Cape Peninsula
University of Technology

Ecological and human health risk assessment of selected endocrine disrupting phenolic compounds in potable water and treated wastewater effluent in the Western Cape, South Africa

By:

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Dissertation submitted in fulfilment of the requirements for the degree Master of Technology Environmental Health in the Department of Environmental and Occupational Studies, Faculty of Applied Sciences, Cape Peninsula University of Technology

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April, 2021

DECLARATION

I, Nkosiyenzile Londiwe Mhlongo, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed _____ **Date**_____

ABSTRACT

Potable water is becoming increasingly scarce in Cape Town, Western Cape- South Africa due to climate change effects such as drought. The region's water resources are under threat by industrial and agricultural pollution due to the release of organic contaminants such as phenol into water bodies. This reduces water quality, thereby decreasing the availability of clean water. Potable water (tap and bottled) as well as effluent from wastewater treatment plants (WWTPs) are among the most important freshwater resources in the Western Cape.

This study focused on two phenolic compounds - 2,4-dichlorophenol and 4-chlorophenol. Samples of a wastewater treatment plant effluent, tap water, and four brands of bottled water were analysed for the two phenolic compounds using the HPLC/DAD. Toxicity assessment of the WWTP effluent was conducted using *Raphidocelis subcapitata* (a primary producer), *Daphnia magna*, a primary consumer that feeds on algae and *Tetrahymena. thermophila* a protozoan (decomposer). The Ames mutagenicity test was conducted using the T98, T100 strain and with the S9 activation enzyme. Two brands of bottled water, tap water and WWTP effluent (found to have the highest concentrations of 2,4-DCP and 4-CP) were tested for mutagenicity. The potential risk of using these water sources for potability was assessed. The phenolic compounds were both below the regulatory limits in all the samples analysed. . The concentrations of 2,4-DCP in the WWTP effluent, tap water and bottled water brands 'A', 'B', 'C' and 'D' ranged from; ND-5.40×10^{-6}, ND- 1.90×10^{-5}, ND-1.31×10^{-5}, 3.68×10^{-6} - 1.37×10^{-5}, ND-6.85×10^{-6} and 6.28×10^{-6} - 1.47×10^{-5} respectively. Corresponding values for 4-CP were 4.04×10^{-6} - 5.61×10^{-5}, 9.96×10^{-6} - 1.90×10^{-5}, ND-5.81×10^{-6}, ND-6.95×10^{-6}, ND-9.78×10^{-6} and 8.90×10^{-7} - 6.74×10^{-6} (mg/L) respectively.

The cumulative immobility of daphnids in a *Daphina Magna* test against the effluent during the test period of 48 h indicated a clear minute concentration-response relationship. Throughout the 48 h test period, there were no significant deaths of the daphnia (i.e 1/20 to a maximum of 6/20 in all different concentrations). For the *Raphidocelis subcapitata*, the results showed a substantial constant increase or growth of algae throughout the 72 h period in all different concentrations (including the 100% undiluted effluent) thus indicating the eutrophic potential of the effluent. The *T. thermophila* toxicity test showed that the diluted effluent samples were more toxic that the whole effluent.

All the samples tested exhibited strong mutagenicity on the T98 strain but slight mutagenicity on the T100 strain. All the samples were classified to have non-carcinogenic adverse effects but not cancer risk.

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ACKNOWLEDGEMENTS

I wish to thank:

- I wish to thank and give all honour and glory to the Lord God Almighty, for being the author and finisher of this work.
- My mother, Mukelisiwe Idah Mhlongo, Indlovukazi. Thank you so much for being so supportive of everything I put my mind into, thank you for constantly and consistently encouraging me to press on until the end and reminding me when my strength runs out that we serve an awesome God who is faithful and will see me till the end.
- My sisters and my best friends; Halalisiwe Mhlongo and Mbaliyezwe Madikizela. There would be times when I would be stressed, frustrated and would cry out to you when things become stagnant and would be encouraged to press on and would always give me a shoulder to cry on.
- My mom (Bongi Madlala) & family for your continues and genuine support. Always checking up on my progress and encouraging me.
- Prof. B.O. Opeolu for being a mother-like figure to me. This work would not be what it is if it was not for your hard work and time. Thank you for going far and beyond to even support me financially. I thank God for you, truly.
- Prof. I Human, Dr. M. Akharame & Dr. B. Fagbayigbo, without you this work would not be what it is.
- My academic peers, Dr. Omoniyi, Sihle Mlonyeni and Komlan Apetogbor, thank you for an incredible working relationship.

The financial assistance of the National Research Foundation towards this research is acknowledged. Opinions expressed in this thesis and the conclusions arrived at, are those of the author, and are not necessarily to be attributed to the National Research Foundation.

DEDICATION

For my mother, Mukelisiwe Idah Mhlongo.

ABBREVIATIONS

Abbreviations	Meaning
2,4-DCP	2,4-dichlorophenol
4-CP	4-chlorophenol
BAC	based activated carbon
CCT	City of Cape Town
DAD	Diode array detector
DWA	Department of Water Affairs
DWAF	Department of Water Affairs and Forestry
EC	European Commission
ECD	Electron Capture Detection
EHRA	Environmental Health Risk Assessment
EPA	Environmental Protection Agency
EU	European Union
FID	Flame Ionization Detection
FLD	Fluorescence detector
GC	Gas Chromatographic
GC/MS	Gas Chromatographic/Mass spectrometry
HPLC-DAD/UV	High Performance Liquid Chromatography- Diode Array Detection- Ultraviolet
LLE	Liquid Liquid Extraction
LOD	Limit of Detection
LOQ	Limit of Quantitation
MS/MS	Mass spectrometry/Mass spectrometry
OD	Optical Density
Ng/g	Nanogram per gram
NIR	Near InfRed
OSRB	Orange-Senqu River Basin
PC	Phenolic Compound
PCBs	polychlorinated biphenyls
POPs	Persistent organic pollutants
PPM	Parts per million
PPB	Parts per billion
S.typhimurium	*Salmonella typhimurium*
SPE	Solid Phase Extraction
SPME	Solid-Phase Micro-Extraction
SS	sewage sludge
USEPA	United States Environmental Protection Agency
UV-vis	Ultraviolet and visible
WCWSS	Western Cape Water Supply System
WWTP	Wastewater treatment plant

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CHAPTER ONE

1.0. Introduction

1.1. Background

Sources of organic contaminants in the environment are largely caused by anthropogenic activities while natural sources represent negligible fractions (Menichini & Bocca, 2003; Michałowicz & Duda, 2007; Swiegelaar & Quinn, 2017). The release of contaminants from industrial processes, agricultural and domestic activities, and air borne chemicals amongst others are published in literature (Barnes *et al*., 2002). Most chemicals generated from human activities ultimately have a way in reaching and accumulating our surroundings and habitats (Morris, 1995; Kogevinas, 2009). Runoff obtained from agriculture, wastewater discharges, unrestrained discharges, industrial plants leakages or landfill sites and accidental discharges during disasters also contaminate both groundwater and surface water (Skupinska *et al*., 2004; Michałowicz & Duda, 2007; Demir & Ergin, 2013). Airborne pollutants are flushed down by rainfall from the firmament and flows across the terrain prior emptying in aquifers, rivers, rills and lakes (Barnes *et al*., 2002).

Phenols are extensively utilised in both industrial and commercial applications. However, they have become a source of concern because of their increasing existence in drinking water coupled with their capability of inducing detrimental health effects (Demir & Ergin, 2013). Phenol and its methyl compounds exhibit great ability for stable matrices to be adsorbed. Furthermore, it has been discovered that some are considerably toxic to fish and other aquatic life. They are a grave threat to the environment, with postulation of apparent endocrine disruptor or carcinogenic effects (Olujimi *et al*., 2010). Many studies have revealed that they are potentially carcinogenic, teratogenic, mutagenic and they possess endocrine disrupting characteristics (El-Amin Bashir *et al*., 2017; Liu *et al*., 2017; Syed *et al*., 2017). Levels in environmental matrices, including atmosphere, soil, sediments, plants, and human bodies have been reported (Olujimi *et al*., 2010; Egeghy & Lorber, 2011; Yang *et al*., 2015).

It was approximated that over 1.3 million metric tons of phenolic compounds are released annually into the marine environment, consequently resulting in an unanticipated presence of toxic subsurface oil, and chronic exposures even at sub-lethal levels (USNRC, 2003; Wetzel & Reynolds, 2006). Woodruff *et al.* (2011) reported the exposure of pregnant women to environmental chemicals that included phenolic compounds in the United States. Exposure of pregnant women to chemicals in the course of foetal evolution is inclined to trigger deleterious health consequences, including unfavourable birth defects, acute and severe medical conditions such as effects on the development of the nervous system and childhood cancer, in adults; cancer and cardiovascular issues (Stillerman *et al*., 2008; Snijder *et al*., 2012).

The presence of phenolic compounds in ecosystems are connected to formulation and degradation of several pesticides, such as generation of municipal and industrial sewage (Michałowicz & Duda, 2007). Owing to the overwhelming manifestation of phenolic compounds present in our food, this has given rise to humans ingesting a substantial measure of these compounds (Morris, 1995). It is noteworthy that diets rich in phenolic compounds reduce the risk of a number of chronic diseases, such as cancer (Huang

& Ferraro, 1992). The detection of Phenols from source water is common, with the maximum median concentration of 10 µg/L (Ge *et al*., 2006). Phenols are classified as significance pollutants by the USEPA (USEPA, 2014). Endocrine disrupting chemicals such as phenolic compounds, can adversely affect estrogenic responses at significantly low concentration of contaminates such as in ppb to parts per trillion. Many of these compounds have been discovered at significant concentrations in environmental mediums such as wastewater, drinking water, surface waters, groundwater, and sediments, in various nations (Olujimi *et al*., 2010). In South Africa, chemicals that seek to disrupt endocrine have been determined in potable and river water, wastewater treatment plants' effluents, sediment and serum, including fish tissue samples at levels that have a potential to trigger endocrine disruption in people and wildlife (Morris, 1995; Olujimi *et al*., 2010; Mathieu-Denoncourt *et al*., 2015).

Drinking water standards of the European Commission (EC), the World Health Organization guidelines and the Netherlands have served as a basis for South African regulations. The USEPA, Health Canada and some other agencies have presented preliminary human health risk assessments on some suspected carcinogenic compounds that include phenolic compounds (ECHA, 2013; Lemieux *et al*., 2015). However, South Africa still lacks detailed coverage of these organic chemicals in water sources (Mamba *et al*., 2008). Wastewater treatment plants are a major source of phenols to man and the environment. Thus, It is imperative that the environmental and health risks of selected phenolic compounds in environmental matrices be assessed. This study will identify and measure levels of phenols and establish environmental and health risks related to exposure. This will contribute to developing a strategy for the management and mitigation of health implications of phenols and facilitate the need for remediation if found at significant levels. Environmental hazard assessment of phenols in the ecosystem will also be necessary.

1.2. Statement of the research problem

Water is a universal solvent that easily dissolves many natural and synthetic substances from the environment. Safe drinking water is also essential to humans and other life forms (Clemencoc, 2005; Pohorille & Pratt, 2012). The presence of organic compounds such as phenolic compounds (PCs) have been reported in environmental samples that include food, air, soil, street dust, rain water, urban runoffs and industrial wastewater (Padhye & Tezel, 2014; DER, 2017; Vinod *et al.,* 2017). Studies have revealed that environmental and health implications of phenolic compounds include teratogenicity, carcinogenicity and mutagenicity (Menichini & Bocca, 2003; Zhang *et al*., 2004; Karyab *et al*., 2013; Abel *et al*., 2014). Currently, concerns about phenols are growing globally due to their distribution, and adverse effects on man and environment. They are famous for their toxicity and exert both harsh and enduring health effects on persons and faunae (Kanaly & Harayama, 2000; Liu *et al*., 2011; Anku *et al*., 2017).

Cape Town, in the Western Cape, is one of the regions of South Africa where potable water is becoming increasingly scarce because of the ongoing drought. The region's water resources are under threat by industrial and agricultural pollution due to the release of organic contaminants such as phenol into water

bodies. This reduces water quality, thereby decreasing the availability clean water (CCT, 2018b; OECD, 2021). Potable water (tap and bottled) as well as effluent from WWTPs are among the most important water resources in the Western Cape (CCT, 2018a). Determination of phenolic compounds in drinking water and wastewater will provide information related to the levels of exposure and risks to humans when consumed. Bio-concentration and bioavailability of these compounds in water will constitute environmental health hazards to both humans and the environment in a city, which is known as one of the most popular tourist destinations in the world (Sharma & Bhattacharya, 2017; Sunkara & Hastings, 2019).

Globally, grave concerns have been voiced out by various health and environmental organizations, including departments of water resources and scientific communities about the safety of potable water (Aoyi et al., n.d.). A threshold limit for suspected carcinogenic phenolic compounds for South African waters is non-existent due to sparse information on phenolic compounds in the country. South African National Drinking Water Standard- SANS (2015) doesn't sufficiently speak to phenolic compounds in terms of drinking water quality management. The current, water quality standard for phenolic compounds for domestic water use needs to be more elaborate and precise. Phenolic compounds are endocrine disruptors and pose threats to human and ecosystem health (Neng & Nogueira, 2014). They have been included in the monitoring and evaluation programme supported by the WHO in Africa (Mamba *et al*., 2008; Ncube, 2009).

1.3. Research questions

1. Is it possible to accurately identify and quantify the selected phenolic compounds in water samples and effluent from a WWTP?
2. What are the levels of the selected phenolic compounds in tap water, bottled water, groundwater samples and WWTP effluents?
3. What are the possible health and ecological risks associated with the occurrence of the phenols in the water samples?

1.4. Research objectives

Aim

The aim of this study is to assess levels and possible human and ecological risks of phenolic compounds (4-Chlorophenol and 2,4-Dichlorophenol) in the tap water, bottled water and WWTP effluent.

Specific Objectives

- To adapt an existing method for qualitative and quantitative determination of selected phenolic compounds (4-chlorophenol (4-CP) and 2, 4-dichlorophenol) in water.
- To assess the levels of 4-chlorophenol (4-CP) and 2, 4-dichlorophenol in tap water, bottled water and WWTP effluent.
- To evaluate possible human and ecological health risk assessment of the phenols in aquatic ecosystems.

CHAPTER TWO

2. Literature review

2.1. Water and wastewater contamination by organic contaminants

Water typically has a dual responsibility, it can be considered as a pivotal sanitation parameter as well as an economic factor (Adeleye, 2016). A secure, reliable, economical, and easily accessible water supply is paramount for sound health *(Hunter et al*., 2010). The removal of micro constituents in a wastewater treatment plant can serve as an imperative component in ensuring safe, reliable, affordable and easy access of water supply because the effluents from the WWTPs are typically discharged into surface waters, such as rivers (Wagner, 2000; Edokpayi *et al*., 2017).

It has been ascertained that contaminants derived from treated wastewater tend to unfavourably affect wildlife and the aquatic environment (Olujimi *et al*., 2010). Organic contaminants such as phenolic compounds are one of the contributors towards pollutants in wastewater and stormwater runoff which have been widely used in agricultural activities (Chowdhury *et al*., 2016). This is due to the hasty growth of population, industry advancement and the extensive employment of diverse chemicals in the industry that is increasing. Thus, improper disposal practices leads to the contamination of soil and groundwater by inadequate treatment of phenolic compounds and thereby contaminating the water system by inflowing wastewater containing these phenolic compounds. This process, compromises the health of living organisms including ecosystems and human health via bioaccumulation, contamination of public water supply and recreational use of contaminated water (Watts, 1998; Mainali, 2020).

The presence of chemicals and micro-pollutants is a serious barrier faced by treatment plants, as pollutants reduce the adequacy of treated wastewater to be used as a source of drinking water. Research has shown that South Africa's new traditional wastewater treatment solutions are insufficient to treat wastewater prior to reuse or discharge. (Adeleye, 2016; Edokpayi *et al*., 2017; Afolabi *et al*., 2018). A vast number of authors have suggested based on their investigations that final effluents of wastewater treatment plants were primarily liable for the increasing estrogenic activity in various aquatic environments (Olujimi *et al*., 2010; Jasim *et al*., 2016). Auriol *et al*. (2006) posited that “endocrine disrupting compounds’ existence in the environment is highly inclined to interrupt the ecosystems and adversely affect the health human beings. Hence, this necessitates the establishment of a reliable detection technique, analysis tools, and adapted wastewater treatment processes is now the subject of a quasi-consensus between the scientific communities.”

Therefore, eliminating organic compounds or lowering their concentrations to the measures legalised by environmental standards remains a daunting task. Though biological treatments are associated with multiple benefits compared to physicochemical treatments (environmentally friendly and energy saving), there are still disadvantaged such as processes being unable to even treat high concentration of contaminants (Ramírez *et al.*, 2017).

2.2. Phenolic compounds in the environment

Phenolic compounds, also known as hydroxybenzenes, are colorless, crystalline substances of characteristic odor and are soluble in water. Their solubility and characteristics owes to their stronger hydrogen bonds when reacting with organic solvents (Michałowicz & Duda, 2007). Phenols were initially isolated in crude type at the conclusion of the eighteenth century; today, they are applied as antiseptics, disinfectants and preservatives (Hugo, 1978; Wade, n.d.). Phenolic substances occur in water bodies owing to the discharge of contaminated wastewater from industrial, agricultural and household applications and can also occur because of natural phenomena (Davì & Gnudi, 1999; Peñalver *et al*., 2002; Balasundram *et al*., 2006; Anku *et al*., 2017). They are understood to be harmful and inflict both dreadful and long-term impacts on both humans and animals (Anku *et al*., 2017; Afolabi *et al*., 2018). Phenols are widely used in domestic substances also as intermediates for industrial synthesis (in little concentrations) including disinfectant in household detergents and in mouthwash (Wade, n.d.; Aljamali *et al*., 2015).

Phenols can be classified as organic compounds having benzene rings linked to a hydroxyl group called carbolic acids (Ho, 1992). Phenols are typically associated with higher boiling points in contrast to other hydrocarbons, possessing the same molecular masses. This is as a result of the existence of intermolecular hydrogen bonds joining hydroxyl groups of phenol molecules (NCERT, 2015). Hydrogen bonds are established between water and phenol molecules thereby making phenol soluble in water (Banat *et al*., 2000). In industrial applications, phenols are employed as a feedstock in the production of plastics, explosives namely picric acid, and drugs namely aspirin (Uddin *et al*., 2007; Huang *et al*., 2010; Malhorta, 2016). Phenol and its derivatives are extremely harsh, they are toxic pollutants that are considered hazardous even at low concentrations hence, the management of wastewater with high concentrations of phenols presents crucial economic and environmental hinderances to the majority of industries (Al-Khalid & El-Naas, 2012).

This study focuses on two phenolic compounds, 4-chlorophenol and 2,4-dichlorophenol. They were chosen due to the ubiquitous occurrence of chlorinated phenolic compounds from various industrial sources. These environmental pathways include manufacturers of preservatives, pesticides and dyes, and pulp and paper industries and other phenol-based compounds (Olaniran & Igbinosa, 2011; Xu *et al*., 2017). The compounds pose some of the most threatening and persistent organic pollutants due to their vast industrial applications which have given rise to accumulation in the environment, and hence, a grave concern (Ghaffar *et al*., n.d.). Majority of these compounds include by-products of industrial processes as well as pharmaceutical, pesticide, paint and solvent production, wood, paper, and pulp processing (Allaboun & Al-Rub, 2016; Xu *et al*., 2017). Due to the toxicity of chlorophenols coupled with their persistence in the environment, methods of their elimination are urgently required (Movahedyan *et al*., 2008).

