THE EFFECT OF ESSENTIAL OILS ON THE GROWTH OF BACTERIA FROM MUNICIPAL WASTEWATER TREATMENT

by

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AUTHOR'S DECLARATION

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ABSTRACT

THE EFFECT OF ESSENTIAL OILS ON THE GROWTH OF BACTERIA FROM MUNICIPAL WASTEWATER TREATMENT

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Bacterial sensitivity to essential oils has been reported in the case of soil isolated bacteria, food isolated bacteria but there is little evidence available to support the fact that wastewater isolated bacteria show sensitivity to essential oils. Keeping in view this fact the present investigation aims to determine the wastewater isolated bacterial strains sensitivity to six commercially available plant essential oils including clove, cinnamon, oregano, tea tree, fennel, and wintergreen. The essential oils were tested against ten laboratory bacterial strains (*Acinetobacter baumanii, Escherichia coli: DH5α, E.coli: AD202, Pseudomonas fluorescens, Pseudomonas poae, Pseudomonas putida, Staphylococcus aureus,* and *Stenotrophomonas maltophilia*) (2) and ten wastewater isolated bacterial strains (*Acinetobacter baumanii, Acinetobacter bouretii, Aeromonas hydrophila, E.coli, Enterobacter cloaceae, Flavobacterium branchiophilum, Klebsiella pneumoniae, Pseudomonas staurtii, Serratia fonticola,* and *Staphylococcus muscae)* using the Kirby-Bauer disc diffusion assay, and the broth tube macrodilution MIC assay. The disc-diffusion assay showed that three of the oils, clove, cinnamon and oregano, were the most effective at inhibiting the growth of all the known single isolates. The broth tube MIC assay found that the WWTP isolated bacterial strains such as *E. coli, Staphylococcus muscae, Enterobacter cloaceae, Acinetobacter baumanii* were most sensitive to clove oil at MIC concentration ≤ 0.52 mg/ml, cinnamon oil at MIC concentration ≤ 0.51 mg/ml, and oregano oil MIC concentration ≤ 0.47 mg/ml. Finally, wastewater microbial community samples from activated sludge, returned sludge and anaerobic digesters were reduced by 0% > 94.24%, 46% > 99%, 70% > 97% percent when tested against clove, cinnamon, and oregano oils.

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DEDICATION

This thesis is dedicated to my Champ Syed Ayaan Raza, Who has been a source of inspiration for me always.

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LIST OF ABBREVIATIONS

AT	Aeration Tank
BOD	Biochemical Oxygen Demand
CBC	Canadian Broadcasting Service
CEPA	Canadian Environmental Protection Act
CFU	Colony Forming Unit
DIG	Digester
DPB's	Disinfection Byproduct
ECCC	Environment and Climate Change Canada
EO's	Essential oils
FDA	Federal Drug and Food Administration
GRAS	Generally recognized as safe
HAAs	Haloacetic acid
HANs	Haloacetonitrile
HKs	Haloketones
MAC	Maximum Acceptable Concentration
MOE	Ministry of Environment
MIC	Minimum Inhibitory Concentration
NCCLS	National Committee on Clinical Laboratory Standards.
NDMA	Nitrosodimethylamine
NOM	Natural Organic Matter

PAA	Peracetic Acid
RS	Return Sludge
TiO_2	Titanium Dioxide
THM's	Trihalomethane
TOC	Total Organic Carbon
TRC	Total Residual Chlorine
TSB	Tryptic Soy Broth
USEPA	United States Environmental Protection Agency
WWTP	Wastewater Treatment Plant
WHO	World Health Organization
WT	Wild-type

CHAPTER ONE

1. INTRODUCTION

1.1 Background

Water is an invaluable natural resource for sustaining life. Globally, 1.8 billion individuals utilize contaminated drinking water sources (WHO 2011). The presence of contamination such as waterborne pathogens and chemical contaminants pose an invisible threat to the human population and can lead to various health complications (Fawell *et al*. 2003; Public Health Agency of Canada 2013). Urban runoff and wastewater treatment plants are primary contributors to the release of pathogenic bacteria and pharmaceuticals such as antibiotics (Rizzo *et al*. 2013). Moreover, the growing use of pharmaceutical products and their subsequent discharge into urban sewers may contribute to the increase in numbers of bacteria that develop resistance to antibiotics and metals due to mutations and horizontal gene transfer under this selective pressure (Davies & Davies 2010). Developing countries are exposed to polluted water sources due to the discharge of approximately 95% of their urban sewage as untreated wastewater directly into surface waters used as drinking water sources for their population (Pimentel *et al*. 2004). In Canada, each year the Great Lakes waterways are recipients of billions of liters of untreated raw sewage which includes pharmaceuticals emerging from both point, as well as non-point sources, as a result of dumping, runoff, wastewater effluent breaches, and combined sewer overflow (Environment Canada 2001; Burton 2013). For example, as recent as November 2015, the City of Montreal dumped 8 billion liters of its raw sewage into the Saint Lawrence River possibly releasing pathogenic, antibiotic-resistant bacteria (CBC News 2015).

Currently, wastewater treatment plants use disinfectants such as chlorine, chlorine dioxide, and chloramines which are powerful oxidants to attenuate pathogenic organisms in the effluent. The standard contact time of chlorine with effluent is 30 min as per standard guidelines (MOE 2008). However, residual chlorine in water can react with natural organic matter (NOM) resulting in the formation of disinfection by-products (DBPs) such as trihalomethane, bromodichloromethane, bromoform, and chloroform. There are about 600 DBPs known so far whereas a countless number of them still need to be identified (Richardson *et al*. 2007; Health Canada 2008; Hrudey, S.E. 2008). During peak flow time primary effluent and secondary effluent

bypass flows are disinfected using chlorine at levels ranging from 2.5 mg/L to 3.5 mg/L (City of Toronto 2010). Canadian Environmental Protection Act (CEPA) and Ontario guidelines, policies require chlorine levels below 0.02 mg/L to eliminate the risk of deleterious effect to aquatic life (Health Canada 1995; CEPA 1999; MOE 2008). The toxicity of very low levels of chlorine residuals to fish and marine life has become a concern to mitigate wastewater effluents a subsection wastewater system effluents regulation enacted under the Fisheries Act which is administered by ECCC (Environment and Climate Change Canada 2016). Studies have reported carcinogenic and deleterious effects of chlorinated wastewater effluents downstream from Canadian sewage treatment plants, demonstrating that levels in the effluent exceeding 0.02 mg/L may cause acute lethality to fish, and changes to the structure of the benthic invertebrate communities (Minister of Supply and Services 1993; Health Canada 1995; MOE 2008; Environment and Climate Change Canada 2016). Feasible alternatives to chlorine such as peracetic acid (PAA), ferrate, bromine compounds, pasteurization, ultrasonic cavitation, electron beam (E-Beam) and gamma irradiation, and photo-catalysis / titanium dioxide (TiO_2) have been studied and are under pilot studies. However, more research is needed to find disinfection products that are less toxic or biocontrol agents that are renewable, cost effective and environmentally friendly. Although chlorine is still considered the first choice for disinfection, it is important to examine additional technologies and additives that can be used to decontaminate water. Furthermore, since chlorine will most likely continue to be used, it is important to look at the synergy that these methodologies could have with chlorine use and the effect on pathogen dissemination and residual chlorine compounds in the water.

1.2 Research Hypothesis

Traditionally we have used chlorine as a primary choice disinfectant for water treatment, however, to increase the options available for water treatment other products need to be examined. Essential oils have been shown through the decades to have antibacterial activity and to be effective for food security and disinfection (Burt *et al* 2003; Winward *et al* 2008). Furthermore, they are natural products that are considered more environmentally friendly than chlorine. The goal of this research project is to determine whether plant essential oils can be used as an antibacterial agent to reduce or eliminate bacteria from the final effluent of an activated sludge treatment process in a municipal wastewater treatment facility.

Hypothesis: It is hypothesized that six plant essential oils would have an inhibitory effect on ten bacterial pure cultures and ten wastewater isolated bacteria due to their antibacterial property. To determine their antibacterial activity against wastewater isolated bacteria standard bioassays were employed as suggested by NCCLS 2014 which are disc diffusion assay (qualitative analysis) and broth tube dilution assay (quantitative analysis). One may predict an alternative or additive antibacterial agent which is naturally driven from renewable resources such as plants.

1.3 Research Objectives

To address the goal of this project, the short-term objectives are:

Objective 1. To determine the antibacterial activity of essential oils against known bacteria. Six essential oils (oil of clove (*Syzygium aromaticum*), oil of cinnamon (*Cinnamomum cassia*), oil of oregano (*Origanum vulgare*), oil of tea tree (*Melaleuca alternifolia*), oil of fennel *(Foeniculum vulgare*) and oil of wintergreen (*Gaultheria procumbens*) were chosen and tested against 10 known wildtype bacterial pure cultures (*Acinetobacter baumanii*, *E.coli*, *Pseudomonas fluorescens*, *Pseudomonas poae*, *Pseudomonas putida, Staphylococcus aureus* and *Stenotrophomonas maltophiles*) and 10 wastewater isolates (*Acinetobacter baumanii, Pseudomonas staurtii. Aeromonas hydrophila, E.coli, Klebsiella pneumoniae, Serratia fonticola, Staphylococcus muscae, Enterobacter cloaceae, Flavobacterium branchiophilum, Acinetobacter bouretii*) that had been previously isolated from the wastewater activated sludge treatment process by our lab and identified. The Kirby-Bauer antimicrobial susceptibility test was used to determine the sensitivity of the bacteria to each oil at a predetermined concentration. The results will indicate the efficacy of each of the oils.

Objective 2: To determine the minimum inhibitory concentration (MIC) of the oils needed to cause a significant inhibition of bacterial growth. This was accomplished by using a 2-fold dilution series of three of the essential oils against the bacterial strains.

Objective 3: To determine the inhibitory activity of the essential oils against mixed wastewater microbial communities collected from various aeration tanks and digesters in the WWTP. The Kirby–Bauer susceptibility assay (objective 1) was used along with the MIC of the oils determined in objective 2 to measure the inhibition of the oils against the mixed bacterial communities from four aeration tanks (AT-2, AT-4, AT-6, AT-8), two anaerobic digesters (DG-1, DG-2) and a return sludge (RS).

CHAPTER TWO

2. LITERATURE REVIEW

2.1 Prevalence of Multidrug Resistant Bacteria in Water Environment

The environmental balance of water bodies is being disturbed due to rapid urbanization in many parts of the world. Since urban populations tend to thrive near water bodies, they are considered responsible for the increasing amounts of contaminants such as pharmaceuticals, personal care products, organic matter, pathogenic bacteria and toxic compounds that enter our water system including wells, streams, lakes, and rivers (Ramaiah *et al.* 2002). In Canada, a recent study has shown that one in every five first nation reserves are affected by boil water advisories due to the unacceptable quality of drinking water contaminated with coliform as well as antibiotic resistant genes (Fernando *et al*. 2016).

Both surface water and groundwater in the province of Ontario are prone to contamination emerging from dumping, runoff, wastewater effluent breaches, and combined sewer overflow due to extensive land use by urban, industrial, rural, and agriculture within the Lake Ontario drainage basin (Hlavinek 2009). Every year the Great Lakes waterways are recipients of billions of liters of untreated sewage (Brubaker 2011). In Walkerton, Ontario, in 2000, a tragedy occurred due to contamination of well no.5 with *Escherichia coli* 0157: H7 and *Campylobacter jejuni* coming from runoff from a nearby cattle farm. About 2,300 individuals experienced gastroenteritis, 65 were hospitalized, 27 developed hemolytic uremic syndrome, and seven died (Hrudey *et al*. 2003). Similarly, the First Nations drinking water crises continue to pose a challenge as figures are gathered based on a water risk analysis model by the government which disclosed facts on 120 Ontario First Nation communities inspected for regional risk summary based on their water source and water treatment classification. The report summarized 158 water systems during an inspection and found that 72 were at high risk; 62 were considered medium risk and only 25 considered low risk (Murphy *et al*. 2015). Overall, many drinking water advisories are issued by Health Canada each year. For example, in 2011 alone, Ontario had 37 advisories, Alberta had 33 advisories, British Columbia had 31 advisories, and Saskatchewan had 20 drinking water advisories (Health Canada 2011). About 80% to 90% of all infectious diseases worldwide are due to waterborne pathogens (Epstein *et al*. 1994; Pimentel *et al*. 2007). Besides pathogens, pharmaceuticals are becoming emerging contaminants due to their excessive use in human and veterinary medicine (Fent *et al.* 2006). Pharmaceutical compounds are designed to target specific metabolic pathways

in humans and animals for therapeutic benefits; however, their impact on non-target organisms has the possibility of becoming deleterious even at very low concentrations (Moldovan 2006). Municipal wastewater is one of the main pathways by which these compounds can enter into the environment (Ternes and Joss 2004). Growing use of pharmaceuticals and their discharge into urban sewage may contribute to the increase in numbers of bacteria that develop antibiotic resistance due to mutations and horizontal gene transfer under this selective pressure (Davies and Davies 2010). In many cases, it has been inferred that bacteria tend to acquire resistance to multiple antibiotics producing multidrug-resistant bacteria or super bugs.

Urban runoff and wastewater treatment plants are hotspots for the release of antibiotics and pathogenic bacteria (Rizzo *et al.* 2013). Bacteria have a tendency to acquire resistance to antibiotics by gene swapping that may transform them into antibiotic resistant cells (Baquero 2008). Currently, developing countries are exposed to polluted water sources due to the discharge of approximately 95% of their untreated urban raw sewage directly into surface waters affecting about 50% of their population (Pimentel *et al*. 2007). As some studies show notable bacteria can be detected in wastewater treatment plant samples such as *Pseudomonas staurtii, Aeromonas hydrophila, E.coli, Klebsiella pneumoniae, Acinetobacter baumanii, Serratia fonticola, Staphylococcus muscae, Enterobacter cloaceae, Flavobacterium branchiophilum, Acinetobacter bouretii, Pseudomonas fluorescence, Staphylococcus aureus, Pseudomonas poae, Stenotrophomonas maltophiles* and *Pseudomonas putida* (Gilbride *et al.* 2006; Helt 2012).

Canada has devised stringent laws and policies to protect water from the source to tap. These laws are in place to reduce the threat of water contamination from pathogenic bacteria emanating as a result of dumping, runoff, wastewater effluent breaches, and combined sewer overflow (Environment Canada 2015; Environment and Climate Change Canada 2016). Based on evidence-based studies it is indicated that surface water and ground water is prone to contamination by pathogenic or antibiotic resistant bacteria which justifies a search for new innovative, alternative, antibacterial agents, novel molecules to be explored which may inhibit the growth of pathogenic and multidrug-resistant bacteria (Djeussi *et al*. 2013; Galvao *et al*. 2012).

2.2 Wastewater Treatment Process

Wastewater is the water after usage from residential, industrial, institutional and commercial sources that enters the wastewater treatment plant for processing. The city of Toronto has four

WWTPs that process nearly 340 million gallons of sewage a day. The increased volume of wastewater being produced in Toronto annually is partially due to the rise in the metropolitan population (City of Toronto, 2010). In conventional wastewater treatment, following an initial screening step, there are three main stages known as primary, secondary, and tertiary treatment (Fig. 1&2).

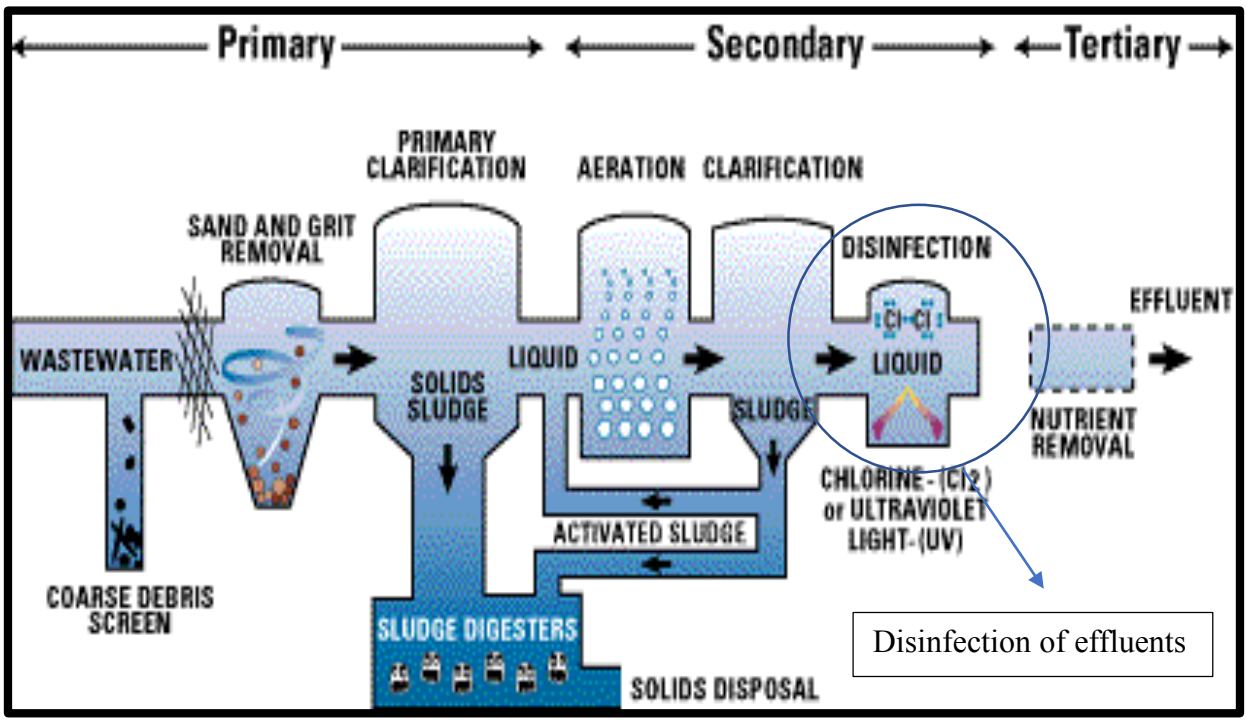


Figure 1. Conventional Wastewater Treatment Flow Diagram (Water Reuse. 2010).

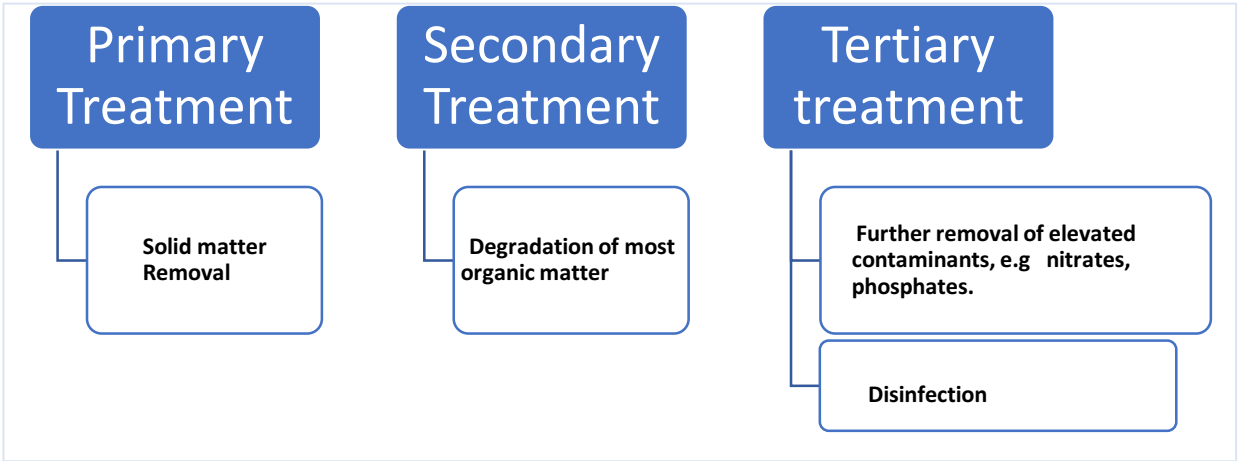


Figure 2. The Wastewater Treatment Plant Processes Stages.

After the wastewater is collected from the various sources, it is carried through a series of pipes to a wastewater treatment plant. Here the wastewater is first subjected to a preliminary process that physically removes large debris using bars and screens. Subsequently, primary treatment can occur in large tanks where the flow of wastewater coming in is reduced to hold the wastewater in these tanks for several hours. Meanwhile, heavier solids such as sand, soil, rocks, grit and other materials will sink to the bottom and are separated and removed. The remaining liquid, called the primary effluent, is then subjected to the secondary treatment (biological treatment). In this stage, the wastewater is blended with a bacteria-rich sludge that can digest dissolved colloidal organic materials which remain in the primary effluent. Oxygen is usually added in this step to aid in microbial growth and metabolism. The main purpose of this step is to reduce the biochemical oxygen demand (BOD) of the effluent. Often the sludge produced during aerobic treatment can be further treated by anaerobic treatment. Tertiary treatment is further applied if the additional removal of nutrients such as phosphorus or nitrogen is needed. If not, the wastewater is subjected to a disinfection method such as chlorination or UV to reduce disease-causing bacteria before being released into surface waters. Roughly 70%- 90% of facilities in North America use chlorine in one form or another (Health Canada 2008). Virtually all of the large plants discharging to Lake Ontario use chlorine, including all of the Toronto wastewater treatment plants (City of Toronto 2010). The disinfection of secondary effluent coming from clarifier in wastewater treatment plant is accomplished by dosing chlorine in any form (gas or liquid) and maintaining adequate contact time between the chlorine and the microorganisms. The chlorine residual remaining in the wastewater after a predetermined contact time can be measured to evaluate the effectiveness of the disinfection process since chlorine will be utilized if microorganisms remain in the effluent. The presence of a chlorine residual demonstrates that the microorganisms have been destroyed. The standard contact time of chlorine with effluent is 30 min as per standard guidelines (MOE 2008). Chlorine is known to have a deleterious effect on aquatic life at relatively low concentrations of 0.02 mg/ml. The toxicity of very low levels of chlorine residuals to fish and marine life has become a concern. Studies have reported effects of chlorinated wastewater effluents downstream from Canadian sewage treatment plants causing acute lethality to fish, and changes to the structure in benthic invertebrate communities when total residual chlorine levels, rises above 0.02 mg/L (Supply and Services Canada 1993; MOE 2008; Health Canada 2013). These effects were

noticeable and spanned up to 500 meters away from the outfall. Based on the deleterious effects on fish habitat, aquatic biota, chlorinated wastewater effluents have been enlisted in the Toxic Substances list of the Canadian Environmental Protection Act, 1999 (CEPA 1999). Accordingly, a guideline to maintain total residual chlorine (TRC) concentrations less than 0.02 mg/L in the wastewater effluent discharged to surface water has been established. During peak flow time primary and secondary effluent bypass flows are disinfected using chlorine. "At that point" the residual chlorine minimum limit of 0.02 mg/L level exceeds from 2.5 mg/L to 3.5 mg/L which is well above the standard set by CEPA and Fisheries Act (the City of Toronto. 2010; Environment and Climate Change Canada 2016).

