CHAPTER ONE

INTRODUCTION AND OBJECTIVES

1.1 INTRODUCTION

A modern dental chair unit (DCU) is a medical device that provides physical support for the entire body of dental patient and facilitates the treatment procedure. A dental unit waterline system (DUWS) refers to the complex inter-connected narrow tubings inside a dental chair in which water passes through. Water supplied by a dental chair during a treatment is used to cool and irrigate tooth surfaces and dental instruments as heat is generated during the process due to friction between the dental tissues and instruments. In addition, water is also used to rinse the oral cavity and flush the DCU spittoon and cuspidor during and after dental treatment.

Over recent years, results of many studies showed that the output water delivered through DUWS is often highly contaminated with bacteria. Although majority of bacteria recovered from DUWS are harmless Gram-negative aerobes, their high presence in DCU water are of concern as it is against the general infection control practice requirement. Moreover, high presence of microbes has often been associated with high concentration of endotoxin and some opportunistic pathogens such as *Pseudomonas aeruginosa*, legionellae and non-tuberculosis mycobacteria in the DUWS.

The possibility of getting infected by an infectious disease during dental treatment has created awareness among the public. In November 2010, it was reported in the Malaysian News that a 20-year-old virgin who has no history of drug abuse or blood transfusion was diagnosed positive for Human Immunodeficiency Virus (HIV). It was believed that she

may have contracted the virus during a visit to her dentist. The Ministry of Health and the Malaysian Dental Council had responded to the news by reassuring the public that the possibility of HIV infection through dental treatment is minimal if a strict standard of infection control is followed. Although this represented a rare case that requires further investigation, it highlighted the importance of maintaining a safe working environment for both dental personnel as well as dental patients.

Most available data on the contamination of DUWS have been based on cases occurring in the developed western countries. In the developing countries, the healthcare provider system is not as extensive as that available in the developed nations. Therefore in these countries, cases of infected DUWS water may expose patients and dental personnel to higher risk of acquiring infectious diseases especially when immune-compromised individuals are involved.

This study was carried out to investigate the microbiological quality of water delivered in DUWS of a dental teaching clinic. Water samples were collected from the clinic and the microbial load in the water was determined and the microbial contaminants were identified. An *in vitro* DUWS model was then designed to assess the effectiveness of silver-coated tubing in reducing biofilm formation on the inner surface of the tubing. The antibiofilm effect of the silver-coated tubing was determined by assessing the number of bacteria adhering to the wall of the tubing out of the total planktonic population of inoculated into the model system. The effectiveness of the silver-coated tubing was compared to that determined for commonly used conventional polyurethane DCUs tubing.

1.2 OBJECTIVES

Objectives of study:

i. To analyse the quality of output water from dental unit waterline system.

ii. To develop an *in vitro* biofilm model and assess the antibiofilm property of silver-coated tubing used for DUWS.

CHAPTER TWO

LITERATURE REVIEW

2.1 DENTAL UNIT WATERLINE SYSTEM AND BIOFILM FORMATION

2.1.1 Dental Unit Waterline System (DUWS)

A dental unit waterline system (DUWS) refers to the complex inter-connected narrow tubing inside a dental chair in which water passes through. The length of the waterline is approximately 6 metres and made up of narrow bore flexible polyurethane or polyvinyl chloride (PVC) plastic tubing. The diameter of the tubing is 1/16 inches (2 mm). Some couplings which are either made up of brass or other non-flexible plastic of 1/8 inches (4 mm) diameter are also included as part of the setup of the DUWS (Walker and Marsh, 2007).

2.1.2 Biofilm Formation

A biofilm may be defined as a community of microorganisms that are irreversibly attached to a surface. A biofilm produces extracellular polymeric substances and exhibits an altered phenotype compared with the corresponding planktonic cells, especially with regard to gene transcription. The microorganisms in a biofilm interacts with each other (Lindsay and Von Holy, 2006). The first biofilm investigation was carried out by van Leeuwenhoek who reported the presence of biofilm on tooth surface (Marsh and Bradshaw, 1995, Slavkin, 1997).

In clinical setting, the accumulation of microorganisms in the form of biofilm in many medical devices have been reported (Costerton et al., 1999). Formation of biofilm on to these medical devices gives rise to many persistent and chronic bacterial infections. In the

form of a biofilm, most microbes are heterogeneous in species as well as morphology and are enveloped in a polysaccharide slime layer known as glycocalyx that protects the organisms within from desiccation, chemical insult, and attacks by the immune systems (Mills, 2000).

There are several reasons why DUWS can become so heavily contaminated despite the fact that DCUs are supplied with well-maintained municipal water. One important factor contributing to the problem lies in the physics of laminar flow. Water passing through the tubing of DUWS results in a maximum flow at the centre of its lumen and minimal flow at the periphery. This encourage deposition of microorganisms on to the interior surface of tubing (Williams et al., 1996c). In addition, stagnation of water within the DUWS for most of the time, that includes during the day, overnight (16 hr) and over weekend (64 hr) periods. Stagnation of the entire water column provides undisturbed environment that further promotes the accumulation of biofilm on the interior side of the tubing. Besides that, the narrow bore tubing provides large surface-to-volume ratio (6:1) and gives biofilm plenty of surfaces on which they can spread. Moreover, bacteria adhere more readily to hydrophobic polymeric plastic tubing that is often used in dental chair units than to those composed of glass or steel (Williams et al., 1996b). Most treated water contains minerals, principally calcium carbonate that is deposited on to water-bearing surfaces. Organic molecules subsequently concentrate on to these surfaces and thus promote the adhesion of bacteria and proliferation of biofilm (Davey and O'toole, 2000). Once a new DUWS is connected to the main municipal source, a biofilm starts its formation within eight hr, even when it is not used for dental treatment (Tall et al., 1995). The biofilm would develop and

reach a climax community embedded in a protective extracellular amorphous matrix as short as six days (Walker et al., 2003).

2.2` MICROBIAL CONTAMINATION AND HEALTH RISKS

The presence of high density of microorganisms, particularly bacterial species in DUWS output water was first described in a report in the early 1960s (Blake, 1963). Over the decades, more and more cases on contaminated DUWS have been reported. Studies had shown that the output water from DUWS is usually heavily contaminated unless appropriate water treatment is employed (Kumar et al., 2010). Table 2.1 demonstrated the types of organisms isolated from studies involving the DUWS.

The majority of DUWS contaminants are Gram-negative aerobic environmental species. Some opportunistic respiratory pathogens occasionally isolated in DUWS output water were *Pseudomonas aeruginosa*, *Legionella* spp., and non-tuberculosis *Mycobacterium* (Pankhurst and Coulter, 2007). However, taking into consideration that the conventional culture techniques often used in water analysis are only capable of recovering around 5 % of the actual total bacterial count, the level of DUWS contamination may thus, be worse than the reported figure (Barer and Harwood, 1999). Despite this fact, documented cross-infection cases caused by contamination of DUWS have been relatively rare. However, these low incidences could also be an issue of under-reporting, whereby individuals seldom relate their illnesses to their visit to the dental clinic. In the recent years however, the increased public awareness on the water quality of DUWS and the increasing number of

immune-compromised patients with higher risk of getting infected via contaminated water from DUWS have raised the attention of health care provider towards this potential route of cross-infection since this group of individuals are more susceptible to the infection than the general population (Walker and Marsh, 2004).

Table 2.1: Types of organisms isolated from dental unit waterline system (Franco et al., 2005, Göksay et al., 2008, Kumar et al., 2010, Stampi et al., 1999, Szymańska, 2005b, Szymańska, 2007).

Bacteria	Fungi	Protozoa
Achromobacter xyloxidans	*Alternaria* spp.	*Acanthomoeba* spp.
Acidovorax spp.	*Aspergillus flavus*	*Cryptosporidium* spp.
Acinetobacter spp.	*Aspergillus* spp.	*Microsporidium* spp.
Actinomyces spp.	*Candida albicans*	*Giardia* spp.
Alicaligenes dentrificans	*Candida curvata*	
Bacillus spp.	*Citromyces* spp.	
Bacteriodes spp.	*Cladosporidium* spp	
Burkholderia cepacia	*Geotrichum candidum*	
Caulobacter spp.	*Penicillum expansum*	
Comamonas acidovorans	*Penicillium* spp.	
Flavobacterium spp.	*Phoma* spp.	
Fusobacterium spp.	*Sclerotium sclerotiorum*	
Klebsiella pneumoniae	*Scopulariopsis* spp.	
Lactobacillus spp.		
Legionella spp.		
Methylophilus spp.		
Micrococcus spp.		
Moraxella spp.		
Mycobacterium spp.		
Nocardia spp.		
Ochromobacterium anthropi		
Pasteurella spp.		
Photobacterium damsela		
Pseudomonas spp.		
Sphingomonas spp.		
Streptococcus spp.		
Staphylococcus spp.		
Xanthomonas spp.		

2.2.1 Exposure to Endotoxin

The cell wall of Gram-negative bacteria that often proliferate in DUWS is a potent source of endotoxin. The USA Pharmacopoeia has set a limit for endotoxin in sterile water used for irrigation at 0.25 endotoxin units (EU)/mL (Mills, 2000). Studies have shown that this requirement is not always met. Putnins, Giovanni and Bhullar (2001) had reported the mean endotoxin levels in water collected from high-speed and air/water lines of DUWS were at 480 and 1008 EU/mL, respectively. This level of contamination greatly exceeded the recommendation of the USA Pharmacopoeia. At this level, endotoxin in DUWS even exceeded the level reported in water samples collected from the adjacent clinic sinks which was only at the mean level of 66 EU/mL. The incidence of high endotoxin levels in DUWS was also reported in dental restorative clinic (Szymańska, 2005c).

Endotoxin level in water is of great concern as it has been associated with localised inflammation, fever and shock. Given the potent inflammatory effects of endotoxin, it is logical to consider the role of endotoxin in the development and exacerbation of asthma (Schwartz, 2001). However, it is important to highlight that not everyone exposed to high concentration of endotoxin develops asthma. Kline et al. (1999) suggested that factors such as genetic, sex differences, or comorbid conditions may play a role in the biologic responses to endotoxin. Based on the prevalence and onset of asthma among dentists in London as well as Northern Ireland and the microbiological quality of DUWS in dental practices, the temporal onset of asthma among the dentists was suggested to be associated with occupational exposure to contaminated DUWS (Pankhurst et al., 2005). Similarly, Mathew et al., (1998) also reported that there was a statistically significant decrease ($P <$

0.05) in lung function and clinically decrease in lung function of approximately 15 % of children subjects with histories of active asthma after routine dental treatment. Scannapieco et al. (2004) however, failed to find an association between exposure to the dental environment and the prevalence of respiratory disease. Further research is required to establish whether microbial allergens and/or endotoxin at levels found in the DUWS can worsen cases of occupational asthma in sensitised individuals.

2.2.2 Exposure to *Pseudomonas aeruginosa*

Several studies have shown that *Pseudomonas aeruginosa* is a common contaminant of dental units (Al-Hiyasat et al., 2007, Barbeau et al., 1996, Walker et al., 2000). Some species of pseudomonads have been known to behave as opportunistic pathogens and become virulent. *P. aeruginosa* for example is highly resistant as it can grow in dilute disinfectants such as chlorhexidine and iodophors. It can also thrive in low nutrient environment including distilled water, which is commonly utilised by dentists to fill independent reservoir of their dental units.

However despite the possibility of being contaminated, cases on infection by *P. aeruginosa* in DUWS water have never been reported. This low virulence status may be due to the low presence of this bacterium in DUWS water. Based on an earlier report, the infective dose for colonisation of this bacterium in healthy human volunteers was determined at more than 1.5×10^6 cfu/mL (Pankhurst, 2003) which is very high and has never been reported in water of DUWS. In addition to that, infection by *P. aeruginosa* very often begins with the colonisation of the gut. Unless a dental patient swallowed a considerable amount of highly

P. aeruginosa contaminated DUWS water while receiving treatment, then the risk of a healthy person to be infected via this route would be very unlikely.

For medically compromised patients however, a couple of infections involving *P. aeruginosa* originating from contaminated DUWS was reported (Martin, 1987). However due to poor identification technique used in both cases, Martin (1987) could only demonstrate, but could not conclude that these patients had acquired the strain of *P. aeruginosa* from DUWS water.

2.2.3 Exposure to Legionellae

Legionellosis is caused by *Legionella* spp. acquired from environmental sources, primarily water sources such as cooling towers. *Legionella* spp. grows intracellularly in protozoa within biofilms in the coolers. Aquatic biofilms such as those found in medical and dental devices are ecological niches in which *Legionella* spp. have been known to survive and proliferate (Atlas, 2001).

Legionella spp. has been isolated from dental water in many studies (Atlas et al., 1995, Challacombe and Fernandes, 1995, Ma'ayeh et al., 2008, Williams et al., 1996a, Zanetti et al., 2000). Based on a recent survey at a dental teaching centre in Jordan, it was highlighted that *Legionella pneumophila* counts ranging from zero to 8.35×10^3 cfu/mL was detected in 86.7 % of the dental units at the beginning of a working day, 40 % after 2 min flushing and 53.3 % at midday. The conservative dentistry clinic recorded the highest contamination

level followed by the periodontics and prosthodontics clinics ($P < 0.05$) (Ma'ayeh et al., 2008).

High prevalence of *Legionella* spp. in DUWS water was explained by their unique survival strategies when in stressful environment. Under an undesirable environment, *Legionella* spp. can enter a viable but non-cultivable (VBNC) state whereby it can multiply intracellularly within a variety of protozoa, such as amoebae. The host-parasite interaction between *L. pneumophilia* and other aquatic protozoa has been shown to be central in its pathogenesis and ecology in water. Singh and Coogan (2005) found that legionella-laden amoebae was isolated from one main tap sample and in 20 % of dental chair units in a clinic of a teaching hospital. This finding led to greater awareness as legionella-laden amoebae might contain levels of *Legionella* spp. well within the infective dose and thus might significantly increase the risk of legionellosis. This bacterium can also survive as free organisms for long periods within biofilms and to be enhanced by the presence of other aquatic bacteria (Borella et al., 2005). The biofilm provides shelter as well as nutrients and in such state it is known to exhibit remarkable resistance to biocide compounds as well as chlorination (Borella et al., 2005).

The infective dose for legionellosis in humans is not known, however outbreaks of infection have been associated with bacterial counts of more than 10^5 cfu/mL (Pankhurst, 2003). A fatal case of legionellosis in a California dentist caused by *Legionella dumoffi* was reported by Atlas et al. (1995). Although the evidence linking the disease to DUWS was circumstantial, more than 10,000 organisms per mL of *L. dumoffi* and other *Legionella* spp.

were recovered from the dentist's surgery waterlines. High levels of *Legionella* spp. in the DUWS may imply that aerosols from the dental units were the source of the fatal *Legionella* infection. However, in a more recent report among general dental practitioners in London and rural Northern Ireland, the prevalence of legionellae was found to be very low at 0.37 %. This gave an indication that the risk to dentists' health from potential exposure to legionellae is very low (Pankhurst et al., 2003).

2.2.4 Exposure to Non-tuberculous Mycobacteria (NTM)

Atypical mycobacteria or non-tuberculous mycobacteria are saprophytic and ubiquitous in the environment especially in water. They differ from those tuberculous mycobacteria namely *Mycobacterium tuberculosis, Mycobacterium bovis* and *Mycobacterium africanum.* Some which are considered potential human pathogens include *Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium chelonae, Mycobacterium kansasii, Mycobacterium marinum, Mycobacterium fortuituma* and *Mycobacterium ulcerans* (Dailloux et al., 1999). NTM such as *Mycobacterium avium, Mycobacterium kansasii,* and *Mycobacterium xenopi* have been frequently isolated from drinking water and hospital water distributions. Biofilm formation, amoeba-associated lifestyle and resistance to chlorine have been recognised as important factors that contribute to the survival, colonisation and persistence of NTM in water distribution systems (Vaerewijck et al., 2006). Although the presence of these microbes in tap water has been linked to nosocomial infections and pseudo-infections, it remains unclear if these NTM provide a health risk for immune-compromised individuals.

In 1995, it was reported that the mean NTM concentration in DUWS water was at 365 cfu/mL, a population that was higher than the mean drinking water concentration by a factor of almost 400 (Schulze-Röbbecke et al., 1995). In biofilm samples, these values was even higher at a mean density amounted to 1165 cfu/cm^2. In addition to biofilms in DUWS, high isolation frequency of *Mycobacterium* spp. was also reported in DUWS water in most countries in the Europe except the UK (Walker et al., 2004a). Although the true extent of the risk posed by NTM in DUWS to immune-compromised patient has yet to be fully elucidated, the likelihood of its occurrence is considered very low for otherwise healthy patients and staff, hence giving an overall low level of risk (Porteous et al., 2004).

2.3 TREATMENTS FOR MICROBIAL CONTAMINATION

2.3.1 Anti-retraction Valves and Retrograde Aspiration of Oral Fluids

Contamination of DUWS is not thought to be entirely environmental. To certain extent, retrograde flow from the oral cavity of patients may contribute towards the contamination. Anti-retraction valves also known as check valves, limit re-aspiration and was reported as the most efficient when fitted immediately distal to a handpiece (Kohn et al., 2004). Autoclaving the handpieces after use and flushing of the units for 30 secs at the end of each patient treatment has been known to increase the action of the anti-retraction and help eliminate any aspirated fluid from the units (Anonymous, 1996). Several studies have however highlighted the inefficiency of using anti-retraction as the only disinfection strategy. Although all handpieces are fitted with anti-retraction valves, they are not completely free of pathogens until the units are flushed internally and disinfected externally

(Allen et al., 2004, Reasoner and Geldreich, 1985). Problems arises because most anti-retraction devices are unable to prevent retractions when the handpieces are not in used, leading to contamination of the DUWS and the possibility of cross-contamination among patients (Karpay et al., 1998).

2.3.2 Flushing

Flushing was introduced as a simple and efficient measure against DUWS contamination. Discharging the stagnant water in DUWS before commencing a treatment improves the quality of effluent water as well as reduces the malodour and bad taste of stagnant and contaminated water (Karpay et al., 1999, Smith et al., 2004, Bartoloni et al., 2006, Fulford et al., 2004). Many studies have shown that flushing can significantly reduce the total viable count of contaminants to the recommended standard of less than 200 cfu/mL. Despite this report, regular 20 min of flushing was impractical in general dental surgery. Furthermore, bacterial density would return to the original level within the pre-flushing range after post-flushing of 30 min (Barbeau et al., 1996, Szymańska, 2005a, Putnins et al., 2001, Huntington et al., 2007).

2.3.3 Filtration

Point-of-use filter offers a physical barrier to the passage of microorganisms and was shown effective in reducing the density of bacteria in output water of DUWS. A filter works best with a pore size of 0.2 μm and inserted just distal to the point of entry into the handpiece (Williams et al., 1996b). Although filters are capable of entrapping microorganism present in the water phase, it has no effect on the biofilm formed on the

interior side of tubing. More recent study has demonstrated the high levels of recontamination of DUWS that may occur within 24 hr due to the result of trapping and growth of bacteria on the filters (Murdoch-Kinch et al., 1997). Filtration is thus considered an uneffective disinfecting method for DUWS water, as bacterial endotoxin cannot be filtered out by the 0.2 μm membrane filters (Putnins et al., 2001).

2.3.4 Independent Clean Water System

The system uses a self-contained or independent sterile water plumbed to the waterlines and bypasses the main connections to the municipal water (Walker and Marsh, 2004). The advantage of using an independent clean water system is to permit the use of water of known microbiological quality rather than relying totally on the incoming municipal water. The cost of installation is low and the waterline cleaner can be easily delivered to remove biofilm formed in the DUWS.

In practice however, the maintenance of an independent clean water system requires routine aseptic replacement of surgical sterile water (Petti and Tarsitani, 2006). Failure to follow disinfection protocol can make the system valueless. Biofilm formation and microbial proliferation can still persist as the residual chlorine in distilled water is significantly lower compared to municipal water. Environmental and human-derived contaminants very often from improper handling of the water during refilling process, have been known to proliferate in the reservoir if it is left undisturbed (Williams et al., 1996b, Murdoch-Kinch et al., 1997).

2.3.5 Chemical Treatment

The most efficient mean of maintaining good quality DUWS output water is the regular treatment of DCU with chemical, biocide or cleaning agent that removes biofilm. This results in significant reduction of total viable counts (TVCs). However, DUWS must be treated regularly (daily or intermittently) as biofilm can re-grow as disinfectant's effect ceases over certain period (normally within a week) (O'Donnell et al., 2006, Tuttlebee et al., 2002). Many studies were carried out on the efficiency of disinfectant *in vitro* but relatively few studies have actually investigated their efficiency on DCUs. In addition, only a small number of studies investigated the long term effect of disinfectant on the DCUs (Coleman et al., 2007).

2.3.5.1 Side Effects and Limitations

A biofilm laboratory model in a controlled environment was established to simulate microbial load of DUWS and to evaluate practical, cost-effective, and evidence-based methods of microbial decontamination (Walker et al., 2003). The assessment of decontamination method was based on the TVCs and using microscopic – image analysis techniques to view the inner surface of tubing. This study highlighted that while many disinfectants are effective in reducing TVCs but they might not able to remove unwanted biofilm from the tubing surfaces. Results obtained from several *in vitro* studies may not be able to replicate in actual clinical practice. Thus, Schel et al. (2006) carried out a longitudinal study to compare the efficiencies of different disinfectants in general dental practice setting across the European Union. This study highlighted that the TVCs reduction

of some disinfectants in GDP to a certain extent, varied from that obtained by Walker et al. (2003) (Table 2.2).

Although various chemical treatments have been successful in reducing bacterial density to an acceptable level, this treatment is not a definite solution to control and reduce microbial contamination due to many possible side effects .One example is Listerine, even in a diluted preparation and Bio 2000 (chlorhexidine) were shown to significantly reduce the bond strength of dentine as compared to BioClear (citric acid) and sodium hypochlorite (Roberts et al., 2000). Chlorine-containing regimens have also been found to release more mercury from amalgam than some other products (Batchu et al., 2006, Roberts et al., 2005). Knowledge such as this is important especially to dental personnel as they should be aware that oxidising disinfectants have the tendency to mobilise mercury ions into solution. If this happens, the ions tend to get deposited in the environment if they are processed improperly. There was a report that iodine-releasing cartridges installed to dental chairs can increase dissolved mercury levels in dental unit wastewater (Stone et al., 2006).

In practice, chemical treatments have many limitations brought about by faulty valves, dead-ends, inappropriate application of chemical concentration or protocol that give rise to compliance issues. All these may lead to inconsistent results. One example was reported by Tuttlebee et al. (2002) who claimed that gradual accumulation of precipitated disinfectant have caused the clogging of DUWS treated with Sterilex Ultra. In another case, O'Donnell, Shore, Russell and Coleman (2007) demonstrated that human error in the handling of disinfectant may lead to the corrosion of equipments. Improper handling of

disinfectants may also favour natural selection of disinfectant-tolerant bacterial species and contribute to the failure of DUWS disinfection. *Exophiala mesophila,* which is an unusual fungus was isolated from output water of DCU's which have been treated by chlorine dioxide (Szymańska, 2007). Such decontamination strategy have been shown to significantly reduce biofilm coverage and at the same time could significantly increase microbial numbers in the water phase (Walker et al., 2000). In other words, an inadequate decontamination regimen might thus increase hazards associated with DUWS output water.

