Bhunia, 2008). Pathogenic *E. coli* are capable of passing through the digestive tract, as would harmless *E. coli*, therefore no special genes are required for pathogenic strains to enter the digestive system (Ingerson-Mahar & Reid, 2011).

Escherichia coli strains that have the ability to cause extraintestinal infection are referred to as ExPEC *E. coli*. ExPEC *E. coli* strains are unable to cause gastrointestinal disease in humans, however they are considered effective intestinal colonisers. Therefore, ExPEC *E. coli* strains may inhabit the intestines of healthy humans in a commensal manner, and only result in disease when they exit the gut and enter another body site (Johnson & Russo, 2002). The pathogenicity of ExPEC *E. coli* strains are linked to the presence of several virulence genes (Pitout, 2012). ExPEC *E. coli* strains are

responsible for causing urinary tract infections (UTI), enteric infections and systemic infections in humans (Pitout, 2012).

Escherichia coli strains can be divided into four main phylogenetic groups including; A, B1, B2 and D, which can then further be divided into seven subgroups including; A₀, A₁, B1, B2₂, B2₃, D₁ and D₂ (Carlos *et al.*, 2010). *Escherichia coli* vary in their ability to cause disease, characteristics and environmental niches, and therefore are divided into different phylogroups (Gordon *et al.*, 2008).

Table 3 Characterisation of the behaviour of the three main *E. coli* groups (Johnson & Russo, 2002)

<i>Escherichia coli</i> pathotype group	Asymptomatic intestinal colonisation	Diarrhoea	Extraintestinal infection
Commensal	+++	-	+
InPEC	-	+++	-
ExPEC	+	-	+++

Pathogens associated with fresh produce

Pathogenic bacteria associated with fresh produce have become a worldwide problem. Pathogenic bacteria frequently associated with fresh fruit and vegetables include: *E. coli* O157:H7; *Listeria monocytogenes*; *Salmonella spp*; and *Shigella spp*. The important pathogenic protozoans include *Cryptosporidium* and *Giardia* (Table 4). Contamination of fruit and vegetables can be associated with manure, soil, contaminated irrigation water, as well as from wild animals such as birds or reptiles (Beuchat, 2006).

Pathogenic bacteria can move into the interior of the plant when water moves inside the plant via capillary action, they can also enter if the surface of the fruit or vegetable is damaged due to bruising or cuts (Janisiewicz *et al.*, 1999). Pathogens such as *E. coli* O157:H7 and *Listeria monocytogenes* can attach to the damaged surface and enter the internal structure of the plant. When a plant has a cut, nutrients are exposed which therefore enables the pathogen to grow (Itoh *et al.*, 1998; Guo *et al.*, 2001). Contaminated

water can also enter the plant through the roots, especially if flooding has occurred (Lynch *et al.*, 2009).

Once the pathogenic bacteria enter the interior of the plant, disinfection and surface washing are not effective (Lynch *et al.*, 2009). The phylloplane refers to the leaf surface of the vegetable and pathogens can survive on the phylloplane and cause disease when consumed (Heaton & Jones, 2008). Studies indicate that *E. coli* O157:H7 inoculated onto lettuce leaves can be isolated 15 days after inoculation indicating the survival of *E. coli* O157:H7 on the lettuce leaf (Beuchat, 1999). *Escherichia coli* O157:H7 and *Salmonella* can survive for up to 177 and 231 days respectively, on parsley, which is considered a long time for survival (Islam *et al.*, 2004a; Islam *et al.*, 2004b). Bacteria, fungi and yeasts can form a complex structure (biofilm) on the plant surface. Bacteria incorporated in the biofilm can survive for longer periods of time due to cells being enclosed by an exopolymeric matrix, which can protect the cells against environmental changes (Montier & Lindow, 2005).

Leafy vegetables are often associated with high levels of mesophilic and psychrotrophic microorganisms. It is important to note that produce does not have to indicate visible signs of deterioration to be considered a food safety risk. Fresh produce may look appealing yet be highly contaminated with pathogenic bacteria and therefore can be deceiving (Abadias *et al.*, 2008). Different pathogenic microorganisms are associated with different fruits vegetables. Vegetables that are grown in direct contact with soil and manure (fertilisers) such as carrots can also be susceptible to contamination. Vegetable surfaces that are not in direct contact with soil can be easily contaminated due to open leaf structure such arugula and spinach (Abadias *et al.*, 2008). Sprouts have been associated with many foodborne outbreaks due to pathogen growth during sprouting. Low levels of bacteria may be present on sprout seeds, however during sprouting (3 – 10 days) bacteria can multiply to high levels. The bacteria can survive during refrigerated temperatures (Harris *et al.*, 2003). Fruits are usually better protected against contamination compared to other fresh produce due to the low pH and low storage temperature. The cell barrier of fruits are often strong enough to protect the fruit from coming into contact with soil or manure (Abadias *et al.*, 2008). However, fruits that do not have strong protective barriers, such as berries, have been associated with many foodborne outbreaks (Heaton & Jones, 2008).

There are many chemical treatment options that may be used to treat high microbial loads associated with fresh produce. However, many of the disinfection processes are not

effective at eliminating high microbial loads associated with fresh produce (Beuchat, 1998).

Water and foodborne outbreaks

The risks associated with consuming fresh produce have increased over the years. These products are consumed with minimal processing and therefore safety is a concern (Harris *et al.*, 2003; Selma *et al.*, 2008). However, consumers are encouraged to consume more fruits and vegetable as part of a healthy lifestyle and as result foodborne outbreaks linked to fresh produce have increased (Matthews *et al.*, 2014). Contamination of fresh produce can occur via animals, human handling, soil or water (Harris *et al.*, 2003). Contaminated irrigation water is seen as a potential source for fresh produce outbreaks (Beuchat, 2002).

An outbreak is defined when two or more cases of a specific infectious illness are linked by microbiological evidence, when an outbreak is thought to be caused by food or water it is considered a foodborne outbreak (Roberts, 2000). Foodborne disease outbreaks are considered a serious problem throughout the world due to negative implications on human health (Dewaai *et al.*, 2010).

Most outbreaks are not reported and therefore the extent of the problem is difficult to understand without sufficient data (Schlundt, 2002). More waterborne outbreaks are documented and reported than foodborne outbreaks. Foodborne outbreaks are often widely spread throughout the world due to importing and exporting, whereas waterborne outbreaks usually originate from a water distribution network and are easier to trace and monitor (Smith *et al.*, 2007). Due to the increase in food and waterborne outbreaks and the insufficient data available, the Centers for Disease Control and Prevention (CDC) was established in the USA, to form an outbreak surveillance system to control and prevent further outbreaks (Shlundt, 2002; Baldursson & Karanis, 2011). Many other countries have formed surveillance agencies to report and document food and waterborne outbreaks, and these include: US Environmental Protection Agency (USEPA), in the USA; in Japan the National Epidemiological Surveillance of Infectious Diseases (NESID) was developed in 1981; the National Notifiable Disease Surveillance System (NNDSS) in Australia in 1990; and the Health Protection Agency (HPA) was developed in 2003 in the United Kingdom (Baldursson & Karanis, 2011). Only a small proportion of outbreaks are reported and investigated, even though systems are implemented. About 500 outbreaks are reported to the CDC every year with many outbreaks not being reported or investigated (Angulo *et al.*, 1998).

The National Institute for Communicable Diseases (NICD), which is a division of the National Health Laboratory Service (NHLS), was implemented in South Africa. Data on reporting of outbreaks was scarce in South Africa and the NICD was implemented to help improve reporting and laboratory testing of outbreaks (NHLS, 2014).

It has been estimated that in the USA, pathogens were responsible 9.4 million foodborne illnesses, of which 3.6 million were a result of bacteria (Scallan *et al.*, 2011). Outbreak associated illnesses are seen as a serious problem in a country like South Africa, as HIV/AIDS has become an epidemic and a large proportion of the population have a compromised immune system. Hence, these food and waterborne microorganisms can lead to severe illness or even death (Paulse *et al.*, 2009; Gemmell & Schmidt, 2010).

Escherichia coli O157:H7 is a pathogenic microorganism responsible for causing 73 000 illness in the USA annually. Between 1982 and 2002, 350 outbreaks were reported to the CDC in the USA, of the 350 reported cases, 52% were foodborne outbreaks and 9% were waterborne outbreaks (Rangel *et al.*, 2005). *Escherichia coli* O157:H7 has become a recognized pathogen that is associated with foodborne outbreaks. The infectious dose for *E. coli* O157:H7 is very low and results in HUS (Harris *et al.*, 2003).

In 2006, 26 States in the USA were affected by an *E. coli* O157:H7 outbreak associated with spinach. The outbreak resulted in three deaths and 199 illnesses (CDC, 2014a). In 2010, shredded Romaine lettuce was linked to an outbreak of *E. coli* O145, 26 people over five states in the USA were infected (CDC, 2014b). In 2011, 852 patients were reported suffering from HUS. Sprouts contaminated with *E. coli* O104:H4 were responsible for the outbreak and resulted in 32 deaths (CDC, 2014c). In 2014, 19 people were infected with *E. coli* O121, after consuming clover sprouts, but no deaths were reported (CDC, 2014d). These are a few examples of pathogens resulting in outbreaks. Thus, it can be concluded that *E. coli* outbreaks linked to fresh produce are a serious problem.

Table 4 Characteristics of specific pathogens associated with fresh produce related illness (Harris *et al.*, 2003)

Microorganisms	Typical incubation period	Symptoms	Infectious dose (number of cells)	Source
Bacteria				
<i>Escherichia coli</i> O157:H7	2 - 5 days	Bloody diarrhoea, abdominal pain, can lead to haemolytic uremic syndrome and kidney failure	10 – 1 000	Animal faeces and cross contamination
<i>Salmonella</i> spp.	18 – 72 hours	Abdominal pain, diarrhoea, chills, nausea	10 – 100 000	Animal and human faeces
<i>Shigella</i> spp.	1-3 days	Abdominal pain, diarrhoea, fever, vomiting	About 10	Human faeces
<i>Listeria monocytogenes</i>	1 – 5 days or weeks	Febrile gastroenteritis in healthy adults, severe septicaemia and meningitis in neonates and immunocompromised adults	Unknown dependent upon health of individual	Soil, food processing environments
Protozoa				
<i>Cryptosporidium</i> spp.	1 – 12 days	Profuse watery diarrhoea, abdominal pain and vomiting	About 30	Animal and human faeces

E. ON-FARM WATER TREATMENT OPTIONS

As irrigation is considered the largest global form of water consumption, there is a need to develop new technologies to improve water decontamination. With the growing population, more pressure is placed on the agricultural sector in order to keep up with the growing food demands (Polo-López *et al.*, 2011).

Studies have been done on the surface-water quality in the Boland region, due to the increasing concerns regarding the deteriorating quality of surface-water in South Africa (Britz *et al.*, 2013). This is of great concern as these rivers are often used for irrigation of fresh produce. The Plankenburg and Eerste Rivers are both used for irrigation in Stellenbosch due to availability and easy accessibility of water (Britz *et al.*, 2013). However, from previous studies it can be seen that the water is of low microbial standard and could lead to water or foodborne outbreaks (Britz *et al.*, 2013). To minimize health risks associated with the use of contaminated river water, on-farm treatment options need to be implemented in order to decrease the high microbial loads present in surface water (Britz *et al.*, 2012). There are many on-farm water treatment options available to decrease high microbial loads present in surface water, these include physical and chemical treatment options (Raudales *et al.*, 2014). Physical treatment options include filtration and ultraviolet radiation. Chemical treatment options include; chlorine, ozone and hydrogen peroxide (H₂O₂) (Raudales *et al.*, 2014).

Physical treatment options

Slow sand filtration

Slow sand filters have been used for many years and were originally used to improve the quality of drinking water. The first sand filter plant was built in London in 1829 and was built in order to treat the water supplies. Other countries also started using sand filters due to cholera outbreaks and deaths due to waterborne outbreaks (GHD, 2005; Parsons & Jefferson, 2006).

Mode of action

Slow sand filtration involves filtering water through a bed of sand, then introducing influent water over the surface of the filter, which is then drained from the bottom. On the surface of the sand, a biologically active layer is formed from algae, protozoa and bacteria found in water. This biologically active layer is referred to as the “*schmutzdecke*” or “dirty skin” (Parsons & Jefferson, 2006). The “*schmutzdecke*” layer is responsible for both the

physical and biological treatment of the water (Gray, 2010). In order to clean the filter, the water is drained below the sand and the “*schmutzdecke*” layer is physically removed, the first 50 cm of sand is also removed and new clean sand is added. The quality of water, time of year and filter loading rate will determine how often the filter will need to be cleaned. In summer, the filter will need to be cleaned more often due to the increase in water temperature and sunlight, which increases the growth of algae. The filter needs to be re-sanded every 1 - 6 months, but the time may vary depending on the quality of the water, it may only need to be done after 2 years if the water quality is good (Binnie *et al.*, 2002).

The design of a sand filter includes; bed of sand, layer of gravel, system of under drains and a flow regulator. The depth of a sand filter is usually about 1 m, with the gravel layer being about 0.3 - 0.5 m. An autotrophic layer develops at the top 2 mm of the sand, which consists algae and nitrifying bacteria (Gray, 2010). Below the autotrophic layer heterotrophic bacteria are present, this is the layer where organic matter is removed (Gray, 2010). Sand filters operate at a flow rate of about 0.12 - 0.25 m.h⁻¹ and the rate is controlled by the flow regulator controls (GHD, 2005).

Advantages and disadvantages

Sand filters are used in many parts of the world, as they are successful at removing protozoans, which are chlorine resistant. The quality of the water after being treated with a sand filter is very good, having a turbidity of about 1 NTU. Most harmful bacteria are removed without the use of chemicals, therefore no disinfectant-by-products (DBP's) are formed. If river water is used for irrigation it may be necessary to include a settlement stage to remove silt, usually water with a turbidity of 30 NTU can be added straight to the filter, otherwise the water must be pre-treated (Binnie *et al.*, 2002).

It is important to note that slow sand filters can take a few hours to effectively remove microorganisms. If river water is used the turbidity as well as algae growth can be problematic with regards to the filtering process. The high turbidity levels and subsequent algae growth can clog the filters (NESC, 2000; Binnie *et al.*, 2002). Although slow sand filtration is not considered an expensive treatment option, a large amount of open land is needed (Gadgil, 1998).

Ultraviolet radiation

Ultraviolet radiation (UV) has been used to disinfect drinking water for many years. The germicidal properties of sunlight was discovered by Downes and Blunt already in 1878

(EPA, 2006). When it was realised that the UV radiation was an effective treatment for the inactivation of *Cryptosporidium* and *Giardia*, the use of UV systems increased (Hijnen *et al.*, 2006).

Ultraviolet radiation refers to radiation in the electromagnetic spectrum, ranging from 100 - 400 nm, and lies between the X and the visible spectrum. The UV spectrum can be divided into four wavelength groups: UV-A (315 - 400 nm); UV-B (280 - 315 nm); UV-C (200 - 280 nm); and vacuum UV (100 - 200 nm) (EPA, 2006; Ratnayaka *et al.*, 2009). Germicidal disinfection takes place between 200 - 300 nm, which includes UV-B and UV-C wavelengths. When referring to DNA, maximum absorption takes place at 260 nm. The best germicidal properties are between wavelengths 220 and 300 nm and these wavelengths are effective against: viruses; bacteria; algae; protozoa; yeasts; and moulds (Guerrero-Beltrán & Barbosa-Cánovas, 2004). Although the sun produces ultraviolet light, ozone prevents large amounts of UV-B and UV-C from reaching the earth (Wright & Cairns, 2013).

A mercury arc lamp is used to generate UV radiation and consists of a hermetically sealed tube containing quartz, and electrodes on opposite ends. An inert gas and mercury are situated inside the tube (Wright & Cairns, 2013). The UV radiation is generated by passing an electrical arc through the mercury vapour resulting in a discharge of photons. UV radiation is released when the excited mercury vapour returns to its lower energy state (EPA, 2006; Ratnayaka, 2009).

Mode of action

UV radiation can inactivate a wide range of microorganisms due to the germicidal properties between 100 - 280 nm. Disinfection takes place by the absorption of UV photons (produced at the different wavelengths) by the DNA (Deoxyribonucleic acid) or RNA (Ribonucleic acid) of the microorganisms and therefore disrupting the nucleic acid structure. As a result of the disruption of the DNA or RNA, the microorganisms are unable to cause disease, as they can no longer replicate (Bolton & Cotton, 2008).

DNA is comprised of sugar molecules and phosphate, which make up the backbone of DNA, and nucleotides (Kalisvaart, 2011). Nucleic acid is present in both DNA and RNA and is made of purines and pyrimidine which are the building blocks of both DNA and RNA, the purines (adenine and guanine) are the same for both DNA and RNA but the pyrimidines differ. For DNA they include thymine and cytosine and for RNA they include uracil and cytosine (EPA, 2006). The sugar and phosphate backbone of the DNA does not absorb UV radiation above 210 nm and therefore the nucleotides are responsible for

the absorption (Kalisvaart, 2011). Proteins absorb most of the UV light at wavelengths below 230 nm, but nucleotides are responsible for most of the UV absorption at wavelengths above 230 nm (Bolton & Cotton, 2008). The aromatic amino acids are capable of absorbing UV light above 210 nm, with an absorption peak at 280 nm. These amino acids include tryptophan, phenylalanine and tyrosine with tryptophan being the strongest absorber at 280 nm (Bolton & Cotton, 2008). Higher UV doses are needed to disrupt proteins and therefore, the disruption of DNA or RNA is the more common mechanism of disruption at lower doses (Bolton & Cotton, 2008). Both purines and pyrimidine's absorb UV light, the absorption of UV light results in the formation of pyrimidine dimers (bonds between adjacent nucleotides are formed). The DNA exposed to the UV radiation results in the electrons in the bases becoming energised and form covalent bonds joined by cyclobutane between the adjacent nucleotides. Pyrimidine dimers are finally formed (Zimmer *et al.*, 2003; Kalisvaart, 2011). The formation of dimers is responsible for 90% of the UV damage (Kalisvaart, 2011).

Advantages and disadvantages

Due to the problems associated with chemical water treatments, alternative methods have been studied. Ultraviolet radiation can be used as an effective method for decreasing the microbial load of irrigation water. The most important advantage of using UV radiation is that it is a physical rather than chemical treatment option (EPA, 1999a). Thus, no toxic by-products are formed during disinfection and there is no need to transport, store or use hazardous chemicals. Other advantages are: the equipment needed for UV systems are reliable and user friendly; and UV radiation can disinfect the water at a rapid rate (20 – 30 s) compared to sand filtration and chemical disinfection methods (EPA, 1999a). Ultraviolet radiation disinfection does not leave any harmful residual that can have a negative impact on humans or aquatic life (EPA, 1999a; Parsons & Jefferson, 2006; Ratnayaka, 2009).

Cryptosporidium is a chlorine resistant protozoan that is associated with many waterborne outbreaks due to the low infective dose with one oocyst enough for infection (Gray 2010). Ultraviolet disinfection is effective in inactivating *Cryptosporidium* and preventing disease. It is effective in inactivating oocysts and cysts when correctly applied (Gray, 2010). Ultraviolet radiation has low operating and capital costs and is more effective than other methods used to treat water-containing protozoa (Bolton & Cotton, 2008).

Although there are many positive aspects regarding UV radiation as a disinfection method, certain disadvantages need to be mentioned. Firstly, repair mechanisms of bacteria can be seen as a disadvantage but the use of medium-pressure UV lamps reduces repair and thus, repair of microorganisms can be avoided (Zimmer & Slawson, 2002). Secondly, it is not easy to measure the UV dose, and therefore secondary measurements are used which include, UV transmittance, flow rate or sensor readings. High UV doses are needed to inactivate certain viruses and consequently, it is important to determine the quality of the water that is going to be disinfected to determine the correct UV dose needed. Thirdly, one of the most important limiting factors regarding UV disinfection is the turbidity and suspended solids present in the water. If the turbidity of the water is high or there is large amount of suspended solids, the effectiveness of the UV may be compromised (EPA, 1999a; Bolton & Cotton, 2008; Ratnayaka, 2009). UV transmission should be greater than 60% for the treatment to be effective (Yiasoumi, 2005).

Chemical treatment options

A biocide refers to an active chemical molecule that is used to control the growth or kill bacteria (SCENIHR, 2009). Biocides have been used in water treatment for many years (Maillard, 2002). They are considered effective disinfectants against vegetative bacteria as they can interact and disrupt the cell wall, cytoplasmic membrane and the cytoplasmic constituents (Denyer & Stewart, 1998). The cytoplasm of bacteria contains ribosomes, enzymes and nucleic acid, which are essential for functioning cells. The most common target site for most biocides is the cytoplasmic membrane. This membrane can be interrupted physically by disrupting the proton motive force, which inhibits active transport across the cell, or by inhibiting certain enzymes essential for the membrane (Denyer & Stewart, 1998; Maillard, 2002). Once the cytoplasmic membrane is disrupted, cytoplasmic constituents like, nucleic acids, ribosomes and potassium may leak out of the cell (Denyer & Stewart, 1998; Maillard, 2002). Biocides rarely have a specific target site and often are effective by acting on multiple target sites (Maillard, 2002). The mode of action depends on the specific target site or target sites (Table 5).

Certain factors can influence the efficiency of a biocide when chosen for water disinfection. These include: type of microorganisms; concentration of biocide; contact time; pH; and organic particles that may be present in the water system (Binnie *et al.*, 2002; Maillard, 2005). Some biocides are more effective against certain microorganisms. If *Cryptosporidium parvum* is a known problem in a water system then chlorine should not

be used due to it being resistant to chlorine (Binnie *et al.*, 2002). The correct biocide concentration should always be considered as well as the formation of residual concentrations (Maillard, 2005). The way in which the biocide reacts with the multiple microbial targets sites depends on the concentration of the biocide used (Beumer *et al.*, 2000). Yet, different target sites are associated with different biocides (Table 5).

The correct contact time also needs to be established for effective disinfection with longer contact times needed if high microbial loads are present (Maillard, 2005). The pH of certain biocides, or the water being treated can influence the efficiency of disinfection. Water pH, for instance, directly influences the efficiency of chlorine (Newman, 2004). Organic matter in a water system may be oxidised by the biocide, therefore less biocide is available to oxidise microbes, as well as being able to protect microorganism by shielding (Maillard, 2005). Thus, these factors need to be considered before selecting a biocide for water treatment.

Chlorine

Chlorine is the most common chemical used to treat water worldwide (Newman, 2004; Gray, 2010). It can be used to disinfect irrigation water as a gas (Cl_2), as a liquid in the form of sodium hypochlorite (NaOCl) or hypochlorous acid (HOCl) or as a solid in the form of calcium hypochlorite ($\text{Ca}(\text{ClO})_2$) (Raudales *et al.*, 2014). Sodium hypochlorite is the most common form of chlorine used for irrigation water disinfection (Anon., 2011). Low doses of chlorine are usually needed to treat irrigation water, doses between 2 and 3 mg.L^{-1} are usually efficient to disinfect water (Anon., 2011).

Mode of action

When chlorine reacts with water, it results in the formation of hypochlorous acid (HOCl) and hydrochloric acid (HCl). Hypochlorous acid is considered a weak acid that readily dissociates to hypochlorite ions (OCl^-) and a hydrogen ion (H^+). Hypochlorous acid is a much stronger oxidising agent compared to OCl^- (Gray, 2010). The reaction of chlorine in water is shown below (WHO, 2004):

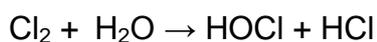


Table 5 Mode of action of chlorine, ozone and hydrogen peroxide (Nakamura & Tamaoki, 1968; Denyer & Stewart, 1998; Bitton, 1999; Maillard, 2002)

Biocide	Target site	Mode of action
Chlorine		
	Outer membrane (Gram-negative bacteria)	Disruption of cell permeability-causing cell lysis (in <i>E. coli</i>)
	Cytoplasmic constituents	Damages nucleic acid and enzymes
	Thiol group	Oxidation of thiol group
Ozone		
	Outer membrane (Gram-negative bacteria)	Disruption of cell permeability
	Cytoplasmic constituents	Disrupts enzyme activity
	Cytoplasmic constituents	Damages nucleic acid core
Hydrogen peroxide		
	Cytoplasmic constituents	Ribosome subunit 70S is dissociated to 30S and 50S (in <i>E. coli</i>)
	Protein thiol groups	Free radical oxidation (OH)
	Enzymes with thiol groups	Free radical oxidation (OH)

When considering chlorine as a disinfectant, pH is an important factor to consider as it determines the amount of HOCl present (Newman, 2004; Parsons & Jefferson, 2006). At pH values >8, OCl⁻ is the predominating species, however at lower pH values (3.5 – 5.5), HOCl is the predominating species. pH values between 5.5 and 9.5 contain both HOCl and OCl⁻ in varying amounts (WHO, 2004). Hypochlorous acid is considered a much stronger, faster acting oxidising agent as opposed to OCl⁻ (Newman, 2004). Consequently, it can be seen that the efficacy of chlorine as a disinfectant decreases with an increase in pH (Dychdala, 2001). At lower pH values all the HOCl is undissociated, but at high pH values dissociation is almost completed (Parsons & Jefferson, 2006). The OCl⁻

and HOCl species are referred to as free chlorine, and are reactive with various cell components (WHO, 2004). Hypochlorous acid is uncharged and has a relatively low molecular weight and consequently, is able to penetrate the cell walls of most microorganisms more efficiently than other chlorine species (Randtke, 2010).

In order to maintain free chlorine in the most active form, the pH should be between 7.4 and 7.6 (Newman, 2004). Hypochlorous acid can affect many cellular processes by producing oxidative, hydrolysis and deamination reactions with chemical substrates. This in turn produces physical lesions, which are damaging to the cell (WHO, 2004). The rate at which chlorine is dosed, depends on the flow rate as well as the desired residual concentration, which is usually 0.2 – 0.5 mg.L⁻¹ (Gray, 2010). The pH, chlorine concentration, temperature, organic content and type of microorganism present can influence the rate of chlorine disinfection (Table 5) (GHD, 2005).

Advantages and disadvantages

Chlorine is a stable disinfectant that has been used for many years, as it is effective over a broad spectrum of microorganism. Compared to other water treatment technologies such as ultraviolet radiation and ozone, chlorine is considered a cost effective treatment option (Hung *et al.*, 2012). Capital cost of chlorine disinfection is lower than of UV disinfection (Anon., 2011). Chlorine is an effective disinfectant as it has a long lasting residual action, unlike other chemical treatments (Gray, 2010; Raffellini *et al.*, 2011). A further advantage is that, residual chlorine can disinfect any newly introduced microorganism (Gadgil, 1998). The residual chlorine is an important aspect when considering disinfection of irrigation water, as the residual helps clean biofilms out of the irrigation system (Yiasoumi *et al.*, 2005).

There are a few disadvantages associated with the use of chlorine as a disinfectant. Firstly, it is important to note that chlorine will react with any organic matter present in the water, therefore residual chlorine may not be long lasting (Gray, 2010). Due to chlorine reacting with organic matter, it may be necessary to provide a higher dose to achieve the desired biocidal concentration (Yiasoumi *et al.*, 2005). Sufficient chlorine should be added to give a chlorine final residue of 0.2 – 0.5 mg.L⁻¹ (Gray, 2010). A relatively long contact time (30 – 60 min) is needed to disinfect water (Gadgil, 1998).

Certain organisms have high resistance levels to chlorine. Protozoa such as *Cryptosporidium*, show very high resistance and standard chlorination levels used in water treatment have been unsuccessful at preventing outbreaks of *Cryptosporidium* (WHO,

2004; Gray, 2010). Hence, other disinfection options should be used to prevent protozoan outbreaks (WHO, 2004).

Bacteria present in biofilms are also more resistant to chlorine disinfection. It has been shown that dosing with 1 or 3 mg.L⁻¹ of chlorine resulted in 99.7% or 99% survival of bacteria present in the biofilm, respectively (Bois *et al.*, 1997). The phytotoxicity of chlorine may have a negative effect on plant health (Raudales *et al.*, 2014). Cayanan *et al.* (2009) reported that 2.4 mg.L⁻¹ of free chlorine resulted in visible phytotoxicity of several shrubs (Cayanan *et al.*, 2009). The efficiency of chlorine disinfection is highly dependent on the water quality, pH and organic matter content (Anon., 2011).

It is evident that chlorine is an effective disinfectant, but it was not until the 1970's that the formation of DBP's was recognised. Disinfectant-by-products are formed when chlorine reacts with naturally occurring organic compounds (Shorney-Darby & Harms, 2010). Trihalomethanes (THM's) and haloacetic acids (HAA's) are the two main groups of DBP's formed during chlorine disinfection (Shorney-Darby & Harms, 2010). Both THM's and HAA's are formed when chlorine reacts with organic material present in the water. These DBP's continue to form throughout the contact time if organic matter is present (Shorney-Darby & Harms, 2010). Although the formation of DBP's depends on the pH of the water, it is difficult to control the formation of DBP's and therefore other disinfectant methods need to be considered, such as ozonation or ultraviolet radiation (Gray, 2010; Shorney-Darby & Harms, 2010).

Chlorine case studies

Chlorination is considered one of the most common wastewater disinfection methods used worldwide (Koivunen & Heinonen-Tanski, 2005). Koivunen & Heinonen-Tanski. (2005) performed a study on the treatment of synthetic wastewater with sodium hypochlorite. Results indicated a less than 1 log reduction of *E. coli* with an 18 mg.L⁻¹ chlorine dose. A 2.6 log reduction of *E. faecalis* was seen when a 12 mg.L⁻¹ chlorine dose was used. When chlorine doses between 6 – 10 mg.L⁻¹ were used, the residual chlorine concentration was below the detection limit of 0.1 mg.L⁻¹. When 12 -18 mg.L⁻¹ chlorine doses were used, results indicated 0.2 – 0.3 mg.L⁻¹ residual chlorine concentrations (Koivunen & Heinonen-Tanski, 2005).