Table 2.1. Wastewater Treatment Plant Residual Chlorine

Year	Average Flow (ML/d)	Average Chlorine Consumption (kg/d)	*E.coli* ([1]CFU/100 mL)	*CEPA/ Fisheries Act ([2]MAC) (mg/L)	Residual Chlorine (mg/L)
2006	701	1,531	3	0.02	0.9
2007	585	1,298	2	0.02	0.9
2008	645	1,434	2	0.02	0.9
2006-2008	644	1421	2	0.02	0.9

Source: City of Toronto. 2010

2.2.1 Disinfection By-Products (DBP)

Chlorine has been the first choice disinfectant for over a century ago. Water supply systems in Canada utilize chlorine as a disinfectant (Health Canada 2008) based on it being cost effective

[1] CFU Colony Forming Unit
[2] MAC Maximum Allowable Concentration. Found in *CEPA, 1999; *Fisheries Act; MOE 2008; wastewater system effluent regulation 2016.

while having good efficacy in removing a wide variety of pathogens. Studies show that chlorine as a strong oxidizing agent can react with natural organic matter (NOM) usually measured as total organic carbon (TOC) during or after the chlorination process of secondary effluent leaving minimum residual chlorine limits between 0.2 mg/L to 4 mg/L (Table.2.1). Perhaps the most challenging and daunting task ever faced by drinking water industry for the past three decades is the formation of DPB's (disinfection byproducts) as a result of disinfection of effluent by using chlorine and its precursors. There are about 600 DBP's known so far whereas a countless number of them still need to be identified (Richardson *et al*. 2007; Health Canada 2008; Hrudy, S.E. 2008). Chlorinated disinfection byproducts are a group of chemical substances that are unintentionally produced as a byproduct of the disinfection process mostly when the disinfectant reacts with naturally occurring organic matter (NOM) in surface water, or drinking water (Health Canada 2016). Some of the more common DBPs are trihalomethane (THMs), bromate, chlorite, nitrosodimethylamine (NDMA) and haloacetic acid (HAAs) which are regulated (Table 2.2). However, other DBPs are also formed that are not regulated in Canada so far such as iodo THMs, haloketones (HK's), haloacetonitriles (HAN's) (Richardson *et al.* 2007). In Canada, guidelines set standards for some of the DBP's with a maximum acceptable concentration (MAC) in water sources such as Trihalomethanes (THM4) 0.1 mg/L, N-Nitrosodimethylamine (NDMA) 0.00004 mg/ml Haloacetic acid (HAA) 0.08 mg/L, Bromodichloromethane (BDCM) 0.01 mg/L, and chlorite 1 mg/L (Health Canada 2014).

Table 2.2. Some Regulated and Nonregulated Disinfection Byproducts in Canada.

Regulated Disinfection By-Products Maximum Acceptable Concentration (MAC) in Canada.		**Non-Regulated Disinfection By-products in Canada. (Maximum Acceptable Concentration (MAC) in US).**	
***Trihalomethanes (THMs)**	**0.10 mg/L**	****Haloacetonitriles (HANs)**	**0.7 ng/L**
***Haloacetic acid (HAA)**	**0.08 mg/L**	****Haloketones (HKs)**	**0.7 ng/L**
***Bromate**	**0.08 mg/L**	****Iodo THMs**	**-------**
***Chlorite**	**1 mg/L**		
***N- nitrosodimethylamine (NDMA)**	**0.00004 mg/ml**		

Source: Regulated by Health Canada 2014*; Regulated by US EPA 2006**

Health Canada and provincial ministries reports are indicative of the fact that formation of disinfection by-products in Canadian water sources (Chowdhury *et al*. 2011). Studies suggest that disinfection by-products might be linked to possible cancer and mutagenic risk to animals and

humans along with other health implications such as miscarriage, stillbirth, low birth weight, pre-term delivery and cardiac anomalies (Ashbolt, 2004; Gopal *et al*. 2007; Richardson *et al*. 2007). Mode of exposure of disinfection by-product may be through ingestion of drinking water, inhalation, or dermal exposure (USEPA, 2006). Demographically approximately 75% of the population in Canada thrives in cities (Statistics Canada, 2008) where the main source of drinking water is municipal water system, therefore, much of the population may have been exposed to disinfection byproducts throughout their lifetime (Health Canada, 2008). The microbiological risk from improperly disinfecting the water, however, is more evident than presence of those disinfection by-products in drinking water (MOE, 2008; WHO, 2000) and therefore chlorine is still the disinfectant of choice. More recently, some municipal water systems have implemented alternative disinfection system such as UV disinfection system to reduce the exposure to DBPs without compromising proper disinfection (Health Canada, 2008; USEPA, 2006).

2.2.2 Quest for Alternative Disinfection

Based on the literature published so far Epstein *et al*. 1994; Fawell *et al*. 2003; Ashbolt, 2004; Gopal *et al*. 2007; Richardson *et al*. 2007; Pimentel *et al*. 2007; Hrudy, S.E. 2008; Brubaker 2011; Chowdhury *et al*. 2011 surface water and groundwater can be contaminated by pathogenic or antibiotic resistant bacteria and high chlorine residual along with formation of disinfection byproducts has become emerging challenge for regulatory authorities and water industries, in view of above facts it is justified to look for alternative water disinfection methods or synergistic constituents that are environmentally friendly (Djeussi *et al.* 2013; Galvao *et al.* 2012). A very few research papers published (1998-2015) have tested plant extracts, plant proteins and essential oils for their antibacterial potential against surface water isolated bacteria and gray water disinfection. For instance, one study was done on river water isolated bacteria "preparation of *Moringa Oleifera* flower to treat contaminated water" (Maiara *et al.* 2011). The researcher used aqueous extract and precipitated protein fraction from *Moringa oleifera* that showed antibacterial activity against gram-positive and gram-negative bacteria derived from the river, lake water. Further, it was concluded that antibacterial activity was due to *Moringa oleifera* flower preparation could be a potential source of the disinfectant agent to treat contaminated water. In a similar paper published in 2008 entitled "Essential oils for the disinfection of greywater" eight essential oils were tested to disinfect gray water collected from bathroom sinks, bath, and shower it was reported that all essential oils

showed effective results. The current research project is based on the same principles that determined plant essential oil antibacterial activity (Bauer-Kirby, 1966, F. Karanagh, 1972 and Brantner *et al.* 1994) against waterborne pathogenic bacteria. This study might add to knowledge to establish the fact that plant essential oils could be another possible alternative solution to inhibit pathogenic and antibiotic resistant bacteria found in water and wastewater. As previous studies have indicated, plant essential oils are well known for their antibacterial properties against a broad range of gram positive and gram negative bacterial pathogens (Edris 2007; Lang and Buchbauer 2012; Teixeira *et al*. 2013).

2.3 Plant Essential Oils

We depend on plants and their components for our fundamental needs such as food, medicine and natural antimicrobial agents (Rai and Kon 2013). Earth has a rich renewable resource of roughly 250,000 higher plant species among which 80,000 species are medicinal (Joy *et al*. 1998). Since the ancient time, it has been recognized that some plant possesses antimicrobial potential (Finnermore 1926). Over the centuries, mankind has used plants and their parts such as leaves, stems, roots, flowers, seeds, and fruits to cure diseases and relieve physical suffering (Trease and Evans 2009). An essential oil is volatile organic oil which is mostly an aromatic oily liquid derived from different plant parts such as root, stem, leaves, flower, fruit, seeds, twigs, bark, buds, wood, resin and peel through steam distillation (Guenther, 1948; Lawless 2013; Sangwan *et al*. 2001). Plants are a valuable natural resource that is not only renewable but is also considered environmentally friendly (Chemat *et al*. 2012). Natural antibacterial components such as plant essential oils, plant extracts, and plant proteins possess antibacterial activity against a broad range of bacteria (Trease and Evans 2009; Maiara *et al.* 2012; Rai). These plants contain chemical constituents such as essential oils, tannins, polyphenols, terpenoids, phytoalexins, isothiocyanates allicins and anthocyanins (Somaatmadja *et al.* 1964; Beuchert *et al.* 1989; Delaquis and Mazza 1995; Lis-Balchin and Deans 1997; Cutter 2000). Nearly 3,000 essential oils are estimated among them with 300 being produced commercially for pharmaceutical purposes as antimicrobial agents (Mendes *et al*. 2010). Various studies have shown the efficacy of essential oils and their constituents as antibacterial, antiviral, antifungal, insecticidal, herbicidal and antiparasitic potential (Bishop 1995; Carson *et al*. 1995; Hammer *et al*. 1999; Burt *et al.* 2004; Campiglia *et al*. 2007; Edris 2007; Ayvaz *et al.* 2010; Stephanie de Rapper *et al*. 2013; Monzote *et al*. 2014).

Further phytochemistry has shown that the antimicrobial potential of plant essential oil is due to compounds such as carvacrol, eugenol, anethole, cinnamaldehyde, cineole, linalool, terpinen-4-ol, and methyl salicylate (Trease and Evans 2009). Essential oils and plant extracts are now gaining acceptance as phytodisinfectants (Yongabi *et al*. 2011), biopesticides (Mohan *et al*. 2011), aromatherapy (Ali *et al*. 2015), pharmaceutical (Edris 2007) and food preservatives (Burt *et al.* 2004).

2.3.1 Historical Context

According to, the medieval forerunner of chemistry also known as Alchemist, the "soul of a plant is its oil, and its spirit is the plant's alcohol or tincture" (Lawless 2013). Historically, fragrant plant oils have been utilized for many years, as incense, perfumes, cosmetics and for their therapeutic and culinary applications. With the advent of human civilization, plant essential oils have been part and parcel of early culture, where their religious and healing roles became inextricably merged. For instance, in the east, springs of juniper are burnt for purification in Tibetan temples, Arabs in middle east use perfumes derived from rose and jasmine in their religious ceremonies. Similarly, in the west, frankincense is used during the Roman Catholic congregation ceremonies (Lawless 2013).

2.3.2 Ancient History

The knowledge of the healing power of plant products and their safe usage is as old as the dawn of human civilization. The history of using plant products goes back to the period of early Romans, Greeks, Egyptians, Indians, Arabs, Australians, Africans, and Europeans (Dias *et al.* 2012). The application of healing power of plants started from the use of poultices and imbibed infusion of thousands of indigenous plants such as hollyhock dating back to the era of Neanderthals (Thomson, W.A.R (ed) 1978; Leroi-Gourhan 1975; Lietava 1992) with actual written evidence documented as far back as the Sumerians (3000-5400 BC) and Akkadians (2270-2083 BC) of ancient Mesopotamia (Costa *et al.* 1999; Sinclair and Hechtman 2011).

The (1470-1670) era witnessed many publications on herbalism including the "Grete Herbal," and the concept of herbal medicine emerged. For the first time, Paracelsus von Hohenheim (1493-1541) used the term essential oil for a natural product and this term refer to alchemist idea of sublime extractive as "Quinta Essentia" material which acts as an active

ingredient of pharmacologically important drugs (Guenther 1948; Haagen-Smit. 1961). By the 16th Century, the use of essential oils and their production by distillation became widespread in Europe.

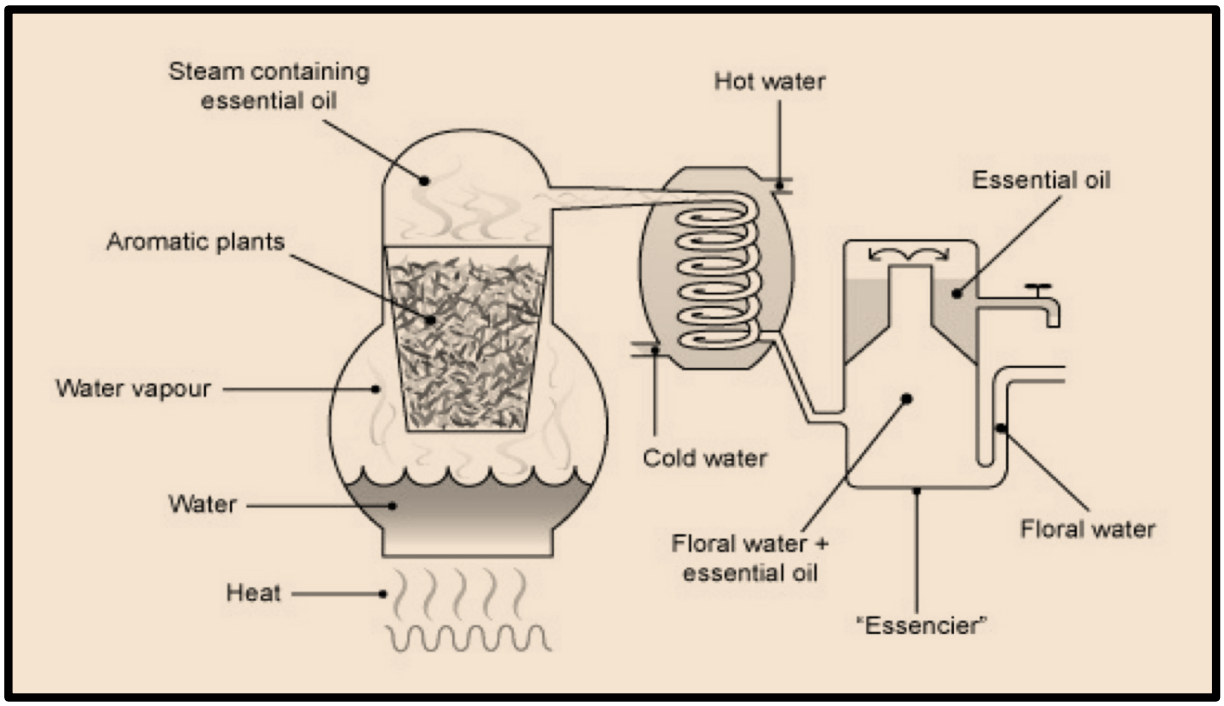
In the 17th Century, the preparation of essential oil was well known, and pharmacies stocked 15-20 different oils (Guenther 1948). One of the traditional herbs that later on became a drug by the end of 17th century was *Digitalis purpurea* L (commonly known as Foxglove) that defined the pathway for modern pharmacology (Goldman 2001). In the 18th Century during colonization of Australia, tea tree was used for a medicinal purpose which was already practiced by the natives of Australia long before. By 19th-20th Century, use of essential oils medicine gradually become less with the passage of time and was used mostly for flavors and aroma. However, researchers are still focusing their attention on the antimicrobial activity of natural products. Recently researchers have extracted 30000 antimicrobial compounds from 1340 plants (Tajkarimi *et al*. 2010).

2.3.3 Current Uses of Essential Oils

The number of species of plants which are aromatic is estimated to be 17500, distributed across approximately 60 plant families some of which are Asteraceae, Lauraceae, Lamiaceae, Myrtaceae, Pinaceae, Rosaceae, Rutaceae, and Umbelliferae (Bakkali *et al.* 2008). The essential oils and their components are used successfully as dental canal sealer, fractions are formulated in shampoos, toothpaste, disinfectants, topical ointments, drugs, multivitamins, and cosmetics, perfumery (Manabe *et al.* 1987), as phytodisinfectants such as *Moringa oleifera* (Yongabi *et al.* 2011), as food preservatives to enhance shelf life, (Burt 2004), as pharmaceuticals such as aspirin, quinine, morphine, and eugenol and as biopesticides such as piperidine (Mohan *et al*. 2011). Due to their relatively safe status "GRAS" Generally recognised as safe (FDA 2016) some essential oils are gaining interest among the consumers based on the fact that they are also considered safe for the environment and mimic the effect of developing resistance to pathogenic bacteria due to diversity of mechanism of action of essential oils their application in packaging material, coated onto polyvinyl surface (Appendini 2002). Highly volatile, essential oils are sometimes lost due to evaporation during the packaging process of finished processed food using microencapsulation technology encapsulating antimicrobial EOs, but they also can be released in products at a controlled rate to deliver effective inhibitory concentrations over extended periods and thereby extend shelf life (Iraj Rasooli 2007).

2.3.4 Commercial Production Process of Plant Essential Oils

The extraction of essential oil is done by steam distillation, cold pressing, or using organic solvents; the oil yield is dependent on environmental conditions and distillation processes. The yield can vary between batches from the same trees and between different sites of their collection (Price 1987). Various techniques for the production of commercial essential oils are used such as the conventional steam distillation method where plant material is placed in a distillation apparatus containing boiling water which produces steam and oil which are recaptured and finally separated out to produce essential oil. An example of steam distillation is seen in the Figure. 3. However, the liquid carbon dioxide or supercritical carbon extraction methods are used when there is less oil present in flowers, and it is performed under low temperature and high pressure. These methods are employed in industrial manufacturing units throughout the world.



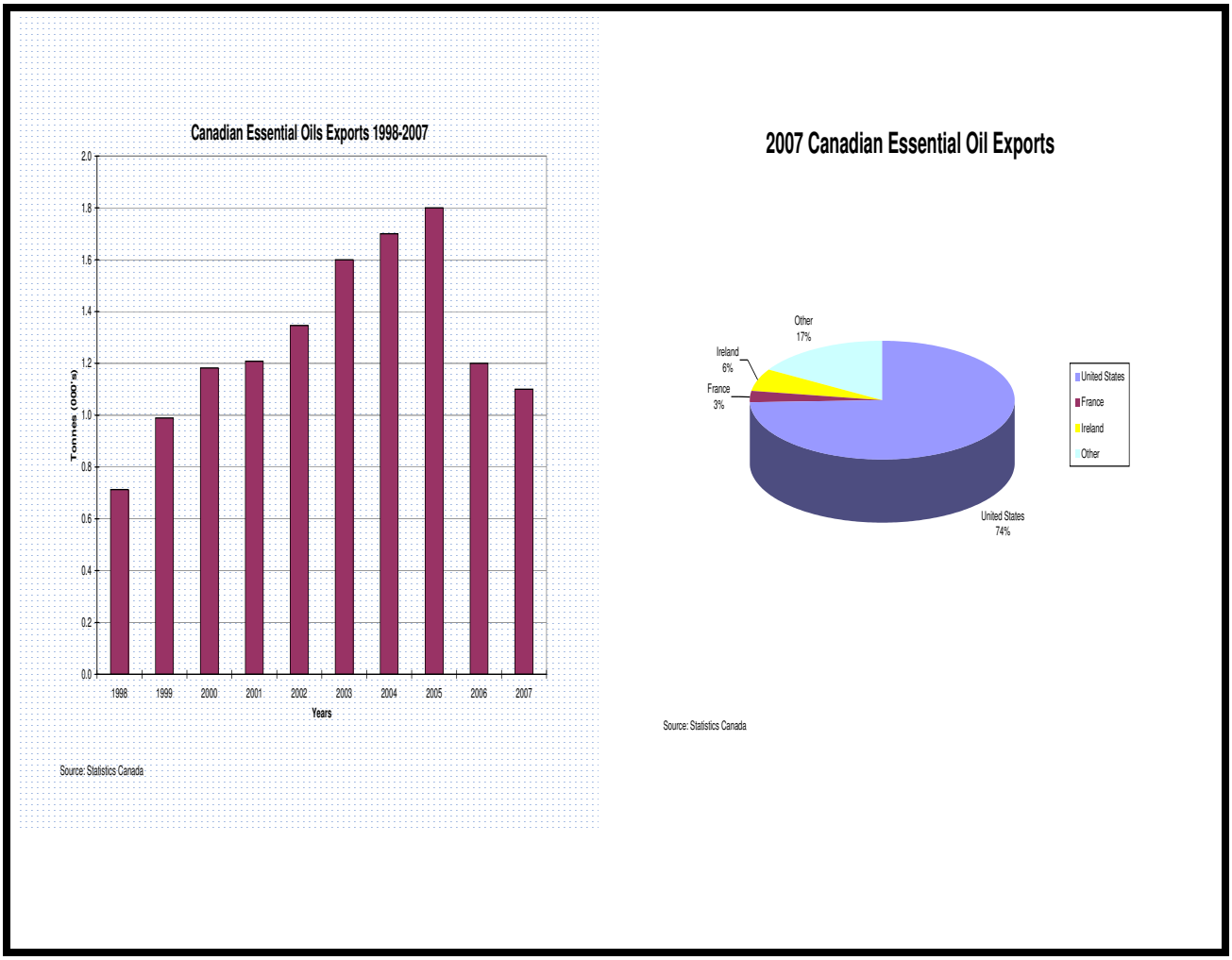
source: (*http://www.union-nature.com*)

Figure 3. Steam Distillation Manufacturing Process Essential oil.

2.3.5. Commercial Production of Plant Essential Oil Analysis

A rough estimate predicts that 3000 plant essential oils are reported in the literature, whereas 300 of them are of commercial importance (Tuley de Silva 1997). In the year 1999, globally the

business of plant essential oil sales around the world reached $15 billion in total among which Europe had $7 billion in sales, Japan's sales reached $2.4, Asia's sales reached $2.7, and North America's sales reach $3 billion. Commercially an approximate production of 40,000 to 60,000 tons per annum has been reported worldwide with an ever increasing trend regarding consumption (Djlani and Dicko. 2012). An overview of world and Canadian exports of plant essential oil depicts its ever growing need within the global market (Fig 4, Table 2.3).



SOURCE: Statistics Canada, 2008

Figure 4 Canadian Essential Oil Exports (1998-2007)

Table 2.3. Production Analysis of Important Essential Oils (2008)

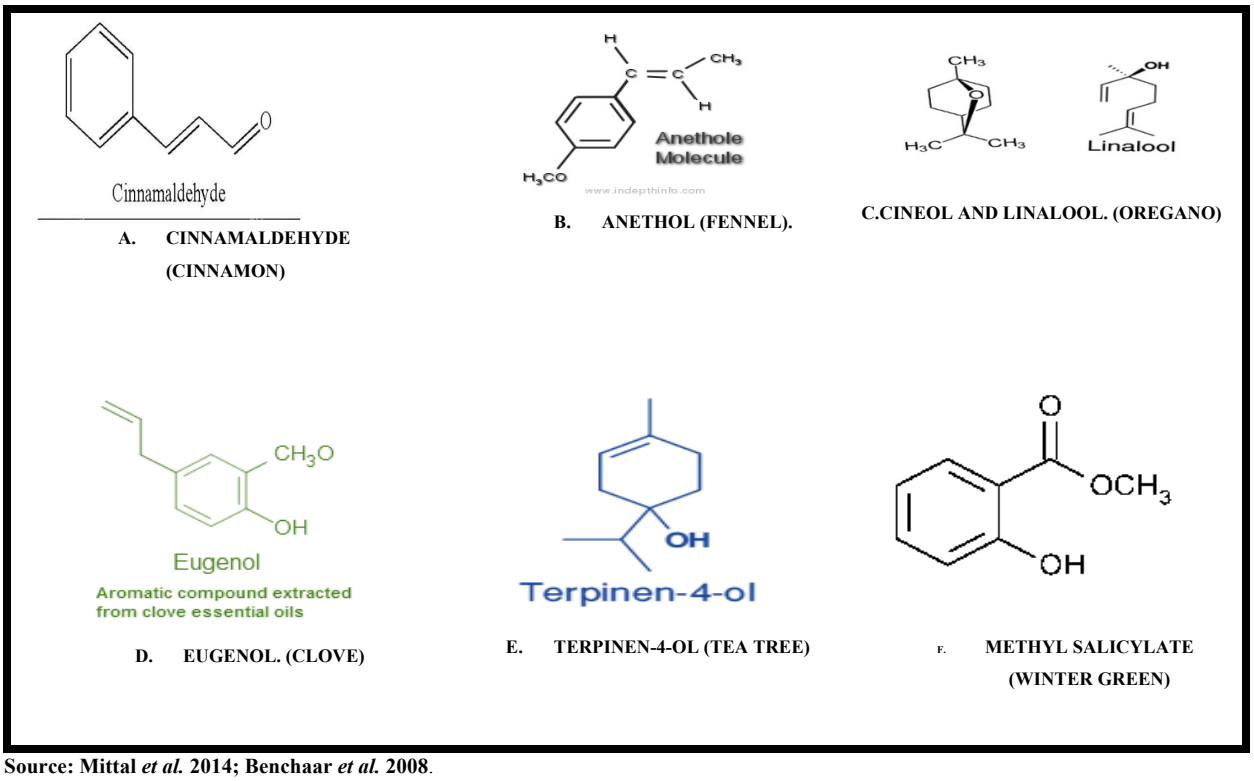
Essential Oil	Production (Metric Tons)	Countries
Orange oils	51000	USA, Brazil, Argentina
Corn mint oil	32000	India, China, Argentina
Lemon oils	9200	Argentina, Italy, Spain
Eucalyptus oils	4000	China, India, Australia, South Africa
Peppermint oil	3300	India, USA, China
Clove leaf oil	1800	Indonesia, Madagascar
Citronella oil	1800	China, Sri Lanka
Spearmint oils	1800	USA, China
Cedar wood oils	1650	USA, China
Litsea cubeba oil	1200	China
Patchouli oil	1200	Indonesia, India
Lavandin oil	1100	France
Corymbia citriodora oil	1000	China, Brazil, India, Vietnam
Oregano oil	15-20	Turkey
Cinnamon oil	90	Sri Lanka
Clove oil	2000	Indonesia, Madagascar, Sri Lanka
Tea tree	2-20	Australia
Fennel oil	0.35	Turkey
Wintergreen	50-80	China

Source: Lawrence 2009; Trease and Evans 2009.