4-chlorophenol (4-CP, also called p-chlorophenol) is a white crystal with an intense phenol odour, is relatively soluble in water, based on the isomer, denser than water and non-combustible (Bien *et al*., 2000). As a result of its aromatic ring configuration and the stability of the C-Cl bond in the ring, this compound is insusceptible to biodegradation (NOAA, n.d.). 4-chlorophenol (4-CP) has been listed as a priority pollutant due to their teratogenic, carcinogenic, mutagenic, persistence and high toxicity (Xu *et al*., 2017). 4-chlorophenol is difficult to the natural and biodegradation because of its low water solubility and vapour pressure. As a consequence of its wide usage and negative health impacts, it is necessary to assess its occurrence in environmental water samples and effects on ecological systems (WHO, 1994; WHO, 1996; WHO, 2003; Villegas *et al*., 2016; Anku *et al*., 2017; Mu'azu *et al*., 2017).

2,4-dichlorophenol (2,4-DCP) can be classified under organic compounds that are known as dichlorobenzenes (T3DB, 2009). 2,4-DCP is a colourless crystal, white or pale-yellow solid with a medicinal odour. It is poorly biodegradable with melting point of 45°C and sinks in water (METI, n.d.). 2,4-dichlorophenol is a predominantly encountered toxic chlorophenol compound that is found in some industrial effluents (Dilaver & Kargi, 2009). 2,4-DCP is a paramount element in the production of phenoxy acid herbicides and is also involved in the synthesis of antiseptics and various pharmaceuticals. Furthermore, It is possible to also discover it the environment as a product of degradation of an antiseptic agent named triclosan (Park & Kisok, 2018)

2.3. Occurrence of 4-chlorophenol and 2,4-dichlorophenol in the environment

Distribution of phenols have been widely reported in environmental matrices such as surface water, wastewater, drains, drinking water, groundwater, biological samples, plant tissues, sediment, soil amongst others (Jafari *et al*., 2009; Olujimi *et al.,* 2010). Occurrence of phenolic compounds in environmental matrices has been attributed to several applications manufacturing processes, application in phenolic resins, organic synthesis and in petroleum products such as coal tar, creosote combustion sites of wood and auto exhausts (ATSDR, 2008a). Wang and Lin (1995) reported levels of selected phenols in drinking water which ranged from 0.01 or 0.04 to 10 μg/L with the potential to develop damaging effects on exposed DNA.

The presence of chlorophenol in potable water, is a consequence of the chlorination of phenols that take place during disinfection, as by-products of the reaction of hypochlorite with phenolic acids, as biocides, or as degradation products of phenoxy herbicides (WHO, 1996). The WHO (1996) stated that data obtained from 40 Canadian treatment plants demonstrate that chlorophenol amount in potable water are relatively low but differ substantially from one region to the next. Hence, most of the common environmental discharges of chlorophenols are to surface water because as previously stated, Industrial waste discharge and seeping of chlorophenols from dumpsites is the main cause of water toxicity by chlorophenols. (Czaplicka, 2004). The rising chlorination results in an inclination of these compounds to segregate distinctively into sediments and lipids and to bioconcentrate (Jensen, 1996). Bruce *et al*, 1987 conducted a study where they found phenol in the surface water of Netherlands having a

concentration of 2.6-5.6 μg/L. In river waters contaminated by sewage derived from petrol processing plants had a phenol concentration of over 40 mg/L.

Moreover, chlorophenols ends up in the atmosphere via volatilization. The mono- and di-chlorophenols are known to have the highest volatility once they discharged into the atmosphere. Chlorophenols experience a number of physical, chemical, and biological metamorphoses. Sorption, volatilization, degradation, and leaching are the primary processes contributing to their fate and transport. More concerns around chlorophenols exposure in the environment's is their ability in contaminating aquatic ecosystems including other organisms incorporated in the food chain of the aquatic Eco biota . The circumstance could possibly be exacerbated by chlorophenols that are so obstinate in maintaining elevated toxicity degrees within the environment for a lengthy period (Jensen, 1996; Watts, 1998; Czaplicka, 2004; Igbinosa *et al*., 2013; Oluwasanu, 2018; Ramírez *et al*., 2017).

In soil, chlorophenol isomers experience biodegradation under aerobic conditions as a result of technological processes, biodegradation of herbicides, pesticides, and from atmospheric deposition. Albanis & Danis, 1999 conducted assessments on arable lands in the environs of Thessaloniki and Ionnina where in the area of Thessaloniki, 2,4-dichlorophenol and PCP were found in concentration of 0.12 and 0.24 ng/g, respectively (Czaplicka, 2004).

More specifically, 4-CP is one of the final-products of the process of biodegradation of polychlorinated phenols by the microorganisms under anaerobic conditions as a result of its stability against biological degradation, highlighting that the biological treatment of industrial wastes having great amounts of 4-CP is immensely challenging. Therefore, the detoxification of 4-CP from industrial aqueous effluents is significant for environmental protection (Bae *et al*., 1996; Wen *et al*., 2006; Liu *et al*., 2007). Exposure to 4-CP could potentially also happen through its use as a root canal packing (Oluwasanu, 2018). The 2,4-DCP compound is a chlorinated phenol derivative used in copious quantities in the manufacture of certain herbicides and preservatives, such as 2,4-dichlorophenoxyacetic acid and pentachlorophenol, readily contained in soils and waste streams. (Theurich *et al*., 1996; Muller & Caillard, 2017). It can also be found in the atmosphere as a by-product of triclosan (a degradation of an antiseptic agent). 2,4-dichlorophenol (2,4-DCP) is the most common organochlorine compounds to be easily found in soils and waste streams (Park & Kisok, 2018). In water, it is very soluble and has a half-life of 14.8 days. In certain drinking water sources, 2,4-dichlorophenol has been detected, but knowledge is very restricted. (EPA, 2015).

2.4. Uses of phenolic compounds

Phenolic compounds amongst other things are reputable for their health advantages related to antioxidant activity, extensively applied as antiseptics (substances that kill microorganisms on living tissue) as well as disinfectants (Zheng & Zhang, 2012; Chemistry, 2017). They are also typically used as constituents in pharmaceuticals, agricultural products, photographic products, lubricating materials, and in other chemical industries (Jewell, n.d.).

Roughly 66% of all the phenol produced globally is used in the preparation of reagents used in plastic manufacturing industries. Phenolic compounds are employed as additives in domestic products and as intermediates for industrial synthesis (Malhorta, 2016). Phenols are also used as constituents in dyes, polymers, drugs and other organic substances during production (Michałowicz & Duda, 2007). They have been found useful as an oral anesthetic in throat lozenges, sunscreens and water-proof sunscreen (Vermerris, 2008).

Phenolic resins are commercially made from polymerisation reactions involving phenol and formaldehyde. (Gattey, 2008). The resin that is obtained is known as phenol –formaldehyde resin, but in the market it is known as Bakelite. Due to its capacity to withstand high temperature levels and resistance to electricity and other chemicals, thus bakelite is commonly used in electric switches and automobiles. (Bakelizer, 1993). Moreover, phenol is also used in the study and extraction of biomolecules. Phenol has application in the extraction of nucleic acids from tissue samples in Molecular Biology techniques(Byju's, n.d.; Faurobert *et al*., 2007; Tan & Yiap, 2009). Medically, concentrated phenol liquids are widely applied to avoid a portion of the toenail from growing back in the surgical treatment of developed toe nails. This technique is called phenolization. (Odhiambo, 2014).

4-CP is used as an intermediate in organic synthesis of dyes, drugs, fabricating insecticides and for preserving wood; although the largest quantities of 4-CPare as a by-product from the paper pulp bleaching process, mainly because of the inherent properties associated to the chlorine presence (Muller & Caillard, 2011; Hernández-Fuerte *et al*., 2014).

2,4-Dichlorophenol is an important intermediate in the 2,4-dichlorophenoxyacetic acid production, the well-known industrial commodity herbicide. It is a chief element in the production of phenoxy acid herbicides and is also used in the synthesis of pesticide, antiseptics and pharmaceuticals (DAS, 2000; Park & Kisok, 2018).

2.5. Health effects of phenolic compounds

Unrestricted release of phenolic compounds into the environment could pose adverse effects on human health and the environment (Nakayama *et al*., 2005; Fair *et al*., 2010; Olak *et al*., 2012) . The route of exposure, bioaccumulation, bio-concentration, duration of exposure and rate of metabolism are among the factors that contribute to the potential health hazards (Abdel-Shafy & Mansour, 2016). Long-term health effects of exposure for phenolic compounds may include cataracts, kidney and liver damage,

and jaundice (Rengarajan *et a*l., 2015). Additionally, long-term exposure to low levels of some phenolic compounds has the potential to cause cancer in laboratory animals (EPA, 1992).

Phenolic compounds routes of exposure include inhalation, dermal and oral exposure; resulting in adverse health conditions such as cardiovascular disease, gastrointestinal damage of liquid products, skin damages and muscle twitching in animals (Vermerris, 2008; ATSDR, 2008a). A study by Baker *et al.* (2013) reported phenol poisoning in contaminated drinking water from a rural area in Southern Wisconsin USA, with an estimated intake of phenol in the range of 10 to 240 mg/person/day. Elevated levels of phenol in the human body have the tendency to lead to muscle tremors, difficulty in walking, and even death (EPA, 1992; Michałowicz & Duda, 2007). Application of phenolic compounds in consumable products in female may lead to adverse impacts on estrogenic activity and hormonal balance which may result in breast cancer in women (Vermerris, 2008).

4-CP causes health disorders such as nausea and vomiting (Kurniawan & Lo, 2007). When undiluted, it whitens & cauterizes the skin & mucous membranes (Hoover & John, 1976). Burning pain in mouth and throat has also been reported, with white necrotic lesions in mouth, oesophagus and stomach. Abdominal pain and bloody diarrhoea have also been reported, along with pallor, sweating, weakness, headache, dizziness and tinnitus. Shock, weak irregular pulse, hypotension, shallow respirations, cyanosis, and a profound fall in body temperature have been associated with phenol poisoning (Goshman, 1985; Sax & Lewis, 2007; Pohanish, 2011; Fanaie *et al*., 2016).

One can be affected by 2,4-Dichlorophenol via inhalation and dermal exposure. Direct contact may cause irritation and burning of the skin and eyes. “Inhalation of 2,4-Dichlorophenol may also irritate the nose, throat and lungs causing coughing, wheezing and/or shortness of breath. 2,4-Dichlorophenol may damage the liver and kidneys. Repeated exposure may affect the nervous system causing headache, dizziness, nausea, vomiting, weakness and coma.” (NJDOHSS, 2016; Park & Kisok, 2018).

2.6. National regulations and international guidelines

Standard guidelines and threshold limits for most carcinogenic compounds in water are still scanty in South Africa. The Department of Water Affairs and Forestry provide a general umbrella guideline of phenolic compounds for wastewater effluent and is set at 0.01 mg/L (DWAF, 1984). According to the screening criteria for Persistent Organic Pollutants (POPs) under the Stockholm Convention, phenolic compounds and their precursors have consequently been listed with restricted use worldwide (UNEP, 2006). Chlorinated phenols are part of a family of compounds damaging to the environment owing to their toxicity even at low concentrations. As a result, Regulatory bodies in some developed states have proposed standard guidelines for phenolic compounds and are listed in the US Environmental Protection Agency (EPA) priority list of pollutants and in the EU Directive 76/464/ECC relating to dangerous substances. The EPA has determined that lifetime exposure to 2 mg/L phenol in drinking water is not expected to cause any adverse effects. The American Food and Drug Administration

(FDA) has determined that the phenol concentration in bottled drinking water should not exceed 0.001 $mg.L^{-1}$.(ATSDR, 2008b)

Table 2. 1: Phenolic compounds included in priority pollutants list of the EC and US EPA (method 604 and 8041)

Commission of the European Communities (directive 76/464/EC)	
Commission of the European Communities (directive 76/464/EC)	
4-Chloro-3-methylphenol	2-Amino-4-chlorophenol
2-Chlorophenol	Pentachlorophenol
3-Chlorophenol	Trichlorophenols
4-Chlorophenol	
US EPA list of priority pollutants (EPA 8041)	
Phenol 2-chlorophenol 2,4-dichlorophenol, 2,4,6-trichlorophenol pentachlorophenol 4-chloro-3-methylphenol 2,4-dimethylphenol 2-nitrophenol 4-nitrophenol 2,4-dinitrophenol 2-methyl-4,6-dinitrophenol	

The standards of eleven phenols are regarded as major pollutants by the EPA and EU Directive 2455/2001/EC sets a maximum concentration of 0.5 µg/L in drinking water and their individual concentration should not exceed 0.1 µg/L (Fattahi *et al*., 2007; Santana *et al*., 2009). The U.S. EPA established that waters should be limited to 0.3 mg phenol per litre of water to protect human health from the possible harmful effects of exposure to phenol through drinking water and/or eating contaminated water plants and animals (Younis & Rafati, 2004). Below the 0.3 mgL^{-1} acceptable level, no harm has been noticed to aquatic life (Khalid, 2011). Also U.S. EPA recommends that the concentration of phenol in surface water (lakes, streams) should be limited to 3.5 $mg.L^{-1}$ (EPA, 2002). In the European Community, for each pollutant, the maximum acceptable concentration in drinking water is 0.1 $\mu g.L^{-1}$ (Khalid, 2011).

Table 2. 2: International Acceptable regulatory limits for 4-CP and 2,4-DCP

Compound	Regulation Body	Drinking Water Limit
4-chlorophenol	US-EPA	5.5 µg/L(EPA, 1990)

2,4-dichlorophenol	US-EPA	0.02 mg/L (EPA, 2018)
2,4-dichlorophenol	Environment Management Act, 1981 of British Columbia, Canada.	0.9 mg/L (Cananada, 1997)

The Drinking Water Standards of the United States Public Health Services recommended less than one part per billion of extractable total phenols in potable water. The WHO suggests a concentration of 0.04 $mg.L^{-1}$ for 2,4- DCP, the latter values were based on taste and odour (Brumsted *et al*., 1965; Pocurull *et al*., 1995; Khalid, 2011).

2.7. Analytical methods for phenolic compounds

Over the years, several techniques have been for the determination of phenolic derivatives in water including wastewater. They include spectrophotometry electrochemical method, capillary electrophoresis, gas chromatographic (GC) method using liquid-liquid extraction and either using flame ionization detection (FID) or derivatization and electron capture detection (ECD) to analyse various phenols at low concentration (Peñalver *et al*., 2002; Zhang *et al*., 2006; Saraji & Marzban, 2010; Gorla *et al.*, 2016). Gas chromatography/mass spectrometric (GC/MS) technique with liquid-liquid extraction (LLE) has been used to determine phenol at high concentrations (Gilala, 2010).

Qualitative and quantitative determination of phenolic compounds are crucial to estimate their levels in various matrices. However, the determination of phenolic derivatives in water or other matrices in the concentrations less than 1 ng/mL is a great challenge (Khalid, 2011). The extraction and pre-concentration of a mixture of phenols is difficult due to their relatively high polarity. Moreover, volatilization may cause losses in pre-concentration owing to their excessive vapor pressure levels. The conventional pre-concentration method is liquid-liquid extraction for aqueous samples (Faraji *et al*., 2009). Advantages of using the LLE include its simplicity, less time required, ease in downstream treatment and the usage of inexpensive equipment (Humbert *et al.*, 2014; Tanaka, 2015). However, LLE has several disadvantages, including emulsion formation, unique extraction efficiencies for different compounds, the usage of high volumes of solvent, slow and hazardous to human health as they involve extremely toxic organic solvents that are also significantly costly when it comes to their disposal (Stevens, n.d.; Lee & Hwang, 2000; Interchim, 2016).

2.7.1. Solid-phase extraction (SPE)

SPE, is an accepted technique that has been employed for increasing the concentration of a sample before analysis for numerous and various classes of compounds in a variety of matrices (Albanis & Danis, 1999; Bagheri *et al*., 2004). For enhancement and clean-up of aqueous samples and extraction from aqueous matrices, the SPE method was used (Chitongo, 2017). How the SPE works, a solution is exposed to a solid sorbent comprising of the analyte(s) of interest that specifically adsorbs the analytes onto the surface. The solids are isolated from the original solution after the sorption of analytes to the surface, and the analytes are eluted with an appropriate solvent from the sorbent.

(Hennion, 1999). Such a method allows; separation, qualitative and quantitative detection (Chitongo, 2017). The extraction technique has been established in the off-line and on-line mode. Both modes have benefits and shortcomings but the on-line approach is preferred due to advantages such as; "higher sensitivity, insufficient organic solvent and less manipulation of the samples, which leads to greater precision, and making it easier to be automated" (Hennion, 1999; Bagheri *et al.*, 2004; Feng *et al.*, 2009). SPE not only requires less sample and solvent, but also removes most of the interferences (Santana *et al*., 2009).

Solid-phase extraction (SPE) and solid-phase micro-extraction (SPME) procedures are regularly utilized more than conventional LLE procedures, hence, decreasing loss of analytes and the use of huge quantities of toxic solvents (Simões *et al*., 2007). Solid phase extraction stops majority of the issues experienced with LLE extraction and enhances quantitative recovery yields. This technique is fast (mostly <30 min), easy to perform and can be automated (Affinisep, n.d.; Stevenson, 2000; Poole, 2015). The downfall of SPE is that it is relatively costly because cartridges are usually thrown away after single use. Furthermore, organic solvents which could threaten health and the environment are still used in extraction (Khalid, 2011). On the other hand, Solid-phase micro extraction (SPME) methods have been extensively used for invasive and non-invasive *in vivo* studies. This technique does not require the use of organic solvents (Lee & Hwang, 2000).

Solid phase micro-extraction (SPME) has been designed to eliminate a few of the shortcomings of the SPE process (Santana *et al*., 2009). The target analytes investigated to date include environmental pollutants, pharmaceuticals, pheromones and metabolites; studies show the versatility and capability of this technique (Musteata & Vuckovic, 2012). The technique reduces prep time, procurement and disposal costs for solvents, and can also increase the LOD. It has been used in conjunction with GC) and GC/MS and has been effectively used in various of compounds widely, particularly for the extraction from water samples of volatile and semi-volatile organic pollutants.(Kataoka *et al*., 2000; Nilsson, 2000). With these methods, Typical detected levels of phenols are in the low µg/L range in river water and industrial effluents while in freshwater sediments they are between 1 and 100,000 ng/g (Petrović *et al.*, 2001).

2.7.2. HPLC-DAD

High-performance liquid chromatography (HPLC) is the most important analytic tool in modern science. Contemporary HPLC proposes quality resolutions which allows the quantitative determination of target analytes within complex matrices by its compatibility accompanied by a single or number of detector(s) (Ornaf & Dong, 2005; Gika *et al*., 2016). High-performance liquid chromatography (HPLC) is offered as another alternative process compared to direct measurement techniques (Ko *et al*., 1977; Mischke & Wickstrom, 1980; Tamaoka & Komagata, 1984). Chromatography has an obvious advantage in that it enables the foundation composition to be determined directly. Therefore, errors can easily be predicted and identified (Mesbah *et al*., 1989).

Presently, HPLC is the famous and dependable technique to analyse phenolic compounds, it is regarded as a powerful tool in analysis (Clark, 2016). Analyte detection often use ultra-violet (UV), electrochemical and colorimetric detectors. HPLC is an extremely upgraded form of column chromatography. The solvent is pushed through under high pressures of up to 400 atmospheres (Moreno-Arribas & Polo, 2003; Ötleş, 2008; Clark, 2016). Thus it makes it possible for the column packing material to employ a much smaller particle size that enables an even larger surface area for interactions between the stationary phase and the molecules that move past it. This makes it easier to isolate the components of the mixture better. (Koester, 2006; Omics, 2014; Pooya, 2017).