Ozone

Ozone is an unstable blue gas that can be found naturally in the earth's atmosphere (Newman, 2004). Ozone is a form of oxygen (O₃) and is produced by passing oxygen

between electrodes by a system referred to as corona discharge. Ozone can be produced by other methods, which include; photochemical, chemical, chemonuclear, electrolytic or electrochemical (Yousef *et al.*, 2011). The oxygen molecule is split resulting in the formation of oxygen atoms, which then bond to other oxygen molecules forming ozone. The gaseous form of ozone is more stable than the aqueous phase, therefore gas is used (Yousef *et al.*, 2011). Ozone is usually injected into irrigation systems, where it can inactivate microorganisms (Newman, 2004).

Mode of action

Ozone is an effective disinfectant due to its strong oxidising properties (Gómez-López, 2012). When ozone is present in water, it can react with microorganisms directly by molecular ozone or by indirect reaction with free radical species formed during the decomposition of ozone (WHO, 2004). The decomposition of ozone results in the formation of superoxides, hydroxyl and hydroperoxyl radicals, and these radicals are responsible for the oxidising effect of ozone (Gómez-López, 2012). Inactivation of microorganisms is due to oxidation of cellular components. Ozone reacts with the genetic material (DNA and RNA), cell envelope (oxidising sulfhydryl groups), enzymes and unsaturated lipids oxidation (Yousef *et al.*, 2011). Thus, ozone is an effective disinfectant as it destroys microorganisms by damaging the cell membrane and other components rendering the microorganisms inactive (Table 5) (Newman, 2004).

Advantages & disadvantages

Ozone is a powerful oxidant, with an oxidation potential of twice that of chlorine (Yiasoumi *et al.*, 2005). It is capable of destroying indicator organisms, pathogens, bacterial spores and even chlorine resistant protozoans such as *Cryptosporidium*. Therefore, it is effective over a broad range of microorganisms (Newman, 2004; Yousef *et al.*, 2011). Contrary to that of chlorine, the use of ozone does not result in the formation of environmentally unfriendly by-products (Yiasoumi *et al.*, 2005). This is an important factor to consider when selecting a water treatment option. Compared to chlorine, ozone is also less affected by pH and temperature (Clarke & Hill, 1977). Ozone is also capable of coagulating natural constituents of water, which may improve filtration (Yiasoumi *et al.*, 2005). Ozone treatment requires a short contact time with a dose of 1 ppm being able to destroy bacteria within 10 min (EPA, 1999b; Gray, 2010). A contact time of 10 - 12 min is required, which is relatively short, compared to other chemical disinfectants (Yousef *et al.*, 2011).

However, there are disadvantages associated with the use of ozone. Ozone has no residual action to prevent recontamination (Gray, 2010). Some bacteria contain flavonoids and carotenoids, which are pigments that can protect the bacteria from the ozone therefore rendering them less susceptible to inactivation (WHO, 2004).

Ozone is considered a chemically unstable gas, consequently, it has to be generated on-site and used immediately (EPA, 1999b). Although ozone is powerful oxidant, it is a very expensive treatment option. It is not recommended as a treatment option in developing countries due to the high cost, as well as operational and maintenance infrastructure (Gadgil, 1998).

Case studies

Martínez *et al.* (2011) conducted a study on using O₃ in wastewater treatment to produce water suitable for irrigation. Results indicated that O₃ at concentrations between 11 – 13 mg O₃.L⁻¹, resulted in effective microbial reductions (3 log reduction). The treatment was considered effective and water was suitable for irrigation based on microbial and chemical reductions (Martínez *et al.*, 2011). Another study by Zhang *et al.* (2005) on the use of O₃ to preserve fresh-cut celery. The celery was dipped in water containing 0.03, 0.08 and 0.18 ppm of ozone. Results indicated that 0.18 ppm O₃ was effective at decreasing microbial loads and ensuring nutritional and sensory quality for 9 days at 4°C (Zhang *et al.*, 2005).

Hydrogen peroxide

Hydrogen peroxide is a metastable, colourless substance that has been used as a disinfectant for many years (McDonnell & Russell, 1999; Labas *et al.*, 2008). Hydrogen peroxide was first recognized as a chemical in 1818, by Louis-Jacques Thernard, although the disinfecting and bleaching properties of H₂O₂ were only recognized in the 1900's (Schumb *et al.*, 1955; Spaulding *et al.*, 1977). In nature, H₂O₂ is formed when rain reacts with ozone in the atmosphere, whereby ozone loses an oxygen molecule to the water, which then forms H₂O₂ (Newman, 2004).

The use of H₂O₂ as a biocide is becoming increasingly common, due to its non-toxic and degradable properties. These properties are considered important when selecting a biocide for environmental use, such as water treatment (Linley *et al.*, 2012). Hydrogen peroxide has been used as a topical disinfection, as well as in the food industry to facilitate sterile packaging (Newman, 2004).

Hydrogen peroxide has also been found to be an effective disinfectant of fresh produce due its oxidising capability. It does not produce carcinogenic compounds, as it does not react with organic compounds present in perishables and H₂O₂ has been classified as Generally Regarded as Safe (GRAS), for certain food applications (Joshi *et al.*, 2013). However, the use of H₂O₂ may result in detrimental quality changes (Joshi *et al.*, 2013). A study was done by Sapers *et al.* (1999) on the effectiveness of sanitizing agents in lowering *E. coli* loads on apples. Results indicated that similar log reductions were achieved when chlorine and other commercial sanitizers were used. However, with 5% H₂O₂ concentration (or combinations of H₂O₂ and other commercial sanitizers), higher log reductions were achieved (3 – 4 log reductions). Both sanitizers were heated to 50°–60°C as slightly better results were seen at 50°C when compared to ambient temperature (Sapers *et al.*, 1999). It is important to note that strain–strain differences were seen in response to the sanitizing agents (Sapers *et al.*, 1999).

Mode of action

The toxicity of H₂O₂ is not due to the direct result of its oxidative properties in its molecular state, but due to the formation of strong oxidant chemical species including; singlet oxygen, superoxide radicals and hydroxyl radicals ([•]OH) (McDonnell & Russel, 1999; Raffellini *et al.*, 2011). Hydrogen peroxide can easily diffuse through the microbes cell membrane, where the H₂O₂ is degraded to form reactive [•]OH radicals (Halliwell & Gutteridge, 1984; Labas *et al.*, 2008). Compared to other reactive oxidant species formed, [•]OH radicals have the strongest potential to damage cellular components (Labas *et al.*, 2008). The damaging action of H₂O₂ is referred to as oxidative stress, resulting from the formation of reactive oxidative species (ROS). These reactive radicals are capable of damaging cellular components such as, sugars, amino acids, phospholipids and DNA bases, resulting in cell death (Halliwell & Gutteridge, 1984; Block, 2001). Deoxyribonucleic acid damage due to oxidants, leads to the modification of purine and pyrimidine bases as well as resulting in breaking of oligonucleotide strands (Asad *et al.*, 2004). Hydrogen peroxide is also capable of dissociating 70S ribosomes into its 30S and 50S subunits of *E. coli* (Nakamura & Tamaoki, 1968). Ribosomes play an essential role in the formation of peptides from amino acids, and are therefore essential for cell functioning (Table 5) (Morató *et al.*, 2003).

Advantages and disadvantages

Hydrogen peroxide is considered an environmentally friendly treatment option, as it readily breaks down into water and oxygen, therefore leaving no residue (EPA, 2002; Joshi *et al.*, 2013). It does not react with organic compounds to form carcinogenic by-products, as occurs when chlorine is used as a disinfectant (Fraise, 1999; McDonnell & Russell, 1999; Joshi *et al.*, 2013). Hydrogen peroxide is considered an effective disinfectant over a broad spectrum of microorganisms including; bacteria, yeasts, fungi, viruses and spores (Spaulding *et al.*, 1977). In addition, it is considered an effective disinfectant over a wide pH range (6 - 10) (Joshi *et al.*, 2013).

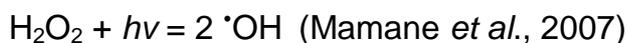
There are disadvantages associated with the use of H₂O₂ as a chemical treatment option. Hydrogen peroxide is a strong oxidant and can be considered harmful if it comes into contact with humans (EPA, 1999c). Due to the fact that H₂O₂ degrades rapidly into oxygen and water, no long term residual can be detected (Raffellini *et al.*, 2011). High levels of organic matter present in water systems could limit the efficacy of H₂O₂ (Newman, 2004). Vargas *et al.* (2013) investigated the use of H₂O₂ on treated domestic sewage. Results indicated that disinfection of the synthetic sewage was faster than that of the treated sewage. This was thought to be due to the presence of other pollutants present in the sewage that may be oxidised by the H₂O₂ (Vargas *et al.*, 2013).

Hydrogen peroxide case studies

Different concentrations of H₂O₂ are available ranging from 3 to 90% (McDonnell & Russell, 1999). Concentrations commonly used for disinfection range between 3 and 25%, with 25% being used when higher levels of disinfection are required (Fraise, 1999). Studies have been also done to determine the most effective concentration and contact time needed (Orta de Velásquez *et al.*, 2008; Ronen *et al.*, 2010). Ronen *et al.* (2010) performed a study on greywater disinfection with the environmentally friendly H₂O₂ Plus (HPP). Different concentrations and contact times were used. The different concentrations included; 25, 50, 125, 250 and 1 000 mg.L⁻¹ and the different contact times included; 0, 30, 60 and 120 min. Results indicated that a concentration of 125 mg.L⁻¹ and a contact of 35 min inactivated 99% of faecal coliforms. The inactivation pattern was determined by using the Chick-Watson model. Orta de Velásquez *et al.* (2008) completed a study on adding silver and copper to H₂O₂ and peracetic acid in the disinfection of an advanced treatment effluent. Hydrogen peroxide concentrations used in the study included; 50, 100, 150, 200, 250 mg.L⁻¹ and contact times used included; 0, 30, 60, 90 and 120 min. The study was done using faecal coliforms and a 2.2 log reduction was seen

after using a concentration of 250 mg.L⁻¹. Hydrogen peroxide and a 120 min contact time. They found that the efficiency of H₂O₂ as a treatment option can be improved by a combination with other treatment methods (Orta de Velásquez *et al.*, 2008). When 50 mg.L⁻¹ of H₂O₂ (CT 1500) was used, a log reduction of 0.6 was achieved for faecal coliforms. However, when H₂O₂ and Cu²⁺ were used in combination (50 + 1 mg.L⁻¹) (CT value of 1530) a 3.9 log inactivation was achieved. When H₂O₂ and Ag were used in combination (50 + 1 mg.L⁻¹) (CT value of 1530), a 1 log inactivation was achieved for faecal coliforms. A 0.6 log reduction was achieved with only H₂O₂ (50 mg.L⁻¹). Thus, from this study it was concluded that the addition of Cu²⁺ to H₂O₂ was very effective. The addition of Ag increased the log inactivation slightly but was not as effective as the addition of Cu²⁺ (Orta de Velásquez *et al.*, 2008).

Hydrogen peroxide has been used in combination with UV disinfection, due to the formation of hydroxyl radicals. These hydroxyl radicals are generated by interactions of UV radiation with a chemical disinfectant that is capable of releasing radicals (Koivunen & Heinonen-Tanski, 2005). The formation of hydroxyl radicals is shown in the following equation:



Results from a study done Koivunen & Heinonen-Tanski. (2005) indicated that using H₂O₂ in combination with UV radiation slightly increased the log microbial reductions when compared to using UV alone. Therefore, H₂O₂ may be used in combination with other treatments or compounds in order to achieve a more effective microbial reduction.

F. RESISTANCE MECHANISMS OF *E. COLI* ASSOCIATED WITH IRRIGATION WATER

Escherichia coli are capable of developing resistance mechanisms in order to protect themselves from biocide disinfectants, oxidative stress and antibiotics (Storz & Imlay, 1999; Cloete, 2003; Davies & Davies, 2010). A further problem associated with *E. coli* is the difference between well-characterised reference strains and wild-type environmental *E. coli* strains. A study done by Mazzola *et al.* (2006), indicated that microorganisms isolated from a water purification system showed higher resistance to chemical treatments than standard reference strains used in the study (Mazzola *et al.*, 2006).

Bacterial resistance to biocide disinfectants

Organisms need to be able to respond quickly to their constantly changing environments in order to survive (Morató *et al.*, 2003). Resistance refers to the capacity of bacteria to withstand the effects of antibiotics or biocides that are intended to kill or control them (SCENIHR, 2009). Bacteria are usually considered resistant when they are no longer susceptible to a concentration of a specific biocide used in practice (Cloete, 2003).

The efficiency of a specific biocide may vary between different microorganisms as well as between strains within a species (Maillard, 2002). Microorganisms show different levels of resistance, with Gram-negative bacteria being more resistant than Gram-positive bacteria (Maillard, 2002). Gram-negative bacteria have a layer consisting of lipoproteins and lipopolysaccharides outside the peptidoglycan layer which makes penetration of biocides more difficult (Hugo, 1992). Differences in biocide resistance between Gram-negative and Gram-positive bacteria are not due to different target sites of the biocide, but rather due to penetration differences (Maillard, 2002).

Bacterial resistance to biocides can be divided into two major mechanisms of resistance: intrinsic resistance; and acquired resistance (Russell, 1998; Meyer & Cookson, 2010). Some bacteria are capable of withstanding higher concentrations of a biocide due to their innate biochemical makeup, which results in intrinsic resistance (White & McDermott, 2001). Intrinsic resistance is usually associated with the impermeability of the outer cell layers and this is seen with Gram-negative bacteria, as the outer cell layers limit the amount of biocide uptake (Russell, 1998; Russell, 2000). Therefore, intrinsic resistance directly influences the uptake of biocides (Beumer *et al.*, 2000). For a biocide to be considered effective, it must be able to penetrate the cell envelope and obtain a high concentration at the specific target site (Cloete, 2003). During intrinsic resistance, the disinfectant is not able to reach the specific target site in high enough concentrations to achieve cell death (Russell, 1998). There are different mechanisms, which enable bacteria to resist biocides. These include: target modification; impermeability; efflux pumps; and alterations of targets or metabolisms (Table 6) (Maillard, 2005). Gram-negative bacteria, mycobacteria and endospores usually display intrinsic resistance. Efflux pumps in Gram-negative bacteria are capable of pumping out a wide variety of substances due to their broad specificity (Russell, 1998; Beumer *et al.*, 2000). Intrinsic resistance also refers to phenotypic changes that influence the susceptibility of an organism, due to changes in the environment (Beumer *et al.*, 2000).

Acquired resistance refers to resistance that is achieved by mutations or acquisition of new genetic material. Mutations in cellular genes can result in the acquisition of foreign resistance genes (White & McDermott, 2001; Chapman, 2003).

Resistance against biocides may develop due to organisms being exposed to sub-lethal biocide concentrations, which may have led to the selection of certain strains which have an increased minimal inhibitory concentration (MIC) (Meyer & Cookson, 2010). Therefore, when organisms are exposed to gradually increasing concentrations of a specific biocide it often results in acquired non-plasmid-encoded resistance (Russell, 1998). To overcome this problem it is important to use concentration levels that will be lethal to all the organisms. If water is going to enter the environment after treatment, it is important that the concentrations be diluted to levels that are considered biodegradable. This ensures that strains are not exposed to biocide concentrations lower than their MIC, thereby preventing resistant strains from developing (Chapman, 2003; Meyer & Cookson, 2010).

Resistance of organisms against biocides usually results from cellular changes, which can impact the accumulation of the biocide, limit uptake or change the expression of the efflux pump mechanism (Poole, 2002). Although biocide resistance because of mutations is rare, target site mutations resulting in increased resistance have been identified (Poole, 2002).

Oxidative stress resistance

Oxidative stress results when cells are exposed to reactive oxidative species, which can be detrimental to cellular constituents. In order for organisms to protect themselves from these reactive species, they are able to express enzymes that can protect and repair the damage caused by these harmful reactive species (Storz & Imlay, 1999; Iwase *et al.*, 2013).

Hydrogen peroxide can be catalysed into water and oxygen by the catalase enzymes hydroperoxidase I (HPI) and hydroperoxidase II (HPH) (Loewen *et al.*, 1985). The *katG* gene is responsible for encoding HPI, and is expressed in response to oxidative stress and controlled by *oxyR* regulon. However, HPH does not respond to *oxyR* regulon (Loewen *et al.*, 1985; Triggs-Raine & Loewen, 1987). If the organism is exposed to excess H₂O₂, the *oxyR* regulon is triggered in order to protect the cell, if O₂⁻ radicals are present in the cell it triggers the *soxRS* regulon.

Table 6 Mechanisms of biocide resistance (Maillard, 2005)

Mechanism	Effect	Example
1. Decrease in biocide concentration		
Impermeability	Decreases amount of biocide that can penetrate into the cell therefore affecting biocide accumulation	Gram-negative bacteria
Efflux pumps	Decrease the amount of biocide in the cell	<i>E. coli</i> (Gram-negative)
Degradation	Inactivation of the biocide within or outside the cell	<i>E. coli</i> (catalase and superoxide dismutase)
2. Alterations and metabolism		
Modification of target	Render the biocide ineffective	<i>E. coli</i> (has only been observed with bisphenol triclosan)
Alteration of metabolism	Decrease the detrimental effect of a biocide	Persists and phenotypic alterations

Both the *oxyR* and *soxRS* regulons play an important role in protecting and repairing damage due to oxidative stress (Dempfle, 1996). Hydroperoxidase II is encoded by *katE* and regulated by *rpoS*. The gene *katF* is a positive regulatory gene for *katE* (Heimberger & Eisenstark, 1988; Storz & Imlay, 1999; Iwase *et al.*, 2013). It is evident that the two *E. coli* catalases do not have the same functions (Heimberger & Eisenstark, 1988). Specific decomposition of H₂O₂ at membrane level is from HPI, whereas HPII decomposes H₂O₂ that may arise in the cytoplasm (Heimberger & Eisenstark, 1988). Thus, catalase is an enzyme that can protect the cell when sub lethal concentrations of H₂O₂ exist. Catalase levels may be increased due to phenotypic adaptation to protect the cell against oxidative stress. Consequently, active resistance against H₂O₂ may develop (Morató *et al.*, 2003).

Cross-resistance may occur between different biocides. This occurs when different biocides have the same target site, and therefore resistance against more than one biocide may exist (Chapman, 2003). A study done by Dukan & Touati (1996), indicated that cross-resistance to oxidants may occur. This was observed for H₂O₂ and HOCl. This is seen as a problem with regards to water treatment, as both H₂O₂ and HOCl are used as to treat irrigation water.

Antibiotic resistance

Antibiotics have been used for many years to treat infectious disease in humans and animals. Antibiotics are effective as they inhibit the growth of pathogenic microorganism in order to protect the host (Davison *et al.*, 2000; Cerf *et al.*, 2010). Antimicrobial agents are used widely throughout the agricultural industry for growth promotion (non-therapeutic use), to prevent infections, as well as being used to treat sick animals (WHO, 2011). Resistance of Gram-negative bacteria to these antimicrobial agents has become a rapidly increasing and major problem (Romanis, 2013)

Antibiotics are categorised according to their importance and there are three main groups including: critically important; highly important; and important antimicrobials (WHO, 2011). There are four main modes of actions by which antibiotics can successfully protect against infectious diseases. These include inhibition of cell wall synthesis, inhibition of protein synthesis, inhibition of nucleic acid synthesis and inhibition of metabolic pathways (Tenover, 2006; Chopra, 2010). The discovery of antibiotics saved many lives, but unfortunately, the use of antibiotics has led to resistant strains present in the environment (Davies & Davies, 2010).

Antibiotics target commensal bacteria present in the gut as well as pathogenic microorganisms. Therefore, the problem associated with antibiotic resistance is that it is not restricted to pathogenic bacteria (Erb *et al.*, 2007). Commensal bacteria may carry resistant genes, which can be transferred to pathogenic strains. The way in which the non-target organisms are affected by the antibiotic depends on the antibiotic used, as well as the resistance properties of the non-targeted organism (Erb *et al.*, 2007; Jernberg *et al.*, 2010). Pathogenic strains acquiring antibiotic resistance genes from other microorganisms are considered a major cause for concern, as resistance genes can be transferred from environmental bacteria to human bacteria (Ozgumus *et al.*, 2007; Jernberg *et al.*, 2010).

Antibiotic resistance may develop when organisms are exposed to certain antibiotics for a prolonged length of time, or may be acquired through horizontal or vertical gene transfer (Giedraitienė *et al.*, 2011; Wright, 2011). When faecal bacteria, including pathogenic strains, enter a water system it is considered a health risk for humans. If these bacteria are antibiotic resistant, there is a much greater risk with regards to human health (Sidrach-Cardona *et al.*, 2014). *Escherichia coli* strains are important organisms to consider with regards to antibiotic resistance in water systems, as they carry resistance genes, which can be then transferred to other bacteria (Coleman *et al.*, 2013).

Antibiotic resistance *E. coli* strains have been isolated from ground and surface water in South Africa. A study done by Phokela *et al.* (2011) reported the presence of antibiotic resistant *E. coli* strains in ground water samples that were taken from two rural communities in the Mafikeng areas. Results indicated *E. coli* strains with resistance to tetracycline, chloramphenicol and streptomycin (Phokela *et al.*, 2011). Another study done by Ademola *et al.* (2009) reported antibiotic resistant *E. coli* in South African surface waters. Surface water was sampled from the Umgeni and Palmiet River located in KwaZulu Natal. Results indicated 91.1% of the Palmiet River *E. coli* isolates carried indicated antibiotic resistance properties, while 71.15% of the Umgeni isolates were multi-drug resistant (Ademola *et al.*, 2009). Romanis (2013) isolated *E. coli* strains from irrigation, contamination and environmental sources. The resistance of these strains were tested against: ampicillin; cephalothin; chlormaphenicol; ciprofloxacin; streptomycin; tetracycline; and trimethoprim. Thirty-five out of the 163 isolates (21.5%) showed resistance to at least one antibiotic. Nine out of 35 *E. coli* isolates (25.7%) exhibited antibiotic resistance, this indicates that these antibiotic resistance *E. coli* strains may be transferred onto fresh produce via irrigation water. These are a few examples of the isolation of antibiotic resistance *E. coli* in South Africa.

Microorganisms can have several mechanisms that enable them to be antibiotic resistant. Antibiotic resistance mechanisms include target modification, molecular bypass, efflux pumps and chemical modification (Table 7). Target modification results rapidly due to mutations in certain genes and from the specificity of target molecules. Even a small change can disrupt antibiotic binding (Giedraitienė *et al.*, 2011; Wright, 2011). Molecular bypass occurs when bacteria are capable of producing an alternative resistant target (Giedraitienė *et al.*, 2011). Efflux pump systems actively remove antibiotics from inside the cell, therefore the antibiotic is not present in high enough concentration to damage the cell (Wright, 2011). Resistance due to efflux pumps is common in Gram-negative bacteria (Drawz & Bonomo, 2010). Multidrug resistance is mediated through the expression of genes, an example being the multiple antibiotic resistance (*mar*) locus in *E. coli* (Levy, 2002). Chemical modification can result in antibiotic resistance by enzyme-catalysed inactivation. The most common mechanism of action in Gram-negative bacteria is the production of β -lactamase enzyme (Drawz & Bonomo, 2010). Genetic transfer has resulted in bacteria becoming resistant to more than one antibiotic class. If bacteria are resistant against three or more antibiotic classes they are considered to be multidrug resistant (Tenover, 2006).

Link between biocide and antibiotic resistance

Both similarities and differences are noted in the mechanisms by which bacteria are resistant against biocides and antibiotics (Russell, 2003). Although similarities between biocide and antibiotic resistance exists, the relationship is not yet understood (White & McDermott, 2001). Biocides do not usually have a specific target unlike antibiotics (Denyer & Stewart, 1998). The resistance of bacteria to biocides is less known than antibiotic resistance of bacteria (Cloete, 2003).

Antibiotic resistance due to chromosomal efflux pumps can also result in biocide resistance as many of the pumps are non-specific (White & McDermott, 2001). Biocide resistance is frequently due to non-specific multidrug pumps and can thus be linked to antibiotic resistance mechanisms (Meyer & Cookson, 2010). Efflux-mediated pumps protect the organism against antibiotics and biocides and are common to most bacteria (Ortega Morente *et al.*, 2013). Chromosomally encoded multidrug efflux pumps in Gram-negative bacteria can occur due to phenotypic adaptations. The expression of these efflux genes can result in increased resistance, which result from mutations or by environmental signals (Beumer *et al.*, 2000; Levy, 2002). In Gram-negative bacteria where resistance is often due to barrier limiting, effects may be seen against biocides and antibiotics (Beumer *et al.*, 2000). Changes in the cell wall have been shown to display cross-resistance between biocides and antibiotics. However, although the exact mechanism is unknown it is probably due to changes in the permeability and therefore preventing uptake (Fraise, 2002).

Antibiotic resistance has also been linked to *soxRS*, which suggests that the same mechanisms may be used to protect organism against antibiotics and oxidative stress (Dempsey, 1996). Oxidative stress can lead to the expression of multidrug efflux systems in order to protect the cell from and these oxidative stress responses are able to contribute to antimicrobial resistance (Poole, 2012). The *soxRS* regulon is induced by oxidative stress in *E. coli*, however it is capable of switching on genes for both oxidative stress and antibiotic resistance (Ariza *et al.*, 1994).

It is however, important to note that there is no consistent evidence to show that there may be a link between antibiotic and biocide resistance (Beumer *et al.*, 2000).

There is a need for further research to monitor the prevalence of antibiotic resistance and biocide resistance. Zoonotic bacteria in food, animals and humans are of great importance when considering the link between antibiotic and biocide resistance (Mavri *et al.*, 2012).

Table 7 Antibiotic resistance mechanisms (Neu, 1992; Chopra, 2010; Davies & Davies, 2010; Giedraitienė, *et al.*, 2011; Wright, 2011).

Antibiotic group	Mode of action	Examples	Resistance type
Inhibition of cell wall synthesis (β – lactams)			
Penicillins	Inhibits enzyme required for peptidoglycan layer	Ampicillin	Target modification, efflux, chemical modification
Cephalosporins	Inhibits enzyme required for peptidoglycan layer	Cephalothin	Target modification, efflux
Inhibition of protein synthesis			
Aminoglycosides	Binds to 30S ribosomal subunit	Streptomycin	Target modification, efflux, enzymatic modification
Chloramphenicol	Binds to 50S ribosomal subunit	Chloramphenicol	Target modification, efflux, enzymatic degradation
Tetracyclines	Binds to 30S ribosomal subunit	Tetracycline	Target modification, efflux
Inhibition of nucleic acid synthesis			
Quinolones & fluoroquinolones	Inhibits DNA synthesis	Ciprofloxacin	Target modification, efflux
Anti-metabolite			
Trimethoprim	Disrupts folate cycle	Trimethoprim	Target modification, efflux

G. CONCLUSION

Previous studies done on the surface water quality in South Africa, have shown that high microbial levels are present in many South African rivers (Paulse *et al.*, 2009; Ackermann, 2010; Gemmell & Schmidt, 2010; Huisamen, 2012; Le Roux *et al.*, 2012). Many farmers are forced to use these surface waters for irrigation due to the water shortages

experienced in South Africa (Ijabadeniyi *et al.*, 2011). Using contaminated irrigation water can lead to the spread of water and foodborne diseases and therefore it is of utmost importance that irrigation water is disinfected prior to use.

When considering chemical and physical treatment options there are many advantages and disadvantages associated with the different options as discussed previously. Water quality factors such as turbidity and the organic matter content can influence the efficiency of chlorine, H₂O₂, sand filters and UV radiation. Therefore, before a disinfection method is selected, the quality of water must be determined in order to ensure effective treatment. The type of microorganisms present in a water system may react differently to the treatment. *Escherichia coli* is a common indicator organism, used for detecting faecal contamination from human and animal sources. Water guidelines and regulations are often based on faecal coliforms, including *E. coli* (Campos, 2008). However, some microorganisms such as *Cryptosporidium* are resistant to chlorine and therefore other treatment options need to be considered if *Cryptosporidium* is a known problem (WHO, 2004; Gray, 2010).

Reference strains have often been used in studies to test the efficacy of certain treatment options, however the growth kinetics are not always the same when compared to the environmental strains (Wojcicka *et al.*, 2007). Environmental strains may be better adapted to adverse environmental conditions. Hence, using standard reference strains during treatment optimisation studies may lead to inaccurate results and in return incorrect parameters may be selected (UV doses or chemical concentrations and contact times).

Antibiotic resistance may be linked to biocide resistance, as similarities are seen between the mechanisms used to protect the bacteria (Russell, 2003). Antibiotic resistant *Escherichia coli* strains have been isolated from rivers in the Boland region (Lamprecht *et al.*, 2014). This indicates that resistant strains are present in the water systems and studies need to be performed in order to determine if these strains are more resistant to chemical treatment options. It is, however still unclear whether antibiotic resistant environmental strains would have a better chance of surviving irrigation water treatments than environmental strains with no antibiotic resistance properties.

Although there are many on-farm irrigation treatment options available to farmers, there are advantages and disadvantages associated with all of them, as well as cost implications. Thus, many important factors need to be considered before selecting an on-farm treatment option in order to ensure that the treatment process is effective.

As part of the larger WRC project (K5/2174/4) this thesis focused on using H₂O₂ as a possible on-farm treatment option. H₂O₂ has been extensively used as a biocide, as it is

environmentally friendly and degrades into hydrogen and water (EPA, 2002; Linley *et al.*, 2012).