2.3.5.2 Common Chemical Disinfectants

There is a broad range of commercially available treatment products for DUWS that have been shown efficient in removing biofilm and thus reduce TVCs to acceptable levels. Table 2.2 summarised the list of proposed disinfectant and their respective efficiency based on the available published data.

Table 2.2: List of proposed disinfectants and their respective efficiency

Biocide/disinfectant (active ingredients)	Efficiency (Published data)	Mode of use
A-dec ICX (sodium percarbonate, silver nitrate & cationic surfactants)	1. ≤ 200 cfu/mL TVCs (McDowell et al., 2004)	Daily

Biocide/disinfectant (active ingredients)	Efficiency (Published data)	Mode of use
Alpron (sodium hypochlorite, citric acid, sodium-*p*-toluolsulfonechloramide, EDTA	1. 87 % treated water is ≤ 200 cfu/mL TVCs (Schel et al., 2006) 2. 100 % TVCs and 100 % biofilm coverage reduction (Walker et al., 2003) 3. < 10 cfu/mL TVCs (Ozcan et al., 2003) 4. ≤ 200 cfu/mL TVCs (Smith et al., 2002)	Continuous/ weekly
Betadine (povidone iodine solution)	1. 100 % TVCs and 97.3 % biofilm coverage reduction (Walker et al., 2003)	N/S
BioBlue (Bio 2000) (ethanol & chlorhexidine)	1. < 100 cfu/mL TVCs (Chate, 2006, Kettering et al., 2002) 2. 74 % treated water is ≤ 200 cfu/mL TVCs (Schel et al., 2006) 3. 100 % TVCs and 53.2 % biofilm coverage reduction (Walker et al., 2003) 4. < 1 cfu/mL (Ozcan et al., 2003)	Intermittent/ weekly
Chlorhexidine	1. 100 % TVCs and 31.77 % biofilm coverage reduction (Walker et al., 2003)	N/S
Chloride dioxide	1. Short term decline in TVCs but did not provide potable quality water (Smith et al., 2001)	N/S

N/S: Not stated

Biocide/disinfectant (active ingredients)	Efficiency (Published data)	Mode of use
Combizyme (proteinases & carbohydrases)	1. 70 % TVCs and 45 % biofilm coverage reduction (Walker et al., 2003)	Weekly
Dentosept (hydrogen peroxide & silver)	1. < 100 cfu/mL TVCs (Jatzwauk and Reitemeier, 2002, Kettering et al., 2002) 2. 91 % treated water is ≤ 200 cfu/mL (Schel et al., 2006)	Continuous/ weekly
Dialox (hydrogen peroxide, peracetic acid, acetic acid	1. 100 % TVCs and 99.77 % biofilm coverage reduction (Walker et al., 2003)	Weekly
Gigasept (Glutaraldehyde, formaldehyde, didecyldimethylammonium chloride)	1. 100 % TVCs and 34 % biofilm coverage reduction (Walker et al., 2003)	N/S
Oxygenal (hydrogen peroxide)	1. 91 % treated water is ≤ 200 cfu/mL (Schel et al., 2006) 2. 100 % TVCs and 99.2 % biofilm coverage reduction (Walker et al., 2003) 3. < 100 cfu/mL TVCs (Szymańska, 2006)	Continuous/ weekly

N/S: Not stated

Biocide/disinfectant (active ingredients)	Efficiency (Published data)	Mode of use
Ozone	1. 65 % TVCs and 57.8 % biofilm coverage reduction (Walker et al., 2003)	Weekly
Parmetol (Butylhydroperoxide)	1. 94.8 % TVCs and 31.3 % biofilm coverage reduction (Walker et al., 2003)	N/S
Planosil/ Planosil Forte (hydrogen peroxide & silver ions)	1. ≤ 200 cfu/mL TVCs for 7 days post-disinfection (O'Donnell et al., 2006)	Weekly
Sanosil (hydrogen peroxide & silver)	1. 83 % treated water is ≤ 200 cfu/mL (Schel et al., 2006) 2. 100 % TVCs and 100 % biofilm coverage reduction (Walker et al., 2003) 3. ≤ 200 cfu/mL TVCs (Tuttlebee et al., 2002)	Intermittent
Sodium hypochlorite (chlorite)	1. 100 % TVCs and 94.4 % biofilm coverage reduction (Walker et al., 2003)	Weekly
Sporklenz (hydrogen peroxide, peracetic acid, acetic acid)	1. 100 % TVCs and 92.6 % biofilm coverage reduction (Walker et al., 2003)	Weekly

N/S: Not stated

Biocide/disinfectant (active ingredients)	Efficiency (Published data)	Mode of use
Sterilex Ultra (alkaline peroxide)	1. 78 % treated water is ≤ 200 cfu/mL (Schel et al., 2006) 2. 100 % TVCs and 97.3 % biofilm coverage reduction (Walker et al., 2003) 3. ≤ 200 cfu/mL TVCs (Linger et al., 2001, Tuttlebee et al., 2002)	Intermittent
Ster4Spray (tetraacetylethylenediamine & sodium perborate)	1. 60 % treated water is ≤ 200 cfu/mL (Schel et al., 2006) 2. ≤ 200 cfu/mL TVCs (Montebugnoli et al., 2004)	Intermittent /daily
Sterilox (superoxidised water)	1. < 1 cfu/mL TVCs (Chate, 2006, Martin and Gallagher, 2005) 2. 100 % TVCs and 99.3 % biofilm coverage reduction (Walker et al., 2003)	Continuous/ weekly
Tegodor (benzalkoniumchloride, formaldehyde, glutaraldehyde)	1. 100 % TVCs and 33 % biofilm coverage reduction (Walker et al., 2003)	Weekly
Tetra-sodium EDTA	1. < 10 cfu/mL TVCs (Percival et al., 2009)	Intermittent

N/S: Not stated

2.3.6 DUWS Cleaning and Disinfection System

Tuttlebee et al. (2002) had reported on the effectiveness of the Planmeca Waterline Cleaning System (WCS) that was used to disinfect DUWS in Planmeca Prostyle Compact DCUs. The WCS was designed to be retrofitted to the existing Planmeca DCU and disinfectant was then delivered to the DUWS from an external container. Two types of hydrogen peroxide-based disinfectants (Sterilex Ultra and Sanosil) were used in this cleaning system. Although both disinfectants were able to reduce TVCs to below the ADA levels, this retrofitted system however had several shortcomings. Among others were the necessity of flushing manually some of the waterline following disinfection, disinfectant leakage from the retrofitted valves and the efficiency of the system strictly relied on the compliance with operating procedure.

As new technology develops, O'Donnel et al. (2006) reported a new Planmeca Compact i DCU equipped with an integrated Planmeca Water Management System (WMS). This fully integrated and automated waterline cleaning system was easy to use. The operator just need to (i) place the instrument hoses into dedicated receivers, (ii) activate the WMS on the DCU console, (iii) add disinfectant to the DCU bottle container, (iv) allow the waterlines to be filled automatically with the disinfectant (v) and lastly switch the unit off to allow the disinfectant to take effect. The whole process took the operator less than 5 min. After pre-set turning off period (15 hr in this study), the WMS was turned on and an automatic flushing cycle with clean water begun. In addition, this WMS was equipped with a vertical air gap that prevented backflow contamination of the mains water supply with water from DUWS. The design of this WMS ensured that every single parts of water distribution

network within the DCU was subjected to disinfection when the cleaning mode was activated.

Although WMS was capable in reducing bacterial density to below ADA levels, in June 2006, a sudden bloom of *Pseudomonas fluorescens* (more than 100,000 cfu/mL) was isolated in the 8000 L mains water storage tank supplying the hospital's DCUs. It was reported that this incident had coincided with an increased level of iron (250 times greater than the average) in the main supply. This sudden phenomenon might have been due to the works carried out on the mains water distribution network by the municipal authorities. The incidence highlighted the requirement of maintaining good water quality throughout the DCU supply water network in dental hospitals and multi-DCU clinic rather than only within the DUWS. Resulting from the incidence, O'Donnell, Boyle, Swan, Russell and Coleman (2009) had decided to enhance the filtration of main waterline supply to DCUs followed by an automated disinfection using an electrochemically activated (ECA) solution, Ecasol^{TM}. Following a two years longitudinal study, Ecasol^{TM} treatment was found successful in maintaining the effluent water from DCUs at a better quality than that of drinking water. No adverse effect was observed on the DUWS and DCU instruments. Ecasol^{TM} was also shown very unlikely to have adverse effects on human oral tissues at levels effective in maintaining clean DUWS output water (Boyle et al., 2010).

2.3.7 Modification of tubing

The existence of biofilm in DUWS is virtually inevitable. This unique microbial aggregation may have originated from the accumulation and multiplication of microbial contaminants in the municipal water supply, contamination of storage bottles and tanks and/or from the active or passive retrograde flow of microorganism from patient sources (Williams et al., 1996c). A simple measure to prevent biofilm formation in DUWS tubing would be to utilise materials with properties that can inhibit the formation of biofilm. Fluoridated resins have been reported to possess inhibitory activity against bacterial colonisation of surfaces. Low surface free energy of fluoride-coated tubing was suggested the factor that contributes to the effective inhibitory property and thus, would hinder biofilm formation (Tsibouklis et al., 1999). Polyvinylidene fluoride (PVDF) tubing was found effective in inhibiting the formation of biofilm on its inner wall surface. The count of bacterial outflow from DUWS using this type of tubing was found to be greatly reduced (Yabune et al., 2005, Yabune et al., 2008). The use of waterline made of polytetrafluorethylene (PTFE) in a similar situation, was reported an effective approach in inhibiting the colonization and growth of *Pseudomonas aeruginosa* (Sacchetti et al., 2007).

2.4 GUIDELINES AND RECOMMENDATIONS ON DUWS

In 1995, the American Dental Association (ADA) has set a goal that by the year 2000, the counts of aerobic heterotrophic bacteria in DCU output water must be less than 200 cfu/mL. At that time, this was equivalent to an existing quality assurance standard for dialysate fluid in haemodialysis units (Anonymous, 1996). The current United States

Centres for Disease Control and Prevention (US CDC) however has recommended a slightly milder guideline that the DCU output water should be less than 500 cfu/mL of aerobic heterotrophic bacteria which was in agreement with the drinking water standard proposed by the United States Environmental Protection Agency (US EPA), the American Public Health Association (APHA), and the American Water Works Association (AWWA) (Kohn et al., 2004).

At present, the Guidelines for Infection Control in Dental Health Care Settings – 2003 is the latest recommendation proposed by the US CDC. This guideline covers several topics such as (i) education and protection of dental health care personnel, (ii) prevention of transmission of bloodborne pathogens, (iii) personal protective equipment, (iv) sterilisation and disinfection of patient-care items and lastly (v) environmental infection control (Kohn et al., 2004). The full details of review regarding the DUWS and dental handpieces are given in Appendix 4.

2.5 METHODS TO DETECT CONTAMINATION

The most common method of assessing microbial contamination is by using the heterotrophic plate count or total viable count (TVC). These heterotrophic bacteria are ubiquitous in nature and its status in posing health risk to the public is not clear due to insufficient clinical evidences (Allen et al., 2004). Thus, despite many studies that have demonstrated the contamination of dental chairs with aerobic environmental bacteria, the significance of these group of bacteria in health risk is still being investigated and need to

be verified. Based on this ambiguity, Petti and Tarsitani (2006) had suggested that TVC was not an effective indicator of oral fluid aspiration. However, US CDC hold different opinion and stated that ‘exposing patients or dental health care personnel to water of uncertain microbiological quality, despite the lack of documented adverse health risk, is inconsistent with the generally accepted infection control principles’ (Kohn et al., 2004).

In practical TVC is considered the most common method used in measuring bacterial contamination. This method however inherits several disadvantages as it is a laborious and time-consuming procedure and requires an inevitable delay before the data are available and can be acted upon. Therefore several attempts had been carried out to overcome this shortcoming. For instance, an user-friendly, economical heterotrophic plate count (HPC) sampler was developed and used in measuring bacterial contamination of DUWS (Bartoloni et al., 2006, Karpay et al., 1999, Karpay et al., 1998, Smith et al., 2004). Karpay et al. (1999) demonstrated that there was a strong correlation between HPC sampler and conventional R2A culture techniques for measuring bacterial contamination (Pearson correlation coefficient = 0.89). However, Bartoloni et al. (2006) cautioned that the underestimated bacterial levels of HPC sampler might result in a false sense of security and a lack of compliance with recognised recommendations for DUWS quality.

Along with the importance of the heterotrophic plate count, bacterial contaminants in DUWS also include the Gram negative environmental bacteria. Upon cell lysis, these type of bacteria release endotoxins into the environment in which in this case, is the DUWS output water. Thus, the estimation of endotoxins released in DUWS may be an alternative

to the TVC as a measure of DUWS contamination. Another simple and rapid adenosine triphosphate (ATP) test can also be carried out at the chairside as an alternative to TVC. However, despite the improved method of using the endotoxin and ATP tests, many researchers did not share the same opinion. Fulford et al. (2004) had failed to find any strong correlation between endotoxin and ATP concentration with the TVC data and thus, suggested that both methods could not be recommended as an alternative to TVC. Similar opinion was also shared by Szymańska (2005a) who suggested that endotoxin concentration was not indicative of Gram-negative bacteria contamination. However, in a more recent study, a statistically significant Spearman correlation coefficient of $P = 0.94$ between endotoxin (EU/mL) and bacterial load (cfu/mL) was reported (Huntington et al., 2007). With this latest research, the reasons of such disagreement between the various studies still remain unclear.

CHAPTER THREE

METHODOLOGY

3.1 RESEARCH DESIGN

This study was divided into the (i) microbial assessment of DUWS and (ii) effectiveness of silver-coated tubing in reducing biofilm formation. In the fomer, the physical parameters (pH and temperature) and microbiological parameters (heterotrophic plate count, total coliform, faecal coliform, *E. coli*, faecal streptococci and *P. aeruginosa* plate counts) of the water samples were determined. In the later, the effectiveness of silver-coated tubing in reducing the adherence of bacteria to its inner surfaces was assessed based on SEM images and the counts of the adhering bacteria. A flow-chart of the research methodology is given in Figure 3.1.

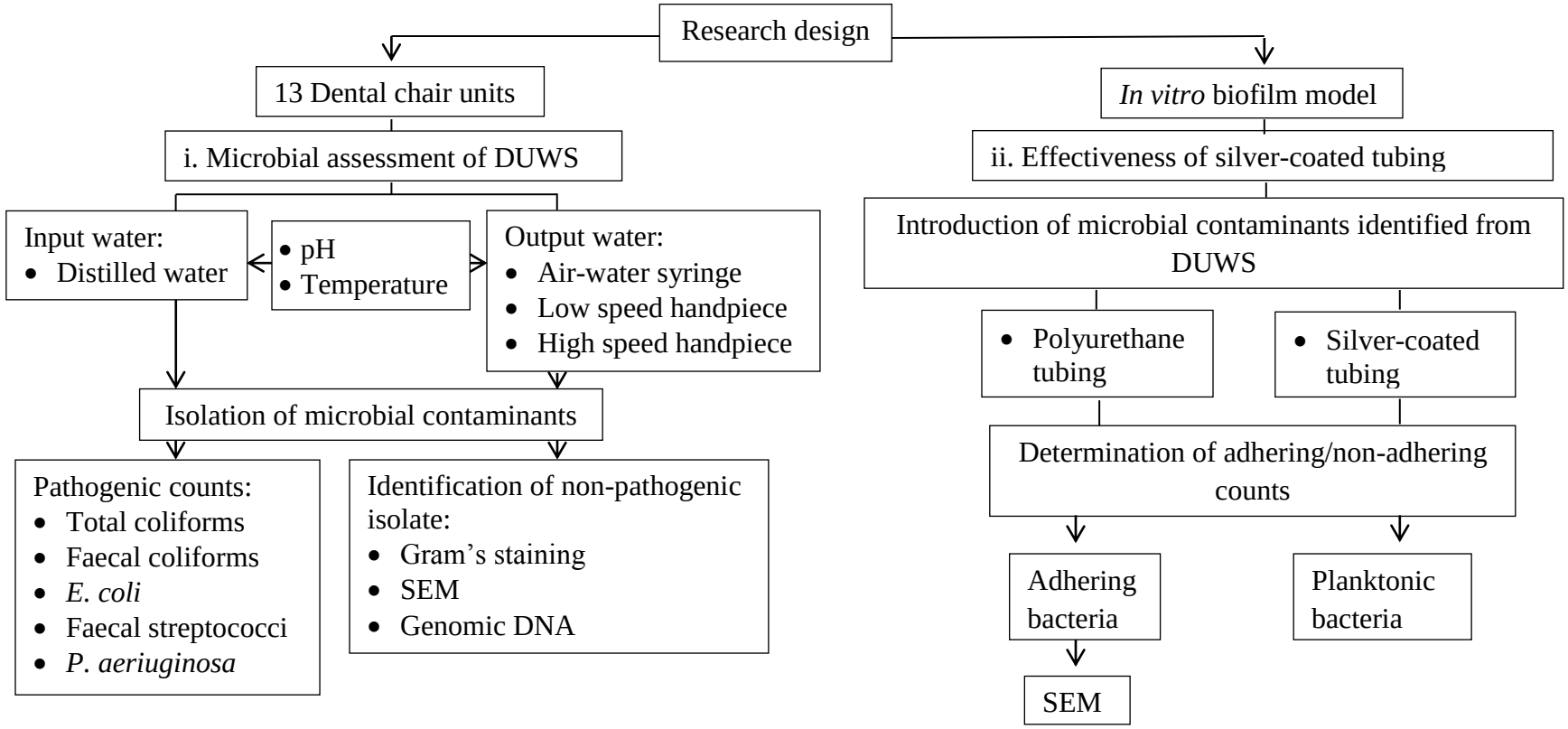

Figure 3.1: Flow chart of the design of the study. The methodology was divided into two parts: (i) the microbial assessment of DUWS and (ii) the assessment of the effectiveness of silver-coated antibiofilm tubing.

3.2 MEDIA, DILUENT, AND APPARATUS

The equipments, apparatus, chemicals and media used in this study together with their brands and places of manufacture are listed in Appendix 1. Sterile media, diluent and apparatus were prepared according to respective manufacturer's instructions and Standard Methods given in Appendix 2 (APHA-AWWA-WEF, 2005).

3.3 SAMPLES COLLECTION

3.3.1 Dental Chair Unit (DCU)

Water samples from 13 DCUs were obtained from a dental teaching clinic, University of Malaya. The DCUs have been routinely in use for more than 10 years for dental treatment during student clinic sessions. This model employed clean water supplied from an independent reservoir filled with distilled water. In other words, water used in these DCUs had bypassed the main connections to the municipal water (Figure 3.2 - 3.5).

Figure 3.2: An overview of a dental chair unit in the dental teaching clinic.

Figure 3.3: The filter unit used to filter municipal water prior to distillation.

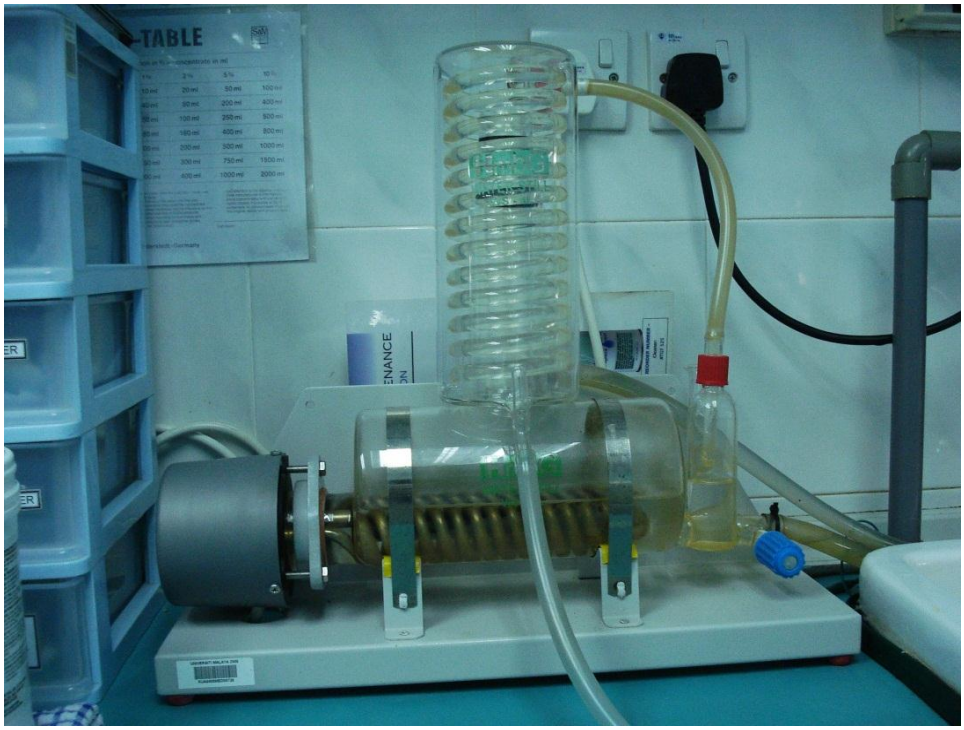

Figure 3.4: The water distiller placed in the clinic.

Figure 3.5: Distilled water collected from the distiller was stored in 20 L plastic container.

3.3.2 Collection of Water Samples

13 collections were made over a period of two months with a total of 572 water samples. Sampling was carried out in the early morning (around 8.30 am) after a nightly disuse of the DCU. In each sampling, water samples were collected from the output and input water. The former consisted of water from the air-water syringe (AWS), low speed handpiece (LSH) and high speed handpiece (HSH) while the later consisted of distilled water (DW) used to fill in the reservoir of each DCU (Figure 3.6 and 3.7).

Before the start of each collection, the tip points of each of the water sources were carefully wiped with alcohol to ensure sterility of their surfaces. Water was then allowed to run to waste for about one min before samples from each of the tips were aseptically collected

into universal and polypropylene bottles for laboratory analyses. The temperature and pH of the water samples were immediately measured using a portable temperature-pH meter before they were transported in cool condition (4 ºC) to the laboratory for microbial analyses. For every 120 mL of water samples, 0.1 mL of sodium thiosulphate (3%) solution was added to neutralise the presence of any residual chlorine in the water.

