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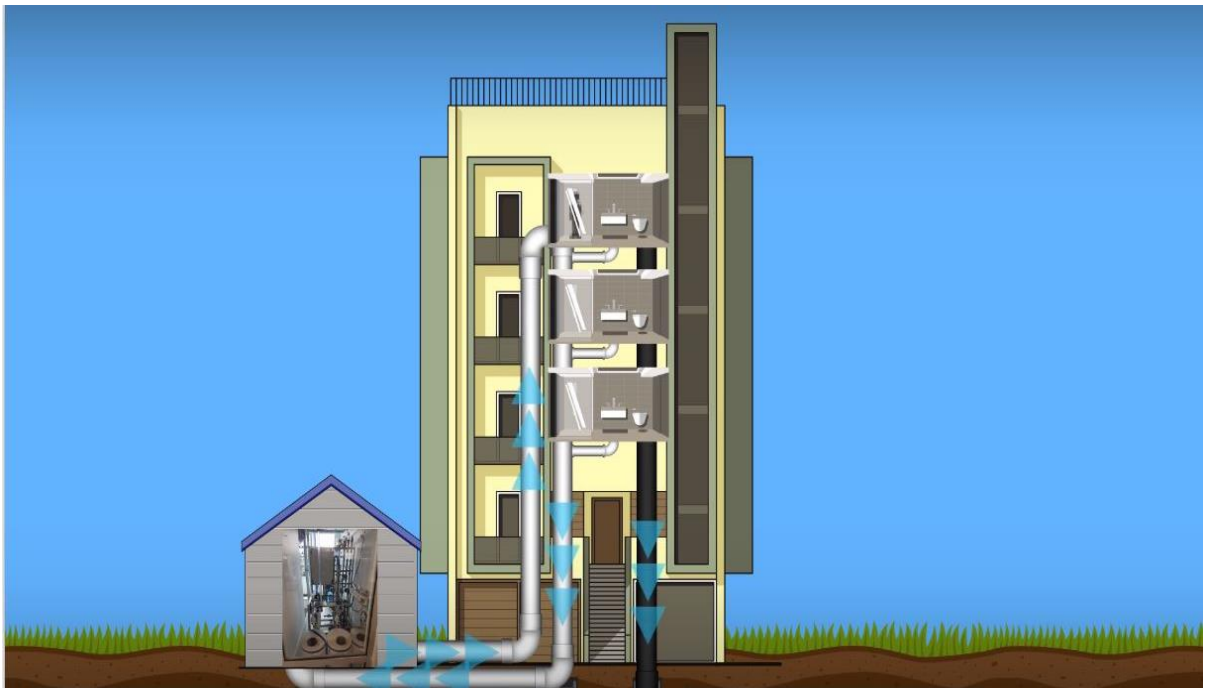

Biofilm formation and microbial quality monitoring in a decentralized greywater collection and treatment system

Master's thesis in Infrastructure and Environmental Engineering

Khamees Zoghbor

Supervisor: Jesper Knutsson (Chalmers University of Technology)

Department of Architecture and Civil Engineering

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MASTER'S THESIS ACEX030

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Supervisor: Jesper Knutsson, *Researcher, Architecture and Civil Engineering, Water Environment Technology*.

Examiner: Sebastien Rauch, *Professor, Architecture and Civil Engineering, Water Environment Technology*.

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UNIVERSITY OF TECHNOLOGY

Department of Architecture and Civil Engineering
Division of Water Environment Technology
Chalmers University of Technology
SE-412 96 Gothenburg

Abstract

With the emerging of water scarcity problems resulted from increasing population and urbanization, reutilized greywater is considered one of the most promising alternative sources that contributes to alleviate stress on municipal drinking water supplies. An onsite decentralized greywater reuse system was installed in a living lab in Gothenburg, Sweden (HSB living lab, HLL) collecting greywater from bathroom sinks and showers in six shared bathrooms. The produced greywater pass through four treatment processes including ultrafiltration, activated carbon filter, ion exchange filter and advanced oxidation processes which produce high quality water intended to be reused for showering as hot water in two shared bathrooms.

Although the available greywater treatment processes remove the majority of microorganisms and organic materials in raw greywater, the produced water is still not sterile and low level of microorganisms may survive and persist when entering the storage tanks. 90% of the total microorganisms in water can be found in biofilms while only 5% can be found as floating planktonic cells in water. The objective of this thesis is to monitor biofilm formation and microbial quality in storage tanks for four weeks under different conditions to ensure the microbial stability of produced water in the tanks in order to not posing any health risks to the users. Therefore, a sampling campaign was conducted to monitor the water microbial quality parameters such as indicator bacteria (*total coliforms*, *E. coli*), *L. Pneumophila*, biofilm formation as well as the water characteristics such as physical parameter, organic and inorganic nutrients concentrations.

The results shows that there is no microbial quality deterioration observed throughout the study under different conditions (different temperatures, different surface materials, water stagnation) since no *L. Pneumophila*, *coliforms* and *E. coli* growth or biofilm formed throughout the study. The results of this study should be viewed as a start point for a long-term monitoring to ensure continuous providing of safe water for showering.

Keywords: water reuse, greywater reuse, decentralized greywater systems, greywater microbial quality, L. pneumophila .

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Finally, I dedicate this work to my lovely family and my friends. My mom, the person who counts most to me and my father, the leader of my life, who have supported me throughout my life. I will always appreciate your presence. A special thanks to my best friend Sarah Bellalouna for her unconditional love and support throughout my last year tough times.

Best regards

Khamees Zoghbor

إهداء

إلى أمي وأبي .. بسببكم ولأجلكم أنا هنا, أحبكم .

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Abbreviations

BOD - Biochemical oxygen demand

BOM - Biodegradable organic matter

COD - Chemical oxygen demand

CLSM - Confocal laser scanning microscopy

EPS - Extracellular polymeric substances

ESEM - Environmental scanning electron microscopy

FISH - Fluorescence in situ hybridization.

HPC - Heterotrophic plate count

HSB LL - HSB Living Lab

ID - infectious dose

LD - Legionnaires' disease

LD -lethal dose

MF - Microfiltration

NF - Nanofiltration

NOM - Natural organic matter

QMRA- Quantitative microbial risk assessment

RO- Reverse osmosis

TN - Total nitrogen

TOC - Total organic carbon

TP - Total phosphorus

TSS - Total suspended solids

UF - Ultrafiltration

USEPA - United States Environmental protection agency

UV - Ultraviolet

WDS – Water distribution system

WWTP - Wastewater treatment plants

XOCs - Xenobiotic organic compound

1 Introduction

Water is a limited natural resource which is considered highly important to human health, food security, and the environment. Urban water demand has been continuously increasing around the world due to many reasons: urbanization, growing global population and increasing specific water demand (Friedler & Hadari, 2006). As a result of this increasing demand, water scarcity has become one of the most pressing problems of today. Today, around 1.42 billion people live in regions affected by high water vulnerability (UNICEF, n.d.). To solve this problem, many countries are seeking water sources alternatives which contribute to water conservation to enhance water security, sustainability, and resilience (National Research Council, 2012). Examples of new water sources are seawater desalination, construction of new dams, and utilization of more distant water (surface water) and deeper (groundwater) sources (Friedler et al., 2005).

Due to these new sources' high cost and expected adverse environmental impacts (Friedler et al., 2005), a revision of the current water cycle is necessary to promote sources' efficient utilization. A combination of different measures such as increasing the efficiency of the water supply system by lowering water losses, increasing the public awareness, and using water savings appliances can be employed to reduce water demand (Friedler & Hadari, 2006). One of the promising alternative options which play an increasingly significant role for augmenting fresh water supplies is water reuse. Water reuse refers to the reclamation of water from different sources such as municipal wastewater, stormwater, industry process and agriculture runoff to be treated and reused for different purposes such as agricultures, potable water supplies, groundwater recharge, and environmental restoration (National Research Council, 2012). For instance, decentralized wastewater treatment systems are commonly installed in suburban areas where people reuse their effluents for non-potable applications such as garden irrigation (Abed et al., 2020).

Greywater recycling has been seen as a promising approach in terms of supporting the sustainability of freshwater resources. Grey water is defined as the urban wastewater generated from domestic activities such as baths, showers, handbasins, and washing machines but streams from toilets are excluded (Li et al., 2009, Ottosson, 2003). These streams are typically moderately contaminated and have a potential to be reused after suitable treatment (Li et al., 2009). Wastewater from Kitchen and dishwashers are considered greywater according to some definitions, while in others considered as black water due to its relatively high load of contaminants (Al-Gheethi et al., 2015). Separation of wastewater from sources results in greywater that contain low fecal contamination and eutrophying substances which simplifying the on-site treatment (Ottosson, 2003).

Furthermore, Greywater production is not seasonal - dependent in contrast to other good quality water sources such as rainfall (Leong et al., 2018). As shown in *table1*, around 60%-70% of the domestic water consumption is transformed into greywater with a discharge ranging between 60 and 120 Liter/capita/day depending on the lifestyle, living standards, population structures (age, gender), customs and habits, water installation and the degree of water abundance (Friedler& Hadari, 2006; Li et al., 2009). In Sweden, greywater average flow was decreased from 150 L person^{-1} day^{-1} in 1995 to 100 L person^{-1} day^{-1} because of the change in

habits and water saving equipment and practices (Ottosson, 2003). However, domestic grey water reuse plans are yet undeveloped in Europe and full-scale systems are not common as well as not tested for long time periods (Eriksson et al., 2009; Friedler,2004).

Table 1 showing approximate percentage of wastewater composition/household (adapted from WHO,2006).

Wastewater type	Total wastewater		Total greywater	
	Total (%)	(L/day)	Total (%)	(L/day)
Toilet	32	186	-	-
Hand basins	5	28	8	28
Baths/shower	33	193	54	193
Kitchen	7	44	-	-
Laundry	23	135	38	135
Total	100	586	100	356

1.1 The project background

This study is based on an on-site greywater reuse system that was installed in a living lab in Gothenburg (HSB Living Lab (HSB LL)) which is described in further detail elsewhere (Knutsson & Marx, 2016). The raw greywater generated from bathroom sinks and showers in six shared bathrooms in HSB Living Lab are collected in separate pipes and subsequentially passing through four treatment barriers to produce high quality water to be reused as hot water for shower purposes in two shared bathrooms.

The current greywater conveyance system is divided into four parts: (1) The raw greywater from six showers and sinks is collected in separate pipes to be discharged to a collection tank. (2) The raw greywater is then pumped to the treatment system. (3) The effluent of the treatment processes is conveyed to a clean storage tank. (4) Treated greywater from the clean storage tank is intended to be returned as hot water for showering.

1.2 Problem description

Although the available greywater treatment processes remove the majority of microorganisms and organic matters found in raw greywater, the resulting water is still not sterile and low level of microorganisms may survive and persist when entering the distribution system. Additionally, the contamination of water contained in tanks can occur through poor handling, unhygienic domestic water handling practices, and ambient contamination from the environment (Peter & Routledge, 2018). As a result, the persisting microorganisms in the water tanks can proliferate under different favorable conditions. These microorganisms can be found in water tanks either suspended in the water or attached to tanks' surfaces. Bacteria tend to form biofilms which provide a habitat and favorable conditions for microbial growth. Over time, this potentially leads to the release of pathogens to the water and pose health risks to the users (Abdel-Nour et al., 2013). Such contaminants make the water not appropriate for the intended reuse scenarios.

In HSB LL, greywater clean tank which collects treated greywater after the treatment process represent the point of entry of water into heating system to be later resupplied for showering purposes. Therefore, bacterial growth and biofilm formation in the clean tank should be evaluated carefully by implementing monitoring programs in order to protect the users from

any harmful impacts resulted from exposure to pathogenic bacteria. *L. Pneumophila* occurrence in domestic water is a cause of concern because of its transmission pathway which occurs via inhalation of contaminated water droplets generated by showering and faucets as well as because of its tolerance to high temperature. *L. Pneumophila* favor to grow in a temperature between 18 and 45 °C, which is typically encountered in the greywater treatment system being examined in the present project. The extent of monitoring procedure is dependent on the objectives of greywater and the severity of the failure impacts (National Research Council, 2012). Therefore, in HSB LL recycling system where treated greywater intended to be resupplied for showering requires rigorous quality assurance in order to not posing any health risks to the users.

1.3 Aim and goal of the thesis

The aim of this study is to evaluate the degree to which treated greywater in clean tanks can potentially support bacterial regrowth (*Coliforms, E. coli* and *L. Pneumophila*) and biofilm formation during four weeks under controlled conditions to ensure that it fulfills the available standards for showering reuse.

1.4 Research questions

1. Does any microbial water quality deterioration (*Coliforms, E. coli, L. Pneumophila* growth, and biofilm formation) occur in the clean tank throughout the study under different conditions?
2. Do the water characteristics in the clean tanks promote the bacterial growth (*Coliforms, E. coli, L. Pneumophila growth*) and biofilm formation?
3. Does the HSB treated greywater quality in the clean tank fulfill drinking water quality standards (LIVSFS 2003:45) throughout the study?
4. Is the current treatment process considered sufficient to prevent bacterial regrowth and biofilm formation in the clean storage tank?

1.5 Limitations

Several limitations were faced throughout the study that might have an impact on the results. Time limitations is the first obstacle since the quality monitoring procedure should be performed for a long period. Furthermore, more tests are needed to be done in the microbial monitoring programs to cover different aspects of the topic and to obtain more precise results. Examples of these tests are AOC test, Molecular biological identification methods. This could not be done due to limited time and budget. These limitations can be considered as recommendations for any future works.

2 Background

2.1 Greywater reuse significance

The driving forces for greywater reuse are diverse throughout the world. Firstly, the on-site greywater reuse for non-potable applications results in a reduction in the overall potable water consumption which, in turn leads to more sustainable urban water utilization (Winward et al., 2008). For example, 51.8% of the toilet flushing consumption was achieved by reusing bathroom greywater in HSB LL (Knutsson & Knutsson, 2021). Secondly, economic, and environmental benefits are the other driving incentives for greywater reuse. As a result of increasing water demand due to growing urbanization coupled with the increasing specific water consumption, investments in developing new water sources such as seawater desalination, and the construction of new dams are required (Friedler & Hadari, 2006). Not only the cost of such new water sources (construction, operation, and maintenance) which in many cases is higher than the conventional sources costs, but adverse environmental impacts are also expected (Winward et al., 2008; Friedler et al., 2005). For instance, increased CO_2 and other pollutant emissions are expected from seawater desalination process which in turn causes disturbance to the adjacent marine environment (Friedler et al., 2005). Therefore, promoting greywater recycling can avoid or delay the new investments costs and the expected negative environmental impacts. Furthermore, the onsite greywater recycling lowers the load of wastewater discharged to the treatment plants leading to minimizing the total cost of wastewater handling (Eriksson et al., 2002). According to Friedler & Hadari (2006), the on-site greywater recycling for toilet flushing can be economically effective not only to state water authority but also to the users as well, depending on the used treatment technologies' costs, the size of served users, and the water price. Finally, the on-site greywater reuse reduces the quantity and the quality of wastewater discharged to the sewage disposal system leading to reduce the stress on the infrastructure, prolong the systems life, save energy (for pumping sweage) and delay capital expenditures required for upgrading and enlargement (WHO, 2006). Furthermore, it is expected that the wastewater discharged to wastewater treatment plants (WWTPs) have a lower load of biodegradable pollutants when excluding streams of greywater which in turn, leading to reduce the consumption of energy and chemicals in WWTPs (Friedler & Penn, 2011).