HPLC makes use of diode arrays for the recording of the absorption spectrum of samples when ultraviolet and visible light passes through them. This enables the gathering of qualitative information about the samples in question (Mizell, n.d.). Diode-Array Detection (DAD) is an analytical technique that can be employed during an HPLC separation to assess the purity of an analyte or associated peak eluting impurity. (Andi, n.d.). A diode array detector (DAD) may simultaneously scan samples at different wavelength as well as provide data about specific spectral characteristics for compound identification, with a lower sensitivity than that of UV quantification detectors. (Zhang *et al*., 2013). Diode Array detectors are most widely used in HPLC systems to record the ultraviolet and visible absorption spectrum (UV-vis) of samples. Pharmaceuticals and life sciences, chemistry, energy and petrochemical sectors, the environment and agriculture are applications for DAD. The capacity to pick the best wavelength for analysis is an advantage of DAD. Resolution, wavelength range, NIR (Near InfRed) ranges, low noise, baseline stability, peak integration and an interchangeable flow cell design are some features to consider when selecting a DAD. (Chromatography Online, n.d.; Mizell, n.d.; Swartz, 2010; Quimica, 2010).

The HPLC technique has been proven to be the most appropriate for the analysis of many organic compounds including phenols. This is due to their structural similarity and diversity, resulting in sufficient precision, selectivity and analysis within a reasonable time. HPLC systems are typically hyphenated with ultraviolet visible (UV), photodiode array (DAD), mass spectrometry (MS), fluorescence, chemiluminescence, refractive index; evaporative light scattering detectors has been the best method of choice for routine analysis of phenolic compounds in most hitherto published studies (Zhang *et al*., 2013).

2.8.0. Ecological health risk assessment

2.8.1. Water toxicity testing

In toxicity assessment of chemicals, humans are regarded as the best breed to conduct the test on because the most precise extrapolation of animal information in conjunction to humans may not be assured since there is an interspecies differences in anatomy, physiology and biological chemistry (Gallagher, 2003). The conventional acute toxicity test, entails the usage of multitude of animals therefore it is being

substituted by other different methods. The methods require smaller quantity of animals or alternatively other ways that require not the usage of animals to be employed (Erhirhie et al., 2018).

Toxicity tests, are a way to try measure the toxicity of a sample by analysing the outcome of the exposure that is produced by the standard test organisms (Kroll, 2009). Toxicity tests are grouped according to the test period, life stage, and final outcome. Minute, short-term tests run for 48- or 96-h on exposures and also quantify the death rate to determine the middle or average value of lethal concentration (LC50), i.e. the exposed test population dies at the concentration of 50% (Anderson & Phillips, 2016). With the toxicity testing, the control compares the response of the organism exposed to a particular chemical in different concentrations to that unexposed (Kress, 2019).

In this study, toxicities of the selected phenols in water and effluents samples on aquatic organisms were assessed. Three aquatic organisms (each representing a trophic level) were used for ecotoxicity testing. *Daphnia magna*, a primary consumer that feeds on algae, *Raphidocelis subcapitata* (a primary producer) and *Tetrahymena. thermophila* a protozoan (decomposer) were exposed to water samples, water contaminated with phenolic compound cocktails and WWTP effluent. The Ames test uses the bacterial strain *Salmonella typhimurium* to assess the possible carcinogenic effect of chemicals (Jain et al., 2018).

2.8.2. Daphnia magna

Daphnia magna, a tiny planktonic crustacean is classified under the subclass Phyllopoda, the adult length can grow from 1.5-5.0 mm. It is present in numerous of freshwater environments (acidic swamps, rivers resulting from snow runoff) that is enormously dispensed all over the Northern Hemisphere and also South Africa (Elenbaas, 2013). They form a vital link to the food chain thus, are easily affected by toxic substances; have short life span, multiply quickly, adjust easily in laboratory condition, can be cultured in a confined space, moreover, the time to measure the outcome is relatively quick (Adema, 1978; Tyagi *et al*., 2007).

In toxicology, *Daphna magna* is used for the monitoring of wastewater treatment systems, quantifying the quality criteria for the determination of permissible concentrations of pollutants, determining the maximum impurity from water in natural effluents, and to find the effectiveness of a substantial sanitation method. This well-known bioassay is used world-wide for toxicity assessment of chemical compounds and the monitoring of industrial effluents (Persoone *et al*., 2009).

This test is useful in an analytical tool for screening of chemical analysis and early warning system to monitor the different operational units of wastewater treatment plants

When evaluating the performance of a wastewater treatment plant (paying special attention to toxicity reduction), the use of *D magna* is vital for the study of possible adverse effect of treated wastewater on the receiving aquatic system. It may assist in detecting environmentally realistic concentrations of pollutants, distinguish separate levels of toxicity and most importantly, the toxicity of the compounds in question. To assess the types of operational units of wastewater treatment plants, this particular test

is useful because it is used as an analytical subject of screening for chemicals. Moreover, it can be used as an early warning system (Tyagi *et al*., 2007; Koçbaş & Oral, 2015).

2.8.3. Raphidocelis subcapitata

Raphidocelis subcapita is a microalgae that is a dominant group of green algae, it displays a c-shape with a twisted appearance, like a sickle. It has a length of 8-14µm, and a width between 2 and 3µm (Heijerick *et al*., 2002). Irrespective of the organism's popularity, the information regarding the organism's cell biology-such as the design of nuclear and cytoplasmic separation at mitotic stage-is minimal. Presently, it's unsurpassed and most constantly used ecotoxicological biological indicator species because; of its high growth rate, sensitivity to toxicants, and good reproducibility in comparison to other types algae (Yamagishi *et al*., 2017a). As a biological measure species, it uplifts the measure of nutrients, toxic substances in freshwater habitat with its sensitivity to the presence of toxic substances and its ubiquitous distribution thus making it ideal for bioassays in toxicological risk assessments due to its elevated growth rate and sensitivity to toxicant. Moreover, it is most importantly natural food for aquatic organisms thus making it an important organism for toxicant analysis (Yamagishi *et al*., 2017b; Silva *et al*., 2018; Suzuki *et al*., 2018).

The motive of the test being conducted is to examine the effects of a particular or certain substance in question in which it might have on the growth of the fresh water microalgae. Substantially growing test algae is often introduced to the test substance in batch cultures over a 72 h period. Despite the relatively short test duration, effects over a number of generations can be determined. The way that the test works; there's a decrease of growth in a serial of algal cultures introduced to levels of different concentrations of a test substance if the substance is toxic to the organism(Crawls, 1996; Heijerick *et al*., 2002). The reaction is determined by how the test organism being exposed to the different levels of the concentration is, compared with the average growth of duplicated, unexposed control cultures. For optimal sensitivity of the systems response to toxic effects, the test organism is permitted unrestricted rapid growth under sufficient nutrient conditions and unlimited light for the required period to quantify reduction of the particular growth rate. The amount of growth is measured from the quantification of the algal biomass solidity as a function of time (OECD, 2002).

2.8.4. Tetrahymena thermophila

An unrestrained living single-cell under the ciliated protozoa, is an ecologically triumphant clade of unicellular eukaryotes. *T. themorphile* have a pear shape and is proximately 30 × 50 µm, the cell thereof is covered by multiple layers of cortex, a bit inflexible and ordered into 15-25 vertical ciliary rows of cortical parts that contain basal bodies in most parts. These are then accompanied by the cilia that allows movement motility of the cells (Juganson, 2018).

Natural habitats for *T.thermophila* are; freshwater lakes, ponds or streams that have vegetation. Their choice of habitat is at the base, closer to degrading vegetation and bacteria where the water temperature

exceeds 13 °C, further research as to how this specie acclimatizes in temperatures lower than 13°C is yet to be conducted (Orias *et al*., 2011; Doerder & Brunk, 2012). Ciliated protozoa are plentyful units in the aquatic ecosystems and play a significant role in the trophic mediatory of the microbial and macrobial parts of the aquatic food webs, revitalizing of nutrients, regulating of bacterial mases and the self-cleansing of the natural aquatic ecosystems (Gilron *et al*., 1999). *T.thermophila,* are known to being used for biological and chemical, genetic and toxicological investigations for over a period of time. They are easily grown axenically in inexpensive medium and will generally achieve sufficient density for experimentation overnight (Pinheiro, 2007). Moreover, *T.thermophila* gives an indication of a well-nourished aquatic environment and has been found to have a role in the activated sludge process (Pauli *et al*., 1993; Juganson, 2018).

2.8.5. Ames mutagenicity

Mutagenicity is known to be one of the traditional toxicological outputs in determining water quality control in the drinking water production process (Zwart *et al*., 2018). Contaminated sources for drinking water production, could bring in mutagens during the production process. Industrial and urban effluents are therefore known to act as potential mutagens (Heringa *et al*., 2011; Zwart *et al*., 2018). Therefore, it is of paramount importance to quantify and identity mutagens in drinking, surface, ground and effluent water, this is in attempts to monitor their discharge in the environment.

The "*Salmonella typhimurium* reverse mutation assay" (Ames Test) is a bacterial short period test to identify carcinogens using mutagenicity in bacteria as an output (Hengster & Oesch, 2001). The test was developed to determine the mutagenicity potential of a chemical; with the assumption that a mutagenic compound may also be carcinogenic (AWQC, n.d.). *S. typhimurium* reverse mutation assay relies on the capability of a mutagenic chemicals to give rise to a reverse mutations in the *S. typhimurium* strains that are defected in the histidine biosynthesis pathway, and are dependent on the histidine for growth, and will not multiply when there is no histidine (Gupta, 2016). The different types of bacterial strains used for the mutagenicity tests are presented in Table 2.3.

Table 2. 3: Bacteria strains commonly used for mutagenicity testing

Strain	**Standard mutagen**	**Type of Reversion mutation**	**Comment**
Salmonella typhimurium: Histidine dependent			

TA97a	Frameshift	9-AA or ICR191	Derived from TA1537, along with plasmid pKM 101 that encourages error-prone DNA repair enzymes to increase sensitivity
TA98	Frameshift	2-NF	Derived from TA1538 and along with plasmid pKM 101which encourage error-prone DNA repair enzymes to increase sensitivity
TA100	Base-pair substitution, oxidative	NaN_3	Derived from TA1535 along with plasmid pKM 101 which encourage error-prone DNA repair enzymes to increase sensitivity
TA1535	Base-pair substitution, oxidative	NaN_3	uvrB repair deficient, rfa mutation increases permeability to mutagens. Sensitive to 3 unique mutagens compared to TA100 (acetaldehyde oxime, 6-mercaptopurine and 1,3-butadiene)
Escherichia coli: Tryptophan dependent			
E.coli WP2 uvrA	Base- pair substitution	4-NQO	uvrA deletion mutation eliminates accurate excision repair mechanism.
E. coli WP2 uvrA (pKM101)	Base- pair substitution	4-NQO	uvrA deletion mutation removes precise deletion repair

			mechanism. Contains plasmid pKM 101 which encourage error-prone DNA repair enzymes and increases sensitivity

(EBPI, 2019)

An individual tester strain comprises of a dissimilar kind of mutation inside a histidine operon (AWQC, n.d.; Levin *et al*., 1982; Gee *et al*., 1994). Because of the mutation, the tester strain is unable to create colonies on agar with or without low histidine constituents. When a mutation is brought about in the histidine needing strain, to give rise to a histidine-independent strain, it will obtain the capability to create colonies on very little agar (Hengster & Oesch, 2001).

Contrary to mammals, this kind of bacteria has insufficient ability to oxidate the enzyme systems to metabolize foreign compounds to electrophilic metabolites which have the ability to react with DNA. Therefore, with the post-mitochondrial supernatant, called 'S9' or 'microsome fraction' (that is made from livers of mammals, usually rats), the bacteria are treated with the test compound. How the S9 activity metabolizes; it's intensified by treating the rats with a very strong inducer of drug-metabolizing enzymes prior killing it, and their livers are removed. Thereafter, the S9 is buffered and accompanied with the crucial co-factors 'NADP' and glucose-6-phosphate to make the 'S9-mix'. This test can therefore be utilized to distinguish mutagenic effects of particular test materials, combinations and extracts (Hakura *et al*., 2005; Carr, 2014; Samiei *et al*., 2015; Vijay *et al.*, 2018).

In this study, two bacterial strains were used: the TA98 bacterial strain and the TA100 bacterial strain. The T98 bacterial strain involves a frameshift mutation. This is a kind of mutation entails the 'insertion or deletion' of a nucleotide where a number of deleted base pairs are not dividable by three. Being able to be divided by three is crucial because the cell only reads a gene in sets of three bases. Each set of three bases identifies with one of the 20 various amino acids used to make a protein. On condition that a mutation disrupts the reading frame, the entire DNA order following the mutation will be read incorrectly (Streisinger *et al*., 1966; Roth, 1974; NIH, n.d.). The TA100 'base-pair substitution' involves a kind of mutation that has to do with the replacement or substitution of a singular nucleotide base with an alternative DNA or RNA molecule. On condition that this mutation occurs in the encouraged order of a gene, the outcome may be evident because the expression of the gene may change (Griffits, 1998; Biology online, 2001; Sturum, 2019; NIH, n.d.). These bacteriological strains are binary generic strains of *S typhimurium* evaluated in Ames testing. Both strains possess; rfa mutations, a defective lipopolysaccharide layer which allows the bacteria to be more pervious to bigger molecules; uvrB mutations, which removes completely the excision repair of DNA damage and the pKM101 plasmid (which increases error-prone repair of DNA damage) (NIH, n.d.).

The EPBI Muta-ChromoPlate^{TM} EBPI (2019) was used to test for mutagenicity of the Stellenbosch WWTP effluent, bottled water and tap water samples. The test makes use of a 96-well microplate of the *Styphimurium.* Ames *et al* (1975) created a test, to examine mutagenic substances in "water soluble extracts of sediment, air, chemicals, food components, cosmetics, wastewater and potable water". The core purpose of this bacterial reverse mutation test is to detect mutations which reverse mutate on the test strains and refurbish the core ability of the bacteria to produce an essential amino acid. The revertant bacteria also has the ability to multiply in the absence of the amino acid needed by the source test strain.

The strain *S. typhimurium* TA98 was used to screen the effluent, tap water and bottled water samples. A minimal medium containing histidine and biotin was allowed for few cell divisions. Bacteria were exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. The T100 strain was also used to screen the selected samples in question. With the T100 strain, bacteria were also subjected to the test substance both in the presence and absence of a suitable metabolic activation set up. The S9 liver homogenate from the male 'Aroclor 1254-induced Sprague Dawley rats' were included because testing a compound for mutagenicity both with and without metabolism expands the detection capabilities of the assay. This is essential for an accurate assessment as mammals exhibit extensive metabolic capabilities in vivo. Both direct and indirect mutagens could be detected in the presence of S9 in the test design. If samples had twice the number of reverse mutations compared to the background mutation rate, then the samples were considered mutagenic.

2.9. Human health risk assessment

Risk assessment involves the order of assessing the prospective effect of a 'chemical, physical, microbiological or psychosocial hazard' on the identified human populace or ecological organization under a particular set up and for a particular time frame (Carpenter , 1995; Australia, 2012; Ahmed, n.d.). Risk assessment is a strict course for assessing the size, possibility, and doubt of environmentally persuaded health effects (Primates, 2003) . It has been indicated as a process to combine scientific data regarding substances that could pose a hazard to the decision-making process in a way which human exposures are monitored (Olujimi *et al.*, 2010).

According to Willet in 1901, he defined risk assessment as the actualized doubt concerning the occurrence of an unsort after event (Suter, 1993). Opinions decades later extended marginally, defining risk assessment as the likelihood of an unwanted effect, expressed in the framework of linked uncertainties (USEPA, 1998). Moreover, risk assessment is defined as a period that represents the wholistic process or method of identifying the hazards and risk factors that may potentially cause harm, this is regarded as 'hazard identification.' Analysing and evaluating the risk linked with that hazard and determining the suitable forms to avoid the hazard. More so, alternatively controlling the risk when the hazard cannot be removed, this is referred to as 'risk control' (CCOHS,2020).

Human health risk assessment is a form of assessing the possible effect of a hazard on the well-being of an individual or a society. Methodological data is utilized to comprehend the possible health consequence. Every human health risk assessment is different to the circumstance and people being evaluated.

There are five broad steps in the human health risk assessment process namely:

1. Issue identification: assessing and identifying the problem or situation
2. Hazard assessment: identifying conceivable adverse health effects related with hazard.
3. Understand the dose-response relationship(s): identify the dose response relationship for a particular identified hostile health effect.
4. Exposure assessment: create a circumstance model linking ways to the sources of each hazards to individuals or population.
5. Characterise the risk: from the above step, the data collected is then used to determine the health risk of either past, present or future risk(s) for individuals or communities. (Primates, 2003; Olujimi, 2012; USEPA, 2016).

The disquiet subsequent arising from the potential exposure to pollutants was the opening idea to improve methods in order to assess the outcomes they have on the environment and human health. The average daily dose, hazard quotient and cancer risk values were utilized in producing anticipated no effect concentrations from environmental exposure for human health from drinking water and wastewater treatment plant effluent.

CHAPTER THREE

3. METHODOLOGY

3.1 Phenolic compounds analysis

3.1.1. Choice and cleaning of glassware

The selection of materials used for the investigation of target analytes was carefully considered. For instance, the choice of amber glass bottles for sample collection, the use of glassware instead of plastic and high purity solvents were necessary measures required for the development of a reliable analytical protocol. All glassware was thoroughly washed with Sunlight dishwashing liquid detergent and rinsed properly with tap water. Then, rinsed thoroughly with distilled water and left to drain and dried placed upside down overnight, prior sampling day.

3.1.2. Chemical and standards

The chemicals, materials, and reagents were purchased from a trusted vendors and suppliers (Supelco and Sigma Aldrich). The standards for the toxicity were purchased from MicroBioTests, Belguim and were prepared according to UCI (2003) and used according to manufacturer's instructions. Phenolic compounds were obtained from (2,4-dichlorphenol (99 %) and 4 chlorophenol) Sigma Aldrich (South Africa).

C 18-E cartridges consisting of 500 mg/12 ml of adsorbent (Seupelco, South Africa)

and a newly launched kinetex C18-100A column (150 mmx4.6 mm i.d., 5µm particle size) was used.

3.1.3 Instrumentation and chromatographic conditions

High performance liquid chromatography (HPLC) manufactured by Waters Corporations (United State of America) includes a terminal solvent delivery system, an auto sampler and photodiode array detector attached to an analytical workspace was utilized for the identification and separations of the phenolic compounds. Separations were achieved using Sulpelco C18-E column (25cm x 0.46 cm i.d) and the elution of the compounds, using binary gradients, were optimized.

Compound identification was conducted against the retention time values and the UV-spectral of the target analytes. A gradient mobile phase of Milli Q water ("A") and 0.1 % phosphoric acid, acetonitrile and 0.1 % phosphoric acid ("B") was used for the chromatographic separation flow-rate of 1.0 ml/min." Detection was conducted at 280 nm for all the target analytes. The chromatographic system was conditioned by allowing the solvents through for 30 minutes so that a stable baseline signal was obtained. Once the chromatographic system was conditioned with mobile phases, the chromatograms were obtained by injecting 20 ml of the standards and analytes in question (while the temperature was maintained at 25°C).

3.1.4 Identification and quantification of Phenols

Compound identification was conducted through the comparison of the retention time values and UV-spectral of the target analytes.

3.1.5 Description of study area and sampling protocol

The research was performed in Cape Town in the Western Cape Province, South Africa. Water samples were collected from taps and four brands of bottled water were procured. Effluents from Stellenbosch WWTP (Figure 3.1) were collected in autumn and winter months. Stellenbosch WWTP previously used together the activated sludge bioreactors and fixed medium (stone) trickling filter systems to treat the influent. The influent is primarily domestic waste and largely industrial component. Due to the aging system, the function of the system was poor, therefore a need to upgrade was necessary for the sake of increasing both the capacity and efficiency. Currently, the plant has upgraded to using the membrane bioreactors for wastewater treatment, this is a combination of a suspended growth biological treatment method and a membrane filtration equipment (the membranes are utilized as a function to critically perform solid-liquid separation function) (Olujimi, 2012; AMTA, 2016; Nqombolo *et al*., 2016). During the rainy winter period (June to August), large cold fronts come from the Atlantic Ocean with strong precipitation and strong north-westerly winds. The average minimum and maximum temperatures during the winter are 7°C and 17.5°C, respectively. The autumn begins in March and ends in May. During this period, a typical autumn will have an average temperature of 20ºC in March and 18ºC in April (Cape Town Magazine, 2018).