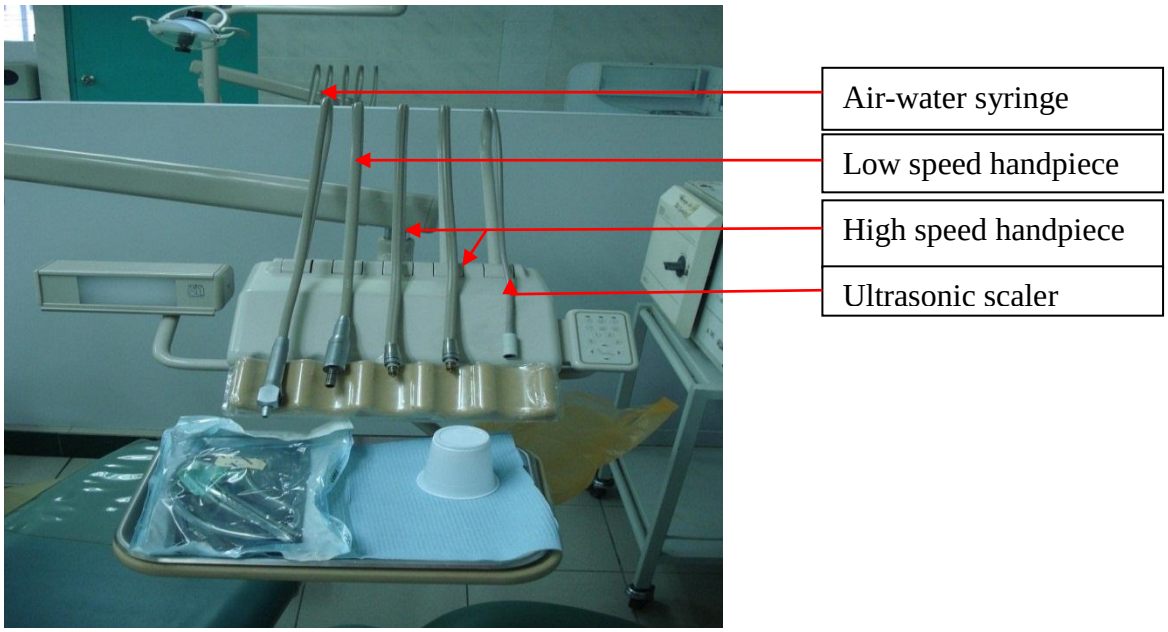

Figure 3.6: The tip points of each handpieces were wiped with alcohol prior to sampling.

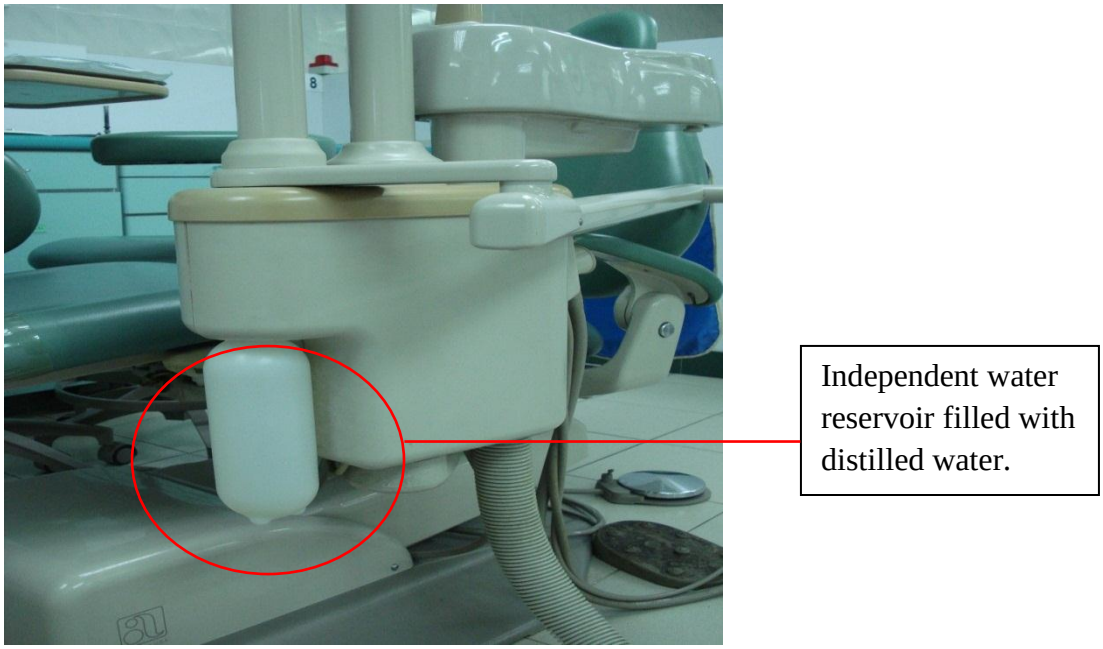

Figure 3.7: Water inlet to dental chair.

3.4 LABORATORY PROCESSING OF WATER SAMPLES

3.4.1 pH and Temperature Measurement

A portable pH-temperature meter was calibrated prior to collection of water samples. The pH and temperature were then immediately measured by putting the pH and temperature probes into the water. Readings were taken in triplicate and their average was recorded.

3.4.2 Heterotrophic Plate Count

Heterotrophic plate count was used to determine the total bacterial load in the water. Each water sample was diluted to 10-fold and 100-fold using buffered water. 0.1 mL of water

sample from each dilution was aseptically spread on to the surface of pre-dried R2A agar plates using a glass spreader. The agar plates were then inverted and incubated at 28 °C for 7 days. Following incubation, the total number of colonies and the relative abundance of each different colony types in each plate were recorded and the average of the triplicate plates was determined. Representatives of each colony types present on each plate were selected and subcultured for subsequent identification.

3.4.3 Total Coliforms Plate Count

A filtration apparatus consisting of glass funnel, filter base and vacuum beaker was correctly assembled and connected to an electronic vacuum pump (Figure 3.8). A piece of sterile gridded cellulose nitrate membrane filter with 0.45 µm pore size and 47 mm diameter (Whatman) was aseptically placed on to filter base (grid side up)(Figure 3.9). 100 mL of water sample was pumped through the membrane filter at a pressure differential of 34 – 51 kPa. Once finished, the filtration apparatus was dissembled and the membrane filter was aseptically transferred and placed on to the surface of m Endo LES agar plate. The agar plates were left to stand for 30 min, then inverted and incubated at 35 ± 0.5 °C for 22-24 hr. Only those plates with 20-80 coliforms colonies were selected for reporting. A typical coliform colony often appeared pink to dark-red colour with metallic sheen while atypical coliforms appeared dark-red, mucoid, or nucledated without sheen. In general, pink, blue, white or colourless colonies lacking sheen were considered as non-coliforms. Both typical and atypical coliforms colonies were verified by transferring each colony into lauryl tryptose broth and brilliant green lactose broth and incubated at 35 ± 0.5 °C for 48 hr. The production of gas inside the fermentation vial placed in the broth would confirm the

presence of coliforms. Results were recorded as coliforms/100 mL based on an average of verified counts.

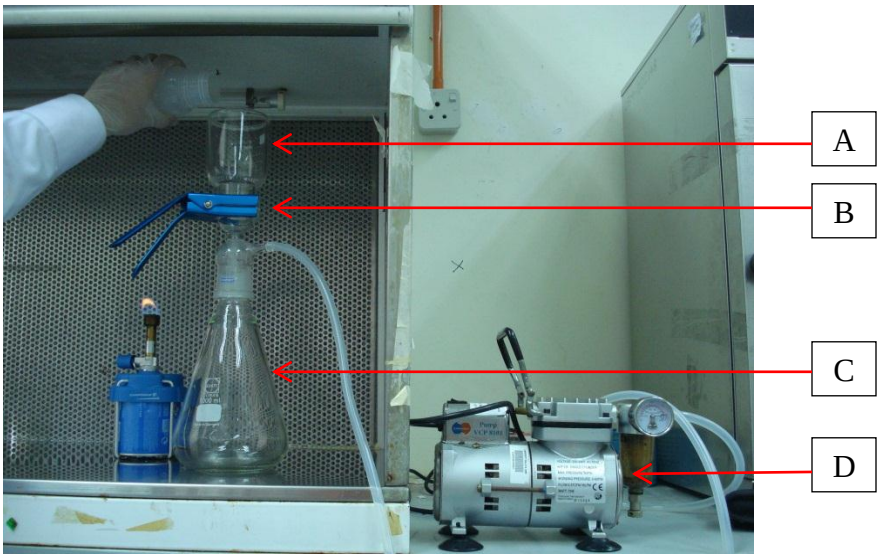

Figure 3.8: A filtration apparatus consisting of glass funnel (A), filter base (B) and vacuum beaker (C) was assembled and connected to an electronic vacuum pump (D).

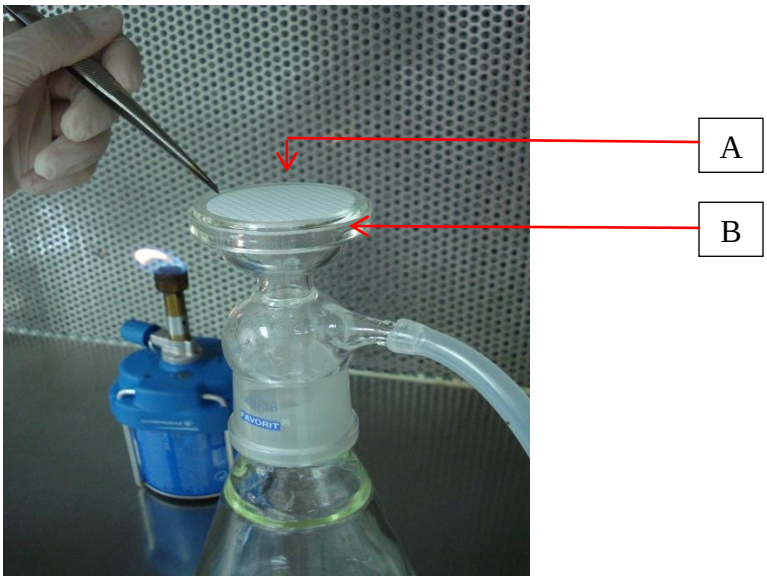

Figure 3.9: A piece of sterile gridded cellulose nitrate membrane filter (A) with 0.45 µm pore size and 47 mm diameter was aseptically placed on to the filter base (B).

3.4.4 Faecal Coliforms Plate Count

Similar to procedure described in Section 3.4.3, 100 mL water sample was pumped through the filtration unit. The membrane filter was then aseptically transferred on to M-FC agar plate. The agar plates were left to stand for 30 min, then inverted and placed in a waterproof plastic bag. The plastic bag containing the petri dishes inside was submerged in water bath and incubated at 44.5 ± 0.5 °C for 24 ± 2 hr. Only plates with 20 – 60 faecal coliforms colonies were selected for reporting. Faecal coliforms colonies appeared in various shades of blue while non-faecal coliforms colonies were gray to cream-coloured. Both of the typical blue colonies and atypical grey to green colonies were verified by transferring each colony into lauryl tryptose broth and incubated at 35 ± 0.5 °C for 48 hr. The production of gas inside fermentation vial placed in the broth would confirm the presence of faecal coliforms. Results were recorded as faecal coliforms/100 mL based on an average of verified counts.

3.4.5 *Escherichia coli* Plate Count

Water samples that were positive for total coliforms were subjected to this analysis. The membrane filters from the filter apparatus were transferred aseptically from the m Endo LES agar plates to a nutrient agar with MUG plates. The nutrient agar with MUG plates were left to stand for 30 min, then inverted and incubated at 35 ± 0.5 °C for 4 hr. The agar plates were examined under long wave length (365 nm) ultraviolet light illuminator. The presence of colonies with blue fluorescence on the periphery (outer edge) was considered a positive response for *E. coli*. The number of *E. coli* were counted and recorded as *E. coli*/100 mL based on an average counts.

3.4.6 Faecal Streptococci Plate Count

Similar to procedure described in Section 3.4.3, 100 mL water sample was pumped through the filtration unit. The membrane filter was then aseptically transferred onto m Enterococcus agar plate. The agar plates were left to stand for 30 min, then inverted and incubated at 35 ± 0.5 °C for 48 hr. Those plates with 20 – 60 faecal streptococci colonies were selected for reporting. All light and dark red colonies were considered as faecal streptococci. Typical colonies were verified by picking them from the membrane and streaking them for isolation on to the surface of a brain heart infusion agar plate. The agar plates were incubated at 35 ± 0.5 °C for 24 – 48 hr. Two individual isolated colonies were transferred to two clean glass slides. These two slides were subjected to catalase test and Gram staining. Faecal streptococci are gram-positive, ovoid cells, mostly in pairs or short chains and reacted negatively to catalase test. Results were recorded as faecal streptococci/100 mL based on an average of verified counts.

3.4.7 *Pseudomonas aeruginosa* Plate Count

Similar to procedure described in Section 3.4.3, 500 mL water sample was pumped through the filtration unit. The membrane filter was then aseptically transferred onto *M*-PA-C agar plate. The agar plates were left to stand for 30 min, then inverted and incubated at 41.5 ± 0.5 °C for 72 hr. Only plates with 20 – 80 *P. aeruginosa* colonies were selected for reporting. Typically, *P. aeruginosa* colonies are 0.8 – 2.2 mm in diameter and flat in appearance with light outer rims and brownish to green-black centres. Results were recorded as presumptive *P. aeruginosa*/100 mL based on an average of verified counts.

3.4.8 Statistical Analysis

Statistical analyses were carried out using Statistical Package for the Social Sciences (SPSS) version 12.0.1. Bacterial loads in different water sources (air-water syringe, low and high speed handpieces and distilled water) were compared to the standard recommended by ADA (≤ 200 cfu/mL) using one sample *t*-tests on a log-transformed heterotrophic plate count. Paired *t* tests were also carried out to compare bacterial load of the input water (distilled water) and output water (air-water syringe, low and high speed handpieces). Statistical significance was assumed at a *P* value of < 0.05.

3.4.9 Control Cultures for Laboratory Processing

Control cultures were essential to ensure accurate laboratory processing. Any abnormal behaviour of both positive and negative controls were used to indicate inaccurate laboratory processing and thus samples collection were repeated. The positive and negative control cultures used in this study were as listed in Table 3.1.

Table 3.1 Cultures used as positive and negative controls in the laboratory processing.

Laboratory processing	Control Culture	
	Positive	**Negative**
Heterotrophic plate count	*Escherichia coli*	-
Total coliforms plate count	*Escherichia coli*	*Staphylococcus aureus*
Faecal coliforms plate count	*Escherichia coli*	*Enterococcus faecalis*
Escherichia coli plate count	*Escherichia coli*	*Enterococcus faecalis*
Faecal streptococci plate count	*Enterococcus faecalis*	*Escherichia coli*
Pseudomonas aeruginosa plate count	*Pseudomonas aeruginosa*	*Escherichia coli*

3.5 IDENTIFICATION OF BACTERIAL ISOLATES

Bacterial isolates that were commonly recovered from the heterotrophic plate count of all water samples were further subcultured and purified for identification purposes. The colony morphology of isolates was first observed under a dissecting microscope. The cells were later Gram-stained and observed under a light microscope at 100 x magnification. The ultrastructure of the bacteria was also observed under a scanning electron microscope at 24,000 x magnification. The definitive identification of isolates was carried out based on the DNA sequence of a segment of the small ribosomal subunit 16S rRNA gene. The bacteria were identified by comparing the sequences to consensus sequences for individual bacterial species in the GenBank nucleotide sequence databases using the Basic Local Alignment Search Tool (BLAST).

3.5.1 Gram Staining

Three randomly picked bacterial colonies were obtained from a R2A agar plate and smeared on to three different glass slides respectively. The smear on each slide was fixed by either air-dried or dried by flipping through gentle flaming for several times. The smear was then flooded by crystal violet solution for one min and followed by washing off using tap water. The smear was then flooded by iodine solution for one min and followed by washing off using tap water. The smear was then decolourised by alcohol for five sec and followed by washing off using tap water. The smear was then counterstained by safranin solution for one min and followed by washing off using tap water. The glass slides were observed under a light microscope at 100 x magnification. The morphology of bacteria and their response to Gram-staining were then recorded. *Staphylococcus aureus* and *Escherichia coli* were used as positive and negative control cultures respectively.

3.5.2 Observation under Scanning Electron Microscope

Several similar colonies of bacteria was randomly picked from the R2A agar plates and suspended in McDowell-Trump fixative prepared in 0.1 M phosphate buffer (pH 7.2) at 4 °C overnight. The suspension was then centrifuged and the supernatant was discarded. The pellet was resuspended in 0.1 M phosphate buffer. The process of centrifuging suspension and discarding supernatant was repeated for three times. The pellet formed after centrifugation was then suspended for one hr in 1 % (w/v) osmium tetroxide prepared in phosphate buffer (post-fixation). After one hour, the suspension was centrifuged and

supernatant was discarded and resuspended in distilled water. The process of centrifuging suspension and discarding supernatant was repeated for two times. The cells were dehydrated in ascending percentage of ethanol from 50 %, 75 %, 95 % up to 100 %. The cells were dehydrated by 100 % ethanol for two times. Lastly the cells were suspended in hexamethyldisilazane (HMDS) for two times. The sample was then left to air-dry at room temperature. The sample was then observed under a scanning electron microscope at 24,000 x magnification.

3.5.3 Identification of Bacterial Genomic DNA

Total genomic DNA from bacterial isolates was prepared using the GeneJET^{TM} Genomic DNA Purification Kit (Fermentas). The isolates were subcultured on R2A agar plates at 28 °C for 2 days. Bacterial genomic DNA was purified based on their reaction to Gram stain.

3.5.3.1 Genomic DNA Purification of Gram-Negative Bacteria

Quantitation of bacterial cells was based on its optical density at 550 nm (Appendix 3). A maximum of 2 x 10^9 bacterial cells (OD_{550} = 2.5) was harvested in a 1.5 mL microcentrifuge tube by centrifugation for 10 min at 5000 x g. The supernatant was then discarded. The bacteria cells that were pelleted down was resuspended in 180 µL of Digestion Solution. 20 µL of Proteinase K Solution was added and the mixture was thoroughly mixed to obtain an uniform assay mixture. The suspension was incubated at 56 °C with occasional vortexing until the cells were completely lysed (~30 min). 20 µL of Rnase A Solution was added, mixed and the assay mixture was incubated for 10 min at

room temperature. 200 µL of Lysis Solution was added to the assay mixture, thoroughly mixed by vortexing for about 15 sec until a homogeneous assay was obtained. 400 µL of 50 % ethanol was added to the assay mixture and mixed by vortexing. The prepared lysate was transferred to a GeneJET^{TM} Genomic DNA Purification Column which has been inserted a collection tube. The column was centrifuged for 1 min at 6000 x g. The collection tube containing the flow-through solution was discarded. The purification column was placed into a new 2 mL collection tube. 500 µL of Wash Buffer I (with ethanol added) was added to the assay mixture, centrifuged for 1 min at 8000 x g. The flow-through was discarded while the purification column was placed back into the collection tube. 500 µL of Wash Buffer II (with ethanol added) was added to the purification column, centrifuged for 3 min at maximum speed (≥12000 x g). The collection tube containing the flow-through was discarded and the purification column was transferred to a sterile 1.5 mL microcentrifuge tube. 200 µL of Elution Buffer was added to the centre of the purification tube membrane to elute genomic DNA. The purification column was incubated for 2 min at room temperature and centrifuged for 1 min at 8000 x g. The purification column was discarded. The purified DNA was used immediately in downstream applications or stored at -20 °C.

3.5.3.2 Polymerase Chain Reaction (PCR)

100 ng of purified DNA was used as template DNA in the PCR. Primers 27F (5'-AGAGT TTGATC/TA/CTGGCTCAG-3') and 1495R (5'-CGGC/TTACCTTGTTACGAC-3') were used to amplify a 1500-bp region of the 16S rDNA gene. The assay mixtures contained 1 X PCR buffer; 2.0 mM $MgCl_2$; 200 µM each dATP, dCTP, dGTP and dGTP; 300 mM each forward and reverse primer; and 0.025 U of *Taq* DNA polymerase (Fermentas). The assay

mixtures were incubated for an initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 50 °C for 30 sec, and 72 °C for 2 min and lastly a final extension at 72 °C for 10 min.

3.5.3.3 PCR Product Purification

Binding buffer was added to the PCR product in 1:1 proportion and thoroughly mixed. The appearance of yellow colour was used to indicate an optimal pH for DNA binding. A maximum of 800 µL of the solution was transferred to the GeneJET^{TM} purification column (Fermentas) and centrifuged for 60 sec at 12,000 x g. The flow-through was then discarded. 700 µL of wash buffer was added to the purification column and centrifuged for 60 sec at 12,000 x g. The flow-through was discarded and the purification column was placed back into the collection tube. The empty purification column was centrifuged for an additional 1 min to completely remove any residual wash buffer. The purification column was then transferred to a clean 1.5 mL microcentrifuge tube. 50 µL of elution buffer was added to the centre of the purification column and centrifuged for 1 min. The purification column was then discarded and the purified DNA was stored at -20 ºC.

3.5.3.4 Agarose Gel Electrophoresis

1 % (w/v) agarose solution was prepared by dissolving 0.5 g of agarose in 50 mL of Tris/Borate/EDTA (TBE) solution. The mixture was heated in a microwave to completely dissolve the agarose and left to cool for several min. 1 µl of ethidium bromide was added to the agarose solution. The agarose solution was then poured to a mini gel casting tray and a 8-welled comb was placed in the gel tray. The gel was left to solidify for 20 min. 3 µl of

DNA ladder was loaded into the first well of the gel. A mixture of 5 µl of purified PCR products from each individual isolates and 1 µl of loading dye was loaded into the gel. A PCR product without DNA template was also loaded into the gel and used as negative control. The gel electrophoresis was then run at 110 V for 50 min. The gel was then visualised under UV lightbox and the image was analysed by Gel-Pro Analyser.

3.5.3.5 DNA Concentration, Yield and Purity Determination

DNA concentration was estimated by measuring the optical absorbance at 260 nm (A_{260}), multiplying with the dilution factor and using the relationship that an A_{260} of 1.0 = 50 µg/mL of pure DNA.

Concentration (µg/mL) = A_{260} reading x Dilution factor x 50 µg/mL

Total yield was obtained by multiplying the DNA concentration by the final total purified sample volume.

DNA yield (µg) = DNA concentration (µg/mL) x Total sample volume (mL)

A_{260}/A_{280} ratio was used as an estimate of DNA purity whereby an A_{260}/A_{280} ratio of between 1.7 and 2.0 was generally accepted as representative of a high-quality DNA sample.

3.5.3.6 Sequencing of the 16S rDNA Gene and Identification of Bacteria

The purified PCR products (A_{260}/A_{280} ratio between 1.7 and 2.0) were sent for commercial DNA sequencing. The identity of individual bacterial isolates was determined by comparing their 16S rDNA sequences to those in the GenBank nucleotide sequence databases using the Basic Local Alignment Search Tool (BLAST) family of computer programmes (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.6 THE ANTIBIOFILM PROPERTIES OF SILVER-COATED TUBING

3.6.1 *In Vitro* Dental Unit Waterline System (DUWS) Biofilm Model

An *in vitro* DUWS model simulating clinical conditions in a DCU was developed to assess the inhibitory effect of silver-coated tubing on biofilm formation. Polyurethane tubing which is the most common tubing used in DCU was used as the control in this study. Both silver-coated tubing and polyurethane tubing were pre-sterilised by ethylene oxide.

This model consisted of four main components, namely water reservoir, tubings, electronic peristaltic pump and waste water container (Figure 3.10 and 3.11). The water reservoir contained distilled water with the bacteria recovered from the water samples collected in the earlier part of this study. The types of bacteria was similar to those determined in Section 3.5 and the proportion of respective bacteria used was calculated based on data obtained in Section 3.4.2. Three tubings were used simultaneously for each model running. The first tubing was the polyurethane tubing connected to water reservoir that contained distilled water which has been inoculated with bacteria and used as the positive control.

The second tubing was the silver-coated tubing which was the tested tubing. The third tubing was the polyurethane tubing connected to the water reservoir that contained sterile distilled water and used as the negative control. The electronic peristaltic pump was used to withdraw water from water reservoir, allowed the water passing through the tubing and lastly allowed the water to waste. The waste water container collected water from the tubings.