2.2 Greywater reuse scenarios: advantages and challenges

The reuse of greywater for toilet flushing has the highest likelihood of being implemented in dense urban areas due to its potential to reduce the urban water demand and alleviate stress on freshwater resources (Friedler & pen, 2011). As shown in *figure 1*, toilet flushing represents 21.5 % of the total consumption in HSB LL building (Knutsson & Knutsson, 2021). The water used for toilette flushing in many countries is at potable water quality (Eriksson et al., 2009) while approximately 10-20% of urban water consumption can be saved by greywater reuse for this purpose (Friedler, 2004). However, the uncertainties of designing the right volume of treated greywater storage tanks for toilet flushing are considered the main challenge due to several reasons: The volume of the storage tanks is dependent on the amount of water

consumption expected to be used for toilet flushing and on the yield of treated greywater (Gross et al., 2015). The estimation of these flow rate is considered challenging because of the daily changes in the user's habits leading to a change in the consumption uses, and the numbers of served users which is directly correlated to the water consumption stability (Gross et al., 2015; Knutsson & Knutsson, 2021).

Consequently, the choice of storage volumes affects the amount of water savings as the increase in the storage tanks volumes leads to more water saving potential (Campisano & modica, 2008). This issue can be solved by choosing large storage tanks and then the excess water may be used for other purposes such as washing cars and garden irrigation (Gross et al., 2015). On the other hand, using larger tanks means more expensive and more required space which could be unavailable.

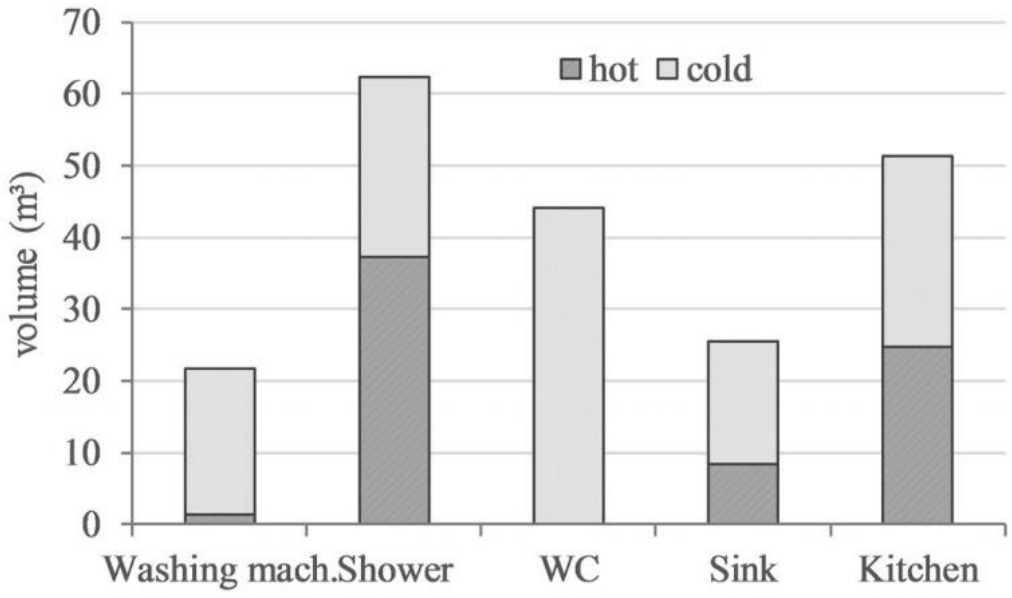


Figure 1. Graphs showing the distribution of domestic water consumption by uses in HSB LL. WC, toilet flushing (adapted from Knutsson & Knutsson, 2021)

The reuse of greywater for irrigation is very common in arid and semi-arid areas to recover nutrients, recharge the aquifers and reduce the resupply cost (Al-Gheethi et al., 2015). The appropriate design of irrigation can increase water savings, reduce the environmental potential negative impacts (Gross et al., 2015). Based on a study done by (Friedler & Penn, 2011), the reuse of the overflow of light greywater streams originating from bathtubs, showers and wash basins for garden irrigation reduces the daily water demand up to 41%. It is noteworthy to mention that to avoid health risks by either exposure to contaminated aerosol generated by water spray or contaminated groundwater due to the percolation through the soil, appropriate treatment and distribution system should be installed (Gross et al., 2015).

Despite the greywater reuse for hygienic purposes such as showering is not common, a good water consumption reduction potential as well as significant energy savings are expected. According to (Knutsson & Knutsson, 2021), 118% of the baseline shower and sinks hot water demand could be satisfied when reusing greywater collected from bathrooms sinks depending

on the tank volumes. Furthermore, around 50% of sinks and showers hot water energy consumption was reduced when reusing hot water (Knutsson & Knutsson, 2021).

2.3 Greywater reuse system configuration

Greywater recycling is usually performed in decentralized systems. Despite the aforementioned advantages of these recycling systems, some obstacles are faced during the design and the operation of the systems. Examples of these obstacles include: (1) Space, maintenance, and monitoring constraints, (2) The fluctuation in generated greywater volumes (3) The variations in greywater quality (Gross et al., 2015).

The greywater reuse distribution system is divided into three parts as shown in *figure 2*: Firstly, the collection system which collects greywater from apartments in separate pipes to the treatment processes. Greywater sources differ in their contamination and their daily produced quantity and hence their intended reuse scenarios (WHO, 2006). Greywater can be divided into light greywater including bathing and rinsing wastewater and dark greywater including kitchen wastewater and sometimes washing machines wastewater (Gross et al., 2015). It is always recommended to recycle the less polluted streams in order to lower the treatment costs, the potential health risks, and the environmental and aesthetic impacts (Friedler, 2004). Furthermore, the implementation of source separation is technologically simple, and its total cost is only the cost of the additional plumbing needed for the dual conveyance system (Gross et al., 2015). Some systems use a collection tank to collect greywater to regulate the water volume entering the treatment system while other discharge the greywater directly to the treatment process (Gross et al., 2015). However, the collection tank can lead to undesired problems such as odor, microbial regrowth, and the need for regular maintenance (Al-Gheethi et al., 2015).

Secondly, a distribution system of treated greywater to the storage tank which is placed either on rooftops or in basements. Finally, a conveyance system to distribute the treated greywater from the storage tank to the apartments to be used for the intended purposes (Friedler & Hadari, 2006).

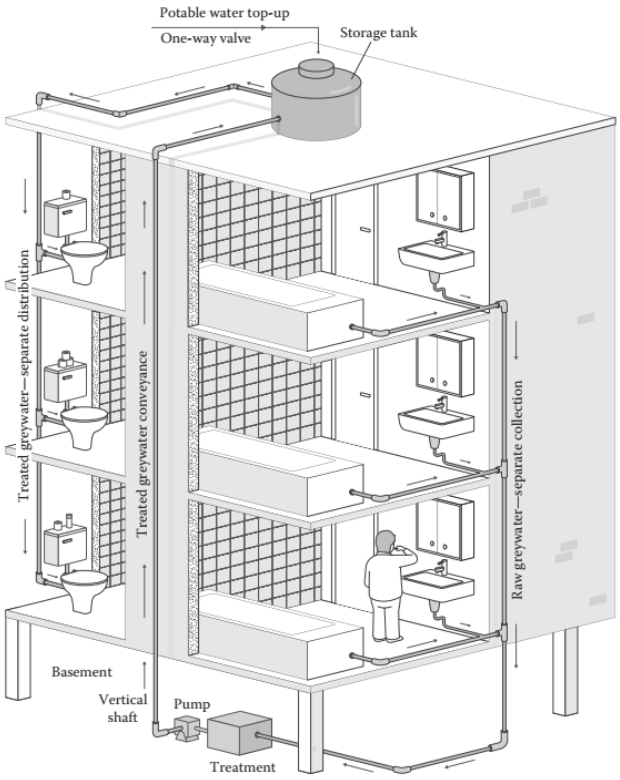


Figure 2 illustrating the collection, treatment, and conveyance of greywater in a building of several floors. (Adapted from Friedler and Hadari, 2006)

2.4 Greywater quality in storage tanks

Treated greywater quality will not be constant after treatment as it may deteriorate during distribution and storage (Ollos et al., 2003; Kilb et al., 2003). Approximately 18% of the outbreaks of waterborne pathogens reported in the United States occurs during the water distribution and storage (Bitton, 2014). Therefore, producing biologically stable water that do not have favorable conditions for microbial regrowth is necessary before distribution and storage (Charnock & Kjønnø, 2000).

As mentioned, the greywater recycling systems may include storage tanks either to collect raw greywater or to collect treated greywater before conveyance to the users. The storage of greywater is a controversial issue in all greywater onsite recycling systems because of the possibility of water quality deterioration during storage (Abed et al., 2020; Liu et al., 2010). For example, although the treatment effluent had low coliform levels (0.3 cfu/100ml), an increase by twentyfold after entering the distribution system was observed (Flemming et al., 2002). Furthermore, an increase in Heterotrophic plate count (HPC) values were reported after storing treated water samples even at low organic compound concentrations (Kooij, 1992).

This microbial quality deterioration can be attributed to several reasons. Firstly, bacterial regrowth in water can occur because of the fact that some disinfection barriers such as ozonation and biofilters do not cause a physical damage to bacterial cells which may lead to bacterial regrowth in the system (Berney et al., 2006). For example, regrowth of some pathogens such as Salmonella spp., S. aureus and E. Fecalis was observed after the storage of disinfected greywater using SODIS for 4 days at 37 °C (Al-Gheethi et al., 2013).

Secondly, even though the tanks could be probably designed and correctly installed and kept in good external order, there is still potential for stored water quality deterioration driven by the storage tanks' internal conditions and the treatment processes (Peter & Routledge, 2018; Al-Gheethi et al., 2015). These conditions such as internal water flow, the tank ambient temperature, the tank construction method, the materials used, and plumbing arrangements can provide favorable conditions for pathogens proliferation (Peter & Routledge, 2018).

However, the potential of microbial quality deterioration of treated greywater during storage is less than that in untreated greywater (Al-Gheethi et al., 2015). Furthermore, some greywater treatment techniques may fail to fulfill the treatment targets because they are designed for freshly untreated greywater without considering the storage effects on untreated collected water quality (Winward et al., 2008). Therefore, residence time should be taken into consideration when designing the system to ensure that the water degrade to an acceptable level before pumping it to the users (Liu et al., 2010). A long residence time can lead to chlorine dissipation, water temperature increases and bacterial growth (Bartram et al., 2003). Other conditions such as the concentration of bacteria leaving the treatment process, biodegradable organic matter (BOM) and disinfectant type can affect bacterial regrowth in the water systems (Bartram et al., 2003).

Therefore, the microbial quality of treated greywater in tanks should be evaluated carefully by adapting standards for monitoring point-of-use and point-of-entry potable water quality (Peter & Routledge, 2018). Monitoring and sampling strategies have been adapted by the United States Environmental protection agency (USEPA) and the European environmental agency to ensure the stable water quality (Figueras & Borrego, 2010).

2.5 Greywater quality

Greywater quality varies daily depending on the users' lifestyle, the products used as well as the greywater source of generation as shown in *table 2* (Li et al., 2009; Eriksson et al., 2002). In general, separated greywater is always less polluted than the total wastewater because it does not contain toilet flushing (Ottoson, 2003). The greywater composition varies significantly in terms of place and time. Despite of this variation, greywater originating from washing machine, dishwasher, and kitchen sinks were always found to be the most contaminated streams, with both organic and inorganic pollutants, while the highest fecal coliform concentrations were detected in the bath and shower streams (Friedler, 2004; Li et al., 2009). Therefore, it is preferable to recycle less polluted greywater streams when the demand of reused greywater is lower than its production (Friedler & Penn, 2011).

Table 2.Showing the range of different greywater generated from different categories (adapted from Li et al., 2009)

	Bathroom	Laundry	Kitchen	Mixed
pH (−)	6.4–8.1	7.1–10	5.9–7.4	6.3–8.1,
TSS (mg/l)	7–505	68 – 465	134–1300	25–183
Turbidity (NTU)	44–375	50 – 444	298.0	29–375
COD (mg/l)	100–633	231 – 2950	26–2050	100–700

BOD (mg/l)	50–300	48 – 472	536–1460	47–466
TN (mg/l)	3.6–19.4	1.1 – 40.3	11.4–74	1.7–34.3
TP (mg/l)	0.11->48.8	ND –171	2.9– >74	0.11–22.8

2.5.1 Physico-chemical quality

Greywater physical parameters of interest are total suspended solids (TSS), turbidity, color, odor, and temperature. Turbidity and suspended solids are important to be measured due to their ability to clog the installations such as pipes and treatment filters (Eriksson et al., 2002). Furthermore, turbidity can be considered a health index due to the protection that TSS and colloidal particles provide to heavy metals and pathogens as well as the toxic by-products formed by organic colloids after disinfections processes such as chlorination (Gross et al., 2015). TSS and biological oxygen demand ($BOD_{t)}$ concentrations in greywater were found to correspond to 55-70% of the common load of municipal sewage's TSS and BOD_t concentrations (Friedler, 2004). The concentrations of TSS in greywater originated from laundry and kitchen sinks were reported to be higher than those in other streams (Eriksson et al., 2002). Greywater temperature varies depending on the ambient temperature and the source type. For example, greywater generated from warm bathing and washing has higher temperatures than the surrounding temperature (Gross et al., 2015). High temperatures are considered a favorable condition for growth of certain pathogenic bacteria, such as *L. Pneumophila* (Ottosson, 2003). Odor is considered a problem especially for raw greywater storage. Therefore, some greywater reuse systems do not prefer to install a collection tank and discharge the greywater directly to the treatment process (Gross et at., 2015).

The content of organic matters in greywater should be reduced to prevent the microbial growth, increase the disinfection efficacy, and avoid the toxic by-products generated from the reaction of disinfectant residuals with organic matters (Gross et al., 2015). Chemical oxygen demand (COD), BOD and nutrients (nitrogen (N) and phosphorous (P)) are necessary to be evaluated because they give an indication on the oxygen depletion rate due to organic matter degradation during transportation and storage (Eriksson et al., 2002). Compared to municipal wastewater's COD: N:P average ratio of 100:20:1 suggested by (Metcalf & Eddy, 1991), bathroom greywater has lower N:P ratio due to the exclusion of urine and faeces while kitchen greywater has a ratio close to that of municipal wastewater (Friedler, 2004). In general, greywater may contain wide range of pollutants, with COD concentrations reaching up to several hundred mg /l (could be 1000 mg /L), 5-15 mg/l nutrients (N, P) and considerable concentrations of salts (boron, sodium, sulfide, and chlorides) (Friedler, 2004).

Furthermore, evaluating the concentration of ions in greywater is important because of its significant role in microbial growth (Leoni et al., 2005). Several ions including sodium (Na^{+2}), magnesium (Mg^{+2}), calcium (Ca^{+2}) and boron (B) may be presented in greywater as shown in *table* 3. Moreover, the concentrations of heavy metals (Cd, Cu, Hg) and xenobiotic organic compounds (XOCs) which are generated from chemical products such as soap and detergents have to be taken into consideration. Ericsson et al., (2002) detected at least 900 XOC's in greywater originated from used products such as soaps, detergents, and pharmaceuticals.

Table 3 showing average physio chemical quality of greywater generated from shower/bath based on several studies (Ericsson et al., 2002)

Study/source / Parameters	Shower / bath	Bathroom	Shower/bath	Shower
pH		6.4-8.1	7.6	6.7 – 7.4
COD mg/l			424	
TOC mg/l			104	30-38
Conductivity (µS cm^{1})		82–250		
Turbidity (NTU)			92	49-69
TN mg/l	17			
Sodium mg/l		7.4–18		
Calcium mg/l		3.5–7.9		
Chloride mg/l		9-18		
Sulfate mg/l		1.2–3.3		
Potassium mg/l		1.5–5.2		
Magnesium mg/l		1.4–2.3		
Ammonium mg/l	2	<0.1-15		
Phosphate mg/l	1		1.63	
Nitrate mg/l	0.4		0.9	
Bromide mg/l		<0.1		

2.5.1.1 The current HSB LL greywater physico-chemical characteristics

Several physico-chemical parameters presented in untreated greywater generated from HSB LL bath/showers are shown in *figure 3*. Compared to physio-chemical quality of shower/bath greywater investigated by several studies as shown in *table 3*, the HSB LL greywater quality is considered better or at the same level. For example, the average COD and total organic carbon (TOC) in HSB LL greywater are 100 and 18 mg/l while it is 424 and 104 mg/l in shower/bath greywater as shown in *table 3*. Furthermore, low inorganic nutrient contents were observed in HSB LL untreated greywater since most of these nutrients are below the treated drinking water standards (SLVFS 2001:30).