2.3.6 Composition of Plant Essential Oil

Antibacterial activity of plant essential oils can be attributed to the chemical constituents and functional groups present in plant essential oils, the proportions in which they are present, and the interactions between them (Dorman and Deans 2000). higher inhibition activity of clove, cinnamon, oregano, essential oil are due to the main constituents, cinnamaldehyde, eugenol,

thymol, and carvacrol, against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Lambert *et al.* 2001). It has been observed that the combination of thymol and carvacrol exhibited higher antibacterial activity than either compound alone and further that the inhibitory effect of oregano is mainly due to the additive antibacterial action of these two compounds (Delaquis *et al.* 1995). The detailed compositional analysis achieved by gas chromatography and mass spectrophotometry of essential oils have shown that phenolic compounds are chiefly responsible for antimicrobial activity (Cosentino *et al.* 1999). A chemical structural formula of the main antibacterial components of plant essential oils is seen in Figure 5. As reported by various researchers from (1999 to 2014) cinnamon essential oil possesses 80 % cinnamaldehyde (Mith *et al.* 2014), clove essential oil possess 85% to 95% eugenol (Moreira *et al.* 2005), oregano essential oil possesses 46% cineole and 26.1 % linalool (Ultee and Smid 2001), fennel essential oil possesses anethol 55% to 75 % anethol (Hammer *et al*. 1999), tea tree essential oil possess 40.1 % terpine- 4-ol (Hammer *et al*. 1999) and winter green possesses methyl salicylate 95% methyl salicylate (Hammer *et al*. 1999).



Source: Mittal *et al.* 2014; Benchaar *et al.* 2008.

Figure 5. Structural Formula of Essential Oils Constituents of Clove, Cinnamon, Oregano, Fennel, Tea tree, Wintergreen.

Table 2.4. Essential oil Distribution, Antibacterial Components, Literature value.

Essential oil	Plant Species	Antibacterial components	Part of Plant	Origin
Cinnamon	*Cinnamomum zeylanicum*	Cinnamaldehyde 65 % - 80%	Bark of tree	Sri Lanka
Clove	*Syzygium aromaticum*	Eugenol 75% - 85%	Bud	Madagascar
Oregano	*Origanum vulgare*	Carvacrol 80% Cineole 46 % Linalool 26.1 %	Leaves	Mediterranean, Europe
Tea tree	*Melaleuca alternifolia*	Terpnen-4-ol 40.1 %	Leaves	Australia
Fennel	*Foeniculum vulgare*	Anethole 55-75 %	Seed	Spain
Wintergreen	*Gaultheria procumbens*	Methyl Salicylate 95 %	Berries	North America

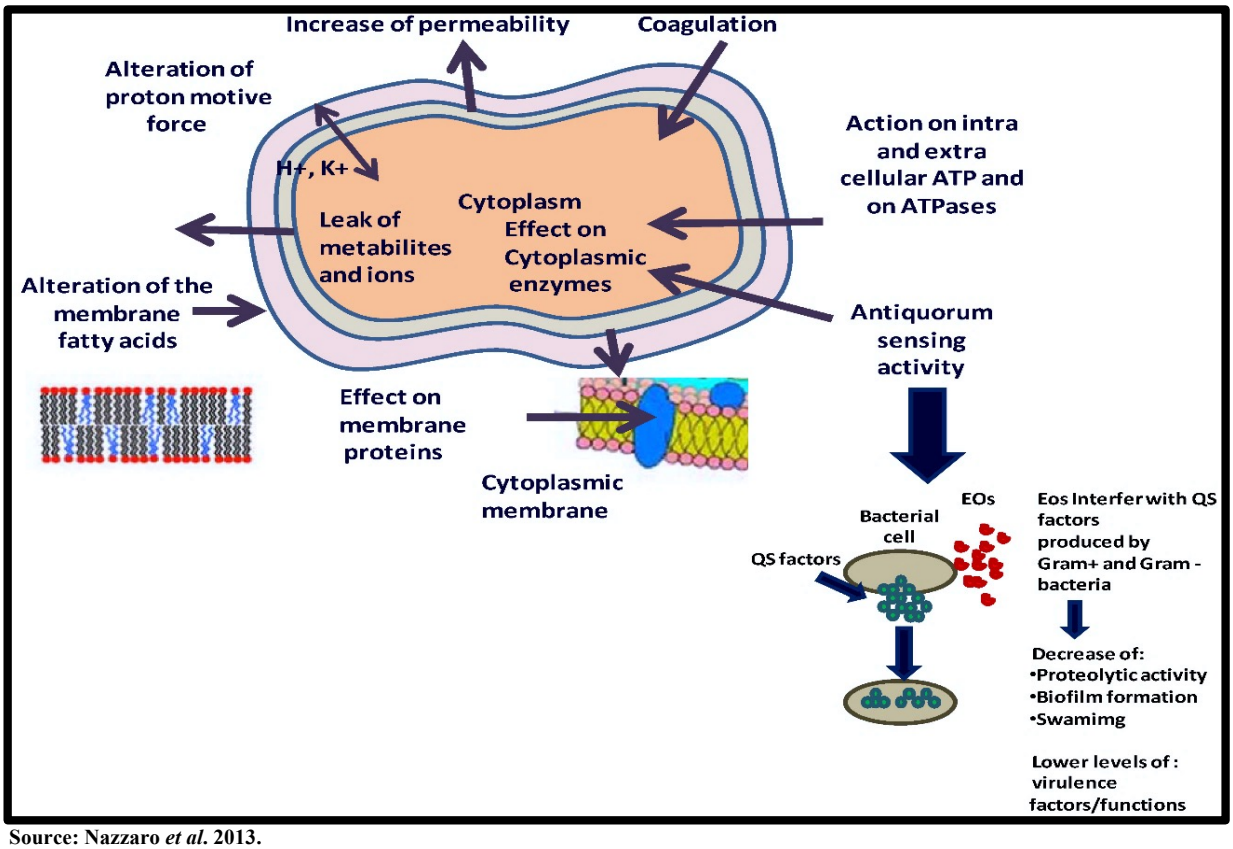
Source: Hammer *et al.* 1999, Ultee and Smid, 2001, Mith *et al.* 2014, Moreira *et al*. 2005

This study examines the antibacterial potential of six plant essential oils by employing Kirby-Bauer disc diffuaion assay against bacterial pure cultures. The three most effective essential oils were then selected for qualitative and quantitative analysis of bacterial communities from a wastewater treatment plant process.

2.4 Antibacterial Mode of Action

Studies show that antibacterial activity of essential oil is due to different modes of action of essential oil on bacterial cell such as cell wall and membrane disturbance, ATP production imbalance, protein synthesis imbalance, pH disturbance, intracytoplasmic changes, bacterial DNA mutation, anti-quorum sensing activity (Oussalah *et al.* 2007; Becerril *et al*. 2012; Burt *et al*. 2004; Bouhdid *et al.* 2009; Turgis *et al*. 2009; Khan *et al*. 2009; Brackman *et al*. 2011). Studies show the antibacterial activity of essential oils is linked to hydrophobicity resulting in greater cell permeability and ultimately leakage of cell constituents (Ultee *et al*. 2000; Lambert *et al*. 2001; Caillet *et al*. 2005). Scanning electron microscopy (SEM) observation of food borne bacteria *E.coli 0157:H7* and *Salmonella enterica* showed the presence of white spots and holes on the cell wall, distorted and unfinished cell shape when exposed to orange, mustard, and spanish oregano

essential oil (Gaunt *et al*. 2005; Turgis *et al*. 2009). Studies reported the action of essential oil components such as carvacrol and p-cymene triggered the production of heat shock protein (HSP) in *E.coli 0157:H7* (Burt *et al*. 2003). Some studies also reported intracellular ATP loss through membrane disturbance (Oussalah *et al*. 2007; Turgis *et al.* 2009) in bacterial strains such as *E.coli 0157:H7*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella enterica.* Bacteria showed a decrease in intracellular ATP when exposed to oil of mustard, oil of oregano and carvacrol at their MIC concentration (Ultee *et al.* 2000; Caillet *et al.* 2005; Turgis *et al.* 2009). Similarly, a decrease in pH has been reported in *E.coli 0157:H7*, *Salmonella enterica, Listeria monocytogenes*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* when exposed to oil of oregano, oil of cinnamon, oil of savory and oil of mustard (Ultee *et al.* 2000; Oussalah *et al.* 2007; Turgis *et al.* 2009).



Source: Nazzaro *et al.* 2013.

Figure 6. Modes of Action of Plant Essential Oils on the Bacterial Cell.

Some studies show anti-quorum sensing activity of cinnamon, clove, geranium, lavender and rosemary (Khan *et al*. 2009; Brackman *et al*. 2011).

2.4.1 Antibacterial Studies

Based on a literature review of articles published (1994-2016 see reference below), six essential oils were selected for study based on their antibacterial properties and their minimal toxicity. The six essential oils were oil of clove (*Syzygium aromaticum* L.), oil of cinnamon (*Cinnamomum zeylanicum.* (Nees & T.Nees, J.Presl), oil of oregano (*Origanum vulgare* L.), oil of tea tree (*Melaleuca alternifolia* (Maiden & Betche), Cheel), oil of fennel *(Foeniculum vulgare* Mill.), and oil of wintergreen (*Gaultheria procumbens.* L.). Previous studies have shown antibacterial properties of the above mentioned essential oils against a wide array of bacteria.

2.4.1.1 Clove Essential Oil

Extraction of clove oil is done from the flower buds of *Syzygium aromaticum* L., which belong to the family Myrtaceae, a tree 10-20m high that is indigenous to melaleuca or clove island; oil of clove is a colorless, pale yellow liquid. The major chemical constituents are eugenol (84-95 %), acetyl eugenol (3%), carvacrol and cinnamaldehyde (Trease and Evans, 1989). Eugenol is utilized widely in perfume and flavoring industries. The FDA considers eugenol as Generally Recognised As Safe ("GRAS"). A literature review of eight relevant articles published between 1998-2014 used clove essential oil against a wide array of bacteria isolated from sources such as food, food spoilage, soil, and hospital. In antibacterial studies, Kirby-Bauer disc diffusion method has been employed to test bacterial sensitivity to clove essential oil the results showed significant antibacterial activity against bacterial strains such as *E.coli, E.coli:* 0157*, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis, Proteus vulgaris, Salmonella enteritis, Campylobacter jejuni* (Smith-Palmer *et al.* 1998; Hammer *et al.* 1999; Burt *et al*. 2003; Dorman and Deans 2003; Carson *et al.* 2006; Roller *et al*. 2009; Rather *et al.* 2012). Furthermore, clove essential oil have reduce the bacterial population completely at or below MIC value of 5000 mg/l level for *Escherichia coli, Staphylococcus aureus, Bacillus cereus, Yersinia enterocolitica* (Siddiqua *et al*. 2014). In another study, de Rapper *et al*. (2013) reported MIC against *Staphylococcus aureus and Pseudomonas aeruginosa* of 1.5 mg/ ml. Similarly, (Burt *et al.* 2003) reported a MIC value of 0.4-2.5 ul/ml each based on microdilution assay for clove essential oil against food isolated pathogens such as *E.coli and Staphylococcus aureus.* It was also reported that MICs against *Klebsiella pneumoniae* and *E.coli* were > 6.4 mg/ml and >1.6 mg/ml

respectively (Prabuseenvasan *et al.* 2006). Moreira *et al*. (2005) reported a MIC value for clove essential oil against *E.coli* ATCC 25158 of 0.25 ml/ 100 ml.

2.4.1.2 Cinnamon Essential Oil

Extraction of Cinnamon oil is from the bark of the shoots grown on the cut stock of *Cinnamon cassia* Blume. It is native to Sri Lanka and cultivated in Ceylon, Madagascar, Jamaica, and Brazil. The major chemical constituents are cinnamic aldehyde and 4-10 % eugenol (Trease and Evans 1989). FDA considers cinnamon essential oil as Substance Generally Recognised As Safe ("GRAS"). A literature review of eight relevant articles published between 1998-2014 used cinnamon essential oil against a broad range of bacterial strains isolated from sources such as food, soil, and hospital. Studies conducted on essential oil of cinnamon have shown significant antibacterial activity during preliminary screening against bacteria such as *Brochothrix thermosphacta*, *E.coli, E.coli:* 0157*, Listeria monocytogenes, Salmonella typhimurium, Staphylococcus aureus, Pseudomonas flourescens, Pseudomonas aeruginosa, Bacillus subtilis, Proteus vulgaris, Salmonella enteritis, Campylobacter jejuni* (Smith-Palmer *et al*. 2002; Prabuseenivasan *et al*. 2006; Oussalah *et al*. 2007 ; Dobre *et al.* 2011; Becerril *et al.* 2012; Akhtar *et al.* 2014; Yap *et al*. 2014 ; Raut *et al.* 2014; Mith *et al*. 2014). Using the Kirby-Bauer disc diffusion method (Mith *et al.* 2014) showed the antibacterial activity of cinnamon essential oil against food-borne, food spoilage bacteria such as *Listeria monocytogenes*, *Salmonella typhimurium, E.coli:* 0157*, Brochothrix thermosphacta, Pseudomonas fluorescens, E.coli, Staphylococcus aureus.* Furthermore, the cinnamon essential oil showed significant inhibition with a MIC value of 0.125 ul/ml, and an MBC (Minimum Bactericidal Concentration) value of 0.25 ul/ml against all five bacteria tested except *Pseudomonas fluorescens* that remained resistant against cinnamon at MIC/ MBC value 1 ul/ml. According to another paper, the MIC of cinnamon essential oil against *Klebsiella pneumoniae* and *E.coli* was 3.2 mg/ml, and >1.6 mg/ml respectively (Prabuseenvasan *et al.* 2006). Similarly, another paper has reported a MIC value of 5 mg/ml of cinnamon essential oil against *E.coli* with broth microdilution assay (Silveria *et al.* 2012).

2.4.1.3 Oregano Essential Oil

Oregano oil extracted from the leaves of *Origanum vulgare* L., which belong to the family Lamiaceae, is a perennial herb that grows from 8-20 cm tall having opposite leaves 1-4 cm long.

Oregano oil chemically consists of cineole 46% and linalool 26.1% (Trease and Evans 1989). A literature review done on relevant articles published between 2000-2012 used oregano essential oil against a wide array of bacteria isolated from sources such as food, food spoilage, soil, and hospital. Studies conducted on essential oil of oregano have shown significant antibacterial activity during preliminary screening by employing disc diffusion method of oregano essential oil against bacteria such as *Acinetobacter calcoacetica, Aeromonas hydrophila, Alcaligenes faecalis, Bacillus subtilis, Beneckea natriegens, Brevibacterium linens, Brocothrix thermosphacta, Citrobacter freundii, Clostridium sporogenes, Enterococcus faecalis, Enterobacter aerogenes, Erwinia carotovora, Escherichia coli, Flavobacterium suaveolens, Klebsiella pneumonia, Lactobacillus plantarum, Leuconostoc cremoris, Micrococcus luteus, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella pullorum, Serratia marcescens, Staphylococcus aureus and Yersinia enterocolitica, Enterobacter cloacae, E.coli* ATCC25158, *E.coli* ATCC32922, *E.coli* CI, *E.coli* CII, (Dorman 2000; Moreira *et al*. 2005; Silviera *et al.* 2012). Furthermore, a MIC assay of oregano essential oil against *Aeromonas hydrophilia* showed MIC value of 2.5 ul/ml when the broth microdilution assay was employed (Azeredo *et al*. 2011). Similarly, another paper showed a MIC value of 0.5 ug/ml against *Enterobacter cloacae* by employing the broth microdilution assay (Sokovic *et al.* 2010). It was also reported in the literature that oregano essential oil possesses a MIC value of 1.8 ml/100ml against *E.coli* based on a broth microdilution assay (Moreira *et al.* 2005).

2.4.1.4 Tea Tree Essential Oil

Tea tree oil is derived from leaves of *Melaleuca alternifolia* which belong to the family Myrtaceae, native to Australia, is a tree or tall shrub about 7 m tall. The major chemical constituents of tea tree oil are terpinin 4-ol 40% (Trease and Evans, 1989). A literature review done on a relevant articles published between 1997-2005 have demostrated tea tree essential oil against few bacteria isolated from sources such as food, food spoilage, soil, and hospital. Studies conducted on essential oil of tea tree have shown least antibacterial activity during preliminary screening by employing disc diffusion method against bacteria such as *Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus, E.coli* ATCC25158, *E.coli* ATCC32922, *E.coli* CI, *E.coli* CII, (Hili 1997; Moreira *et al*. 2005).

2.4.1.5 Fennel Essential Oil

Sweet fennel oil is extracted from seeds of *Foeniculum vulgare* L. subsp. *vulgare*, var. *dulce*, ("Umbelliferae") the fruit resembles a bitter variety but has a sweet taste and low volatile content. Cultivated in many parts of Europe and imported from India, China and Pakistan (Trease and Evans 1989). A literature review done on a relevant article published in 2006 used fennel essential oil against few bacteria isolated from sources such as food, food spoilage, soil, and hospital. Studies conducted on essential oil of fennel have shown some antibacterial activity during preliminary screening by employing disc diffusion method against bacteria such as *Bacillus subtilis, Bacillus cereus, Escherichia coli, Pseudomonas sp and Staphylococcus aureus* (Helal *et al.* 2006).

2.4.1.6 Winter Green Essential Oil

Wintergreen oil is extracted from leaves of *Gaultheria procumbens* which belong to the family Ericaceae. It is a small, low-growing shrub, typically reaching 10-15 cm tall. The leaves are elliptical to ovate 2-5 cm long and 1-2 cm broad. The major chemical constituent of the oil of wintergreen is methyl salicylate. A literature review done on a relevant article published in 2006 used wintergreen essential oil against few bacteria isolated from sources such as food, food spoilage, soil, and hospital. Studies conducted on essential oil of wintergreen have shown some antibacterial activity during preliminary screening by employing disc diffusion method against bacteria such as *Proteus vulgaris*, *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus cerevisiae, Bacillus subtilis.* There was no activity recorded against *Klebsiella pneumonia* (Prabuseenivasan *et al.* 2006).

CHAPTER THREE

3. MATERIAL AND METHODS

3.1 Source of Experimental Bacterial Cultures

Both wild-type and wastewater isolated bacterial cultures were previously isolated from the wastewater activated sludge treatment process by our lab and identified.

3.1.1 Bacterial Pure Cultures

The bacterial pure cultures listed in Table 3.1 were utilized for preliminary screening testing. Frozen stocks of all the strains, made with 40% glycerol and 60% of a 5% w/v trisodium citrate solution in water, were maintained at -80˚C ± 1˚C and routinely subcultured onto Nutrient Agar (Difco Laboratories, MI, USA) plates. The cultures were stored at 4˚C and sub-cultured every week to maintain viability and avoid contamination.

3.1.2 Wastewater mixture sample collection.

The wastewater samples were taken from the activated sludge tanks (AT-2, AT-4, AT-6, AT-8) the digestors, (DIG-1 DIG-2) and the returned sludge (RS). WWTP bacterial aeration tank samples were obtained from the Humber Wastewater Treatment Plant and used to test the efficacy of the oils against a mixed culture.

3.2 Preparation of Inoculum

Broth suspension of the wild type pure bacterial cultures and wastewater isolated bacteria were prepared by inoculating 3 ml of Tryptic Soy Broth (Scott Laboratories Inc., CA, USA) with a single colony from the respective R2A Agar plate culture. and vortexed; colonies were added until the suspension matched a 0.5 McFarland standard.

Table 3.1. Inventory of wild type and wastewater isolated bacterial cultures.

Name of Bacteria	Lab Frozen Stock No	Source of strain (Lab Isolated)
Acinetobacter baumannii	27	[3]Wild type
Acinetobacter baumannii	12K	WWTP
Acinetobacter bouretii	10A	WWTP
Aeromonas hydrophila	7A	WWTP
E. coli: DH5∞	17	Wild type
E. coli: AD202	36	Wild type
E. coli: DH5∞	9	Wild type
E. coli		WWTP
Enterobacter cloacae	12E	WWTP
Flavobacterium branchiophilum	8I	WWTP
Klebsiella pneumoniae	11A	WWTP
Pseudomonas fluorescens	8	Wild type
Pseudomonas poae	26	Wild type
Pseudomonas putida	BBC- 443	Wild type
Pseudomonas staurtii	14D	WWTP
Serratia fonticola	4B	WWTP
Staphylococcus aureus	16	Wild type
Staphylococcus muscae	15D	WWTP
Stenotrophomonas maltophilia	13	Wild type
Stenotrophomonas maltophilia	33	WWTP

[3] A *wild type* is the original strain of an organism which has not undergone any form of mutation genetically or phenotypically

3.3 Selection of Essential Oil

The commercial essential oils such as clove, cinnamon, oregano, fennel, teatree, and wintergreen manufactured by Now essential oils were purchased from Noah Natural Food Canada. These essential oils were claimed 100 % pure extracted through steam distillation process. For broth tube, macro-dilution MIC assay each essential oils were dissolved in 5% Tween, a stabilizing and dispersing agent, as required. All essential oils used in this experiment are listed in Table. 3.2

Table 3.2. Plant Essential oils Inventory with Scientific Name, Common Name, Specific Gravity, Concentration and Color.

Scientific Name	Common Name	Specific gravity	[4]Concentration in mg/ml of 50 uL of E.O taken	Color
Cinnamomum cassia (Nees & T.Nees) J.Presl	Cinnamon	1.03	51.5 mg/ml	Pale yellow
Foeniculum vulgare Mill.	Fennel	0.96	48 mg/ml	Pale yellow
Gaultheria procumbens L.	Winter Green	1.18	59 mg/ml	Pinkish yellow
Origanum vulgare L.	Oregano	0.94	47 mg/ml	Pale yellow
Melaleuca alternifolia (Maiden & Betche) Cheel	Tea Tree Oil	0.89	44.5 mg/ml	Colorless
Syzygium aromaticum (L.) Merrill & Perry.	Clove	1.04	52 mg/ml	Colorless

Source: Hili 1997; Hammer *et al*. 1999; Dorman. 2000; Helal *et al*. 2006; Prabuseenivasan *et al*. 2006; Mith *et al*. 2014

3.4 Antibacterial Assay

The antibacterial assay was divided into a two-tiered testing process.

1. The qualitative analysis involved preliminary screening of plant essential oils against bacterial pure cultures using the Kirby-Bauer disc diffusion method.
2. The quantitative analysis which included the determination of MIC by employing broth tube macro-dilution assay.

3.4.1 Kirby-Bauer disc diffusion Assay

The Kirby-Bauer disc diffusion method was adopted from National Committee on Clinical Laboratory Standards for preliminary screening of essential oils which is principally modified from testing of antibiotics (Hammer *et al*. 1999; NCCLS 2014). Testing was done with cultures that had been grown in a Tryptic Soy Broth (TSB) suspension standardized to a 0.5 McFarland standard representing approximately 1 x 10^6 CFU.

[4] Assuming Each E.O's are100 % pure as claimed by manufacturer. The concentration is calculated based on Specific Gravity of each E.O's multiplied by the volume used for each Assay.

3.4.2 Preparation of Discs

To complete the Kirby-Bauer disc diffusion assay as suggested by National Committee on Clinical Laboratory Standards., the necessary antibacterial susceptibility discs containing the essential oils were inoculated with 50uL of the essential oil and left to diffuse into the disc for 2 hours.

3.4.3 Experimental Design

3.4.3.1 Preliminary Screening of Essential Oils

Each plant essential oil was tested for antibacterial activity (Fig. 7A) against all the selected wild type bacterial cultures and wastewater isolated bacteria (Table 3.1). A bacterial suspension was prepared by inoculating 3 ml of TSB with pure colonies, and vortexed; colonies were added until the suspension matched a 0.5 McFarland standard. A sterile cotton swab was then dipped into the bacterial suspension and swabbed onto the R2A agar plate. Prepared filter discs (13 mm or 6 mm) immersed with the essential oils, were placed onto the surface of the agar. The Petri plates were then incubated in an upright position at 37˚C for 72 hours. Petri plates were sealed with paraffin or plastic tape to reduce evaporation of essential oil component.