The model was run to waste for one min in the early morning (simulated flushing of handpiece), run for 8 hr of simulated chair side use (alternating 15 min at 80 mL/min, dispensing around 5 mL of water) and followed by 16 hr (overnight) of stagnation. Cycles of flushing and stagnation was repeated over seven days.

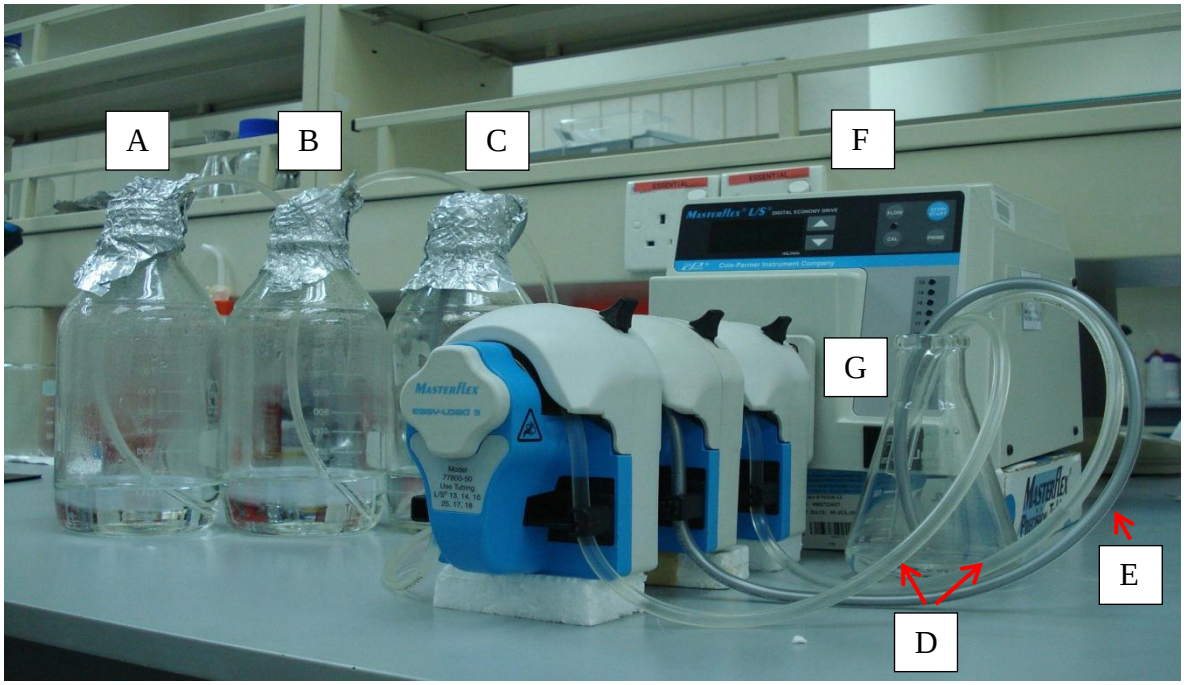

Figure 3.10: *In vitro* model for DUWS biofilm formation

A & B: Water reservoir contained distilled water that has been inoculated with bacteria recovered from previous water samples in the earlier part of study

C: Water reservoir contained sterile distilled water.

D: Polyurethane tubing (white tubing)

E: Silver-coated tubing (grey tubing)

F: Peristaltic pump

G: Waste water container

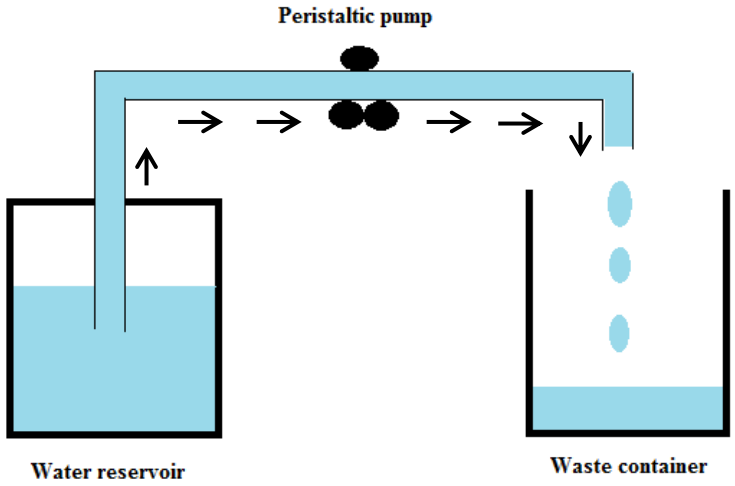

Figure 3.11: Schematic diagram of *in vitro* model for DUWS biofilm formation.

3.6.2 Assessment of Antibiofilm Properties of Tubing

In this experiment, (i) the number of adhering bacteria to the inner wall of tubing, and (ii) the number of planktonic bacteria present in the output water samples were determined, and (iii) observation of the adhesion under a scanning electron microscope were made. The inhibitory effect of silver-coated tubing was assessed by comparing (i), (ii) and (iii) between the commonly used polyurethane tubing and the silver-coated tubing.

3.6.2.1 Determination of Number of Adhering Bacteria

The model was left to run over a period of seven days to allow for biofilm formation. In the early morning of each day of the period, the run was stopped and the end of tubing samples was wiped with alcohol to remove potential contaminants on their surfaces. 10 mm of the tubing was aseptically cut and placed into a sterile microcentrifuge tube containing buffered water. The tube was then sonicated and vortexed for 1 min respectively to completely detach the bacteria from the wall of the tubing. 0.1 mL of the buffered water containing the detached bacteria was plated onto R2A agar plates as described in Section 3.4.2. After seven days of incubation at 28 °C, the number of colonies formed was counted and an average count of triplicates was recorded.

3.6.2.2 Determination of Number of Planktonic Bacteria

Following section 3.6.2.1, the output water sample from the tubings was also aseptically collected into sterile universal bottles. 0.1 mL of water samples was evenly spread on to R2A agar plates as described in Section 3.4.2. After seven days of incubation at 28 °C, the number of colonies formed on the plates was counted and an average count of triplicates was recorded.

3.6.2.3 Observation under Scanning Electron Microscope (SEM)

Following section 3.6.2.1, another 10 mm of the tubing was also cut and sectioned longitudinally. The section was immersed overnight at 4°C in McDowell-Trump fixative prepared in 0.1M phosphate buffer (pH 7.2). The section was washed in phosphate buffer for three times before it was post-fixed in 1 % (w/v) osmium tetroxide at room temperature

for two hr. After post-fixation, the section was dehydrated in ascending percentage of ethanol from 50 %, 75 %, 95 % up to 100 %. Dehydration by 95 % and 100 % ethanol was repeated two times and three times, respectively. The dehydrated section was then immersed in hexamethyldisilazane (HMDS) for 10 min twice. The HMDS solution was decanted and the section was left to air-dry at room temperature before it was mounted onto the SEM stub. The section was then observed under a scanning election microscope at 2,500 x magnification.

3.6.3 Statistical Analysis

Statistical analysis was carried out using Statistical Package for the Social Sciences (SPSS) version 12.0.1. The number of adhering bacteria to the inner wall of silver-coated tubing was compared to that of polyurethane tubing using Mann-Whitney U test. Similarly, the number of planktonic bacteria was also compared between silver-coated tubing and polyurethane tubing using Mann-Whitney U test. Statistical significance was assumed at a *P* value of < 0.05.

CHAPTER FOUR

RESULTS

4.1 LABORATORY PROCESSING OF WATER SAMPLES

4.1.1 pH and Temperature Measurement

The pH and temperature of water samples were measured on site and the means of pH and temperature of water samples were shown in Figure 4.1 and 4.2 respectively. On average, the pH of water samples was between 5.4 and 5.5 which were slightly acidic. The average temperature of water samples was found to be around 23 °C which was around the room temperature.

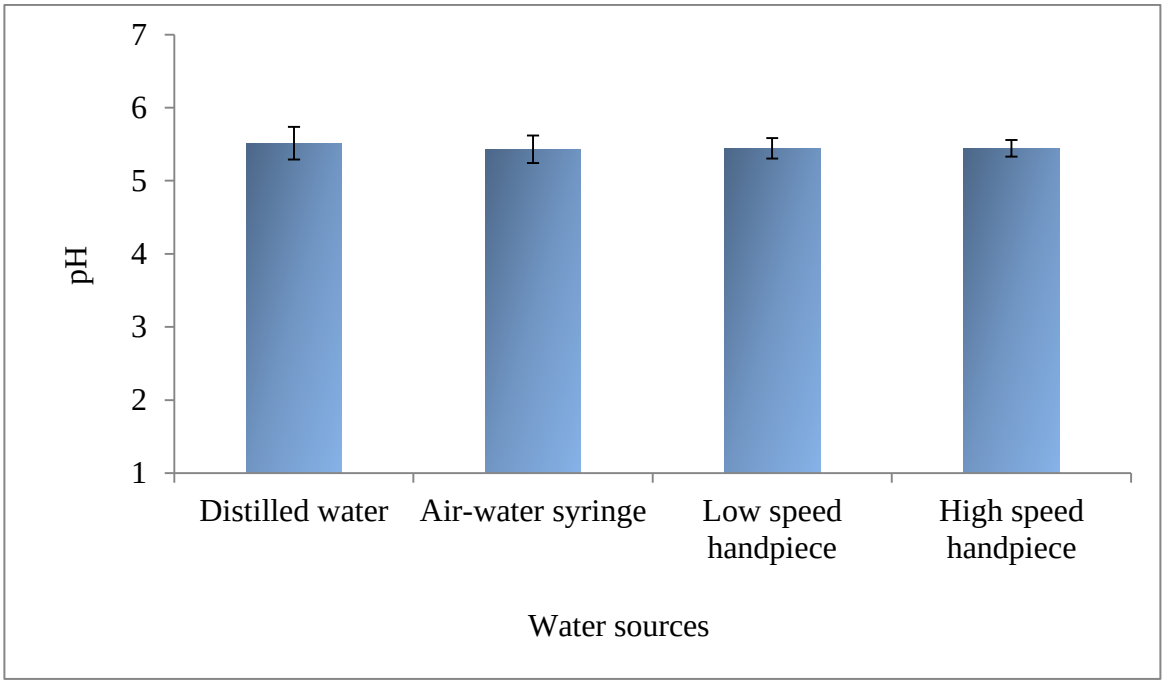

Figure 4.1: A bar chart showing the pH of water collected from four different water sources. The values plotted were the means of readings from 13 DCUs (n=39).

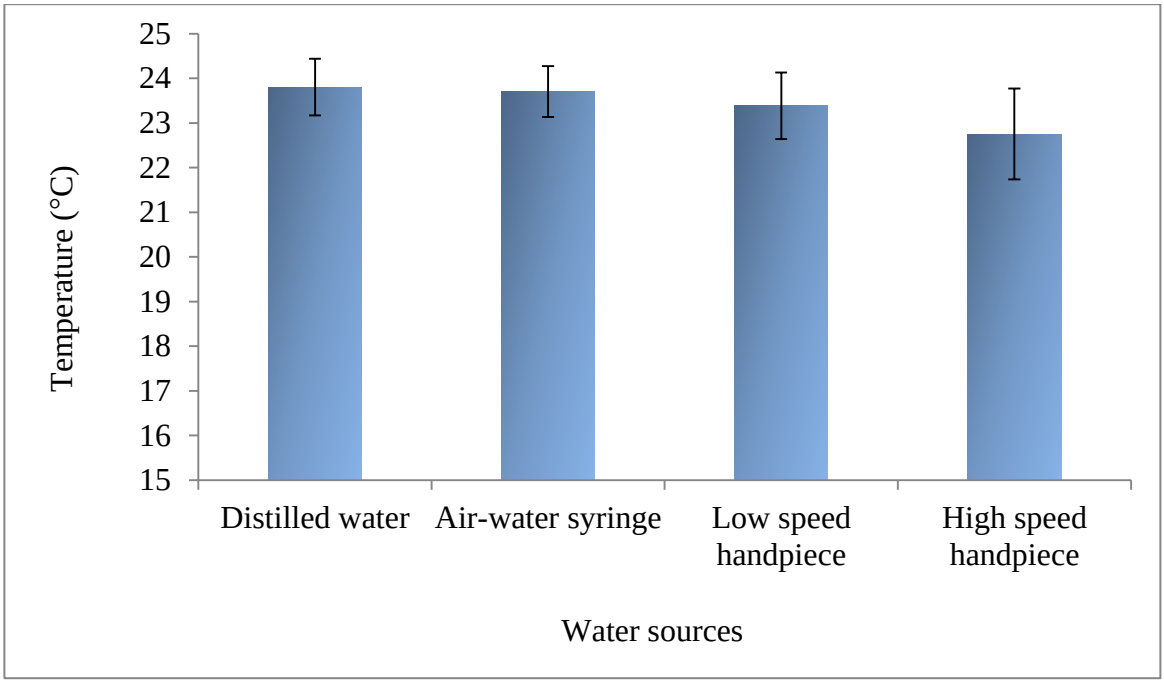

Figure 4.2: A bar chart showing that the temperature of water collected from four different water sources. The values plotted were the means of readings from 13 DCUs (n=39).

4.1.2 Heterotrophic Plate Count

The heterotrophic plate count (cfu/mL) which indicated the microbial load in the water samples was expressed in $\log_{10}$. As shown in Figure 4.3, means of microbial load in distilled water, air-water syringe, low speed handpiece and high speed handpiece were determined at 3.92 $\log_{10}$ cfu/mL (8256 cfu/mL), 3.81 $\log_{10}$ cfu/mL (6469 cfu/mL), 3.92 $\log_{10}$ cfu/mL (8290 cfu/mL) and 3.92 $\log_{10}$ cfu/mL (8356 cfu/mL) respectively. All water samples were found to contain microbial population that significantly exceeded the standard recommended by ADA which is at less than 200 cfu/mL ($P < 0.001$) (Table 4.1). The population of microorganism in the air-water syringe was found to be significantly lower than that of the distilled water ($P = 0.005$) (Table 4.2). No significant difference was found between the microbial load in the low speed and high speed handpieces, and that of

the distilled water respectively (P = 0.888 and P = 0.652, respectively) (Table 4.2). Representative plates of water samples as well as positive and negative controls were as shown in Figure 4.4.

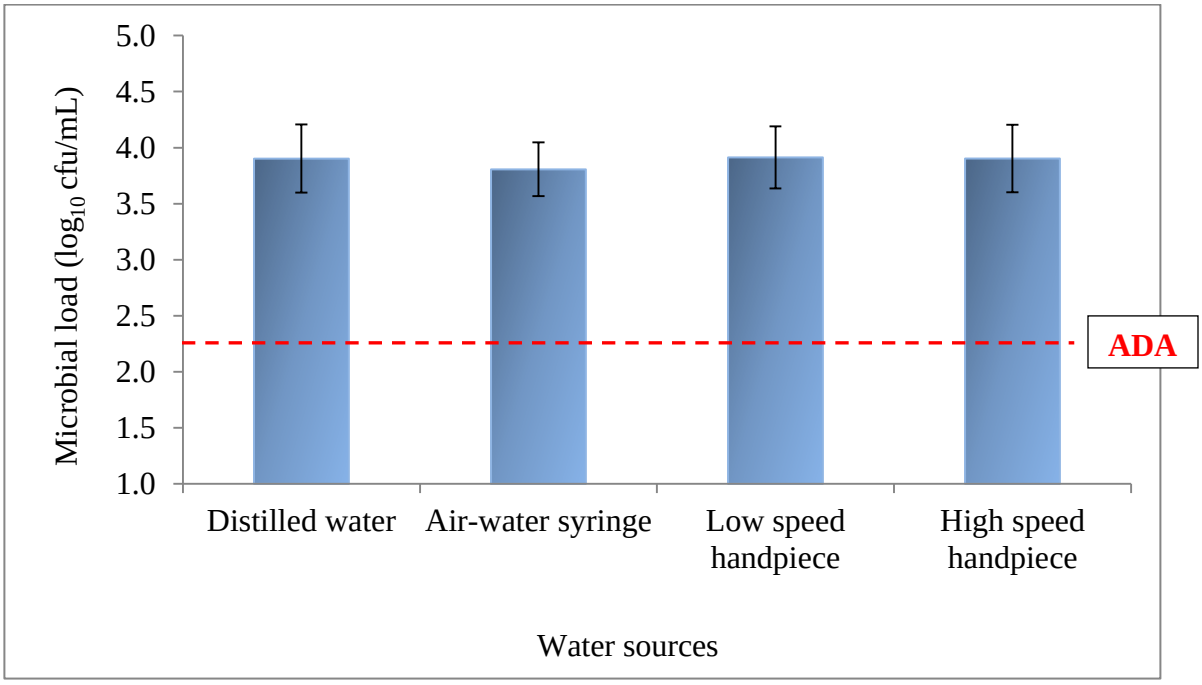

Figure 4.3: The mean of microbial load from different water sources. The dotted line drawn across the bar chart indicated the level of microbial load below which is recommended by the American Dental Association (ADA) as acceptable for DUWS (≤ 200 fu/mL).

Table 4.1: Comparison of microbial load from different water sources to the standard recommended by ADA (200 cfu/mL= 2.301 $\log_{10}$). Comparison was made using one sample *t* test and the *P* values are as indicated in the table.

Water sources	**Microbial load (SD)**	**Mean diff. (95 % CI)**	***t*-statistic (df)**	***P* value[a]**
Distilled water	3.9168 (0.3049)	1.6158 (1.5402,1.6913)	42.720 (64)	< 0.001
Air-water syringe	3.8109 (0.2203)	1.5099 (1.4553, 1.5645)	55.247 (64)	< 0.001
Low speed handpiece	3.9186 (0.2779)	1.6176 (1.5487, 1.6865)	46.926 (64)	< 0.001
High speed handpiece	3.9220 (0.3030)	1.6210 (1.5459, 1.6961)	43.137 (64)	< 0.001

a. level of significance was set as $P < 0.05$

Table 4.2: Comparison of microbial load between output water from the air-water syringe, low and high speed handpieces to that of distilled water source. Comparison was made using paired *t* test and the *P* values are as indicated in the table.

Water sources	**Microbial load (SD)**	**Mean diff. (95 % CI)**	***t*-statistic (df)**	***P* value[a]**
Distilled water	3.9168 (0.3049)	-	-	-
Air-water syringe	3.8109 (0.2203)	0.1059 (0.0325,0.1793)	2.881 (64)	0.005
Low speed handpiece	3.9186 (0.2779)	-0.0185 (-0.0280, 0.0243)	-0.141 (64)	0.888
High speed handpiece	3.9220 (0.3030)	-0.0052 (-0.2826, 0.1780)	-0.454 (64)	0.652

a. level of significance was set as $P < 0.05$

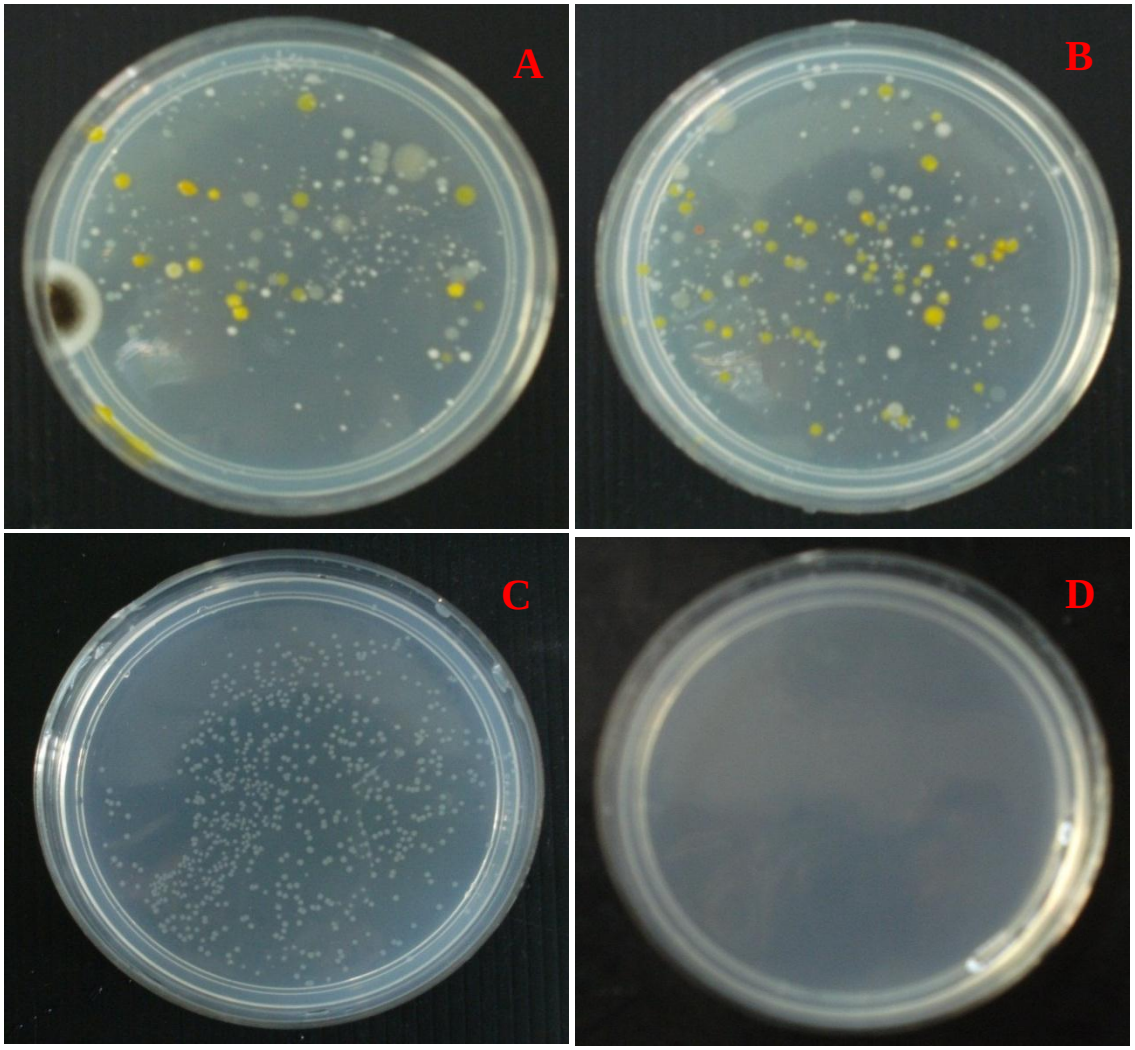

Figure 4.4 Images of heterotrophic plate counts. (A) & (B) Representative plates of water samples; (C) *Escherichia coli* (positive control culture); (D) Sterile distilled water (negative control culture).

4.1.3 Microbiological Tests (Total Coliforms Count, Faecal Coliforms Count, *Escherichia coli* Count, Faecal Streptococci Count and *Pseudomonas aeruginosa* Count)

All water samples were shown negative in total coliforms counts, faecal coliforms counts, *Escherichia coli* counts, faecal streptococci counts and *Pseudomonas aeruginosa* counts (Table 4.3). Water samples from all 13 DCUs were found to be free of pathogenic bacteria. Representative plates as well as positive and negative control cultures of all tests were shown in Figure 4.5, 4.6, 4.7, 4.8 and 4.9, respectively.

Table 4.3: Microbiological tests of distilled water, air-water syringe, low speed handpiece and high speed handpiece. All water samples showed no presence of bacterial indicators and thus pathogenic microorganisms.