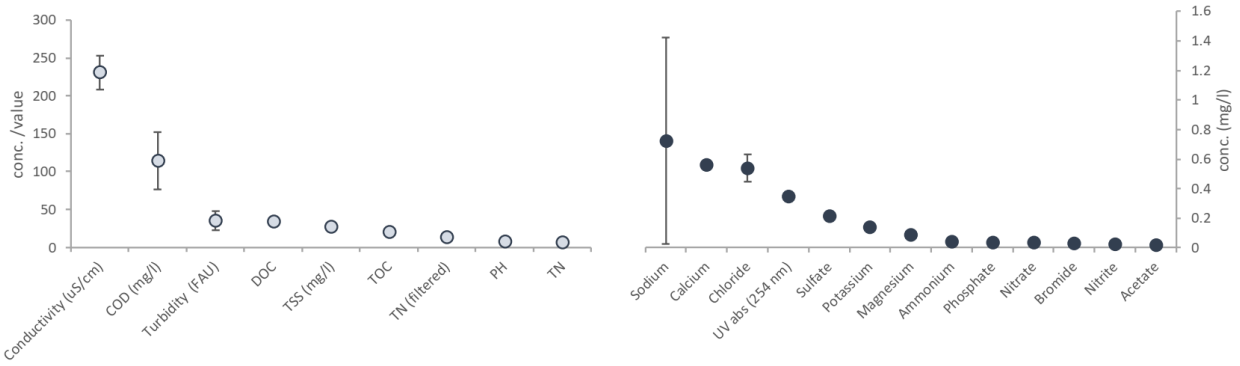


Figure 3. Graphs showing untreated greywater physico-chemical quality parameters as measured in HSB LL (adapted from Knutsson & Ericsson, 2019)

2.5.2 Microbial quality

The microbial quality of greywater varies depending on the greywater source, temperature, and users' hygiene behaviors (WHO, 2006). Although greywater may not contain fecal contamination, the concentrations of pathogens generated from different sources could be higher and more serious (Al-Gheethi et al., 2015). Bacteria, viruses, protozoa, and worm's presence in greywater can be generated from different sources such as dirty laundry and childcare, infected bodies (fecal pathogens), external users' bodies (skin, nose etc.) (Ottoson & Stenström, 2003; Gross et al., 2015).

Bacteria is considered the most common pathogen in greywater due to its ability to proliferate and survive for a long time even under harsh conditions (Al-Gheethi et al., 2015) but some viruses such as enteroviruses could be detected in fecally contaminated greywaters (Eriksson et al., 2002). A wide range of opportunistic pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Pseudomonas* spp., *Mycobacteria* or *L. Pneumophila* spp., could be presented in greywater as shown in *table 4.* Traditionally, greywater microbial quality is assessed by testing the fecal contamination using bacterial indicators such as *E. coli*, but other pathogenic bacteria should be regularly evaluated to ensure not posing any health risks to the users (Gross et al., 2015).

Table 4 showing Concentrations of bacteria and viruses found in light and mixed greywater (adapted from Gross et al.2015).

Etiological agent	Light greywater	Mixed greywater	Etiological agent	Light greywater	Mixed greywater
Total coliforms	1.7-7.4	7.2-8.8	*S. aureus sp*	4.0-5.7	4.0-5.7
Fecal coliforms	1.0-6.9	3.0-8.0	*L. Pneumophila sp.*	0-3.5	1.5-2.9
Enterococci/fecal streptococci	1.0-3.4	2.4-4.6	*Clostridium perfringens sp.*	0.66	
Heterotrophic plate count	5.6-8.3	5.0-7.0	*Cryptosporidium spp.*		0-8.3
P. aeruginosa	0-3.5	2.3-4.3	*Giardia spp.*		0-7.9

F-RNA phages		5.6	*Somatic phages*		3

Greywater pathogens can be transmitted from the reservoir to a host through different transmission routes as shown in *figure 4*. These transmission routes can be divided into different categories such as Gastrointestinal illness, respiratory, skin, mucous membranes, wound and eyes.

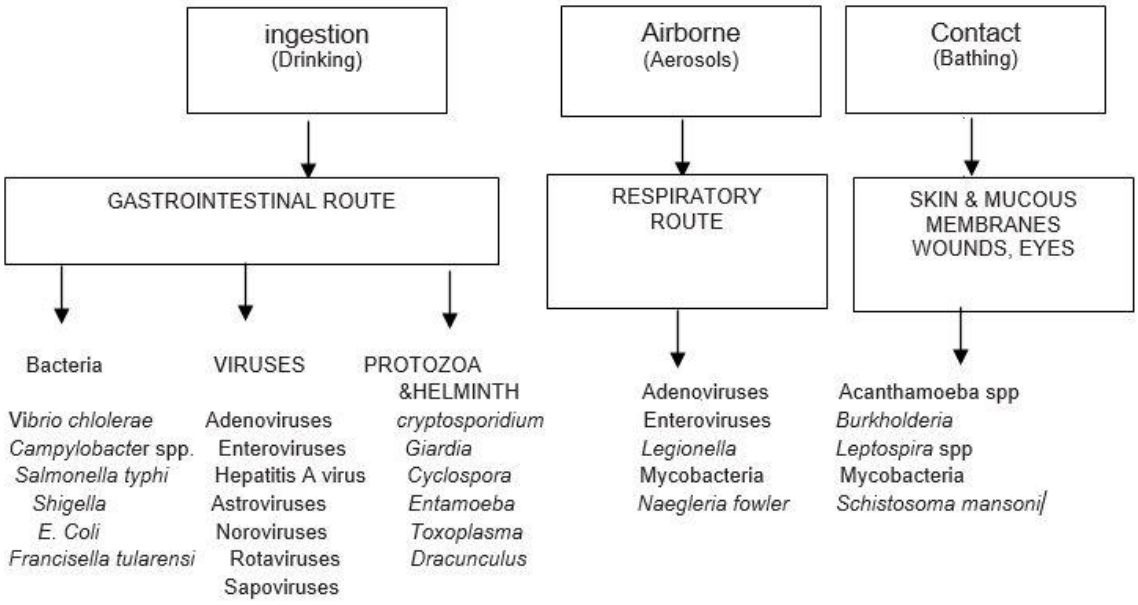


Figure 4 showing the transmission pathways of pathogens in water.

2.5.2.1 Indicator bacteria (*Coliform bacteria* and *E. coli*)

Due to the difficulty of pathogenic bacteria enumeration in greywater, fecal contamination has historically been measured by using a group of bacterial indicators. The selection of these indicators is based on several reasons such as: the indictor should present in high concentrations in humans and warm-blooded animals' faeces, the survival rate of indicators should be higher than that of pathogens, high survival in different kinds of water (NRMMC & EPHC, 2006).

The concentration of different bacterial indicators such as *coliform bacteria, E. coli, Enterococci, Bacteriophages, Spores of sulphate reducing anaerobic* detected in different greywater sources is shown in *table 5* (Ottoson, 2003). *E. coli* bacteria is one of the most reliable fecal contamination indicators which excreted in densities ranging from 105 to 108 c.f.u /g (Geldreich, 1978). It is worthy to mention that the absence of these indicators does not mean that the greywater does not contain pathogens, as well as their presence does not mean that pathogens are presented in greywater (Gross et al., 2015). Nevertheless, using *E. coli* to assess microbial contamination is still widely common to investigate the presence of pathogenic bacteria.

Despite most of *E. coli* are not pathogenic, some types can pose health risks and cause gastroenteritis in humans (Bartram et al., 2003). The infectious dose (ID) of *E. coli* was reported to be between 10^6 to 10^8 (Kothary & Babu, 2001).

Table 5 showing reported numbers of indicators bacteria in greywater (log/100ml) (adapted from Ottosson, 2003)

Wastewater source	*Total coliforms*	*Thermotolerant coliforms*	*E. coli*	*Fecal enterococci*
Bath, hand basin			4.4	1–5.4
Laundry	3.4–5.5	2–3		1.4–3.4
Shower, hand basin	2.7–7.4	2.2–3.5		1.9–3.4
Greywater	7.9	5.8		2.4
Shower, bath	1.8–3.9	0–3.7		0–4.8
Laundry, wash	1.9–5.9	1– 4.2		1.5–3.9
Laundry, rinse	2.3–5.2	0–5.4		0–6.1
Greywater	7.2–8.8			
Hand basin, kitchen sink		5.0		4.6
Greywater		5.2–7.0	3.2–5.1	
Greywater, 79% shower	7.4	4.3–6.9		
Kitchen sink		7.6	7.4	7.7
Greywater		5.8	5.4	4.6

2.5.2.2 L. Pneumophila

The number of cases infected with Legionnaires' disease (LD) has been increasingly reported during the last decade in Europe and US since it reached to more than 10,000 cases in 2010 (Van der Kooij et al., 2013). The fatality rate of this disease for the hospitalized cases is estimated to be 10% in Europe particularly to elderly and immunocompromised persons (Bitton, 2014). Therefore, investigating the source of causative bacteria and removing the agent's mode of transmission is imperative to avoid LD infections. Legionella Pneumophila (*L. Pneumophila*) is the causative bacteria of 90% of LD (Koubar et al., 2013).

L. Pneumophila is a common gram-negative bacterium living freely in anthropogenic and natural aquatic environments or attached into biofilms (Abdelnour et al., 2013; Koubar et al., 2013). Its common presence in water is due to its tolerance to heat and low nutritional needs to survive and persist (Leoni et al., 2005) despite of its fastidious nature (Kuiper et al., 2004). *L. Pneumophila* is mainly transmitted in the form of aerosols that are generated via showering, toilette flushing, faucets, irrigation etc. (Bitton, 2014; Van der Kooij et al., 2013; Koubar et al., 2013) and human become infected by inhaling aerosols from contaminated water (Koubar et al., 2013). A conceptual exposure model describing the transmission of *L. Pneumophila* by aerosols generated by shower head is described in *figure 5*.

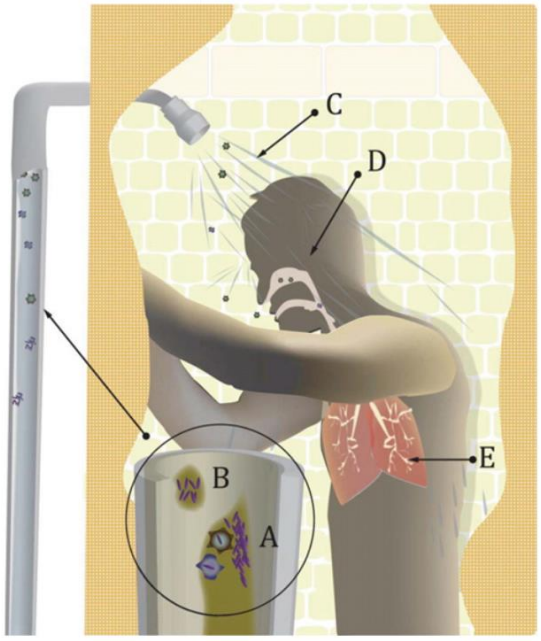


Figure 5. Schematic visualizing conceptual model of L. Pneumophila transmission via inhalation of shower aerosol containing L. Pneumophila. (Adapted from Bitton, 2014).

It has been observed that *L. Pneumophila* resistance to harsh conditions such as chlorine and high shear turbulent flow is higher than *E. coli* resistance (Bitton, 2014). Temperature and other water quality parameters such as the availability of specific nutrients (organic matter, Fe^{+2}, Ca^{+2}, Mg^{+2}), biofilm formation, sediments and scale, disinfectant type and concentration, and the protective protozoa hosts affect the growth of *L. Pneumophila* (Bitton, 2014).

2.5.2.2.1 Critical concentration

The infectious dose of *L. Pneumophila* varies depending on the virulence of the bacteria species and the conditions of the exposed person (Van der Kooij et al., 2013). Quantitative microbial risk assessment (QMRA) done by Armstrong and Haas (2005) for exposure to *L. Pneumophila* determined the dose-response relationships for the lethal dose (LD) and infectious dose (ID) which indicates $LD_{50\%}$ of 6200 CFU and $ID_{50\%}$ of 12 CFU. Furthermore, a quantitative model developed by Schoen and Ashbolt (2011) to estimate the infectious dose of *L. Pneumophila* during 15 minutes of showering exposure revealed that levels of $3.5 \times 10^6 – 3.5 \times 10^8$ CFU/liter are necessary for infection.

2.5.3 Limiting factors for *L. Pneumophila* growth

2.5.3.1 Substrate presence

Biodegradable organic matters (BOM) presence in water is the most significant factor that controls the growth of bacteria. *L. Pneumophila* is considered as a picky organism which utilizes a limited range of compounds as energy source (Bitton, 2014). Therefore, it cannot compete with oligotrophic bacteria which can consume a wide range of biodegradable organic compounds at low concentration levels in water (Abdel-Nour et al., 2013). Amino acids such as cysteine, methionine, arginine, leucine, and isoleucine, are necessary for *L. Pneumophila* growth while others such as alanine, aspartate, glutamate, serine, and proline can be utilized as sources of energy and carbon (Van der Kooij et al., 2013). *L. Pneumophila* occurrence in the hot water system was found to be directly correlated to TOC concentration in water (Leoni et al., 2005).

Assimilable organic carbon (AOC) is associated with the regrowth of microorganisms in bulk water (Kooij, 1992). The concentration of AOC is increased when using oxidation processes such as ozonation due to the incomplete oxidation of organic compounds leads to the formation of biodegradable compounds (Kooij et al., 1982). AOC cannot be assessed by simple chemical methods because it is difficult to detect many biodegradable compounds at low concentrations (Kooij et al., 1982). In unchlorinated system, Van der Wielen and Van der Kooij, (2013) observed that *L. Pneumophila* was detected more often at AOC concentration above 10 µg C/L than AOC concentration below 5 µg C/L. Therefore, AOC lower than 10 µg c /L has been derived as a reference value for controlling bacterial growth without maintaining disinfectant residuals (Kooij, 1992). Despite the bacterial growth was hardly observed at these low concentrations (Kooij, 1992), some bacteria such as Mycobacteria and P. aeruginosa can grow at lower AOC because they can outcompete other bacteria in oligotrophic stagnant water (Bartram et al., 2003). Therefore, it is considered complicated to define the water stability parameters. AOC typically represent 0.1% to 9% of TOC (Escobar & Randall, 2001) and it can reach to 13% (Zacheus et al., 2000) but it is usually below 1% as observed from the results of Van der Wielen and Van der Kooij (2013).

The presence of inorganic nutrients such as phosphorus, nitrogen, iron, zinc, calcium, and magnesium are crucial for microbial regrowth. Iron and Zinc are important elements for bacterial growth especially for *L. Pneumophila* which considered iron dependent bacteria and contain Fe superoxide dismutase and a zinc metalloprotease (Koubar et al., 2013; Liu et al., 2016). For example, high *L. Pneumophila* growth was reported in hot water tanks that are rich in iron and zinc sediments (States et al., 1985). Manganese (Mn^{+2}) concentration below 3 µg/l was found as a good indication to *L. Pneumophila* absence (Borella et al., 2003). Copper at high concentration were considered as inhibitor for *L. Pneumophila* growth since, at copper > 50µg/l and temperature >55 °C less *L. Pneumophila* contamination was observed (Bargellini et al., 2011). Some contradictions were found regarding the effect of Mg^{+2} and Ca^{+2} on *L. Pneumophila* growth. For instance, 9 of 15 of elevated water temperature samples in a hospital was tested positive for *L. Pneumophila* correlated to concentrations of magnesium and calcium (Vickers et al., 1987) while no *L. Pneumophila* occurrences differences were noticed between softened water and unsoftened water by Leoni et al., (2005). Phosphorus in its different formulas is considered indispensable for bacterial growth in water (Liu et al., 2016). Nitrogen is also considered crucial for microbial growth particularly for autotrophic nitrifying bacteria which utilize nitrogen-based compounds such as nitrite, nitrate, and ammonium (Kowalchuk & Stephen, 2001).