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CHAPTER 3

IMPACT OF HYDROGEN PEROXIDE ON ENVIRONMENTAL *ESCHERICHIA COLI* STRAINS AND FACTORS THAT INFLUENCE THE EFFICACY OF HYDROGEN PEROXIDE AS A TREATMENT OPTION

SUMMARY

The impact of H₂O₂ was determined on river water, as well as on environmental and ATCC reference *E. coli* strains. Three H₂O₂ concentrations (250, 300 and 350 mg.L⁻¹), for 30, 60, 90 and 120 min time intervals were used. The *E. coli* strains reacted differently to the H₂O₂ treatment, which resulted in different log reductions after the 120 min contact time. The environmental strains were generally more resistant than the reference strains, with the exception of ATCC 35218, which was found to be a resistant strain. A resistant environmental strain (M53) and a potential pathogenic strain (W1371) were used to determine the effect higher (and a lower) H₂O₂ concentrations of 50, 350, 700 and 1 000 mg.L⁻¹ on the microbial inactivation. Differences were seen between the different concentrations used, with 50 mg.L⁻¹ resulting in low microbial inactivation and 1 000 mg.L⁻¹ resulting in an effective microbial inactivation. A difference in microbial reduction was not observed at H₂O₂ concentrations between 350 and 700 mg.L⁻¹. The influence of organic matter (COD) on the H₂O₂ treatment was determined by inoculating autoclaved river water with *E. coli* strains before H₂O₂ treatment. It was observed that the organic matter, at the levels investigated, did not have an effect on the H₂O₂ treatment. A difference was only seen in the first 30 min, with regards to the strain indicating H₂O₂ resistance. Catalase activity was also measured to determine if catalase activity and H₂O₂ resistance are linked. Different methods, indicated similar results, with a trend seen between H₂O₂ resistance and an increased amount of catalase activity. However, this was not always the case, as two of the most resistant *E. coli* strains indicated low catalase activity. It was concluded that resistant environmental *E. coli* strains are capable of surviving high H₂O₂ concentrations (1 000 mg.L⁻¹), for long time periods (up to 90 min) provided microbial loads are present at high concentrations.

INTRODUCTION

Water scarcity is a global problem, and is a major problem in South Africa, where it is a reality (NWRS, 2004; Norton-Brandão *et al.*, 2013). In developing countries water scarcity can be linked to poverty, due to the decrease in food production (FAO, 2007). Almost every industry relies heavily on water to function productively (Tarver, 2008). Thus, in a

country like South Africa where water scarcity exists, the quality of natural water resources is of utmost importance (Le Roux *et al.*, 2012).

In South Africa the agricultural sector demands the most water compared to other sectors (industrial, urban and rural) and is responsible for 62% of the total water withdrawals. The agricultural industry is of utmost importance to keep up with the growing food demands, as well as playing a critical role in the South African economy. Surface water, including rivers are the main source of irrigation water for the agricultural sector (NWRS, 2004).

A study done by Britz *et al.* (2013) indicated that the surface water in the Boland region is highly contaminated on a microbial level. These rivers are often used for irrigation, as the water is readily available (Britz *et al.*, 2013). Using contaminated river water can lead to foodborne disease outbreaks, as irrigation water can be an important source of contamination for fresh produce. The contamination of fresh produce has increased over the years (Martínez-Vaz *et al.*, 2014). This can lead to foodborne diseases, which in turn can cause the ban of fresh produce being exported out of South Africa, thus negatively affecting the economy (Britz *et al.*, 2012). On-farm treatment options of water are needed in order to decrease the high microbial loads prior to irrigation.

The disinfectant properties of H_2O_2 have been known for many years (Labas *et al.*, 2008). H_2O_2 has been used for microbial inactivation of pathogenic microorganisms, vegetative bacteria and even viruses (Labas *et al.*, 2008). It acts as a disinfectant due to the formation of strong oxidant chemical species including; singlet oxygen, superoxide radicals and hydroxyl radicals (McDonnell & Russell, 1999). However, it is important that the suitable concentrations and operating conditions are used (Labas *et al.*, 2008). The lack of toxicity followed by the degradation of H_2O_2 in water, is the main advantage associated with the use of H_2O_2 , as it is degraded into non-toxic by-products (oxygen and water). This is seen as a significant advantage for farmers when considering water treatment options.

Faecal coliforms and *E. coli* are seen as good indicator organisms as they are present in faeces of humans and animals. They can be detected by simple methods, and they are not naturally present in water (Campos, 2008). According to the WHO (World Health Organization) (WHO, 1989) and DWA (Department of Water Affairs) faecal coliforms must not exceed 1000 faecal coliforms per 100 mL, this is stipulated in the guidelines for irrigation of fresh produce (DWAF, 1996) as well as the regulations for the

use of wastewater intended for irrigation use (DWA, 2013). Therefore *E. coli* was selected as the organism of choice for this study.

It is evident that variation between strains exists with regards to survivability after exposure to treatment options. Environmental strains do not always have the same inactivation kinetics as reference strains. A study done by Cherchi & Gu (2011) on chlorine disinfection indicated that *E. coli* O157:H7 (pathogenic strain) was more sensitive to chlorine disinfection compared to *E. coli* K12 (commensal strain) (Cherchi & Gu, 2011). Wojcicka *et al.* (2007) found results that differed slightly, with *E. coli* O157:H7 being slightly less sensitive than reference strains (after a free chlorine and monochloramine treatment). However, other heterotrophic environmental strains were also included in the study, and the results indicated that they were easily inactivated compared to reference strains (Wojcicka *et al.*, 2007). Different results were found by Mazzola *et al.* (2006), where environmental strains isolated from a water purification system showed higher resistance against chemical disinfectants compared to standard reference strains (Mazzola *et al.*, 2006). It is, however important to understand strain-strain variation in order to optimise treatment options to determine the correct treatment parameters, such as concentration and time.

Many factors can influence the efficacy of H_2O_2 as a treatment option, including: concentration; contact time; organic matter content; and type of microorganisms. There is limited literature available on H_2O_2 concentrations used for irrigation water treatment, thus literature on wastewater treatment was used. Orta de Velásquez *et al.* (2008) found that using 250 mg.L^{-1} H_2O_2 resulted in a 2.2 log reduction after 120 min. The organic matter content in water can also decrease the efficiency of H_2O_2 as a treatment option. Vargas *et al.* (2013) reported that H_2O_2 is not as effective in water containing pollutants, as they are oxidised by the H_2O_2 . It is furthermore, unknown if strain-strain variation will occur during H_2O_2 treatment, as was reported for chlorine. Pathogenic *E. coli* strains have been isolated from surface water in the Boland region (Lamprecht *et al.*, 2014). It is also unknown whether *E. coli* strains with virulence factors are more resistant against H_2O_2 treatment.

Catalase is an enzyme produced by microorganisms in order to protect them from H_2O_2 . Catalase levels may be increased due to phenotypic adaptation to protect the cell against oxidative stress. Consequently, resulting in active resistance against H_2O_2 (Morató *et al.*, 2003). It is unknown to what extent catalase production can influence the effectiveness of H_2O_2 treatment of water.

Thus, the aim of this study was to determine the efficacy of H₂O₂ against both environmental and reference *E. coli* strains, as well as determining the influence different factors could have on H₂O₂ as a treatment option. The study will focus on: the effect of different H₂O₂ concentrations at different contact times; the differences (if any) between the log reductions achieved for environmental *E. coli* strains compared to reference ATCC *E. coli* strains; the effect of H₂O₂ on a potential pathogenic *E. coli* strain; the effect that organic matter content has on the efficacy of H₂O₂ as a treatment option; determining to what extent catalase activity can protect individual *E. coli* strains from the H₂O₂ treatment.

MATERIALS AND METHODS

Design of experimental treatments

The experimental design for the five studies (A, B, C,D and E) is summarised in Figs.1a and b. Study A was performed on river water sampled from the Plankenburg River. Three concentrations of H₂O₂ and five time intervals were tested in order to determine the effect that H₂O₂ had on the ACC (aerobic colony counts), total coliforms and *E. coli* counts in the river water. River water was sampled and ACC, total coliforms and *E. coli* counts were analysed before and after the addition of H₂O₂ in order to determine the log reduction achieved after specific time intervals.

Study B focused on eight environmental *E. coli* strains (M29, M53, MJ58, MJ56, F11.2, E12.1, E11.1 and E22.1) that had previously been isolated from river water and fresh produce. Three ATCC reference *E. coli* strains (ATCC 25922, ATCC 35218 and ATCC 11775) (Table 1) were also studied. The sensitivity of the environmental and reference *E. coli* strains to different H₂O₂ treatments (see Figs. 1a and b) were tested in Physiological Saline Solution (PSS). *Escherichia coli* counts were determined before and after the addition of H₂O₂ in order to determine the log reduction achieved at specific time intervals (Experiments were done in triplicate)

In study C, two environmental *E. coli* strains were studied. One strain showing resistance to the H₂O₂ treatment was selected from study B. A second strain was selected to include a strain carrying virulence factors, this was used to indicate potential pathogenicity. The strains were exposed to lower and higher H₂O₂ concentrations (compared to the concentrations used in the study B) to determine the effect of different H₂O₂ concentrations, as well as determining the effect that H₂O₂ had on a strain expressing virulence factors (Experiments were done in duplicate)

For study D (Fig. 1b), a resistant environmental strain, a sensitive environmental strain and a sensitive ATCC strain to the H₂O₂ treatment were selected. These strains

were then inoculated into autoclaved river water, (instead of PSS) in order to establish if the organic matter (expressed as COD) present in the river water had an influence on the efficacy of the H₂O₂ treatment. River water from two different rivers (Plankenburg and Veldwagters) was sampled in order to achieve water samples with different COD levels. *Escherichia coli* enumeration was done before and after the addition of H₂O₂ in order to determine the log reduction achieved. This was then compared to the results of the *E. coli* strains done in the PSS (Experiments were done in duplicate)

For study E (Fig. 1b), the catalase activity of all 12 *E. coli* strains (Table 1) was measured. The catalase activity was measured to determine if the *E. coli* strains with increased catalase activity were better protected against the H₂O₂ treatment. Two different methods were used in this study. The first method, described by Chen *et al.* (2013), was considered subjective (as it was a visual method) as well as only being able to determine the total catalase activity. The method described by Iwase *et al.* (2013) was included as HPI (hydroperoxidase I), HPII (hydroperoxidase II) and total catalase was measured (Experiments were done in duplicate)

Table 1 *Escherichia coli* strains used in this study, source of isolation and the different experimental studies they were used in

Strain	Source	Experimental study the strain was used in (Figs. 1 a and b)
M29	Mosselbank River	Study B and E
MJ58	Parsley	Study B and E
MJ56	Parsley	Study B, D and E
M53	Eerste River	Study B, C, D and E
E12.1	Plankenburg River ₍₁₎	Study B and E
E22.1	Plankenburg River ₍₁₎	Study B and E
E11.1	Plankenburg River ₍₀₎	Study B and E
F11.2	Plankenburg River ₍₂₎	Study B and E
W 1371	Plankenburg River ₍₂₎	Study C and E
ATCC 11772	Reference	Study B and E
ATCC 25922	Reference	Study B, D and E
ATCC 35218	Reference	Study B and E

(1), (0), and (2) indicate different locations of the Plankenburg River as reported by Britz *et al.* (2013)

STUDY A

River water sample

Determine the impact of three H₂O₂ concentrations (250, 300 and 350 mg.L⁻¹ at five intervals (0 (initial counts), 30, 60, 90 & 120 min) on Aerobic Colony Count and total coliforms *E. coli*

Standard Microbial Methods

Enumeration on Plate Count Agar (PCA) and Violet Red Bile Agar (VRBA)

Water quality parameters

pH

Temperature

COD

Alkalinity

STUDY B

11 *E. coli* strains

Determine the impact of three H₂O₂ concentrations (250, 300 and 350 mg.L⁻¹ at five intervals (0 (initial counts), 30, 60, 90 & 120 min) on *E. coli* strains in PSS

Standard Microbial Methods

Enumeration on VRBA

API 20E

Gram-staining

Oxidase and catalase activity

Oxidative-fermentative

Motility

Antibiotic resistance test (Kirby Bauer)

Molecular Methods

Multiplex PCR (pathotype)

Triplex PCR (genotype)

Figure 1a Experimental design of studies A and B and analyses done.

STUDY C

***E. coli* strain (with high H₂O₂ resistance) and a pathogenic *E. coli* strain**

Determine the impact of four H₂O₂ concentrations (50, 350, 700 & 1000 mg.L⁻¹) at five time intervals (0 (initial counts), 30, 60, 90 & 120) on one of the resistant environmental *E. coli* strains as well as a pathogenic *E. coli* strain

Standard Microbial Methods

Enumeration on VRBA
API 20E
Gram-staining
Oxidase and catalase activity
Oxidative-fermentative
Motility

Molecular methods

Multiplex PCR (pathotype)
Triplex PCR (genotype)

STUDY D

3 *E. coli* strains

Determine the impact of 350 mg.L⁻¹ of H₂O₂ on *E. coli* strains inoculated into autoclaved river water sampled from the Plankenburg and Veldwagters rivers.

Standard Microbial Methods

Sampling
Enumeration on VRBA
Oxidase activity
COD

STUDY E

Catalase activity of all 12 *E. coli* strains

The catalase activity of all 12 *E. coli* strains was measured using two different methods.

Figure 1b Experimental design of studies C, D and E and analyses done.

Site selection and sampling

River water samples for study A were collected over a three-week period in October 2013 (two samples were taken). Samples were taken from the Plankenburg River (P₁) (Britz *et al.*, 2013), which is situated downstream from the Kayamandi Informal Settlement as well as the Plankenbrug Industrial area.

River water samples for study D were also collected from P₁ as well as from the Veldwagters River. This river is situated downstream from the Waste Water Treatment Works in Stellenbosch. For study D, the river water was autoclaved, and inoculated separately with the three environmental strains used in the study.

Water sampling was done according to the SANS 5667-6 method (SANS, 2006). Samples were collected in sterile 1L reagent bottles. The sample was taken as near to the middle of the river as possible. Bottles were submerged about 30 cm below the surface prior to being opened. All samples were kept at 4°C until analysed in the laboratory as soon as possible (Study A).

Water sample analysis

Environmental parameters

pH and Temperature

The pH and temperature were measured simultaneously at the sampling site. pH and temperature was measured according to Standard Methods (APHA, 2005) with a WTW pH 320 digital pH - meter (Xylem Inc, Germany).

Alkalinity

Alkalinity of the samples was measured according to Standard Methods (APHA, 2005), by titrating 20 mL of sample with 0.1 N H₂SO₄ until the pH of the sample reached 4.3.

Conductivity

The conductivity of the water samples was measured with a HI 8633 conductivity meter (Hanna Instruments, South Africa), according to the instruction manual. Samples were measured in duplicate.

Chemical Oxygen Demand

The chemical oxygen demand (COD) was measured colorimetrically using a DR 2000 HACH spectrophotometer (Hach Co. Loveland, CO) set at 585 nm, according to Standard Methods (APHA, 2005). Samples were measured in duplicate.

Microbial analysis

Aerobic colony count (ACC)

The ACC enumeration was performed according to the SANS 4833 (SANS, 2007) method. A dilution series was prepared in PSS. Plate Count Agar (PCA) (Biolab, Merck) was used for the enumeration of the aerobic colony count and the plates were incubated at 30°C for 48 h. All dilutions were plated in triplicate.

*Total coliform and *E. coli* enumeration*

The total coliform and *E. coli* enumeration was performed according to the method described in Standard Methods (APHA, 2005). A dilution series was prepared in PSS and Violet Red Bile Agar (VRBA) (Biolab, Merck) was used for the plating. Plates were incubated at 30°C for 24 h as stipulated in the Merck Manual (Merck, 2005). Red colonies that were surrounded by a red precipitation zone were counted as *E. coli* as described in the Merck Manual (Merck, 2005).

Escherichia coli cultures

For this study, three ATCC strains and nine environmental *E. coli* strains were used (Table 1). The environmental *E. coli* strains had been isolated from previous studies (Department of Food Science, Stellenbosch University) and stored in 40% (v.v⁻¹) glycerol (Fluka Analytical, Germany) at -80°C. After the strains had been removed from the -80°C freezer, 100 µL of each culture was inoculated into 5 mL Nutrient Broth (NB) (Biolab, Merck) and incubated at 35°C for 24 h. A loop-full of this inoculum was then streaked out on Levine Eosin Methylene-Blue Lactose Sucrose Agar (Levine) (L-EMB) (Oxoid, South Africa) to obtain single colonies. *Escherichia coli* colonies have a metallic green sheen on L-EMB agar (Merck, 2005).

Characterisation and confirmation of *E. coli* identification

A series of confirmation tests were done in order to confirm that the test strains were *E. coli* strains. API 20E as well as the additional tests recommended in the API manual were

performed and the results were recorded in order to generate an API code that can be used to identify the strains.

Oxidase

The oxidase test was performed on all the *E. coli* strains and a purple colour change was recorded as a positive result (Gerhardt *et al.*, 1981).

Catalase

The catalase test was performed on all the *E. coli* strains according to the method described by Gerhardt *et al.* (1981). A colony was transferred from Nutrient Agar (Biolab, South Africa) onto a glass slide and covered with three drops of H₂O₂ (3%). The immediate formation of bubbles was recorded as a positive result.

Gram-staining

Gram-staining was performed on all the *E. coli* isolates according to the method described by Gerhardt *et al.* (1981).

Oxidative-Fermentative (O-F) test

Oxidative-fermentative test was performed in O-F basal medium (Merck, South Africa) according to Gerhardt *et al.* (1981). Both aerobic and anaerobic environments were created by adding a layer of mineral oil to the test tubes. All the tubes were incubated at 35°C for 48 h. In both the aerobic and anaerobic tubes, a yellow colour indicated fermentative degradation. A yellow colour in the aerobic test tube after incubation indicated that the glucose had been broken down due to oxidation.

Motility

Motility testing was done according to the hanging drop method described by Gerhardt *et al.* (1981). The slides were examined under the microscope (100 x lens was used) for motility was observed, it was recorded as a positive result.

MacConkey agar

Cultures were streaked out on MacConkey agar (Oxoid, South Africa) and incubated at 37°C for 24 h. Growth on MacConkey agar was recorded as a positive result.

API 20E identification

Each culture was streaked out on Nutrient Agar (Biolab, South Africa) and incubated at 35°C for 24h. An API 20E (BioMèrieux, South Africa) test strip was then inoculated for

each of the *E. coli* strains used in this study. The API test strips were incubated at 37°C for 18 – 24 h. The APIweb™ (BioMérieux, South Africa) database was then used as confirmation that the isolated strains were *E. coli* strains. After the strains had been confirmed as *E. coli*, each strain was stored in 40% (v.v⁻¹) glycerol and stored at -80°C.

H₂O₂ treatments

H₂O₂ solutions

H₂O₂ 30% (v.v⁻¹) (Merck, South Africa) was used to prepare the H₂O₂ concentrations (50, 250, 300, 350, 700 and 1 000 mg.L⁻¹) that were used in this study. The H₂O₂ concentrations were confirmed by a Spectroquant® Hydrogen Peroxide Cell Test (2.0 – 2-mg.L⁻¹) (Merck, South Africa).

Escherichia coli strain preparation and enumeration

Escherichia coli enumeration was done according to the method prescribed in Standard Methods (APHA, 2005). After the strains had been revived from the -80°C freezer, a single colony was inoculated in 5 mL NB (Merck) and incubated at 35°C for 8 h. The NB was then removed from the incubator and aseptically transferred to 80 mL NB (Merck) and then further incubated for 12 h. This NB culture was then used to inoculate the PSS volume required for each experiment to achieve a cell concentration equal to 0.5 McFarland standard (BioMérieux, South Africa) (Fig. 2). *Escherichia coli* enumeration was done before and after the H₂O₂ treatment. A dilution series were prepared before the addition of H₂O₂ (control series), and then the dilution series were prepared after the H₂O₂ was added at 30, 60, 90 and 120 min in order to observe the log reduction at the specific time intervals (Fig. 3)

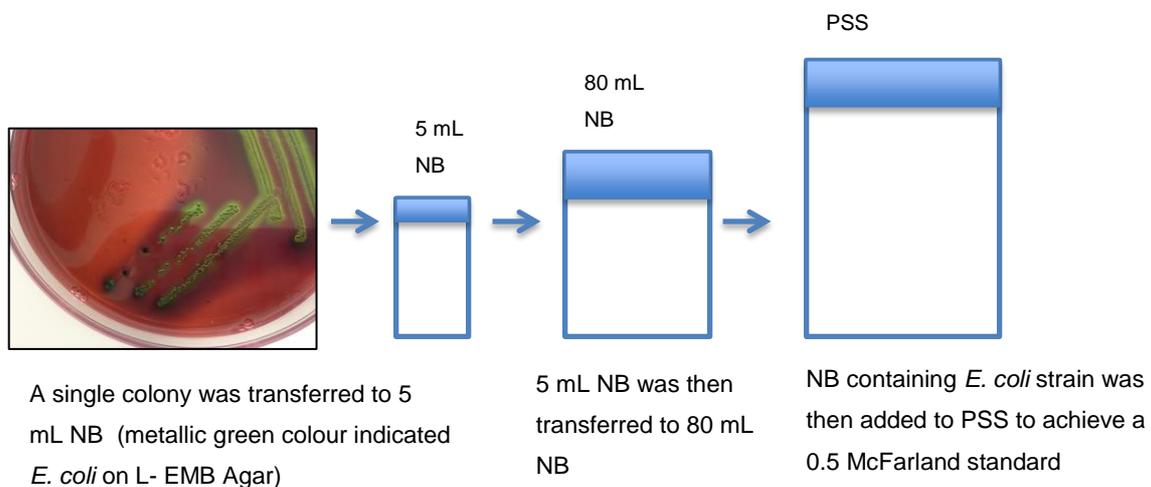


Figure 2 Inoculating PSS with specific *E. coli* strains

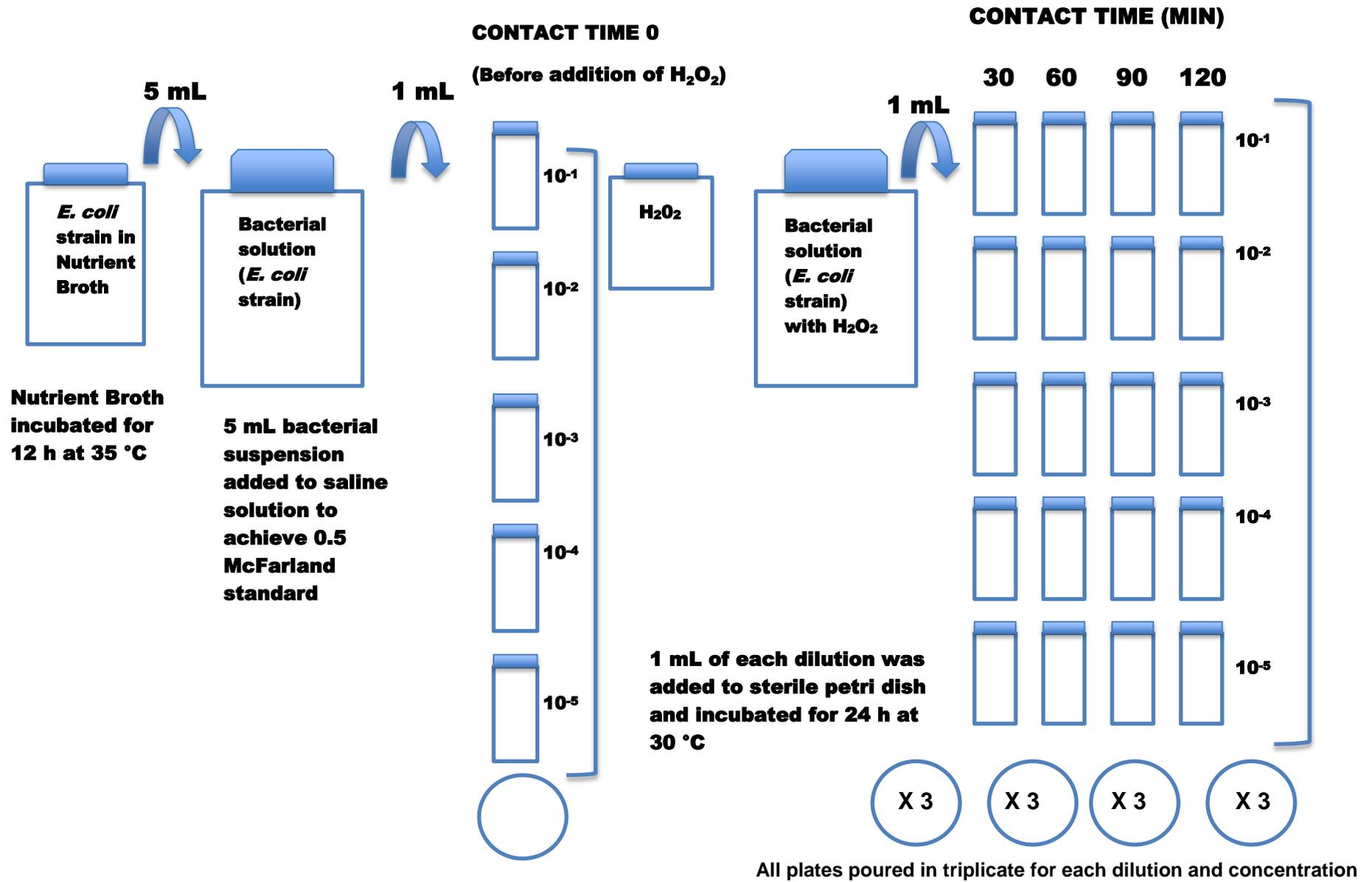


Figure 3 Diagram of experimental set-up used to determine *E. coli* counts before and after each individual H₂O₂ treatment

Antibiotic resistance of *E. coli*

Antibiotic susceptibility tests were done on all 12 *E. coli* strains (Table 1), using to the Kirby-Bauer disc diffusion assay (Andrews, 2009; CLSI, 2009). *Escherichia coli* strains were revived after being stored in the -80°C freezer, this was done by inoculating sterile nutrient broth with 100 µL bacterial suspension and incubating at 37°C for 24 h. After incubation the bacterial suspensions were streaked out on Eosine Methylene-Blue Lactose Sucrose Agar (L-EMB agar) (Oxoid, South Africa) to achieve single colonies. Plates were incubated at 37°C for 24 h. After incubation, colonies displaying a metallic green sheen denoting *E. coli* growth were considered to be *E. coli* (Merck, 2005). Bacterial suspensions were prepared by inoculating sterile nutrient broth with a typical *E. coli* colony and incubating at 37°C for 24 h. The bacterial suspension was used to inoculate PSS and the turbidity was adjusted to achieve a 0.5 McFarland standard. Mueller-Hinton (MH) plates were inoculated with 100 uL of the adjusted bacterial suspension and prepared according to the spread plate method. After the suspension had been added to the MH plates, the plates were dried in the laminar flow cabinet for 3 - 5 min before the antibiotic discs were added. Antibiotic discs (MAST, South Africa) were applied to the surface of the plates with a disc dispenser (MAST, South Africa). Seven antibiotics were chosen for this study (Table 2). All strains were tested in duplicate.

Escherichia coli ATCC 25922 was included as a susceptible control in all the antibiotic resistance tests (ATCC 25922 was susceptible to all the antibiotics used in the study). A quality control plate containing only antibiotics and no bacterial suspension was included to ensure that contamination did not occur during the drying phase in the laminar flow cabinet.

After incubation the plates' inhibition zones were measured (mm) and interpreted (Table 2) and the strains were considered either susceptible, intermediate or resistant against the antibiotics used in the study. Each *E. coli* strain was done in duplicate and average diameter reading values were calculated and used.

Catalase activity of *E. coli* strains

Catalase test

Catalase activity of all 12 *E. coli* strains was measured according to the method described by Chen *et al.* (2013). Strains were streaked out on Nutrient Agar (Biolab, South Africa) and incubated for 37°C at 12 h. A loop-full of culture was then placed on a glass slide and one drop of 30% (v.v⁻¹) H₂O₂ was added to the culture. Results were recorded according

to the robustness of the bubbling, ‘- (no bubble formation)’, ‘+ (little bubble formation)’, ‘++ (average bubble formation)’ and ‘+++ (immediate, strong bubble formation)’.

Table 2 Inhibition zone criteria for determining whether *E. coli* strains are sensitive, resistant or intermediate against the specific antibiotics tested (Andrews, 2009; CLSI; 2009)

Antibiotic	Concentration	S \geq (mm)	I (mm)	R \leq (mm)
Ampicillin	10 μ g	17	14-16	13
Cephalothin	30 μ g	18	15-17	14
Chloramphenicol	30 μ g	18	13-17	12
Ciprofloxacin	5 μ g	21	16-20	15
Streptomycin	10 μ g	15	12-14	11
Tetracycline	30 μ g	15	12-14	11
Trimethoprim	2.5 μ g	17	11-15	13

S= susceptible, I= intermediate and R= resistant

Quantification of catalase activity

The catalase activity of all 12 *E. coli* strains was measured according to the method described by Iwase *et al.* (2013). Strains were streaked out on Nutrient Agar (Biolab, Merck) and incubated at 37°C for 12 h. A sterile plastic loop was used to transfer 0.010 g of wet weight cells to a 200 μ L PCR tube, 100 μ L of PSS was added to each 200 μ L PCR tube and vortexed. The bacterial solution in the PSS was then added to a test tube, after which 100 μ L Triton X-100 was added, followed by the addition of 100 μ L H₂O₂ solution (30% v.v⁻¹). The height of the foam was measured after the foam height had remained constant for 15 min. This value was then used as an indication of total catalase activity.

In order to distinguish between the two types of catalase, HPI and HPII, a second aliquot in PSS was also prepared. This aliquot underwent a heat treatment of 55°C for 15 min. The heat treatment was done before adding the cell suspension to the test tube with Triton-X and H₂O₂. Physiological saline solution was added to the bacterial cells. The heat-stable catalase (HPII activity) was determined by measuring the height of foam that had been stable for 15 min of the heat treated aliquots. The heat-labile (HPI activity) catalase activity was then determined by subtracting the heat-stable catalase activity from the total catalase activity.