Water sources	**Total coliforms count (cfu/100 mL)**	**Faecal coliforms count (cfu/100 mL)**	***Escherichia coli* count (cfu/100 mL)**	**Faecal streptococci count (cfu/100 mL)**	***Pseudomonas aeruginosa* count (cfu/100 mL)**
Distilled water	ND	ND	ND	ND	ND
Air-water syringe	ND	ND	ND	ND	ND
Low speed handpiece	ND	ND	ND	ND	ND
High speed handpiece	ND	ND	ND	ND	ND

ND: not-detected

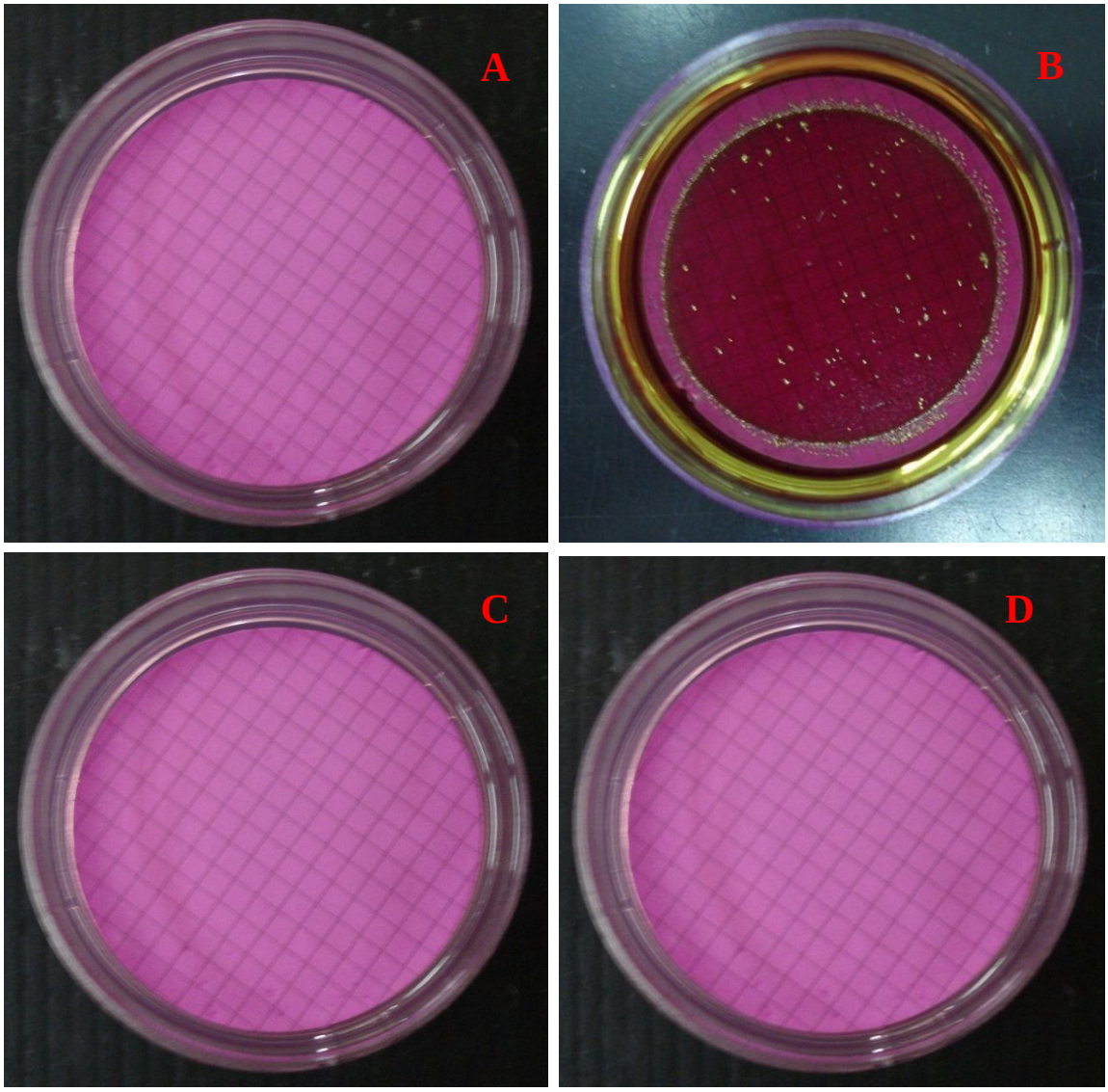

Figure 4.5 Images of total coliforms counts. (A) Representative plate of water samples; (B) *Escherichia coli* (positive control culture); (C) *Staphylococcus aureus* (negative control culture); (D) Sterile distilled water (negative control).

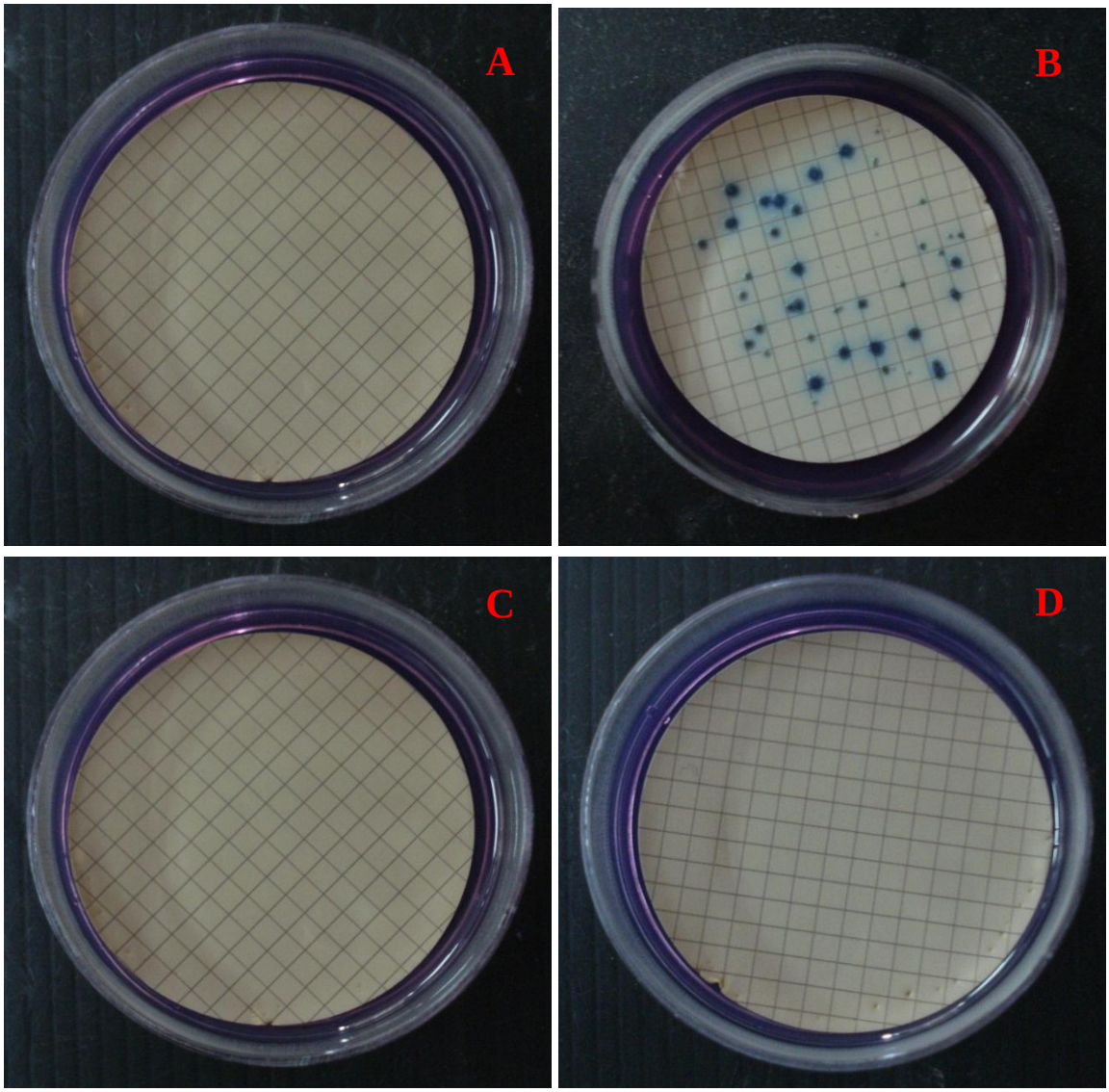

Figure 4.6 Images of faecal coliforms counts. (A) Representative plate of water samples; (B) *Escherichia coli* (positive control culture); (C) *Enterococcus faecalis* (negative control culture); (D) Sterile distilled water (negative control).

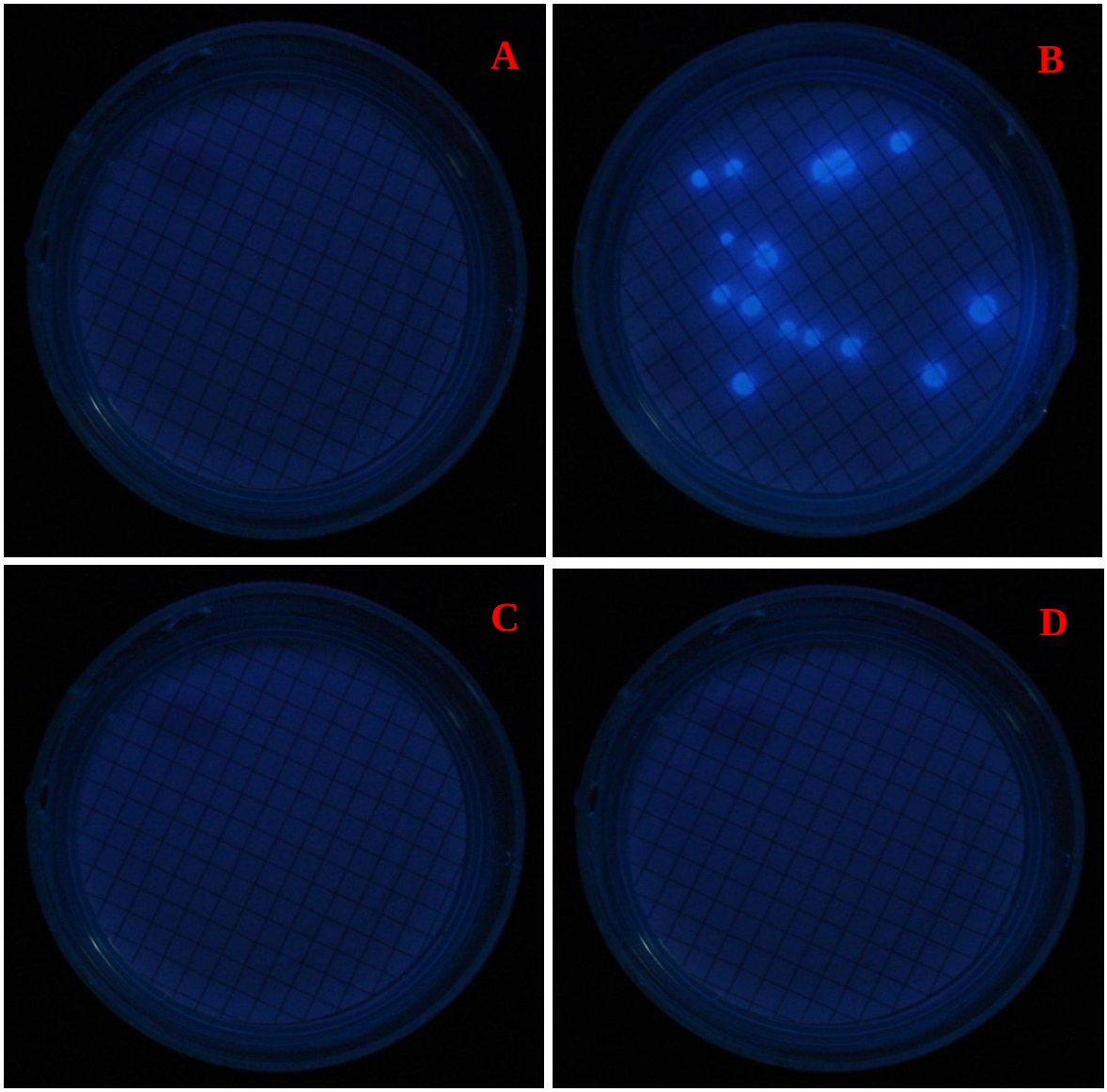

Figure 4.7 Images of *Escherichia coli* counts. (A) Representative plate of water samples; (B) *Escherichia coli* (positive control culture); (C) *Enterococcus faecalis* (negative control culture); (D) Sterile distilled water (negative control).

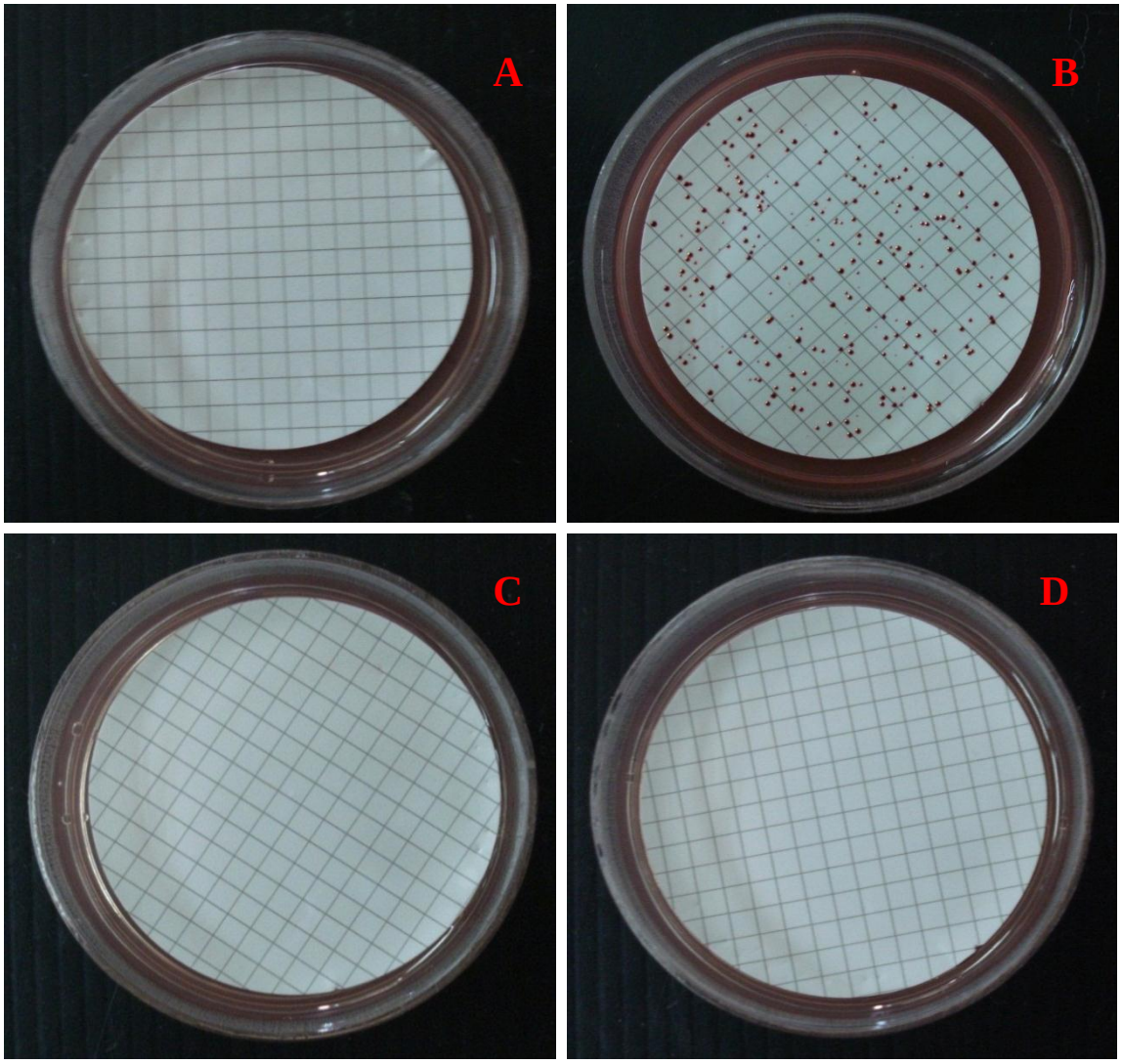

Figure 4.8 Images of faecal streptococci counts. (A) Representative plate of water samples; (B) *Enterococcus faecalis* (positive control culture); (C) *Escherichia coli* (negative control culture); (D) Sterile distilled water (negative control).

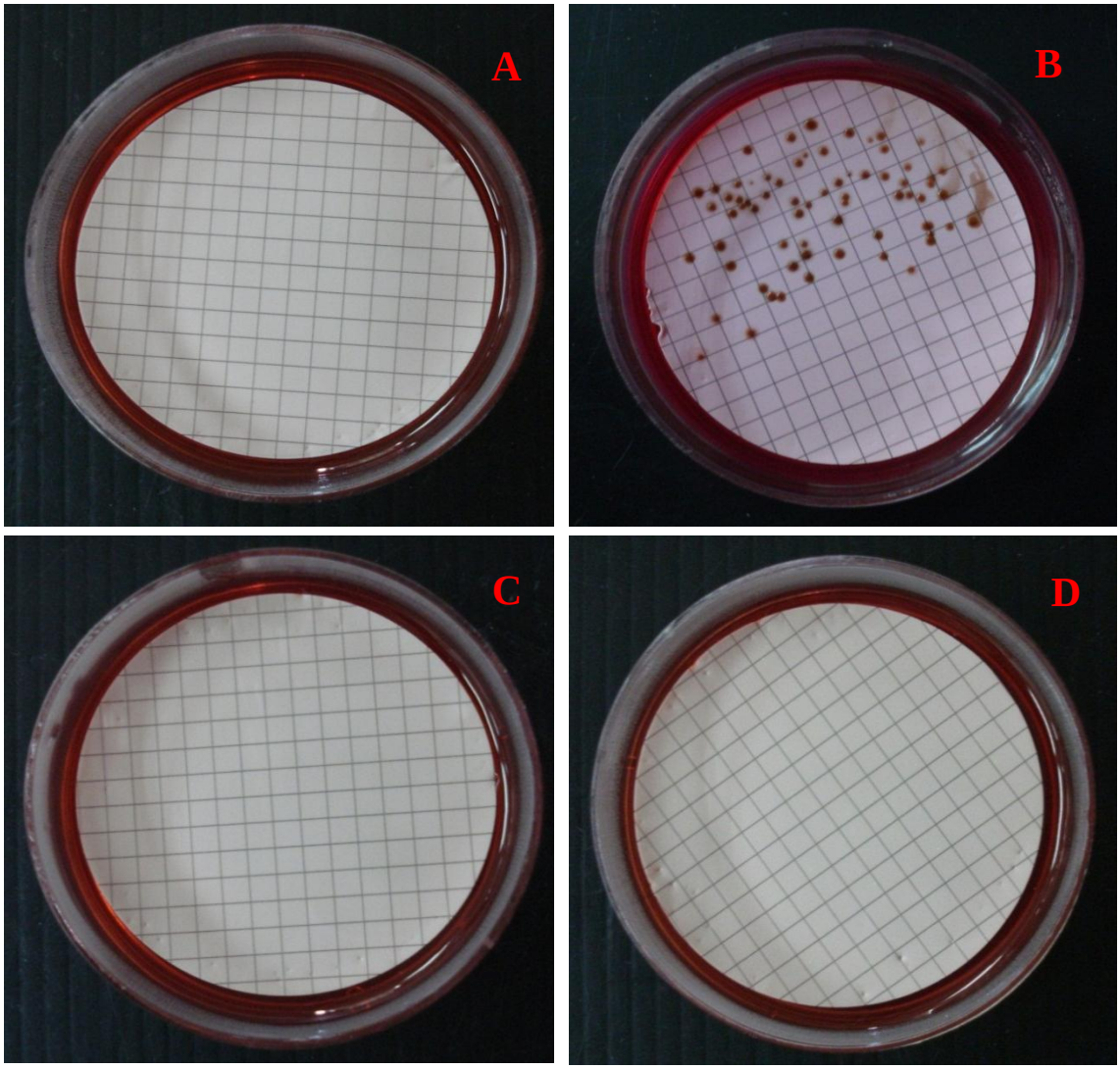

Figure 4.9 Images of *Pseudomonas aeruginosa* counts. (A) Representative plate of water samples; (B) *Pseudomonas aeruginosa* (positive control culture); (C) *Escherichia coli* (negative control culture); (D) Sterile distilled water (negative control).

4.2 IDENTIFICATION OF BACTERIAL ISOLATES

4.2.1 Colony, Cells and Ultrastructural Characteristics

Four colonies displaying different characteristics with regards to size and colour were isolated from the water samples. They were named as isolate A, isolate B, isolate C and isolate D. Their colony morphology was observed under a dissecting microscope. Their cells structure was observed under a light microscope at 100 x magnification after they were stained by Gram's staining while their ultrastructural characteristic was observed under a scanning electron microscope at 24,000 x magnification. Table 4.4 showed the colony characteristics of each isolate. Figure 4.10, 4.11, 4.12 and 4.13 showed the pure culture, colony morphology, cell morphology and ultrastructural morphology.

Table 4.4: Colony characteristics of isolates recovered from water samples.

Characteristics	**Isolate A**	**Isolate B**	**Isolate C**	**Isolate D**
Colony colour	Yellow	Creamy white	Brown	Pink
Colony size[a]	2-5 mm	2-4 mm	2-10 mm	2-4 mm
Form	Circular	Circular	Filamentous	Circular
Elevation	Convex	Convex	Raised	Convex
Margin	Entire	Entire	Lobate	Entire
Cell morphology	Rod	Rod	Rod	Rod

a. colony size was determined by measuring three representative colonies on the plate.