2.5.3.2 Temperature

Temperature is in fact one of the factors that plays a significant role in bacterial growth in water systems. As shown in *figure 6*, LD cases are notably increased in warm seasons which indicate that the environmental temperature has a direct impact on *L. Pneumophila* growth (Bartram et al., 2003). Water temperatures between 20-45°C were reported to encourage *L. Pneumophila* proliferation in water and could grow at much higher temperatures as well (Leoni et al., 2005). In average, the occurrences of coliform bacteria in water systems were reported to be higher at temperature above 15°C (Bartram et al., 2003). Studies have shown that increasing the water temperature above 55°C reduces the detectable amount of *L. Pneumophila* even in the presence of organic carbon (Proctor et al., 2017). The cold-water tanks temperature was reported to be above 20°C in united states and Europe (Peter & Routledge, 2018). This indicates that there is a potential for *L. Pneumophila* growth in the tanks especially if the temperature factor is coupled with other *L. Pneumophila* growth enhancing factors. *Figure 7* illustrates a decision

tree that combines different growth factors thresholds where the probability of coliform detection is increased.

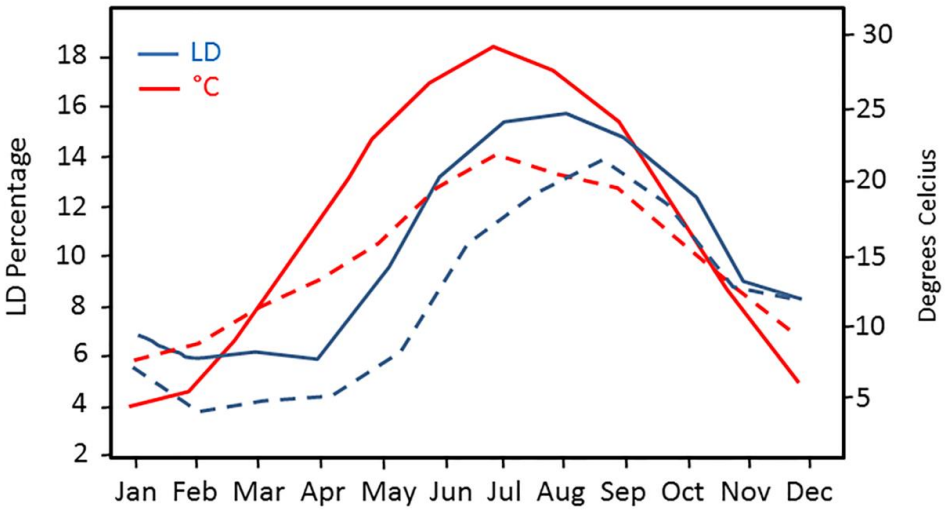


Figure 6 showing comparison of LD cases occurring in the United States and United Kingdom UK annually by month (adapted from Bartram et al., 2003)

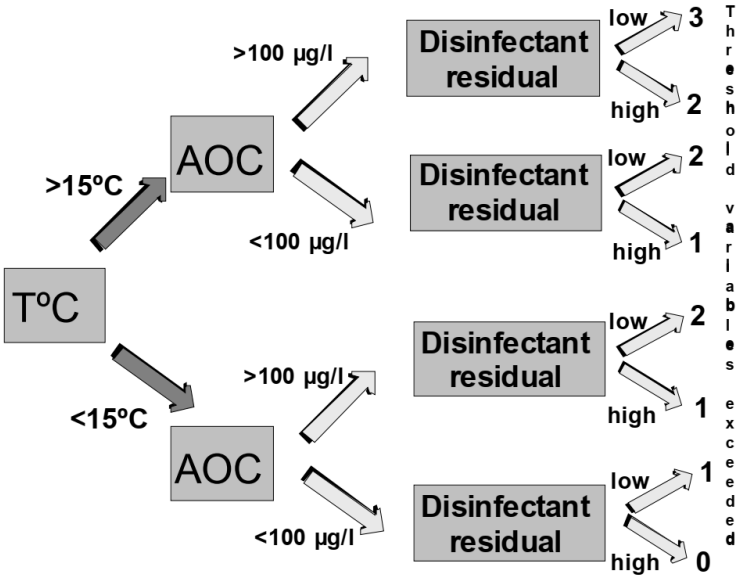


Figure 7 illustrating a decision tree that combine different growth factors thresholds where the probability of coliforms detection is increased (Adapted from Bartram et al., 2003)

2.5.3.3 Water stagnation

The stagnation of water in tanks and within any closed pipes may arise the bacterial proliferation (Bartram et al., 2003). LD cases were increasingly reported after the exposure to water systems of hotels where there is high risk of water stagnation due to intermittent use of the water (Leoni et al., 2005). According to a survey regarding the growth in Swedish water systems, *L. Pneumophila* was detected in all hot water tanks without circulation at temperature

below 50 °C (Ottosson, 2003). The water stagnation in water tanks can be attributed to two reasons: (1) the inlet (incoming mains) and outlets are at the same side which reduce the recirculation of water inside the tanks. (2) The inlet has an oversized pipe combined with small outlets Europe (Peter & Routledge, 2018). The recirculation of water in the tanks promote the buildup of organic matters and fairly distribute the contamination throughout the tank (Leoni et al., 2005). Furthermore, it was reported that the microbial growth in the locations with long residence time is corelated to decreased disinfectant residuals and the increase of water temperature in summer (Bartram et al., 2003). Therefore, reducing residence time and stagnation in the storage tanks is one of the significant measures to reduce the microbial activity in the tanks (Bartram et al., 2003).

2.5.3.4 Disinfectants effects

Disinfectant residuals type and concentration can affect the suspended bacteria occurrence in water (Bitton, 2014). A significant reduction in coliforms occurrences were reported when free chlorine was changed with chloramine (Bartram et al., 2003). It was also reported by (Cunliffe, 1990) that suspended *L. Pneumophila* was more sensitive to monochloramine than *E. coli* since 99% of *L. Pneumophila* inactivation was achieved at 1 mg/l chloramine exposure for 15 mins compared to 37 mins required for *E. coli* under same conditions. Regarding the disinfectants concentration, coliforms regrowth is likely to occur in systems that maintained free chlorine below 0.2 mg/L or monochloramine below 0.5 mg/L (LeChevallier et al., 1996).

2.5.3.5 Biofilms and protozoa

Protozoa mostly amoeba and ciliates species such as Acanthamoeba spp., Hartmannella spp., Naegleria and Valkampfia can serve as a host for pathogens in water (Van der Kooij et al., 2013). The bacterial growth inside protozoa provides a protection against any unfavorable conditions such as low pH, heat, and disinfectants (Abdel-Nour et al., 2013; Liu et al., 2016). For example, *L. Pneumophila* inside thermotolerant amoebae can survive and grow even after heat treatment (Abdel-Nour et al., 2013). Furthermore, biofilms formation in water systems enhance the coliforms and *L. Pneumophila* survival, pathogenicity, and proliferation (Bitton, 2014). It was found that *E. coli* could resist 2,400 times more chlorine in the statues of attached cells than as planktonic cells in water (Flemming et al., 2002). Furthermore, the pathogens released from biofilms were found more toxic than planktonic cells. For instance, studies showed that the cytotoxicity of *L. Pneumophila* released from biofilms is higher than other strains (Abdel-Nour et al., 2013).

2.6 Water quality monitoring

Water quality monitoring procedures should be followed to ensure that specified finished water criteria are met. The extent of monitoring procedure is dependent on the project's specific water quality objectives and the severity of the failure impacts (National Research Council, 2012). The monitoring programs consist of online monitoring devices such as turbidity, pH, and chlorine residuals as well as conducting discrete measurements such as *E. coli*, TOC using grab or composite samples (Korak & Arias-Paic, 2016). Combination of monitoring measurements are summarized in *table 6.* The online monitoring enables collecting data in short time intervals while its main disadvantage that it can conduct only limited types of measurements (Korak & Arias-Paic, 2016). More advanced measurements require samples to be measured either on-site or off-site laboratory (National Research Council, 2012). The time lag between sample collection, data analysis and results hinder rapid process control (Korak & Arias-Paic, 2016). Furthermore, appropriate water reuse monitoring requires proper selection of indicator and surrogate measurements depending on project objectives (National Research Council, 2012).

Recently, the monitoring approaches have been consisted of bulk water parameters (i.e., surrogates) and indicators (i.e., *E. coli*) to ensure optimal performance of the system (National Research Council, 2012). For example, TOC is one of surrogate parameters which its reduction in water reduces the concentration of hazardous unidentified organic compounds (Crook et al., 1991). Conducting only bulk water microbial quality monitoring may lead to unexpected failures because bulk water samples do not represent the attached microorganisms on surfaces (Douterelo et al., 2016). Therefore, water monitoring program could fail since unanticipated contaminants were detected in final water products even after state- of-the-art treatment processes and monitoring program (National Research Council, 2012).

Table 6 showing commercial water quality monitoring techniques (refers to Korak & Arias-Paic, 2016).

Monitoring technique	Parameters	Tool
Online Sensor	Conductivity	Electrode
	Temperature	Thermocouple
	Oxidation Reduction Potential (ORP)	Electrode
	Ammonium	Ion selective sensor
	pH	Electrochemical sensor
	Nitrate	Ion selective probe Optical sensor
	Dissolved oxygen	Optical sensor Galvanic sensor
	Suspended solids.	Light scattering
	Dissolved carbon dioxide	Membrane with thermal
	Natural organic matter (NOM)	UV254 (single wavelength) UV-VIS spectroscopy (full scan) Fluorescence sensor (limited wavelengths)
Online Analyzer or Sensor	Particles	Turbidimeter Dynamic light scattering
	Oxidants (Free chlorine, chlorine dioxide, ozone)	Amperometric electrode Colorimetric analyzer
	Total and Dissolved Organic Carbon (TOC, DOC)	Online organic carbon analyzer
	NOM characterization	Absorbance spectroscopy
	Inorganic ions (nitrate, chloride, etc.) Ammonium Ammonia monochloramine	Ion selective electrode Ion selective electrode Online analyzer

	Fluoride Phosphate Silica Sodium Hardness	Ion selective electrode Colorimetric analyzer Online analyzer Ion selective electrode Automatic
	Trace Metals (e.g., arsenic, chromium, selenium)	Voltammetry
Offline/Grab Sample Analysis	NOM quantity (e.g., TOC, DOC)	Organic carbon analyzer UV254
	NOM quality (e.g., molecular size, optical properties, molecular chemistry)	Absorbance spectroscopy Fluorescence spectroscopy Size exclusion chromatography High resolution mass spectroscopy Nuclear magnetic resonance Radical formation
	Contaminants of emerging concern (CEC) (e.g., pharmaceuticals, endocrine disrupting compounds, personal care products, pesticides, algal toxins)	Gas chromatography-mass spectrometry Liquid chromatography-mass spectrometry
	Major anions and cations (e.g., nitrate, sulfate, calcium, magnesium, chloride)	Ion chromatography Flow injection analysis
	Trace metals (e.g., iron, manganese, chromium, arsenic, uranium)	Inductively coupled plasma-mass spectroscopy Atomic emission spectroscopy
	Microorganisms (e.g., *E. Coli*, Giardia, Cryptosporidium, *L. Pneumophila*, Norovirus, Cyanobacteria)	Microscopy Microbial cultures Bioassays Genotoxicity and cytotoxicity assays

2.7 Biofilm formation

2.7.1 Occurrence and characteristics of biofilms

Water quality parameters should be assessed not only at the end of treatment processes, but also at consumers end point because there is a potential for water quality deterioration during distribution and storage. Biofilm formation is considered one of contamination sources affecting the water microbial quality in water distribution systems (WDSs) (Codony et al.,

2002). The formation of biofilms is a ubiquitous phenomenon which is irreversibly associated with water solid interfaces in natural aquatic environments as well as engineered systems (Bitton, 2014). 90% of the total biomass in WDSs can be found in biofilms while only 5% can be found as floating planktonic cells in the water (Flemming et al., 2002). These biofilms are considered a concerning phenomenon due to its ability to deteriorate water quality by detaching biofilms microorganisms into water phase, affecting the physical water characteristics such as turbidity, taste, odor, and color of the water as well as promoting the corrosion of pipes (Douterelo et al., 2016; Abu Khweek & Amer, 2018). Biofilms consist of microbial communities embedded in self-produced gelatinous matrix of extracellular polymeric substances (EPS) (Bitton, 2014). EPS constitute 50-90% of biofilms' organic matters and mainly composed of polysaccharides and proteins as well as varying amounts of nucleic acids, lipids, phospholipids, and Humic substances (Liu et al., 2016). EPS is a slim like substance, functioning as glue to encourage the attachment of cells together and on surfaces, the trapping of nutrients and providing a protective habitat for pathogens against unfavorable conditions such as low nutrients concentrations and disinfestation and variations in temperature (Else et al., 2003; Abdel-Nour et al., 2013). 99% of the total biofilm's volumes in WDSs is water from the gelatinous matrix while microorganisms occupy only 2-5% of the volumes (Liu et al., 2016). Other matters such as corrosion by-products, organic detritus, and inorganic particles such as silt and clay can incorporate also to biofilms increasing their mechanical strength and biomass accumulation (Bitton, 2014). The stages of biofilms formation are illustrated in *figure 8*. Microorganisms within biofilms build microcolonies separated by open channels where the transmission of nutrients, oxygen and antimicrobial agents into the cells are occurred (Bitton, 2014). Biofilms stop growing once it reaches to a critical thickness (approximately 100-200 µm) where the nutrients start limiting (Liu et al., 2016). Days to weeks are needed for biofilms to be formed on surfaces depending on nutrients availability in water and other conditions such as temperature, surface materials, flow rate and pH variation and disinfectant residuals etc. (Bitton, 2014). Furthermore, the diversity of biofilm microorganisms' assemblages depends on the chemical compositions of the surfaces, the finished water quality, and oxide-reduction potential in biofilms (Van der Kooij et al., 2013).