Calibration curve

A series of standard catalase solutions were prepared (Sigma, South Africa). Each catalase solution (100 µL) was added to a test tube, after which 100 µL of Triton-X and 100 µL of H₂O₂ (30% v.v⁻¹) was added and mixed. The height of the foam was measured after the foam height had remained constant for 15 min. A calibration curve was plotted with the defined catalase activity (5, 10, 15, 17.5 and 20 catalase activity units). The height of the foam formation was then used to determine the catalase units.

Molecular methods

DNA extraction method

Whole cell extracts were prepared according to the method described by Altalhi & Hassen (2009). The strain was cultivated on Tryptone Soya Agar (TSA) (Oxoid, South Africa) and incubated at 37°C for 24 h. A single colony was then transferred to 100 µL sterile nuclease-free water and boiled for 13 min in a 1.5 mL microcentrifuge tube. This was done to lyse the cells and release its contents. The tubes were cooled on ice and centrifuged for 15 min at 14 000 × g. The supernatant, containing the DNA was then transferred to a sterile 0.6 µL PCR tube and stored at -20°C.

Multiplex Polymerase Chain Reaction (PCR) for pathotype detection

Multiplex PCR (m-PCR) was done on strain W1371 to confirm the presence of pathogenic gene sequences. The m-PCR method was done as described by Lamprecht *et al.* (2014). The reaction volume for the m-PCR was 12.5 µL, which consisted of 0.2 µM of each primer (Table 3), 1 × KAPA 2G fast multiplex PCR master mix (KAPA Biosystems, South Africa), and 0,25 µL of the template DNA. A Standard Culture Mix (SCM) was prepared in advance and used as the positive control. The SCM consisted of DNA from each pathotype investigated (EPEC, EAEC, EHEC, EPEC, EIEC), and was then added in place of the DNA template as the positive control. A negative control was also prepared by replacing the DNA template with nuclease-free water. The tubes were then placed in the G-storm thermal cycler (Vacutec, South Africa), and reactions outlined in Table 4 were applied. The PCR products were analysed, using gel electrophoresis with a 1.75% agarose (Seekem, Switzerland) gel containing 1 µg.mL⁻¹ ethidium bromide (Sigma, Germany). Gel electrophoresis was performed for 90 min at 120 V. Band patterns were then observed under UV light.

Table 3 m- PCR primers for detecting *E. coli* pathotypes

Pathotype	Primer	Primer sequence	Size (bp)	Reference	
Commensal (<i>mdh</i>)	Mdh01	GGTATGGATCGTTCCGACCT	300	Tarr <i>et al.</i> , 2002	
	Mdho02	GGCAGAATGGTAACACCAGAGT			
EPEC/EHEC (<i>eaeA</i>)	L- <i>eaeA</i> (F)	GACCCGGCACAAGCATAAGC	384	Lopez-Suacedo <i>et al.</i> , 2003	
	L- <i>eaeA</i> (R)	CCACCTGCAGCAACAAGAGG			
EHEC (<i>stx1</i> , <i>stx2</i>)	Stx1 (F)	ACACTGGATGATCTCAGTGG	614	Moses <i>et al.</i> , 2006	
	Stx1 (R)	CTGAATCCCCCTCCATTATG			
	Stx2 (F)	CCATGACAACGGACAGCAGTT		779	Moses <i>et al.</i> , 2006
	Stx2 (R)	CCTGTCAACTGAGCACTTTG			
EIEC (<i>ial</i>)	L- <i>ial</i> (F)	GGTATGATGATGATGATTCCA	650	Lopez-Suacedo <i>et al.</i> , 2003	
	<i>ial</i> (R)	GGAGGCCAACAATTATTTC			
EAEC (<i>eagg</i>)	Eagg (F)	AGACTCTGGCGAAAGACTGTATC	194	Pass <i>et al.</i> , 2000	
	Eagg (R)	ATGGCTGTCTGTAATAGATGAGAAC			
ETEC (LT, ST)	LT (F)	GGCGACAGATTATACCGTGC	450	Lopez-Suacedo <i>et al.</i> , 2003	
	LT (R)	CGGTCTCTATATTCCCTGTT			
	ST (F)	TTTCCCCTCTTTTAGTCAGTCAACT	160	Omar & Barnard, 2010	
	ST (R)	GGCAGGATTACAACAAAGTTCACA			

Table 4 Summary of m-PCR reaction conditions

Step	Temperature (°C)	Time (mm:ss)
Initial denaturation	95	15:00
Denaturation	94	00:45
Primer annealing	55	00:45
Elongation	68	02:00
Final elongation	72	05:00
Cooling	4	00:30

Triplex Polymerase Chain Reaction (t-PCR) for genogroup detection

The t-PCR method was done to confirm the phylogenetic grouping of strain W1371 and was done according to the method described by Clermont *et al.* (2000). The t-PCR had a reaction volume of 12 μL , which consisted of 6.25 μL 1 \times KAPA 2G fast multiplex PCR master mix (KAPA Biosystems, South Africa), 0.2 μM of each primer (Table 5) and 0.25 μL of the template DNA. ATCC 25922 was used as a positive control, as it contains the following genetic markers; *chuA* and *yjaA* and the DNA fragment TspE4.C2. A negative control was also included, and this was prepared by replacing the DNA template with nuclease-free water. All the tubes were then placed in the G-storm thermal cycler (Vacutec, South Africa). The reaction conditions in Table 6 were applied. The PCR products were analysed, using gel electrophoresis with a 2% agarose (Seekem, Switzerland) gel containing 1 $\mu\text{g.mL}^{-1}$ ethidium bromide (Sigma, Germany). Gel electrophoresis was performed for 20 min at 210 V. After the gel electrophoresis was complete, band patterns were observed under UV light, in order to determine which combination of the three DNA fragments were amplified. Based on band patterns, the strain could be assigned to one of the four main phylogenetic groups (A, B1, B2 or D). The four main groups could be subdivided into seven phylogenetic subgroups (A₀, A₁, B₁, B₂, B₃, D1 or D₂) (Table 7).

Table 5 List of primer sequences and amplicon sizes used for t-PCR (Clermont *et al.*, 2000)

Primer	Primer sequence	Size (bp)
chuA (F)	GACGAACCAACGGTCAGGAT	
chuA (R)	TGCCGCCAGTACCAAAGACA	279
yjaA (F)	TGAAGTGTCAGGAGACGCTG	
yjaA (R)	ATGGAGAATGCGTTCCTCAAC	211
TSPE4.C2 (F)	GAGTAATGTCGGGGCATTCA	
TSPE4.C2 (R)	CGCGCCAACAAAGTATTACG	152

(F) – Forward primer; (R) – Reverse primer

Table 6 Summary of t-PCR reaction conditions

Step	Temperature (°C)	Time (mm:ss)
Initial denaturation	95	3:00
<i>30 cycles of:</i>		
Denaturation	95	00:30
Primer annealing	60	00:30
Extension	72	00:30
Final extension	72	05:00

Table 7 Distribution of genetic markers *chuA*, *yjaA* and DNA fragment TSPE4.C2 denoting each of the seven phylogenetic subgroups (Carlos *et al.*, 2010)

Phylogenetic group	<i>chuA</i>	<i>yjaA</i>	TSPE4.C2
A ₀	-	-	-
A ₁	-	+	-
B ₁	-	-	+
B _{2₂}	+	+	-
B _{2₃}	+	+	+
D	+	-	-
D ₂	+	-	+

Statistical analysis

Main effects ANOVA using Statistica 12.5 (Statsoft, 2014) was used, with *E. coli* strain and time as the two effects. The main purpose of doing the ANOVA was to determine significant differences between means. A mixed model repeated measures ANOVA was used for the analysis in study B. A mixed model repeated measures ANOVA was used to analyse the *E. coli* counts and a two-way ANOVA was used to analyse the log reductions achieved in study and C. In study D, a mixed model repeated measures ANOVA was used to analyse the *E. coli* counts and a one way ANOVA was used to analyse the log reductions achieved in the PSS, Plankenburg and Veldwagters River samples. Post hoc tests were conducted using Fisher least significant difference (LSD) testing. A 5%

significance level ($p < 0.05$) was used as guideline for determining significant results. If no *E. coli* growth was observed it was recorded as 30 cfu.mL^{-1} ($1.48 \log \text{ cfu.mL}^{-1}$) (as the lower limit) for all the statistical analyses done in studies B, C and D.

RESULTS AND DISCUSSION

STUDY A: Effect of H_2O_2 on the microbial loads in river water

Physico-chemical analysis

The chemical data obtained for the two samples from Plankenburg River site 1 (P_1) are summarised in Table 8. The pH measured in both river water samples were within the DWA regulations, which stipulates that the pH must be between 5.5 and 9.0 (DWA, 2013). The temperature of the water was similar to temperatures reported for the Plankenburg River in a previous study done by Britz *et al.*, (2013). According to Britz *et al.* (2013) water temperatures varied between $9.9 - 21.8^\circ\text{C}$, with colder values ($< 15^\circ\text{C}$) seen between May and October (Britz *et al.*, 2013). The alkalinity and COD values determined previously at this site ranged between $14 - 1\ 125 \text{ mg.L}^{-1}$ and $0 - 69 \text{ mg.L}^{-1}$, respectively (Britz *et al.*, 2013). The alkalinity and COD values in this study were slightly lower than previously reported by Britz *et al.*, (2013), although they were within the same range. All the physico-chemical parameters were within the DWA guidelines and therefore the water can be used for the irrigation of fresh produce (DWAF, 1996; DWA, 2013).

Table 8 Physico-chemical data for the two river water samples taken from P1- during October 2013

Sample	pH	Temperature ($^\circ\text{C}$)	Alkalinity (mg.L^{-1})	Electrical conductivity (mS.m^{-1})	COD (mg.L^{-1})
Sample 1	6.95	15.1	150	0.46	5.4
Sample 2	6.71	14	100	0.47	9

Microbial analysis – H₂O₂ treatment

The effect of H₂O₂ on river water was tested using ACC, total coliforms and *E. coli*. This was done in order to determine the impact that 250, 300 and 350 mg.L⁻¹ H₂O₂ had on the microbial reduction after 0, 30, 60, 90 and 120 min exposure times.

Effect of H₂O₂ on ACC

The ACC counts in the river water samples are shown in Figs. 4a and b. In river water Sample 1 (Fig. 4a), the counts decreased (only to 60 min) over the 120 min contact time for all three H₂O₂ concentrations (250, 300 and 350 mg.L⁻¹). Initial counts were 4.57 log cfu.mL⁻¹ and after the 120 min contact time, the counts had decreased to 3.45, 3.30 and 2.95 log cfu.mL⁻¹, respectively. Thus, achieving a 1.12, 1.27 and 1.61 log reduction at 250, 300 and 350 mg.L⁻¹, respectively. At 30 min similar counts were seen at each concentration. Yet, at 60, 90 and 120 min lower counts were seen when the higher H₂O₂ concentration (350 mg.L⁻¹) was used (Fig. 4a). It was expected that the higher H₂O₂ concentration would result in a greater log reduction.

In river water Sample 2, a 2.05, 1.92 and 1.57 log reductions were achieved at 250, 300 and 350 mg.L⁻¹, respectively after 120 min. The log reductions achieved differed between river Sample 1 and 2, with river Sample 2 being more sensitive to the H₂O₂ treatment. The two river water samples probably contained different bacterial strains and species that could have reacted differently to the H₂O₂ treatment. This may explain why differences were seen in the microbial decrease between the two samples. The initial ACC counts were lower for sample 2. This could also influence the microbial decrease. The samples were taken three weeks apart and variations in the microbial counts have been observed in the Plankenburg River over time (Britz *et al.*, 2013).

Effect of H₂O₂ on total coliforms

The impact of 250, 300 and 350 mg.L⁻¹ H₂O₂ on the total coliforms over the 120 min contact time is shown in Fig. 5a and b for river Sample 1 and 2, respectively. A rapid microbial decrease was seen in the first 20-30 min thereafter the decrease was slow. For river water Sample 1, the total coliforms decreased from 3.8 log cfu.mL⁻¹ to 2.09, 2.21 and 1.92 log cfu.mL⁻¹ at 250, 300 and 350 mg.L⁻¹, respectively after 120 min. Thus, a 1.73, 1.60 and 1.90 log reduction was achieved at 250, 300 and 350 mg.L⁻¹, respectively, after 120 min. The highest log reduction was achieved at 350 mg.L⁻¹, which was expected.

For river Sample 2, the initial total coliform counts were slightly lower than the counts observed in Sample 1. After 120 min, a 2.63, 2.69 and 2.25 log reduction was

achieved for 250, 300 and 350 mg.L⁻¹, respectively. The initial total coliform counts were in the same range for 250 and 300 mg.L⁻¹, but they were slightly lower for the 350 mg.L⁻¹ sample. However, after the 120 min contact time coliform counts were similar for all three H₂O₂ concentrations. It was observed that lower log reductions were achieved in river water Sample 1 when compared to Sample 2. This could indicate a variation of bacterial strains and species, which could react differently to the H₂O₂ treatment. The different initial coliform counts could influence the treatment option. The regulations and guidelines stipulated by DWA and WHO refer to faecal coliforms, there are no regulations referring to total coliforms and therefore *E. coli* counts were also measures (as a faecal coliform) (WHO, 1989; DWAF, 1996; DWA, 2013).

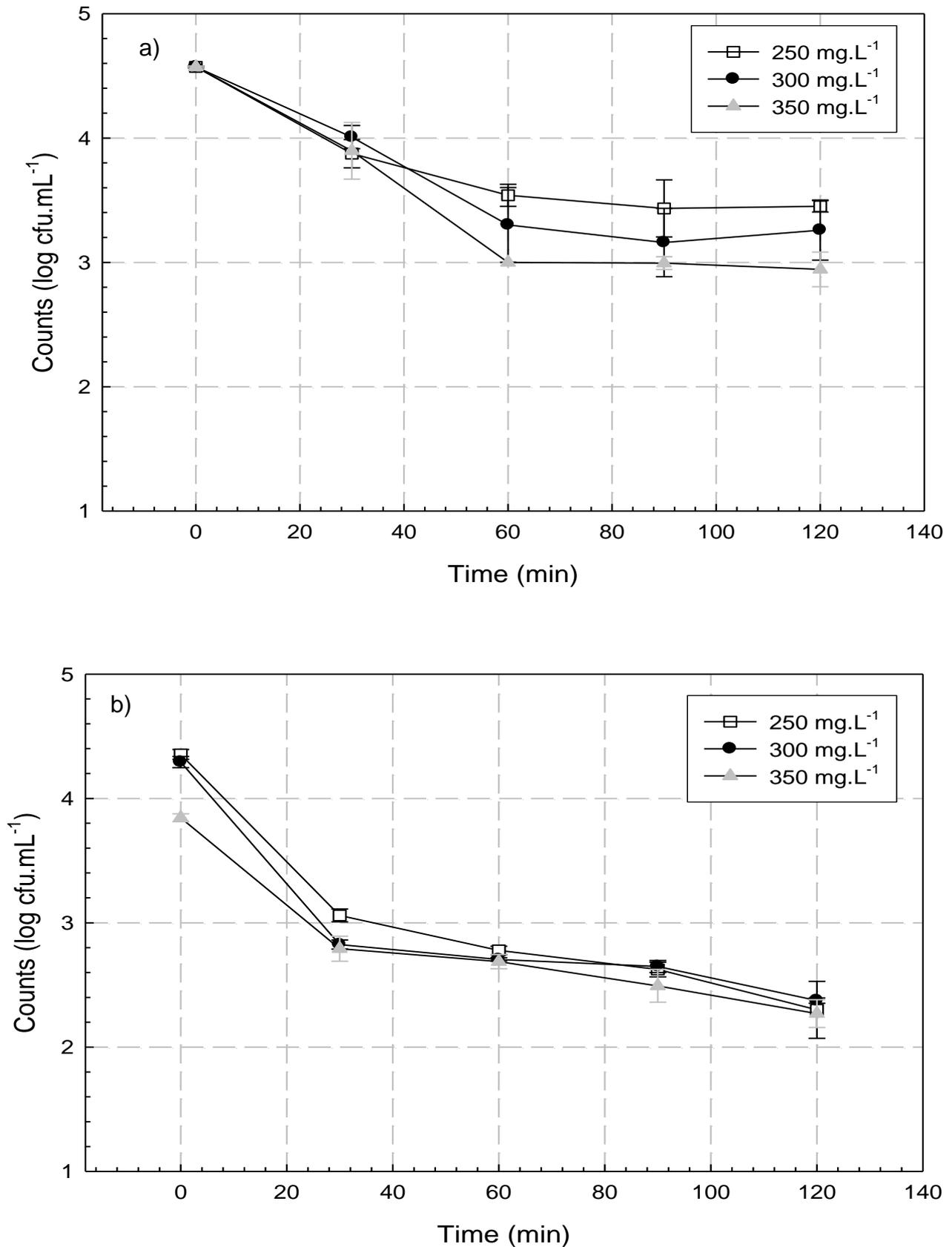


Figure 4 Effect of H₂O₂ on ACC levels in sampled river water at specific time intervals (a) Sample 1; (b) Sample 2. Error bars represent error at a 95% confidence interval.

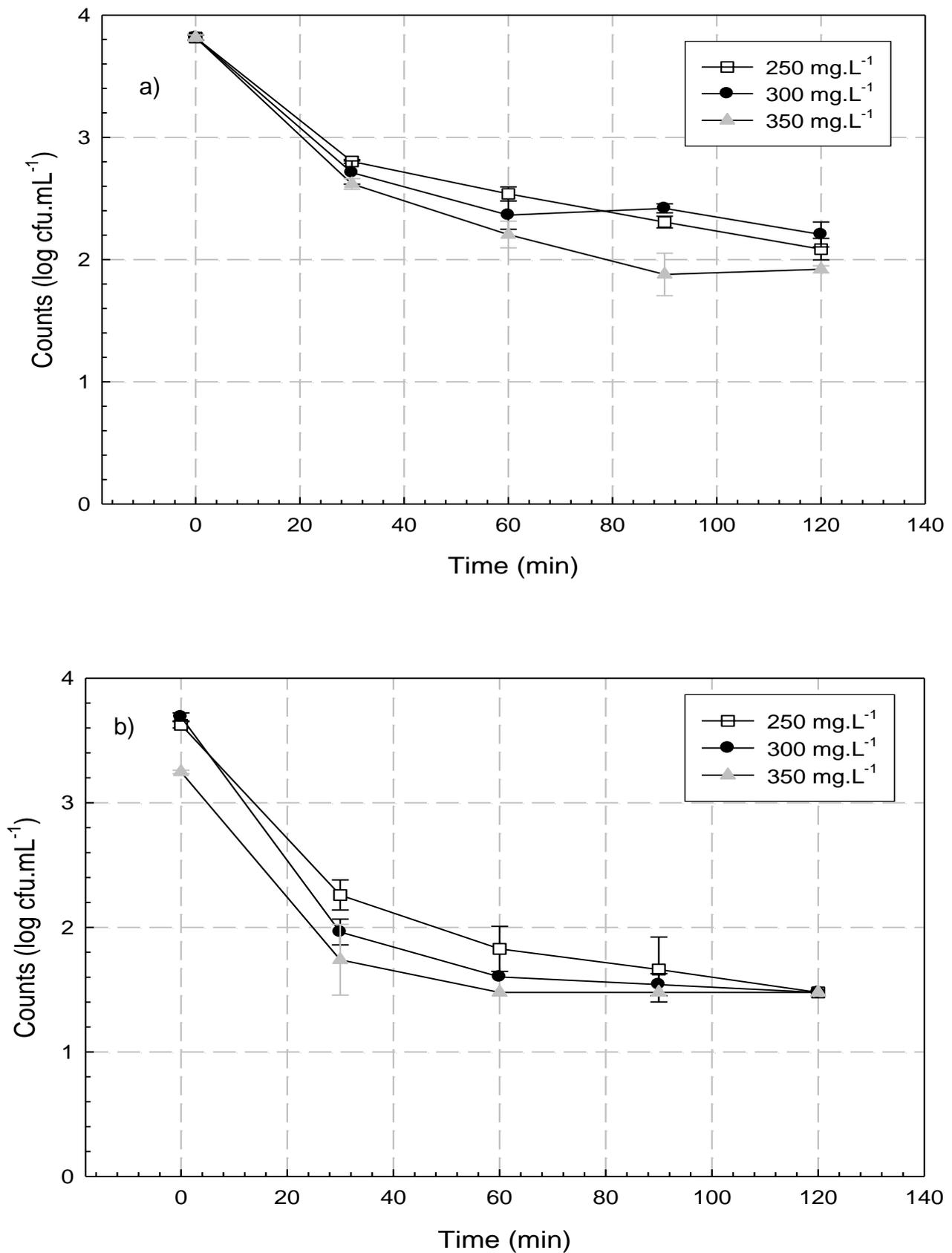


Figure 5 Effect of H₂O₂ on total coliforms in sampled river water at specific time intervals (a) Sample 1; (b) Sample 2. Error bars represent error at a 95% confidence interval.

Effect of H₂O₂ on *E. coli*

The impact of 250, 300 and 350 mg.L⁻¹ H₂O₂ on the *E. coli* counts over the 120 min contact time is shown in Fig. 6a and b. A rapid microbial decrease was observed in the first 30 min. For river water Sample 1, the initial *E. coli* counts were all around 3.79 log cfu.mL⁻¹, which decreased to 1.98, 2.11 and 1.75 log cfu.mL⁻¹ after 120 min with 250, 300 and 350 mg.L⁻¹ H₂O₂, respectively. After 30 min the *E. coli* counts had decreased to values in the same range for all three H₂O₂ concentration (Fig. 6a). Yet, a final 2.04 log reduction was achieved with 350 mg.L⁻¹, whereas a 1.81 and 1.68 log reduction was achieved with 250 and 300 mg.L⁻¹, respectively after 120 min. Although it was expected that the higher H₂O₂ concentration would result in a higher log reduction, a slightly higher log reduction was seen at 250 compared to 300 mg.L⁻¹. Similar results for faecal coliforms were reported by Vargas *et al.* (2013) where a greater microbial reduction was seen at 200 mg.L⁻¹ compared to 300 mg.L⁻¹ (Vargas *et al.*, 2013). There are many different *E. coli* strains present in the Plankenburg River (Lamprecht *et al.*, 2014), which may react differently to the H₂O₂ treatment.

For river water sample 2, the initial *E. coli* numbers were lower than Sample 1 and differed slightly for each treatment (Fig. 6b). After the 120 min, 2.43, 2.54 and 2.23 log reductions were achieved for 250, 300 and 350 mg.L⁻¹, respectively (Fig.6). The initial *E. coli* counts were not the same for the three samples and therefore the log reductions after 120 min were calculated in order to compare the microbial decrease between the H₂O₂ concentrations.

The log reductions achieved after the 120 min contact time, differed between the river water samples. In this study the *E. coli* counts were measured per 1 mL, whereas the regulations according to DWA and WHO are based per 100 mL. Therefore, for discussion purposes, the counts were multiplied by 100 to give an estimate of what could be present in a 100 mL sample. With regards to the *E. coli* counts in river water Sample 1 it was estimated that, 9 666, 13 000 and 5 666 cfu per 100.mL⁻¹ were present after the 120 min contact time for 250, 300 and 350 mg.L⁻¹, respectively. According to WHO and DWA, faecal coliforms (*E. coli*) must be less than 1 000 in 100 mL sample of irrigation water (WHO, 1989; DWA, 1996; DWA, 2013). It was concluded that this water could not be used for irrigation of fresh produce, even after it had been treated with H₂O₂.

The *E. coli* counts in river water sample 2 ranged between 1 and 3.60 log cfu.mL⁻¹. After the H₂O₂ treatment counts were around 1 log cfu.mL⁻¹ (this would be estimated to be 1 000 cfu per 100 mL). However, enumeration of *E. coli* would need to be done on a 100 mL sample to determine if the water was safe to use.

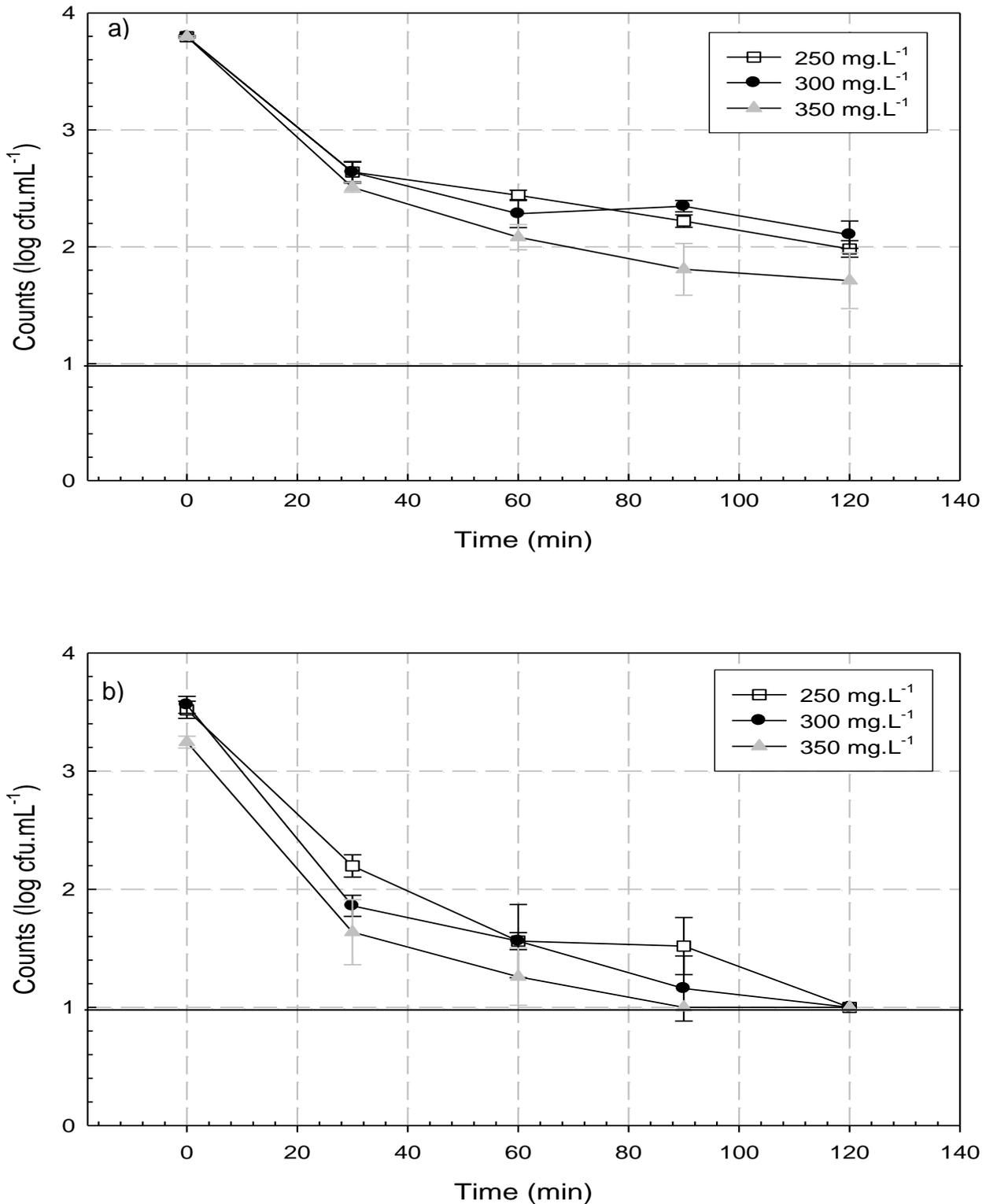


Figure 6 Effect of H₂O₂ on *E. coli* in sampled river water at specific time intervals (a) Sample 1; (b) Sample 2. The solid line represents the DWA guidelines of less than 1000 faecal coliforms per 100 mL. The method only required a 1 mL sample, therefore, if more than 1 log was present in 1 mL the estimated value would be above the limit of 1 000 faecal coliforms in 100 mL (DWA, 2013). Error bars represent error at a 95% confidence interval.

Overall, a trend was seen which indicated that the higher H₂O₂ concentrations resulted in a higher log reduction, which was expected. It was observed that a 120 min contact time would be needed to achieve better log reductions, since the counts were still decreasing (slowly) in the last 30 min for most of the treatments. When comparing the overall microbial inactivation from river sample 1 and 2, it was observed that the same log reductions were not achieved in the different samples. There are three reasons that could explain why this was observed. Firstly, the initial microbial counts differed for sample 1 and 2 and this could influence the log reductions achieved, where a higher log reduction was achieved in the sample with the lower initial counts. Secondly, the two river samples probably contained different bacterial strains and species, which could have reacted differently to the H₂O₂ treatment. The samples were not sampled on the same day and therefore the time lapse may have influenced the microbial levels present in the sample. Thirdly, other unknown substances may be present in the water (from runoff) that may interact or interfere with the H₂O₂ treatment.

***Escherichia coli* strain characteristics**

A collection of previously isolated environmental *E. coli* strains was chosen to represent a heterogeneous *E. coli* population that might be present in irrigation water. These strains were then applied in studies in B, C, D and E.

Analytical Profile Index (API 20E)

The API 20E system uses 20 miniature biochemical tests along with an extensive database APIweb™ (BioMérieux, South Africa) to achieve accurate identification of microorganisms. All the strains used in the study were confirmed as *E. coli* by the API 20E test (Table 9).

All the API profiles observed for the environmental *E. coli* strains used in this study, have been reported before for *E. coli* isolated from soil and surface water (Brennan *et al.*, 2010; Janezic *et al.*, 2013). It can be seen that M29, MJ56 and E12.1 had the same biochemical profile (5144572). The same was observed for MJ58 and F11.2 (5044552), as well as M53, E11.1, ATCC 11775 and ATCC 25922 (5144552). MJ58 and MJ56 were both isolated from fresh parsley and M29, F11.2. The other environmental strains were isolated from river water. However Janezic *et al.* (2013) reported that for their study the 5144572 and 5144552 were the most abundant profiles isolated from surface water. Three environmental strains in this study were identified as having 5144572 as a

biochemical profile and two environmental and two ATCC strains were identified as having 5144572 as a biochemical profile.