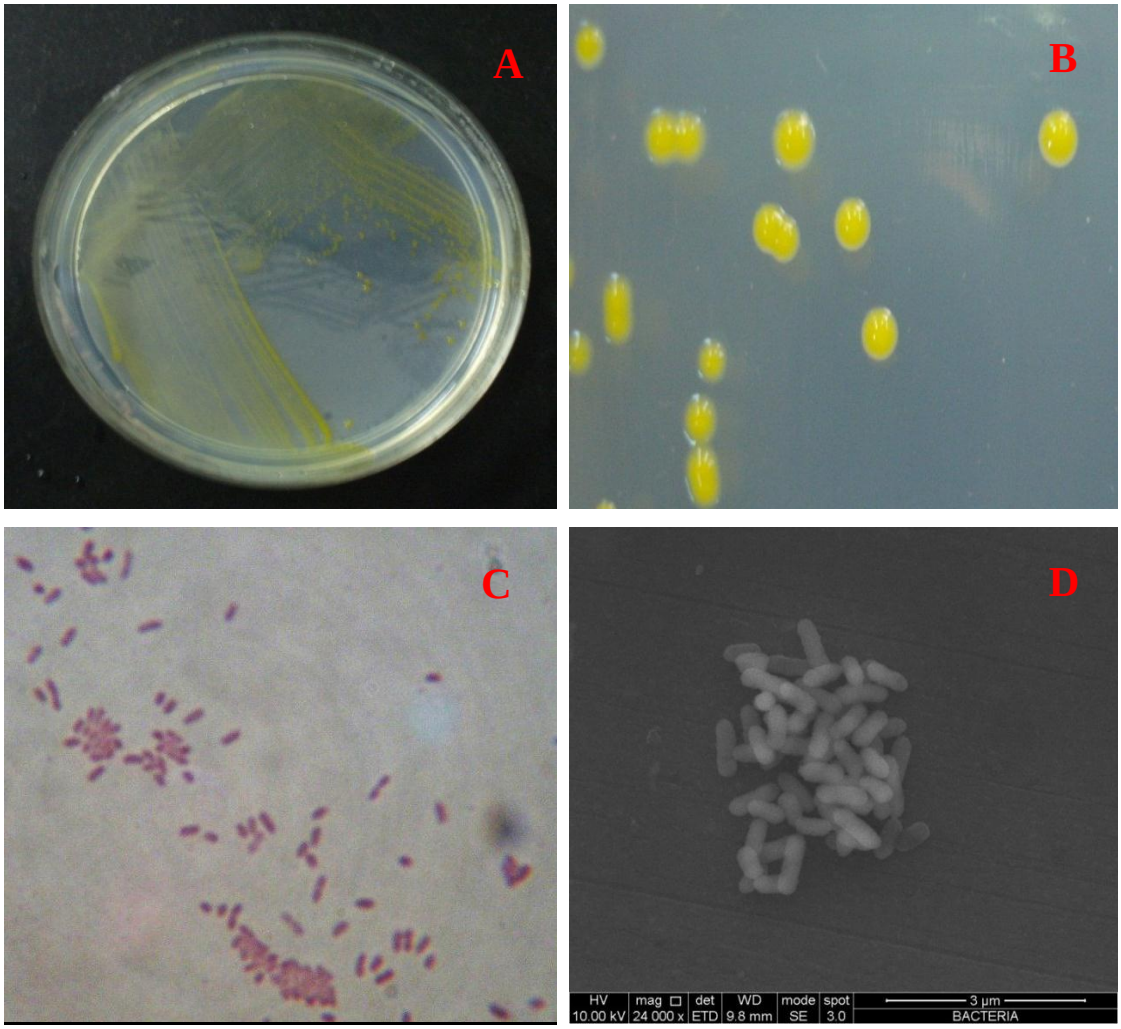

Figure 4.10: Images of isolate A. (A) Pure cultures on R2A agar plate. (B) Closer view to individual colony. (C) Gram-stained bacteria cells observed under a light microscope at 1,000 X magnification. (D) Ultrastructural observation under a scanning electron microscope at 24,000 X magnification.

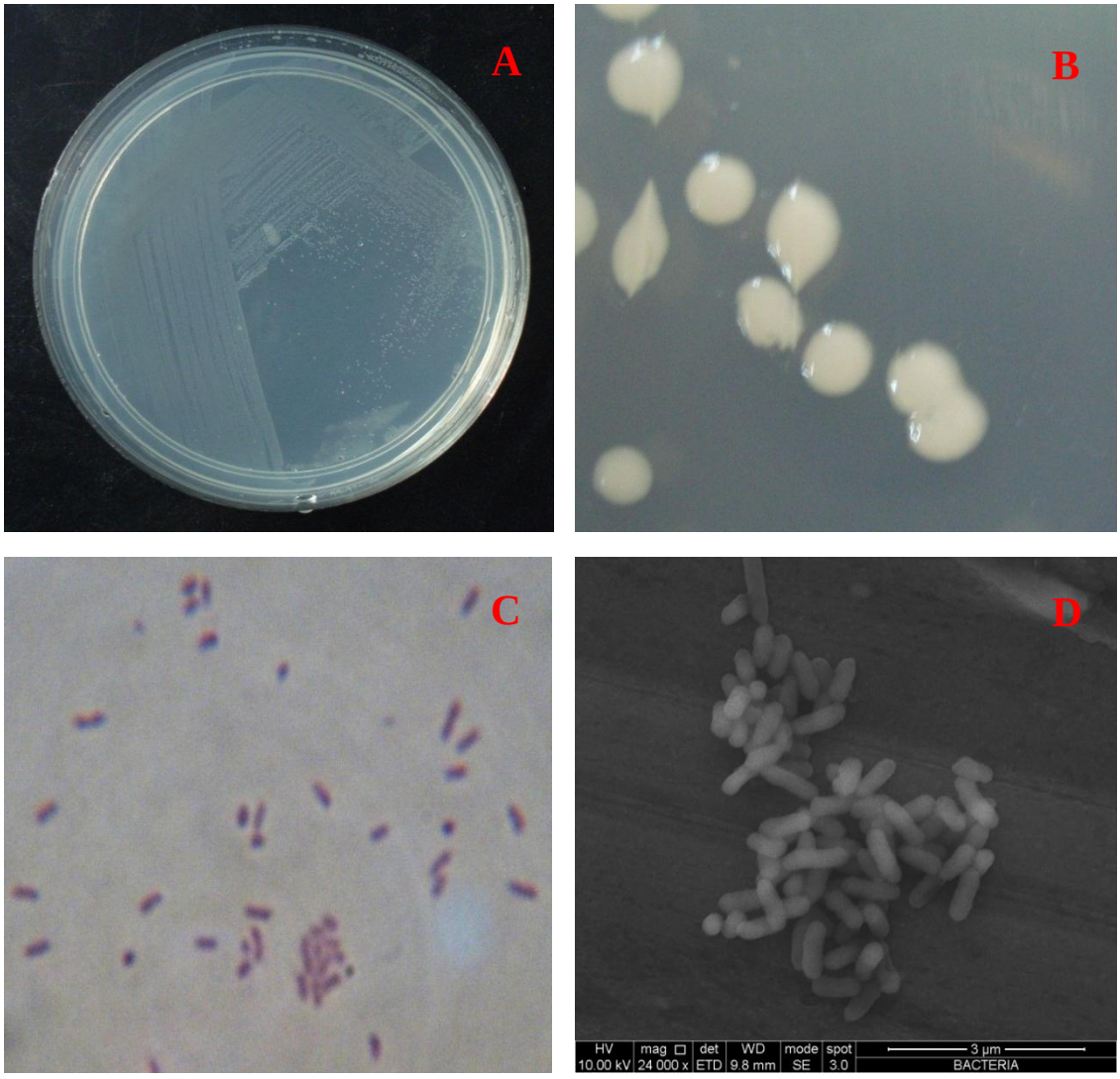

Figure 4.11: Images of isolate B. (A) Pure cultures on R2A agar plate. (B) Closer view to individual colony. (C) Gram-stained bacteria cells observed under a light microscope at 1,000 X magnification. (D) Ultrastructural observation under a scanning electron microscope at 24,000 X magnification.

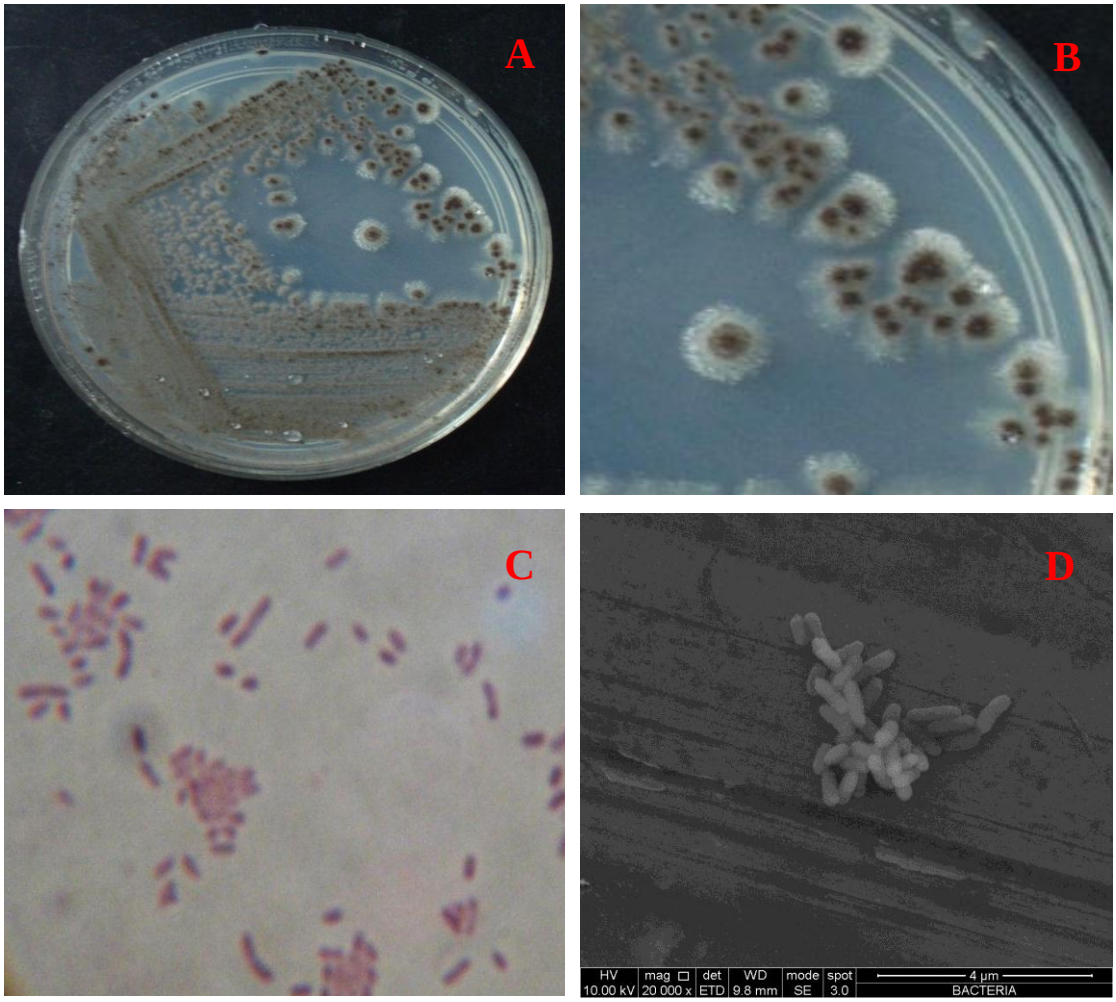

Figure 4.12: Images of isolate C. (A) Pure cultures on R2A agar plate. (B) Closer view to individual colony. (C) Gram-stained bacteria cells observed under a light microscope at 1,000 X magnification. (D) Ultrastructural observation under a scanning electron microscope at 24,000 X magnification.

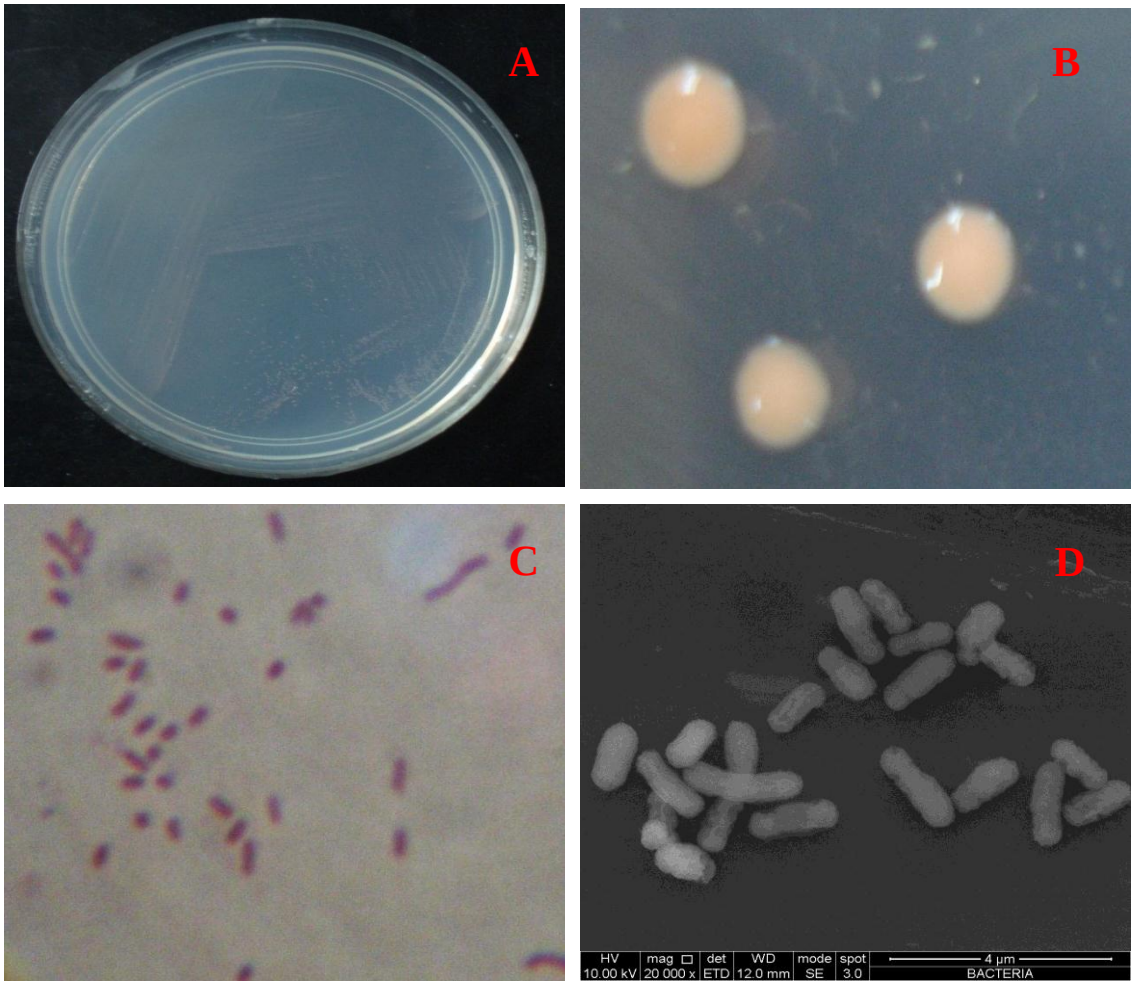

Figure 4.13: Images of isolate D. (A) Pure cultures on R2A agar plate. (B) Closer view to individual colony. (C) Gram-stained bacteria cells observed under a light microscope at 1,000 X magnification. (D) Ultrastructural observation under a scanning electron microscope at 24,000 X magnification.

4.2.2 Gel Electrophoresis Visualisation

After the 16S rRNA gene of each bacterium was amplified by PCR and purified, the purified DNA samples were loaded into agarose gel and visualised under ultraviolet illumination. The appearance of a single band in each lane indicated that the DNA sample was free from other DNA contaminants at correct length size (1,500 bp) and ready for commercial sequencing.

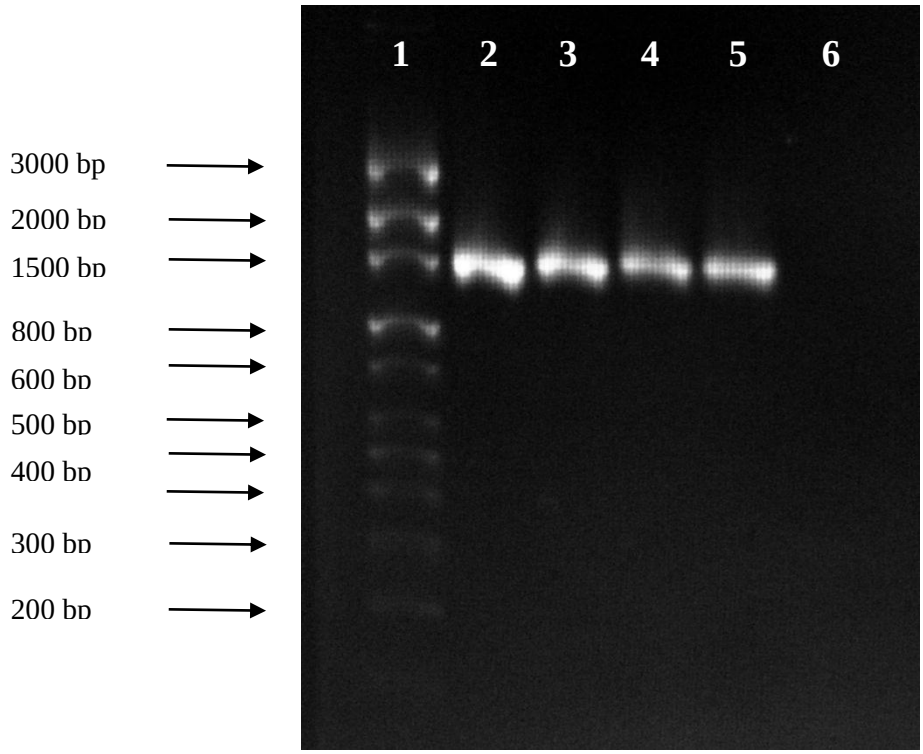

Figure 4.14: Image of gel electrophoresis. (Lane 1) DNA ladder, (Lane 2) Isolate A, (Lane 3) Isolate B, (Lane 4) Isolate C, (Lane 5) Isolate D, (Lane 6) PCR mixture without template DNA (negative control).

4.2.3 Nucleotide Sequence Comparison with GenBank Database and Microbial Abundance Percentage

The identification of each isolate was based on their 16S rDNA sequence compared to the homologous sequence in the National Center for Biotechnology Information (NCBI) database using BLAST software. A high similarity (≥ 98 %) to the homologous sequence in the database confirmed the identity of the bacteria. Table 4.5 showed the identity of each isolate and their abundance percentage in water samples.

Table 4.5: Identity of common bacteria isolated from water samples as determined by comparing their 16S rDNA gene sequences with that of GenBank database. The number of colonies formed and abundance percentage of these bacteria in the water samples are presented.

Isolate	**Bacterial species (GenBank accession no.)**	**Number of colonies formed (SD)**	**Abundance percentage (%)**
A	*Sphingomonas rhizogenes* (AY962684.2)	1278.79 ± 1084.93	17.90
B	*Sphingomonas dokdonensis* (DQ178975.1)	5701.51 ± 2829.60	79.50
C	*Sphingomonas mucosissima* (AM229669)	78.79 ± 141.98	1.10
D	*Methylobacterium radiotolerans* (GU294334.1)	107.58 ± 294.73	1.50

4.3 THE ANTIBIOFILM PROPERTIES OF SILVER-COATED TUBING

4.3.1 Determination of the Number of Adhering Bacteria

In Figure 4.15, the curves demonstrated the binding pattern of bacteria to the inner surface of silver-coated tubing and polyurethane tubing. Minimal adhering bacterial count was displayed on the first and second day of the trial. Accumulation of adhering bacteria cells to the tubing began after the second day resulting in a uniform increase in plate count reading. A maximum population at 10^5 cfu/cm^2 was achieved on the seventh day of trial (Table 4.6). Examination of the both types of tubing surface before trial (day 0), on second, fourth and seventh day under a scanning electron microscope showed gradual bacterial colonisation of the tubing surface (Figure 4.16 and 4.17). No significant difference in the bacterial colonisation to the inner surface of both types of tubing was observed ($P > 0.05$) (Table 4.6).

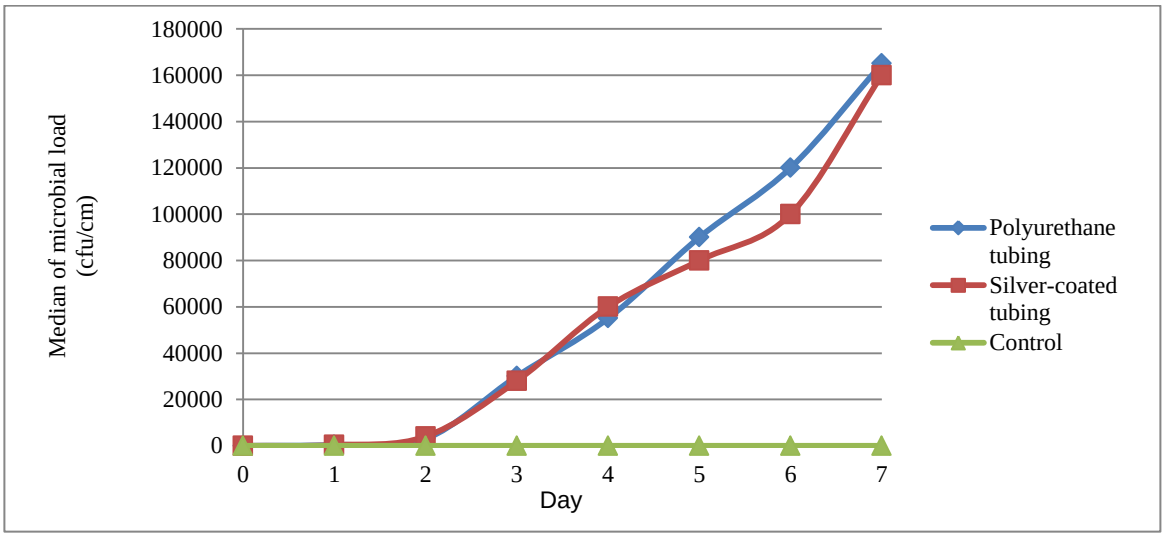

Figure 4.15: The number of adhering bacteria (cfu/cm) to the inner surface of silver-coated tubing compared to the conventional polyurethane tubing.

Table 4.6: The number of adhering bacteria (cfu/cm) to the inner surface of silver-coated tubing compared to the conventional polyurethane tubing. Comparison was made using Mann-Whitney Test and the *P* values are as indicated in the table.