The experiments were carried out in triplicate for each essential oil against each of the wild type bacterial cultures. Simultaneously controls were tested by using 50 µl of vegetable oil and 5% acetone to compare the efficacy of antibacterial constituents found in each essential oil. Testing of antibacterial activity of 5% acetone or vegetable oil such as 5% olive oil are used as diluent in some essential oils. The susceptibility of bacteria to the inhibitory effects of the selected essential oils was assessed based on the Kirby-Bauer disc diffusion assay (Hammer *et al.* 1999; NCCLS 2014). All the steps involved in the antibacterial assay were performed in sterilized/ aseptic condition.

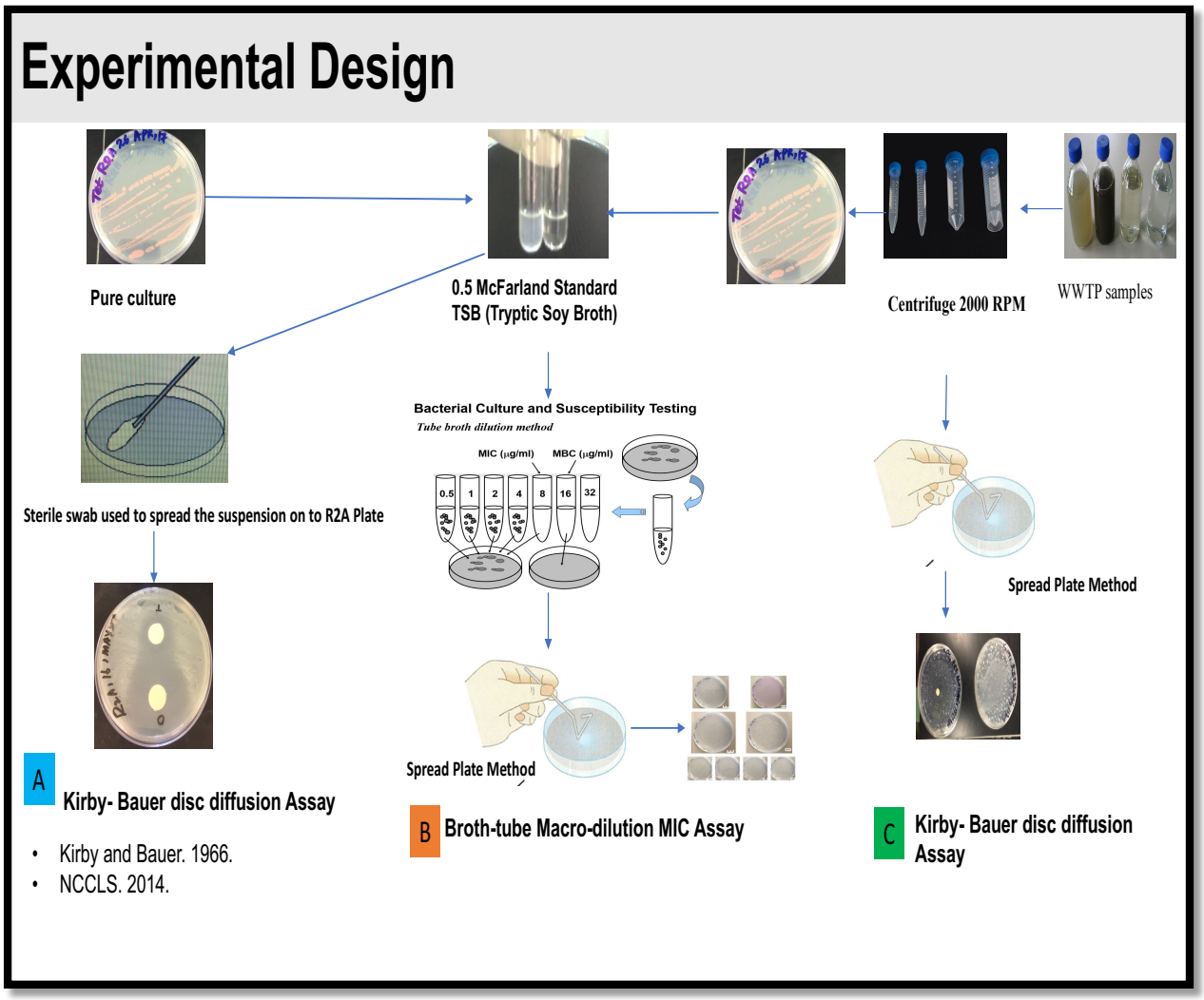


Figure 7. Experimental Design (A) Kirby-Bauer Disc Diffusion Assay (B) Minimum Inhibitory Concentration (C) Kirby-Bauer Disc diffusion assay for wastewater mix bacterial cultures.

3.4.3.2 Zone of Inhibition

The zone of inhibition was determined by measuring the diameter of the zone in mm minus the diameter of the disc. The bacterial sensitivity and diameter of the zone of inhibition was determined with slight modification to interpret the results. The zones of inhibition produced by the essential oil were recorded by using a scale (Fig. 8).

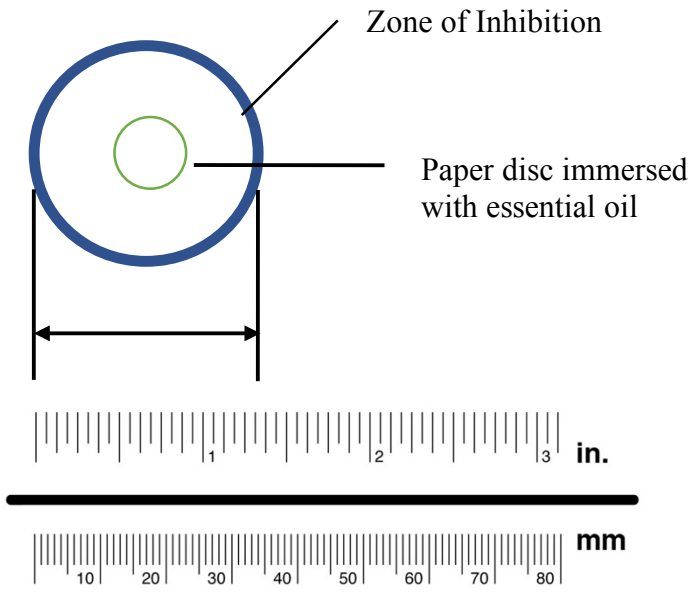


Figure 8. Zone of Inhibition Measurement

Table 3.3. Bacterial Sensitivity and Diameter of Zone of Inhibition (13 mm*; 6 mm)**

Sensitivity	Zone of inhibition in mm
(-) Not Sensitive.	• Zone of inhibition less than or equal to 14 mm* • Zone of inhibition less than or equal to 7 mm**
(+) Intermediate	• Zone of inhibition of diameter 15mm-17mm* • Zone of inhibition of diameter 8mm-10mm**
(++) Sensitive.	• Zone of inhibition of diameter 19mm-23mm* • Zone of inhibition of diameter 12mm-14mm**
(+++) Very Sensitive	• Zone of inhibition of diameter greater than 24 mm* • Zone of inhibition of diameter greater than 15 mm**

Source: Moriera *et al*. 2005

* measurement used with larger 13 mm discs

** measurement used with smaller 6 mm discs

3.5 Determination of MIC (Minimum Inhibitory Concentration)

Minimum inhibitory concentration (MIC) is the lowest concentration of a compound required to inhibit visible growth of bacteria after 72 hrs incubation time. Based on the results obtained from the preliminary screening assay of the six essential oils, three oils (clove, cinnamon, and oregano) were subjected to quantitative analysis. The MIC assay was carried out by employing the broth tube macro-dilution method with modification (Ericsson and Sherris, 1971; Wiegand *et al*. 2008). Wastewater isolated bacterial cultures (Table 3.1) were tested against clove, cinnamon and oregano oils (Table 3.2) to determine the MIC (Minimum Inhibitory Concentration) of each essential oil. Broth tube dilution method as suggested by CLSI adopted from Stephanie de Rapper *et al.* 2013 with some modifications. The stock solution was prepared for each essential oil by taking 32 ul of essential oil was diluted in 1 ml of 5% acetone (v/v) at the starting dilution volume of 32 ul based on specific gravity essential oil actual concentration was computed in mg/ml by

assuming essential oils are 100% pure (Table 3.2). Further two-fold serial dilutions of the stock essential oil solution yielded dilution volumes of 16, 8, 4, 2, 1, and 0.5ul (Fig. 7B). A control tube of 5% Tween+ 5% acetone+ bacterial suspension without essential oil was used as a reference. The bacterial culture in a log phase growth was diluted in Tryptic Soy Broth (TSB) to achieve 0.5 McFarland standard and then spread plated onto the R2A plate. All plates were incubated at room temperature for 72 hours, and all experiments were done in triplicate.

3.6 Wastewater Sample Test Assay

The seven wastewater samples such as aeration tank (AT-2, AT-4, AT-6, AT-8), digesters (DIG-1, DIG-2), and Return Sludge (RS) containing mixed population of different bacteria was tested against three essential oils such as clove oil, cinnamon oil, and oregano oil. The three essential oils were screened for their bacterial growth inhibitory activity. A 200 ul aliquot of each raw wastewater sample was mixed with 100 µl distilled water. The mixture (100 µl) was spread plated on R2A and prepared filter discs, (6 mm) immersed with the essential oil at their MIC cons. clove 0.52 mg/ml, cinnamon oil 0.51 mg/ml and oregano 0.47 mg/ml were placed on it as described by the Kirby-Bauer Disc diffusion assay with slight modifications (Fig. 7C). The Petri plates were then incubated in an upright position at room temperature for 72 hours. The experiment was conducted in triplicate.

CHAPTER FOUR

4. RESULTS

4.1 Preliminary Screening of Plant Essential Oils.

4.1.1 Wild Type Bacterial Pure Cultures

The susceptibility of ten wild type bacterial pure cultures was determined against six plant essential oils and the results are shown in Table 4.1. The degree of sensitivity was determined by using the Kirby-Bauer disc diffusion assay as standardized by NCCLS 2014 and the susceptibility measurements provided by the manufacturer (BBL^{TM}Taxo^{TM} 13 mm disc) were adopted as resistant to very sensitive. Results showed that *Staphylococcus aureus* was very sensitive (+++) to five of the essential oils and sensitive (++) to tea tree oil with zones of inhibition halos recorded based on triplicate results (Table 4.1; Appendix A, C, D, E, F & G). *Acinetobacter baumanii* showed sensitivity to clove oil, cinnamon, and oregano oil and showed lesser to no sensitivity to fennel, tea tree, and wintergreen oils. Three *E.coli* strains were used and showed some variation in sensitivity. *E.coli*: *AD202* was very sensitive to clove and cinnamon oils, less inhibited by oregano and fennel oils and less to no inhibition to tea tree and wintergreen oils. The two strains of *E.coli*:*DH5α* were very sensitive to clove and cinnamon oils but showed less inhibition to the other four oils. *Pseudomonas fluorescens* was found to be very sensitive (+++) to sensitive (++) to clove oil, cinnamon oil, and oregano oil but less so to the other three oils. *Pseudomonas poae* was very sensitive to cinnamon oil, oregano oil, clove oil and tea tree oil. Similarly, *Pseudomonas putida* also was very sensitive (+++) to sensitive (++) against cinnamon oil, wintergreen oil, and clove oil. The two strains of S*tenotrophomonas maltophilia* were sensitive to cinnamon and clove and a lesser extent to tea tree oil for one and oregano for the other. Based on these results, it appeared that clove oil, cinnamon oil, and oregano oil were the most effective at inhibiting the growth of a variety of both Gram positive and Gram negative bacteria. The control 5% acetone and vegetable oil did not show any antibacterial activity (Table 4.1; Appendix J).

Table 4.1. Wild Type Bacterial Sensitivity to Clove, Cinnamon, Oregano, Fennel, Tea tree and Winter green Essential Oils Determined by a Disc Diffusion Assay.

Bacterial Strains	Essential Oils Used Zones of Inhibition in (mm)							
	Clove (52mg/ml)	Cinnamon (51.5 mg/ml)	Oregano (47mg/ml)	Fennel (48 mg/ml)	Teatree (44.5 mg/ml)	Winter Green (59 mg/ml)	Control	
							Veg Oil	5% Acetone
***Acinetobacter baumanii* (27)**	++	++	+++	+	-	+	-	-
***E.coli*:*DH5∞* (17)**	++	+++	-	++	-	-	-	-
***E.coli:AD202* (36)**	+++	+++	+	+	-	-	-	-
E.coli:DH5∞	++	+++	-	+	-	-	-	-
***Pseudomonas fluorescens* (8)**	+++	+++	++	+	-	-	-	-
***Pseudomonas poae* (26)**	+++	+++	+++	+	+++	+	-	-
***Pseudomonas putida (*BBL-443)**	++	++	+	+	+	+++	-	-
***Staphylococus aureus* (16)**	+++	+++	+++	+++	++	+++	-	-
***Stenotrophomonas maltophilia* (13)**	++	+	+	++	++	+++	-	-
***Stenotrophomonas maltophilia* (33)**	++	+++	+	+	+	++	-	-
Total Sensitive (ZOI> 15 mm) **Total Resistant (ZOI< 15 mm)**	**S-10** **R-0**	**S-10** **R-0**	**S-8** **R-2**	**S-10** **R-0**	**S-5** **R-5**	**S-6** **R-4**	**S-0** **R-10**	

Sensitivity Index

- Bacteria Not Sensitive
+ Intermediate Sensitive
++ Bacteria Sensitive
+++ Bacteria Very Sensitive

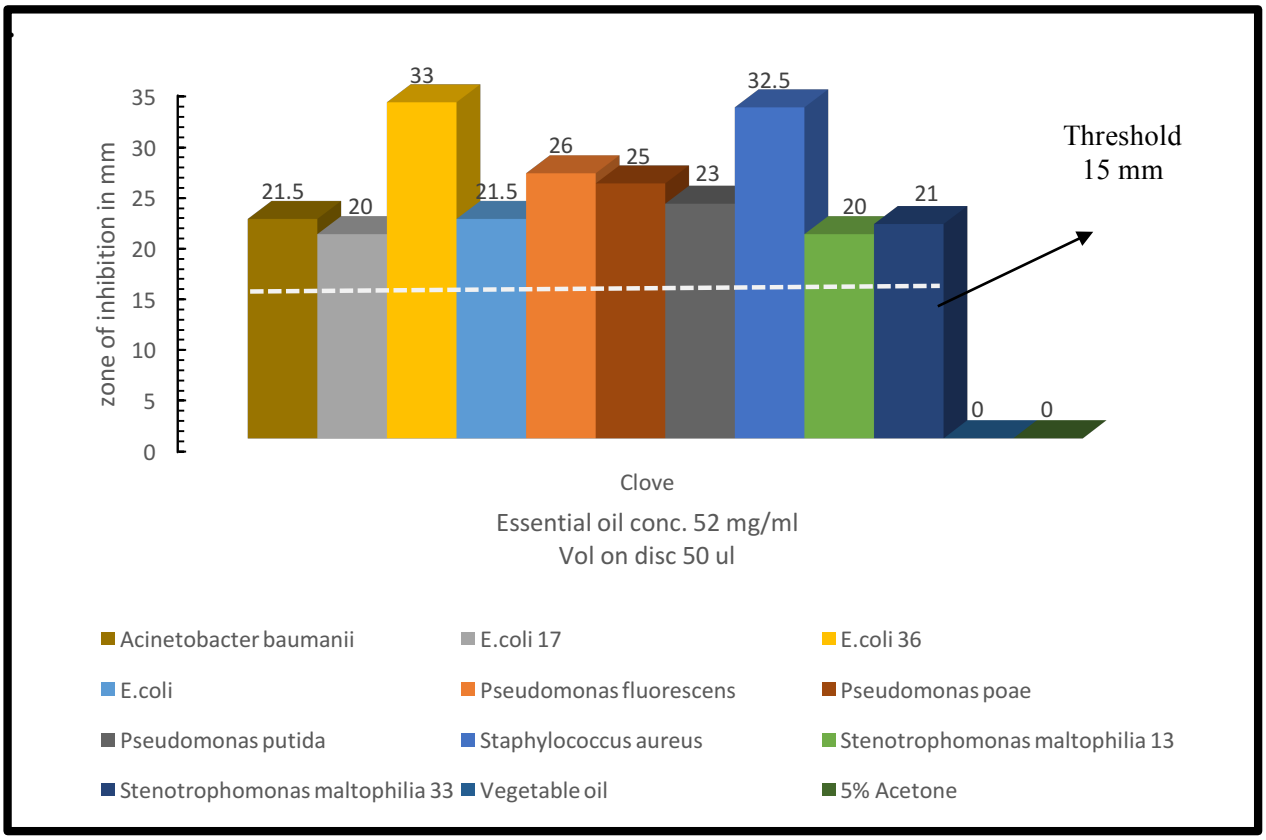


Figure 9. Wild type Bacterial Strain Sensitivity to Cinnamon Essential Oil Determined by Disc Diffusion Assay.

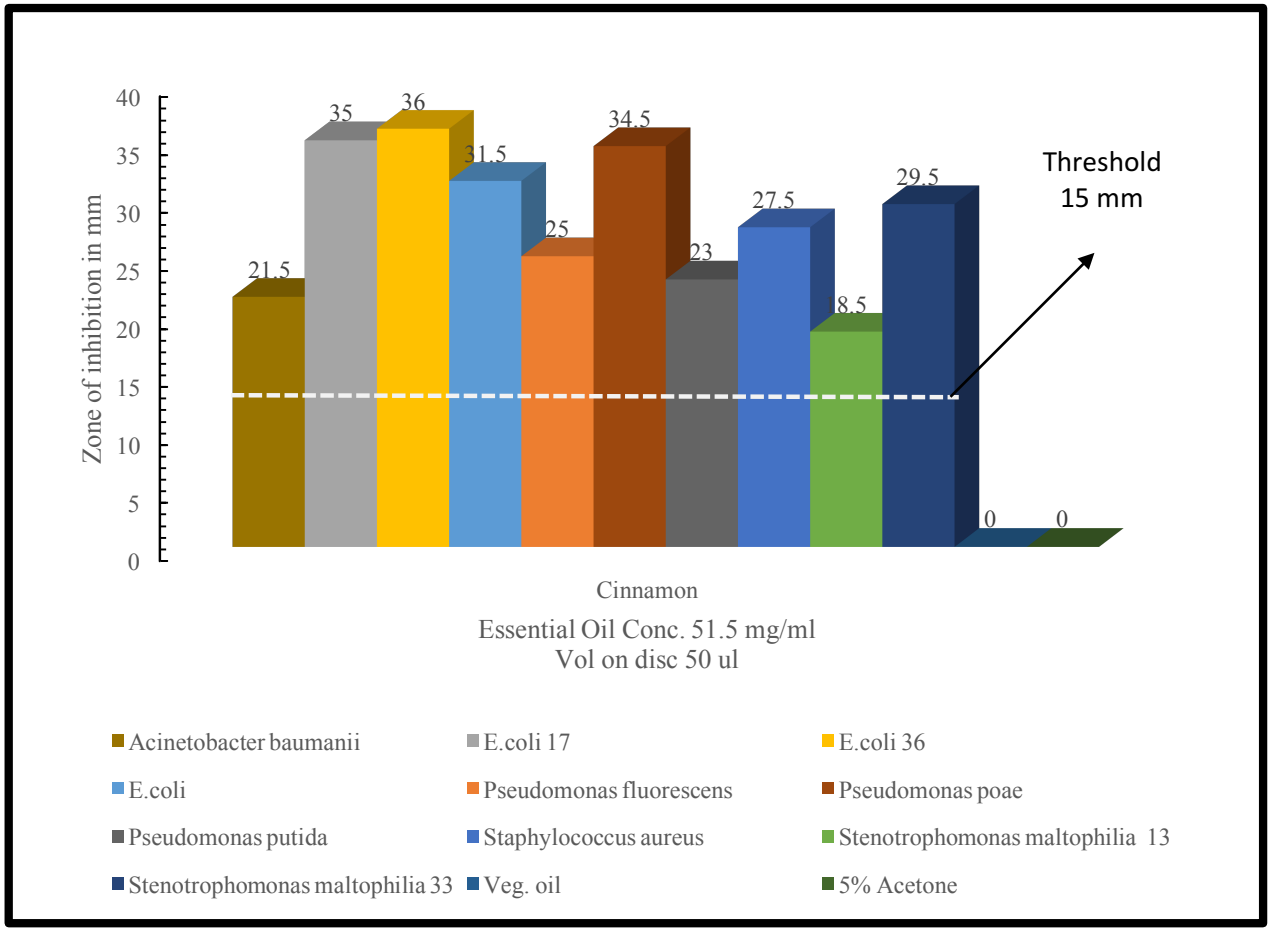


Figure 10. Wild type Bacterial Strain Sensitivity to Cinnamon Essential Oil Determined by Disc Diffusion Assay.

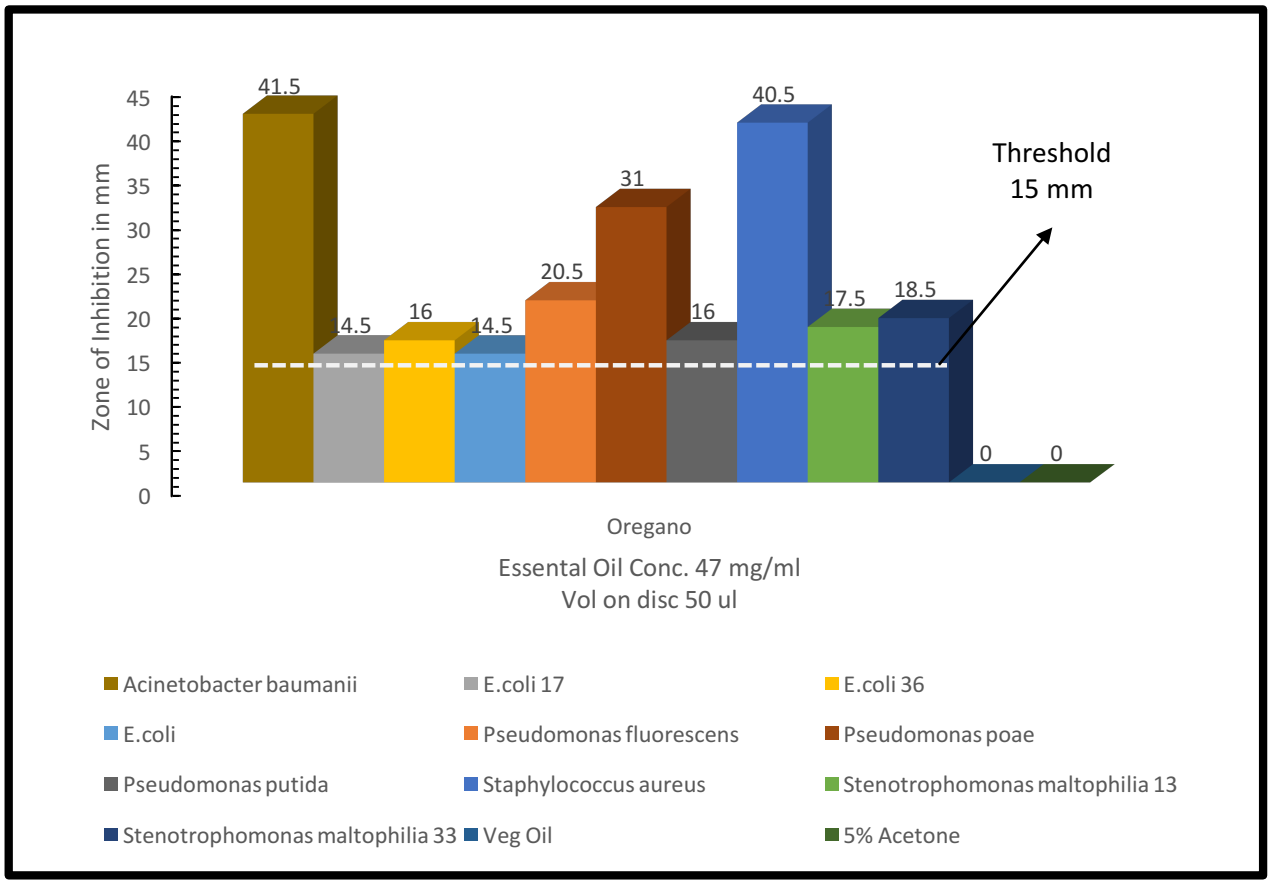


Figure 11. Wild type Bacterial Strain Sensitivity to Oregano Essential Oil Determined by Disc Diffusion Assay.