Table 9 API codes and % certainty for each *E. coli* strain used in the study

Strain	API code	% Certainty	Profile description
M29	5144572	99.5	Very good identification
MJ56	5144572	99.5	Very good identification
E12.1	5144572	99.5	Very good identification
MJ58	5044552	99.9	Excellent identification
F11.2	5044552	99.9	Excellent identification
M53	5144552	99.9	Excellent identification
E11.1	5144552	99.9	Excellent identification
ATCC 11772	5144552	99.9	Excellent identification
ATCC 25922	5144552	99.9	Excellent identification
ATCC 35218	5144570	99.5	Very good identification
W1371 (potential EPEC)	50441*72	99.1	Very good
E22.1	5044542	99.9	Excellent identification

* sorbitol negative

The biochemical profiles of the parsley isolate MJ58 and MJ56 differed by a single biochemical test. *Escherichia coli* strain with the biochemical profile, 5144572 (MJ56) was able to ferment saccharose (sucrose) whereas, 5144552 was not able to ferment saccharose (Janezic *et al.*, 2013). Fermentation of saccharose has been linked to plant-associated *E. coli*. Méric *et al.* (2013) found that a large proportion of plant-associated bacteria utilise saccharose as a carbon source. The following *E. coli* strains were also able to ferment saccharose: M29, E12.1, ATCC 35218 and W1371. Therefore, theoretically speaking, these strains (and other *E. coli* with similar properties) might have a better chance to survive on fresh produce if transfer does occur during irrigation. Inactivation of these strains is thus very important during irrigation water treatment.

Escherichia coli strain W1371 was the only strain unable to ferment sorbitol, this was also reported in *E. coli* isolated from soil before (Brennan *et al.*, 2010). Ojeda *et al.* (1995), identified five out of 40 (12.5%) sorbitol negative EPEC *E. coli* strains, thus this was a good representative of a sorbitol negative EPEC *E. coli* strains, as an EPEC strain was included in this study.

Phylogenetic subgroups

The phylogenetic group of W1371 was determined as a B2₃ according to triplex PCR. (Fig. 7). Where *chuA*, *yjaA* and TSPE4.C2 genes were present for W1371 (Fig. 7). The phylogenetic groups of all the *E. coli* strains are presented in (Table 9). Most of the isolated environmental strains were from the A and B1 genogroups, whereas only one strain was from the D genogroup (Table 10).

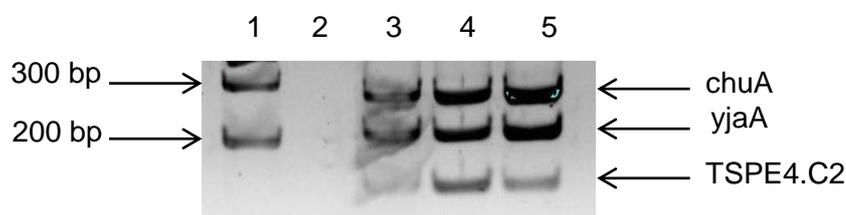


Figure 7 Agarose gel (2% agarose gel and $1\mu\text{g.L}^{-1}$ ethidium bromide) with triplex PCR amplicons for W1371. Lane 1 = 100 bp marker. Lane 2 = negative control. Lane 3 and 5 = duplicate of sample W1371. Lane 4 = positive control.

Table 10 Summary of phylogenetic groups of all the 12 *E. coli* strains used in the study

<i>E. coli</i> strain	Phylogenetic group (genogroup)	Confirmation
M29	B1	Romanis, 2013
MJ58	A ₀	Jordaan, 2013
MJ56	D ₂	Jordaan, 2013
M53	B1	Romanis, 2013
E12.1	B1	Schoeman, 2013
E22.1	A ₁	Schoeman, 2013
E11.1	B2 ₃	Schoeman, 2013
F11.2	A ₁	Schoeman, 2013
W1371	B2 ₃	In this study
ATCC 11772	B2 ₃	Romanis, 2013
ATCC 25922	B2 ₃	Romanis, 2013
ATCC 35218	B2 ₃	Romanis, 2013

A study done by Hamelin *et al.* (2007) on the genogroup distribution of environmental *E. coli* isolated from aquatic systems, observed that most *E. coli* belonged to genogroups A and B1. Only a few were identified as D. Carlos *et al.*, (2010) also reported on a variety of *E. coli* strains with regards to their genogroups. In their study, *E. coli* strains grouped in the subgroup B2₃, were only isolated from human samples, and therefore could be considered an indicator of human faecal contamination. If Carlos *et al.* (2010) conclusions are applied in this study the presence of E11.1 and W1371 could potentially be considered to be from human faecal contamination.

Méric *et al.* (2013) reported that the majority of the plant-associated *E. coli* belonged to phylogenetic group B1, whereas animal-associated *E. coli* were found to belong to A and B2. This indicates that *E. coli* belonging to the B1 phylogenetic group may survive for longer periods of time on fresh produce if transferred during irrigation. *Escherichia coli* strains M29, M53 and E12.1 may therefore survive better on fresh produce, as they belong to the phylogenetic group B1. *Escherichia coli* strains M29 and E12.1 are also capable of fermenting saccharose, which may also increase the chance of survival on fresh produce.

Multiplex PCR

Escherichia coli W1371 tested positive for the *eaeA* gene, and thus it was confirmed to carry EPEC (Enteropathogenic *E. coli*) virulence factors (Fig. 8). The *eaeA* gene is located on the locus of enterocyte effacement (LEE). EPEC strains are capable of producing attaching and effacing (A/E) lesions, enabling the *E. coli* to attach tightly to the cell membrane. The cell surface can be disrupted which leads to the bacteria effacing the microvilli (Nataro & Kaper, 1998; Ochoa & Contreras, 2011). EPEC strains are responsible for causing diarrhoea, especially in young children (Vallance & Finley, 2000).

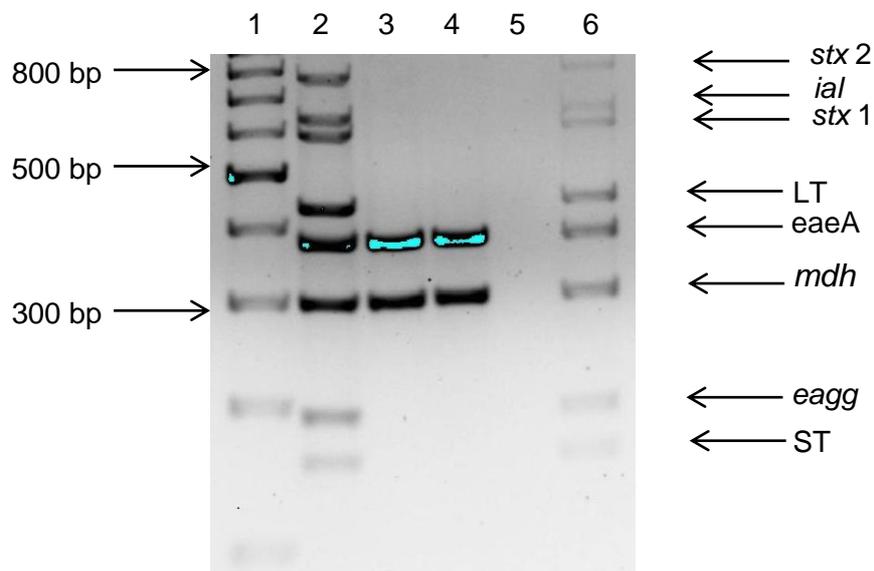


Figure 8 Agarose gel (1.75% agarose and $1\mu\text{g}\cdot\text{L}^{-1}$ ethidium bromide) with multiplex PCR amplicons. Lane 1 contains 100 bp marker. Lane 2 and 6 = positive control containing virulence genes. Lane 3 and 4 = duplicate of sample W1371 (EPEC strain). Lane 5 = negative control.

Antibiotic resistance of *E. coli* strains

The Kirby Bauer test results indicated that five out of the 12 *E. coli* strains showed antibiotic resistance to the antibiotics tested in this study (Table 11). Four of the environmental *E. coli* strains and one of the ATCC reference strains showed antibiotic resistance (Table 11). *Escherichia coli* strains M29, M53, ATCC 35218 and W1371 showed antibiotic resistance against more than one antibiotic whereas F11.2 only showed antibiotic resistance against a single antibiotic.

Antibiotic resistant *E. coli* strains have been isolated before from ground and surface water in South Africa. Phokela *et al.* (2011) isolated *E. coli* strains from ground water samples, indicating resistance to tetracycline, chloramphenicol and streptomycin. Ademola *et al.* (2009) isolated *E. coli* strains from surface water from the Umgeni River (Kwazulu-Natal) with 71.15% of the *E. coli* strains showing multi-resistance to antibiotics. Antibiotic resistant *E. coli* can be transferred from irrigation water via produce to consumers and this can have a negative impact on human health. These *E. coli* are thus difficult to treat due to their antibiotic resistance.

Table 11 Antibiotic resistance of 12 *E. coli* strains used in the study

Strain	Resistance
M29	C, TM
MJ58	None
MJ56	None
M53	T, TM, AMP, S
E12.1	None
E22.1	None
E11.1	None
F11.2	T
W1371	S, TM, A
ATCC 11772	None
ATCC 25922	None
ATCC 35218	AMP, C, STR

C= chloramphenicol, T= tetracycline, TM= trimethoprim, AMP= ampicillin, STR= streptomycin

STUDY B: Effect of H₂O₂ on environmental *E. coli* strains

After the effect of H₂O₂ treatment on river water was tested in study A, it was observed that the log reductions achieved in the two river water samples differed. One possible reason for this can be that the different strains in the river water do not necessarily react in the same way to the H₂O₂ treatment.

In Study B environmental *E. coli* strains and ATCC reference *E. coli* strains were tested in order to determine if the selected environmental strains react differently to the H₂O₂ treatment. It is also important to determine if environmental strains react in the same

way to the H₂O₂ treatment as the ATCC strains. ATCC strains are often used for studies on biocide efficiency, yet they do not always have the same growth patterns as environmental strains (Wojcicka *et al.*, 2007).

The effect of the different H₂O₂ concentrations (250, 300 and 350 mg.L⁻¹) as well as the effect of the contact time was thus studied. Koivunen & Heinonen-Tanski (2005) investigated the effect that H₂O₂ had on *E. coli* during a 10 min contact time and results indicated a less than 0.2 log reduction at 150 mg.L⁻¹. Thus, longer contact times were selected.

In an attempt to achieve higher log reductions longer contact times and higher H₂O₂ concentrations were selected for this study. From a previous study by Britz *et al.* (2013), it was concluded that at least a three log reduction would be required to obtain irrigation water with less than 1 000 faecal coliforms in 100 mL, as stipulated by DWA and WHO (WHO, 1989; DWAF, 1996; DWA, 2013). The contamination is not always as high, but counts can vary as observed in study A. This is due to the fact that high counts have been reported in the rivers in the Boland region before (Britz *et al.*, 2013) therefore, a three log reduction was considered to be the minimum target to achieve adherence to the guidelines.

Effect of H₂O₂ concentration and contact time on selected E. coli strains

The effect of three H₂O₂ concentrations (250, 300 and 350 mg.L⁻¹) at 0, 30, 60, 90 and 120 min was determined on 11 *E. coli* strains (pure cultures) (three ATCC strains and eight environmental strains) and results are presented in Fig. 9. The highest H₂O₂ concentration (350 mg.L⁻¹) was the most effective and resulted in a higher log reduction over 120 min (Fig. 9). When comparing the 250 and 300 mg.L⁻¹ treatments, 300 mg.L⁻¹ resulted in a slightly higher log reduction (Fig. 9). This indicated a trend that the higher H₂O₂ concentration would result in a greater log reduction. However, although a trend was observed when comparing results at the different H₂O₂ concentrations, a significant difference could not be proven ($p < 0.01$). Similar results were seen in a study done by Vargas *et al.* (2013), where little statistical significant variation was found between the following H₂O₂ concentrations: 100, 150, 200, 250 and 300 mg.L⁻¹.

Due to the fact that a significant difference was not observed between the H₂O₂ treatments at all the time intervals, the data obtained at the different concentrations were pooled (average colony counts at all three H₂O₂ concentrations) for discussion purposes throughout this study.

The highest log reduction of 1.64 was achieved in the first 30 min, this indicated that most microbial inactivation occurred in the first 30 min (Table 12). However, *E. coli* inactivation is evident throughout the 120 min.

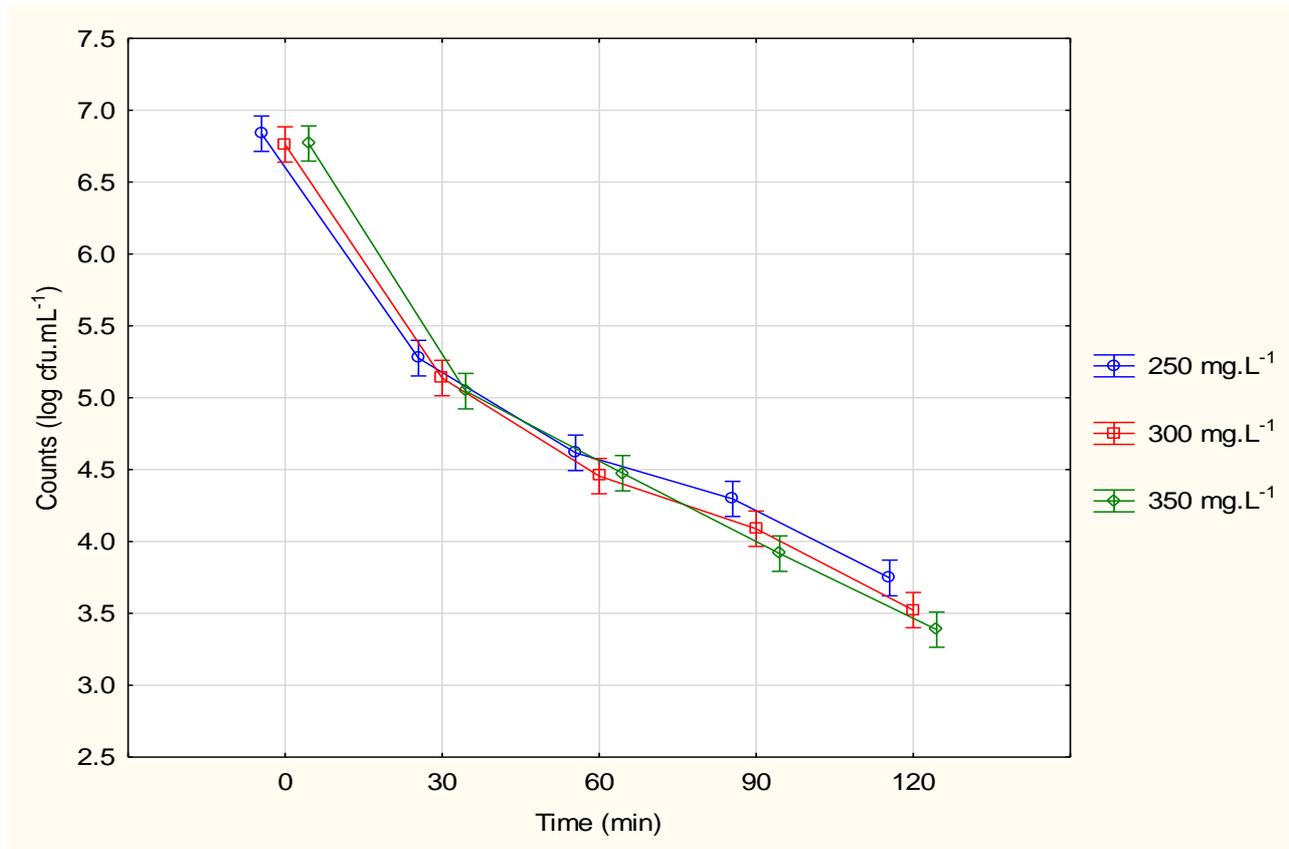


Figure 9 Effect of H₂O₂ concentration and contact time (0, 30, 60, 90 and 120) on the 11 *E. coli* strains used in study B, the *E. coli* strains were pooled in order to observe only the effect of H₂O₂ concentration and contact times on the *E. coli* strains. Error bars represent error at a 95% confidence interval. Statistical analysis: mixed model repeated measures ANOVA.

Table 12 Average log reductions achieved for all the *E. coli* strains at the specific time intervals (pooled for all concentrations).

Time intervals	Log reduction
0 – 30	1.64
30 – 60	0.64
60 - 90	0.42
90 - 120	0.55

Effect of H₂O₂ on ATCC *E. coli* strains

The decrease in numbers for each of the 11 selected *E. coli* strain at the specific time intervals can be seen in Fig. 10. The log reductions achieved after the reference ATCC *E. coli* strains (25922, 11775 and 35218) had been exposed to H₂O₂ for the 120 min contact time varied between 2.13 – 5.48 (Fig. 11). It was observed that ATCC 11775 was the most sensitive to the H₂O₂ treatment, with initial *E. coli* counts decreasing from of 6.97 to 1.49 log cfu.mL⁻¹, resulting in a 5.48 log reduction (Figs 10 and 11). ATCC 25922 resulted in a 5.14 log reduction after 120 min. A significant difference was not seen between the log reductions achieved for ATCC 11775 and ATCC 25922 at 120 min ($p = 0.19$). However, the *E. coli* counts decreased at a faster rate for ATCC 25922 than for ATCC 11775 (Fig. 10). At 30 and 60 min, a difference can be seen between ATCC 25922 and 11775, where ATCC 25922 is more sensitive to the H₂O₂ treatment after 30 and 60 min (Fig. 10). After the 120 min contact time, similar results were seen for these two strains. These strains were considered sensitive to the all the H₂O₂ treatments tested.

ATCC 35218 was the most resistant ATCC strain to H₂O₂ treatment. *Escherichia coli* counts decreased from 6.74 to 4.60 log cfu.mL⁻¹, achieving a 2.14 log reduction after 120 min (Figs. 10 and 11). ATCC 35218 is also resistant to three antibiotics including ampicillin, chloramphenicol and streptomycin (Table 10). Therefore it can be seen that the most resistant ATCC reference strain is also the only ATCC strain of the three strains tested, which showed antibiotic resistance. Log reductions obtained for ATCC 35218 differed significantly ($p < 0.05$) from both those obtained for ATCC 11775 and ATCC 25922. Thus, variations exist between the log reductions achieved after the 120 min contact time for the different ATCC *E. coli* strains. This indicates strain-to-strain variation with regards to the H₂O₂ treatment,

Effect of H₂O₂ on environmental *E. coli* strains

The log reductions achieved after the environmental *E. coli* strains had been exposed to the H₂O₂ treatment for the 120 min contact time varied between 2.17 – 3.93, indicating variation in strain resistance to the H₂O₂ treatment (Fig. 11)

Escherichia coli strains MJ58 and M53 showed the same reduction rate when exposed to the H₂O₂ treatment, with these strains being two of the most resistant environmental strains observed in this study. *Escherichia coli* strains MJ58 and M53 had initial counts of 6.85 and 6.91 cfu.mL⁻¹, respectively, and after the first 60 min, counts had decreased to 5.1 log cfu.mL⁻¹ for both strains (Fig. 10). After the 120 min contact time the counts had decreased to 4.68 and 4.74 log cfu.mL⁻¹ respectively, therefore both achieving

a 2.17 log reduction after 120 min (Fig. 10). The log reductions achieved for MJ58 and M53 did not differ significantly ($p = 0.73$) after 120 min. M29 had initial *E. coli* counts of $6.93 \text{ log cfu.mL}^{-1}$, which decreased to $4.66 \text{ log cfu.mL}^{-1}$, resulting in a 2.27 log reduction after 120 min (Fig. 11). Therefore, a large difference was not seen between MJ58, M53 and M29, as the log reductions only differed by 0.10. For MJ58, M53 and M29 it was clear that growth could still be detected after 120 min, thus indicating that H_2O_2 concentrations between 250 and 350 mg.L^{-1} would not achieve an effective (3 - 4) log reduction. Data from a previous study by Britz *et al.* (2013) indicated high faecal microbial contamination in this river water and thus a 3 – 4 log reduction would be recommended to achieve irrigation water of a safe quality. A significant difference was not seen between M53, MJ58 and M29 ($p > 0.05$) after 120 min, and these strains were considered the most resistant of the environmental *E. coli* strains evaluated in this study.

Escherichia coli strains M53 and M29 also exhibited antibiotic resistance (Table 10). M29 is resistant to chloramphenicol and trimethoprim, while M53 is resistant to tetracycline, trimethoprim, ampicillin and streptomycin. Therefore, it can be seen that M29 and M53 are resistant to three and four antibiotics, respectively, as well as being the most resistant strains to the H_2O_2 treatment.

Escherichia coli strains F11.2, E22.1 and E11.1 indicated log reductions in the same range after the H_2O_2 treatment, resulting in a 2.82, 3.08 and 2.9 log reduction after 120 min, respectively. A significant difference was not observed between F11.2, E22.1 and E11.1 ($p > 0.05$), although these strains did however, differ significantly from, M53, MJ58 and M29 ($p < 0.05$) (Fig. 11).

Since F11.2 had a slightly lower average log reduction compared to E22.1 and E11.1, it was considered the fourth most resistant strain to the H_2O_2 treatment in this study. Strain F11.2 was found to be resistant against a single antibiotic, tetracycline. Thus, out of the four most resistant *E. coli* strains to the H_2O_2 treatment, three showed antibiotic resistance, with two of these strains indicating resistance to more than one antibiotic. This may indicate a link between H_2O_2 resistance and antibiotic resistance.

A 3.52 log reduction was achieved after the 120 min contact time for E12.1. Consequently, *E. coli* E12.1 was considered one of the more sensitive environmental strains to the H_2O_2 treatment. E12.1 differed significantly from M29, M53, MJ58, F11.2 and E11.1, however a significant difference was not seen between E12.1 and E22.1 ($p = 0.08$) (Fig. 11).

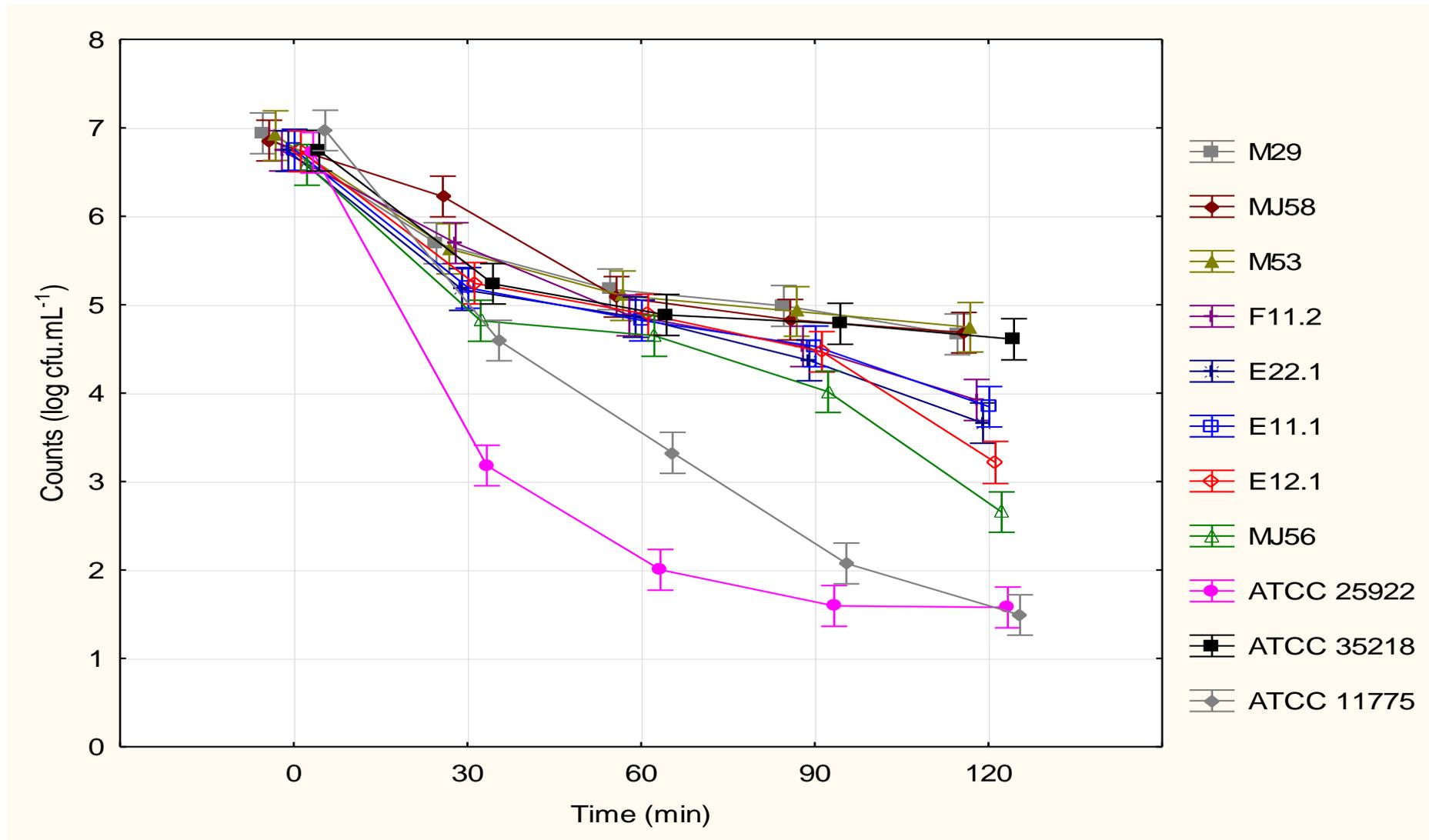


Figure 10 Effect of the H₂O₂ treatment on the 11 *E. coli* strains used in study A, the H₂O₂ concentrations were averaged (pooled) as the concentration interaction was not significant. No growth was recorded as 30 cfu.L⁻¹ (1.48 log cfu.mL⁻¹) as that was the chosen lower limit in this study. Error bars represent error at a 95% confidence interval. Statistical analysis: mixed model repeated measures ANOVA.

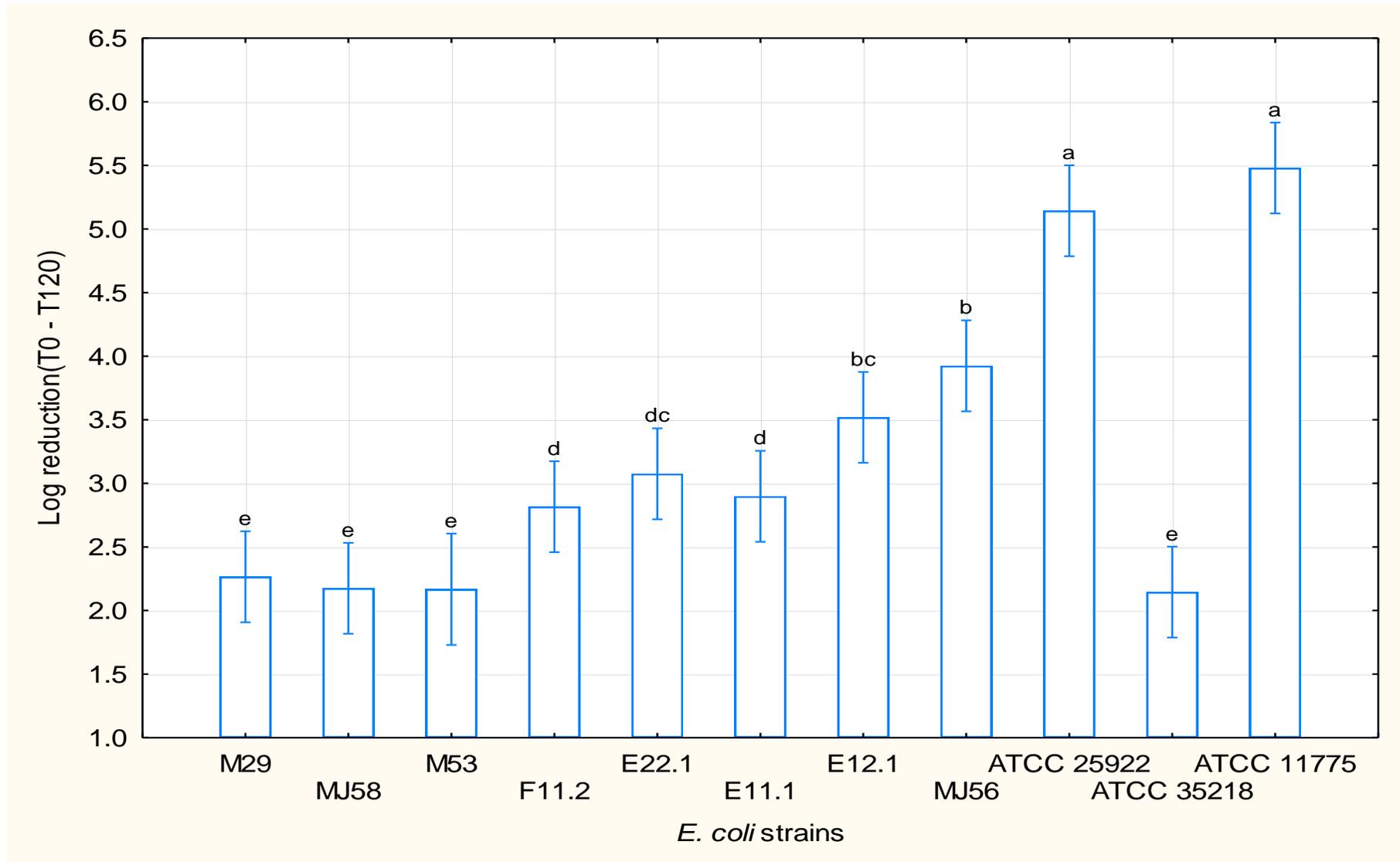


Figure 11 Log reductions achieved for the 11 *E. coli* strains after the 120 min contact time (T0 – T 120) (Pooled H₂O₂ concentrations). Error bars represent error at a 95% confidence interval. Statistical analysis: mixed model repeated measures ANOVA.