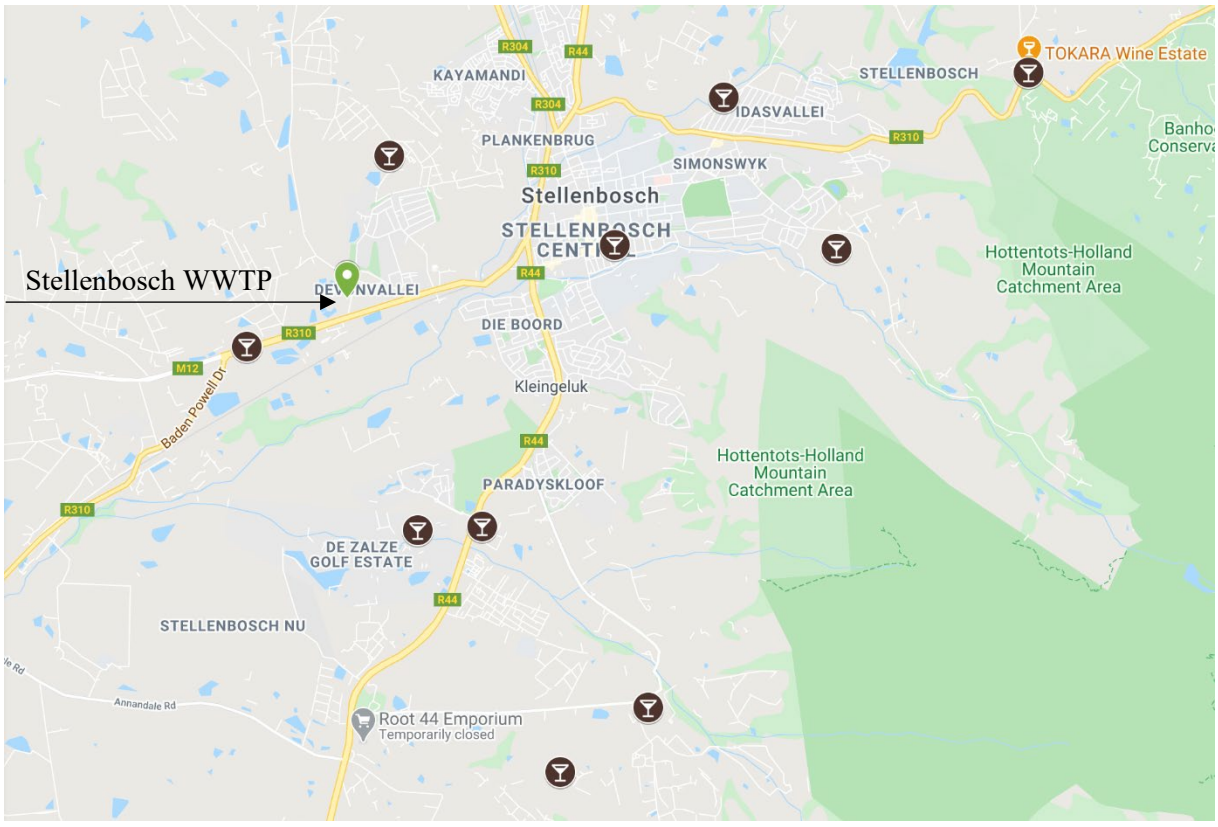

Figure 3.1: Map showing Stellenbosch Wastewater Treatment plant surrounded by wine farms (Google, 2021).

3.1.6 Sampling procedure and storage

Tap water samples were collected from the Cape Peninsula University of Technology, Bellville Campus laboratory and four brands of bottled water were bought from local grocery stores. Samples were collected in sterile 2,5 L amber bottles from the sampling stations at the Stellenbosch WWTP and preserved in the ice chest to maintain the integrity of the samples. To minimize water quality changes between sampling and analysis, the samples reached the laboratory within 24 h, refrigerated at 4^0C in the laboratory and were analysed within seven days. Water samples were collected in two replicates from the WWTP including one blank sample that served as the control sample. Water samples were filtered by passing through 0.22 µm polyethersulphone membrane syringe filters to remove possible debris and particles before storage in the refrigerator (at 4 ^{0}C) in the laboratory.

3.1.7 Phenol extraction materials and chemicals

For the filtration of the effluent prior extraction process, 0.22 µm filter was required. This was to avoid any blockages in the SPE. For the conditioning of the cartridges; 8.5 ml n-haxane:acetone (50:50 v/v), 8.5 ml methanol and 15 ml Milli-Q purified water, 5ml and 10ml glass pipette were required, along with the pipette bulb. C18-E cartridges for the extraction of the analyte was used along with hydrochloric acid for the adjusting of the pH and the nitrogen to blow to dryness the analytes under gentle pressure. The vacuum and pump were used for the analyte extraction process environment and sample filtration along with the vial glass for the collection of the analyte of interest. A hot plate was used to assist in the process of drying effectively.

3.1.8 Extraction procedures and analysis for phenols in water

Prior to extraction, collected samples were passed through 0.22 µm nylon filters to remove possible suspended particulates present in the water to avoid the clogging of the SPE cartridges (in the exception of bottled water). The pH of the water samples was reduced to a pH of 2.5 with hydrochloric acid prior to channelling it through the conditioned cartridge. Water samples were spiked with a mixture of phenolic standard with a known concentration. After allowing the samples to go pass the cartridges, 5 ml of Milli-Q water was passed through and left on the vacuum manifold for 30 minutes to dry (-70kPa). Thereafter, the desired analyte was held back then eluted with 3.5 ml of methanol, 3.5 ml of n-hexane: acetone (50:50 v/v) into a glass flask respectively. Following this, it was blown to dryness using a gentle flow of nitrogen. Aliquots from the solution were analysed by direct injection into the HPLC system in single injection. Recovery studies will be conducted as well, while the concentration of the target analytes were determined by external calibration standards.

3.1.9. Quality assurance and quality control

To ensure the quality of data and the accuracy and precision of results in the study, the following quality assurance steps was taken into consideration:

- Analytical grade reagents, distilled/deionized/milli-Q water was used to control external contributions.

- Reduction and correction for external contributions through analysing of blank samples.
- Recovery studies to provide information on the degree of possible error and accuracy efficiency of the results obtained.
- Analysis of control samples to ensure instrument consistency.
- Analytical methods and instrumentation were chosen based on their detection limit, sensitivity of methods and supervision advice.
- Strict adherence to recommended standard methods during sampling, sample handling, preservation, and analysis.
- Assessment of reproducibility of analytical procedures by analysing samples in triplicate.
- Prevention of sample contamination and maintaining sample integrity during and after sampling.

3.2 Toxicity assay materials and methods

3.2.1 *Daphnia magna* acute toxicity testing

This screening bioassays was developed by Prof. Dr. G. Persoone and his research team at the Laboratory for Environmental Toxicology and Aquatic Ecology at the Ghent University in Belgium. This particular test was done according to the prescribed test procedures national and international organizations (OECD and ISO 6341). The test kit was bought from the MicroBioTests Inc. (ISO, 2012a).

3.2.1.1 Exposing the standard freshwater to air and hatching of the ephippia

The freshwater water was pre-aerated over night by leaving it uncapped for hatching (done 3 days prior the toxicity test) for dormant eggs and for the preparation of the toxicant. The important aspect to remember when hatching the ephippia was to incubate for 72h, at 20-22°C under continuous illumination of 6000 lux (minimum).

3.2.1.2 Preparation of the toxicant dilutions

According to 1: 1 serial dilution with standard freshwater, a dilution sequence of the effluent sample was prepared. Five 100 ml standardized flasks marked from Concentration 1 to Concentration 5 (with Concentration 1 being 100% effluent to Concentration 5 being the maximum dilution). From Flask C1, filled with 100 ml of effluent, Concentration 2-Concentration 5 filled with 50 ml standard freshwater was diluted respectively according to table 3.1.

Table 3. 1: Dilution series of the effluent

Flask	**Effluent concentration**
Concentration 1	100%
Concentration 2	50%
Concentration 3	25%
Concentration 4	12.5%
Concentration 5	6.25%

3.2.1.3 Pre-feeding of the neonates and filling of test plate

The neonates had previously been fed (with a spirulina microalgae suspension) 2 h before. Subsequently, 10 ml of dilution water was poured into the control wells and 10 ml of the respective toxicant concentrations were poured into each well in the respective rows, according to cumulative toxicant concentrations.

3.2.1.4 Transferring neonates into test wells

For better visibility, a light table was used to move strictly 5 neonates from the individual rinsing well into the 4 wells of each row. This transition was carried out in the order of rising test concentrations. On completion, the parafilm strip was used to cover the multi-well plate to seal it tightly. The multi-well was then incubated at 20°C, in darkness.

3.2.1.5 Recording of the outcomes

After 24 h and 48 h incubation, the multi-well plate was scored to determine the number of dead or immobilized daphnids under the light table. The neonates found to not be swimming after making small movements of the liquid were considered immobile even if they could move their antennae. Mortality was recorded and data generated was analysed using the ToxRat Professional 3.2® Software for the determination of mortality, statistical significance and critical concentrations .

3.2.2 *Raphidocelis subcapitata* growth inhibition test

With *Raphidocelis subcapitata* de-immobilized from algal beads, a 72 h algal growth inhibition test was performed. The OECD Algal Growth Inhibition Test and the ISO (ISO Standard 8692) 'Water Quality - Freshwater Algal Growth Inhibition Tests' were used to conduct the algae test (ISO, 2012b).

3.2.2.1 Materials

Two tubes of *Raphidocelis subcapitata* microalgae in a form of small beads were used. A glass with a specific matrix to disperse the content in the microalgae. A matrix dissolving medium and 5 bottles of concentrated solutions of various chemicals for the 2L of algal culturing medium. Two sets of 18 disposable cells (and two additional cells for zero calibration of the Jenway 6300 spectrophotometer and scoring of the optical density of the concentrated algal suspension) and two plastic strips to be slid in between the cells and their lids to allow gaseous exchange.

3.2.2.2 De-immobilization of the algae and preparation of the concentrated algal inoculum

The liquid was poured out from the algal beads. 5 ml of the "Matrix dissolving medium" was transferred into the glass vial, capped and shook vigorously up till the matrix immobilizing the algae disintegrates completely. The glass vial was centrifuged for ten minutes at 3000 rpm. The supernatant was poured out and replaced by 10 ml deionized water, then, capped and shook aggressively to redistribute the algae

evenly. Again, the glass vial was centrifuged at 3000 rpm for 10 minutes. then discarded the liquid medium. Finally, the algae cells were re-suspended in 10 ml algal culturing medium, then transferred directly to a 25 ml calibrated flask and finally toped with the algal culturing medium to make the 25 ml mark. 25 ml algal culturing medium was filled in the long cells labelled "Calibration long cell" and "Algal Stock cell" for zero-calibrating the Jenway 6300 spectrophotometer. The algal suspension was then transferred into the Algal Stock cell for the reading of the optical density (OD) in the Jenway 6300 spectrophotometer. The algal suspension was transferred from the Algal Stock cell into a 100 ml flask and added with the volume of algal culturing medium for the optical density value estimation.

3.2.2.3 Arranging for serial dilution

It was of utmost importance to ensure that the samples are well filtered through a 0.45 µm membrane filter of to eliminate turbidity. Six 200 ml calibrated flasks labelled, Concentration 0 to Concentration 5; Concentration 0 -the control, Concentration 1- non-diluted effluent and "Concentration 5" the most diluted as indicated in table 3.2. Flask labelled "Concentration 1" was filled with filtered effluent and added 2 ml of nutrient stock solution "A" and 0.2 ml of solutions "B-D". 100 ml algal culturing medium was put in flasks Concentration 0, Concentration 2, Concentration 3, Concentration 4 and Concentration 5. 100 ml of the contents of flask Concentration 1 was transferred into flask Concentration 2 to make up the first 1:1 dilution (50% effluent) and shaken thoroughly to mix the contents. The operation was repeated for flasks Concentration 3, Concentration 4 and Concentration 5 as indicated in table 3.2. From flask Concentration 5 100 ml of the solution was removed and discarded. Then, 1 ml of the 1.10^6/ml of the algal suspension was added into each flask, in order to obtain an initial algal concentration of 1.10^4/ml in each effluent flask.

Table 3. 2: Dilution series of effluent

Flask	Effluent concentration
Concentration 1	100%
Concentration 2	50%
Concentration 3	25%
Concentration 4	12.5%
Concentration 5	6.25%

3.2.2.4 Pouring of algae-toxicant dilutions into respective test vials and its incubation thereof.

25 ml of the algae toxicant were poured respectively in each flask into the subsequent 3 (a, b, c) long cells (five different dilutions-three for each cell which makes 18 cells), the lids were lifted slightly to allow the plastic strip in for the purpose of gas exchange. The cells were incubated for 72h with a uniform illumination provided by cool white fluorescent lamps . The incubator was controlled to room temperature of 23°C for the duration of the three-day test period, to achieve an acceptable algal growth during the 3-day test period.

3.2.2.5 Scoring of the results

Measurements for the OD at 670 nm of the algal growth comparative to the control were measured daily at; 24 h, 48 h and 72 h period. Everyday the outcomes for each cells were written on the results sheets and generated through ToxRat software for interpretation.

3.2.3 *Tetrahymena thermophile* growth inhibition test

The protozoan growth inhibition bioassay derived from the research group of Dr. W. Pauli at the Institute of Biochemistry and Molecular Biology, Free University of Berlin, Germany. The method followed to conduct this experiments is in accordance with the Protoxkit F, 1998.

3.2.3.1 Materials

- 3 ml of living *Tetrahymena* suspension (kept in ambient temperature)
- 6 sterile, disposable syringes- to draw a small amount of stock culture
- Six small tubes of food substrate
- Six small tubes with reconstitution solution for food preparation
- Six disposable 1 cm polystyrol spectrophotometric cells of 1.5 ml contents to determine ciliate density
- Six 5 ml disposable tubes to dilute stock culture
- Eighty disposable 1 cm polystyrol spectrophotometric cells
- Two cell holders in cardboards
- Sheets for data scoring

3.2.3.2 Planning for serial dilution

Serial dilution was arranged according to Table 3.3. 15 ml glass tubes were labelled Concentration 1 to Concentration 5. Concentration 1 contained the undiluted effluent, Concentration 5 the highest dilution. 10 ml of the original concentrated sample was poured in tube Concentration 2 and filled with 5 ml distilled water each. Thereafter, 5 ml were transferred from Concentration 1 to Concentration 2 and mixed and repeated respectively from Concentration 2- Concentration 5.

Table 3. 3: Dilution series of effluent

Flask	**Effluent concentration**
Concentration 1	100%
Concentration 2	50%
Concentration 3	25%
Concentration 4	12.5%
Concentration 5	6.25%

3.2.3.3 Preparation of ciliate inoculum

The stock culture was shaken gently to distribute evenly the contents. A 500 µl culture was drawn from the ciliate stock culture and transferred into a 1.5 ml stock-culture cell and topped with 1 ml distilled water. The stock-culture cell was then closed, shaken tenderly and the "OD" was measured at 440 nm. The dilution factor required to accomplish a 'theoretical' OD value of 0.040 was calculated using the formula:

F = ODvalue / 0.040 Equation (1)

V = 0.5 x (F-1)

500 µl of the diluted ciliate stock was transferred into the ciliate inoculum tube and Added with V ml distilled water and mixed gently.

3.2.3.4 Preparation of the food suspension and inoculation of the test cells

In the food substrate tube, the vial with reconstitutive medium was mixed. Twelve test cells were labelled in pairs from Concentration 0 to Concentration 5, 2 ml distilled water was added into the two control cells (Concentration 0). Then, 2 ml from the dilution tubes, 'Concentration 1 to Concentration 5' was added to Concentration 1 to Concentration 5 test cells respectively. The food substrate tube was mixed properly with the substances, then, 40 µl food suspension was added to every 12 test cells. The ciliate inoculum tube was then homogenized with the contents. 40 µl was transferred into all twelve test cells.

3.2.3.5 Measuring of the Optical Density and incubating the test cells

For the zero calibration of the spectrophotometer at 440 nm, a test cell filled with 2 ml distilled water was inverted a few times, then inserted in the spectrophotometer. The OD of every test cell was measured and recorded at 440 nm. At the end of the first optical density reading (T0), cells were returned in their holding tray and incubated (in darkness) at 30 °C for 24 h. After a 24 h incubation, the measuring equipment would be recalibrated using a test cell filled with 2 ml distilled water. Then, each cell was gently shaken, the OD determined and recorded at 440 nm (T24, day after experiment).

3.2.3.6 Scoring of results

The OD was determined and recorded at 440 nm at T0 and T24. Every day the outcomes for every cell would be recorded on the results sheets and generated through ToxRat software for interpretation.

3.3 Human Health Risk Assessment

3.3.1. Mutagenicity potential testing (Ames test)

Reagents :

'A': Davis-Mingoli salts 22 mL

'B': D-glucose 10 mL

'C': Bromocresol Purple 7 mL

'D': D-Biotin 4 mL

'E': L-Histidine 200 mL (WP2 strains substitute L-Tryptophan, 100 µL)

'F': Sterile distilled water, 120 mL

'G': Growth Medium 5 mL

'V': Ampicillin 100ml

Table 3. 4: Composition of S9 Mix

S9A :$MgCl_2$ + KCl solution	0.96 mL
S9B: Glucose-6-phosphate	0.22 mL
S9C: NADP	1.94 mL
S9D: Phosphate buffer	23.96 mL
S9E: Sterile water	20.32 mL
9F: S9 fraction (hydrate with 2.1 mL of sterile H_2O)	0.60 mL
Total	**48.00 mL**

Lyophilized Test Strains

- T100
- T98

Standard Mutagens

- NaN_3, 110 mL – for use with TA 100.
- 2-Nitrofluorene (2-NF, 110 µL) – for use with TA 98
- 2-Aminoanthracene (2-AA, 110 µL) – for use with S9 activation kits

Disposables

- 96-well sterilized microplates
- Sterilized multi-channel pipette reagent boats
- 50 mL sterilized tubes
- Sterile 120 mL reaction mixture container
- A membrane filter (0.22 μm) unit for sample sterilization
- Zip-lock bags
- 1-10 ml micropipette and tips
- 200ml micropipette, multi-channel pipette and tips
- 1000ml micropipette and tips

3.3.1.1 Hydrating dried bacteria and it's incubation

hydrating the dried bacteria and incubation was performed late at night prior the assay. 10ml of Reagent 'V' (Ampicillin) was transferred into reagent "G" (Growth Media) before mixing with the lyophilized bacteria (T98 and T100). Using aseptic technique, all the Growth Media (G) was transferred into the vials of bacteria and mixed. The mixed lyophilized bacteria was incubated at 37 °C overnight (16 to 18 h). Before commencing with the test, the bacterial growth was visually affirmed with the existence of turbidity.

3.3.1.2 Aqueous sample dilutions

The sample to be tested was filter-sterilized using a 0.22 µm membrane filter. Sample dilutions with sterile distilled water in the 50 mL sterile tubes were prepared according to Table 3.4.