Day	**Polyurethane tubing median (IQR)**	**Silver-coated tubing median (IQR)**	**Z Statistic**	***P* value[a]**
0	0 (0)	0 (0)	0.00	1.00
1	4×10^2 (2.5×10^2)	3.5×10^2 (1.1×10^2)	-0.627	0.531
2	3×10^3 (1×10^3)	4×10^3 (1.4×10^3)	-1.398	0.162
3	3×10^4 (1.25×10^4)	2.8×10^4 (1.65×10^4)	-1.202	0.229
4	5.5×10^4 (1.55×10^4)	6×10^4 (2.5×10^4)	-1.245	0.213
5	9×10^4 (1.5×10^4)	8×10^4 (1.85×10^4)	-1.763	0.078
6	1.2×10^5 (3.5×10^4)	1×10^5 (2×10^4)	-1.542	0.123
7	1.65×10^5 (3×10^4)	1×10^5 (5×10^4)	-1.562	0.118

a. level of significance was set as $P < 0.05$

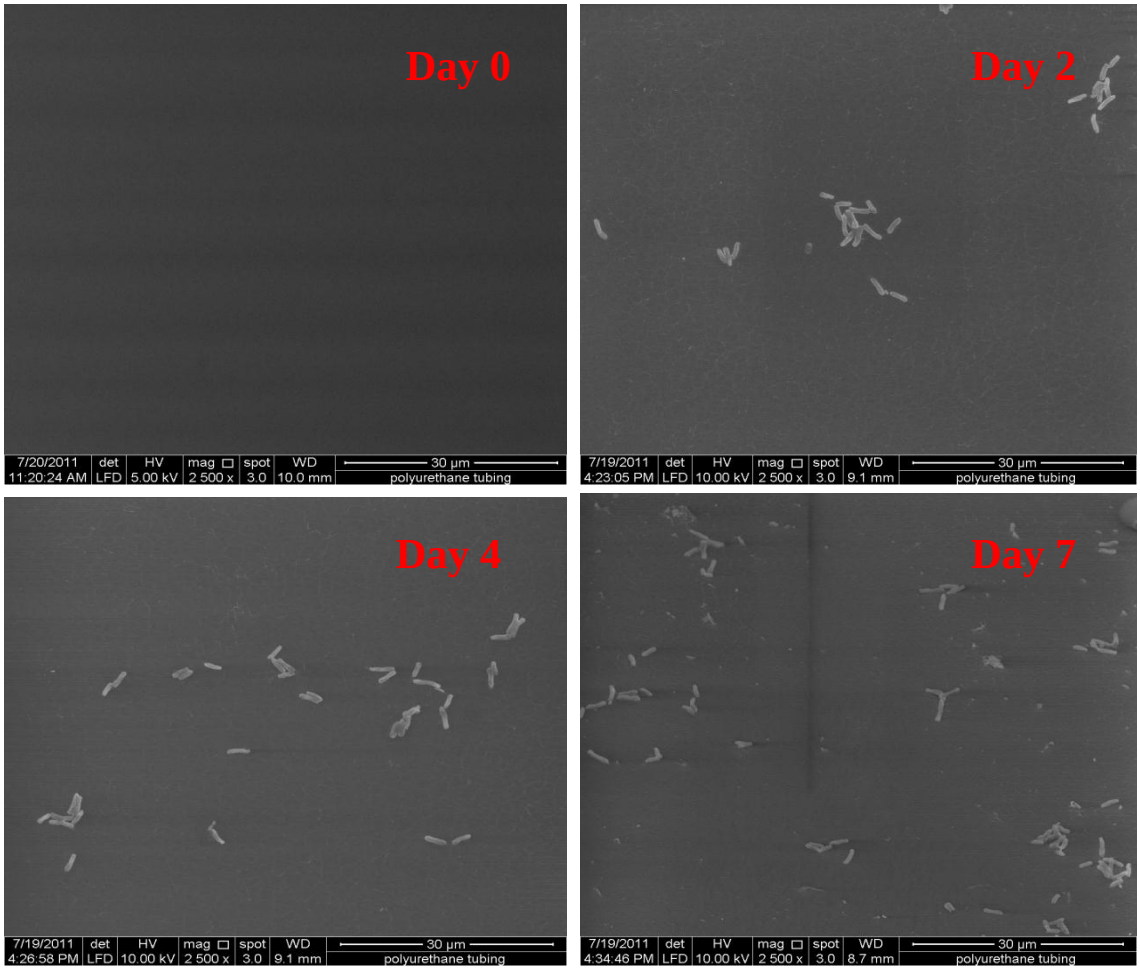

Figure 4.16: Electron micrograph showing the gradual increase in the bacterial colonisation population to the inner surface of polyurethane tubing (2,500 X magnification).

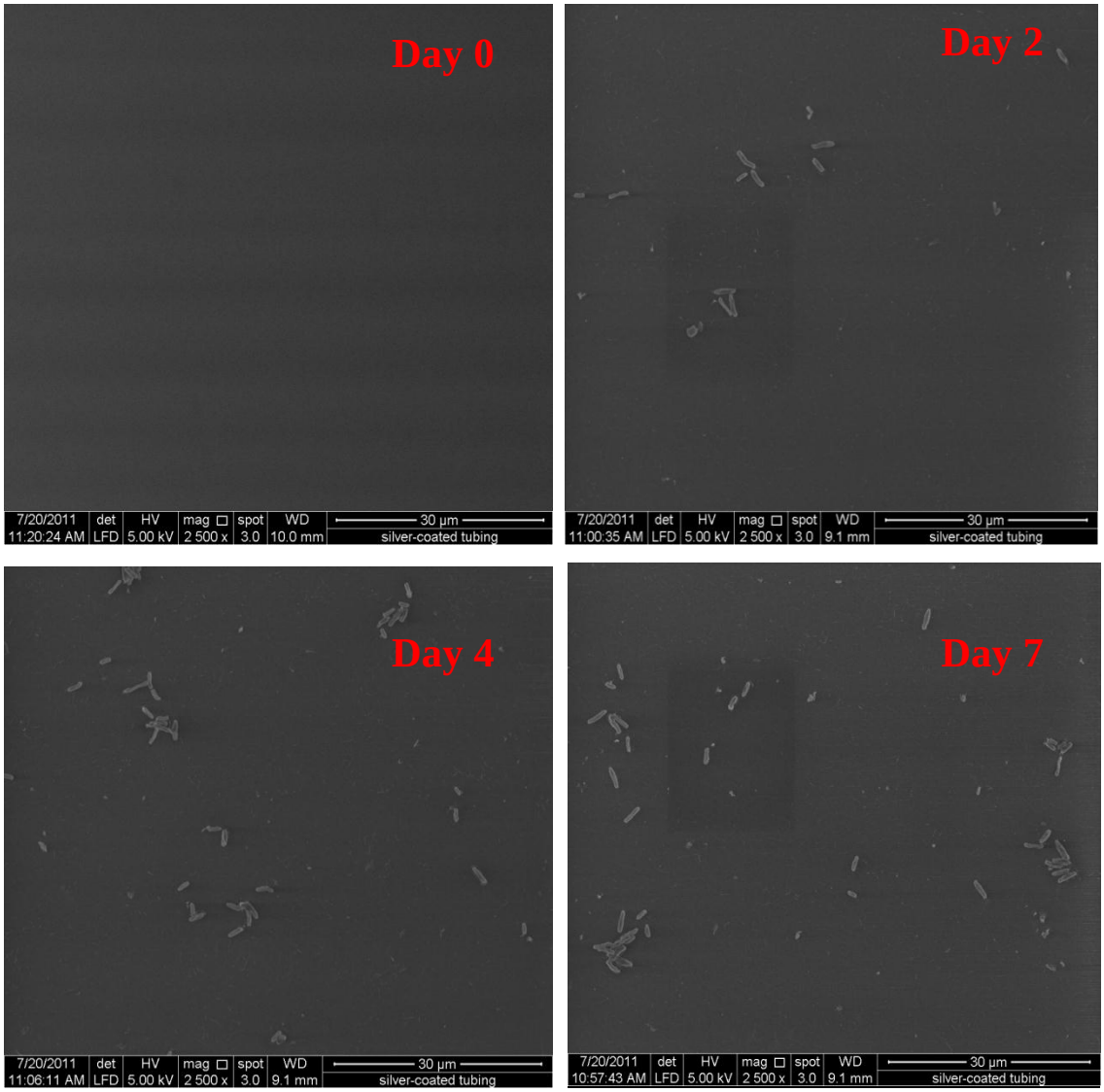

Figure 4.17: Electron micrograph showing the gradual increase in the bacterial colonisation population to the inner surface of silver-coated tubing (2,500 X magnification).

4.3.2 Determination of the Number of Planktonic Bacteria

In Figure 4.18, the curves demonstrated the number of planktonic bacteria present in water samples collected from silver-coated tubing as well as polyurethane tubing. Minimal adhering bacterial count was displayed on the first and second day of the trial. Planktonic bacteria counts constituted of the non-adhering bacteria together with the detached bacteria from the biofilm. A maximum population at 10^6 cfu/mL was attained on the seventh day of trial (Table 4.7). No significant difference in the planktonic bacterial counts in water samples collected from both types of tubing was observed ($P > 0.05$) (Table 4.7).

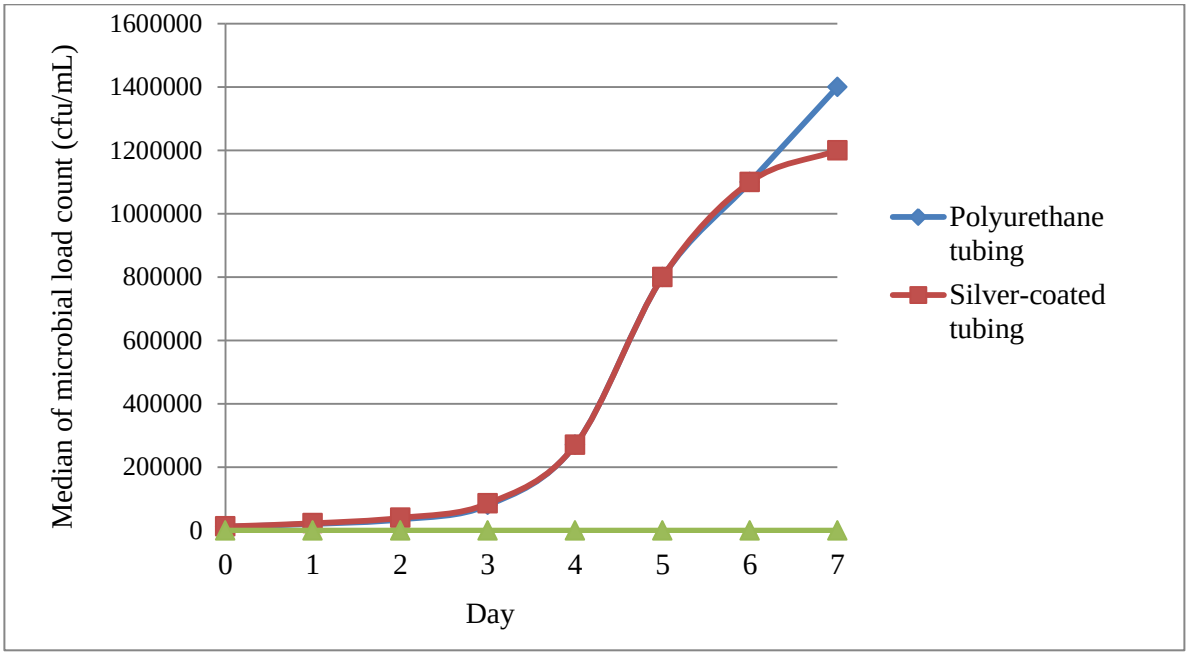

Figure 4.18: The number of bacteria found in water samples collected from silver-coated tubing compared to that of the conventional polyurethane tubing. The number of bacteria passed through the model was set at 1 x 10^4 cfu/mL as previously determined in the early part of the study.

Table 4.7: The number of planktonic bacteria (cfu/mL) found in water samples collected from silver-coated tubing compared to that of the conventional polyurethane tubing. Comparison was made using Mann-Whitney Test and the *P* values are as indicated in the table.

Day	Polyurethane tubing median (IQR)	Silver-coated tubing median (IQR)	Z Statistic	*P* value[a]
0	1.2×10^4 (3.5×10^3)	1.3×10^4 (3×10^3)	-0.091	0.928
1	2.1×10^4 (4.5×10^3)	2.3×10^4 (8×10^3)	-1.472	0.141
2	3.5×10^4 (1×10^4)	4×10^4 (1.5×10^4)	-1.215	0.224
3	8×10^4 (1.75×10^4)	8.5×10^4 (1×10^4)	-1.526	0.127
4	2.7×10^5 (5.5×10^4)	2.7×10^5 (6×10^4)	0.000	1.000
5	8×10^5 (1.3×10^5)	8×10^5 (1.75×10^5)	-0.224	0.823
6	1.1×10^6 (2.5×10^5)	1.1×10^6 (2×10^5)	-0.092	0.927
7	1.4×10^6 (3×10^5)	1.2×10^6 (3×10^5)	-1.839	0.066

a. level of significance was set as $P < 0.05$

4.3.3 Gradual Changes in the Number of Bacteria over Period of Trial

In Table 4.8 and 4.9, the total number of bacteria that included adhering bacteria and planktonic bacteria was compared over the period of trial. As the number of day increased, the number of bacteria also increased. A maximum of 130 x fold of increase was observed on the seventh day of trial for polyurethane tubing while 100 x fold of increase was observed for silver-coated tubing.

Table 4.8: The number of adhering bacteria and planktonic bacteria from polyurethane tubing was compared over the period of trial.

Day	Adhering bacteria (cfu/cm)	Planktonic bacteria (cfu/mL)	Total number of bacteria (cfu)	Fold
0	0	1.2×10^4	1.2×10^4	-
1	4×10^2	2.1×10^4	2.14×10^4	1.78 x
2	3×10^3	3.5×10^4	3.8×10^4	3.17 x
3	3×10^4	8×10^4	1.1×10^5	9.17 x
4	5.5×10^4	2.7×10^5	3.25×10^5	27.1 x
5	9×10^4	8×10^5	8.9×10^5	74.2 x
6	1.2×10^5	1.1×10^6	1.22×10^6	101.7 x
7	1.65×10^5	1.4×10^6	1.56×10^6	130 x

Table 4.9: The number of adhering bacteria and planktonic bacteria from silver-coated tubing was compared over the period of trial.

Day	Adhering bacteria (cfu/cm)	Planktonic bacteria (cfu/mL)	Total number of bacteria (cfu)	Fold
0	0	1.3×10^4	1.3×10^4	-
1	3.5×10^2	2.3×10^4	2.34×10^4	1.8 x
2	4×10^3	4×10^4	4.4×10^4	3.38 x
3	2.8×10^4	8.5×10^4	1.13×10^5	8.7 x
4	6×10^4	2.7×10^5	3.3×10^5	25.4 x
5	8×10^4	8×10^5	8.8×10^5	67.7 x
6	1×10^5	1.1×10^6	1.2×10^6	92.3 x
7	1×10^5	1.2×10^6	1.3×10^6	100 x

CHAPTER FIVE

DISCUSSION

5.1 LABORATORY PROCESSING OF WATER SAMPLES

This cross-sectional study was carried out to assess the microbial contamination of output water from dental unit waterline system (DUWS). The water samples were collected from one of the teaching clinics in the Faculty of Dentistry, University of Malaya. The outcome of this study may provide some insights and understanding on the output water quality as well as the efficiency of infection control practices in local dental clinic setups.

In this study, a combination of physical and microbiological properties of output water from DUWS was assessed. pH and temperature were two simple physical properties that were easily measured at the sampling site but provided some useful information on the microbial environment of water samples. Other parameters included were the heterotrophic plate count (HPC), total coliforms count, faecal coliforms count, *Escherichia coli* count, faecal streptococci count and *Pseudomonas aeruginosa* count. These were the common microbiological approaches employed in assessing the microbial contamination of the water samples.

The term 'heterotrophic bacteria' includes all bacteria that use organic nutrients for growth, including those living in all types of water, food, soil, vegetation and air. Although clinical and epidemiological evidences of HPC bacteria in water posing a health risk is still insufficient, its presence remains a useful tool in the monitoring the effectiveness of water treatment processes and undesirable changes in the quality of water especially during storage and distribution (Allen et al., 2004).

The quality of treated water against microbial contamination is usually determined by the presence of certain bacterial indicators in the water. Among the main common indicators include *Escherichia coli*, faecal coliforms and the total coliforms. These indicators are used to assess the potential health risk of treated water. Hence, their presence or absence are important key elements considered in most drinking water quality guidelines (Annie et al., 2002). Faecal streptococci for example have been shown to outlive faecal coliforms in water samples. This thus, justify the inclusion of faecal streptococci counts in the guideline as its presence may provide a better picture of the water quality (Pinto et al., 1999). *Pseudomonas aeruginosa* is another bacterium that has pathogenic potential and has been associated with infections in immuno-compromised patients. Several reports have identified the presence of *P. aeruginosa* in water that passed through DUWS (Al-Hiyasat et al., 2007, Oliveira et al., 2008). The inclusion of *P. aeruginosa* counts is thus, essential to provide an insight into the purity of output water from DUWS against pathogenic microorganisms.

In this study, the standard for DUWS was compared to that used for drinking water. Although it is only occasionally that water is swallowed by a patient while receiving dental treatment, the fact that the mouth represents an opening to the whole body system warrants upmost precaution steps to be taken. According to the World Health Organisation (WHO), the optimum pH of water for drinking purpose is recommended in the range of pH 6.5-9.5 (WHO, 2006). In this study however, the pH of water that flow into the DCU as well as those coming out through the handpieces was found to be around pH 5.4-5.5 (Figure 4.1). Acidic water in DUWS varying in a range of pH 6.80-7.74 has also been reported by other researchers in Turkey (Tűretgen et al., 2009). However, despite the tendency that some

patients might swallow some water during dental treatment, health risks associated with short exposures to slightly acidic water during dental treatment have never been reported. Nevertheless, the point of concern here is that the pH of these output water is not potable and does not conform to a good general infection practice. Among the reasons suggested causing this acidity was the accumulation of excretions and metabolic end-products of microorganisms in the water. In this study, an exceedingly high microbial load at levels above that recommended by ADA was determined in both the input and output water of the DCUs under study (Figure 4.3).

Malaysia is a tropical country with temperature ranging from 21 °C to 32 °C throughout the year. Due to the warm weather, most dental clinics are air-conditioned to around 22-25 °C. This is thus shown by the temperature of the water samples which was recorded at around 23 °C, warmer than 18.8 °C which was often reported in DUWS (Tűretgen et al., 2009). In general, the temperature of DUWS water is relatively cool at around 20 °C. This temperature was however, reported as the normal growth temperature of environmental contaminants (Membre et al., 2005). A warmer environment such as that determined in this study would have facilitated the growth of these microbes. This may explain for the bloom of these contaminants as indicated by the large number of heterotrophic plate counts from the water samples under study.

Heterotrophic plate count employed in this study provides an indication as to the number of total microbial population in a DUWS and is a standard method used in the assessment of water quality. DUWS water of the dental clinic in this study was found to carry a microbial

load of 3.8-3.95 $\log_{10}$ cfu/mL (equivalent to 6300-8900 cfu/mL) that far exceed the level recommended by the ADA at ≤ 200 cfu/mL ($P < 0.001$) (Table 4.1). Similar cases have also been reported in other studies (Szymańska, 2007, Walker et al., 2000, Walker et al., 2004b). Problems associated with high microbial load in the waters of DUWS are also common in other developing countries such as Brazil and Turkey (Göksay et al., 2008, Souza-Gugelmin et al., 2003, Tűretgen et al., 2009). Walker et al., (2004a) have noted that up to 51 % of 237 of DUWS across seven European countries, including the United Kingdom, Ireland, Greece, Spain, Germany, Denmark and the Netherlands exceeded the level recommended by ADA. These findings confirm that a substantial proportion of DUWS have high levels of microbial contamination, irrespective of country, type of equipment and source of water. In other words, it can be generally suggested that it is difficult to exclude microbial contamination in output water from DUWS unless an effective water treatment system is employed and a strong compliance of dental personnel's to recommended regulations are met.

In this study, all the water samples showed some level of microbial contamination. It was determined that the means of heterotrophic plate count from the stored distilled water, air-water syringe, low speed handpiece and high speed handpiece were 3.92 $\log_{10}$ cfu/mL (8256 cfu/mL), 3.81 $\log_{10}$ cfu/mL (6469 cfu/mL), 3.92 $\log_{10}$ cfu/mL (8290 cfu/mL) and 3.92 $\log_{10}$ cfu/mL (8356 cfu/mL), respectively. It was a practice in the clinic under study that distilled water was collected and stored in a large 20 L tank for clinical usage. As the containers were seldom cleaned and water was left stagnant for a long period of time, it might contribute to the presence of microorganism in the water samples. Since the

incoming water into the DUWS in which case is the distilled water was highly contaminated itself, it is thus logical that the output water was also found to be highly contaminated. This finding is in agreement with several others reports (Szymańska, 2007, Tűretgen et al., 2009). However, incidences of highly contaminated output water despite receiving clean incoming water have also been reported (Pasquarella et al., 2010, Souza-Gugelmin et al., 2003).

Although the count of cells determined from heterotrophic plate represents an important indicator of water contamination, the value at times may underestimate the true microbial load to which a patient may be exposed to. This is because it has been demonstrated that only 3-4 % of the microscopically visible bacteria produces colonies on agar plates. Other bacteria may be either in temporarily non-culturable state or may represent the large fraction of the microflora from many natural habitats which remain as 'yet uncultured' (Barer and Harwood, 1999).

Contamination of DUWS water has always been attributed to the biofilm accumulated on the inner surface of the DUWS tubings. In this study however, biofilm may not be the sole agent for microbial contamination in DUWS water. Based on the heterotrophic counts of microorganisms in the sample of distilled water fed to the DCU (Section 4.1.3), the water was shown to be heavily contaminated. This high contamination could be probably due to poor hygiene maintenance by the dental personnel. The water reservoir should be regularly cleaned and sterilised and filled with only sterile distilled water at a temperature not exceeding 20 °C (Szymańska et al., 2004). The unprofessional practice of dental personnel

has also been reported in Italy. In general they did not abide to the principal procedures of infection control and their knowledge of disinfection mechanisms and procedures are not very comprehensive (Monarca et al., 2000).

Some dentists believe that certain types of DUWS may be less prone to microbial contamination than the rest. However, there were no significant differences between different DUWS systems, regardless of whether systems were main, bottle, or header tank fed or whether the water supplied to them was hard, soft, deionised, or distilled. Thus, no DUWS can be considered superior in microbiological terms to any other (Walker et al., 2000).

All water samples were shown negative in the total coliforms, faecal coliforms, *Escherichia coli*, faecal streptococci and *Pseudomonas aeruginosa* counts (Table 4.3). According to WHO, *Escherichia coli* and faecal coliforms bacteria must not be detectable in any 100 mL sample for drinking purpose (WHO, 2006). Total coliforms bacteria and faecal streptococci are not included in the drinking water quality guidelines. However, the constant absence of all total coliforms, faecal coliforms, *E. coli* and faecal streptococci in all water samples in this study suggested that the water was totally free of pathogenic bacteria and thus on this account, the possibility of the water used in the DUWS to cause infection among patients would be very unlikely. Furthermore, although *P. aeruginosa* has been isolated from DUWS in the previous literature, but no *P. aeruginosa* was found in this study (Al-Hiyasat et al., 2007, Oliveira et al., 2008). Therefore, the output water from DUWS in this study could be said as non-potable water (exceeded ADA recommendation) but still free of true

pathogenic and opportunistic pathogenic bacteria. Short exposure to this output water during dental treatment generally would not pose any significant health risk to patients as confirmed by no infection case has been reported in the clinic.

In Poland, Szymanska et al. (2004) assessed microbiologically the water contained in dental unit reservoirs as the water reservoir is the initial part of DUWS similar to the dental chair involved in this study. They found that up to 63.1 % of the water samples significantly exceeded the acceptable values of heterotrophic plate counts and coliforms bacteria. In contrast, no coliform was detected in this study.

Dentist and their assistants might have a slightly higher risk of exposure to the respiratory pathogens such as *Pseudomonas, Legionella* and non-tuberculosis *Mycobacterium*. Bennett et al. (2000) showed that there was always at least one peak of aerosol concentration with much higher number of bacteria in dental treatment rooms. The peak concentrations were often associated with mechanical scaling procedures (47 % of procedures giving rise to a peak) and to a lesser extent by cavity preparations (11 %). Pasquarella et al. (2010) demonstrated that microbial air contamination showed the highest levels during dental treatments and tended to decrease at the end of the working activity ($P < 0.05$). J. Szymańska and Dutkiewicz (2008) also reported similar result of high concentration of bacteria in the air of dental operation area.

5.2 IDENTIFICATION OF BACTERIAL ISOLATES

There were a total of four different types of isolates recovered from the water samples. Their identification was determined by comparing their 16S rRNA gene sequence with the homologous sequence in the public nucleotide database. 16S rDNA gene sequencing is used today in clinical laboratories for routine identifications, especially for slow-growing, unusual or fastidious bacteria, but also for bacteria that are poorly differentiated by conventional biochemical methods (Mignard and Flandrois, 2006).