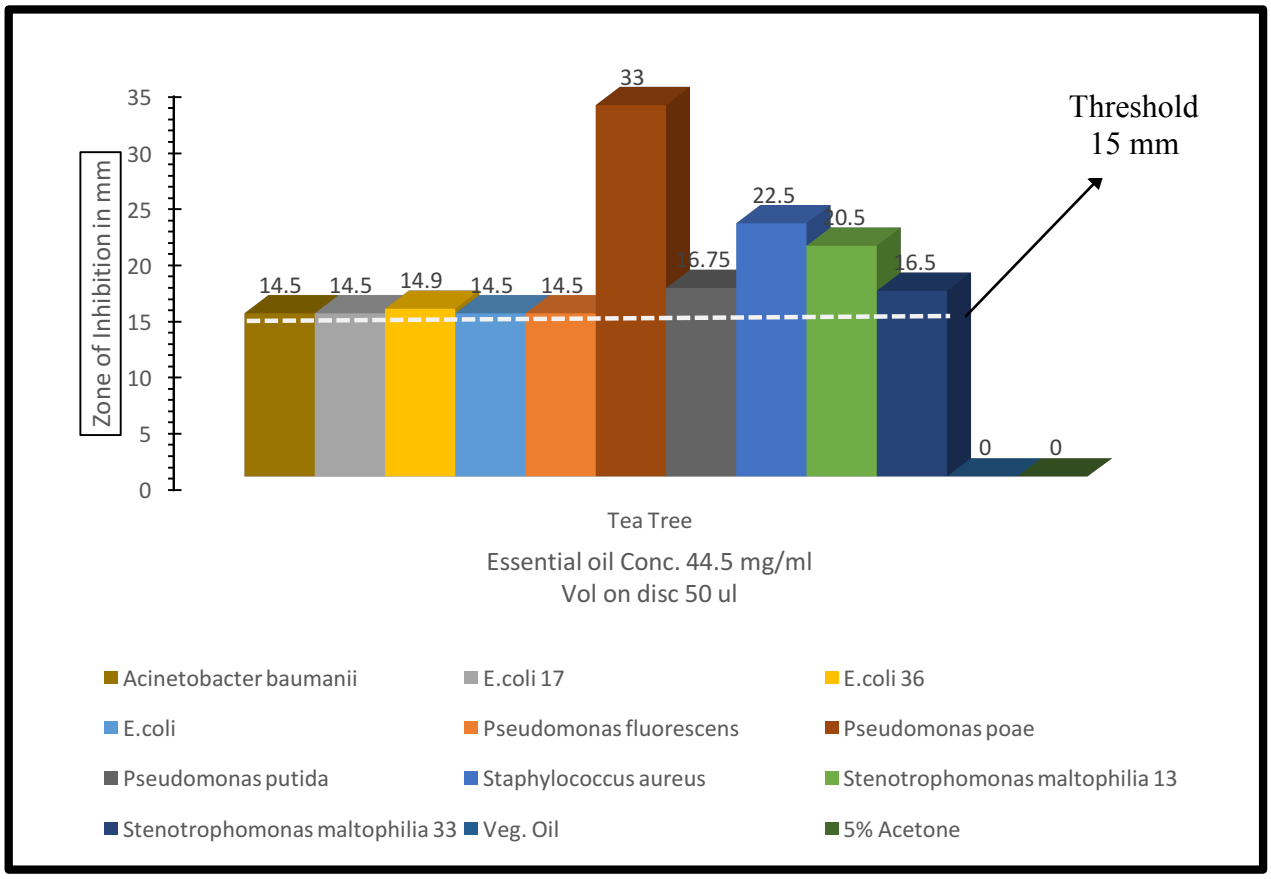


Figure 12. Wild type Bacterial Strain Sensitivity to Tea tree Essential Oil Determined by Disc Diffusion Assay.

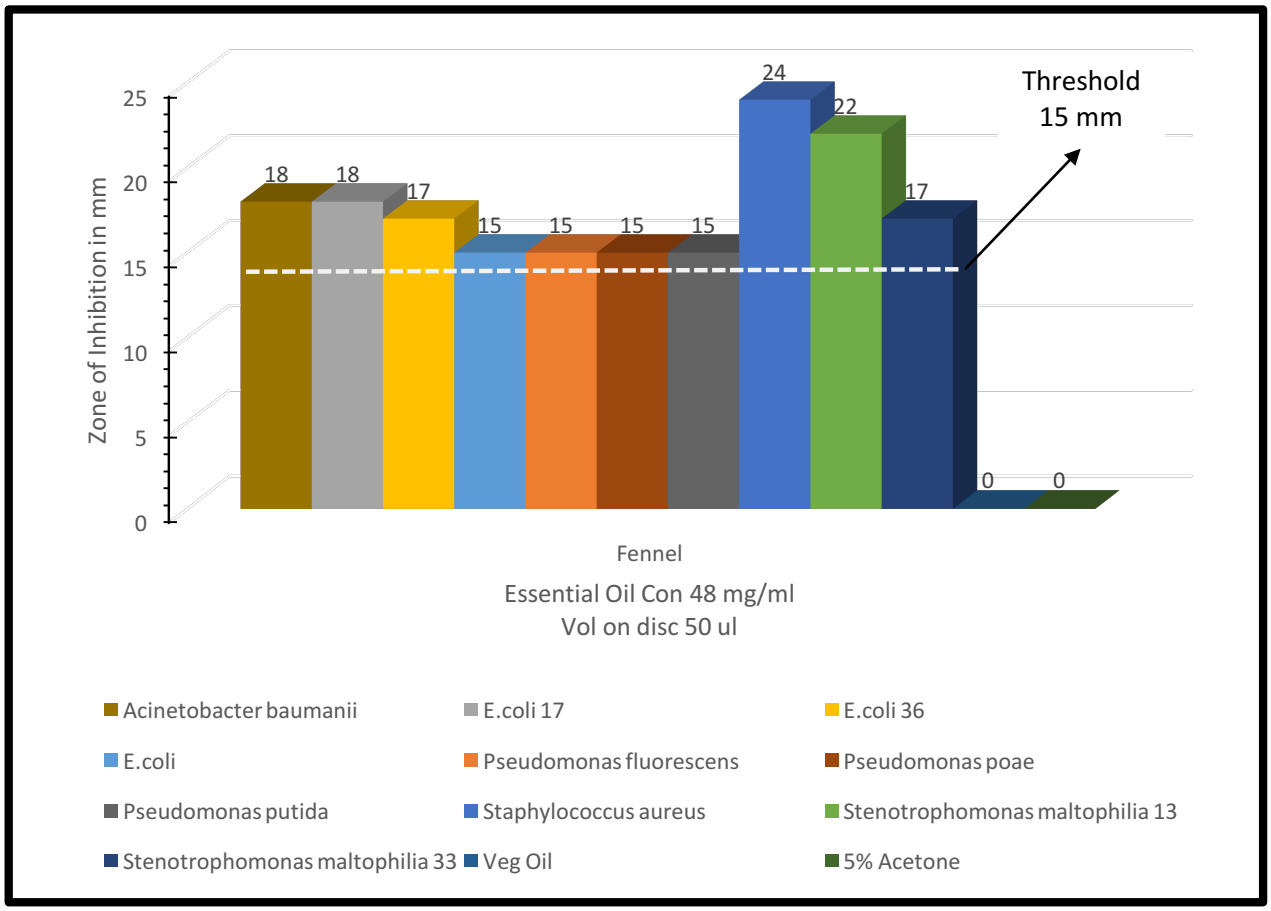


Figure 13. Wild type Bacterial Strain Sensitivity to Fennel Essential Oil Determined by Disc Diffusion Assay.

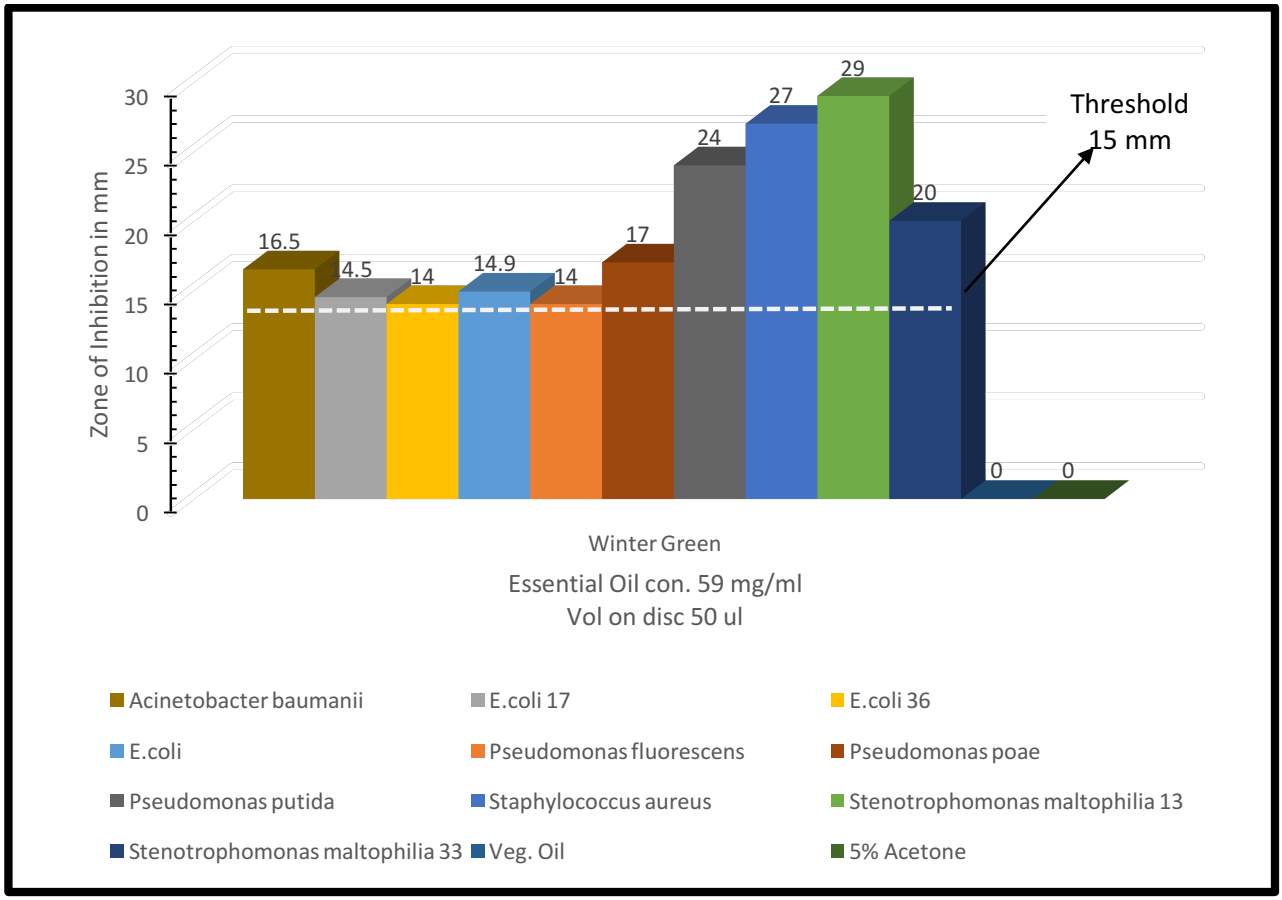


Figure 14. Wild type Bacterial Strain Sensitivity to Wintergreen Essential Oil Determined by Disc Diffusion Assay.

4.1.2 Wastewater isolated bacterial strains

In the case of ten wastewater isolated bacterial strains, sensitivity was determined against three of the plant essential oils such as clove, cinnamon and oregano, results are shown in (Table 4.2; Appendix B, H, I and J). The degree of sensitivity was determined by using the Kirby-Bauer method as standardized by NCCLS 2014.

The wastewater isolated bacterial strains such as *Acinetobacter baumanii*, *Acinetobacter bouretii*, *Aeromonas hydrophilla, E. coli*, *Flavobacterium barchiophillum, Klebsiella pneumonii, Serratia fonticola, Staphylococcus muscae*, *Pseudomonas staurtii* were very sensitive to all three essential oils. One exception was *Enterobacter cloaceae* that was found to be very sensitive (+++) to clove oil, cinnamon oil but only sensitive (++) to oregano oil. Based on these results, it appeared that clove oil, cinnamon oil, and oregano oil were the most effective at inhibiting the growth of a variety of both Gram positive and Gram negative bacteria. All experiments were done in triplicate with the mean ± S.E (Standard Error) values of the zones of inhibition halos in mm to clove, cinnamon, oregano. The complete results are presented in the Appendix B.

Table 4.2. Wastewater Isolated Bacterial Sensitivity to Clove, Cinnamon and Oregano Essential Oils as Determined by Disc Diffusion Assay.

Bacteria	Essential Oils Used Zones of Inhibition in (mm)				
	Clove (52 mg)	Cinnamon (51.5 mg)	Oregano (47 mg)	Control	
				Veg Oil	5% Acetone
***Acinetobacter baumanii* (12K)**	+++	+++	+++	-	-
***Acinetobacter bouretii* (10A)**	+++	+++	+++	-	-
***Aeromonas hydrophilla* (7A)**	+++	+++	+++	-	-
***E. coli* (36)**	+++	+++	+++	-	-
***Enterobacter cloaceae* (12E)**	+++	+++	++	-	-
***Flavobacterium branchiophilum* (8I)**	+++	+++	+++	-	-
***Klebsiella pneumoniae* (11A)**	+++	+++	+++	-	-
***Pseudomonas staurtii* (14D)**	+++	+++	+++	-	-
***Staphylococcus muscae* (15D)**	+++	+++	+++	-	-
***Serratia fonticola* (4B)**	+++	+++	+++	-	-
Total Sensitive (ZOI> 15 mm) **Total Resistant (ZOI< 15 mm)**	**S-10** **R-0**	**S-10** **R-0**	**S-10** **R-0**	**S-0** **R-10**	**S-0** **R-10**

Sensitivity Index

- \- Bacteria Not Sensitive
- \+ Intermediate Sensitive
- ++ Bacteria Sensitive
- +++ Bacteria Very Sensitive

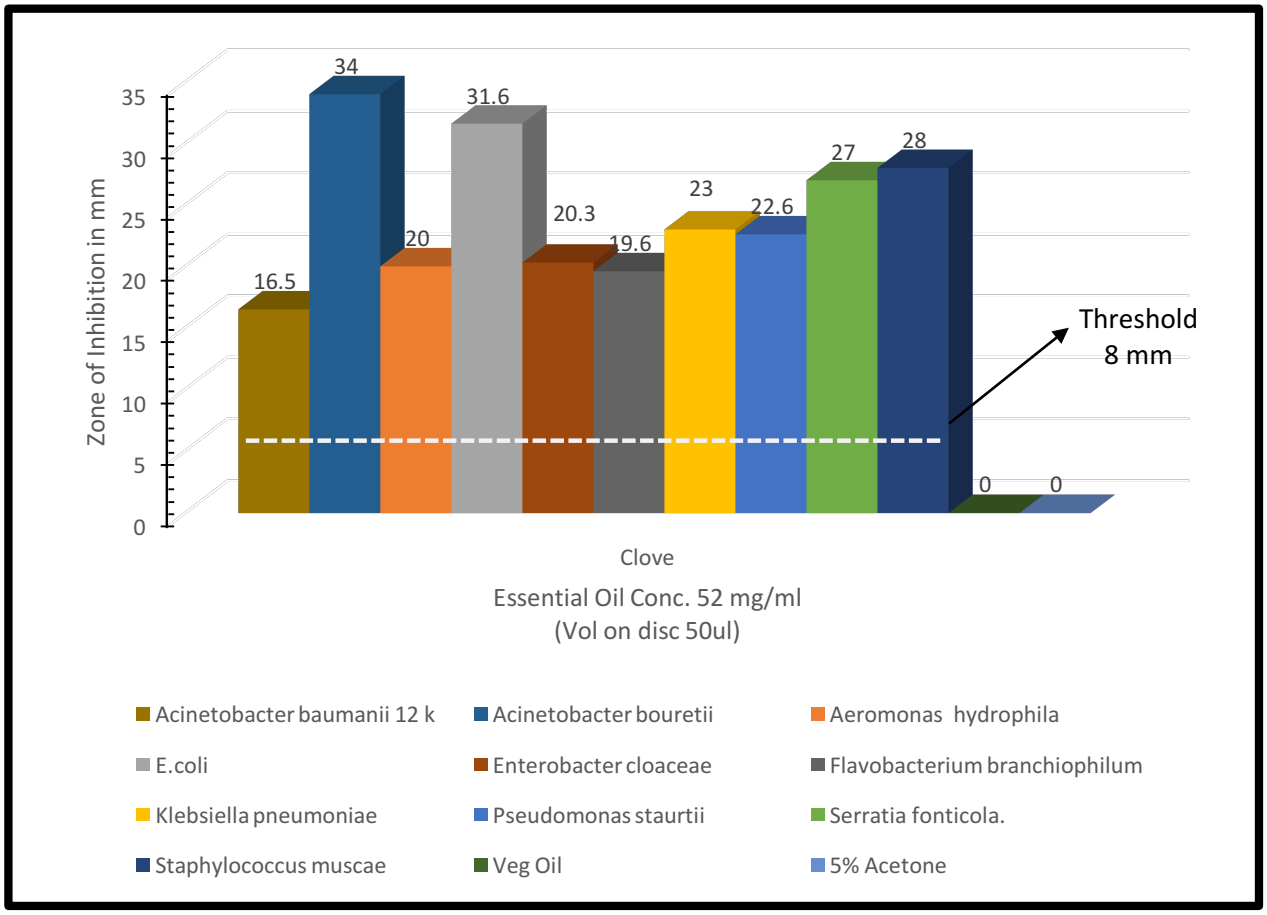


Figure 15. Wastewater Isolated Bacterial Strains Sensitivity to Clove Essential Oil Determined by Disc Diffusion Method.

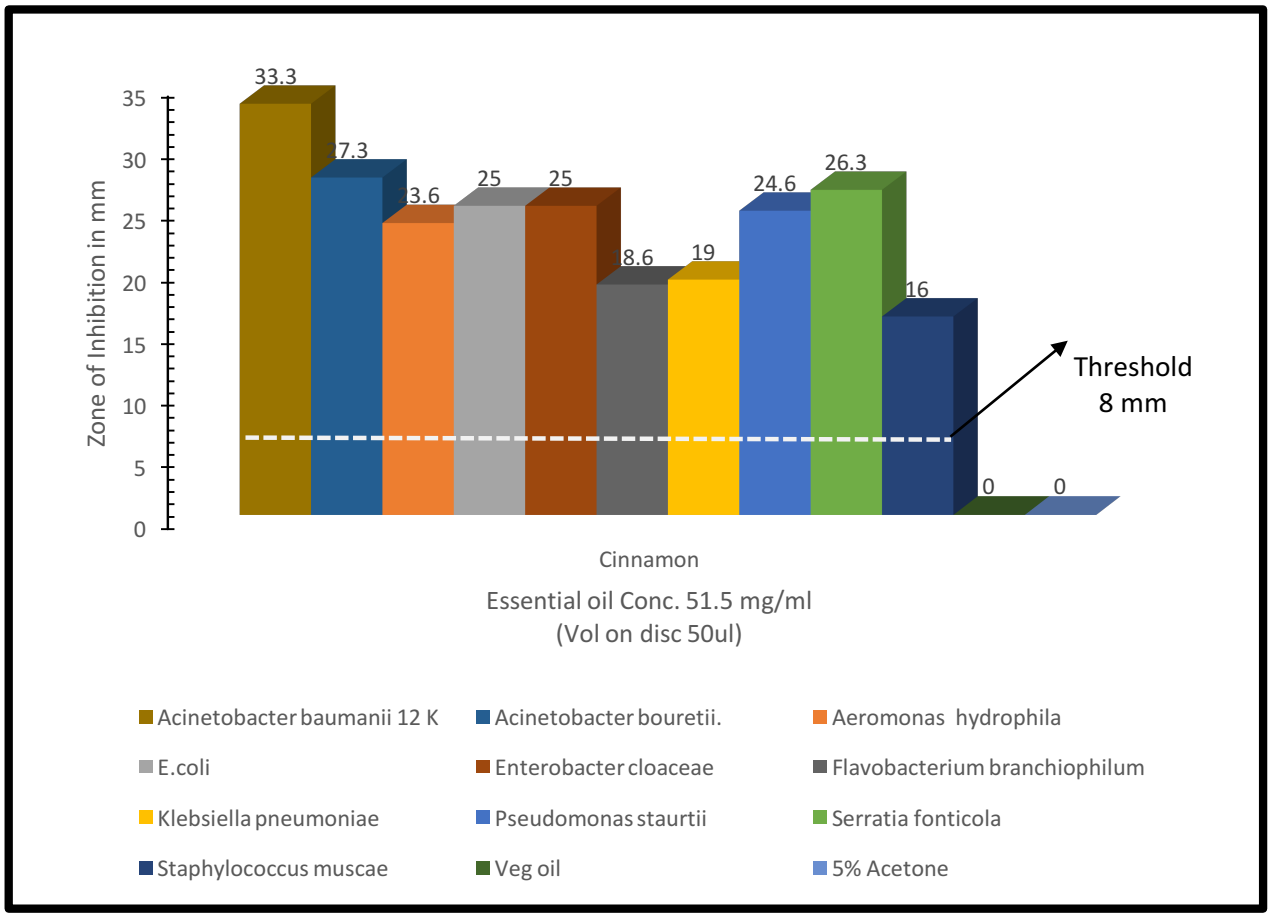


Figure 16. Wastewater Isolated Bacterial Strains Sensitivity to Cinnamon Essential Oil Determined by Disc Diffusion Method.

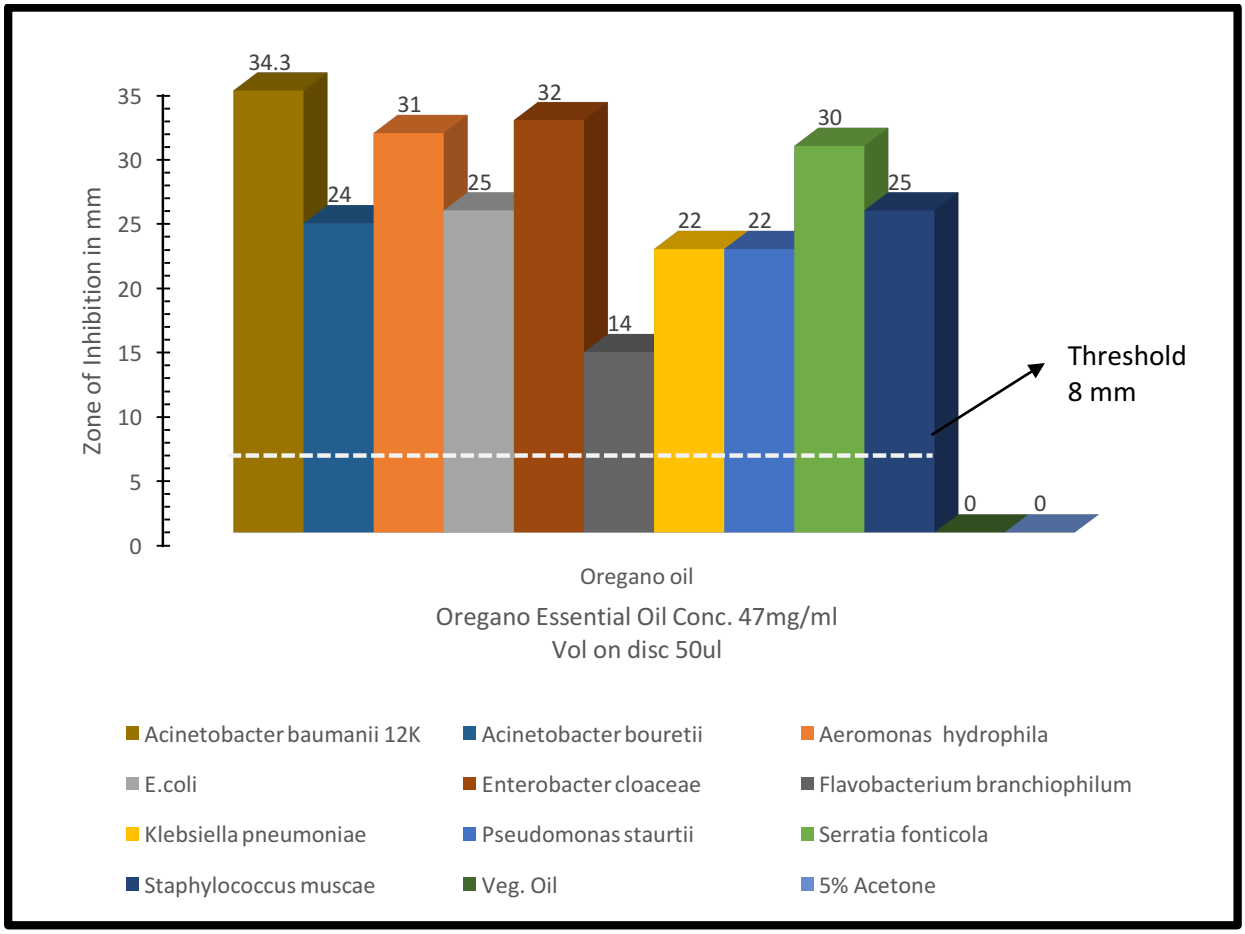


Figure 17. Wastewater Isolated Bacterial Strains Sensitivity to Oregano Essential Oil Determined by Disc Diffusion Method.