Table 3. 5: Experimental setup of the Muta-ChromoPlateTM Assay with S9 activation for T100 and T98 bacterial strains

Treatment plate (1-12)	Standard	Sample	Water	Reaction mix	S9 mix	Bacteria (5µL)
Blank (sterility check w S9)	-	15.5	0	2.5	2.0	-
Background w S9	-	-	15.5	2.5	2.0	+
Positive control indirect	0.1	-	15.5	2.5	2.0	+
WWTP effluent I (w S9)	-	15.5	0	2.5	2.0	+
WWTP effluent I	-	15.5	0	2.5	0.0	+
WWTP effluent II	-	3	12.5	2.5	2.0	+
Tap water I (w S9)	-	15.5	0	2.5	2.0	+
Tap water I	-	15.5	0	2.5	0.0	+
Tap water II	-	3	12.5	2.5	2.0	+
4-CP	-	15.5	0	2.5	2.0	+
Bottled water A-I (w S9)						
Bottled water A-I	-	15.5	0	2.5	0.0	+
Bottled water A-II	-	3	12.5	2.5	2.0	+
2,4 DCP	-	15.5	0	2.5	2.0	+
Bottled water B-I (w S9)						
Bottled water B-I	-	15.5	0	2.5	0.0	+
Bottled water B-II	-	3	12.5	2.5	2.0	+

3.3.1.3 Preparation of the reaction mixture

The reaction mixture comprises of components "A" to "E" combined:

A) 43.24 mL

(B) 9.50 mL

(C) 4.76 mL

(D) 2.38 mL

(E) 0.12 mL (Tryptophan for WP2 strains)

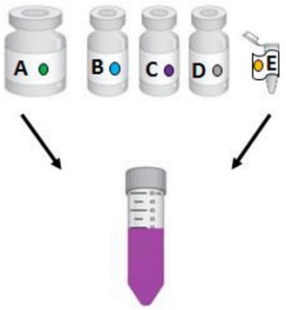

Figure 3.2: Reaction mixture(EBPI, 2019)

TOTAL: 60.00 mL Reaction Mixture (an additional 60 mL was required for both bacteriological strain).

3.3.1.4 Preparation of treatments (with and without S9 activation enzyme)

2.5 mL of the reaction mixture was sterilely transferred in all aseptical tubes that contained test samples. Sterile filtered material or dilutions to be tested (15.5 mL) was added. The S9-activation enzyme experiments, 2.0 mL of S9 mix was added to each of the tubes requiring S9 activation only. Reaction mixture (2.5 mL) and 15.5 mL of the sample to undergo testing was added to the tubes as presented in Table 3.5.

3.3.1.5 Preparing the Muta-ChromoPlateTM Assay

Every treatment tube that has the material to be assessed, 5 mL of bacterial test strain broth culture (eg. *S. typhimurium* TA100 and TA98) was added ensuring that the bacteria was fully suspended in the vial before withdrawing. The content of every tube was poured directly into a sterile mixture boat and 200 mL of the mixture was dispensed in all 96-well sterile microplate with a multi-channel pipette. Plates were labelled for facile identification and separation of bacterial strains and incubated in an aseptic sealed plastic bag at 37 °C for 3-6 days.

3.3.1.6 Analysis of the results

The scoring of the plates were done visually. Yellow and partial yellow wells were scored as positive. Purple wells were scored as negative.

The test was regarded valid in the following manner:

a) The `Blank' (sterility assessment) wells is observed.

b) If the average score for negative or background control was ≥ 0 and ≤ 30 revertant wells per 96-well section on day 6.

c) If the average score for positive (standard mutagen) controls was ≥ 50 revertant wells per 96-well section on day 6.

The number of positive wells for each plate were recorded using the Muta Assay report template (Table 3.6).

Table 3. 6: Ames test experimental design

Number	Plate/treatment	Pos. Control	Bacteria	Day 4	Day 5	Day 6
1	Blank	-	-			
2	Background	-	+			
3	Pos. Control	+	+			
4	WWTP effluent I (w S9)	-	+			
5	WWTP effluent II (w S9)	-	+			
6	WWTP effluent III (w S9)	-	+			
7	Tap water I (w S9)	-	+			
8	Tap water II (w S9)	-	+			
9	Tap water III (w S9)	-	+			
10	Bottled water A-I (w S9)	-	+			
11	Bottled water A-II (w S9)	-	+			
12	Bottled water A-III (w S9)	-	+			
13	Bottled water B-I (w S9)	-	+			
14	Bottled water B-II (w S9)	-	+			
15	Bottled water B-III (W S9)	-	+			

For each treatment-plate, the statistical significance of the difference was determined using a Table 3.7. The results were interpreted using the method of Mortelmans & Zeiger (2000).

Table 3.7. Scoring of 96-well microplates for mutagenicity

No. Wells Positive in Background Plate	No. Wells Positive in Treatment Plate			No. Wells Positive in Background Plate	No. Wells Positive in Treatment Plate		
	0.05	0.01	0.001		0.05	0.01	0.001
0	3	6	10	36	48	53	58
1	5	8	12	37	49	54	59
2	7	10	14	38	50	55	60
3	9	12	16	39	51	56	61
4	10	14	19	40	52	57	62
5	12	15	20	41	53	58	63
6	13	17	21	42	54	59	64
7	15	18	23	43	55	60	65
8	16	20	25	44	56	61	66
9	17	21	26	45	57	62	67
10	19	23	27	46	58	63	68
11	20	24	29	47	59	64	69
12	21	25	30	48	60	63	70
13	22	27	32	49	61	66	70
14	24	28	33	50	62	67	71
15	25	29	34	51	63	67	72
16	26	30	36	52	64	68	73
17	27	32	37	53	65	69	74
18	28	33	38	54	66	70	75
19	30	34	39	55	67	71	76
20	31	35	40	56	68	72	77
21	32	36	42	57	68	72	77
22	33	38	43	58	69	74	78
23	34	39	44	59	70	75	79
24	35	40	45	60	71	75	80
25	36	41	46	61	72	76	81
26	37	42	47	62	73	77	71
27	39	43	49	63	74	78	82
28	40	44	50	64	75	79	83
29	41	45	51	65	76	80	84

30	42	47	52	66	77	80	84
31	43	48	53	67	78	81	85
32	44	49	54	68	78	82	86
33	45	50	55	69	79	83	87
34	46	51	56	70	80	84	87
35	47	52	57	71	81	84	88
72	82	85	89	84	91	94	95
73	83	86	89	85	92	94	96
74	83	87	90	86	93	94	96
75	84	87	90	87	93	95	-
76	85	88	91	88	94	95	-
77	86	89	92	89	94	96	-
78	87	89	92	90	95	96	-
79	87	90	93	91	96	-	-
80	88	91	93	92	96	-	-
81	89	91	94	93	96	-	-
82	90	92	94				
83	90	93	95				

(Source: Gilbert, 1980)

3.3.2 Cancer risk assessment for 4-CP and 2,4-DCP exposure

The health risk assessment equations that are mostly used for estimating exposure to phenols was based on a previously developed method by USEPA and those reported in the literature (Rand & Mabury, 2017; Olujimi, 2012). Humans exposure to toxic effects is expressed as an Average Daily Dose (ADD) and defined as the quantity of a substance consumed on a daily basis during the exposure period. For cancer risk, the Average Daily Dose (ADD) and the life-time average daily dose (LADD) was estimated by averaging the total exposure over the lifetime of the individual (expected 70 years and 365 days for daily dose). The concentrations of 4-CP and 2,4-DCP measured in water and wastewater samples were used for the estimation of cancer risk.

Risks were calculated using equations 2-4.

ADD= (IR*C*EF*ED)/ (BW*AT) ……equation 2 where

IR=Ingestion rate, C= Concentration, EF= exposure frequency, ED= Exposure duration, BW= Body weight, AT= Averaging time (Life expectancy)

Hazard Quotient (HQ)= (ADD)/(RfD)……equation 3 where RfD is the Reference Dose (IRIS-USEPA)

HQ>1.0 ...non-carcinogenic adverse effect, HQ<1.0 ….no adverse effect

Cancer risk = SF*ADD ……. equation 4 where SF is slope factor.

Table 3. 7: Exposure values used in dose calculation

Exposure parameter	Value
Exposure duration (ED)- years	10
Body weight (bw) kg	70
Lifetime (LT) years	70
Exposure frequency (EF) days/years	45
Exposure time (ET) hour/event	1
Events/day EV	1
Water intake rate L/hr	0.071

3.4. Statistical analysis

To establish the effects of levels of different samples, the significant differences among the sample types was determined using the SPSS Statistics v27. The Microsoft Excel office tool was used to conduct the environmental hazard assessments of the investigated samples.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1. Phenols occurrence in WWTP effluent and potable water samples

A typical chromatogram, the calibration curves and data for the method used are presented in Figures 4.1, 4.2a, 4.2b and Table 4.1 respectively. The retention times were 11.7 and 14.1 for 4-CP and 2,4-DCP, respectively. The R^2 values for both calibrations were >0.99 indicating the suitability of the method for analysis (Table 4.1). In this study, selected phenolic compounds -4-CP and 2,4-DCP in Stellenbosch wastewater effluent, tap water and four brands of bottled water were analysed. The results obtained from the analysis of potable water samples and wastewater treatment plant effluent are presented in Table 4.2.

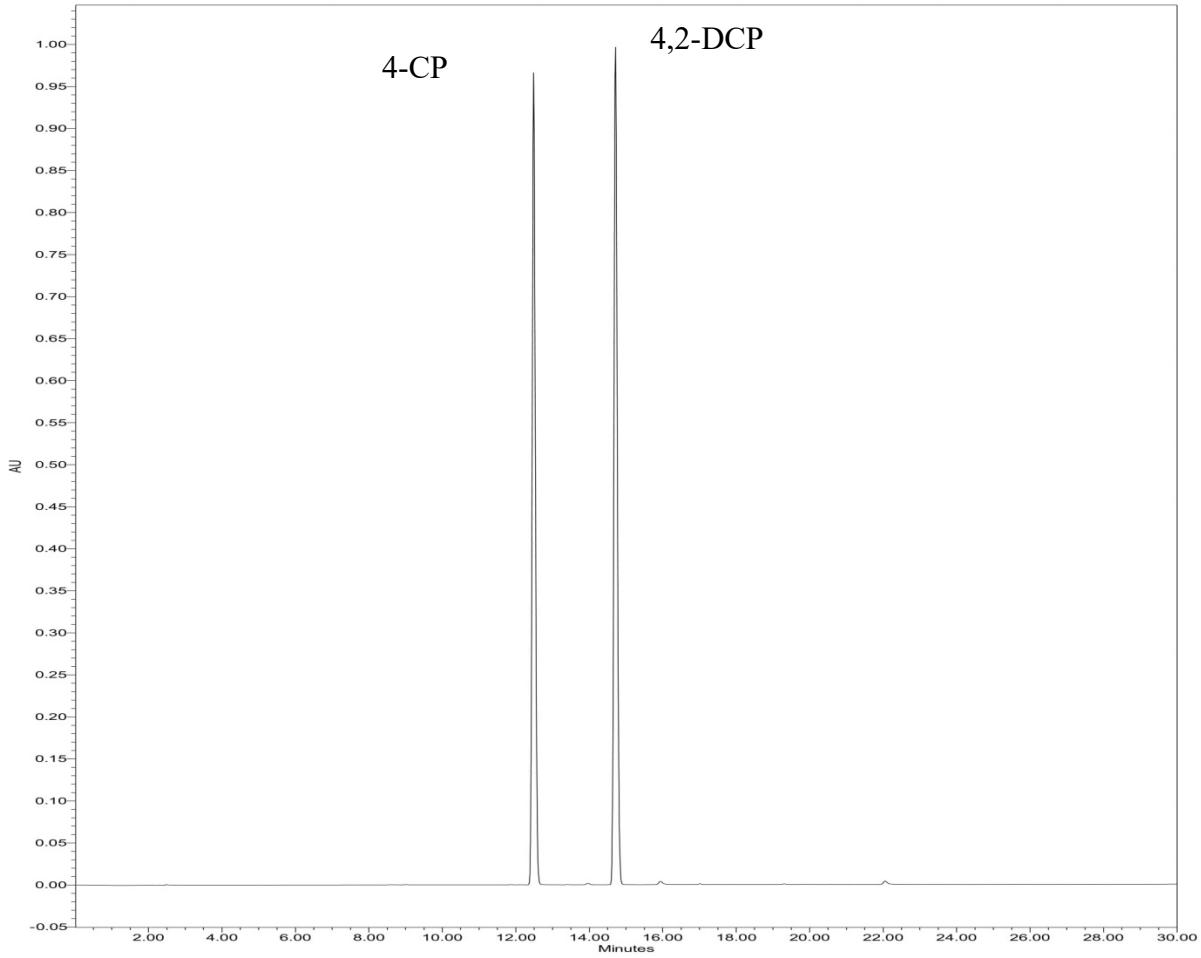

Figure 4. 1: Chromatogram of 4-CP and 2,4-DCP

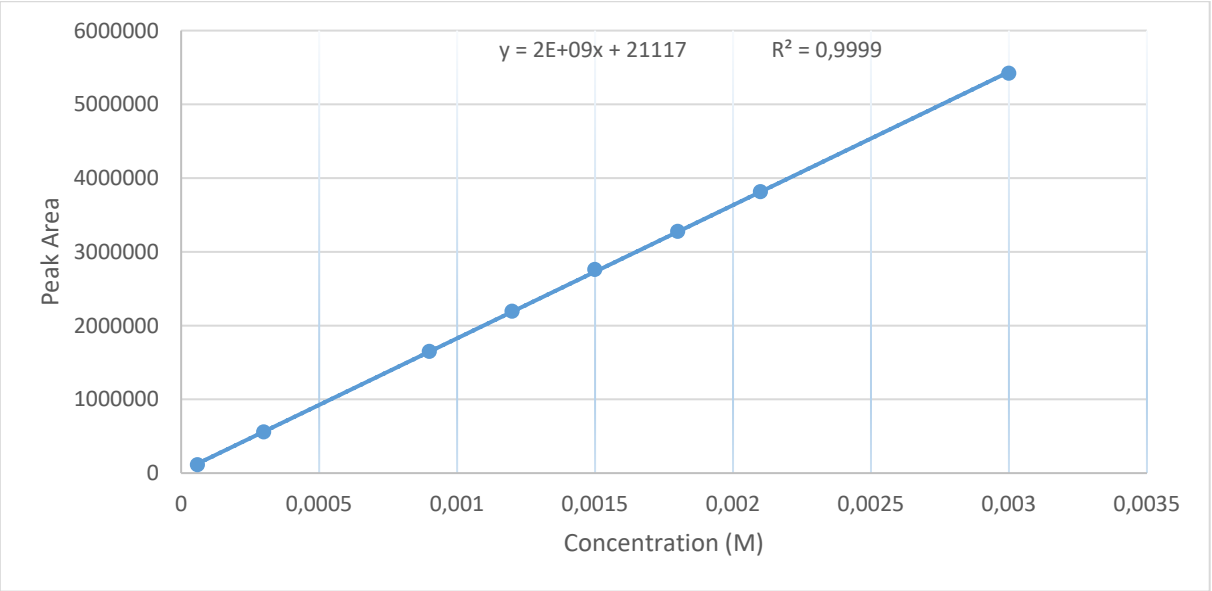

Figure 4.2a: Calibration curve for 4-CP

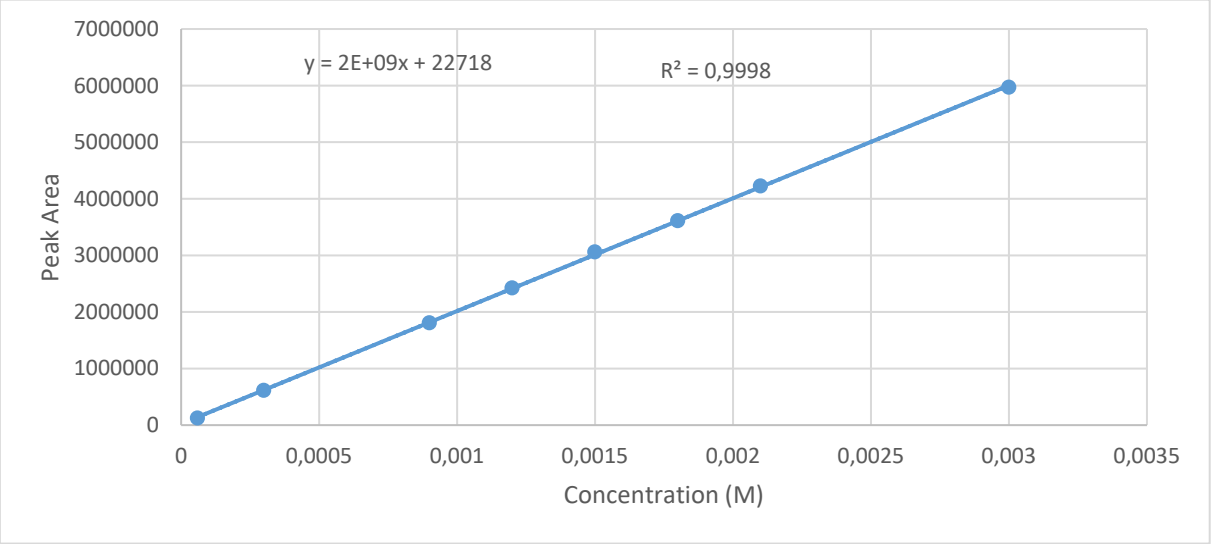

Figure 4.2b: Calibration curve for 2,4-DCP

Table 4. 1:Calibration data for 4-CP and 2,4-DCP

4-CP			**2,4-DCP**		
Concentration (M)	**Peak Area**	**Retention Time**	**Concentration (M)**	**Peak Area**	**Retention time**
0.00006	113374	11.715	0.00006	123054	14.059
0.0003	557496	11.767	0.0003	614290	14.122
0.0009	1648622	11.761	0.0009	1805903	14.119
0.0012	2193595	11.762	0.0012	2421714	14.118
0.0015	2759050	11.723	0.0015	3058599	14.073
0.0018	3276298	11.739	0.0018	3610620	14.7
0.0021	3815336	11.708	0.0021	4224544	14.07
0.003	5420296	11.719	0.003	5969507	14.08

Table 4. 2: Levels of phenolic compounds in potable water and WWTP effluent samples in mg/L (mean ± SD, n=3)

Sample	**4 CP**			**Mean**	**SD**	**2,4 DCP**			**Mean**	**SD**
	Batches					Batches				
	1	2	3			1	2	3		
WWTP influent	1.04×10^{-4}	1.149×10^{-4}	6.43×10^{-5}	9.43×10^{-5}	2.66×10^{-5}	9.81×10^{-5}	1.72×10^{-4}	6.39×10^{-5}	1.11×10^{-5}	5.51×10^{-5}
WWTP effluent	5.61×10^{-5}	1.27×10^{-5}	4.04×10^{-6}	2.43×10^{-5}	2.79×10^{-5}	ND	ND	5.40×10^{-6}	1.80×10^{-6}	3.12×10^{-6}
Bottle water Brand A	ND	9.32×10^{-7}	5.81×10^{-6}	$2,25\times10^{-6}$	3.12×10^{-6}	ND	3.68×10^{-6}	1.31×10^{-5}	5.60×10^{-6}	6.77×10^{-6}
Bottle water Brand B	ND	6.95×10^{-6}	3.42×10^{-6}	$3,46\times10^{-6}$	3.48×10^{-6}	5.56×10^{-6}	1.37×10^{-5}	3.68×10^{-6}	7.66×10^{-6}	5.35×10^{-6}
Bottle water Brand C	ND	ND	9.78×10^{-6}	3.26×10^{-6}	5.64×10^{-6}	8.80×10^{-7}	ND	6.85×10^{-6}	2.58×10^{-6}	3.73×10^{-6}
Bottle water Brand D	1.97×10^{-6}	8.90×10^{-7}	6.74×10^{-6}	3.20×10^{-6}	3.11×10^{-6}	1.47×10^{-5}	8.37×10^{-6}	6.28×10^{-6}	9.77×10^{-6}	4.36×10^{-6}
Tap water	9.96×10^{-6}	1.90×10^{-5}	ND	9.65×10^{-6}	9.50×10^{-6}	6.23×10^{-6}	1.90×10^{-5}	5.97×10^{-6}	9.27×10^{-6}	5.49×10^{-6}

For wastewater treatment, the Stellenbosch wastewater plant uses membrane bioreactors. This system uses a combination of biological treatment methods for suspended growth, generally activated sludge, with membrane filtration equipment, typically membranes for low-pressure microfiltration (MF) or ultrafiltration (UIF). To carry out the critical solid-liquid separation function, the membranes are used. (AMTA, 2016; Nqombolo *et al*., 2016). Therefore, the levels of 2,4-DCP in WWTP effluent are expected to be low due to this process of treatment used because the 2,4-DCP compound in drinking and wastewater is a by-products of water treated by chlorination (Park & Kisok, 2018) .