MJ56 was the most sensitive environmental strain to the H₂O₂ treatment, resulting in a 3.92 log reduction after 120 min. MJ56 differed significantly ($p < 0.05$) from all the other environmental *E. coli* strains used in the study, except for *E. coli* E12.1 ($p = 0.11$).

The *E. coli* counts for E12.1 and MJ56 were in the same range as F11.2, E22.1 and E11.1 at 30, 60 and 90 min (Fig.8). However, after 120 min E12.1 and MJ56 were more sensitive to the H₂O₂ treatment.

It can also be noted when comparing the API codes, MJ58 and F11.2 have the same biochemical profile, as do M29, MJ56 and E12.1 (Table 8). However, similar log reductions were not seen between MJ58 and F11.2 or between M29, MJ56 or E12.1. Therefore the resistance mechanisms are probably not related to the biochemical profiles of the *E. coli* strains.

Therefore from the results it can be seen that the log reductions achieved after the 120 min contact time varied between the environmental *E. coli* strains, indicating that certain strains are better adapted to protect themselves against the H₂O₂ treatment. The strains could be grouped according to the log reduction achieved after the 120 min contact time. M29, M53 and MJ58 were considered resistant, F11.2, E11.1 were considered slightly less resistant to the H₂O₂ treatment and E12.1 and MJ56 were considered sensitive to the H₂O₂ treatment.

Orta de Velásquez *et al.* (2008) reported similar results in a study done using H₂O₂ to treat wastewater. They found that faecal coliforms decreased by 2.2 log reduction after 120 min, using 250 mg.L⁻¹ H₂O₂. This was similar to the log reductions achieved for the resistant *E. coli* strains (M53, MJ58 and M29) in this study. However it must be noted that the treatment was done in wastewater, and therefore high levels organic matter present in the water could reduce the efficiency of the H₂O₂ treatment (Orta de Velásquez *et al.*, 2013). High organic matter may limit the efficiency of H₂O₂ (Newman, 2004). Faecal coliforms were tested and not just *E. coli*, hence this could also influence the results. It was expected that higher log reductions would be achieved at these concentrations. Thus, indicating that the treatment was not that effective.

Comparing ATCC strains to environmental strains

Overall, the ATCC strains tested in this study were more sensitive compared to the environmental strains, except for ATCC 35218, which was the most resistant strain included in the study. ATCC 25922 and ATCC 11775 were the most sensitive strains and they differed significantly from all the other *E. coli* strains used in the study, including MJ56, which was the most sensitive environmental *E. coli* strain. Growth was observed for

all the environmental *E. coli* strains after 120 min, however for ATCC 11775 no growth was present after 120 min and therefore a statistical lower limit of 30 cfu.mL⁻¹ (1.48 log cfu.mL⁻¹) was used for the data analysis. The antibiotic resistant *E. coli* strains were usually more resistant to the H₂O₂ treatment as seen with M53, M29, F11.2 and ATCC 35218. Therefore the same trend (*E. coli* strains with antibiotic resistance being more resistant to the H₂O₂ treatment) was seen with both the environmental *E. coli* strains and the ATCC *E. coli* strains. However, antibiotic resistance and H₂O₂ resistance were not always associated. MJ58 was seen as a resistant *E. coli* strain to the H₂O₂ treatment but did not indicate antibiotic resistance to the antibiotics tested in this study. MJ58 might show antibiotic resistance to other antibiotics, as only seven antibiotics were used (Table 2).

The ability of a small sub-population of a genetically homogenous population of bacteria can survive antibiotic exposure and these cells are referred to as persisters (Lewis, 2010). Persisters are slow-growing or can enter a dormant, non-dividing state, which subsequently protects them from harmful antibiotics (Gefen & Balaban, 2009; Lewis, 2010). MJ58 could be a persister or it could employ other resistance mechanisms enabling it to survive in high H₂O₂ concentrations (such as increased catalase activity). This may explain why MJ58 was better adapted to survive in the H₂O₂ treatment when compared to the other *E. coli* strains.

Escherichia coli is known to synthesise catalase enzymes that can degrade H₂O₂ into non-toxic by-products therefore, rendering it harmless to the organism. The amount of catalase produced may differ between the *E. coli* strains, which may explain why certain strains are better adapted to survive in the H₂O₂ treatment.

Overall, a trend was seen between the H₂O₂ concentrations, with the highest H₂O₂ concentration resulting in the highest log reduction. Variation was seen between the log reductions achieved for the ATCC strains, indicating that they did not react in the same way to the H₂O₂ treatment. Variation was also seen between the environmental *E. coli* strains, thus indicating the same trend. A difference was also observed between the ATCC and environmental strains, indicating that environmental strains are usually better adapted to withstand the H₂O₂ treatment.

For the environmental strains, counts were still present after 120 min for all the strains. Thus, it can be concluded that using 250, 300 and 350 mg.L⁻¹ over a 120 min contact time does not result in effective (3 – 4) log reductions and higher concentrations would need to be used to treat contaminated irrigation water.

Other factors may influence the efficacy of the H₂O₂ treatment. This study was done in PSS, however, organic matter present in irrigation water may have a negative impact on the H₂O₂ treatment, as the H₂O₂ oxidises the organic matter. It cannot be assumed that similar log reduction would be achieved in river water.

STUDY C: Effect of different H₂O₂ concentrations on a H₂O₂ resistant *E. coli* strain and a potential pathogenic *E. coli* strain

Two *E. coli* strains were exposed to four H₂O₂ concentrations (50, 350, 700 and 1 000 mg.L⁻¹) as shown in Fig. 12. Based on the data obtained in the previous study (Study B), M53 was selected as one of the most resistant environmental *E. coli* strain to H₂O₂ treatment. The second *E. coli* strain included in this study was W1371. This strain was selected as it carries virulence factors, indicating potential pathogenicity. *Escherichia coli* strains displaying virulence factors have been isolated from the Plankenburg River before (Lamprecht *et al.*, 2014). Thus, a strain with virulence factors was included in the study in order to determine how sensitive a potential pathogen is to H₂O₂ treatment compared to the other environmental and reference *E. coli* strains analysed in this study.

The four concentrations used in this study (50, 350, 700 and 1 000 mg.L⁻¹), were chosen to determine the effect that both low and high H₂O₂ concentrations would have on the two selected *E. coli* strains. A study done by Linley *et al.* (2012) hypothesised that at lower H₂O₂ concentrations, bacterial inactivation is a result of DNA damage, and is closely related to the Fe²⁺ ions associated with the DNA molecule. They argued that DNA-associated iron may be the limiting factor when H₂O₂ reacts with DNA, thus indicating that increasing the H₂O₂ concentration may not result in an increased rate of DNA damage. It was hypothesised that the biocidal effect of H₂O₂ at higher concentrations is a result of oxidation of proteins and lipids (Linley *et al.*, 2012). A low and high H₂O₂ concentration was thus included to determine if it would result in at least a three log reduction.

Effect of time

The average of all four H₂O₂ concentrations (50, 350, 700 and 1 000 mg.L⁻¹) were pooled in order to determine the effect of the different contact times on M53 and W1371. These are presented in Fig. 13.

From the results shown in Fig.13 it can be seen that in the first 30 min, the log reduction achieved for M53 was higher than W1371. A 1.03 and 0.62 log reduction was achieved in the first 30 min, for M53 and W1371, respectively. This was especially evident at 700 mg.L⁻¹, where a significant difference was seen between the log reductions

achieved after 30 min ($p < 0.01$) (Fig. 14). It was thus concluded that W1371 was more resistant to the H_2O_2 treatment in the first 30 min. However, after the 120 min contact time similar log reductions were achieved for both strains (Fig. 13). Thus, if resistant strains are present in a water system, longer contact time may be needed. This is an important factor for farmers to consider.

E. coli strain M53

When $50 \text{ mg.L}^{-1} H_2O_2$ was used, a significant difference was not seen between the counts obtained at 0, 30, 60, 90 and 120 min (Fig. 12) ($p > 0.05$). Counts decreased from 6.75 to $6.53 \text{ log cfu.mL}^{-1}$, after the 120 min contact time, thus achieving a 0.22 log reduction. When the concentration was increased to 350 mg.L^{-1} , the log reduction achieved after the 120 min contact time increased to 2.06 (Figs. 12 and 14). However, when the concentration was doubled to 700 mg.L^{-1} only a slight increase in the log reduction was seen. The log reduction increased from 2.06 to 2.27, and no significant difference was seen between the log reductions achieved at 350 and 700 mg.L^{-1} (Fig. 14) ($p = 0.24$). At $1\ 000 \text{ mg.L}^{-1}$ growth was observed at 90 min (Fig. 12), where a 4.3 log reduction was achieved. However, no growth was present after the 120 min contact time. To facilitate statistical analyses no growth was recorded as 30 cfu.mL^{-1} ($1.48 \text{ log cfu.mL}^{-1}$). Thus, indicating that shorter contact times are needed to achieve a minimum of three log reductions if the H_2O_2 concentration is increased to $1\ 000 \text{ mg.L}^{-1}$.

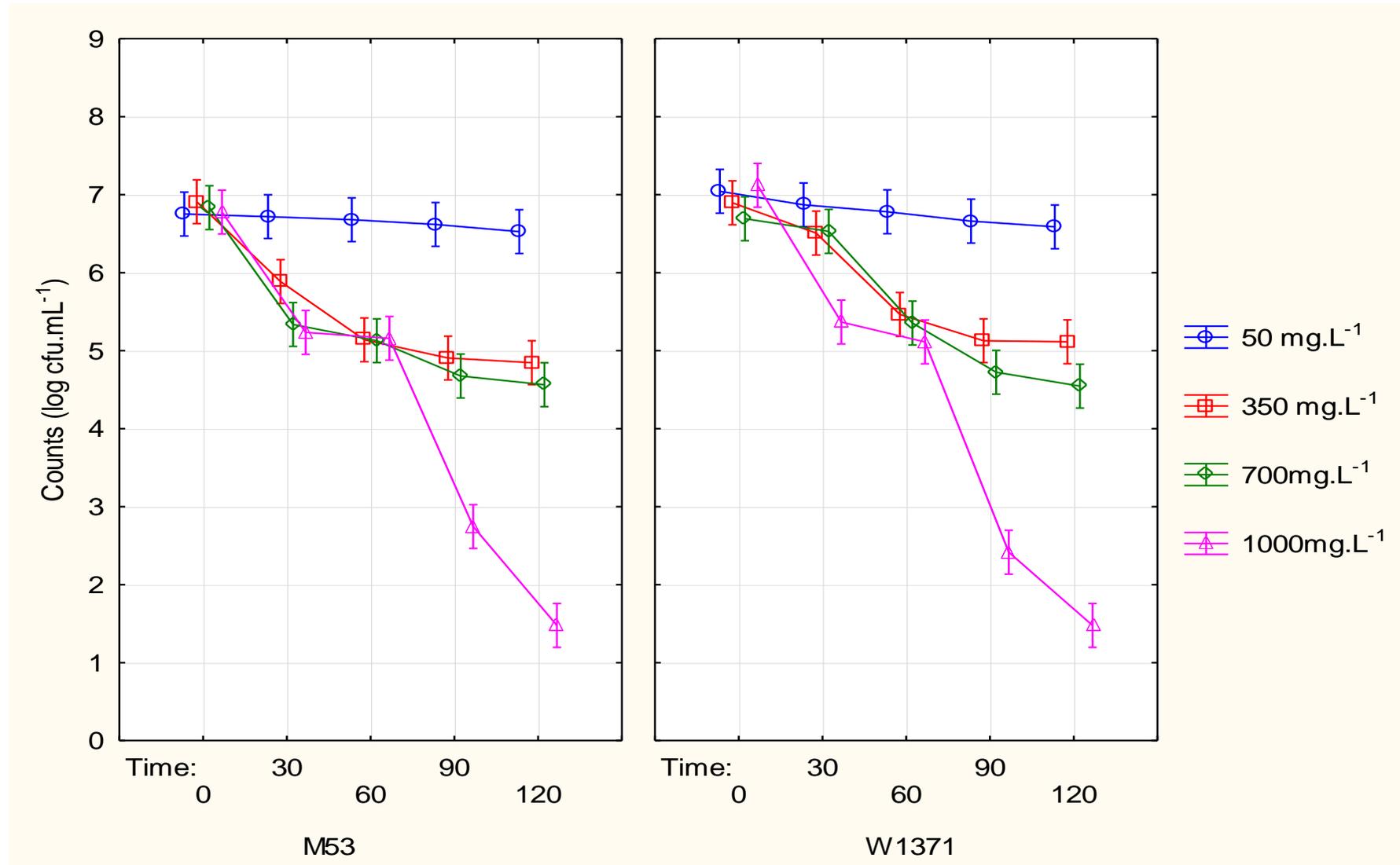


Figure 12 Effect of four H₂O₂ (50, 350, 700 and 1000 mg.L⁻¹) concentrations on M53 and W1371. No growth was recorded as 30 cfu.mL⁻¹ (1.48 log cfu.mL⁻¹). Error bars represent error at a 95% confidence interval. Statistical analysis: mixed model repeated measures ANOVA.

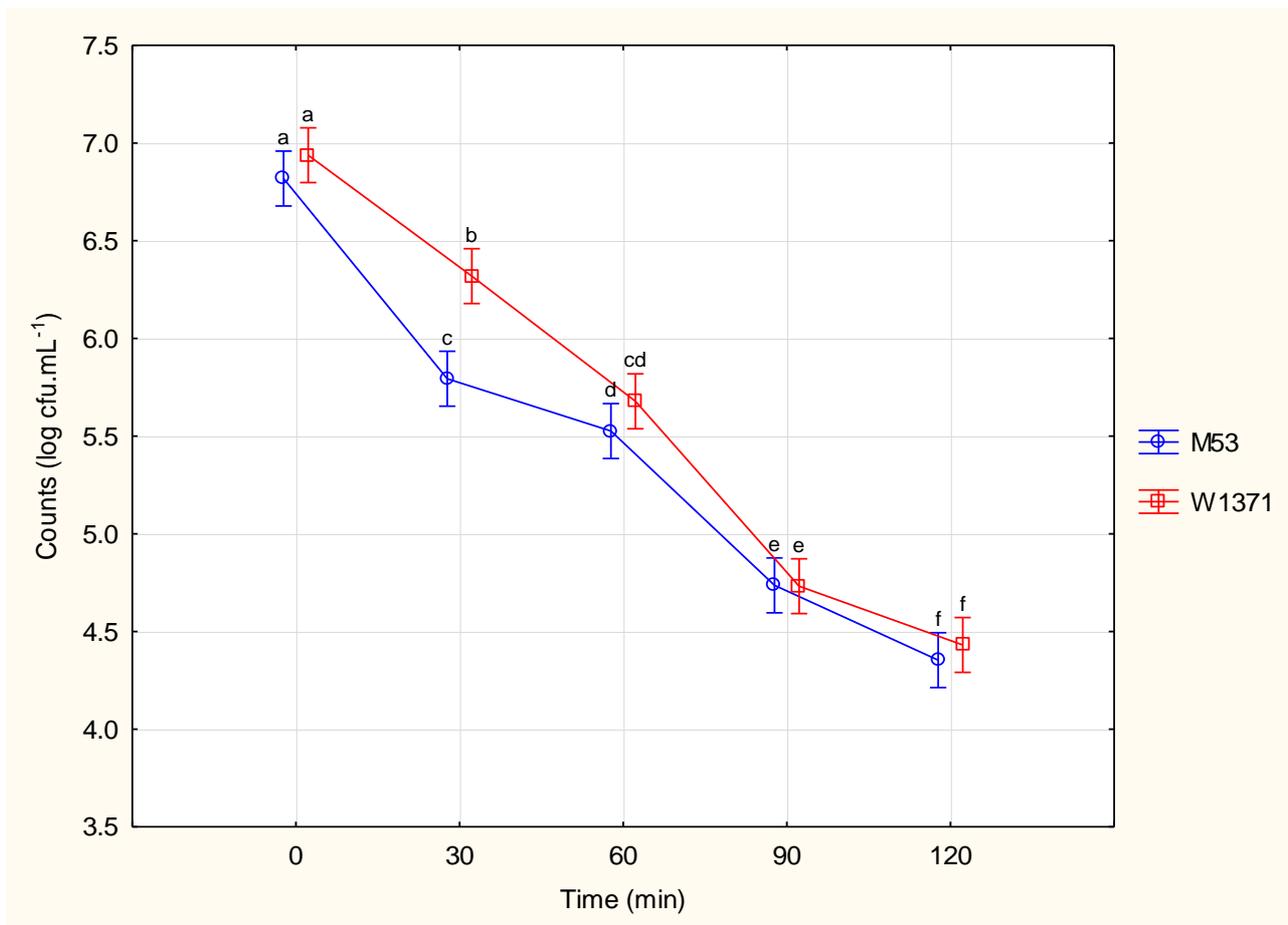


Figure 13 Pooled H₂O₂ concentrations, to observe the effect of time on M53 and W1371. Error bars represent error at a 95% confidence interval. Statistical analysis: mixed model repeated measures ANOVA.

***E. coli* strain W1371**

When 50 mg.L⁻¹ was used, a significant difference was seen between the counts present at 0 and 120 min (Fig. 12) ($p = 0.03$). After the 120 min contact time a 0.45 log reduction was achieved (Fig. 14). When the concentration of H₂O₂ increased to 350 mg.L⁻¹, the log reduction achieved after 120 min, increased to 1.78. However, when the H₂O₂ concentration was increased from 350 to 700 mg.L⁻¹ the log reduction only increased to 2.15 (Fig. 14). Thus, as for M53, no significant difference was seen between the log reductions observed at 350 and 700 mg.L⁻¹ ($p = 0.05$). At 1 000 mg.L⁻¹ growth was observed at 90 min, where a 4.7 log reduction was achieved. However, no growth was present after the 120 min contact time. No growth was measured as 30 cfu.mL⁻¹ (1.48 log cfu.mL⁻¹).

Comparing M53 and W1371

Comparable log reductions were achieved at all the concentrations used for both *E. coli* strains. At 50 mg.L⁻¹ a slightly higher log reduction was achieved for W1371, but a significant difference was not seen (Fig. 14) ($p = 0.19$). However, using 50 mg.L⁻¹ of H₂O₂ was not considered an effective concentration, as not even a 1 log reduction was achieved after 120 min. Mamane *et al.* (2007) tested the effect that 25 mg.L⁻¹ of H₂O₂ had on *E. coli*, *B. subtilis* spores and sampled surface water.

Results indicated that no inactivation was observed at 25 mg.L⁻¹ after 60 min (Mamane *et al.*, 2007). In this study, increasing the concentration to 50 mg.L⁻¹ resulted in low microbial inactivation, indicating that low H₂O₂ concentrations were not effective for inactivation of microorganisms.

At 350 and 700 mg.L⁻¹ a slightly higher log reduction was achieved for M53 (Fig. 14). But once again a significant difference was not seen between the log reductions achieved at either H₂O₂ concentration. It was expected that a greater log reduction would be achieved when the concentration was increased from 350 to 700 mg.L⁻¹. However a significant difference was not seen for either strain (M53, $p = 0.24$) (W1371, $p = 0.05$). This indicated that both the *E. coli* strains were capable of withstanding higher H₂O₂ concentrations. This is an important factor to consider, as the concentration selected to use on-farm has cost implications. It would not be worth doubling the concentration of H₂O₂ and in turn the chemical cost if similar log reductions were achieved at both concentrations.

At 1 000 mg.L⁻¹, a slightly higher log reduction was achieved for W1371, however this is probably due to the fact that the initial counts were slightly higher for W1371 (Fig. 12). A significant difference was not seen between the log reductions achieved for M53 and W1371, at 1 000 mg.L⁻¹ ($p = 0.07$). Thus, using 1 000 mg.L⁻¹ results in a sufficient log reduction, however this concentration might not be feasible from an economic point of view, depending on the quantities of water to be treated.

Overall it can be concluded that using 50 mg.L⁻¹ H₂O₂ did not result in an effective disinfection (less than 1 log reduction achieved). Interestingly, a significant difference was not observed between the log reductions achieved using 350 and 700 mg.L⁻¹, thus the lower concentration can be used. Using 1 000 mg.L⁻¹ resulted in an effective disinfection for the *E. coli* loads present in this study, as a 3-4 log reduction was achieved after 90 min, and this was not achieved at any other concentration. Cost implications need to be determined as using 1 000 mg.L⁻¹ H₂O₂ can be expensive.

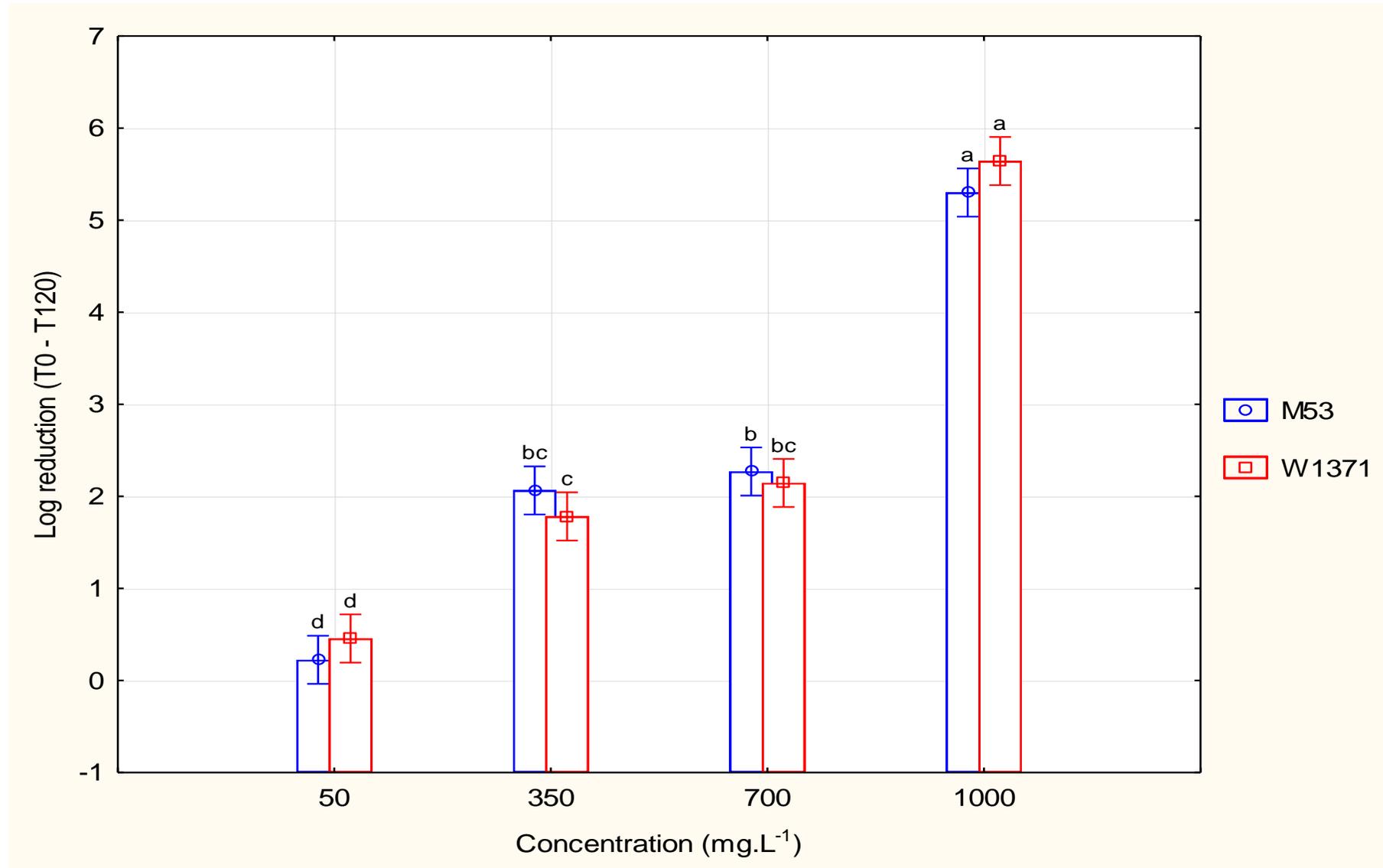


Figure 14 Log reductions achieved after 120 min for M53 and W1371 at 50, 350, 700 and 1 000 mg.L⁻¹. Error bars represent error at a 95% confidence interval. Statistical analysis: two-way ANOVA with Fisher LSD post hoc test.

The strain having virulence factors (potential pathogenic strain) reacted in the same way to the H₂O₂ treatment as did M53. Both these strains were considered resistant to the H₂O₂ treatment. This could have health implications, as W1371 has the potential to causing illness and is resistant to the H₂O₂ treatment. Therefore, these resistant strains could survive the treatment and be transferred onto fresh produce.

STUDY D: Effect of organic matter (COD) on the efficacy of the H₂O₂ treatment

From study B, *E. coli* strains: ATCC 25922; MJ56; and M53 were selected, respectively to represent a sensitive reference strain, a sensitive environmental strain and a resistant environmental strain to the H₂O₂ treatment. River water was sampled from both the Plankenburg and Veldwagters Rivers, and autoclaved before the samples were inoculated with the selected strains. A comparison was made between the log reductions achieved after the H₂O₂ treatment (350 mg.L⁻¹ was used for this study) in PSS, Plankenburg and Veldwagters River waters. This was done to observe if the H₂O₂ treatment, was influenced by different COD values.

COD values

The COD values for the Plankenburg and Veldwagters Rivers were 18 and 55 mg.L⁻¹, respectively (after the water had been autoclaved). The COD values were not very high, however they were similar to previous studies done on at the same site of the Plankenburg River. Huisamen (2012) found that COD values ranged between 0 – 75 mg.L⁻¹ (Huisamen, 2012). Van Blommenstein (2012) found COD values ranging from 4 – 44 mg.L⁻¹, at a different site further along the Plankenburg River. There is no regulation or guideline stipulating acceptable COD values for irrigation water. However, according to DWA (2013), the COD levels must not exceed 75 mg.L⁻¹ for wastewater intended to be used for irrigation.

ATCC 25922

For ATCC 25922, a significant difference was not seen (Fig. 15), when comparing the H₂O₂ treatments of the PSS, Plankenburg River (18 mg.L⁻¹) and Veldwagters River samples (55 mg.COD.L⁻¹). Similar *E. coli* counts were observed at 30, 60, 90 and 120 min in the PSS, Plankenburg and Veldwagters River (Fig. 15). A 5.52, 5.41 and 5.17 log reduction was achieved in the PSS, Plankenburg and Veldwagters River samples, respectively (Fig. 16). A significant difference was not observed between the log reductions achieved in the different samples (PSS and Plankenburg, p = 0.79; PSS and

Veldwagters, $p = 0.39$; Plankenburg and Veldwagters, $p = 0.52$). Although not statistically different, a slightly higher log reduction was achieved in the PSS, and the lowest log reduction was achieved in the Veldwagters River sample. It was expected that a higher log reduction would be achieved in the PSS, as no organic matter is present to react with the H_2O_2 . However, results indicated that the COD concentrations in this study did not influence the H_2O_2 treatment, significantly.

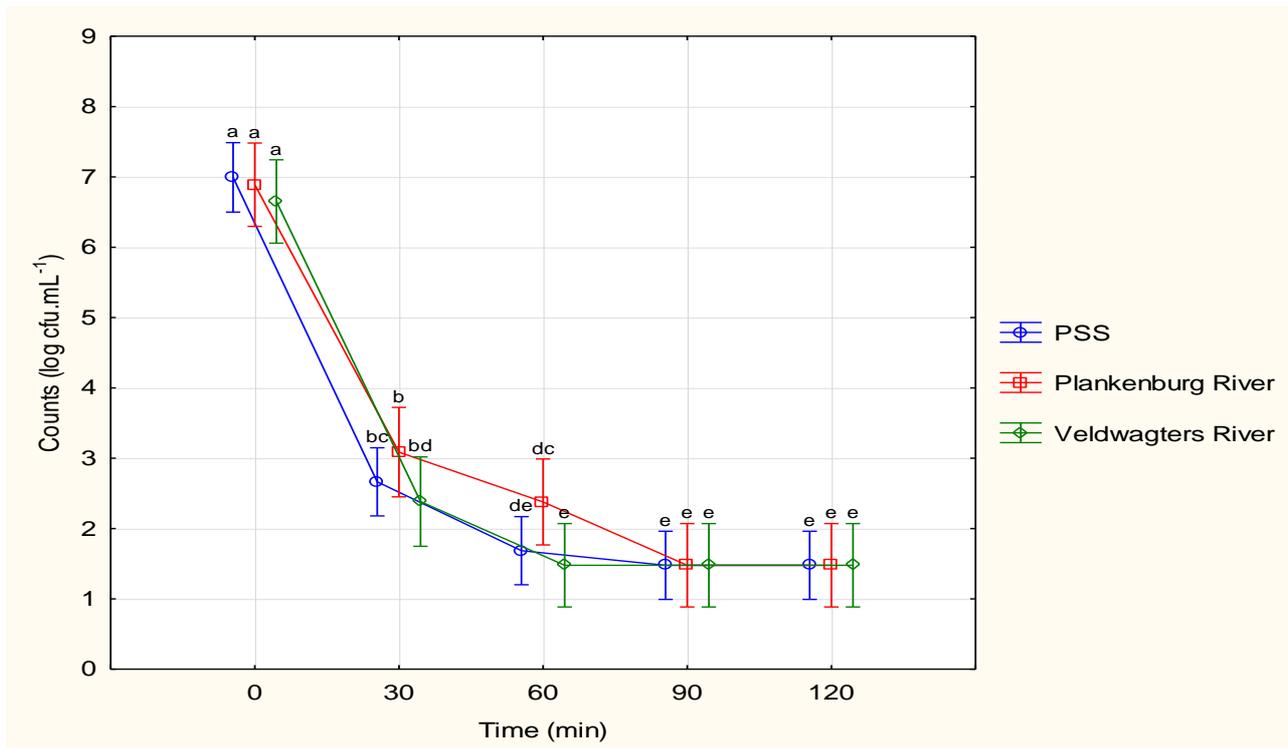


Figure 15 Effect of $350 \text{ mg.L}^{-1} H_2O_2$ on ATCC 25922 in PSS, Plankenburg and Veldwagters River water samples. Error bars represent error at a 95% confidence interval. Statistical analysis: mixed model repeated measures ANOVA.