4.2 Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration required to show inhibition of bacterial growth was determined for the three essential oils that were effective against the wastewater bacterial strains. The MIC for clove, cinnamon, oregano oils was determined by employing broth tube macro dilution method as outlined by NCCLS 2014. The results are shown in Tables 4.3, 4.4, 4.5 and 4.6.

4.2.1 Minimum Inhibitory Concentration of Clove Essential Oil

Among the wastewater isolated bacterial strains assayed *E. coli, Staphylococcus muscae, Enterobacter cloaceae, Acinetobacter baumanii* were found to be most sensitive to clove oil with a minimum inhibitory concentration of 0.52 mg/ml followed by *Klebsiella pneumoniae* with a MIC of 1.04 mg/ml. *Aeromonas hydrophila and Acinetobacter bouretii* both showed a MIC of 2.08 mg/ml whereas *Pseudomonas staurtii, and Serratia fonticola* were comparitively less sensitive with a minimum inhibitory concentration of 4.16 mg/ml (Tables 4.3 and 4.6; Appendix K, L).

4.2.2 Minimum Inhibitory Concentration of Cinnamon Essential Oil

Among the wastewater isolated bacteria assayed *E. coli, Enterobacter cloaceae, Acinetobacter baumanii* were found to be most sensitive to Cinnamon with a MIC of 0.51 mg/ml followed by *Acinetobacter bouretii* with a MIC of 1.03 mg/ml. *Aeromonas hydrophila, Pseudomonas staurtii* were comparitively less sensitive with MIC of 4.12 mg followed by *Staphylococcus muscae, Serratia fonticola, Klebsiella pneumoniae* with MICs of 8.24 mg/ml respectively (Tables 4.4 and 4.6; Appendix K, L).

4.2.3 Minimum Inhibitory Concentration of Oregano Essential Oil

Among the wastewater isolated bacteria assayed *E. coli, Staphylococcus muscae, Enterobacter cloaceae, Acinetobacter baumanii* were found to be most sensitive with a MIC of 0.47 mg/ml followed by *Serratia fonticola, Klebsiella pneumoniae, Acinetobacter bouretii* with MICs of 3.76 mg/ml whereas *Aeromonas hydrophila* was the least sensitive of all other bacterial strains with a MIC of 30.08 mg/ml (Tables 4.5 and 4.6; Appendix K, L).

Table. 4.3. Wastewater Isolated Bacterial Strains Sensitivity to Clove Oil by Employing Broth Tube Macro-dilution MIC Assay.

Bacteria	Clove Oil Concentration/ Volume Taken							
	33.28 mg/ml (32ul)	16.64 mg/ml (16ul)	8.32 mg/ml (8ul)	4.16 mg/ml (4ul)	2.08 mg/ml (2ul)	1.04 mg/ml (1ul)	0.52 mg/ml (0.5ul)	Control 5%Polysorbate 80
***Acinetobacter baumanii* (10A)**	-	-	-	-	-	-	-	+
***Acinetobacter bouretii* (12K)**	-	-	-	-	-	+	+	+
***Aeromonas hydrophila* (7A)**	-	-	-	-	-	+	+	+
***E. coli* (36)**	-	-	-	-	-	-	-	+
***Enterobacter cloaceae* (12E)**	-	-	-	-	-	-	-	+
***Flavobacterium branchiophilum* (8I)**	-	-	-	-	-	-	-	+
***Klebsiella pneumoniae* (11A)**	-	-	-	-	-	-	+	+
***Pseudomonas staurtii* (17D)**	-	-	-	-	+	+	+	+
***Serratia fonticola* (4B)**	-	-	-	-	+	+	+	+
***Staphylococcus muscae* (15D)**	-	-	-	-	-	-	-	+
Total Sensitive **Total Resistant**	**S-10** **R-0**	**S-10** **R-0**	**S-10** **R-0**	**S-10** **R-0**	**S-8** **R-2**	**S-6** **R-4**	**S-5** **R-5**	**S-0** **R-10**

- **No Bacterial Growth**
+ **Bacterial Growth**

Table. 4.4. Wastewater Isolated Bacterial Sensitivity to Cinnamon oil by Employing Broth Tube Macro-dilution MIC Assay.

Bacteria	Cinnamon Oil Concentration/ Volume Taken							
	32.96 mg/ml (32ul)	16.4 mg/ml (16ul)	8.2 mg/ml (8ul)	4.12 mg/ml (4ul)	2.06 mg/ml (2ul)	1.03 mg/ml (1ul)	0.51 mg/ml (0.5ul)	Control 5%Polysorbate 80
***Acinetobacter baumanii* (10A)**	-	-	-	-	-	-	-	+
***Acinetobacter bouretii* (12K)**	-	-	-	-	-	-	+	+
***Aeromonas hydrophila*(7A)**	-	-	-	-	+	+	+	+
***E. coli* (36)**	-	-	-	-	-	-	-	+
***Enterobacter cloaceae* (12E)**	-	-	-	-	-	-	-	+
***Flavobacterium branchiophilum*(8I)**	-	-	-	-	-	-	-	+
***Klebsiella pneumoniae* (11A)**	-	-	-	+	+	+	+	+
***Pseudomonas staurtii* (17D)**	-	-	-	-	+	+	+	+
***Serratia fonticola* (4B)**	-	-	-	+	+	+	+	+
***Staphylococcus muscae*(15D)**	-	-	-	+	+	+	+	+
Total Sensitive **Total Resistant**	**S-10** **R-0**	**S-10** **R-0**	**S-10** **R-0**	**S-7** **R-3**	**S-5** **R-5**	**S-5** **R-5**	**S-4** **R-6**	**S-0** **R-10**

- **No Bacterial Growth**

\+ **Bacterial Growth**

Table. 4.5. Wastewater Isolated Bacterial Sensitivity to Oregano Oil by Employing Broth Tube Macro-dilution MIC Assay.

Bacteria	Oregano Oil Concentration/ Volume Taken							
	30.08 mg/ml (32ul)	15.04 mg/ml (16ul)	7.52 mg/ml (8ul)	3.76 mg/ml (4ul)	1.88 mg/ml (2ul)	0.94 mg/ml (1ul)	0.47 mg/ml (0.5ul)	Control 5%Polysorbate 80
***Acinetobacter baumanii* (10A)**	-	-	-	-	-	-	-	+
***Acinetobacter bouretii* (12K)**	-	-	-	-	+	+	+	+
***Aeromonas hydrophila* (7A)**	-	+	+	+	+	+	+	+
***E. coli* (36)**	-	-	-	-	-	-	-	+
***Enterobacter cloaceae* (12E)**	-	-	-	-	-	-	-	+
***Flavobacterium branchiophilum* (8I)**	-	-	-	-	-	-	-	+
***Klebsiella pneumoniae* (11A)**	-	-	-	-	-	+	+	+
***Pseudomonas staurtii* (17D)**	-	-	-	-	+	+	+	+
***Serratia fonticola* (4B)**	-	-	-	-	-	+	+	+
***Staphylococcus muscae* (15D)**	-	-	-	-	-	-	-	+
Total Sensitive **Total Resistant**	**S-10** **R-0**	**S-9** **R-1**	**S-9** **R-1**	**S-9** **R-1**	**S-7** **R-3**	**S-5** **R-5**	**S-5** **R-5**	**S-0** **R-10**

- **No Bacterial Growth**

\+ **Bacterial Growth**

Table 4.6. Wastewater Isolated Bacterial Sensitivity to Clove, Cinnamon and Oregano oils based on Broth Tube Macro-dilution MIC Assay.

Bacteria	Essential Oils Used				
	MIC in mg/ml				
	Clove	Cinnamon	Oregano	Control	
				Veg Oil	5 % Acetone
***Acinetobacter baumanii* (12K)**	0.52 ≤	0.51≤	0.47 ≤	-	-
***Acinetobacter bouretii* (10A)**	2.08	1.03	3.76	-	-
***Aeromonas hydrophila* (7A)**	2.08	4.12	30.08	-	-
***E. coli* (36)**	0.52 ≤	0.51 ≤	0.47 ≤	-	-
***Enterobacter cloaceae* (12E)**	0.52 ≤	0.51 ≤	0.47 ≤	-	-
***Flavobacterium branchiophilum* (8I)**	0.52 ≤	0.51 ≤	0.47 ≤	-	-
***Klebsiella pneumoniae* (11A)**	1.04	8.24	1.88	-	-
***Pseudomonas staurtii* (14D)**	4.16	4.12	3.76	-	-
***Serratia fonticola* (4B)**	4.16	8.24	1.88	-	-
***Staphylococcus muscae* (15D)**	0.52 ≤	8.24	0.47 ≤	-	-

4.3 Growth of Bacteria from Wastewater Samples Percentage Reduction Analysis

The antibacterial activity of the essential oils Clove, Cinnamon, Oregano were tested directly against wastewater samples (AT-2, AT-4, AT-6, AT-8, DIG-1, DIG-2, Return Sludge) that include a diversity of bacterial cultures. The results revealed the inhibitory potential of all three essential oils against all seven wastewater samples on R2A agar plate to a varying degree (Table 4.7; Appendix M). clove oil reduced the mixed wastewater bacterial population to the largest degree, relative to other tested essential oils. in the anaerobic digesters, less against the bacteria in the aerated sludge samples and not at all to the return sludge sample. cinnamon oil showed a reduction of bacterial colonies consistently across all three types of samples. Similarly, oregano oil was

effective at reducing the numbers of bacteria in all three types of samples. However, none of the essential oils at the concentrations (even lowest MICs) tested were able to completely inhibit all of the bacteria in the samples.

Table 4.7. The Percent Reduction in Colony Forming Unit of mixed Wastewater Bacterial Cultures due to Inhibition by Clove oil, Cinnimon oil and Oregano oil.

Wastewater Samples	Mixed Culture Percentage Reduction Analysis			[5]
	Clove % Reduction	Cinnamon % Reduction	Oregano % Reduction	Control WWTP sample
AT-2	**75%**	**99%**	**82.5%**	100% (660)
AT-4	**80%**	**97.7 %**	**70%**	100% (308)
AT-6	**46%**	**46%**	**97%**	100% (295)
AT-8	**59.8 %**	**99.7%**	**94.7%**	100% (430)
DIG-1	**78.1%**	**84%**	**87.7%**	100 % (345)
DIG-2	**94.2%**	**96.4%**	**88.9%**	100% (267)
Return Sludge	**0%**	**82.7%**	**75.35%**	100% (920)

[5] 100 ul of raw wastewater sample mixed with 100 ul of distilled water.

CHAPTER FIVE

5. DISCUSSION

5.1 Kirby-Bauer Disc Diffusion Assay

In this study, the main objective was to determine the effectiveness of essential oils against wastewater bacteria. This study is the first to determine the effectiveness of essential oils against wastewater bacteria.

5.1.1 Clove oil

Previous studies have shown that clove oil can be quite inhibitory against bacterial strains by employing Kirby-Bauer disc diffusion assay. Moreira *et al*. (2005) reported that clove essential oil had strong bactericidal and bacteriostatic action against *E. coli* (Babu *et al*. 2011) further reported extreme sensitivity of *E. coli* to clove with zones of inhibition ranging from 21 mm to 61 mm by employing disc diffusion method with Muller-Hinton agar. (Burt *et al*. 2002) who reported clove being antibacterial against *E. coli 0157: H7* with a zone of inhibition halos 15.7 mm. In the case of *Staphylococcus aureus* (Babu *et al*. 2011) who reported zone of inhibition halos 25 mm, I found zone of inhibition halos of greater than 32.5. Further *Aeromonas hydrophilla* (Deans *et al*. 1987) reported zone of inhibition halos 16.5 mm while in this study zone of inhibition were found to be greater than 20 mm. Deans *et al*. (1987) also reported zone of inhibition of 7 mm for *Klebsiella pneumonii* whereas this study recorded zone of inhibition greater than 23 mm. The difference in results can be attributed to the fact all above reported experiments had used different essential oil concentrations and different growth media so it is not possible to compare results of current study with reported results in literature. However, this was the first study to examine the effect of essentail oils on *Pseudomonas fluorescence, Stenotrophomonas maltophilies* (2)*, Pseudomonas poa, Pseudomonas putida, Acinetobacter baumanii, Pseudomonas staurtii, Serratia fonticola, Staphylococcus muscae, Enterobacter cloaceae, Flavobacterium brachiophillum,* and *Acinetobacter bouretii* and therefore this study adds to the existing information in the literautre.

5.1.2 Cinnamon oil

The previous studies based on Kirby-Bauer disc diffusion assay of cinnamon oil without added stabilizers (Prabuseenivasan. 2006) reported that cinnamon oil could be a of antibacterial agent based on zone of inhibition from 16.2 to 29.8 mm for *E.coli, Staphylococcus aureus, Klebsiella*

pneumonii whereas this study found zones of inhibition halos between 19 and 36. Further, Silveria *et al*. (2012) reported a zone of inhibition of 17.4 mm and 11.5 mm against *Staphylococcus aureus* and *E.coli* respectively, whereas this study reported zone of inhibition from 27.5 mm to greater than 36 mm. Zhang *et al*. 2016 who reported that cinnamon oil produced significant result against gram positive, and gram-negative bacteria and it exhibited good potential for application in food products, based on zones of inhibition of 19.2 to 28.7 mm, whereas this study reported a zone of inhibition from 27.5 mm to greater than 36 mm against *Staphylococcus aureus* and *E.coli*. The difference in results can be attributed to the fact all above reported experiments had used different essential oil concentrations and different growth media so it is not possible to compare results of current study with reported results in literature. To the best of my knowledge the literature is silent on the effectiveness of cinnamon oil against bacteria in the species, *Pseudomonas fluorescence, Stenotrophomonas maltophilies* (2)*, Pseudomonas poa, Pseudomonas putida, Acinetobacter baumanii, Pseudomonas staurtii, Serratia fonticola, Staphylococcus muscae, Enterobacter cloaceae, Flavobacterium brachiophillum,* and *Acinetobacter bouretii*.

5.1.3 Oregano oil

The previous studies based on disc diffusion assay of oregano oil (Burt *et al*. 2003) reported oregano oil zones of inhibition of 24.3 mm against *E.coli* whereas this study recorded zone of inhibition between 16 mm and 25 mm, Marira *et al.* (2010) reported oregano oil zones of inhibition of 32 mm, 26 mm, 35 mm against *Staphylococcus aureus, E.coli* and *Enterobacter cloceae* whereas this study recorded zones of inhibition halos from 16 to greater than 40.5 mm. Similarly Mith *et al.* (2014) reported oregano oil zones of inhibition of 15.9 mm, and 15.6 mm against *Pseudomonas flourescence, E.coli* whereas this study reported zones of inhibition between 16 mm and 20 mm. (Moreira *et al.* 2005) reported zones of inhibition of 10-12 mm, against different strains of *E.coli* whereas this study found zone of inhibition from 16 to 25 mm. Dobre *et al*. (2011) reported zones of inhibition of 43.5mm against *Staphylococcus aureaus* whereas I recorded zones of inhibition of 40.5 mm. Rusenova *et al*. (2009) reported zone of inhibition of 17.7 mm, 35 mm, 29.3 mm against *Staphylococcus aureus, E.coli, Klebsiella pneumonii* whereas this study recorded zone of inhibition of 40.5 mm, 16 mm and 22 mm. The difference in results can be attributed to the fact that all above reported experiments had used different essential oil concentrations and

different growth media so it is not possible to compare results of current study with reported results in literature. To the best of my knowledge, the literature is silent on the effectiveness of oregnao against *Stenotrophomonas maltophilies, Pseudomonas poa, Pseudomonas putida, Acinetobacter baumanii, Pseudomonas staurtii, Serratia fonticola, Staphylococcus muscae, Flavobacterium brachiophillum,* and *Acinetobacter bouretii.*

5.1.4 Tea tree oil

Previous studies (Rusenova *et al*. 2009) have reported teatree oil producing zones of inhibition of 27 mm against *Staphylococcus aureus* whereas this study reported zones of inhibition of 22 mm Rusenova *et al*. (2009) and Moreira *et al*. (2005) reported that tea tree oil produced zones of inhibition between 26 mm, and 32 mm against *E.coli* whereas this study reported zones of inhibition of 14.5 mm. The difference in results can be attributed to the fact all above reported experiments had used different essential oil concentrations and different growth media so it is not possible to compare results of current study with reported results in literature. Whereas to the best of my knowledge literature is silent in case of *Stenotrophomonas maltophilies* (2)*, Pseudomonas poa, Pseudomonas putida, Acinetobacter baumanii*. This study showed that tea tree oil was inhibitory to some of the bacteria but not to all of them. This additional information adds the dispository of information about the usefulness of this essential oli in antibacterial inhibition

5.1.5 Fennel oil

The previous studies based on disc diffusion assay (Silveria *et al*. 2012) reported fennel oil zones of inhibition of 12.1 mm, and 11.1 mm against *Staphylococcus aureus, E. coli,* whereas this study reported zones of inhibition of 24 mm and 18 mm respectively. Similarly, Deans *et al*. (1987) reported fennel oil zones of inhibition between 0 mm, and 7.5 mm for *E. coli,* and *Staphylococcus aureus* and Diao *et al*. (2014) who reported zones of inhibition of 19.1 mm against *E. coli*. The difference in results can be attributed to the fact all above reported experiments had used different essential oil concentrations and different growth media so it is not possible to compare results of current study with reported results in literature. To the best of my knowledge literature is silent in case of *Stenotrophomonas maltophilies, Pseudomonas poa, Pseudomonas putida,* and *Acinetobacter baumani.* Overall Fennel oil did not exhibit strong antibacterial activity againsts the selected bacteria and therefroe was not included in further testing.

5.1.6 Wintergreen oil

The previous studies based on disc diffusion assay (Prabuseenivasan 2006) reported zones of inhibition between 0 mm, and 8.9 mm for *Staphylococcus aureus* and *E. coli,* whereas this study reported zones of inhibition between 14.9 and 27 mm. The difference in results can be attributed to the fact all above reported experiments had used different essential oil concentrations and different growth media so it is not possible to compare results of current study with reported results in literature. Whereas to the best of my knowledge literature is silent in case of *Stenotrophomonas maltophilia* (2)*, Pseudomonas poae, Pseudomonas putida,* and *Acinetobacter baumanii.*

5.2 Minimum Inhibitory Concentration Assay

The three essential oils (clove, cinnamon and oregano), which showed comparatively greater inhibitory activity during preliminary screening by employing disc diffusion assay, were selected for determination of the minimum inhibitory concentration (MIC) using broth tube dilution method.

5.2.1 MIC of clove oil

The literature suggested that clove essential oil at different concentration was inhibitory against different bacterial strains. Clove essential oil was able to reduce the bacterial population completely at or below MIC value of 5000 mg/L level for *Escherichia coli, Staphylococcus aureus, Bacillus cereus, Yersinia enterocolitica* (Siddiqua *et al*. 2014). In another study, (Stephani de Rapper *et al*. 2013) reported MIC values against *Staphylococcus aureus and Pseudomonas aeruginosa* of 1.5 mg/ml. Similarly, Burt *et al.* (2003) reported a MIC value of 0.4-2.5 ul/ml each based on microdilution assay for clove essential oil against food isolated pathogens such as *E.coli and Staphylococcus aureus.* It was also reported that MICs against *Klebsiella pneumoniae* and *E.coli* were > 6.4 mg/ml and >1.6 mg/ml respectively (Prabuseenvasan *et al.* 2006). Moreira *et al*. (2005) reported a MIC value of clove essential oil against *E.coli* ATCC 25158 of 0.25 ml/ 100 ml. This study found the MIC of the clove essential oil to be 0.52 mg/ml against *E.coli*, *Staphylococcus muscae, Enterobacter cloaceae, Acinetobacter baumanii* and *Flavobacterium branchiophilum.*

The MIC concentration was 1.04 mg/ml against *Klebsiella pneumoniae*, 2.08 mg/ml against *Aeromonas hydrophila*, *Acinetobacter bouretii,* and 4.16 mg/ml against *Pseudomonas staurtii, and Serratia fonticola*.The MIC in this study was found to be in the same range as other studies, although it demonstrated that different bacteria were susceptibile to varying concnetrations of the essential oil.

5.2.2 MIC of cinnamon oil

The literature suggested that cinnamon oil at different concentration was inhibitory against bacteria isolated from different sources. In one paper, cinnamon essential oil showed significant inhibition with a MIC value of 0.125 ul/ml and an MBC (Minimum Bactericidal Concentration) value of 0.25 ul/ml against all five bacteria tested except *Pseudomonas fluorescence* that remained resistant against cinnamon at MIC/ MBC value 1 ul/ml. According to another paper, the MIC of cinnamon essential oil against *Klebsiella pneumonii* and *E.coli* was 3.2 mg/ml, and >1.6 mg/ml respectively (Prabuseenvasan *et al.* 2006). Similarly, another paper has reported a MIC value of 5 mg/ml of cinnamon essential oil against *E.coli* with broth microdilution assay (Silveria *et al.* 2012).

In this study cinnamon oil had a MIC of 0.51 mg/ml against *E.coli, Enterobacter cloaceae, Acinetobacter baumanii, Flavobacterium branchiophilum,* a MIC of 1.03 mg/ml against *Acinetobacter bouretii,* a MIC of 4.12 mg/ml against *Pseudomonas staurtii, Aeromonas hydrophila,* and a MIC of 8.24 mg/ml against *Klebsiella pneumoniae, Serratia fonticola,* and *Staphylococcus muscae*.

5.2.3 MIC of oregano oil

The MIC of oregano essential oil against *Aeromonas hydrophilla* has been reported to be 2.5 ul/ml when the broth microdilution assay was employed (Azeredo *et al*. 2011). Similarly, another paper showed a MIC value of 0.5 ug/ml against *Enterobacter cloacae* by employing the broth microdilution assay (Sokovic *et al.* 2010). It was also reported in the literature that oregano essential oil possesses a MIC value of 1.8 ml/100ml against *E.coli* based on a broth microdilutrion assay (Moreira *et al.* 2005). The literature suggested that cinnamon oil at different concentration was inhibitory against bacteria isolated from different sources. This study found the MIC of oregano to be 0.47 mg/ml against *E.coli*, *Staphylococcus muscae, Enterobacter cloaceae,*

Acinetobacter baumanii and *Flavobacterium branchiophilum,* to be 1.88 mg/ml against *Klebsiella pneumoniae, Serratia fonticola,* to be 3.76 mg/ml against *Pseudomonas staurtii, Acinetobacter bouretii*, to be 30.08 mg/ml against *Aeromonas hydrophila.* Although the MICs to the common bacteria were similar to that previously found, this was the first report of oregano's effect towards *A. hydrophila,* which appears to be quite resistant to the oil.