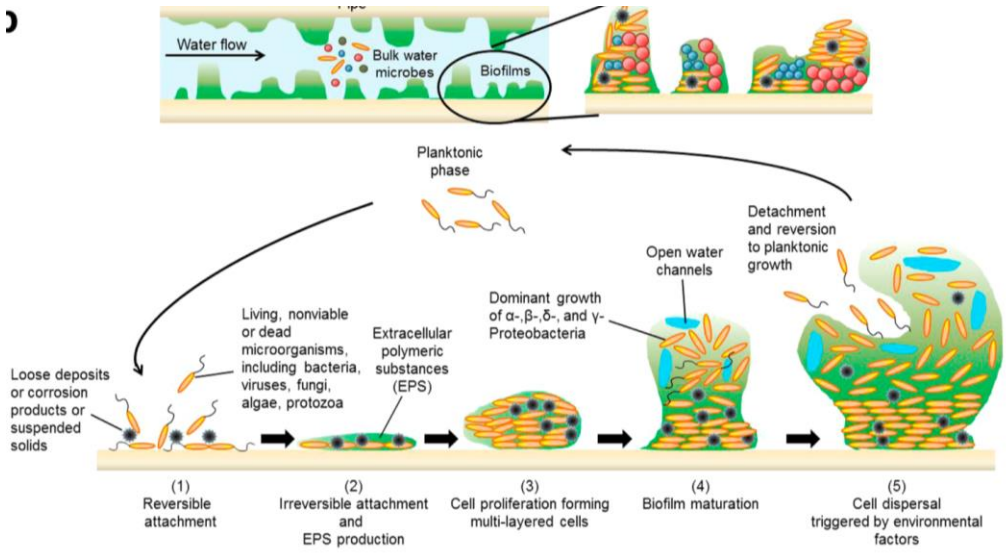


Figure 8 showing biofilm formation life cycle in water (adapted from Liu et al., 2016)

2.7.2 Biofilm accumulation factors.

According to several studies, the development of biofilms in WDSs is enhanced by several interrelated factors as following:

2.7.2.1 Organic and inorganic nutrients

Total cell concentration in water is one of the significant factors controlling the formation of biofilms in water. The presence of biodegradable organic matter (BOM) in water even at low level concentrations enhance the growth of biofilm organisms in water (Ollos et al., 2003). The organic carbon consumed by microorganisms during their growth phase is converted into CO_2 (dissimilation) to provide energy to cells and new cellular materials (assimilation) (Kooij et al., 1982). Microorganism's access to unsaturated aliphatic compounds such as simple carbohydrate, organic acids, low molecular weight proteins is easier than hydrophobic aromatic compounds such as humic substance, phenolic compounds, and aromatic carboxylic acids (Liu et al., 2016). Unfortunately, the capability of identifying the type of carbon presented in water is not well developed in water industry (Kooij, 1992).

It has been found that using oxidative water treatments such as ozonation, ultraviolet (UV) radiation or combination of UV and H_2O_2 change the organic matter structure in water by increasing the biodegradability of organic carbon (assimilable organic carbon AOC) (Zacchaeus et al., 2000, Kooij et al., 1982). To reduce biofilm formation, the AOC concentration should be maintained below 10µg of acetate carbon equivalent per liter and biodegradable dissolved organic carbon less than 0.15 mg C/l (Kooij, 1992).

Indispensable inorganic nutrients such as phosphorus, nitrogen can play a significant role in bacterial and biofilm growth in water system (Bitton, 2014). Autotrophic nitrifying bacteria which utilizing nitrogen-based compounds such as ammonia, nitrate and nitrite as energy sources are considered prevailing microorganism in biofilms (Kowalchuk & Stephen, 2001). In addition to the often presence of ammonia in the untreated water, it could be resulted from the reaction of nitrate with metal surfaces (Liu et al., 2016). Phosphorus in its different formulas is considered indispensable for cellular metabolism such as formation of ATP, DNA, RNA etc. (Van veen, 1997).

Furthermore, changes in biofilm structure such as thicker, more heterogeneous biofilms with more microcolonies were reported after adding phosphate to water to passivate metal surfaces (Liu et al., 2016). Furthermore, the microbial composition of biofilms was reported to be changed based on the change of nitrogen content as the formation of autotrophic bacteria biofilms occurred at high nitrogen to carbon ratio (Ohashi et al., 1995). Chu et al., (2005) observed that the concentration of ammonium or nitrate below 0.1 mg/L and phosphate below 0.005 mg/L has an effect on biofilm formation. Chu et al., (2005) also noticed a significant change in biofilm formation when ammonium increased to 0.5 mg/l and phosphate increased to 0.01 mg/L. Furthermore, adding low concentration of phosphate (1-5 µg/L PO_4^{-3}) was found to increase microbial growth in water and biofilms (Lehtola et al., 2002). As a result, low concentration of phosphate starting from 1 µg/L can promote microbial growth in both water and biofilms.

A study regarding the impact of copper and manganese on *L. Pneumophila* colonization done by Borella et al., (2003) revealed that the colonization was promoted by manganese (> 3 µg/l) and inhibited by copper (>50 µg/l). Nevertheless, copper at high concentrations were found to promote bacterial aggression which behave as a protective response against stress (Liu et al., 2016). Iron and Zinc are important elements for bacterial growth and biofilm formation (Koubar et al., 2013). The presence of iron can enhance the utilization of AOC (Van der Kooij et al., 2013) while zinc is able to increase the ability of *L. Pneumophila* to attach to host cells

such as human lung epithelial cells (Abdel-Nour et al., 2013). Zinc levels below 20 mg/L were found protective against *L. Pnuemophila* colonization (Borella et al., 2004). Furthermore, calcium and magnesium were reported to increase the adherence of *L. Pneumophila* (Koubar et al., 2013). Calcium presence in water enhances producing higher biofilm biomass, EPS, and adhesion forces (Kannan et al., 2016). Therefore, the presence of various minerals in water are considered as an indicator to potential risks related to microbial growth and biofilm formation.

2.7.2.2 Influence of temperature on biofilm formation

Temperature changes has an impact on the attachment of cells on surfaces through number of mechanisms. Temperature fluctuations can change the expressions of many microbial genes which could affect the microorganisms EPS production leading to increase the adherence capability of bacterial cells (Else et al., 2003). Compared to high temperatures, the tendency of microorganisms to form biofilm at low temperature is weak due to the extended lag time, the growth rate reduction, and the length of time before starting the proliferation (Liu et al., 2016). For instance, L. monocytogenes' EPS production was reported at 21 °C but not at 10 or 35 °C (Norwood & Gilmour, 2001) and three-dimensional biofilms were observed at 37 °C and 20 °C while only monolayer of biofilms was noticed at 8°C (Chavant et al., 2002).

2.7.2.3 Surface materials

A range of materials such as iron (stainless steel, galvanized steel), copper, cement-based materials, and polymer-based materials are used in WDSs. The biofilm formation is affected depending on the materials used in the water systems as shown in *figure 9*. The accumulation of biofilms on corroding iron surfaces is reported to be higher than those on noncorrosive materials such as PVC-U, PE, stainless steel (Ninquette et al., 2001; Ollos et al., 2003). This is primarily because the protection provided by the pitted surface against physical disturbance and chemical disinfection (Liu et al., 2016). Furthermore, the growth of biofilms can be attributed to the microorganism's utilization of released corrosion products which reacts with disinfectant residuals (Liu et al., 2016; Kooij et al., 1982). Furthermore. release of biodegradable matters (volatile organic compounds VOCs from polymeric materials), or inorganic compounds (iron, lead) to water can enhance the proliferation of planktonic bacteria by metabolizing small molecular weight plasticizer or antioxidants leading to biofilm formation (Liu et al., 2016). For example, it was found that the corrosion products are able to adsorb and store humic substances which are then utilized for microbial growth (Van der Kooij et al., 2013). Furthermore, surface materials can affect the type of microorganisms involved in the biofilms. Bitton, (2014) states that 74% of the bacteria in PVC pipes was Stenotrophomonas where more different bacterial species diversity such as Nocardia, Acidovorax, Xanthobacter, Pseudomonas, and Stenotrophomonas was noticed on iron pipes. Additionally, *L. Pneumophila* can attach well to several plastic surface while copper inhibits its adhesion (Abdel-Nour et al., 2013). Other surface properties such as increased surface roughness, iron corrosion, and iron surfaces porosity increase biofilm formation (Bitton, 2014).

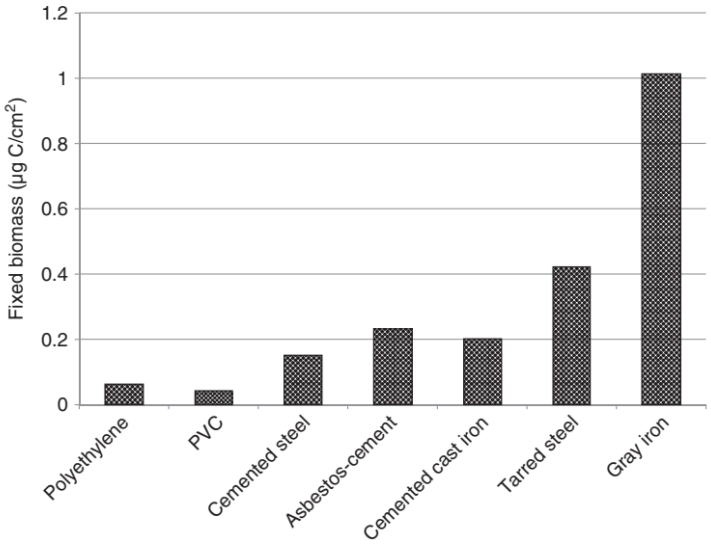


Figure 9 showing biofilm formation depending on the surface materials (adapted from Bitton,2014).

2.7.2.4 Effect of flow rate variations

The biofilm growth can be affected by flow rate variations in WDSs. Increasing the flow rates can facilitate transmission of microorganisms, nutrients or even biocides into the biofilm, resulting in biofilm growth stimulation (Bitton, 2014). For example, flow velocity of 0.1-1 m/s in pipe diameter of 100-500mm was found to create an intensive water-surface contact allowing for a rapid exchange of bacteria and nutrients (Kooij, 1992). Furthermore, high shear force was reported to have a direct impact on microorganisms EPS production in biofilms as well as promoting the nutrients adhesion to microorganisms (Liu et al., 2016). Furthermore, the characteristics of formed biofilms can be changed depending on flow rate variations. The biofilms formed at low flow rates are characterized with less attachment and more porosity because of the decrease of microorganisms and nutrients transmission (Liu et al., 2016). Furthermore, a higher microorganism's diversity was observed in a WDS with a high flow fluctuation (Bitton, 2014). Additionally, biofilm detachment and dispersion were reported because of Sudden hydraulic fluctuations leading to bulk water quality deterioration (Bitton, 2014). The water stagnation was found to enhance the biofilm formation. For example, the higher level of *L. Pneumophila* in centralized systems can be attributed to the stagnation of water in storage tanks which enhances to biofilm formation (Leoni et al., 2005).

2.7.2.5 pH effect on biofilm formation

The importance of adjusting pH at specific ranges in WDSs is not only to facilitate the treatment processes but also to reduce the enhancement of cells attachment to surfaces (Cam & Brinkmeyer, 2019). At pH 7, the net charge of many biofilm forming bacteria is negative due to the presence of anionic groups (e.g., carboxyl) on their surfaces (Palmer et al., 2007). For example, negative charged surfaces take place on PVC surfaces at pH 7 which in turn leading to electrostatic repulsion with the cells surfaces (Liu et al., 2016). pH drops in WDSs to isoelectric pH (e.g., isoelectric pH for PVC is 5.4, stainless steel 2.4-3) could reduce the electrostatic repulsion status which enhance the attachment of the cells to surfaces (Liu et al., 2016).

2.7.2.6 Disinfectant residuals

Using disinfectant residuals such as chlorine in WDSs is able to inhibit biofilm formation. Based on a study done by Codony et al., 2002, no biofilm was developed when bacteria were added to water containing 0.5 mg/l of free chlorine while biofilm formation was observed in

less than 24 hours in case of unchlorinated water. Furthermore, using chloramine was reported to be more efficient in decreasing biofilm and suspended microorganisms than using chlorine because of its slow reactivity (Bitton, 2014). Nevertheless, biofilms formation could occur even in the presence of disinfectants and is capable of decaying the disinfectant residuals (Liu et al., 2016). This in turn leads to increase the disinfectant residuals concentrations which may lead to increase toxic byproducts concentrations in addition to taste and odor problems (Kooij, 1992). Other environmental factors such as Hardness, redox potential, can affect biofilm growth on the surfaces (Bitton, 2014).

2.8 Monitoring of biofilms in water systems

Water microbial quality monitoring based on bulk water fecal indicators does not represent the microbial attached communities (Douterelo et al., 2016). Therefore, to ensure providing safe water to consumers, continuous biofilm monitoring in WDSs is useful. Obtaining representative biofilm samples is difficult because the biofilms are not uniformly developed on the surfaces as well as the difficulty of accessing the internal surfaces of the water networks' components such as pipes and tanks (Liu et al., 2016; Douterelo et al., 2016). Several devices such as Pennie water group coupons, corporation sampling devices and biofilm samplers have been developed to collect and monitor biofilm formation throughout the WDSs (Liu et al., 2016). These devices contain coupons with standardized surface area to stimulate the water systems conditions and facilitate biofilm sampling. However, further improvements are still needed for these devices because the obtained samples contain contamination which make the assessment of biofilms complicated (Liu et al., 2016). Recently, optical fiber-based biofilm sensors have been developed to be installed in WDSs for in situ and nondestructive monitoring of biofilm formation (Fischer et al., 2012). Furthermore, the effect of biocorrosion generated by microorganisms have been detected by using electrochemical sensors (Liu et al.2016). New electrochemical sensors (ALVIM) have been recently used to give fast and accurate information about biofilm formation especially at early stages of colonization (Pavanello et al., 2011). Additionally, these sensors can hinder the growth of biofilms by optimizing the treatment processes (i.e., concentrations, timing) (Pavanello et al., 2011). Furthermore, microelectrode probes which give information about the biofilms' physicochemical properties (pH, temperature, O_2, NO_3, NH_4, NO_2, H_2S, CH_4) have been widely used in biofilms microenvironment (Bitton, 2014)

2.9 Microscopic characterization of biofilms

A wide range of methods used in biofilm formation studies are shown in *table 7*. Biomass in biofilms can be measured by determining the cells biochemical components such as RNA, DNA, proteins, lipid markers or photosynthetic pigment (Bitton, 2014). Epifluorescence microscopy using fluorogenic dyes (i.e., acridine orange, 6-diamidino-2-phenylindole (DAPI)) can provide a rapid visualization of the biofilms especially at the early stage of biofilm formation as well as enumeration of total cell counts (Liu et al., 2016; Bitton, 2014). Using modern techniques such as confocal laser scanning microscopy (CLSM) can give a visualization and quantification of more mature biofilms with thickness more than 3 to 4 µm by doing optical sectioning of biofilms (Liu et al., 2016). The combination of CLSM and 16S rRNA-targeted oligonucleotide probes was reported to give information about the diversity of biofilms microorganisms as well as the activities of specific microorganisms in biofilms (Bitton, 2014). Furthermore, fluorescence in situ hybridization (FISH) have been used to visualize, identify, and quantify the constituents of biofilm community to explain the interactions between biofilm microorganisms (Liu et al., 2016). Other nondestructive methods

such as transmission electron microscopy (TEM), scanning electron microscopy (SEM) and 'biofilm friendly' environmental scanning electron microscopy (ESEM) which has a much lower magnification compared to conventional SEM and allow the observation of biofilms without dehydration (Liu et al., 2016; Bitton, 2014). On the other hand, ESEM has several limitations such as the resolution reduction, the beam damage resulted from the absence of metal coating, as well as the obscured surface topography (Liu et al., 2016).