All samples were initially spiked with a known concentration of 0.0009 M of the analytes; the value was then subtracted from the result to obtain the actual concentration of phenolic compounds in the water samples. The concentrations of both phenolic compounds detected in the Stellenbosch WWTP effluent f were below the limit set by the DWAF (0.01 mg/L). The concentration ranged between 4-CP4.04×10^{-6} mg/L - 5.61×10^{-5} mg/L and 0-5.40×10^{-6} mg/L for 4-CP and 2,4-DCP, respectively. The occurrence of the compounds at trace levels could be due to the compounds having been used either as raw materials or intermediate products in the agro-chemical industry and wood preservation (Santana *et al*., 2002; Ozkaya, 2005). Chlorophenols are also produced in pulp bleaching processes as metabolites of agricultural pesticides, due to inefficient removal of these congeners from wastewater treatment plants waste effluent and as by-products of the chlorination of drinking water (Heberer & Stan, 1997). The Stellenbosch WWTP is in the Boland region of Western Cape, South Africa- an area that is popular for its agricultural prowess as numerous commercial farms abounds. These farms mostly grow grapevines used to produce different types of wines. The presence of the low levels 2,4-DCP may possibly be from the agro-chemical usage from these farms as there was no chlorine treatment taking place in the WWTP. The actual possible sources of 2,4-dichlorophenol contamination into water sources may need further investigation.

There is limited information in the literature 4-CP and 2,4-DCP occurrence in WWTP effluent (Buchholz & Pawliszyn', 1993; Kurniawan & Lo, 2007; Dilaver & Kargi, 2009; Saraji & Marzban, 2010; Olujimi, 2012). However, in the case of 4-CP the Wine production in the vicinity of the WWTP is a possible major contributor to the levels of phenolic compounds detected in effluent samples. Due to the toxicity of phenolic compounds in drinking and surface waters to aquatic and human lives, the European Commission (EC) and the United State Environmental Protection Agency (USEPA) have classified some of them as EDCs (Olujimi, 2012). Four brands of bottled water were investigated for 4-CP and 2,4-DCP. The American Food and Drug Administration (FDA) has established that in bottled drinking water, the phenol concentration does not surpass 0.001 mg/L (ATSDR, 2008b). Standard guidelines for general phenolic compounds were set at 2 mg/L by WHO for drinking water (Enderlein *et al*., 1996; ATSDR, 2006). The concentrations of 4-CP and 2,4-DCP in the bottled water denoted as brand "A" to brand "D are presented in Table 4.2. Brand "A" in the first month of sampling 4-CPneither of the two compounds were detected in the samples analysed. Subsequent analyses showed that water samples were tainted with the two phenolic compounds (9.32×10^{-7} and 5.81×10^{-6} mg/L -4-CP and

3.68×10^{-6} and 1.31×10^{-5} mg/L -2,4 DCP respectively). The values were however below the regulatory limits of the FDA for phenols in bottled water. For Brand "B" in the 4-CP was not detected in the samples but 2,4-DCP (0.00000556) was present at levels below the FDA limits. The second and thirdbatches had 4-CP and 2,4-DCP levels ranging from 0.00000342 mg/L - 0.0000137mg/L; the values were also below the FDA regulatory limits. For Brand "C", 4-CP was not detected in the first two batches, but the last batch was contaminated at levels the FDA. Concentrations of 2,4-DCP below the FDA limit were detected in the first (0.000000880 mg/L) and the third (0.00000685 mg/L) batches of samples analysed but not in the second batch. In Brand "D", both 4-CP and 2,4 DCP were detected in all samples for the three batches, but the levels (ranging from 0.000000890 mg/L to 0.0000147 mg/L for both compounds) were below the FDA regulatory limit Olujimi (2012), reported the occurrence of USEPA 11 priority phenols in three brands of bottled water and found a mean concentration of 5.13 µg/L. However, Steiner *et al*. (2007) for the priority pollutants reported that 2,4-DCP did not occur in both the spiked and un-spiked samples. For 4-CP, The USEPA's guideline for potable water is ≤ 0.3 mg/L phenol, protecting human health from the potential adverse effects of phenol exposure by drinking water and/or consuming contaminated plants and animals (Younis & Rafati, 2004). The limit surface water (lakes, streams) sources is 3.5 mg L^{-1} (EPA, 2002). In the European Community, for every pollutant the maximum allowable concentration of phenols in drinking water is 0.1 µg.L^{-1} (Khalid, 2011). For all samples analyzed, the levels of both 4-CP (ND-0,0000190 mg/L) and 2,4 DCP (0,00000597 -0,0000190 mg/L) were found to be below the set limits and standards. A similar study by Izawa *et al*. (2015) reported that the concentrations of phenols in tap water ranged from 0.01–0.20 mg/L (there were no specific values for the individual phenolic compounds). Possible sources of the low level 4-CP and 2,4-DCP in the tap water may be from the chlorination process carried out to disinfect the water. Moreover, it may come from the water source which is obtained from dams that may have been contaminated with agricultural run-offs. These dams are mostly filled by run-offs during rainfall in the winter seasons when farming activities are at their peaks.

4.2.1. Acute toxicity test of WWTP effluent on *Daphnia Magna*

Five concentrations (100%, 50%, 25%, 12.50% and 6.25%) were used to assess the effect of acute immobilization on the test organism; *D magna*. Experimental results were considered valid only if the control treatment mortality rate was ≤10%. In order to estimate the concentration that would trigger a x% response in the test species, the experimental data was analysed using a regression model; LCx (e.g., LC50, LC20 or LC10). The cumulative immobility of daphnids during the test period (48 h) indicated a minimal concentration-response relationship. For the first batch of samples analysed, there was no significant immobility in the first 24 h. At 48 h, only 5% of the test organisms were immobile in both the 12.25% and at 100% treatments. At 95% confidence limit, the lowest observed effect concentration (LOEC), no observed effect concentration (NOEC) values were ≥100% indicating that the effluent samples would potentially have no effect on *Daphnia magna* in surface waterbodies. The summary of results obtained is presented in Table 4.3.

Table 4. 3: Toxicity metrics of Stellenbosch WWTP effluent on Daphnia magna

Month	Assay duration	LC10	LC20	LC50	LOEC	NOEC
April	24 h	ND	ND	ND	>100.000	>=100.000
	48 h	ND	ND	ND	>100.000	>=100.00
June	24 h	ND	ND	ND	>100.000	>=100.000
	48 h	25.891	20.522	41.814	>100.000	>=100.00
July	24 h	ND	ND	ND	>100.000	>=100.000
	48 h	ND	ND	ND	>100.000	>=100.00

LC: Effective concentration for xx% reduction; 95%-CL: 95% Confidence limits; LOEC: Lowest observed effect concentration; NOEC: No observed effect concentration; ND: not determined due to mathematical reasons

At the end of the 48 h exposure duration of experiments, immobility of *Daphnia magna* in response to the WWTP effluent showed a mortality rate that was ≤10% for all treatments as presented in Figure 4.3.

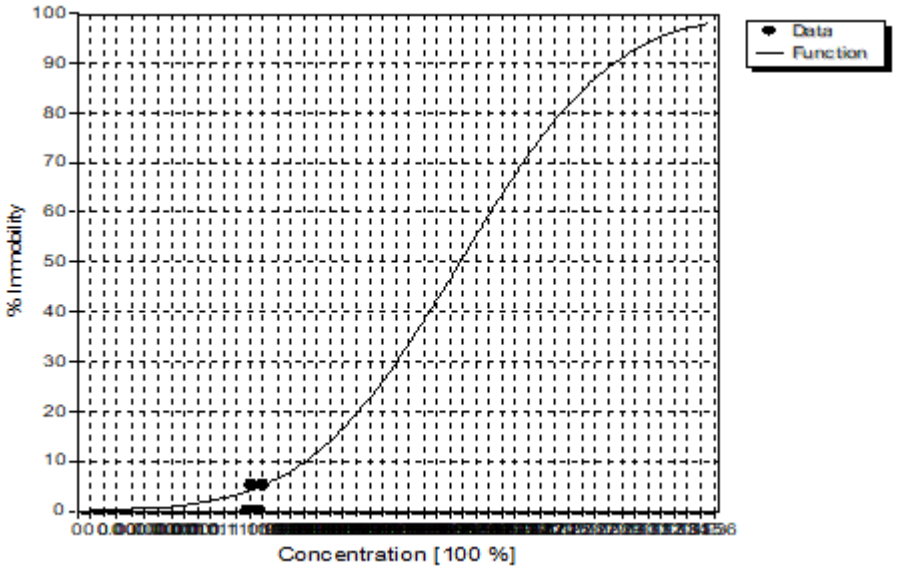

Figure 4. 2: Concentration-effect curve showing the influence of the WWTP effluent sample on immobility of the introduced Daphnia magna as observed after 24 h

There was no immobility observed, the samples collected in June in the first 24 h (Figure 4.3). However, at 48 h, there was slight increase in immobility in the treatments. 12.5% where there was 5% immobility, 30% at 25% concertation and 25% (5 out of twenty) at the 50% concentration only 25% (five out twenty) were found to be immobile. 95% Confidence limits at the lowest observed effect concentration (LOEC) showed no effect in terms of immobility and no observed effect concentration (NOEC) at 100% or greater than the total

concentrated sample was there or potentially be an effect on Daphnia Magna in the water sample. At the end of the exposure period, immobility of *Daphnia magna* being dependent on each dilution series concentration of the test treated effluent at 48h, were recorded as shown in Figure 4.4.

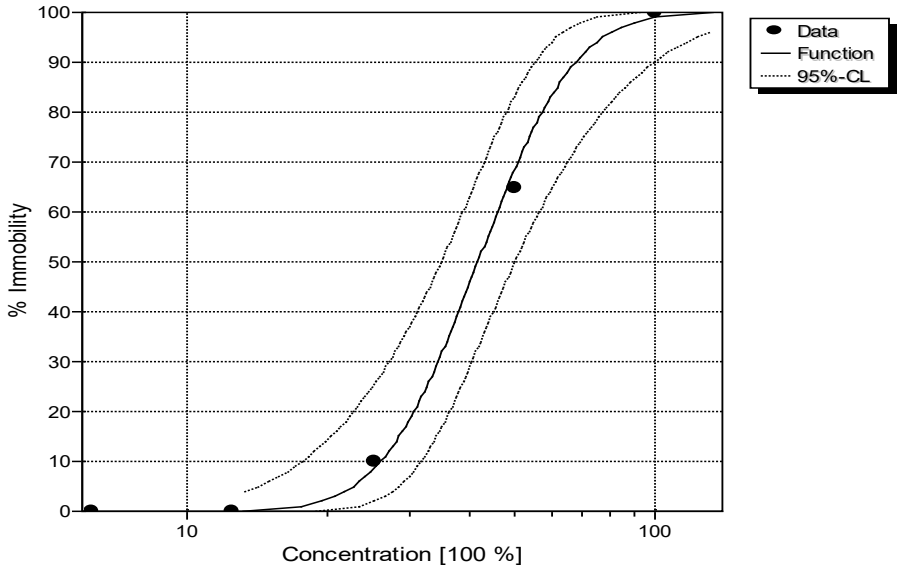

Figure 4. 3: Concentration-effect curve showing the influence of the WWTP effluent on immobility of the introduced Daphnia magna as observed after 48 h

The overview Immobility at 48 h of exposure to the WWTP effluent plotted on the concentration-effect curve was below 10% for overall concentration.

Table 4. 4: Daphnia magna immobility in response to WTTP exposure after 48 h

Treatment	% Immobility		
	April	June	July
Control	0	5	0
6.25	0	0	10
12.5	5	0	0
25	0	10	0
50	0	65	0
100	0	100	0

In the third month (July 2019), there was no significant immobility caused. In the first 24 h at 6.25% there was 5 % (one out of twenty) immobility and that one immobile daphnia was the only one throughout the 48 h testing that remained immobile making the total in the 48 h period to be 5% at 6.25%. 95% Confidence limits at the lowest observed effect concentration (LOEC) showed no effect in terms of immobility and No observed effect concentration (NOEC) at 100% or greater than the total concentrated sample was there or potentially be

an effect on *Daphnia Magna* in the water sample. These results are similar to those of Bakopoulou *et al*. (2011), with the exception of the autumn results for one out of three WWTP tested, which showed 100% mortality.

4.2.2. Algae (*Raphidocelis subcapitata)* growth inhibition test using WWTP effluent

In a 72 h static test, green algae, S *Raphidocelis subcapitata* was exposed to whole and serially diluted (100%, 50%, 25%, 12.50% and 6.25%) WWTP effluent. The endpoint measured was algal biomass produces using cell density/cell counts. The 72 h algae growth inhibition test of the WWTP effluent was performed using the OECD 201 method. Growth inhibition, algal biomass yield and growth rate were used to determine toxicity metrics of the experiments. Test validity was affirmed using the ToxRat Software that utilized exposure time, biomass factor, mean growth rate of treatments and coefficient of variation of replicates over time.

Samples of effluent from the Stellenbosch wastewater treatment plant were collected over three months that spanned through two seasons-winter and autumn. A typical sectional growth rate curve for Raphidocelis *subcapitata* in the WWTP effluent over time is time are shown in figure 4.5. Number of cells increased from the start of the experiment until its expiration. This indicates that growth was not inhibited but the algal cells rather bloomed. An overview of algal yield after each 24 h until the end of experiment is presented in the curve.

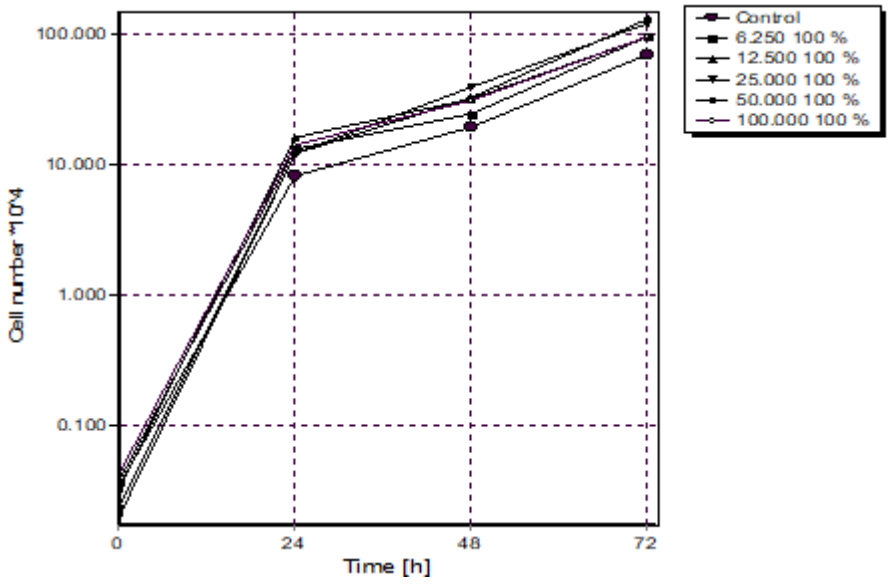

Figure 4. 4: Cell number in Raphidocelis subcapitata as dependent on test item concentration and time

The results obtained revealed a substantial consistent increase in algal growth over the 72 h exposure period all treatments. The results of biomass yield are presented in Table 4.5. An overview over the LOEC and NOEC determination using biomass yield as the end point is presented in the table. Arithmetic means and significance results are computed for yield of all inspection intervals are presented. The LOEC and NOEC values obtained with indication of statistical test used Williams multiple sequential t-test procedure, significance level was

0.050, one-sided smaller are also presented as footnotes to the table. Williams multiple sequential t-test was used to test statistical significance of results.

The negative values on the table denote increased growth rather that inhibition. This suggests that the effluent will potentially support algal growth in water -algal bloom. The environmental implication of this is observation is that the effluent may be eutrophic in the receiving water body.
The values for LOEC and NOEC were >100 and ≥100 respectively for biomass yield after 72 h exposure period. The values were same for algal growth rate for the duration of experiments. The EC10 for biomass sectional growth rate at the expiration of the experiment was 168.8. Both the LOEC and NOEC values were significantly different from the control experiment.

Table 4. 5: Algal biomass yield in WWTP effluent over 72 h

Treatm. [100 %]	**0-24 h**	**0-48 h**	**0-72 h**
6.250	13.0 -	24.8 -	94.3 -
12.500	16.4 -	31.7 -	96.4 -
25.000	12.2 -	39.5 -	119.5 -
50.000	12.2 -	32.5 -	130.3 -
100.000	14.3 -	31.1 -	94.3 -

LOEC >100.000 *wl >100.000 *wl>100.000 *wl; NOEC >=100.000 *wl >=100.000 *wl >=100.000 *wl
+: Significant difference to control (p <=0.050)

The Acute (TUa) and chronic (TUc) toxicity units were therefore not calculated for the samples because the LC50 values were not obtained due to limited/undetected toxicity observations. Current study is similar to that of Bailey (2000), who reported slight adverse effects on *Raphidocelis subcapitata*. Only two (out of eighteen) of the samples tested resulted in reduced algal growth, were the rest of the samples demonstrated algae growth indicating no effect on the algae.
However, literature provides data that is commonly related to samples tested from wastewater treatment plants using treatment processes such as gas chlorination, activated sludge treatment and rotating biological contactor and wastewater treatment plants treat water from domestic, industrial and agricultural use just like Stellenbosch (as it is an area predominately utilised for agriculture-mainly wine production), which uses the membrane treatment method thus, methods of treatments of the wastewater treatment are suspected and questioned if they contribute to the outcome of prohibiting algae growth due to improperly treating and or moving chemical compounds in water though the discharged effluent meets the requirements set (Lanciotti *et al*., 2004; Ra *et al*., 2007; Bohórquez-Echeverry *et al*., 2012; Miashiro *et al*., 2012).

4.2.3. Chronic toxicity testing on *Tetrahymena thermophila*

The effect of the Stellenbosch wastewater effluent on the protozoa *Tetrahymena thermophila* was tested on samples collected in autumn and winter months. The test was a 24 h toxicity test using the protozoan *T.thermophila.*

The test revolves around the turnover of substrate into ciliate biomass. Standard proliferating cell cultures clears the substrate suspension in 24 h but inhibited culture growth would remain turbid. Optical density measurements of the turbidity correlate with degree of inhibition. The clearer the culture, the lesser the toxicity and hence, reduced growth inhibition.

The *T. thermophila* chromic test is a multi-generational growth test that includes 5-6 generations in 24 h. The optical density of the inoculum suspension in sample was measured at 440 nm at the start of the experiment and after 24 h. The test validity was affirmed if the optical density of control treatments after 24 h showed a decrease of at least, 60% after 24 h. The results of the test are presented in Table 4.6. For all samples tested, the treatment at 25 % concentration showed the most growth inhibition. The least percentage inhibition was observed in the 6.25% dilution treatment.