In this study, the isolates recovered were *Sphingomonas rhizogenes, Sphingomonas dokdonensis, Sphingomonas mucosissima*, and *Methylobacterium radiotolerans*. As shown in Table 4.6, up to 98.5 % of bacteria isolated from water samples were *Sphingomonas* spp. and only 1.5 % were *Methylobacterium* spp.

The genus *Sphingomonas* is referred to a group of Gram-negative, rod-shaped, chemoheterotrophic, strictly aerobic bacteria that possess ubiquinone 10 as the major respiratory quinine, contain glycosphingolipids instead of lipopolysaccharide in their cell envelopes (Takeuchi et al., 2001). These sphingomonads are widely distributed in nature, having been isolated from many different aqueous and terrestrial habitats, as well as from plant root systems, clinical specimens, and other sources. In general, most sphingomonads are not clinically important except *Sphingomonas paucimobilis* can cause a range of mostly nosocomial infections. Perola et al. (2002) demonstrated that the *S. paucimobilis* present in

a leukaemic patient was directly linked to bacterial colonisation of the hospital water system. Similarly, *S. paucimobilis* also has been isolated from DUWS in the previous literature (Meiller et al., 1999, O'Donnell et al., 2006).

Another bacterium that contributed to the high microbial load of the water samples was identified as Gram-negative, strictly aerobic, rod-shaped bacteria belonging to the genus *Methylobacterium*. This group of bacteria can grow on one-carbon compounds more reduced than carbon dioxide as sole carbon and energy sources. The colonies are usually pink to red because the cells are pigmented with carotenoids (Hiraishi et al., 1995). *Methylobacterium mesophilicum* and *Methylobacterium zatmanii* were the two most commonly reported species isolated in clinical samples. In the hospital environment, tap water has been implicated as a possible mode of transmission for these bacteria (Rice et al., 2000). In addition to the sphingomonads, the occurrence of *Methylobacterium* spp. in potable water and DUWS has also been reported in previous literature (Barbeau et al., 1996, Reasoner et al., 1989, Wirthlin et al., 2003). Due to its common presence, the level of these bacteria was suggested to be monitored in hospital water distribution system especially for the immune-compromised patients (Hornei et al., 1999).

5.3 THE ANTIBIOFILM PROPERTIES OF SILVER-COATED TUBING

Over decades, many approaches have been suggested to reduce the microbial load in the output water and thus minimise the risk of acquisition of infectious disease among dental personnel as well as patients. To date, chemical water treatment is the most widely approach used in the general dental practices to remove biofilm formed at the interior side of tubing (Walker and Marsh, 2007). The biofilm is often recognised as a reservoir of bacteria and thus makes the output water heavily contaminated. However, Meiller et al. (1999) cautioned that the chemical agents eliminated the planktonic bacteria but did little to destroy the biofilm matrix and thus recurrence of bacteria occurred after treatment. Chemical agents might potentially be trapped in the matrix and represent an additional risk to the patient. Intensive use of chemical agents can also create resistant bacteria and thus results in failure of water treatment (Liaqat and Sabri, 2009). In addition, Sherman et al. (1997) also cautioned that the chemical agents might be corrosive to the metal components in a dental chair and thus resulted in the increase concentrations of metal ions in the output water.

A simple but novel approach to inhibit biofilm formation at the interior side of tubing is the use of tubing material with antimicrobial effect. This study was carried out to develop an *in vitro* biofilm model and to assess the inhibitory effect of silver-coated tubing. The biofilm model was designed to mimic the working environment of a dental clinic. According to the review written by J.T. Walker and Marsh (2004), the flow rates of DUWS are typically 60-

100 mL/min and dispense very small amounts of fluid (~5 mL) per patient. Assuming there were 30 patients every day, the biofilm model dispersed 150 mL of fluid per day, stagnant overnight (16 h) and similar dispersion and stagnation was repeated everyday over seven days. The advantage of model is that it allows the experiment to be monitored without disturbing clinical schedule. This model also allows the tubings to be cut for SEM examination which could not be done when using a real dental chair. However, despite all these advantages, the absence of handpieces in this model was a disadvantage as the effect of retraction which are often reported to contribute DUWS contamination could not be analysed.

Figure 4.16 and 4.17 showed the progressive increase of bacterial adherence to the walls of conventional polyurethane tubing and silver-coated tubing. At day 0 (before model was run), both types of tubings showed completely no attachment of bacteria to the walls. However, as the model run, the number of bacteria attached to the walls of both types of tubing was increased. As shown in Table 4.5, the difference in number of adhering bacteria to both types of tubings was not significant ($P > 0.05$). Similarly, the number of bacteria collected from the water samples dispersed from both types of tubings was also similar ($P > 0.05$). In other words, the silver-coated tubing did not exert its antimicrobial effect and thus failed to inhibit the biofilm formation and reduced microbial load in the dispersed water samples.

The manufacturer of silver-coated tubing claimed that the antimicrobial properties of silver-coated tubing is based on the silver ions which is contained within a zirconium phosphate complex that acted as an ion exchange reservoir and to ensure a controlled release at the surface. The reason behind the failure of silver-coated tubing to inhibit biofilm formation remained unclear. Further research is essential to fully understand the interaction mechanism between the bacteria and the silver-coated tubing.

In medical settings, catheter-associated urinary tract infections (CAUTIs) are commonly acquired by patients which needs prolonged hospitalisation, which substantially increase hospital cost and promote the emergence of resistant organisms (Davenport and Keeley, 2005). Silver-coated catheter is currently recognised as one of the methods used in an attempt to decrease the incidence of CAUTIs. Rupp et al. (2004) suggested the introduction of a silver alloy, hydrogel-coated urinary catheter was associated with a significant decline in nosocomial urinary tract infections (UTIs) and cost savings over the range of cost estimates. However, other study claimed that there was not enough evidence to conclude that catheters coated with silver salts and hydrogel give greater protection than classical catheters (Thibon et al., 2000). In a large randomised clinical trial, silver oxide-coated urinary catheter showed a significantly increased incidence of bacteriuria in male patients and a significantly increased occurrence of staphylococcal bacteriuria (Riley et al., 1995). These contrasting findings of the efficacy of silver-coated catheter suggested the need for caution and for similar large-scale trials before silver-containing compounds are widely used for preventing device associated infections.

CHAPTER SIX

CONCLUSION

6.1 CONCLUSION

The microbial load in the output water from DUWS in the dental teaching clinic under study was high and failed to meet the recommendation by American Dental Association (ADA). *Sphingomonas dokdonensis* was the dominant species among the contaminants. An effective approach in the control of water quality is necessary in the effort to achieve better quality water for use in DUWS. Such measure is important to eliminate potential cross-infection among dental personnel and patients in the dental clinic. Silver-coated tubing was found to be ineffective in preventing biofilm formation at the inner surface of tubing. Future research is warranted to discover a novel method to remove biofilm formation at the wall of tubing and thus able to deliver water with better water quality.

6.2 FUTURE RECOMMENDATIONS

Some future research should be carried out as the followings:

i. Conduct a microbiological survey in a larger scale on DUWS collaborating with other institutions, agencies and clinics in Malaysia
ii. Examine and characterise the DUWS biofilm community in order to have a deeper understanding on the development of biofilm
iii. Discover a novel dental material in replacement with the conventional polyurethane tubing that is able to inhibit biofilm formation at the inner surface of tubing
iv. Conduct an epidemiological studies on DUWS in order to assess the risk of acquisition of infectious disease among dental personnel and patients

CHAPTER SEVEN

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APPENDIX

Appendix 1: List of Equipments

Autoclave machine	Hirayama HVE 50, Japan
Hand-held pH meter	Thermoline, Australia
Hot plate stirrer	Cimarec, Australia
Incubator	Memmert
Laminar flow cabinat	ERLA/CFM 4, Malaysia
Light microscope	Olympus BX61, Japan
Oven	Binder FD53, United States
Peristaltic pump	Cole-Parmer MasterFlex, United States
Refrigerated microcentrifuge	Eppendorf, Germany
Refrigerator	Hitachi, Japan
Scanning electron microscope	Quanta FEG 205, Holland
Spectrophotometer	Shimadzu UV-1700, Japan
Thermo printer	Mitsubishi Electric, Japan
Vacuum pump	Harmony VCP 8101, Malaysia
Vortex mixer	Stuart, United Kingdom
Water deioniser	Elga Purelab Classic UVF, France
Water distiller	J. Bibby Ment, Malaysia
Weighting balance	OHAUS, United States

Appendix 2: Preparation of Media, Diluent and Appratus

1. Brain heart infusion agar (BBL)

Approximate formula per litre

Brain heart, infusion from (solids)...	8.0 g	Peptic digest of animal tissue……	5.0 g
Pancreatic digest of casein……...	16.0 g	Dextrose…….	2.0 g
Sodium chloride……...	5.0 g	Disodium phosphate……..	2.5 g
Agar…….	13.5 g		

52 g of brain heart infusion agar powder was added to 1 L of deionised water and thoroughly mixed. The mixture was boiled for 1 min with frequent agitation. The mixture was then autoclaved at 121 °C for 15 min. The finished product was light to medium amber, slightly opalescent with a flocculent precipitate and was in pH 7.4 ± 0.2. The sterile agar mixture was then dispensed into sterile petri dishes (100 x 15 mm) and left to be solidified. The agar plates were used immediately one day after preparation or stored in sealed plastic bags and kept at 4 °C for 2 weeks of maximum holding time.

2. Brilliant green lactose bile broth 2 % (Difco)

Approximate formula per litre

Peptone……....	10.0 g	Lactose……...	10.0 g
Oxgall……...	20.0 g	Brilliant green……	13.3 mg

40.0 g of brilliant green lactose bile broth 2 % powder was added to 1 L of deionised water and thoroughly mixed. The mixture was warmed slightly in order to completely dissolve the powder. 10 mL of media was dispensed into tubes containing inverted fermentation

vials. The trapped gas inside each fermentation vial was emptied by inverting the test tubes back and forth several times. The tubes were then autoclaved at 121 °C for 15 min and cooled down as quickly as possible after sterilization. The broth was emerald green and clear in appearance as well as was in pH 7.2 ± 0.2. The test tubes were used immediately one day after preparation or stored and kept at 4 °C for 2 weeks of maximum holding time.

3. m Endo Agar LES (Difco)

Approximate formula per litre

Yeast extract…….	1.2 g	Casitone…….	3.7 g
Thiopeptone……..	3.7 g	Tryptose…….	7.5 g
Lactose……..	9.4 g	Dipotassium phosphate……..	3.3 g
Monopotassium phosphate……...	1.0 g	Sodium chloride……	3.7 g
Sodium desoxycholate……..	0.1 g	Sodium lauryl sulphate……..	0.05 g
Sodium sulphite……	1.6 g	Basic fuchsin…….	0.8 g
Agar……..	15.0 g		

51 g of m Endo Agar LES powder was added to 1 L of sterile deionised water containing 20 mL of 95 % ethanol and thoroughly mixed. The mixture was then boiled for 1 min with frequent agitation aseptically. The mixture could not be sterilized by autoclaving. The media was rose coloured and slightly opalescent with the presence of precipitate at pH 7.2 ± 0.2. The media was dispensed into petri dishes (50 x 12 mm). The agar plates were used immediately one day after preparation or stored in the dark and kept at 4 °C in sealed plastic bags. Unused agar plates were discarded after 2 weeks or sooner if there was

evidence of moisture loss, medium contamination, or medium deterioration (darkening of the medium).

4. m Enterococcus Agar (Difco)

Approximate formula per litre

Tryptose……	20.0 g	Yeast extract……	5.0 g
Dextrose……	2.0 g	Dipotassium phosphate……	4.0 g
Sodium azide……	0.4 g	Agar……..	10.0 g
2,3,5-Triphenyl tetrazolium chloride	0.1 g		

42 g of m Enterococcus agar powder was added to 1 L of sterile deionised water and thoroughly mixed. The mixture was boiled for 1 min with frequent agitation aseptically. The mixture could not be sterilized by autoclaving. The finished product was light amber and slightly opalescent with pH 7.2 ± 0.2. The media was dispensed into petri dishes (50 x 9 mm). The agar plates were used immediately one day after preparation or stored in the dark and kept at 4 °C in sealed plastic bags for 2 weeks of maximum holding time.

5. m FC Agar (Difco)

Approximate formula per litre

Tryptose……	10.0 g	Proteose peptone No. 3……	5.0 g
Yeast extract…….	3.0 g	Lactose…….	12.5 g
Bile salt No. 3……...	1.5 g	Sodium chloride……..	5.0 g
Aniline blue……..	0.1 g	Agar…….	15.0 g

52 g of m FC agar powder was added to 1 L of sterile deionised water and thoroughly mixed. The mixture was boiled for 1 min with frequent agitation. 10 mL of 1 % solution of rosolic acid (Sigma) in 0.2 M sodium hydroxide (NaOH) which has been filtered with 47 mm membrane filter with pore size of 0.45 µm was then aseptically added into the mixture and heating was continued for another 1 min. The mixture could not be sterilized by autoclaving. If necessary, the finished product was adjusted to pH 7.4 with 1 N hydrochloric acid (HCl). The finished product was cranberry red and slightly opalescent. The media was then dispensed into petri dishes (50 x 12 mm). The agar plates were used immediately one day after preparation or stored and kept at 4 °C in sealed plastic bags for 2 weeks of maximum holding time.

6. Lauryl tryptose broth (Difco)

Approximate formula per litre

Tryptose……	20.0 g	Lactose……..	5.0 g
Dipotassium phosphate……	2.75 g	Monopotassium phosphate……	2.75 g
Sodium chloride……...	5.0 g	Sodium lauryl sulphate……..	0.1 g

35.6 g of lauryl tryptose broth powder was added to 1 L of deionised water and thoroughly mixed. The mixture was warmed slightly in order to completely dissolve the powder. 10 mL of mixture was dispensed into test tubes containing inverted fermentation vials. The trapped gas inside each fermentation vial was emptied by inverting the tubes back and forth several times. The tubes were then autoclaved at 121 °C for 15 min and quickly cooled down after sterilization. The broth was light to medium amber and clear to very slightly

opalescent in appearance with pH 7.2 ± 0.2. The test tubes were used immediately one day after preparation or stored and kept at 4 °C for 2 weeks of maximum holding time.

7. Nutrient agar with MUG (Difco)

Approximate formula per litre

Beef extract……..	3.0 g	Peptone……..	5.0 g
Agar……..	15.0 g	MUG (4-Methylumbelliferyl-β-D-glucoronide)……..	0.1 g

23.1 g of nutrient agar with MUG powder was added to 1 L of deionised water and thoroughly mixed. The mixture was then boiled for 1 min with frequent agitation. The finished product was autoclaved at 121 °C for 15 min. The finished product was light amber and clear to slight opalescent in appearance with pH 6.8 ± 0.2. The media was then dispensed into petri dishes (50 x 12 mm). The agar plates were used immediately one day after preparation or stored in sealed plastic bags and kept at 4 °C for 2 weeks of maximum holding time.

8. *M*-PA-C Agar (BBL)

Approximate formula per litre

Yeast extract…….	2.0 g	L-Lysine HCl…….	5.0 g
Sodium chloride……..	5.0 g	Xylose……	1.25 g
Sucrose…….	1.25 g	Lactose……..	3.3 g
Phenol red…….	0.08 g	Ferric ammonium citrate……...	0.8 g

Sodium thiosulphate…….	5.0 g	Magnesium sulphate…….	1.5 g
Kanamycin……	8.0 mg	Nalidixic acid……	37.0 mg
Agar……..	12.0 g		

35 g of *M*-PA-C agar powder was added to 1 L of sterile deionised water and thoroughly mixed. The mixture was boiled for 1 min with frequent agitation aseptically. The mixture could not be sterilized by autoclaving. The finished product was orange-red to rose red and clear to slightly hazy with pH 7.2 ± 0.1. The media was dispensed into petri dishes (50 x 12 mm). The agar plates were used immediately one day after preparation or stored in sealed plastic bags and kept at 4 °C for 2 weeks of maximum holding time.

9. R2A Agar (Difco)

Approximate formula per litre

Yeast extract…….	0.5 g	Proteose peptone No. 3……..	0.5 g
Casamino acids…….	0.5 g	Dextrose…….	0.5 g
Soluble starch……...	0.5 g	Sodium pyruvate……	0.3 g
Dipotassium phosphate…….	0.3 g	Magnesium sulphate……..	0.05 g
Agar……..	15.0 g		

18.2 g of R2A agar powder was added to 1 L of deionised water and thoroughly mixed. The mixture was then boiled for 1 min with frequent agitation. The mixture was then autoclaved at 121 °C for 15 min. The finished product was light amber and slightly opalescent with a slight precipitate with pH 7.2 ± 0.2. The media was then dispensed into petri dishes (100 x 15 mm). The agar plates were used immediately one day after preparation or stored in sealed plastic bags and kept at 4 °C for 2 weeks of maximum holding time.

10. Buffered water

Approximate formula per litre

Monopotassium phosphate……. 42.5 mg Magnesium chloride……. 405.5 mg

34.0 g of monopotassium phosphate (KH_2PO_4) was dissolved in 500 mL deionised water and the pH was adjusted to pH 7.2 ± 0.5 with 1 M sodium hydroxide (NaOH). The mixture was then diluted to 1 litre with purified deionised water to make a stock of phosphate buffered solution (34.0 g/L)(PBS). 81.1 g of magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) was dissolved in 500 mL purified deionised water and then diluted to 1 litre (81.1 g/L) to make stock magnesium chloride solution. Buffered water was prepared by adding 1.25 mL of PBS and 5 mL of stock magnesium chloride solution and further diluted to 1 litre with deionised water. The buffered water was dispensed in 9 mL volume into universal bottles and autoclaved at 121 °C for 15 min.

11. Sodium thiosulphate solution (3 %)

Approximate formula per litre

Sodium thiosulphate……. 30.0 g

30 g of sodium thiosulphate ($Na_2S_2O_3$) was dissolved in 500 mL of deionised water and then diluted to 1 litre (30 g/L). Sodium thiosulphate solution was autoclaved at 121 °C for 15 min. Sodium thiosulphate solution was used immediately one day after preparation or stored at 4 °C.

12. Filtration Apparatus

Filtration apparatus which consists of a glass funnel, a filter base and a 1000 L vacuum beaker was washed thoroughly with Decon 90 and rinsed with deionised water. Each of the components was covered with a sheet of aluminium foil and then autoclaved at 121 °C for 15 min. The sterile filtration apparatus was used immediately one day after preparation.

13. Sampling Bottles

All universal bottles and polypropylene bottles (250 mL and 500 mL) were washed thoroughly with Decon 90 and rinsed with deionised water. The caps of bottles were covered with a sheet of aluminium foil and bottles were then autoclaved at 121 °C for 15 min. The sterile bottles were used immediately one day after preparation.

Appendix 3: Quantitation of Bacterial Cells

The number of bacterial cells could be estimated based on its optical density. A series of *Escherichia coli* suspension was determined for their optical density at 550 nm using a spectrophotometer. The *E. coli* suspension with known optical density was then plated on to R2A agar and incubated overnight at 28 °C. The number of colonies formed in each plate was then recorded. A standard curve was drawn with microbial density (cfu/mL) against its corresponding optical density. The number of bacterial cells could be estimated by using the linear equation generated by the standard curve.

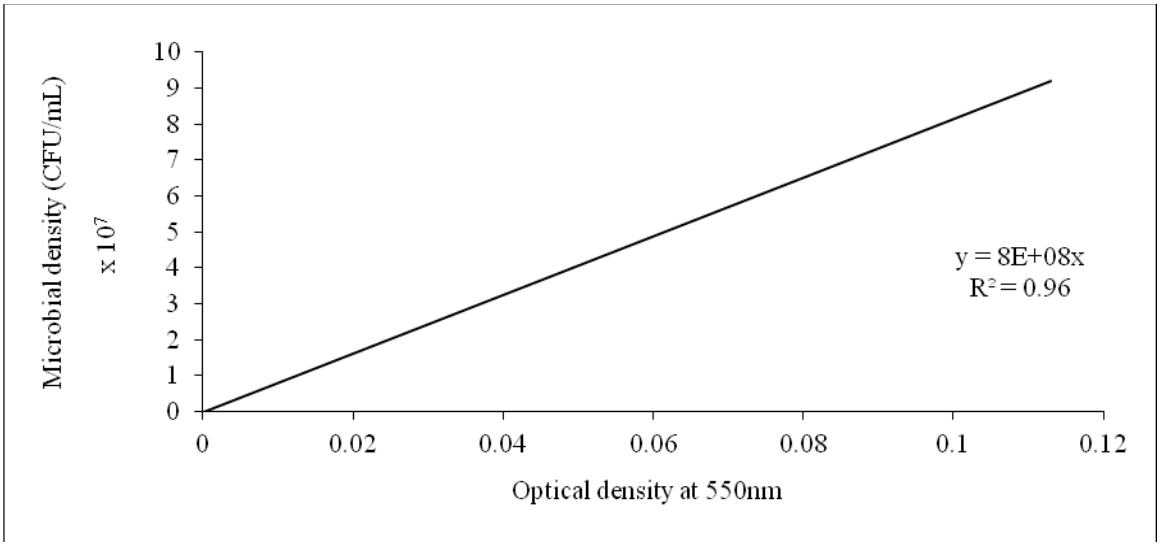

Figure: A standard curve drawn with microbial density against its corresponding optical density.

Appendix 4: Dental Unit Water Lines, Biofilm and Water Quality

A. General recommendations

1. Use water that meets United States Environmental Protection Agency (US EPA) regulatory standards for drinking water (i.e. ≤ 500 cfu/mL of heterotrophic water bacteria) for routine dental treatment output water.
2. Consult with the dental unit manufacturer for appropriate methods and equipment to maintain the recommended quality of dental water.
3. Follow recommendations for monitoring water quality provided by the manufacturer of the unit or waterline treatment product.
4. Discharge water and air for a minimum of 20 – 30 secs after each patient, from any device connected to the dental water system that enters the patient's mouth (e.g. handpieces, ultrasonic scalers and air/water syringes).
5. Consult with the dental unit manufacturer on the need for periodic maintenance of antiretraction mechanisms.

B. Boil – water advisories

1. The following apply while a boil-water advisory is in effect:
    a. Do not deliver water from the public water system to the patient through the dental operative unit, ultrasonic scaler or other dental equipment that uses the public water system.
    b. Do not use water from the public water system for dental treatment, patient rinsing or handwashing.

c. For handwashing, use antimicrobial – containing products that do not require water for use (e.g. alcohol – based handrubs). If hands are visibly contaminated, use bottled water, if available, and soap for handwashing or an antiseptic towel.

2. The following apply when the boil – water advisory is cancelled:
   a. Follow guidance given by the local water utility on adequate flushing of waterlines. If no guidance is provided, flush dental water lines and faucets for 1 – 5 min before using for patient care.
   b. Disinfect dental water lines as recommended by the dental unit manufacturer.

Special Considerations

A. Dental handpieces and other devices attached to air and waterlines
   1. Clean and heat sterilise handpieces and other intraoral instruments that can be removed from the air and waterlines of dental units between patients.
   2. Follow the manufacturer's instructions for cleaning, lubrication and sterilisation of handpieces and other intraoral instruments that can be removed from the air and waterlines of dental units.
   3. Do not surface – disinfect or use liquid chemical sterilants or ethylene oxide on handpieces and other intraoral instruments that can be removed from the air and waterlines of dental units.
   4. Do not advise patients to close their lips tightly around the tip of the saliva ejector to evacuate oral fluids.