Table 7 showing some methods used for biofilm study (Adapted from Bitton, 2014)

Type	Analytical method
Microscopy	Use of light microcopy, fluorescence microscopy, scanning confocal laser microscopy, scanning electron microscopy, environmental scanning electron microscopy, transmission electron microscopy, atomic force microscopy.
Image analysis Direct	Image structure analyzer, time lapse imaging. Biofilm thickness (using optical methods, image analysis, thermal resistance, quartz crystal microbalance) Biofilm mass (total cell count via staining with Acridine Orange or DAPI, crystal violet assay)
Indirect measurement of biofilm quantity	Extracellular polymeric substances (EPS) Specific biofilm constituents (polysaccharides, proteins, nucleic acids) Total organic carbon (TOC) Total proteins Peptidoglycans Lipid biomarkers
Microbial activity within biofilms	- Viable cell count (plate counts, preferably in low nutrient medium such as R2A agar, Live/Dead BacLight^{TM}) Active bacteria: direct viable count (DVC) DVC-FISH ATP Lipopolysaccharides Substrate removal rate Dehydrogenase activity (e.g., TTC, INT, or CTC dyes) or esterases such as carboxyfluorescein diacetate (CFDA). Oxygen uptake rate (OUR) DNA rRNA mRNA
Microbial observation and identification	Immunofluorescence (monoclonal or polyclonal antibodies) FISH mRNA amplified by polymerase chain reaction Green fluorescent protein (GFP) Use of commercial fluorochromes such as DAPI, acridine orange, SYTOX Green, PicoGreen and propidium iodide, or FUN-1 (for fungi)
Microenvironment	Fluor conjugates for determining diffusion and permeability in biofilms. Use of microelectrodes for determining pH, temperature, O2, NH_4, NO_3, NO_2, H_2S, and CH_4 within the biofilm

Other methods	Mass spectroscopy Infrared spectroscopy X-ray spectroscopy

2.10 Biofilm control

Water safety plans published by world health organization (WHO) In 2004 included risk assessment and preventative measures to prevent and control biofilm formation (WHO, 2004). Biofilms in WDSs can be controlled by mechanical, chemical or enzymic measures (Bitton, 2014). The worldwide strategy used for biofilm mitigation is to maintain disinfectants residuals in the system (Van der Kooij et al., 2013). Chlorine is a cheap, efficient, highly reactive oxidizing agent and most widely used to minimize biofilm accumulation throughout the water systems (Liu et al., 2016). Biofilm HPC levels was reported to be decreased by about four orders of magnitude after increasing chlorine residuals from 0 to 0.5mg/L (Ollos et al., 2003). Chlorine decreases the incorporation of microorganisms to surfaces by destroying cell membranes functional groups (Liu et al., 2016). Chlorine is rapidly neutralized by electron donors such as organic matters and corrosion products located on the surface layer of biofilms which lead to its exhaustion before diffusing into cell clusters (Ollos et al., 2003). However, increasing free chlorine residuals in the system is undesired because of several reasons such as consumers dissatisfactions, disinfectants by products as well as high operating costs (Van der Kooij et al., 2013). Furthermore, the detachment of biofilms microorganisms can be enhanced by using chlorine (Liu et al., 2016). A weaker oxidizing agent such as chloramine react slowly with electron donors resulting in penetrating deeper into biofilm matrix and maintaining the disinfectant for longer period as well as generating fewer harmful byproducts (Liu et al., 2016; Van der Kooij et al., 2013). Therefore, both bulk heterotrophic and biofilm bacteria were significantly decreased following using monochloramine (Ollos et al., 1992). Mechanical measure such as flushing, pigging, or air-water scouring used to control biofilms or to remove biomass killed via chemical disinfection (Liu et al., 2016). Nutrients control practices such as reducing the BOM, AOC and inorganic electron donors as well as the concentration of microorganisms prior to distribution is another measure to control biofilms (Ollos et al., 2003). Other practices such as temperature, pH control, and surface materials used in WDSs can be used to inhibit biofilms.

2.11Greywater treatment technologies

Greywater treatment technologies must be robust to handle variations in contaminants concentrations in order to hinder posing health risks and negative aesthetic and environmental effects. The fluctuations in generated greywater quality depending on the users' habits and the used products represent a challenge for selecting appropriate treatment technologies (Gross et al., 2015). The quality degree of treated greywater is dependent on the intended reuse scenario. For instance, water salinity is important in case of reusing greywater for irrigation (Huertas et al., 2008). Three types of greywater treatment technologies are required: physical, chemical, biological.

2.11.1 Physical treatment

Physical treatment such as coarse sand and soil filtration as well as membrane filtration aims to remove suspended solids. Suspended solids (SS) removal is important due to its role in providing shield to microorganisms against disinfection (Winward et al., 2008). Mechanical filtration has been reported to be efficient for pathogens removal without producing toxic

byproducts or antibiotics resistance (Al-Gheethi et al., 2015). Many types of membranes such as microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) membrane can be used in greywater treatment processes (Gross et al., 2015). The treatment performance varies depending on the pore size of the filtration. UF was reported to be efficient in treating medium greywater strength by reducing COD and BOD from 451mg/l and 274 mg/L to 117 mg/l and 53 mg/l, respectively while 56% of BOD reduction was reported when using UF for laundry greywater stream (Li et al., 2009). TOC reduction by 83.4%, low turbidity (below 1 NTU), free of suspended solids and *E. coli* in addition to excellent physical appearance were recorded in permeate after using UF but no soluble ammonia and phosphorus reduction were noticed (Li et al., 2009). NF was able to achieve 93% of organic removal in low strength greywater (Ramon et al., 2004) while 98% BOD removal was achieved by using RO filtration after UF (Sostar-Turk et al., 2005). The key factors limiting the viability of the membrane systems are the fouling of the membrane and the high energy costs (Al-Gheethi et al., 2015; Liu et al., 2010).

2.11.2 Chemical treatment

Different chemical treatments such as coagulation and flocculation, photo-catalytic oxidation, ion exchange, and granular activated carbon can be used to treat greywater. A combination of coagulation, sand filter and granular activated carbon installed to treat low strength laundry greywater showed a COD, BOD, suspended solids reduction from 280 mg/l, 195 mg/l and 35 mg/l in the influent to 20 mg/l, 10 mg/l and less than 5 mg/l respectively in the effluent (Sostar-Turk et al., 2005). Another combination including coagulation with aluminum salt and magnetic ion exchange resin process was studied by Pidou et al., (2008) to treat shower greywater. The reduction of COD, the BOD, turbidity, TN and PO_4^3 was reported from 791 mg/l, 205 mg/l, 46.6 NTU, 18 mg/l and 1.66 mg/l in the influent to 287 mg/l, 23 mg/l, 4.28 NTU, 15.7 mg/l and 0.09 mg/l respectively as well as the *total coliform*s, the *E. coli* and the fecal enterococci in the effluent were all less than 1 cfu/100 ml. Moreover, 90% organic removal and 6 logs reductions of *total coliform* were reported resulted from the installation of an oxidation process by using photo-catalytic oxidation combined with titanium dioxide and UV to treat greywater (Li et al., 2009)

2.11.3 Biological treatment

Biological treatment, ranging from membrane bioreactor (MBRs) to low technical wetlands, has been applied for greywater treatment due to their efficient role in organics removal (Winward et al., 2008). 99.99% *total coliform*s and *fecal coliforms* removal was reported by Bani-Melhem et al., (2015) when using submerged membrane biofilm reactors (SMBRs). The most feasible and economic greywater reuse treatment is the combination of aerobic biological process with physical filtration and disinfection (Friedler & Penn, 2011). In rural areas where there are available spaces, natural structures such as constructed wetlands can be used for greywater treatment (Li et al., 2009).

2.11.4 Disinfection processes

The disinfection processes aim to remove the microbial loads of greywater to avoid posing any health risks to the users. Several technologies include chemical (chlorination and ozone), physical or mechanical (filtration), and radiation disinfection (UV) can be used for greywater microbial removal (Al-Gheethi et al., 2015). Chlorination was the most common disinfection in twentieth century due to its low cost and simplicity (Bitton, 2014). The efficiency of chlorine to remove microbial content is dependent on water turbidity and the concentration of organic particles that may provide protection against chlorine (Al-Gheethi et al., 2015). The drawbacks

of using chlorination are the toxic and carcinogenic by-products formed by the reaction with organic matter as well as the possibility of pathogens regrowth due to chlorine high reactive characteristics leading to its rapid depletion (Liu et al., 2016). The formation of toxic by-products can be hindered if the water is treated using biological oxidation processes to degrade organic matter (Bitton, 2014). Alternatively, ozone has been used as oxidant when the water contains large humic molecules causing taste and odors (Zacheus et al., 2000). It has been reported that ozonation changes the structure of organic matter in water by increasing the concentration of AOC (Kooij, 1992). This leads to increase the possibility of bacterial regrowth in water (Kooij et al., 1982). Irradiation disinfections such as UV can be used in greywater treatment. Although UV irradiation is considered safer in terms of by-products formation but bacteria after UV still have the potential to regrow (Al-Gheethi et al., 2015). Advanced oxidation processes (AOP) have been widely used in wastewater treatment due to its simple operation and high efficiency in mineralizing TOC without adding any chemicals, inactivating pathogens, and producing low toxic by-products (Chong et al., 2016). Among different AOPs, H_2O_2/ UV was found the best in terms of technical, economic, and environmental advantages (Chong et al., 2016). Mechanical disinfection based on filtration would be more effective to remove pathogenic bacteria, but the removed bacteria are still alive (Al-Gheethi et al., 2015). Consequently, the selection of optimal disinfection processes should be based on the potential of by-products formation as well as the possibility of bacterial regrowth after treatment (Al-Gheethi et al., 2015).

2.11.5 Treatment processes combination in HSB LL

A combination of ultra-filtration, activated carbon, ion exchange filter and advanced oxidation process was installed in HSB LL to treat showers greywater. As a result, TOC and COD concentration were reduced by 92% and 99%. Other parameters removal efficiency is shown in *figure 10*. The microbial quality of the treated water fulfills the drinking water standards SLVFS 2001:30. As shown in *figure 11*, no *coliforms, E. coli*, or *L. Pneumophila* was detected in the effluent while other types of bacteria such as slow growing bacteria were detected.

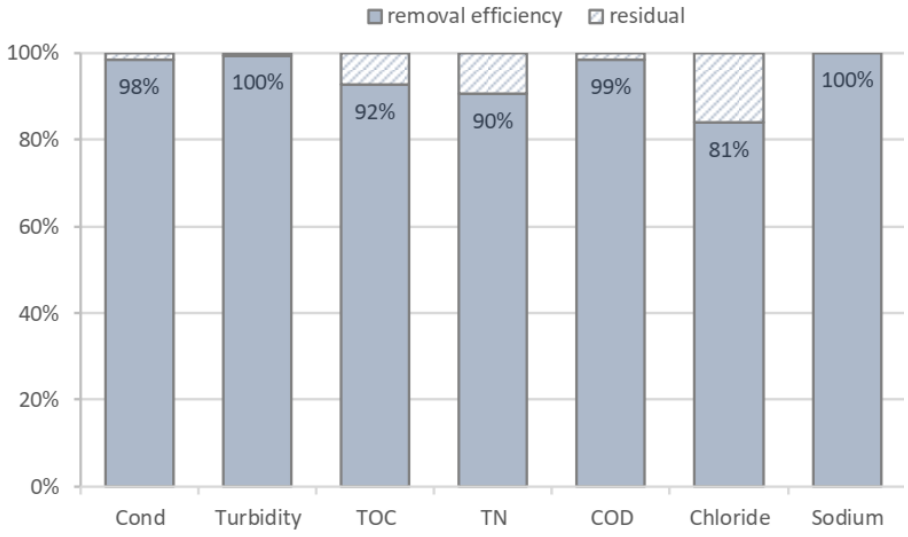


Figure 10. Graph showing the efficiency of HSB greywater treatment system on chosen parameters (adapted from Knutsson & Ericsson, 2019)

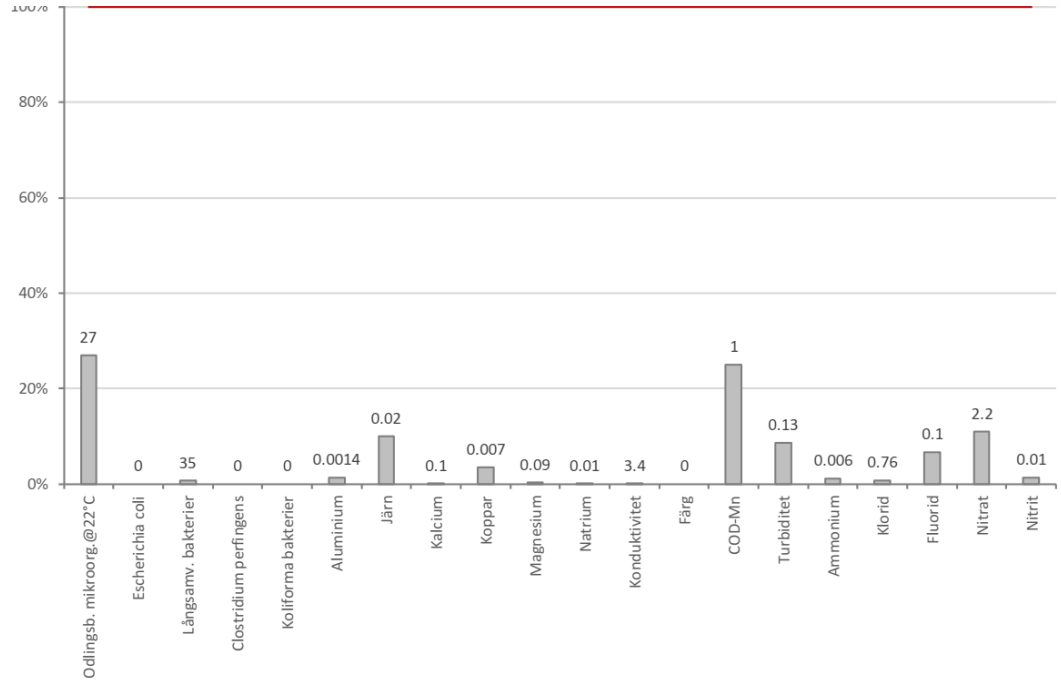


Figure 11. Graph showing the concentration of different parameters of the treated greywater in HSB LL (adapted from Knutsson & Ericsson, 2019)

2.12 Greywater reuse standards

In general, four criteria (hygienic safety, aesthetics, environmental tolerance, and economic feasibility) should be met in order to achieve efficient greywater reuse (Nolde, 2000). Greywater reuse has been impeded because no enforceable international regulations are available (Li et al., 2009). In general, different reuse applications require different quality standards. Very few reuse standards are made especially for greywater for different purposes. Nationally, greywater guidelines established by local authorities vary from country to country in terms of identifiable values, and the limited parameters (Li et al., 2009). Most countries have regulated their own standards focusing mainly on microbial aspects as shown in *table* 8 for irrigation purposes, while other parameters such as heavy metals, disinfection by-products or pharmaceutically active compounds are rarely mentioned (Al-Gheethi et al., 2015). Furthermore, urban water reuse guidelines strictness varies worldwide ranging from strict guidelines of USEPA and Korea (undetectable FC for urban reuse), to moderate regulations in Germany (less than 1000 CFU/100ml), to absence of national guidelines in the UK (based on EU standards for recreational waters) (Al-Gheethi et al., 2015). Moreover, no available standards for greywater recycling for hygienic purposes such as showering, and wash basins have been developed. Therefore, drinking water standards SLVFS 2001:30 was used to evaluate the treated greywater in HSB LL (Knutsson & Ericsson, 2019).

Table 8 showing greywater guidelines for irrigation (adapted from al gheethi et al.2015).

Country/organization	*Total coliforms*	*fecal coliforms*	Pseudomonas aeruginosa
USA (California)		2.2 MPN/100 mL (23 MPN/100 mL in 30 days)	
Australia		<30 CFU/100 mL	

Australia		<10 CFU/100 mL	
Germany	<10^4 CFU/ 100 mL	<1,000 CFU/100 mL	<100 CFU/100 mL
WHO		Class A (<1,000 CFU/100 mL) Class B (No standard recommended)	
Mexico		2,000 CFU/100 mL	
Korea		Must not be detected	
UK	10 CFU/100 mL		
Portugal	104 CFU/ 100 mL	200 CFU/100 mL	

Restricted and unrestricted non potable applications guidelines were presented based on several studies are shown in *table 9*.

Table 9 showing guidelines for greywater reuse (adapted from Li et al., 2009).