5.3 Wastewater samples bacterial growth reduction percentage analysis

In the literature, one study done by Moura *et al*. (2011) suggested a possible use of plant compounds and oils for the disinfection of water. Antibacterial activity of the *M. oleifer* flower preparations were evaluated and found to be active against Gram-negative and Gram-positive bacteria and impair the growth of microorganisms from environmental lake water. This study tested the antibacterial activity of six essentail oils. It was found that three of the essential oils, clove, cinnamon, and oregano were effective against seven different wastewater mixture samples including both aerated sludge (AT-2, AT-4, AT-6, AT-8), anaerobic digesters (DIG-1, DIG-2) and Return Sludge (RS) samples.

6. Conclusion

This study opens a new window to essential "oils" antibacterial activity against municipal wastewater bacteria. The research on antibacterial activity of clove, cinnamon and oregano oil did not only confirm their antibacterial activity but also fill a void in scientific literature. This study aimed to identify antibacterial activity of these essential oils against wide array of municipal wastewater isolated bacteria and muncipal wastewater samples augments objectives of the research project. None of the oils completely inhibited bacteria growth in the samples; however, all the oils had a significant effect on both the aerobic (aerated sludge samples) and anaerobic (digester samples) bacterial population. The inhibition test performed at the lowest MIC was found to inhibit the individual strains and therefore higher concentrations may produce even further reductions in bacterial growth. Finally, the use of essential oils in conjunction with current chlorine treatment may improve the ability to eliminate all bacteria from water samples. On the other hand, the use of an essential oil treatment may allow a reduction in the chlorine usage and consequent reduction in the production of disinfection byproducts.

Future Study

- This study is limited in scope as only bacteria isolated from wastewater were tested against these essential oils, but a broader investigation should include enteric bacteria such *Compylobacter jejuni, Leptospira* spirochete enteric viruses, the fungus *Aspergillus*, the protozoans *Giardia* and *Cryptosporidium,* and the tapeworm *Hymenolepis* respectively.
- Plant essential oils are screened and quantified for their antimicrobial activity, but so far their application as a disinfectant in the water reuse system have not been explored extensively (Winward *et al*. 2008). Based on these findings the application of plant essential oils in foodborne, food spoilage opens a new window towards the water, wastewater and water reuse disinfection potential that needs to be explored fully by further study.
- Electron microscopic analysis of bacterial strains inhibited by the activity of essential oils would help in understanding the mode of action of effective essential oils against bacteria.

APPENDIX A

Preliminary Screening results of the antibacterial potential of *Syzygium aromaticum* (Clove), *Cinnamomum zeylanicum* (Cinnamon), *Origanum vulgare* (Oregano), *Melaleuca alternifolia* (Tea tree), *Foeniculum vulgare* (Fennel), *Gaultheria procumbens* (Wintergreen) against bacterial pure cultres (Disc size 13mm).

Bacteria	**Essential Oils Used**							
	Zones of Inhibition in (mm)							
	Clove (CL) (52 mg)	**Cinnamon (CN)** (51.5 mg)	**Oregano (ORG)** (47 mg)	**Fennel (FN)** (48 mg)	**Tea tree (TT)** (44.5 mg)	**Winter Green (WG)** (59 mg)	**Control** Veg Oil	5% Acetone
***Staphylococus aureus* (16)**	**32.5 ± 3.5**	**27.5± 3.5**	**40.5 ± 0.7**	**24 ± 0.86**	**22.5± 3.5**	**27± 2**	**0**	**0**
***Pseudomonas fluorescens* (8)**	**26 ± 1.5**	**25± 0.46**	**20.5± 1.8**	**15± 1**	**14.5± 0.5**	**14± 0.5**	**0**	**0**
***E.coli: DH5∞* (17)**	**20 ± 2.8**	**35± 7.0**	**14.5± 3.5**	**18± 1**	**14.5± 0.7**	**14.5 ± 0.7**	**0**	**0**
***E.coli: AD202* (36)**	**33 ± 2.8**	**36± 8.4**	**16± 1.4**	**17± 3.6**	**14.9 ± 0.7**	**14± 0.5**	**0**	**0**
E.coli:DH5∞	**21.5 ± 0.5**	**31.5± 2.1**	**14.5± 2.12**	**15 ± 0.86**	**14.5± 0.7**	**14.9 ± 0.87**	**0**	**0**
***Stenotrophomonas maltophilia* (13)**	**20 ± 0.5**	**18.5 ± 0.7**	**17.5 ± 0.7**	**22± 3.6**	**20.5± 0.7**	**29± 6**	**0**	**0**
***Stenotrophomonas maltophilia* (33)**	**21± 2**	**29.5 ± 0.7**	**18.5 ± 0.5**	**17± 2.64**	**16.5± 1.32**	**20± 2**	**0**	**0**
***Pseudomonas poae* (26)**	**25 ± 2.5**	**34.5 ±1.75**	**31 ± 1.4**	**15± 1**	**33± 4.2**	**17± 1.5**	**0**	**0**
***Acinetobacter baumanii* (27)**	**21.5± 1.75**	**21.5 ± 0.5**	**41.5 ± 2.12**	**18± 1**	**14.5± 1.32**	**16.5± 1.5**	**0**	**0**
***Pseudomonas putida (*BBC-443)**	**23 ± 1.75**	**23± 7.0**	**16± 0.7**	**15± 0.86**	**16.75± 4.5**	**24± 2**	**0**	**0**

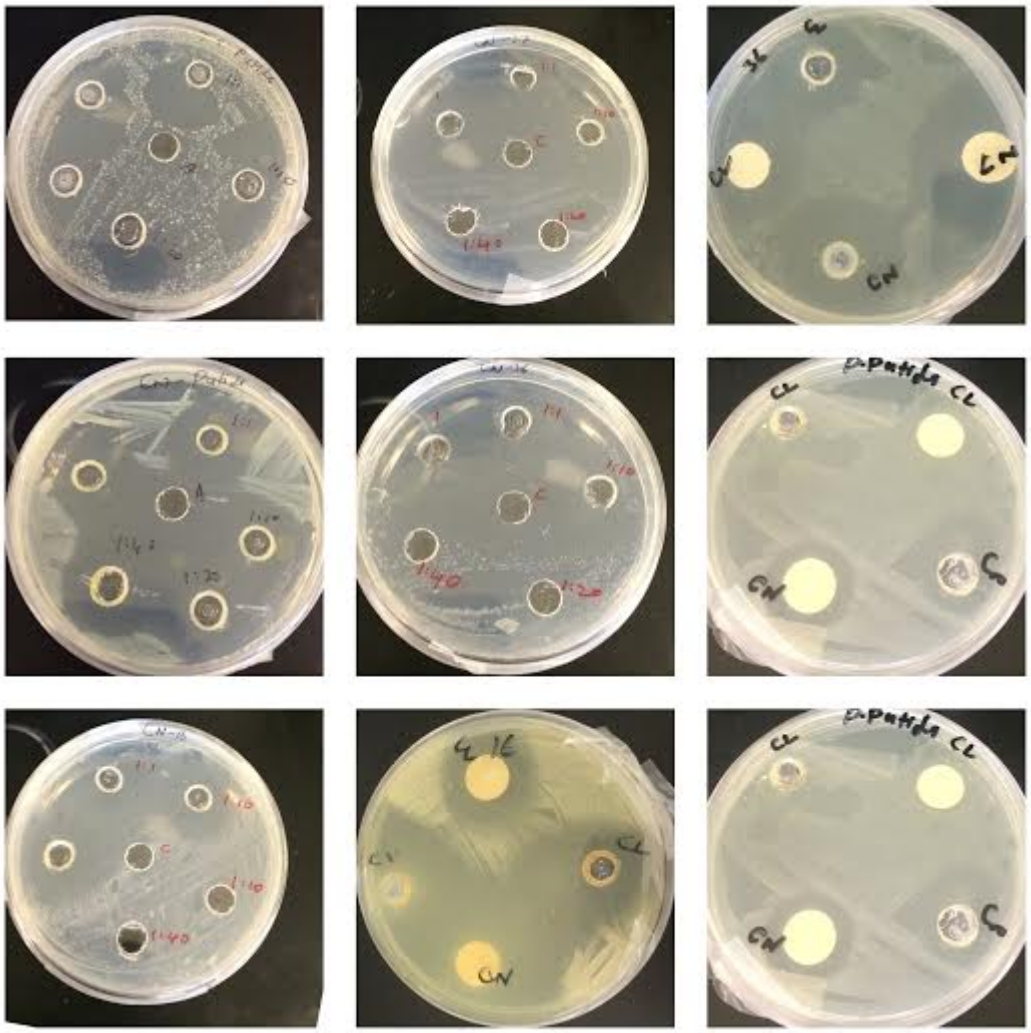
- CL represents Clove oil, CN represents Cinnamon oil, ORG represents Oregano oil, FN represents Fennel oil, TT represents Tea tree oil, WG represents Wintergreen.
- All the results were taken in triplicates.

APPENDIX B

Preliminary Screening results of the antibacterial potential of *Syzygium aromaticum* (Clove), *Cinnamomum zeylanicum* (Cinnamon), *Origanum vulgare*(Oregano) against Wastewater isolated bacterial strains (Disc 6 mm).

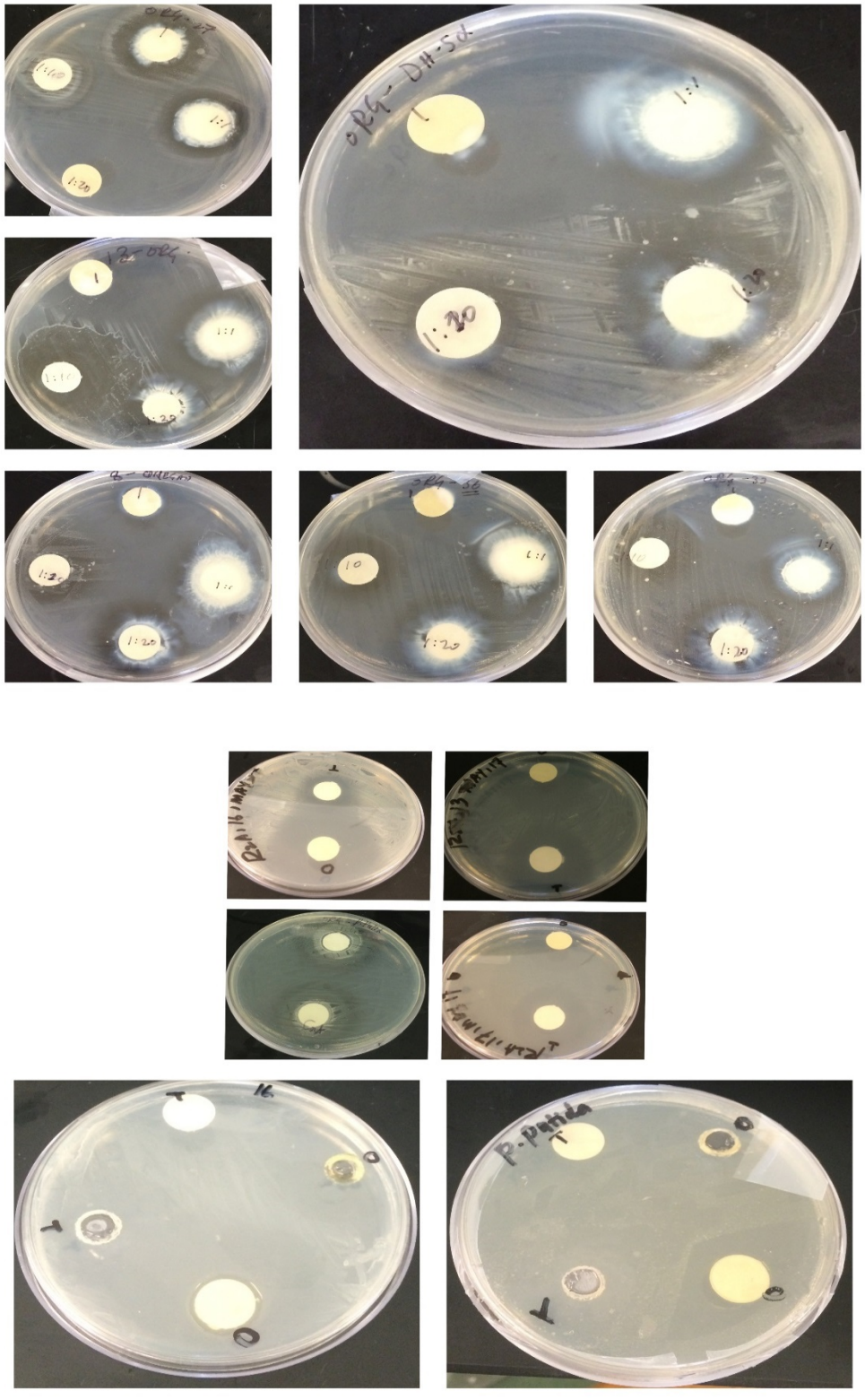
Bacteria	Essential Oils Used Zones of Inhibition in (mm)				
	Clove (CL) (52 mg)	Cinnamon (CN) (51.5 mg)	Oregano (ORG) (47 mg)	Control	
				Veg Oil	5 % Acetone
***Pseudomonas staurtii* (14D)**	22.6 ± 3.5	24.6 ± 1.52	22 ± 4	0	0
***Aeromonas hydrophila* (7A)**	20 ± 2	23.6 ± 3.5	31 ± 1	0	0
***E. coli* (36)**	31.6 ± 1.5	25 ± 5	25 ± 5	0	0
***Klebsiella pneumoniae* (11A)**	23± 3	19 ± 1	22 ± 1	0	0
***Acinetobacter baumanii* (2K)**	19.3 ± 3.5	26.3 ± 3.2	30 ± 5.5	0	0
***Serratia fonticola* (4B)**	27 ± 2.6	16 ± 3.6	25 ± 5	0	0
***Staphylococcus muscae* (15D)**	28 ± 2	25 ± 5	32 ± 2	0	0
***Enterobacter cloaceae* (12E)**	20.3 ± 1.5	18.6 ± 3.5	14 ± 5.2	0	0
***Flavobacterium branchiophilum* (8I)**	19.6 ± 2.08	33.3 ± 7.6	34.3 ± 4.04	0	0
***Acinetobacter bouretii* (10A)**	34 ± 1.6	27.3 ± 3.7	24 ± 2	0	0

APPENDIX C



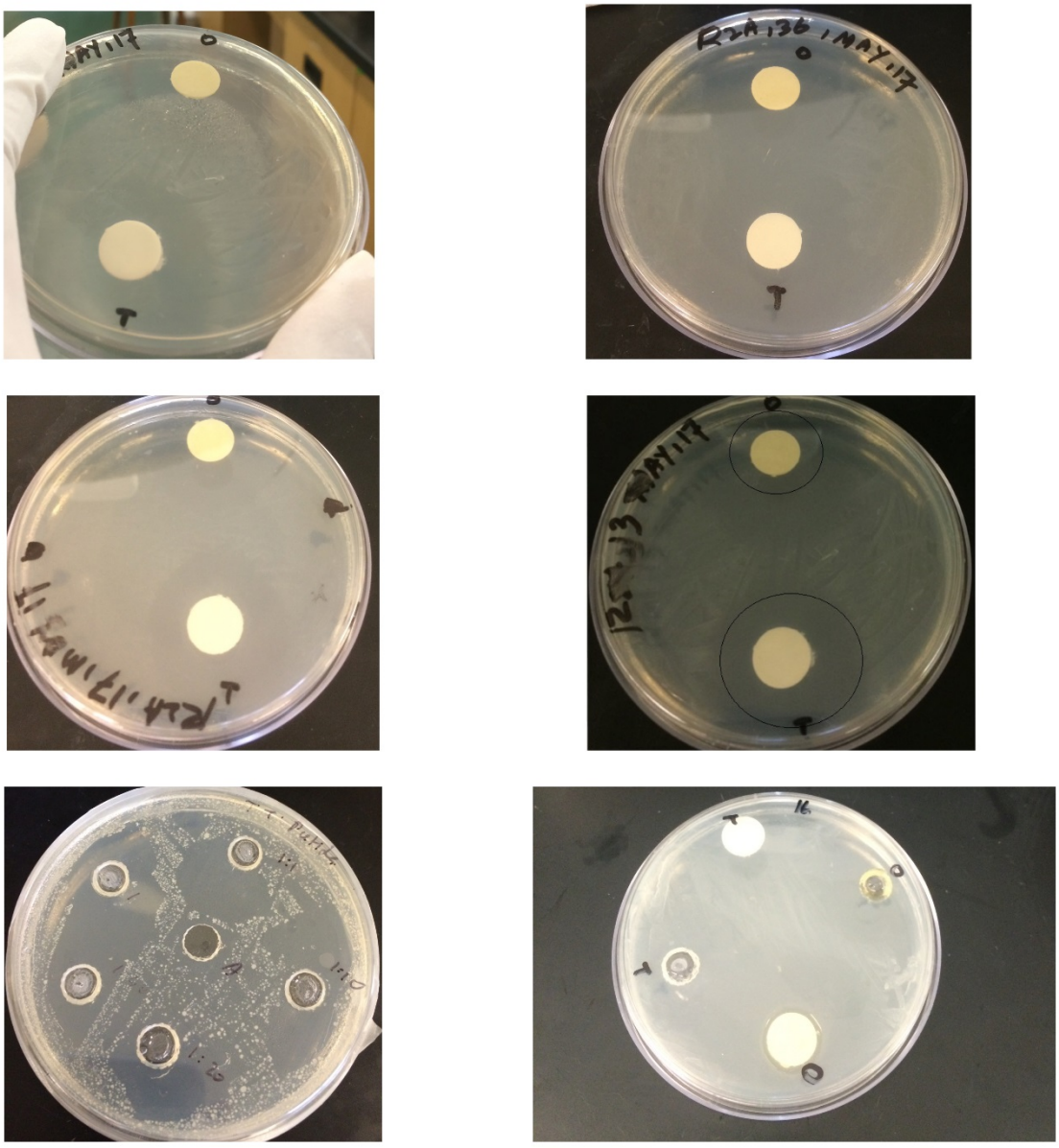
Plates Wild-type Bacterial Pure Cultures Sensitivity to Clove and Cinnamon Essential Oil.

APPENDIX D



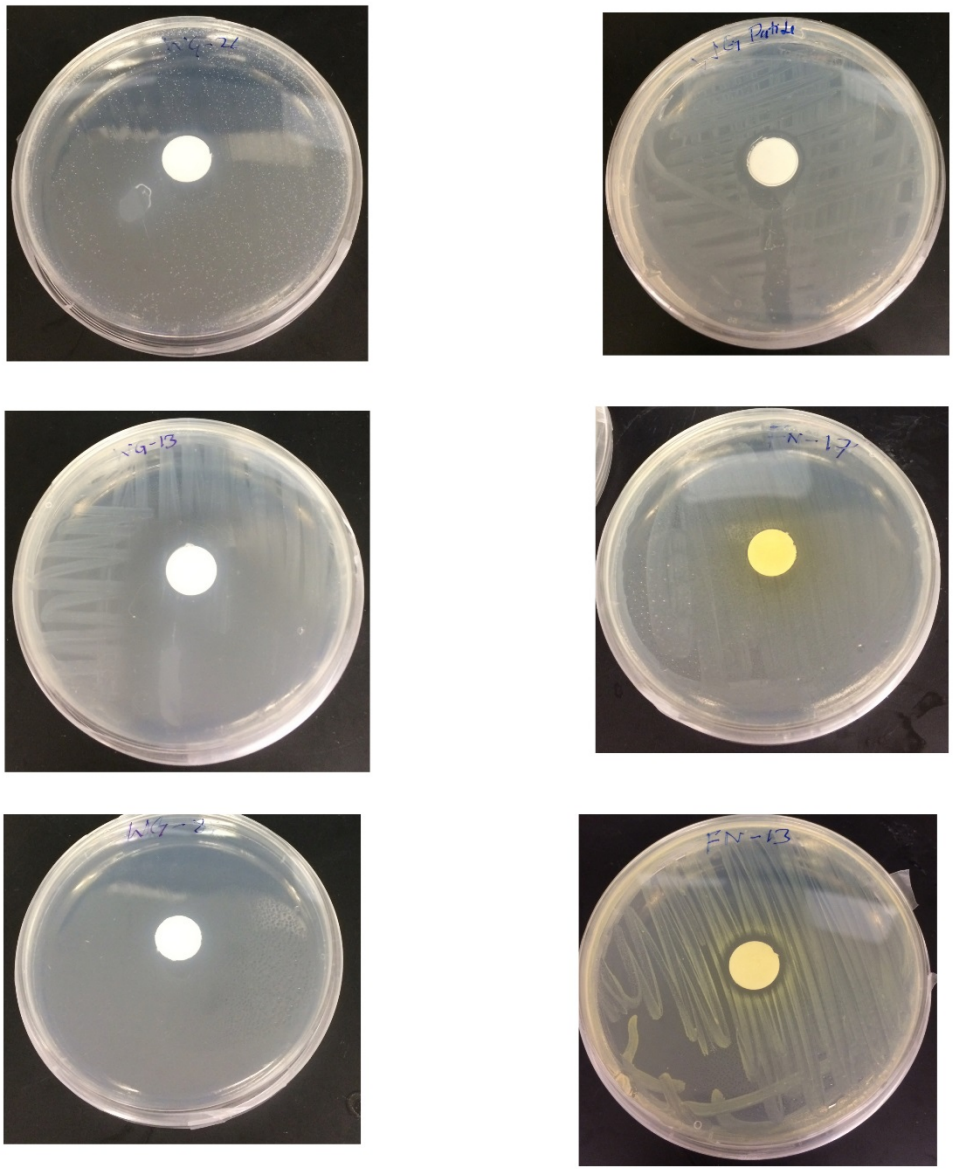
Plates Wild-type Bacterial Cultures Sensitivity to Oregano and Tea Tree Essential Oil.