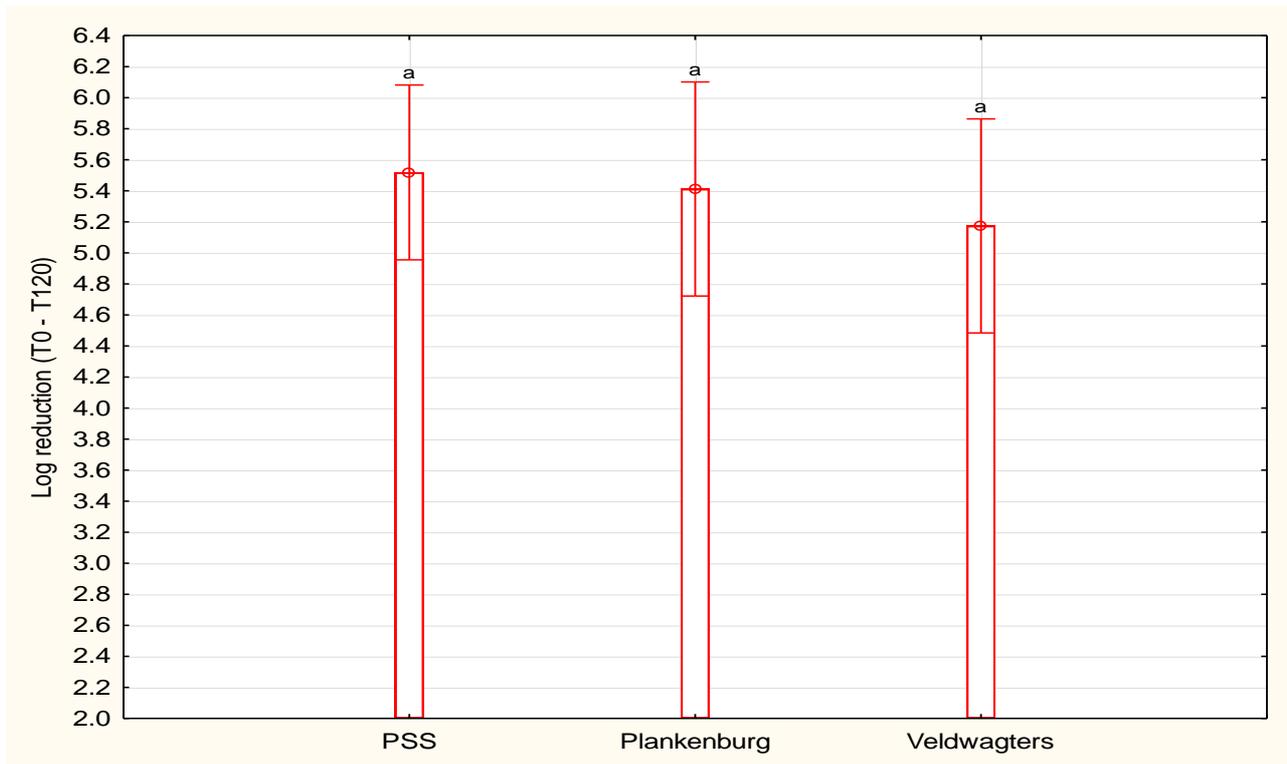


Figure 16 Log reductions achieved after 120 min for ATCC 25922 in PSS, Plankenburg and Veldwagters River water samples. Error bars represent error at a 95% confidence interval. Statistical analysis: one-way ANOVA with Fisher LSD.

MJ56

The *E. coli* counts present in PSS, Plankenburg and Veldwagters is shown in Fig. 17. A similar trend was seen with MJ56, where a significant difference was not seen between the log reductions achieved between the PSS, Plankenburg and Veldwagters River samples, after the 120 min contact time (Fig. 18). The *E. coli* counts followed the same trend at 30, 60, 90 and 120 min. Similar *E. coli* counts were observed in the PSS, Plankenburg and Veldwagters river samples at 30, 60, and 90 (Fig. 17). A 4.36, 4.40 and 4.95 log reduction was achieved in the PSS, Plankenburg and Veldwagters samples, respectively (PSS and Plankenburg, $p = 0.92$; PSS and Veldwagters, $p = 0.15$; Plankenburg and Veldwagters, $p = 0.22$) (Fig. 18). A significant difference was observed between the counts after 120 min for the Veldwagters sample (compared to PSS and Plankenburg samples) and yet no significant difference was observed between the initial counts (at time 0) (Fig. 17). In Fig. 18 (where a mixed model repeated measures ANOVA was used to analyse the data) no significant differences were observed in the log reductions achieved between the different samples. However, when a one-way ANOVA was used to analyse the log reductions for

the data used for Fig. 17, it was not supported by the ANOVA analysis which was performed on the log reduction data.

A slightly higher log reduction was achieved in the Veldwagters River sample, however no significant differences were observed. It is important to note that the exact composition of the river samples was unknown and certain compounds may be present that could influence the H₂O₂ treatment. Compounds might be pumped into the river or enter the river through run-off that may either influence the efficacy of the H₂O₂ treatment, or the sensitivity of the strain tested.

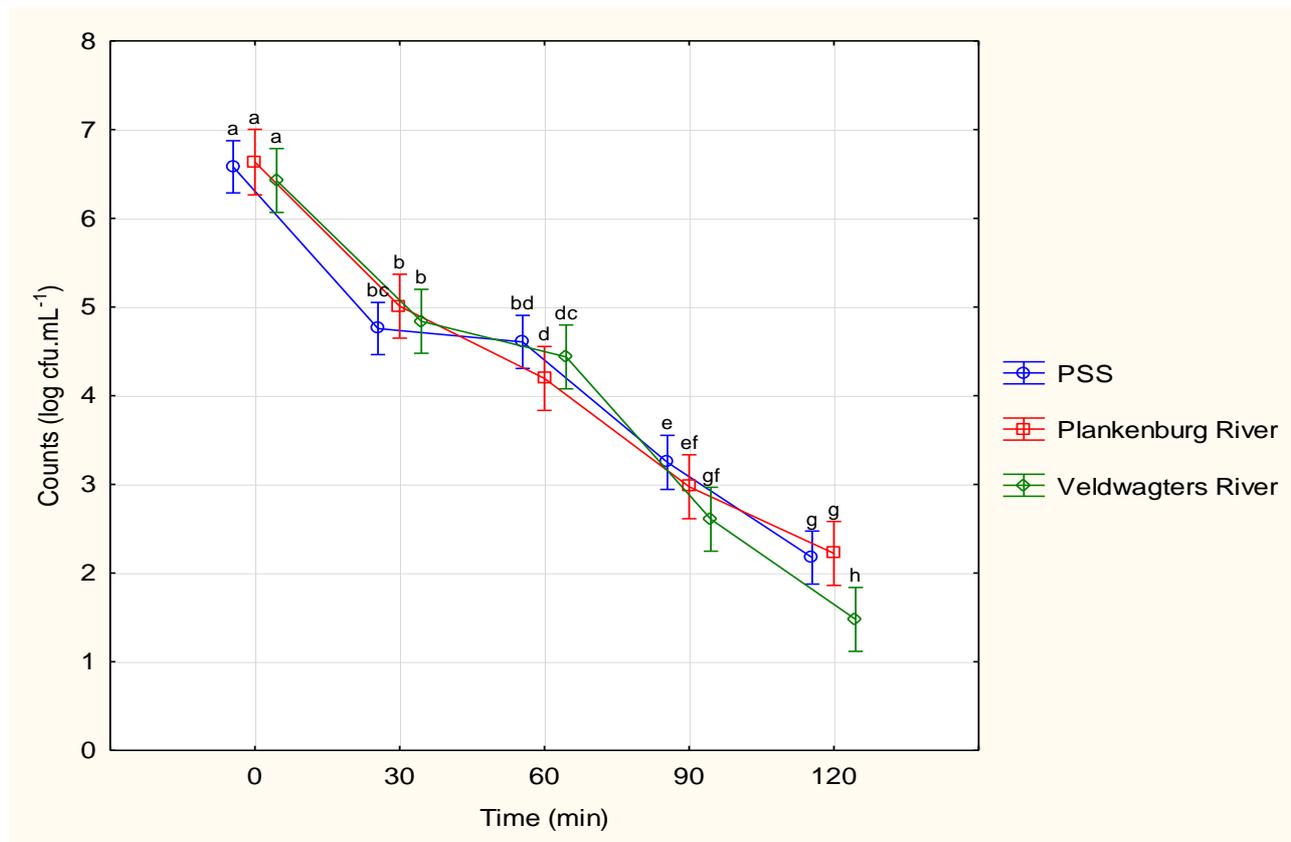


Figure 17 Effect of 350 mg.L⁻¹ H₂O₂ on MJ56 in PSS, Plankenburg and Veldwagters River water samples. Error bars represent error at a 95% confidence interval. Statistical analysis: mixed model repeated measures ANOVA.

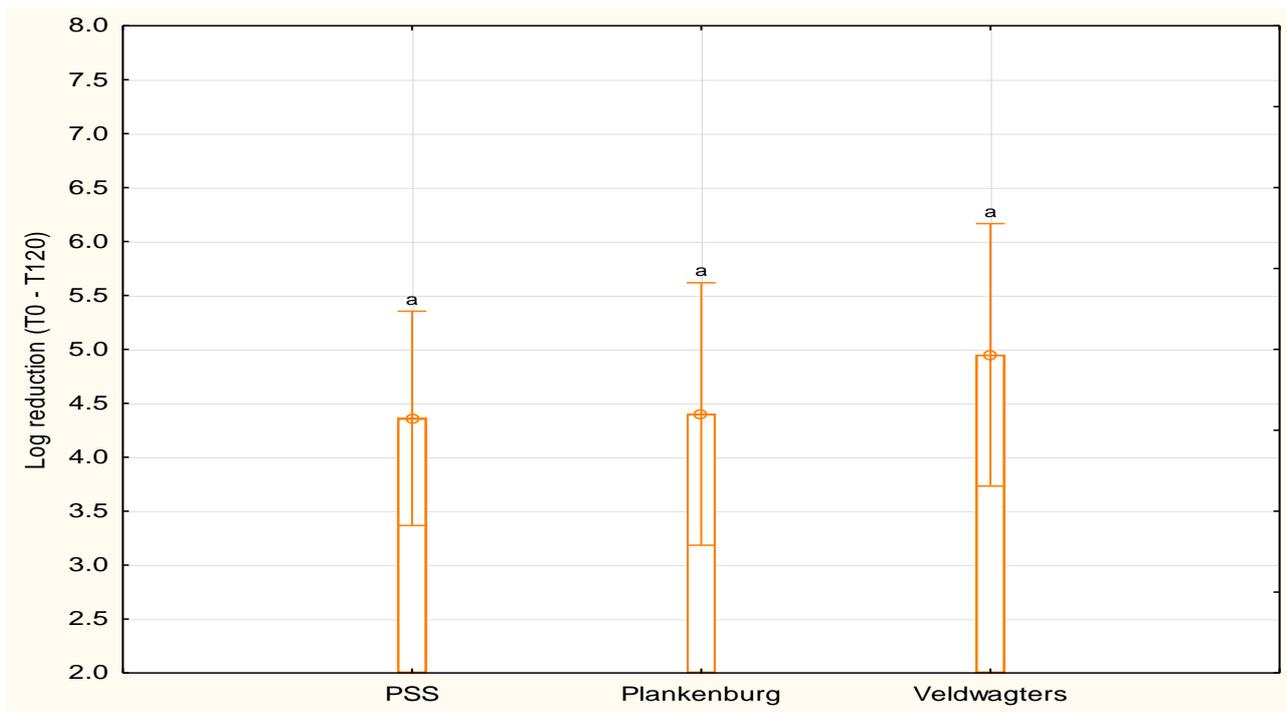


Figure 18 Log reductions achieved after 120 min for MJ56 in PSS, Plankenburg and Veldwagters River water samples. Error bars represent error at a 95% confidence interval. Statistical analysis: one-way ANOVA with Fisher LSD

M53

The *E. coli* counts present in the PSS, Plankenburg and Veldwagters River samples can be seen in Fig. 19. The log reductions achieved after 120 and 30 min can be seen in Fig. 20 and 21, respectively. For M53, a significant difference was not seen between the log reductions achieved in the PSS, Plankenburg and Veldwagters River samples after the 120 min contact time (Fig. 20). A 2.12, 2.18 and 2.28 log reduction was achieved in the PSS, Plankenburg and Veldwagters samples, respectively (PSS and Plankenburg, $p = 0.90$; PSS and Veldwagters, $p = 0.70$; Plankenburg and Veldwagters, $p = 0.80$). However, although a significant difference was not seen after 120 min, at 30 min a higher log reduction was achieved in the PSS (Fig. 21). A significant difference was seen between the log reductions achieved in the PSS and Plankenburg, as well as the PSS and Veldwagters (PSS and Plankenburg, $p = 0.01$) (PSS and Veldwagters, $p = 0.02$). It can be seen that the *E. coli* counts present at 30 min in the PSS are less than when compared to the Plankenburg and Veldwagters River (Fig. 19). This could indicate that organic matter (COD) does influence the H_2O_2 treatment during the first 30 min. In the PSS, there is no suspended matter present and therefore the H_2O_2 is only reacting with the *E. coli* strains. Whereas, in the river water, H_2O_2 can also react with any organic matter present. This was only observed with the strain indicating resistance to the H_2O_2 treatment. This

indicated that longer contact times may be needed if mixed strains of *E. coli* are present, as farmers would not be able to determine the ability of strains to resist the H₂O₂ treatment.

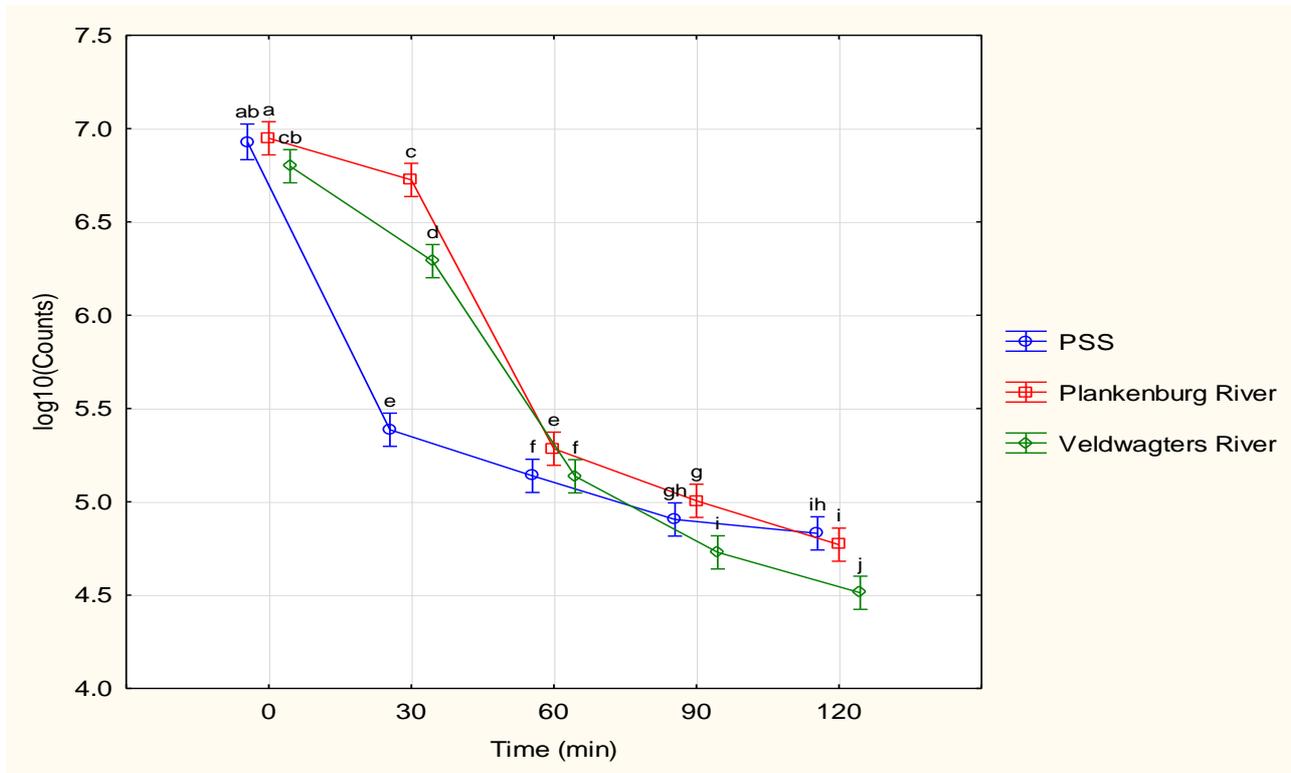


Figure 19 Effect of 350 mg.L⁻¹ H₂O₂ on M53 in PSS, Plankenburg and Veldwagters River water samples. Error bars represent error at a 95% confidence interval. Statistical analysis: mixed model repeated measures ANOVA

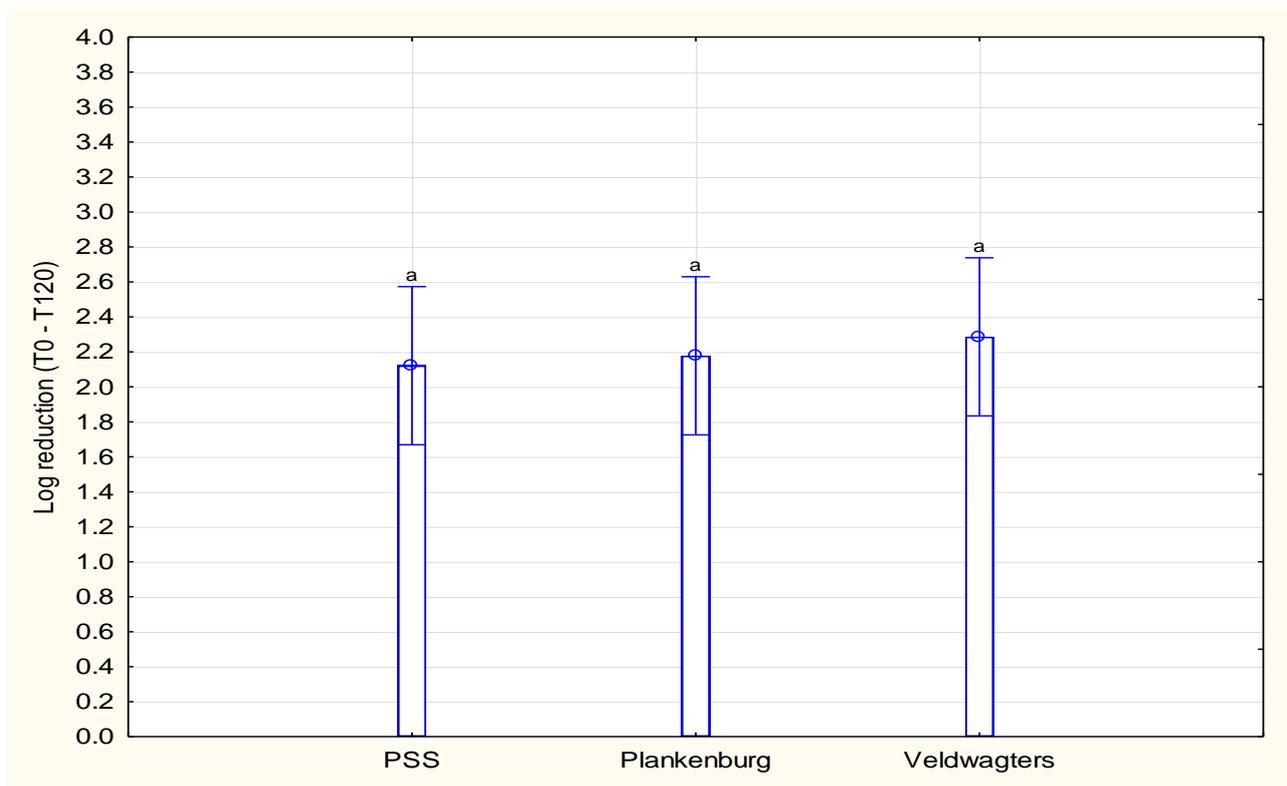


Figure 20 Log reductions achieved after 120 min for M53 in PSS, Plankenburg and Veldwagters River water samples. Error bars represent error at a 95% confidence interval. Statistical analysis: one-way ANOVA with Fisher LSD.

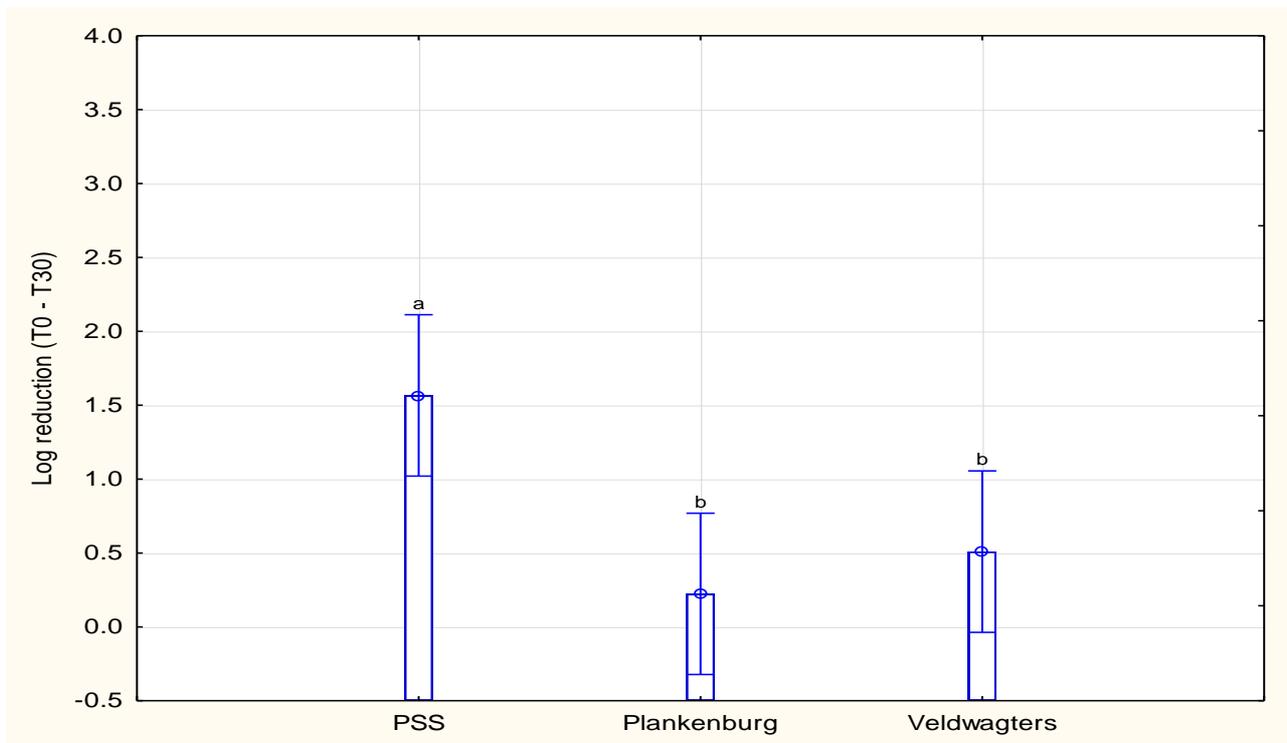


Figure 21 Log reductions achieved after 30 min for M53 in PSS, Plankenburg and Veldwagters River water samples. Error bars represent error at a 95% confidence interval. Statistical analysis-one-way ANOVA with Fisher LSD.

Effect of COD

Overall, a low COD value (18 – 55 mg.L⁻¹) did not appear to influence the efficiency of the H₂O₂ treatment over the 120 min exposure time. This was seen for all three strains tested. When the sample with the higher COD value was used (55 mg.L⁻¹), significant differences were still not observed between the PSS and Veldwagters samples. For strain M53, a significant difference in the log reduction was observed between PSS and Plankenburg (18 mg.L⁻¹) and Veldwagters (55 mg.L⁻¹) after 30 min. However, after the 120 min contact time significant differences were not seen between the log reductions achieved in the PSS, Plankenburg and Veldwagters samples. Thus, the COD present at these two sites did not influence the H₂O₂ treatment after 120 min. The COD would therefore not influence the efficacy of H₂O₂ as a treatment option for irrigation water. Although, a difference was seen in the first 30 min for one strain, contact times longer than 30 min are generally needed for effective treatment.

A study performed by Vargas *et al.* (2013) tested the efficiency of H₂O₂ in treated domestic sewage (20 - 110 mg.L⁻¹ COD) and synthetic wastewater (COD value not mentioned). ATCC 25922 (the same strain used in this study) was used as the test organism in their study for the synthetic wastewater. They concluded that the disinfection of treated sewage was slower than that of synthetic wastewater, and they mentioned that it could be due to the presence of pollutants in the sewage that were oxidised by the H₂O₂.

The surface water used in this study (Study D) was exposed to sewage contamination and it was expected that lower log reductions would be achieved in the river water samples. This was not observed however, as similar log reductions were achieved in the PSS, Plankenburg and Veldwagters River water samples. The outcomes from the Vargas *et al.* (2013) study might have been, due to using ATCC 25922 in the synthetic wastewater, whereas environmental strains would have been present in the sewage samples. From the data in Study B, it is clear that ATCC 25922 is more sensitive to the H₂O₂ treatment than any of the environmental strains. This can influence the parameters such as concentration and contact times, as longer contact times or higher H₂O₂ concentrations may be needed to achieve similar results for both reference (ATCC) strains and environmental strains.

It was expected that the COD content in the river water samples would have had a negative impact on the H₂O₂ treatment. Yet, no significant differences were seen between the river water and PSS samples. This indicated that the COD range between these two river samples (18 – 55 mg.L⁻¹) would not significantly influence the treatment parameters, and would not need to be considered for optimising H₂O₂ as a treatment option.

STUDY E: Catalase activity of all 12 *E. coli* strains

H₂O₂ can be catalysed into water and oxygen by the catalase enzymes hydroperoxidase I (HPI) and hydroperoxidase II (HPII), therefore reducing the toxicity of H₂O₂ (Loewen *et al.*, 1985). *KatG* genes are responsible for encoding HPI and *KatE* genes are responsible for encoding HPII (Loewen *et al.*, 1985; Heimberger & Eisenstark, 1988; Storz & Imlay, 1999; Iwase *et al.*, 2013). These two catalase enzymes have different functions. HPI is induced by low H₂O₂ concentrations, whereas HPII is induced during the stationary phase or other stresses (Mulvey *et al.*, 1990; Visick *et al.*, 1997). Thus, catalase is an enzyme that can protect the cell against sub-lethal concentrations of H₂O₂. Catalase levels may be increased due to phenotypic adaptation to protect the cell against oxidative stress. Consequently, active resistance against H₂O₂ may develop (Morató *et al.*, 2003).

It was thus important to determine if a link could be established between the *E. coli* strains with resistance to the H₂O₂ treatment, and strains indicating high catalase activity. This would in turn determine if catalase is a mechanism by which *E. coli* strains can protect themselves against H₂O₂ treatment.

Catalase method 1

The total catalase activity was measured for all 12 *E. coli* strains using method 1 (Table 13). MJ58, M53 and M29 had the highest catalase activity (+++) according to the amount of bubbles formed. F11.2, E12.1, E11.1, MJ56, W1371 and ATCC 35218 showed a slightly lower catalase activity (++). The ATCC strains 25922 and 11775 had the lowest catalase activity (+) in comparison to all the other *E. coli* strains tested.

It was expected that the *E. coli* strains that showed resistance to H₂O₂ treatment would have the highest catalase activity present. This was seen for MJ58, M53 and M29, as these strains all indicated resistance to the H₂O₂ treatment as well as showing high amounts of catalase activity. F11.2, E12.1, E11.1, MJ56, were *E. coli* strains showing intermediate resistance to the H₂O₂ treatment. These strains also indicated less bubble formation compared to MJ58, M53 and M29, therefore indicating a lower total catalase activity. ATCC strains 25922 and 11775 were the most sensitive *E. coli* strains to the H₂O₂ treatment. These strains also indicated the lowest amount of catalase activity, compared to the other *E. coli* strains.

However, not all the *E. coli* strains tested followed the same trend where a clear link between catalase activity and H₂O₂ resistance could be observed. *Escherichia coli* strain E22.1 indicated a higher catalase activity than F11.2, however F11.2 showed a lower log reduction after the H₂O₂ treatment (Fig. 11). It could be argued that the higher catalase

activity would protect the cell better and therefore result in a lower log reduction. Results indicated that E22.1 had a higher catalase activity, although F11.2 appeared to be better protected against the H₂O₂. E22.1 indicated the same amount of catalase activity as MJ58, M53 and M29, although a significant difference was seen between the log reductions (Fig. 11) achieved between E22.1 and MJ58, M53 and M29 ($P < 0.05$). W1371 and ATCC 35218 were considered the most resistant *E. coli* strains, however high amounts of catalase activity were not observed for these two *E. coli* strains. This could indicate these strains have other mechanisms protecting them against the H₂O₂ treatment (Maillard, 2005). Both W1371 and ATCC 35218 showed multiple antibiotic resistance, thus, the mechanisms protecting them against the H₂O₂ may be linked the antibiotic resistance mechanisms.