Categories		Treatments goals	Applications
Recreational impoundments, lakes	Unrestricted reuses	BOD_5: ≤10 mg/l TN: ≤1.0 mg/l TP: ≤0.05 mg/l Turbidity: ≤2 NTU pH: 6–9 Fecal coliform: ≤10/ml *Total coliform*s ≤100/ml	Ornamental fountains; recreational impoundments, lakes, and ponds for swimming
	Restricted reuses	BOD_5: ≤30 mg/l TN: ≤1.0 mg/l TP: ≤0.05 mg/l TSS: ≤30 mg/l pH: 6–9 Fecal coliforms ≤10/ml *Total coliform*s ≤100/ ml	Lakes and ponds for recreational without body contact
Urban reuses and agricultural irrigation	Unrestricted reuses	BOD_5: ≤10 mg/l Turbidity: ≤2 NTU pH: 6–9 *Fecal coliform*: ≤10 / ml *Total coliform*s ≤100/ ml Residual chlorine: ≤1 mg/l	Toilet flushing; laundry; air conditioning, process water; landscape irrigation; fire protection; construction; surface irrigation of food crops and vegetables (consumed uncooked) and street washing

	Restricted reuses	BOD_5: ≤30 mg/l Deterge t (anionic): ≤1 mg/l TSS: ≤30 mg/l pH: 6–9 *Fecal coliforms* ≤10/ml *Total coliform*s ≤100/ml Residual chlorine: ≤1 mg/l	Landscape irrigation, where public access is infrequent and controlled, subsurface irrigation of non-food crops and food crops and vegetables (consumed after processing)

3 Methods and materials

This chapter describes the method that has been followed to answer the research questions. The structure of the methodology is visualized in *figure 12*.

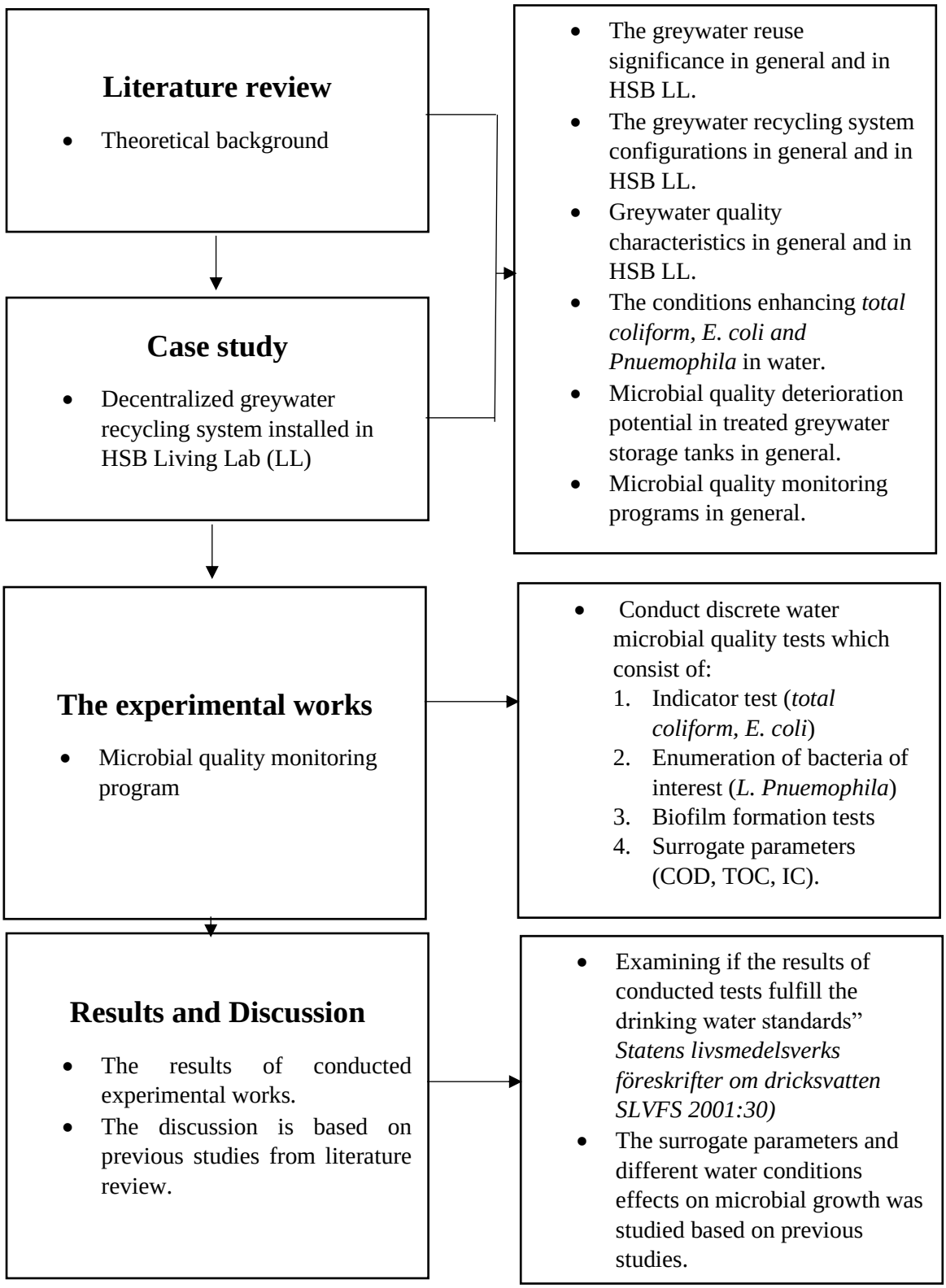


Figure 12. Illustration of methodology structure in combination with research questions.

3.1 Literature review

The study will start with a literature review where books, articles, reports, journals are read and sorted into different research fields. The aim of literature review is to gain knowledge regarding the greywater recycling systems in general and in HSB LL. Different categories and trends related to the topic are sought with focusing mainly on the greywater microbial quality especially in storage tanks. The literature review is considered the base of designing the experimental work as well as discussing the results of the experiments and drawing the conclusion.

3.2 Greywater recycling system in HSB living lab

As shown in *figure 13*, an in-building greywater reuse system was installed in a living lab in Gothenburg, Sweden (HSB living lab, HLL). The living lab building has 29 apartments and studios apartments occupied by 18 persons. The greywater treatment system installed in the LL is connected to only six bathrooms, collecting greywater from showers and bathroom sinks in separate pipes as shown in *figure 14*. In this system, a collection tank was installed to receive raw greywater coming through separate pipes.

After filling the collection tank, the excess water is discharged directly to the existing wastewater disposal system. The treatment system comprising of ultrafiltration, activated carbon filtration, ion exchange filter and advanced oxidizing process collect the greywater from the collection tank to be treated and pumped to the clean tank. A sensor was installed to measure several parameters such as pH, redox potential, conductivity, temperature, and turbidity.

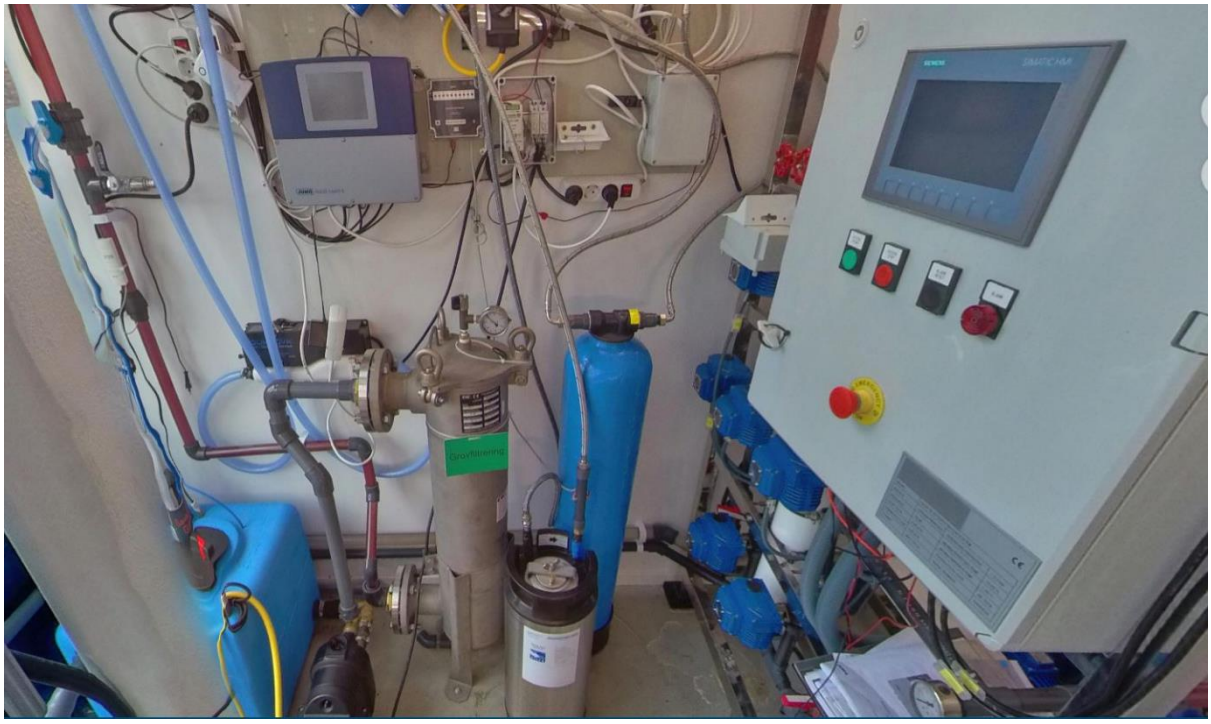


Figure 13 showing an inbuilding greywater treatment system installed in HSB LL.

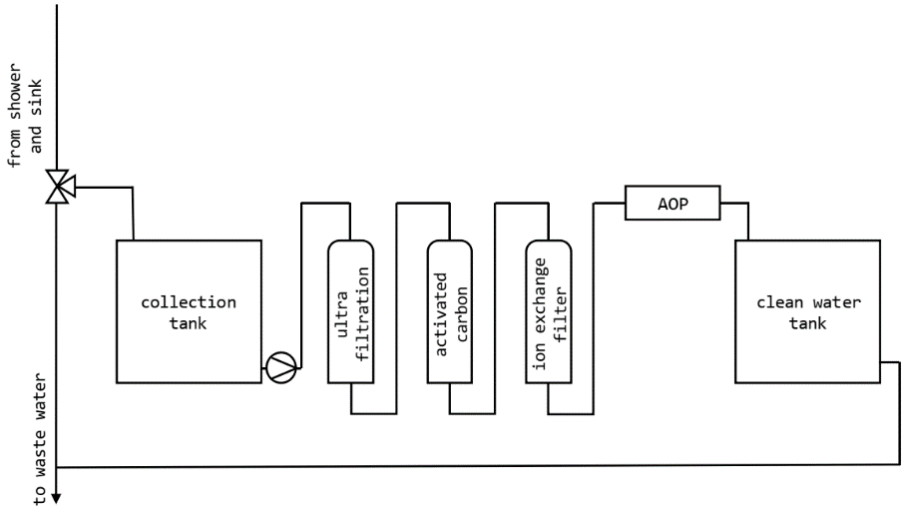


Figure 14 showing schematic drawing of the current system installed in LL (adapted from Knutsson & Ericsson, 2019)

3.3 The experimental work

3.3.1 Preparation and materials

The experiments took a place in two places: HSB LL and Chalmers WET lab. The microbial quality of treated water contained in HSB clean tank was monitored throughout the study. To test the impact of different storage conditions on the microbial quality, other experiments were done in the WET lab. The whole experiment setup is described in detail in *table 10*.

3.3.1.1 Waters and biofilms in HSB LL

100 L clean water storage tank were sterilized with 75% ethanol followed with distilled water before starting the experiment. The tank was filled with treated greywater passed through the forementioned treatment barriers. During the experiment, around 50% of the clean tank's water was regularly emptied three times a week by draining the water to the drain system and then the treatment processes were operated each time to refill the emptied part of the clean water tank. This was done to create water circulation to build up new organic matter and stimulate the realistic scenario when the system regularly operates.

Sterilized 24 stainless steel and zinc galvanized coupons were installed inside the tank by hanging them with sterilized fishing lines to collect biofilms. The coupons were installed at three different levels as shown in *figure 15*. The top level which represented the most aerobic conditions, the bottom level which represented the most anaerobic conditions and had the most sedimentation while the intermediate level has intermediate conditions.

3.3.1.2 Waters and biofilms in WET Lab

The whole experiment configuration is shown in *figure 15*. Two 2.5 L jars were filled with 2L treated greywater collected from source tap in HSB LL and transported to WET lab to perform the experiment under different conditions (see *table 10*). One jar, Jar 30C, was contaminated with106 ml, about 5%, of untreated greywater collected at the same day from the collection tank to study the effects of untreated greywater on the treated water quality if any treatment errors or bypass occur during the system operation. One treated Jar 30 and the contaminated Jar 30C were placed in a water bath at 30 ˚C as shown in *figure 16*, while another treated Jar 7 were placed at 7 °C. 50% of the jars water were regularly emptied 3 times a week and refilled

with freshly treated greywater. It is noteworthy that the contaminated Jar 30C was regularly contaminated with 5% of untreated greywater at the same day of the refilling process. Other two jars (Jar S4, Jar S7) were filled with treated greywater intended to study the effect of storage on greywater quality. JS7 was placed at 7 °C to study the storage effect after 7 weeks and another jar was placed at 35 °C to study the effect of storage after 4 weeks. One untreated jar was placed in 35°C to compare the formation of biofilms with other coupons. Five stainless steel coupons each with a surface area 200 mm^2 were installed in each jar by hanging them with sterilized fishing lines attached to the jar cap (see *figure 15*).

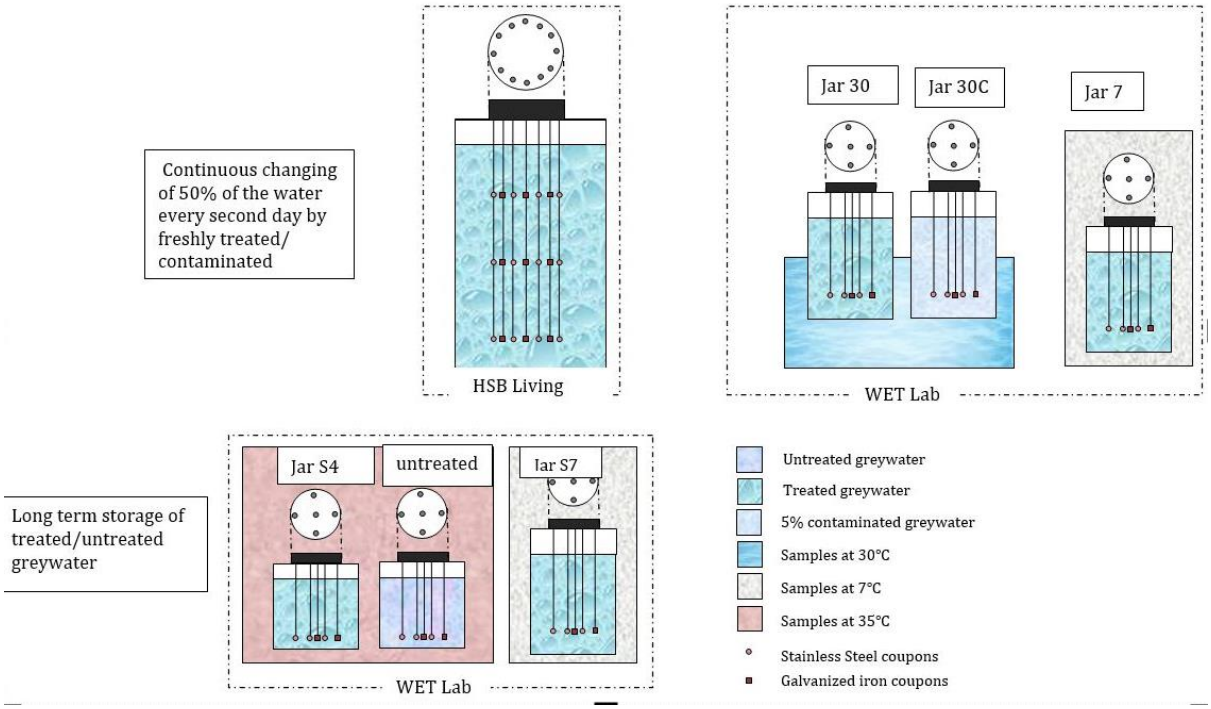


Figure 15. Schematic drawing describing the experiment in HSB LL and in WET Lab.