Table 4. 6: Initial and 24 h optical density values of *T. thermophila* growth inhibition in WWTP effluent

Treatment	Time	Replicate 1	Replicate 2	Replicate 3	Mean	Std. dev.	Coefficient variation (%)
Control	to	0.779	0.813	0.730	0.774	0.041	5.30
	t24	0.691	0.841	0.243	0.5916	0.311	52.60
6.250	to	0.818	1.611	0.598	1.009	0.532	52.73
	t24	0.557	0.757	0.226	0.513	0.268	52.24
12.500	to	0.620	0.810	0.597	0.675	0.116	17.19
	t24	0.599	0.598	0.213	0.47	0.222	47.23
25.000	to	0.793	0.800	0.660	0.751	0.788	104,9
	t24	0.640	0.624	0.223	0.495	0.236	47.68
50.000	to	0.904	0.738	0.649	0.763	0.129	16.91
	t24	0.873	0.646	0.267	0.595	0.306	51.43
100.000	to	1.152	0.896	0.709	0.919	0.222	24.16
	t24	1.072	0.832	0.249	0.717	0.423	59,0

The effective concentration values (ECx) for EC10, EC20, EC50, EC70 and EC90 were 3.294, 4.390, 10.386, 18.442 and 32.747 respectively. Further studies on the factors that are responsible for the variable growth inhibition of the test organisms in the treatments are being planned.

4.2.4. Mutagenicity test- 'Ames test'

The Ames test is a biological test that tests the mutagenic ability of chemical compounds. It makes use of bacteria to test the potential of chemicals that could and have the potentially to cause mutations in the DNA of the test organism. Tap water, bottled water and WWTP effluent were tested for mutagenicity. Two strains were used- the T98 and the T100. Many carcinogens are known to require metabolic conversion to reactive metabolite before they interact with DNA, therefore, testing a compound for mutagenicity both with and without metabolism expands the detection capabilities of the assay and is essential for an accurate assessment as mammals exhibit extensive metabolic capabilities in vivo (EBPI, 2019). Both direct and indirect mutagens can be detected if S9 is included in the test design; hence the S9 was included in the current study. The study was performed according to the 'Muta-ChromoPlate Bacterial Strain Kit with S9 Activation TM Version 2.1' for a period of six days. Wells that remained purple at the time of observations were considered negative and the yellow wells positive. The results were recorded every 24 h and are presented in Tables 4.7 and 4.8.

Table 4. 7: Test scores of samples' mutagenicity using the T98 strain

#	Plate	Concentration	Bacteria	Day 4	Day 5	Day 6
1	Blank –ws9 (tap water)	100%	-	0	0	0
2	Background wS9	-	+	0	0	0
3	Positive control	-	+	95	96	96
4	WWTP effluent I	100%	+	91	91	91
5	WWTP effluent-I ws9	100%	+	95	95	95
6	WWTP effluent-II	19%	+	96	96	96
7	Tap water I	100%	+	95	95	95
8	Tap water-I ws9	100%	+	95	95	95
9	Tap water –II	19%	+	95	95	95
10	Bottled water 'A'-I	100%	+	96	96	96
11	Bottled water 'A'- I wS9-	100%	+	96	96	96
12	Bottled water 'A'-II	19%	+	96	96	96
13	Bottled water 'B'-I	100%	+	96	96	96
14	Bottled water 'B'-I wS9	100%	+	96	96	96
15	Bottled water 'B'-II	19%	+	96	96	96

Table 4. 8: Test scores of samples' mutagenicity using the T100 strain

#	Plate	CONCENTRATION	Bacteria	Day 4	Day 5	Day 6
1	Blank –wS9 (tap water)	100%	-	0	0	0
2	Background wS9	-	+	19	21	23
3	Positive Control	-		54	64	68
4	WWTP effluent I	100%	+	30	35	38
5	WWTP effluent-I ws9	100%	+	26	29	30
6	WWTP effluent-II	19%	+	25	32	35
7	Tap water I	100%	+	29	33	39
8	Tap water-I ws9	100%	+	19	21	24
9	Tap water –II	19%	+	21	33	35
10	Bottled water 'A'-I	100%	+	16	19	24
11	Bottled water 'A'- I wS9-	100%	+	30	37	39
12	Bottled water 'A'-II	19%	+	13	20	23
13	Bottled water 'B'-I	100%	+	25	30	32
14	Bottled water 'B'-I wS9	100%	+	19	23	26
15	Bottled water 'B'-II	19%	+	15	17	23

The results for the T98 bacterial strain were regarded valid if all three criteria were met. The criteria are:

1. The blank wells were sterile (purple).
2. Average score for negative or background control was ≥ 0 and ≤ 30 revertant wells per 96-well section on day 6.
3. Average score for positive (standard mutagen) controls was ≥ 50 revertant wells per 95-well section on day 6.

From the results above, the background had no (0) positive wells, however, the Stellenbosch WWTP effluent, tap water, bottle water 'A' and bottle water 'B' of the 100% undiluted concentration had 95,95, 96, 96 positive wells respectively on day 6. Based on the table for scoring the number of positive wells in a 96-well microplate demonstrating clear significance in the Fluctuation Test, the number of positive wells in the background with those in samples, the waters displayed strong mutagens. The treatments are all undiluted and are far greater than 10 (95, 95, 96 and 96 respectively). There is a <0.001 chance that 0 and 95 and 96 are the same results therefore the treatment plates produced significant difference in reverse mutation rate from that observed in the control. Therefore, all the samples tested demonstrated mutagenicity on the T98 strain.

From the results above, the background had 23 positive wells more so, the Stellenbosch WWTP effluent, tap water, bottle water 'A' and bottle water 'B' showed 30,24,39,26 positive wells respectively on day 6. Based on the table for scoring the number of positive wells in a 96-well microplate demonstrating clear significance in the Fluctuation Test, the number of positive wells in the background with those to the samples, the waters displayed weak mutagens. The treatments are all undiluted at a 100% concentration and are greater than 23 (30, 24, 39 and 26 respectively). From the table below, we see that there is a <0.05 chance that 23 and 30, 24 and 26 (respectively) are the same results and number, suggesting that the sample is not mutagenic under these conditions. For Bottled water (brand) "A," there is a <0.001chance that 23 and 39 are the same results and number thus, suggesting a possible chance of mutagenicity.

Because the treatments are more than 30, Bottled water brand "A" being less than (38, 39, 24 and 32 respectively), the outcome would still suggest that there is a <0.05 chance that "30" and 38,39, 24 and 32 are the same results therefore the treatment plates would have not produced significant difference in reverse mutation rate from that observed in the control, suggesting that the samples have no mutation present.

According to Mortelmans & Zeiger (2000), a compound is regarded a mutagen if, in one or more strains, it induces are reproducible, dose-related rise in the number of reverting colonies. If it induced a reproducible, dose-related increase in the number of reverting colonies in one or more strains, a compound is considered a poor mutagen, but the number of reverting colonies is not double the background number of colonies. Positive bacterial reverse mutation test results suggest that a substance

causes point mutations in the genome of either *Salmonella typhimurium* by base substitution or frameshift (Bonnaz & Koch, 1998). Negative findings suggest that the test material is not mutagenic in the tested organisms under the test conditions.

As previously discussed regarding the T98 (frameshift mutation) and T100 (base-pair substitution), from these results, it is evident that the samples of the T98 strain are a strong mutagen and have a possibility of causing a mutation towards the insertion or deletion of a nucleotide. However, on the contrary the T100 bacterial strain when exposed to the treatment samples, showed slight mutagenicity potential. The samples had little to no effect on the T100 bacterial strain and may not cause mutation involving replacement or substitution of a single nucleotide base with another in DNA or RNA molecule.

4.2.5. Non-carcinogenic and carcinogenic risk assessment

Human health risk assessment was conducted to provide an indication of possible carcinogenic effects of the phenolic compounds to exposed humans. The methodology used has been presented in the previous chapter. The calculations assumed exposure duration of 10 years, a body weight of 70 kg and life expectancy of 70 years (Table 3.7). Hazard quotient (HQ) was calculated and used to determine the non-carcinogenic health. A sample was considered to possess non-carcinogenic adverse effects if the HQ > 1 and non- carcinogenic adverse effect when the value is <1. The mean values of both phenolic compounds measured in the respective water samples were used for exposure concentration. The average daily dose (ADD), hazard quotient (HQ) and cancer risk of samples are presented in Tables 4.10. The HQ value for all samples were <1. All samples therefore are classified to possess non-carcinogenic adverse effects risk for a lifetime exposure.

Table 4.9: Cancer risk assessment using mean concentrations of 4-CP and 2,4-DCP of samples

Sample	ADD	RfD	HQ	SF	CR	Comment
4-CP						
WWTP Influent	$6.15x10^{-7}$	$3.00x10^{-1}$	$2.05x10^{-6}$	$1.10x10^{-2}$	$6.76x10^{-9}$	Non-carcinogenic adverse effect
WWTP Effluent	$1.58x10^{-7}$	$3.00x10^{-1}$	$5.28x10^{-7}$	$1.10x10^{-2}$	$1.74x10^{-9}$	Non-carcinogenic adverse effect
BW A	$2.35x10^{-5}$	$3.00x10^{-1}$	$7.82x10^{-5}$	$1.10x10^{-2}$	$2.58x10^{-7}$	Non-carcinogenic adverse effect
BW B	$3.61x10^{-5}$	$3.00x10^{-1}$	$1.20x10^{-4}$	$1.10x10^{-2}$	$3.97x10^{-7}$	Non-carcinogenic adverse effect
BW C	$3.40x10^{-5}$	$3.00x10^{-1}$	$1.13x10^{-4}$	$1.10x10^{-2}$	$3.74x10^{-7}$	Non-carcinogenic adverse effect
BW D	$3.34x10^{-5}$	$3.00x10^{-1}$	$1.11x10^{-4}$	$1.10x10^{-2}$	$3.67x10^{-7}$	Non-carcinogenic adverse effect
Tap Water	$1.01x10^{-4}$	$3.00x10^{-1}$	$3.35x10^{-4}$	$1.10x10^{-2}$	$1.11x10^{-6}$	Non-carcinogenic adverse effect
2,4-DCP						
WWTPInfluent	$7.24x10^{-8}$	$3.00x10^{-1}$	$2.41x10^{-7}$	$1.10x10^{-2}$	$7.96x10^{-10}$	Non-carcinogenic adverse effect
WWTPEffluent	$1.17x10^{-8}$	$3.00x10^{-1}$	$3.91x10^{-8}$	$1.10x10^{-2}$	$1.29x10^{-10}$	Non-carcinogenic adverse effect
BW A	$5.84x10^{-5}$	$3.00x10^{-1}$	$1.95x10^{-4}$	$1.10x10^{-2}$	$6.42x10^{-7}$	Non-carcinogenic adverse effect
BW B	$7.9x10^{-5}$	$3.00x10^{-1}$	$2.66x10^{-4}$	$1.10x10^{-2}$	$8.79x10^{-7}$	Non-carcinogenic adverse effect
BW C	$2.69x10^{-5}$	$3.00x10^{-1}$	$8.97x10^{-5}$	$1.10x10^{-2}$	$2.96x10^{-7}$	Non-carcinogenic adverse effect
BW D	$1.02x10^{4}$	$3.00x10^{-1}$	$3.40x10^{-4}$	$1.10x10^{-2}$	$1.12x10^{-6}$	Non-carcinogenic adverse effect
Tap water	$9,67x10^{-5}$	$3.00x10^{-1}$	$3.22x10^{-4}$	$1.10x10^{-2}$	$1.06x10^{-6}$	Non-carcinogenic adverse effect

The cancer risk values for all samples tested were also before the regulatory limits set by national and international bodies. The regulatory limit set by DWAF (1984) for phenols in WWTP effluent is 0.01 mg/L. A lifetime exposure of 0.02 mg/L is not expected to cause an adverse health effect (EPA, 2018). The US FDA advisory limit is 0.001m/L in bottled water (ATSDR, 2008b). The European Union limit is 0.5 µg/L for total phenols and 0.1 µg/L for individual compounds (Fattahi *et al.,* 2007; Santana *et al.,* 2009). Khalid (2011) reported that below 0.3 mg/L, no harm to aquatic life was observed. The regulatory limits by the US-EPA for 4-CP is 5.5 µg/L (EPA, 1990) and 0.02 mg/L for 2,4-DCP (EPA, 2018). The WHO limit is 0.04 mg/L in water. None of the samples analysed had a cancer risk (CR) value that exceeded any of the regulatory limits. The possibility of the samples causing cancer is therefore slim.

4.3. Statistical analysis

4.3.1. Phenolic compound statistical test analysis

H0: There is a significant difference between the 4-chlorophenol and 2,4-dichlorophenol results.

H1: There is no significant difference between the 4-chlorophenol and 2,4-dichlorophenol results.

An Independent-sample test was conducted to compare the two means of the phenolic compounds of 4-chlorophenol and 2,4-dichlorophenol. There was no significant difference (*t(df) =1.034, p=<0.049*) in the mean for 4-chlorophenol (*M=2.0x10^{-5}, SD=3,4x10^{-5})* and 2,4-dichlorophenol (*M=7.0X10^{-6}, SD=4.0X10^{-6}).* The magnitude of the difference in the means (mean difference is 1,3x10^{-5}, 95% CI : 1,8x10^{-5} to 4.4x10^{-5}) . Therefore , H0 was rejected.

4.3.2. Ames mutagenicity statistical test analysis

H0: There is a significant difference between the T98 and the T100 test strain results.

H1: There is no significant difference between the T98 and the T100 test strain results.

An Independent-sample test was conducted to compare the two means of the test strains; T98 and T100. The test results show a significant difference (*t(df) =5.963, p=>0.066*). The mean for the T98 test strain (*M=82.58, SD=33.55)* and for the T100 test strain (*M=27.067, SD=13.21).* The magnitude of the difference in the means (mean difference is 55.51, 95% CI : 36.44 to 74.58) . Therefore , H0 was supported.

Table 4.10. Independent sample t-test results for 4-CP and 2,4-DCP

				Levene's Test for equality of variance		t-test for Equality of Means						
		Mean	SD	F	SIG	t	df	Sig(2-tailed)	Mean difference	Std. Error difference	95% Confidence interval of the difference	
											Lower	Upper
EI	4-CP	2.0x10-5	3,4x10-5	4.812	0.49	1.034	6.138	0.340	$1.3x10^{-5}$	$1.3x10^{-5}$	$1.8x10^{-5}$	$4.4x10-^{5}$
	2,4-DCP	7,0x10-6	4.0x10-6									

Table 4.11. Independent sample t-test results for the two test strains of T98 and T100

				Levene's Test for equality of variance		t-test for Equality of Means						
		Mean	SD	F	SIG	t	df	Sig(2-tailed)	Mean difference	Std. Error difference	95% Confidence interval of the difference	
											Lower	Upper
EI	T98	82.58	33.55	3.648	0.066	5.963	28	2.0x10-6	55.51	9.31	36.44	74.58
	T100	27.07	13.21									

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

A previously reported method was optimized and adapted for the qualitative and quantitative determination of two phenolic compounds, 4-CP and 2,4 DCP in WWTP effluent, tap water and four brands of bottled water. The WWTP treatment process showed considerable effectiveness for removal; the membrane bioreactors system replaces the old system fixed medium (stone) trickling filter systems to treat the influent. The influent was also tested for any of the phenolic compounds in question, and it also displayed low concentrations of the phenolic compounds.

The compounds were detected at trace levels in all samples analyzed. *D.magna* acute toxicity test results of the WWTP effluent revealed that both the lowest observed effect and no observed effect concentrations were greater than the whole effluent concentration. Samples collected in early winter (June) exerted some significant toxic effect on test organisms and the effective concentrations were obtained.

The WWTP effluent demonstrated eutrophic potential; algal bloom was observed for the test organism suggesting the potential of the effluent to trigger eutrophication in the receiving waterbody. This has implications for the ecosystem health and stability. In the *T. thermophila* chronic tests, the whole had the least toxicity to the test organisms. The diluted effluent samples were more toxic than the whole effluent. This means that dilution exacerbated toxicity with possible similar scenario in the receiving environment.

All the samples tested exhibited strong mutagenicity on the T98 strain but slight mutagenicity on the T100 strain. All the samples were classified to have non-carcinogenic adverse effects but not cancer risk.

This investigation is an introductory study into the ecological and human health risk assessment of selected endocrine disrupting phenolic compounds in potable water and treated wastewater effluent samples. The study has provided some insight into the possible ecological and human health risks associated with the occurrence of the 4-CP and 2,4-DCP in potable water and effluent samples. However, other questions such as the actual reasons for the toxicities observed remain unanswered. The ecological health studies were carried out at population and organism levels. Cellular and molecular level studies may provide greater clarity on some of these questions.

A comparative study of the ecological risk assessment of effluent samples from different treatment plants using different wastewater treatment technologies is desirable. It will provide information about

the effectiveness of the different wastewater treatment options available. The sustainability issues around freshwater resources make such studies inevitable now. Wastewater reuse may be one of the very few options available to humanity in the age of global warming and climate change effects.

Ecotoxicology and human health risk assessment studies need more funding because very little information is available in the literature in this important field. Several exposure studies that report levels of phenols (and several other chemicals) in different matrices are available but not chemical risk assessment studies.

The test for significant difference for the two phenolic compounds; 4-CP and 2,4-DCP reveal that the presence of the two phenolic compounds is more less the same and that there's no significant difference in the levels of concentrations. On the other hand, the test for mutagenicity using test strain T98 and T100 reveals that the strains are independent of one another and that the mutagenicity for each strain is significantly different from one another.

Finally, it is recommended that the relevant structures in South Africa strive to set up limits for the individual phenolic compounds and other endocrine disrupting chemicals.