APPENDIX E



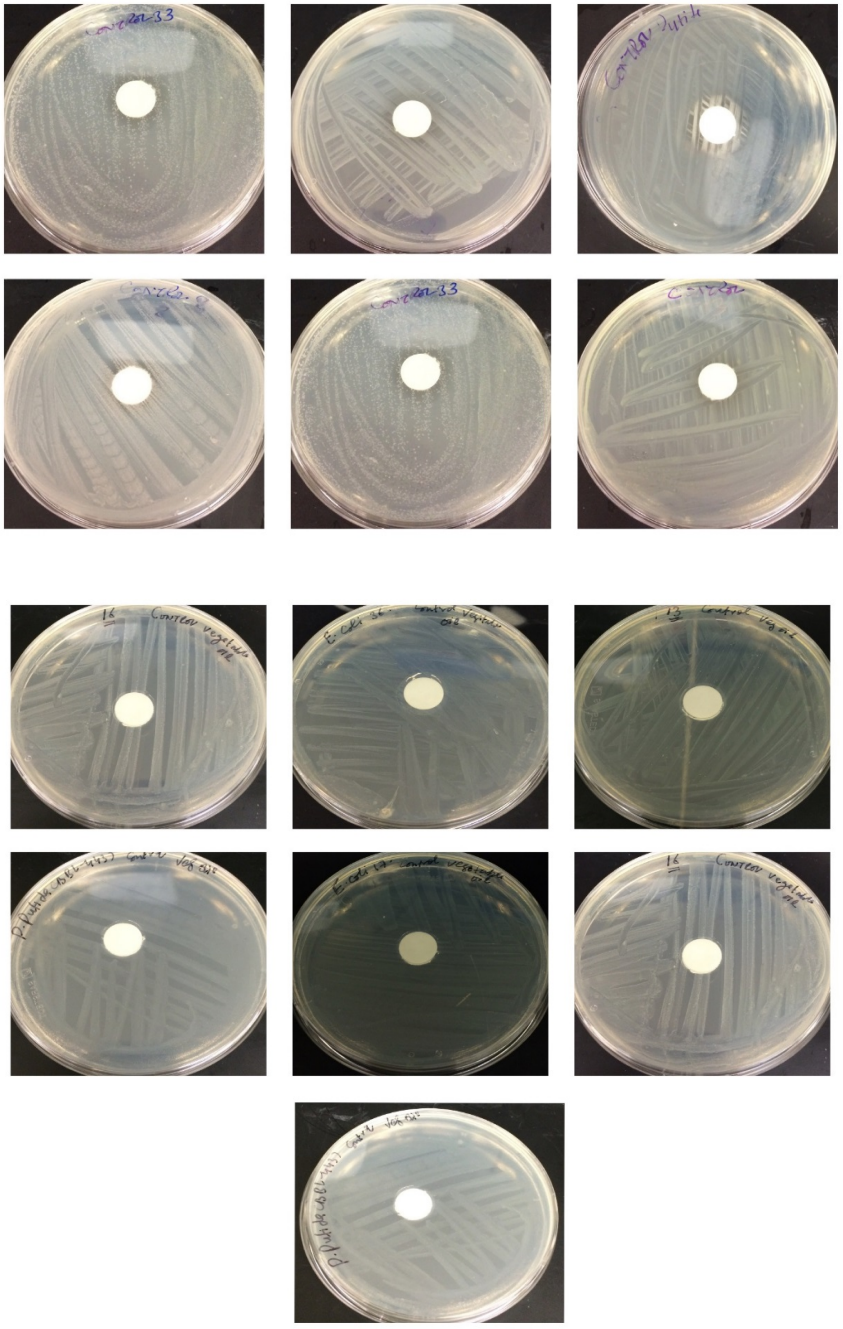
Plates Wild-type Bacterial Pure Cultures Sensitivity to Oregano and Tea Tree Essential Oil

APPENDIX F



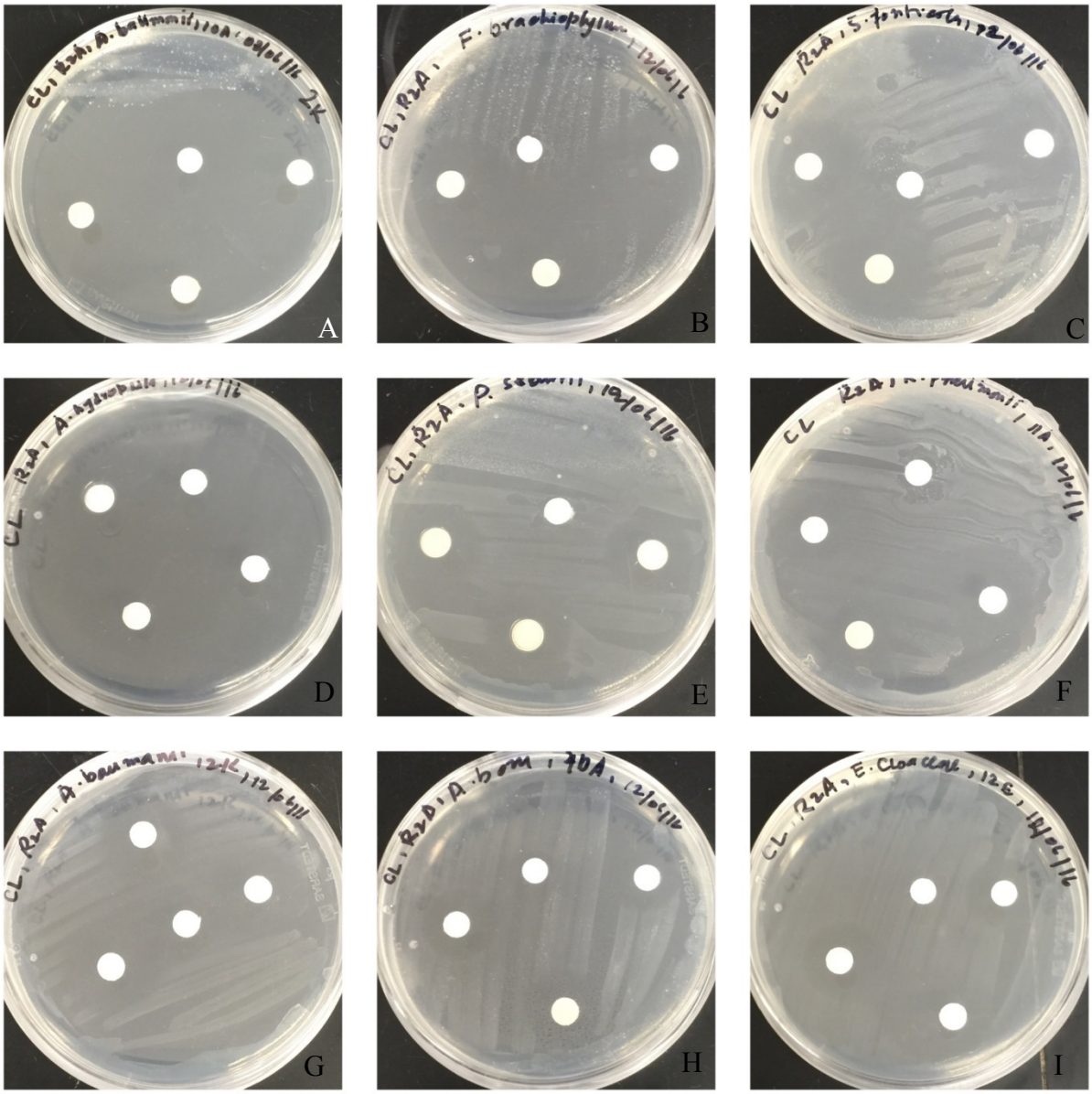
Plates Bacterial Pure Cultures Sensitivity to Fennel and Winter Green Essential Oil.

APPENDIX G



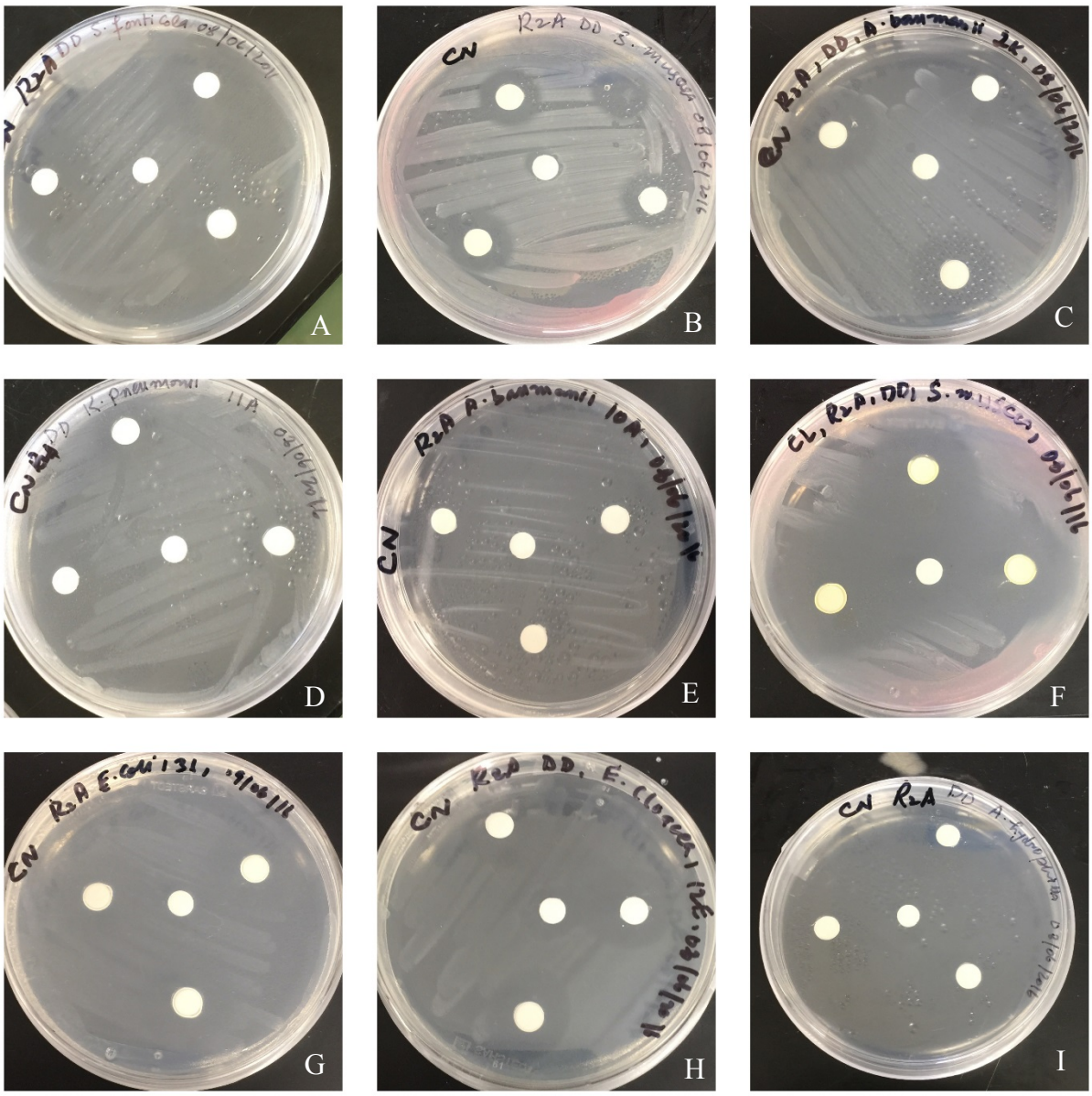
Plates Wild-type Bacterial Pure Cultures Sensitivity to Control Vegetable Oil and 5% Acetone.

APPENDIX H



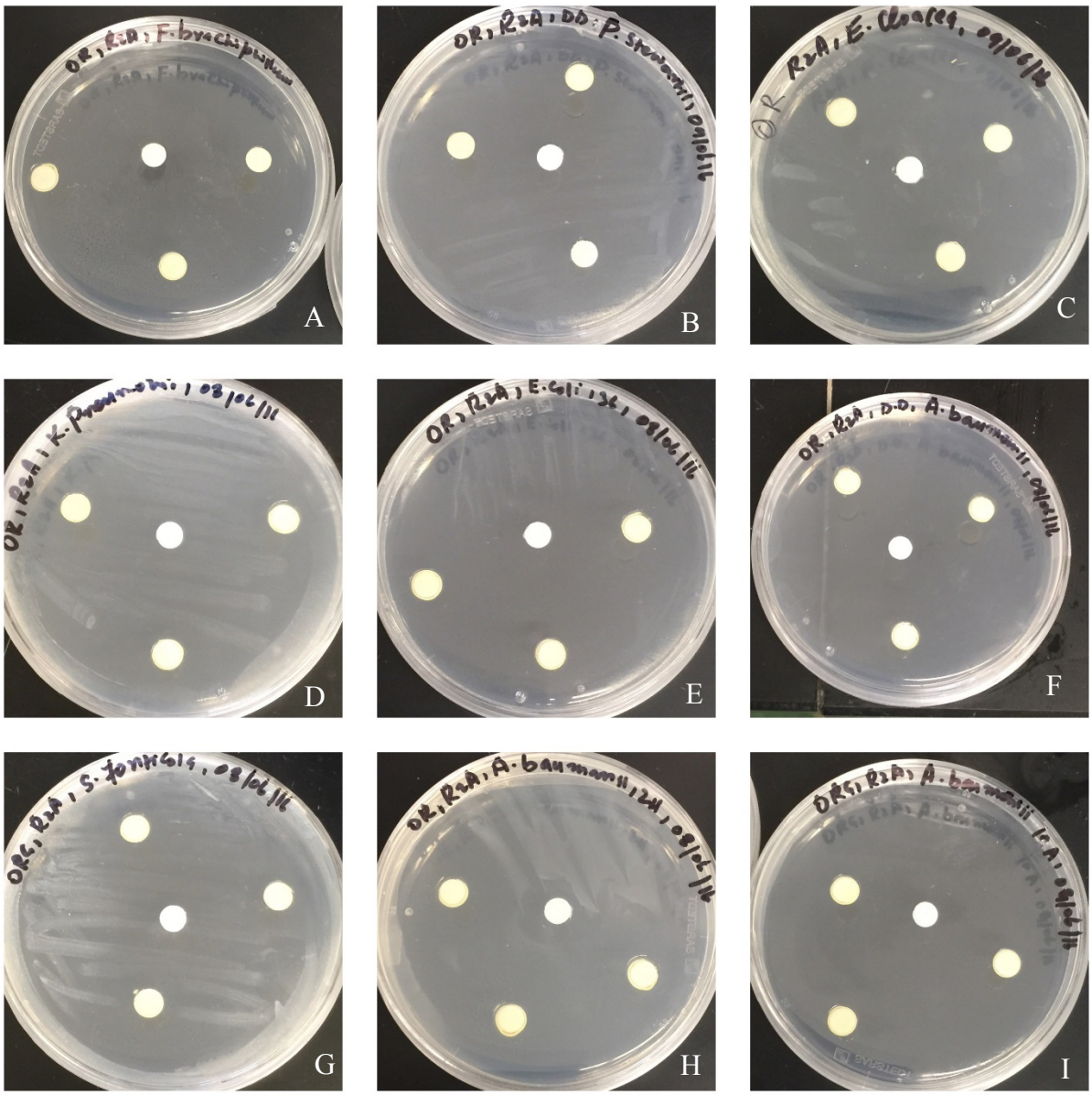
Plates Wastewater isolated Bacterial Sensitivity to Clove Essential Oil.

APPENDIX I



Plates Wastewater Isolated Bacterial Sensitivity to Clove Essential Oil.

APPENDIX J



Plates Wastewater Isolated Bacterial Sensitivity to Clove Essential Oil.

APPENDIX K

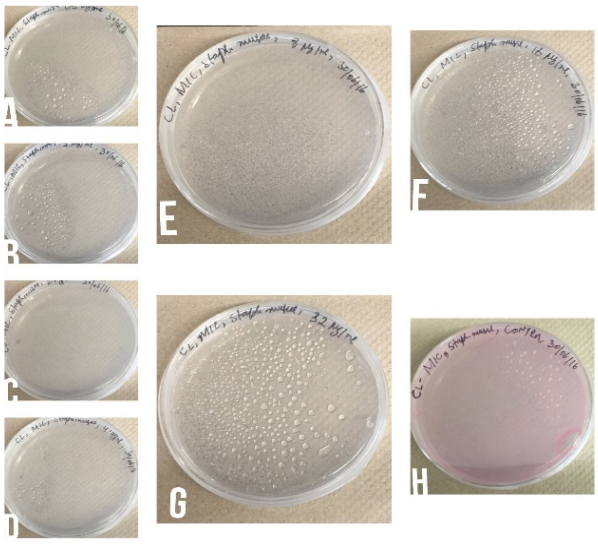


Plate Clove Essential Oil MIC (Minimum Inhibitory Concentration) against *Staphylococcus muscae.*

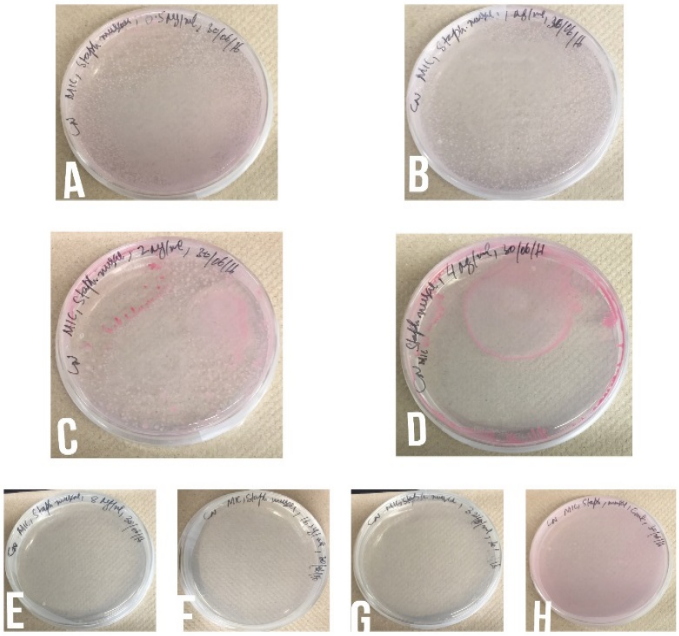


Plate Cinnamon Essential Oil MIC (Minimum Inhibitory Concentration) against *Staphylococcus muscae.*

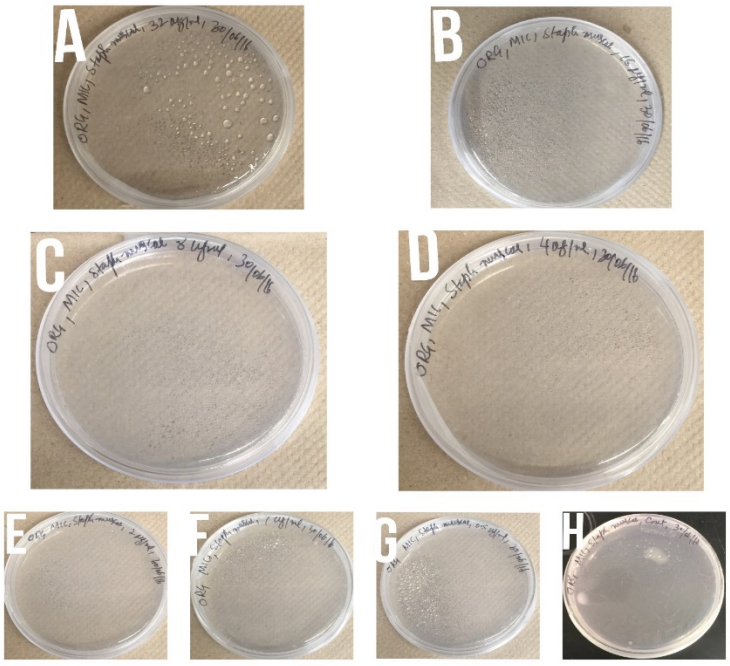


Plate Oregano Essential Oil MIC (Minimum Inhibitory Concentration) against *Staphylococcus muscae* A-G 32 mg/ml, 16 mg/ml, 8 mg/ml, 4 mg/ml, 2 mg/ml, 1 mg/ml, 0.5 mg/ml, H Control.

APPENDIX L

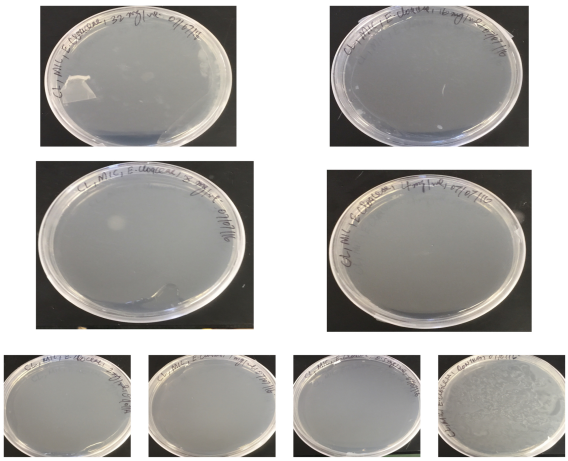


Plate Clove Essential Oil MIC (Minimum Inhibitory Concentration) against *Enterobacter cloaceae.*

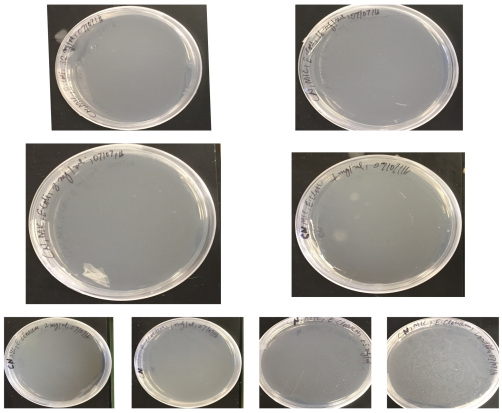


Plate Cinnamon Essential Oil MIC (Minimum Inhibitory Concentration) against *Enterobacter cloaceae.*

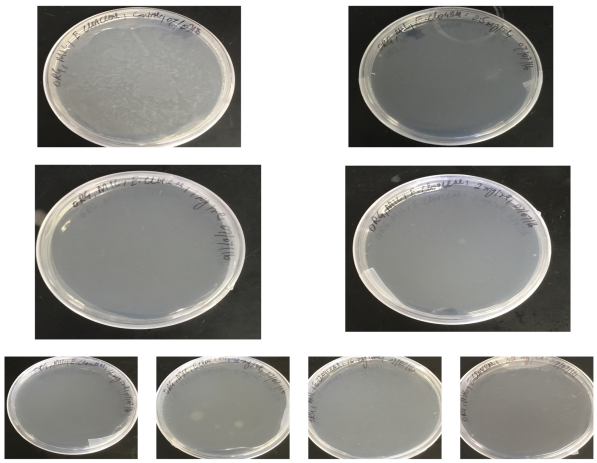
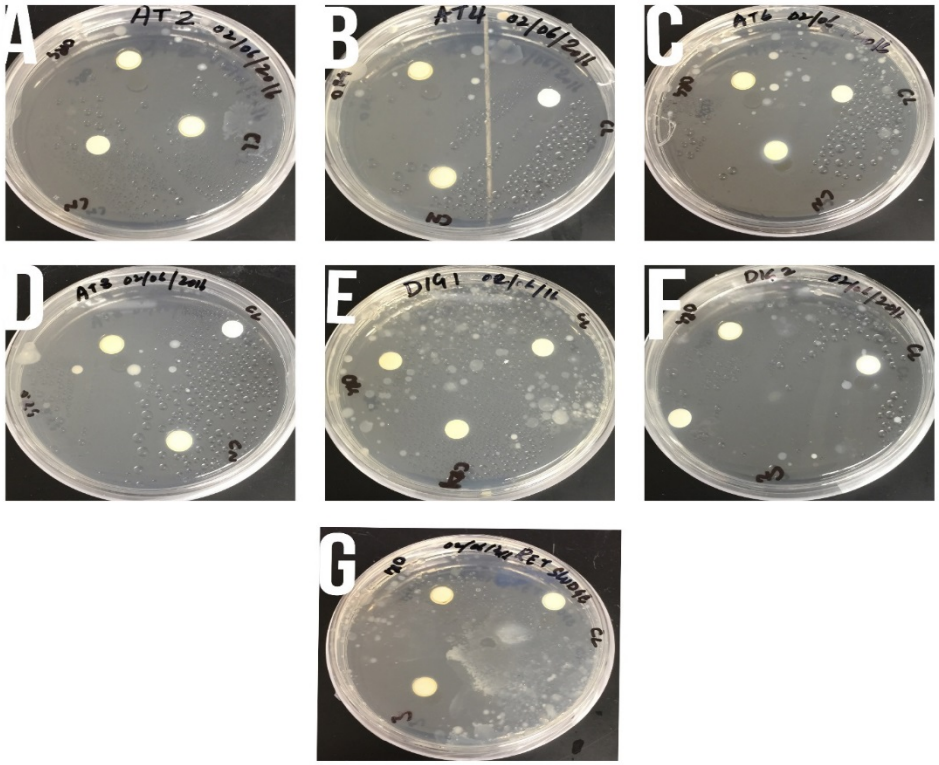
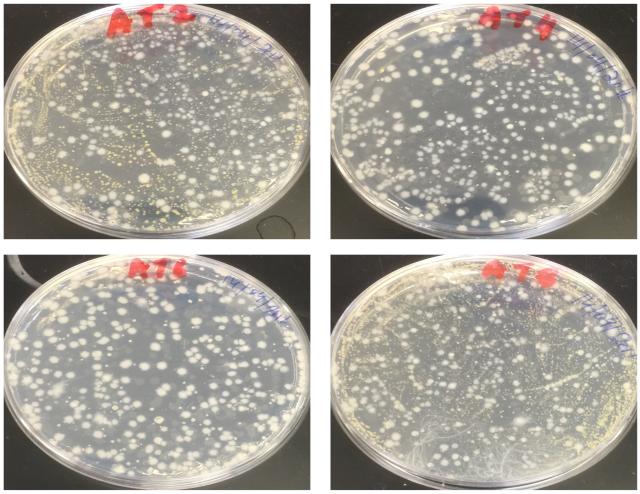


Plate Oregano Essential Oil MIC (Minimum Inhibitory Concentration) against *Enterobacter cloaceae*

APPENDIX M





Plates Wastewater Mixture Bacterial Growth Inhibition A-D Aeration Tank (AT-2, AT-4, AT-6, AT-8), E-F Digester (DIG-1, DIG-2), G Return Sludge (RS) and Control.

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