This method for catalase assessment has been used before (Chen *et al.*, 2013). The interpretation of the results obtained with this method might, however, be subjective due to the fact that results were recorded after visually assessing the amount of bubbles formed for each *E. coli* strain. In an attempt to quantify the catalase activity, another method (Iwase *et al.*, 2013) was evaluated in the next section.

Catalase method 2

Table 13 Catalase activity of all 12 *E. coli* strains, based on the intensity of bubble formation

<i>E. coli</i> strain	Catalase activity*
MJ58	+++
M53	+++
M29	+++
F11.2	++
E12.1	++
E11.1	++
E22.1	+++
MJ56	++
W1371	++
ATCC 11775	+
ATCC 25922	+
ATCC 35218	++

* - indicates no bubbles, + indicates little bubble formation, ++ indicates average bubble formation, +++ indicates immediate, strong bubble formation

The catalase activity of all 12 *E. coli* strains was measured using the method described by Iwase *et al.* (2013). In this method the catalase activity is quantified by measuring the foam formed by trapped O₂, which is released during the decomposition of H₂O₂ by catalase. The Triton X-100 traps the bubbles, allowing the height of the bubbles to be measured. The catalase activity units were measured in duplicate (Table 14). After which a calibration curve was plotted using the defined unit of catalase (Fig. 22). Different catalase solutions were used to create the calibration curve to link the height of foam to enzyme units (U) before the *E. coli* cells were analysed.

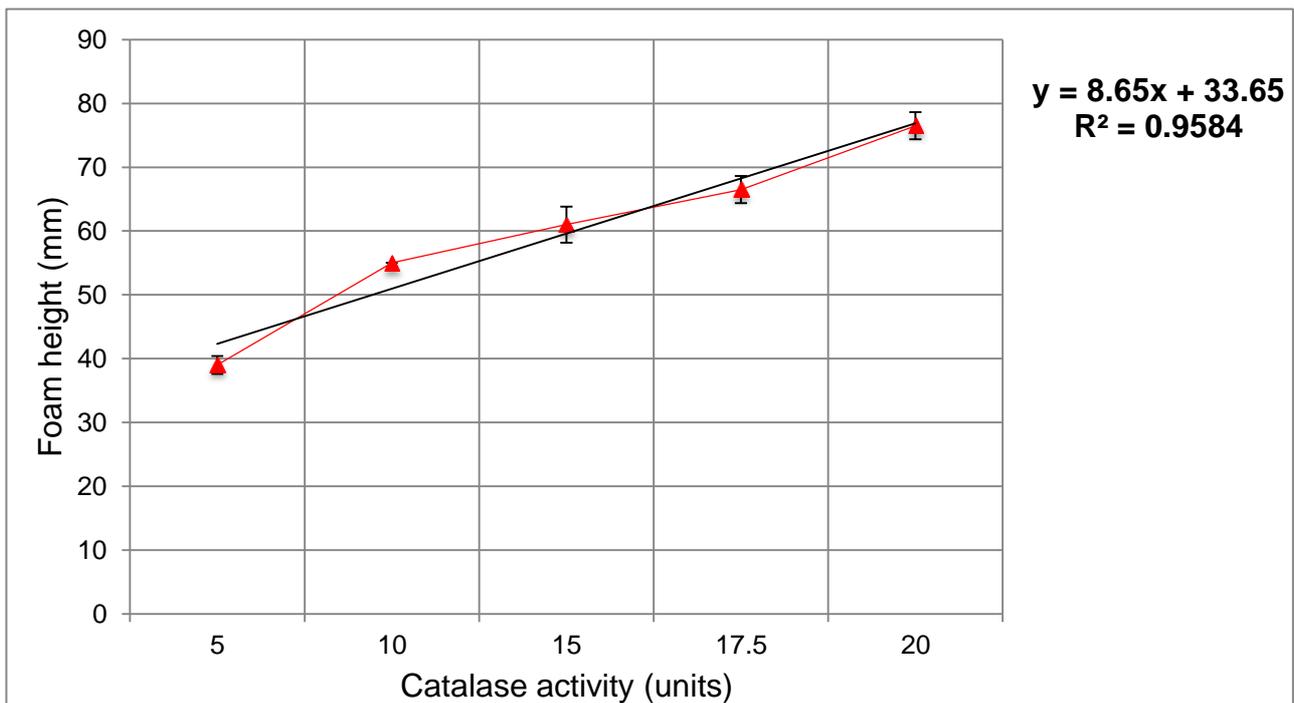
The amount of total catalase activity varied between 120.2 - 557 units (U per 1 mg wet weight) for all strains tested (Fig. 23). This method was also able to distinguish between HPI (*KatG*) and HP II (*KatE*) activity. The HP II catalase is heat stable, whereas HPI is heat labile. For HP II activity the cell extracts were heated to 55°C prior to H₂O₂. The HP II was subtracted from the total catalase to determine HPI activity.

The HPI activity varied between 0 – 224 U catalase for all strains tested (Fig. 23). It was observed that resistant strain (W1371) indicated no HPI activity and ATCC 25922, a sensitive strain to the H₂O₂ treatment, had 224 U HPI catalase activity. Thus, no trends were seen between the HPI activity and the resistance of the strains to the H₂O₂ treatment.

M53, M29 and MJ58 had 557, 548.3 and 513.7 U total catalase, respectively (Fig. 23). These strains had the highest amount of catalase activity present. This was expected, as these strains were considered resistant to the H₂O₂ treatments tested in Study B. This could explain why MJ58 was the only strain showing resistance to the H₂O₂ treatment, without antibiotic resistance. That is, if a link indeed exists between antibiotic resistance and H₂O₂ resistance. Thus, the catalase activity could be responsible for protecting the strain from the H₂O₂ treatment. These strains all had higher amounts of HP II compared to HPI activity present. A trend was seen between the amount of HP II activity and resistance to H₂O₂. M53, MJ58 and M29 were all considered resistant to the H₂O₂ treatment, showing 546.2, 500.8 and 418.6 U HP II activity, respectively (Fig. 23). Therefore it can be seen that some of the most resistant strains to the H₂O₂ treatment in Study B, indicated high amounts of total catalase activity and HP II catalase activity (Fig. 23).

Table 14 Height of foam (in mm), used to construct a calibration curve to determine units of catalase activity

	Catalase activity (U)				
	5	10	15	17.5	20
Height of foam formation (mm)	38	55	59	68	78
	40	55	63	65	75

**Figure 22** Calibration curve calculated from the line of best fit ($y = 8.65x + 33.65$, $r^2 = 0.9584$)

Escherichia coli strains, E.11.1, E12.1 and MJ56 had lower amounts of total catalase and HP11 catalase activity. These strains had similar total catalase activities, although the HP11 activities differed slightly. These strains could be grouped as indicating medium resistance to the H₂O₂ treatment.

ATCC strains 25922 and 11775, had 224 and 120.2 U total catalase activity, respectively (Fig. 23). These strains were the most sensitive strains to the H₂O₂ treatment, as well as being the only strains exhibiting no HP11 activity. This indicated, that the total catalase activity present in these strains must have been only due to HP1 catalase activity. The lack of HP11 may explain why these strains were sensitive to the H₂O₂ treatment. It was observed that HP11 is an important enzyme to protect the cell against high H₂O₂ concentrations.

ATCC 35218 and W1371 were considered the most resistant strains to the H₂O₂ treatment, although these strains only indicated 176.4 and 288.8 U HP11 activity, respectively (Fig. 23). These values were considered low when compared to the other strains. These strains may have another mechanism enabling them to withstand high H₂O₂ concentrations.

Both these strains display multiple antibiotic resistance (Table 10), therefore the mechanisms enabling them to resist certain antibiotics may aid in protecting the strains against high H₂O₂ concentrations.

Loui *et al.* (2009) tested if protein synthesis was important for bacterial survival and if the inhibition of protein synthesis is damaging to bacteria under reactive oxygen stress. The resistance of *E. coli* to H₂O₂ was determined in the presence of chloramphenicol, which inhibits protein synthesis by inhibiting peptide bond formation (Loui *et al.*, 2009). Results indicated that chloramphenicol enhanced the effect of H₂O₂, as greater bacterial inactivation was seen with the use of H₂O₂ and chloramphenicol than either one of the substances alone. It was concluded that the synergistic effect of using H₂O₂ and chloramphenicol, indicates that protein synthesis is important for *E. coli* resistance to H₂O₂. Both M29 and ATCC 35218, showed antibiotic resistance against Chloramphenicol, and both these strains were considered resistant to the H₂O₂ treatment. Thus, the resistance of the *E. coli* strains may not be due to catalase activity alone, especially in the strains indicating H₂O₂ resistance, yet displaying low catalase activity (W1371 and ATCC 35218). Other mechanisms might be protecting the strain from the H₂O₂ treatment, such as efflux pumps (Chapman, 2003).

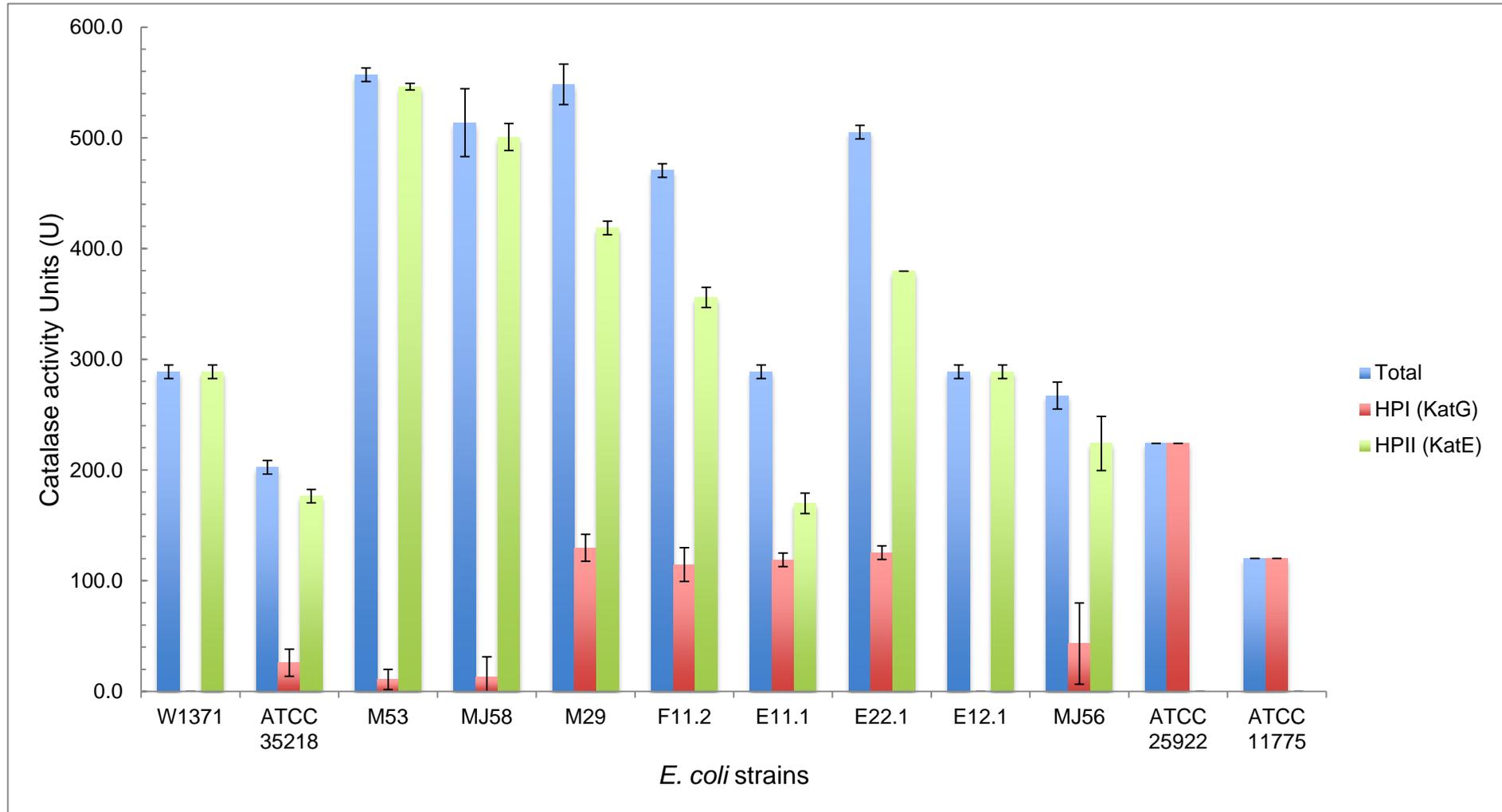


Figure 23 Catalase activity of all 12 *E. coli* strains in the study. Error bars represent error at a 95% confidence interval.

The amount of total catalase or HP11 catalase activity did not always correlate with H₂O₂ resistance. As was observed with catalase method 1, E22.1 had higher amount of total catalase activity than F11.2. Also, E22.1 had 505.1 U total catalase activity, which was more than F11.2 (470.5 U). The HP11 activity for E22.1 and F11.2 was 379.7 and 355.9 U catalase, respectively, indicating similar HP11 activities for these strains. However, F11.2 was more resistant to the H₂O₂ treatment, despite indicating lower amounts of total and HP11 catalase activity (Fig. 23). F11.2 was antibiotic resistant and E22.1 was not, this may explain why F11.2 was better protected against the H₂O₂ treatment.

Overall, a trend was seen between H₂O₂ resistance and the amount of total and HP11 catalase, although there were exceptions. E22.1 had more catalase activity than expected (when considering its resistances to H₂O₂), whereas E11.1, ATCC 35218 and W1371 had less catalase activity than expected (when considering the resistance to H₂O₂).

There were two interesting facts to note with regards to these observations. Firstly, although E22.1 had more total catalase activity compared to F11.2, the HP11 activities for these strains were similar (379.7 and 355.9 U, respectively). A significant difference was not observed between the log reductions achieved after the H₂O₂ treatment for these two strains (study B, Fig. 11), and these strains showed similar HP11 catalase activities. This could indicate that HP11 could be responsible for protecting the strains against H₂O₂. Secondly, two of the most resistant H₂O₂ strains (ATCC 35218 and W1371) showed low catalase activity, indicating that other mechanisms could be protecting them against H₂O₂ treatments.

Iwase *et al.* (2013) determined the catalase activity of pathogenic *E. coli* strains, and their results indicated that more HP1 was present for some strains and more HP11 was present for other strains. In this study, two strains did not exhibit HP1 catalase activity, and two of the strains (ATCC strains 25933 and 11775) indicated no HP11 activity. Interesting, the two strains that did not indicate HP1 activity (W1371 and E12.1) had similar total and HP11 catalase activity, however these strains differed significantly with regards to H₂O₂ resistance. Once again, a trend was observed where the antibiotic resistant strain (W1371) was more resistant to the H₂O₂ than E12.1 which had no antibiotic resistance.

According to literature, *KatG* (HP1) is induced in the response to low amounts of H₂O₂ (Visick & Clarke, 1997). Whereas, *KatE* (HP11) is responsible for survival during stationary phase and other stresses (Mulvey *et al.*, 1990). *KatE*, is not induced by peroxide, yet a trend was seen between HP11 activity and H₂O₂ resistance, while no trend was seen between *KatG* and H₂O₂ resistance in this study. It could be that the high H₂O₂

concentrations used, triggered *KatE* expression due to the increased cellular stress levels. This was evident with ATCC 25922 and 11775 being the only strains where no HPII (*KatE*) was present as well as being the most sensitive strains to the H₂O₂ treatment used in this study.

Comparison between catalase methods 1 and 2

Although the first method used to determine the catalase activity was considered to be more subjective, similar results were achieved for both methods. Both methods indicated that M53, MJ58 and M29 had the highest catalase activity, while F11.2, E12.1, E11.1 and MJ56 had lower catalase activity. Both methods indicated that E22.1 had high amounts of catalase activity, yet this strain was not considered resistant to the H₂O₂ treatment. Low catalase activity was observed in both methods for ATCC 25922 and 11775, which correlated with low H₂O₂ resistance. W1371 and ATCC 35218 both indicated lower amounts of catalase activity, although they were both considered resistant to the H₂O₂ treatment.

CONCLUSION

When considering H₂O₂ as a treatment option, certain factors need to be considered in order to improve the treatment. In this research each study was performed to identify important aspects that may influence the impact of H₂O₂. After the river water samples from the Plankenburg site had been treated with 250, 300 and 350 mg.L⁻¹ H₂O₂ (Study A), it was clear that the two samples did not react in the same manner to the H₂O₂ treatment. This showed that the microbiological content of the river water could be highly variable as a result of the variety of strains being present in the water system and that these strains reacted differently to the H₂O₂ treatment. More resistant strains might survive H₂O₂ treatment, which could result in transfer of *E. coli* to fresh produce and in turn result in illness. Careful optimisation of treatment options and conditions are therefore important.

From the results in Study B, it was seen that the environmental *E. coli* strains did not react in the same manner to the H₂O₂ treatment. Large variations were observed in the log reductions achieved after the 120 min contact time. This indicated, that certain strains were better adapted to survive in the presence of H₂O₂. The environmental *E. coli* strains were generally more resistant than the ATCC reference strains tested in this study, except for ATCC 35218, which was considered a resistant strain. Environmental *E. coli* strains are exposed to adverse conditions and are constantly adapting in order to survive in these conditions, which could explain why these strains were more resistant to H₂O₂.

Thus, it would be advised that if an ATCC reference strain is used for treatment optimisation then ATCC 35218 should be used, as it was more resistant ATCC 11775 and 25922. It is important that the most resistant strains be used for optimisation of treatment options and conditions to reduce the risk of transferring *E. coli* from irrigation water onto fresh produce.

Most of the strains that showed high resistance to the H₂O₂ treatment also exhibited antibiotic resistance, thus indicates that there might be a link between the mechanisms that protects them from H₂O₂ and antibiotics. However, MJ58 was a resistant strain not showing antibiotic resistance, which shows that a link between antibiotic and H₂O₂ resistance does not always exist. Strains not showing antibiotic resistance cannot be assumed to be sensitive to the H₂O₂ treatment.

From Study C it was observed that a low concentration of H₂O₂, was considered ineffective for treating resistant *E. coli* strains, as it resulted in a less than 1 log reduction after 120 min. At higher concentrations (350 and 700 mg.L⁻¹) a maximum log reduction of only 2.27 was achieved, therefore 3 log reductions were still not achieved after 120 min. A significant difference was not seen between the log reductions achieved at 350 and 700 mg.L⁻¹. In this study under conditions where a lower than 3 log reduction would be sufficient, the lower concentration should be selected, as it would be more cost effective and result in similar log reductions. Using 1 000 mg.L⁻¹, was effective at achieving a greater than 3 log reduction after 90 min, however using such high concentrations might not be economically feasible for some large-scale applications.

From the results in Study D, it was concluded that the organic matter (COD content) present in the water did not influence the H₂O₂ treatment. However if resistant strains are present longer contact times may be necessary. Farmers would need to increase the contact time if the quality of the water is unknown.

The amount of catalase activity was measured in order to determine if catalase activity could be linked to H₂O₂ resistance. Although a trend was seen between catalase activity and H₂O₂ resistance, high catalase activity alone did not automatically indicate H₂O₂ resistance, as two of the most resistant strains showed low amounts of catalase activity.

Overall, it can be concluded that if H₂O₂ is selected as a treatment option certain factors need to be considered. It is important to note that certain strains may be more resistant to the H₂O₂ treatment than other strains. Thus, high H₂O₂ concentrations may be needed in order to ensure that resistant strains are also inactivated and will not survive the treatment.

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CHAPTER 4

GENERAL DISCUSSION AND CONCLUSION

Surface water including river water is the main source of irrigation water available for farmers in South Africa. Unfortunately, surface water is highly susceptible to contamination from human and animal sources. However, farmers are often forced to use water of a compromised quality, which in turn can lead to foodborne outbreaks. These rivers often have faecal coliform levels above the Department of Water Affairs (DWA) and World Health Organization (WHO) guidelines, of 1 000 faecal coliforms per 100 mL, and therefore can be seen as a health risk. Using contaminated surface water for irrigation, can lead to the increase of foodborne disease outbreaks. Certain microbes are capable of surviving on fresh produce and thus can lead to illness. Foodborne illness are considered a serious problem in a country like South Africa, where a large percentage of the population is suffering from HIV/AIDS, thus these illnesses can often result in death. Not only are foodborne illnesses a health risk, but can also have a negative impact on the South African economy as outbreaks can lead to the ban of exporting fresh produce (Britz *et al.*, 2012).

A study by Britz *et al.* (2012) indicated that some of the rivers in the Boland region is highly contaminated, and thus unsuitable to be used for irrigation. Therefore, surface water needs to be treated in order to decrease the high microbial loads prior to irrigation. Hydrogen peroxide (H_2O_2) has been used as a disinfectant for many years, due to the many advantages associated with its use. The main advantage associated with the use of H_2O_2 as a water treatment option is formation of non-toxic by-products, as H_2O_2 decomposes into hydrogen and water. It is important that the correct concentrations and contact times are used to ensure effective disinfection.

The first section of this study (Study A) focused on the effect that H_2O_2 had on microbial quality of river water, including aerobic colony counts (ACC), total coliforms and *Escherichia coli* (*E. coli*). From the data, a variation was observed between the different samples, indicating different bacteria present in the different river samples reacted differently to the H_2O_2 treatment. The higher H_2O_2 concentration (350 mg.L^{-1}) generally resulted in a higher log reduction achieved, but overall a large difference was not observed between the different H_2O_2 concentrations used. From a study done by Britz *et al.* (2013) it can be seen that high faecal coliforms levels have been found in the rivers in the Boland region and at least a 3 log reduction would be needed to ensure that the water is below

the DWA and WHO guidelines for faecal coliforms. None of the H₂O₂ concentrations in this study resulted in a 3 log reduction after the 120 min contact time.

Study B was included in order to determine the effect that (250, 300 and 350 mg.L⁻¹) H₂O₂ had on the *E. coli* strains over a 120 min contact time. The second aim was to determine if a difference was observed between the log reductions achieved between environmental and reference *E. coli* strains. From the data obtained in study B it was confirmed that when 250 - 350 mg.L⁻¹ H₂O₂ was used, similar log reductions were achieved, although a trend was observed where the highest H₂O₂ concentration (350 mg.L⁻¹) resulted in the highest log reduction. The most microbial inactivation took place in the first 30 min, but *E. coli* counts kept decreasing up to 120 min, where counts were still present for the environmental *E. coli* strains. It can be concluded that a 120 min contact time would be needed if farmers were to use H₂O₂ as a treatment option, if initial microbial counts are high. Overall, it was thus observed that the ATCC *E. coli* strains were generally more susceptible to the H₂O₂ treatment, compared to the environmental *E. coli* strains. It is important to note that variations were also seen between the log reductions achieved for the different environmental *E. coli* strains, indicating that some strains are better adapted to withstand high H₂O₂ concentrations than others.

Overall, it was found that *E. coli* strains displaying antibiotic resistance were usually better adapted to survive when exposed to H₂O₂ (Study B). Most of the *E. coli* strains indicating resistance to the H₂O₂ treatment also indicated antibiotic resistance. This could indicate a potential link between antibiotic resistance and H₂O₂ resistance

Based on the results of Study B, a strain indicating H₂O₂ resistance as well as a strain expressing virulence factors (potential pathogenic strain) were selected for Study C to determine the impact of four H₂O₂ concentrations (50, 350, 700 and 1 000 mg.L⁻¹). It was concluded that low H₂O₂ concentrations were not effective, as it resulted in a less than 1 log reduction after 120 min. When the concentration was increased to (350 and 700 mg.L⁻¹) similar log reductions were achieved at both these concentrations, thus it might not be economical to use the higher concentration (700 mg.L⁻¹) if similar log reductions are achieved using 350 mg.L⁻¹. It can be concluded that in this study 1 000 mg.L⁻¹ H₂O₂ resulted in an effective microbial inactivation of the high initial concentrations and the *E. coli* strain indicating potential pathogenicity also showed resistance to the H₂O₂ treatment.

In Study D, it was found that the chemical oxygen demand (COD) did not affect the H₂O₂ treatment, as similar log reductions were found in the autoclaved river water and physiological saline solution (PSS) samples, after 120 min. It is important to note that for

one of the *E. coli* strains, it was found that the COD did affect the H₂O₂ treatment, but only in the first 30 min.

Escherichia coli strains synthesise catalase enzymes, which degrade H₂O₂ into hydrogen and water, consequently rendering it non-toxic. It was expected that the *E. coli* strains displaying resistance to the H₂O₂ treatment, would have high catalase activity. However, although a trend was seen (Study E) between H₂O₂ resistance and high catalase activity, this was not always evident. Two of the most resistant *E. coli* strains to the H₂O₂ treatment indicated low amounts of catalase activity, which indicated that another mechanism may be protecting them against the H₂O₂ treatment. Both the strains that displayed low catalase activity were also antibiotic resistant, and this may indicate a link between H₂O₂ resistance and antibiotic resistance. Consequently, it can be concluded that catalase activity alone cannot indicate if the strains will be resistant or sensitive to the H₂O₂ treatment.

Certain implications can be highlighted from the results obtained in this study. The river water samples did not react in the same manner to the H₂O₂ treatment. After the H₂O₂ treatment a less than 3 log reduction was achieved, indicating that if farmers used these conditions to treat surface water with high microbial counts it could result in the transfer of these microbes to fresh produce (which could in turn result in microbial levels on the produce at higher levels than regulations allow). When testing the efficacy of water treatment options it is important that environmental *E. coli* strains are used, as they might be better adapted to adverse environmental conditions. Using ATCC *E. coli* strains to determine concentration or contact times may result in parameters being selected, which will not reduce the microbial contamination to desired levels, permissible for irrigation. Determining the correct H₂O₂ concentration to use to treat irrigation water is a difficult task, due to the variation in log reductions achieved for the different *E. coli* strains. Strains indicating antibiotic and H₂O₂ resistance have been isolated from rivers in the Boland region and this needs to be considered when evaluating other possible treatment options as resistance to other compounds may differ. Farmers will not always be aware of the types of microorganisms present in the water system, and thus higher concentrations and contact times may be needed to ensure effective disinfection to achieve levels in compliance with WHO or DWA guidelines (WHO, 1989; DWAF, 1996; DWA, 2013). The strain indicating potential pathogenicity showed resistance to the H₂O₂ treatment and this could have health implications as this strain may survive treatment and be transferred to fresh produce where it may cause illness. Farmers would want to ensure that they are dosing the water with a high enough concentration of any disinfectant (within allowable

levels) to ensure that these strains (indicating pathogenicity) are inactivated prior to irrigation, thereby preventing potential illness.

When observing the effect that different H₂O₂ concentrations had on the *E. coli* strains it was noted that 1 000 mg.L⁻¹ resulted in an effective disinfection, however, using such a high H₂O₂ concentration might, not be economically viable, depending on the type of application.

The organic matter content in the water did not influence the H₂O₂ treatment, however, it can be concluded that if H₂O₂ is used to treat river water, farmers may need to slightly increase the contact time, especially if the water is highly contaminated or represents a higher organic load.

From a practical point of view farmers cannot sample their water continuously to determine *E. coli* characteristics such as: antibiotic resistance; amount of catalase activity; or resistance of strains to the treatment option. Therefore, slightly higher concentrations and contact times would need to be implemented in order to ensure that strains indicating resistance are effectively inactivated. It is important to note that the initial contamination levels will influence the treatment conditions selected.

From the data it was observed that H₂O₂ might not be an effective treatment option to treat surface water, especially if the water is highly contaminated. Log reductions of more than three logs were not achieved for resistant *E. coli* strains for most of the treatments below 1 000 mg.L⁻¹. Using H₂O₂ as a treatment option alone therefore, would not be recommended for highly contaminated surface water. Further studies would need to be done in order to determine if using H₂O₂ as part of a combination treatment would result in better log reductions and in turn present an effective treatment option. Thus, it might be valuable to explore the possibility of using H₂O₂ in a combination treatment with ultraviolet (UV) radiation or sand filtration. Using H₂O₂ in combination with other treatment options may result in more effective log reductions, and more cost-effective applications.

It is recommended that further studies be done in order to understand how these *E. coli* strains develop resistance to H₂O₂. It would be interesting to observe if the same log reductions would be achieved after the same treatment was applied to the surviving fraction of cells after 120 min at 350 mg.L⁻¹ (as seen in studies B and C). This would determine if the *E. coli* cells that survived are better adapted to survive the H₂O₂ treatment in the future. The effect of H₂O₂ on more antibiotic resistance strains could also be evaluated to determine whether a trend can be observed between antibiotic and H₂O₂ resistance over a larger collection of strains.

River water samples with higher amounts of organic material should be evaluated in order to determine if higher amounts of organic material negatively influence the log reductions achieved with the H₂O₂ treatment, or even other chemical disinfectants. No significant differences were observed in this study, however, the COD values were not very high (18 – 55 mg.L⁻¹)

Finally, the effect of other biocides should be evaluated on *E. coli* strains to determine whether strain-to-strain variations would be observed. It would be important to evaluate whether environmental *E. coli* strains would be more resistant to other biocides (such as ozone or chlorine) compared to ATCC *E. coli* strains. It would also be important to observe if a link between antibiotic resistance and other biocide (such as chlorine resistance) resistance exists. In this study, although a link was observed, there was one *E. coli* strain indicating resistance that did not have antibiotic resistance, therefore, resistance is not always linked. It also cannot be assumed that if *E. coli* strains are resistant to H₂O₂ that they will display resistance to other biocides. Thus, these factors need to be considered with regards to other biocides in order to develop effective disinfection conditions and practical recommendations.

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