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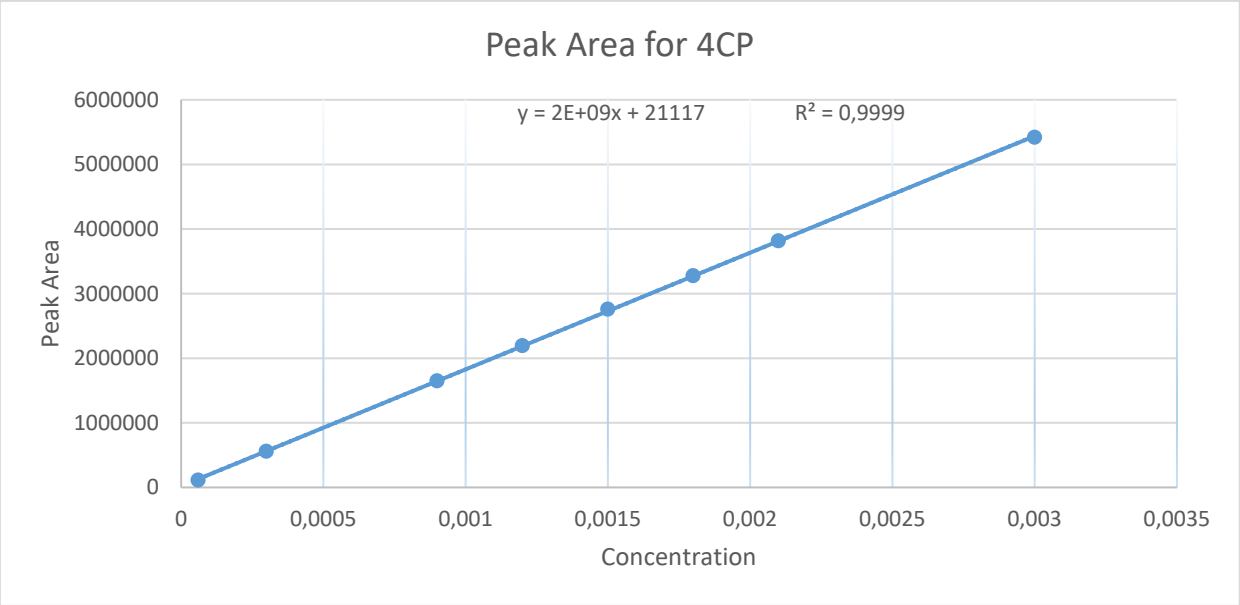
Chapter 2 APPENDICES

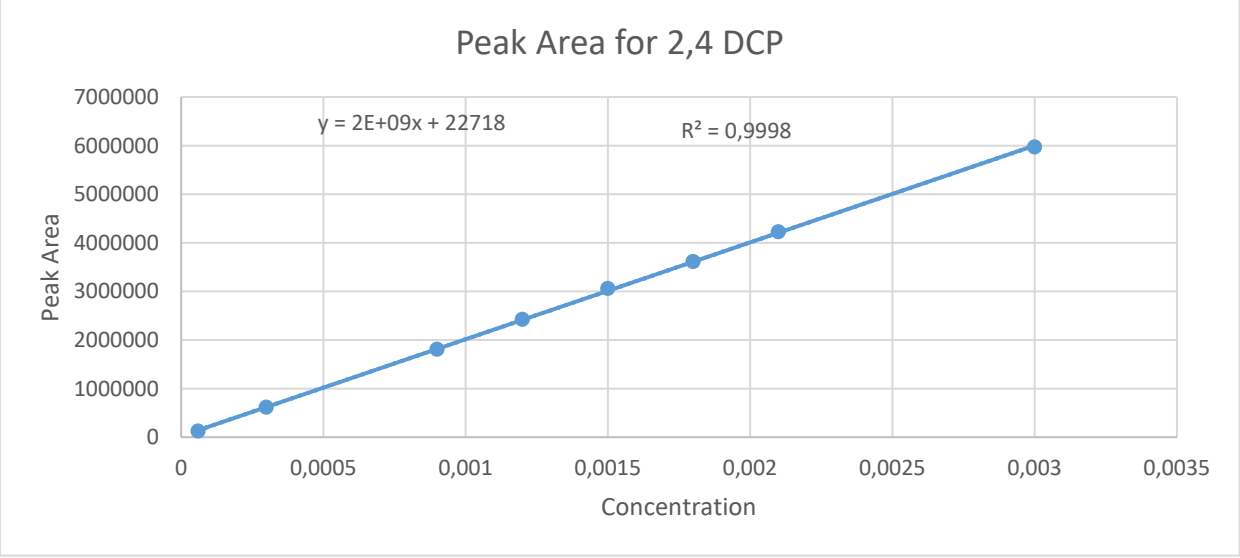
Appendix 1: Calibration table for phenolic compounds

Concentration (M)-4-CP	Peak Area	Retention Time	Concentration (M)-2,4DCP	Peak Area	Retention time
0.00006	113374	11.715	0.00006	123054	14.059
0.0003	557496	11.767	0.0003	614290	14.122
0.0009	1648622	11.761	0.0009	1805903	14.119
0.0012	2193595	11.762	0.0012	2421714	14.118
0.0015	2759050	11.723	0.0015	3058599	14.073
0.0018	3276298	11.739	0.0018	3610620	14.7
0.0021	3815336	11.708	0.0021	4224544	14.07
0.003	5420296	11.719	0.003	5969507	14.08

Appendices 2a and 2b: Calibration curves for 4-CP and 2,4 DCP



2a



2b

Appendices 3a and 3b: Images of the T98 and T100 bacterial strain incubation and growth on day six.

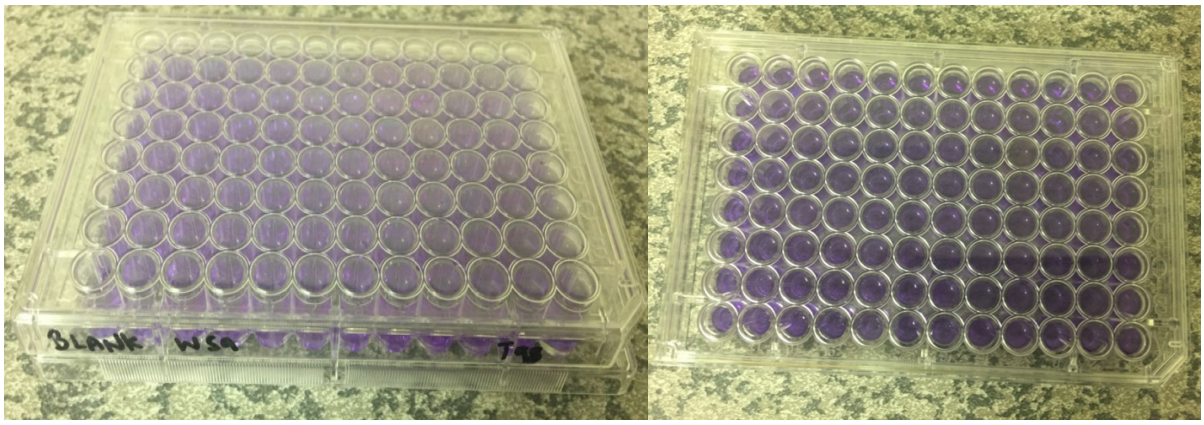
Bacterial strains prior incubation

3a

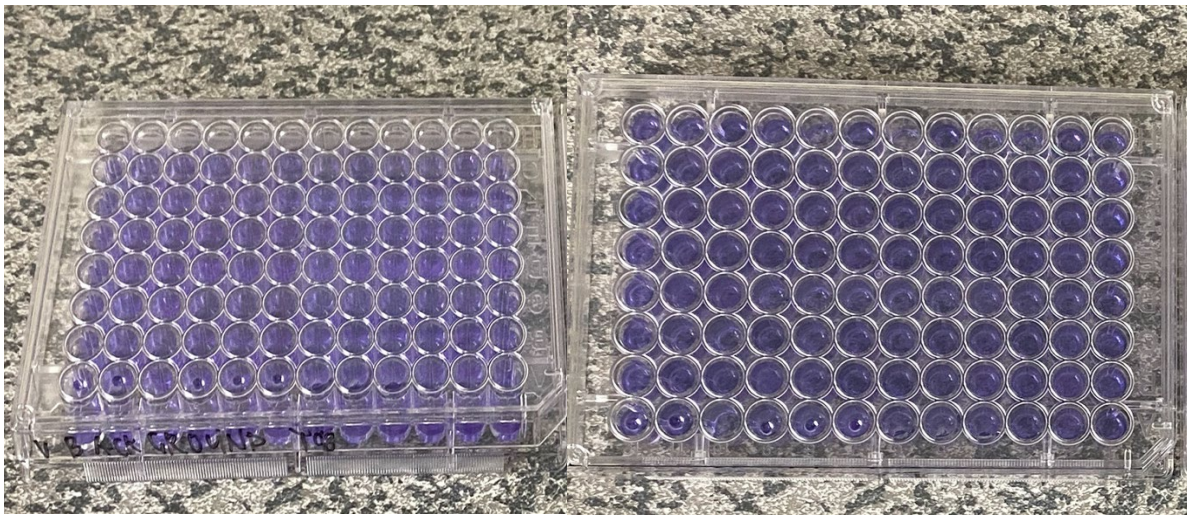
Bacterial strains after incubation demonstrating turbidity

3b

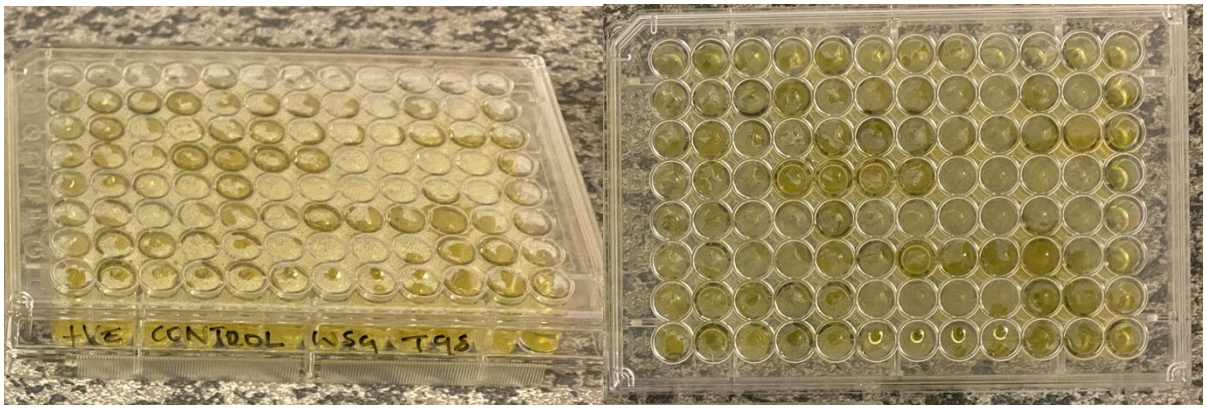
Appendices 4a -c: Ames mutagenicity test wells of blank, background and positive control of T98



Blank (a)

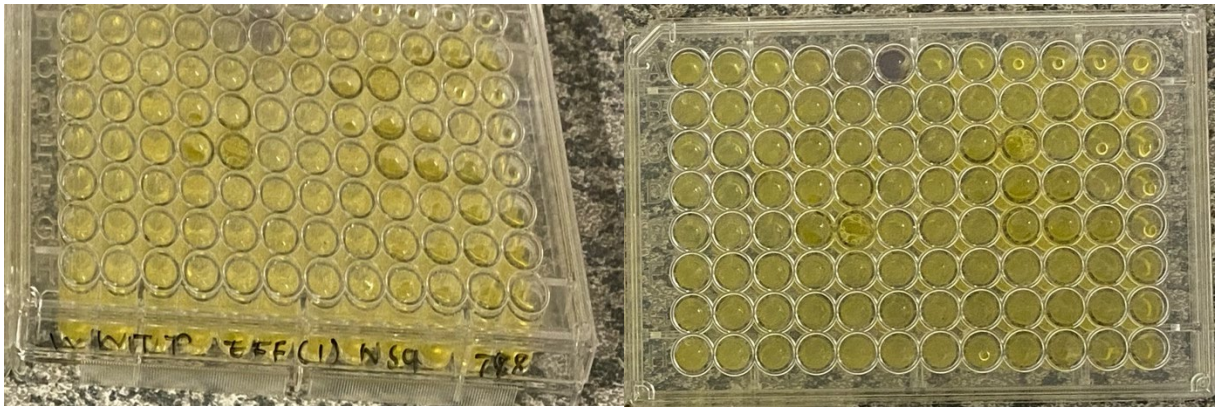


Background (b)

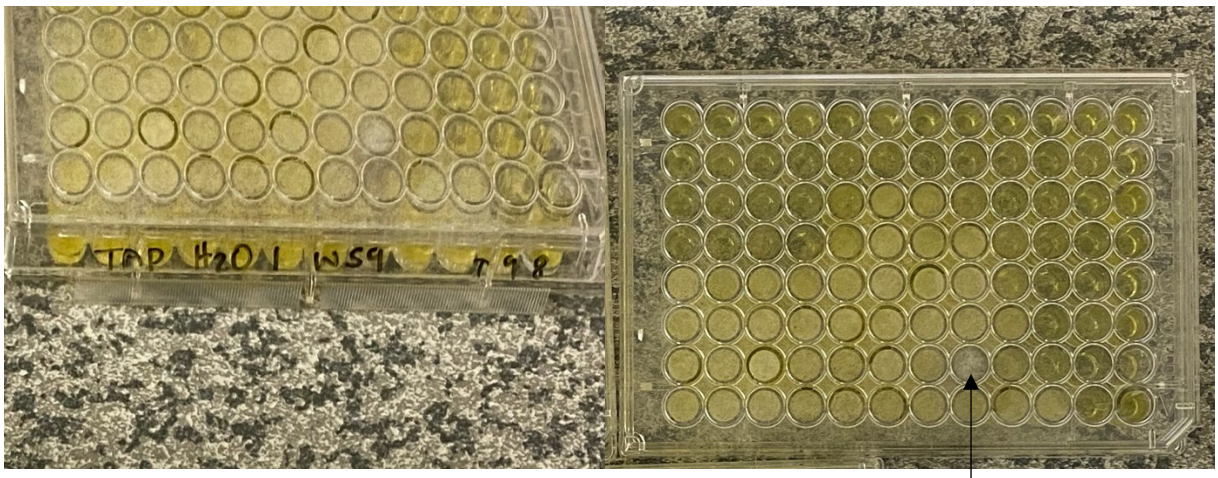


Positive control (c)

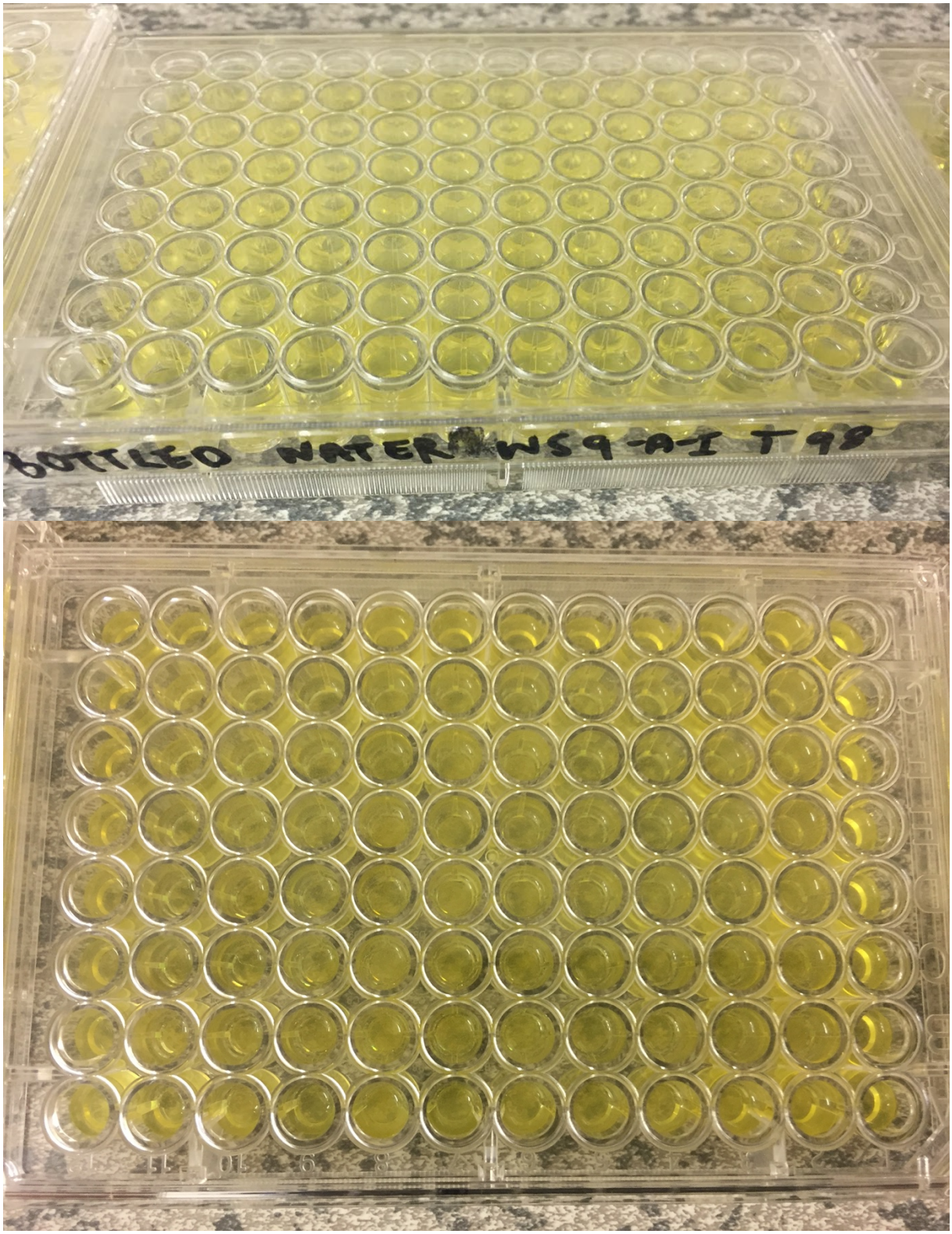
Appendices 5a-d: Ames mutagenicity test wells of T98 in WWTP effluent, tap water and bottled water samples



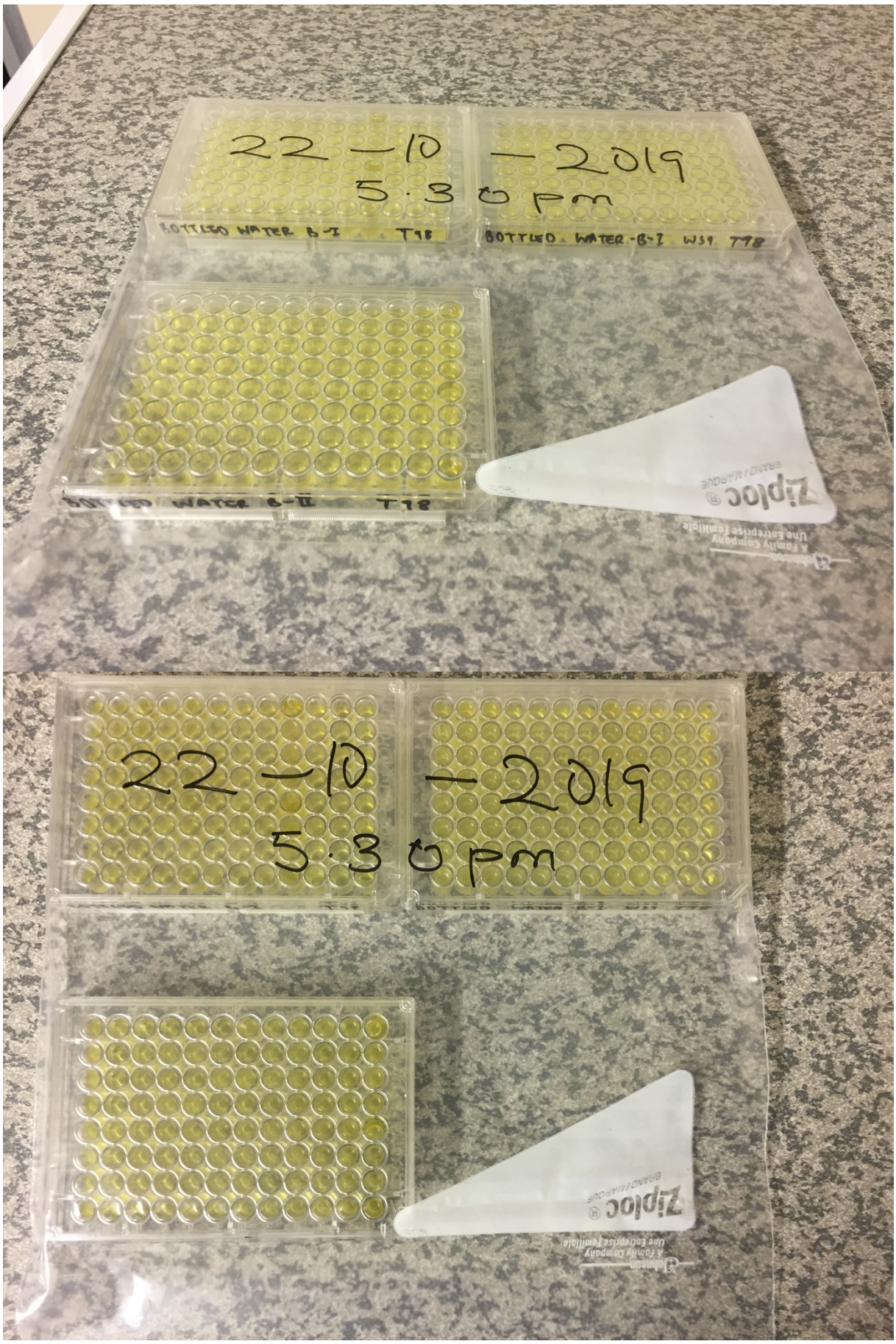
Stellenbosch WWTP effluent (a)



Tap water (b)



Bottled water brand ‘A’ (c)



Bottled water brand ‘B’ (d)