Figure 16 showing the Jar 30 and Jar 30C placed in water bath at 30 °C.

Table 10 Outlining the experiment setup including different storage conditions.

	The HSB and WET lab experiment					
Conditions	HSB clean tank	Jar 30[1]	Jar 7[2]	Jar C30[3]	Jar S4[4]	Jar S7[5]
Temperature °C	22	30	7	30	35	7
Coupon's material	Stainless steel, Zinc galvanized	Stainless steel	Stainless steel	Stainless steel	Steel	Steel
Disinfectant residuals	No	No	No	No	No	No
Flow variations	50% of water replaced 3 times/week	50% of water replaced 3 times/week	50% of water replaced 3 times/week	50% of water replaced 3 times/week	Stagnant water	Stagnant water
Total organic carbon	No externa addition	No external addition	No external addition	5% of contaminated greywater 3 times/week	Ethanol 75 %	Ethanol 75%

1: Jar 30: treated water jar at 30 ○C, 2: Jar 7: treated water jar at 30 ○C, 3: Jar C30 contaminated jar at 30○C, 4: Jar S4: storage jar for 4 weeks, 5: jar S7: storage for 7 weeks.

3.3.2 Water and biofilms samplings

3.3.2.1 HSB LL

At day 0, 250ml of treated greywater used to fill the clean tank was collected to evaluate the water quality at the start of the experiment. 250 ml water samples from the clean tank collected from pipe located at the tank bottom in autoclaved plastic containers was performed every week at day: 7, 14, 21, 28.

Two strings of the coupons were extracted at each sampling point so a total of 6 coupons (3 stainless steel and 3 galvanized coupons) were extracted. Biofilm formation were monitored over 30 days where the coupons were extracted from the tanks at these intervals: Day 7, Day 14, Day 21, Day 28. The samples were transported to the Chalmers WET lab in sterile aluminum dishes to be prepared for ESEM.

3.3.2.2 WET LAB

Using autoclaved 250ml plastic containers, 250 ml was collected from Jar 30, Jar 7 and Jar 30C at days: 7, 14, 21 and 28 respectively. Another 250 ml were collected from Jar S4 and Jar S7 after 4 and 7 weeks.

One coupon was extracted from each jar at days 7,14, 21,28 and placed in sterile aluminum dishes to be prepared for ESEM. One coupon was extracted from Jar S4 and Jar S7 after 4, 7 weeks, respectively.

3.3.3 Water and biofilms analysis

3.3.3.1 Water analysis

The monitoring approach adapted in this study consist of bacterial indicators which are *total coliform, E. coli* and bacteria of interest *L. Pneumophila*, as well as biofilm formation. Surrogate parameters of bulk water which are chemical oxygen demand (COD), total organic carbon (TOC), inorganic nutrients (Ion chromatography (IC)) were tested as well.

a. Enumeration of total coliforms and E. coli by Colilert ®

100 ml of sampled water was used for Colilert ® test (manufactured by IDEXX) which approved by U.S. EPA. Colilert ® tests were performed according to the manufacturer instructions. The procedure includes four steps as shown in *figure 17*. These steps are adding reagents to sample, then Pouring into Quanti-Tray/2000 and then Seal in Quanti-Tray Sealer. All Quanti-Tray*/2,000 trays were then incubated at 35 °C for 24 h and finally reading the results this way: yellow wells = *total coliform*s, yellow/fluorescent wells = *E. Coli*. MPN/100 ml values were recorded according to a tabulation of 95% confidence intervals provided by the manufacturer.

Figure 17 showing the procedure of IDEXX. Total coliform and E. coli enumeration test.

b. Enumeration of L. Pneumophila by Legiolert ®

100 ml of sampled water was used for Legiolert ® test (manufactured by IDEXX). Legiolert ® tests were performed according to the manufacturer instructions. The procedure includes four steps as shown in *figure 18*. These steps are adding reagent to 100ml water sample and then shaking it, then Pouring sample into Quanti-Tray/Legiolert, following by Sealing using the Quanti-Tray Sealer PLUS and incubate for 7 days at 35°C and after 7 days reading results: Any brown and/or turbid wells are positive for *L. Pneumophila*. MPN/100 ml values were recorded according to a tabulation of 95% confidence intervals provided by IDEXX which state that this test accuracy is greater than the traditional spread plate culture methods.

Figure 18 showing the procedure of IDEXX L. Pneumophila enumeration test.

c. Chemical oxygen demand (COD)

Hach reactor digestion method was used to measure COD. According to this method, 2ml of the samples was added to a COD digestion reagent vial and then being inverted gently to mix the content. The vial was then placed in a preheated DRB 200 reactors. A blank vial was prepared with the same procedure. The vials then were heated for 2 hours at 150 °C. After 2 hours, the vials were cooled down to 120 °C and then inverted several times while still warmed. The COD values in mg/l were determined using colorimetric device.

D. Ion chromatography (IC)

6 ml of each sample were filtered through 0.45 µm pore size filter. These samples were filled in clean vials and then closed with cap. The chromatograph used in the experiment is shown in *figure 19*. Then after tuning on the autosampler, anion chromatography and cation chromatography, the pumps were started. The samples were then placed in autosampler and then the run was started. After it finished, the data were analyzed.

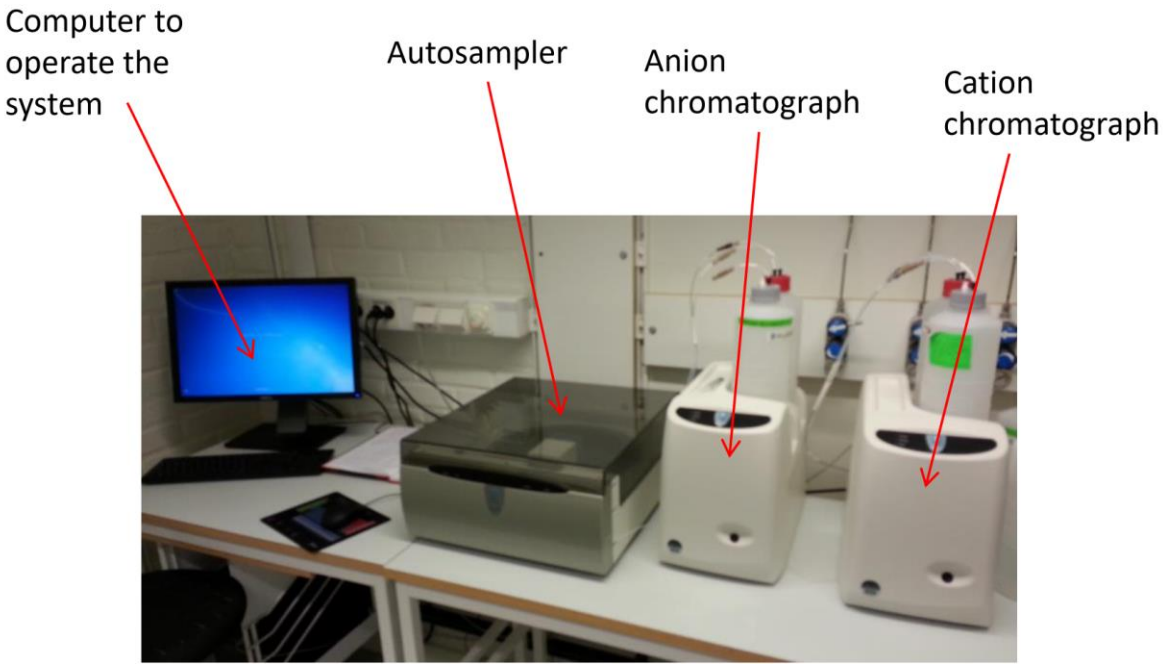


Figure 19 showing Chromatograph used to measure anions and cations.

D. Total organic carbon (TOC)

TOC analyzer was used to measure TOC. The samples were filled in TOC vials, and then covered with cap. The vials were placed in the instrument sample carousel.

E. Physical parameters

Several physical parameters such as oxidation/reduction, conductivity, turbidity and temperature were measured by a sensor installed in HSB LL as shown in *figure 20*. pH was measured for all samples by using pH meter.

Figure 20 showing physical parameters monitoring sensor installed in HSB LL.

3.3.3.2 Biofilm formation analysis

- *Biofilm samples fixation*

Biofilms were fixed using two methods: chemical fixation and freeze-drying method. Several chemical solutions were used to fix biofilms on coupons to test which solution gives the best images quality.

Some biofilms on the coupons were chemically fixed according to Dassanayake et al., (2020) procedure by using methacarn fixative solution (60% methanol, 30% chloroform, and 10% glacial acetic acid) for 1 hour. Following fixation, biofilms on the coupons were rinsed by 0.01M phosphate buffer, post fixed in 1% osmium tetroxide in 0.01 M phosphate buffer for 30 minutes, rinsed in water (four times, 5 mins each) and then dehydrated in a gradual ethanol (70% ethanol for 10 mins, 90% ethanol for 15 mins, 100% ethanol for 20 mins). This was followed by critically drying in well closed desiccators. Another procedure suggested by Merwe et al., (2013) by using 4% paraformaldehyde solution was used for 1 hour and then followed by gradual ethanol dehydration (30% ethanol for 10 mins, 70% ethanol for 10 mins and 100% ethanol for 10 mins). Freeze drying method suggested by Angerer et al., (2016) was also used.

- *Environmental Scanning electron microscopy ESEM*

The biofilm formation was investigated ESEM. The model used was Quanta 200 ESEM FEG from FEI (*figure 21*). The fixed samples were mounted on SEM stubs using double sided carbon tape. The settings used in ESEM was changed as following: operational vacuum mode: High vacuum, operating voltage :10 KV, spot size 4.

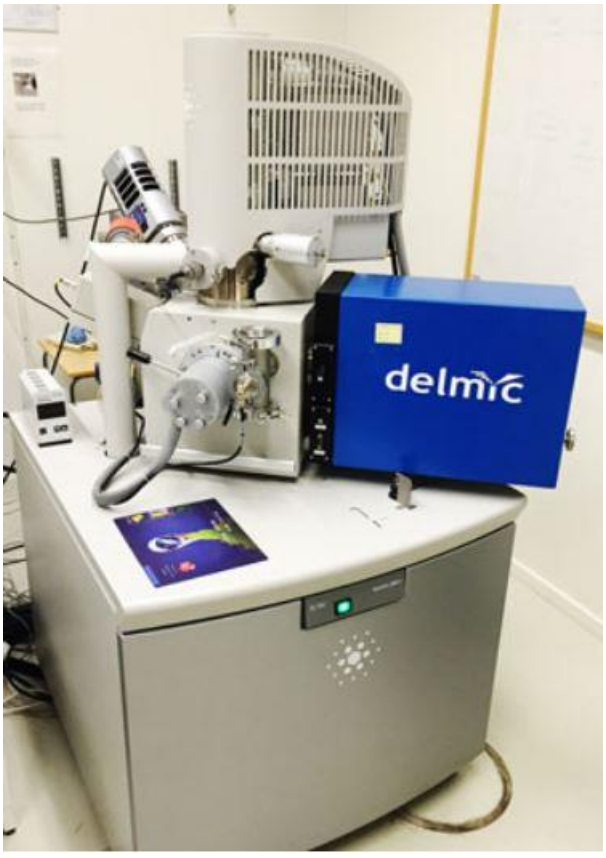


Figure 21 showing Quanta 200 ESEM used for imaging biofilms on the coupons.

- *Biofilm quantification*

This biofilm quantification method was implemented to confirm the results of ESEM. Biofilm quantification was performed by taking the weight of the coupons extracted from the HSB tank and WET jars after 4 weeks after drying at 105 °C for 1 hour and then taking the weight again after burn the coupons in the furnace at 550 °C for 1 hour. The difference between initial and final weight was recorded as weight of the biofilm formed on the coupon. This procedure was done according to Obifu et al., (2018).

3.4 Experimental results and greywater reuse standards

As mentioned in 2.12, no available specific standards for greywater reuse for hygienic purposes such as showering. Therefore, the drinking water standards SLVFS 2001:30 is used to test whether the treated water is appropriate for showering or not. Furthermore, previous studies regarding the impact of organic and inorganic nutrients in water on microbial growth especially *L. pneumophila* are used to test the potential of microbial growth under different conditions.

4 Results and discussion

4.1 *L. Pneumophila* occurrence in water

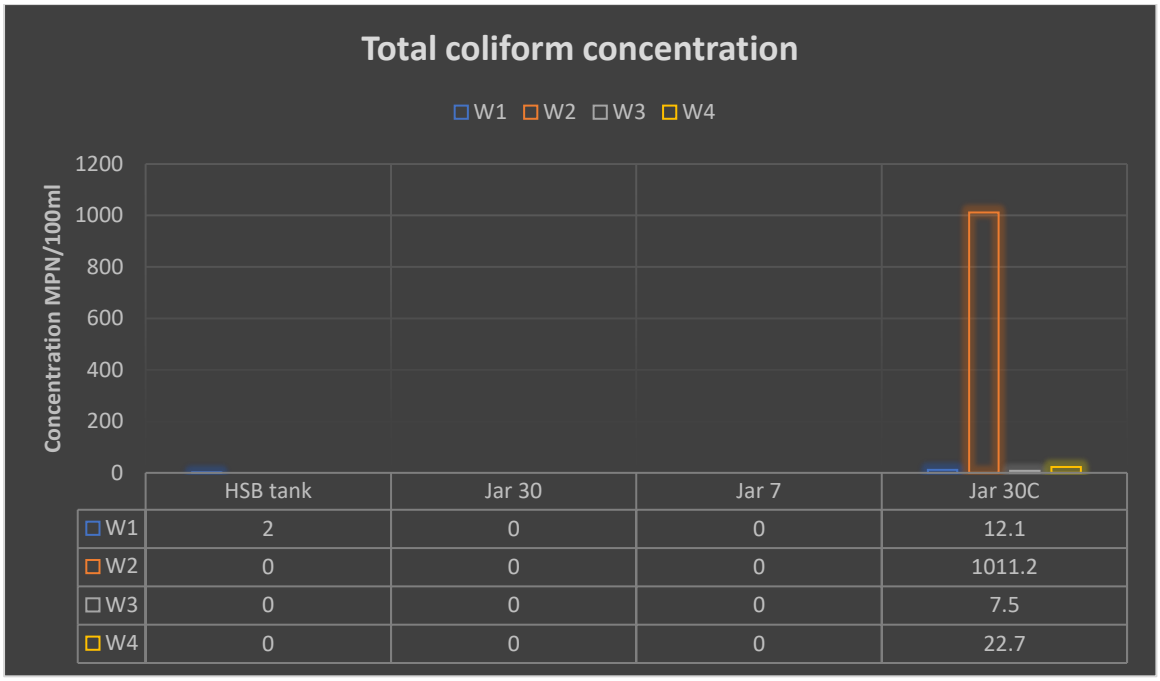
The results *of L. Pneumophila* enumeration test in HSB clean tank and the lab jars are presented *figure 22*. The treatment effluent showed high quality as no *L. Pneumophila* were detected in the reference sample. This test was taken as a reference at the start of the experiment, but the treatment effluent quality could vary throughout the study depending on the raw greywater quality. All the water samples from the HSB tank, Jar30, Jar7, Jar S4, Jar S7 tested negative for *L. Pneumophila* throughout the study except 2.3 MPN/100ml was found in a sample from HSB tank in the week 2 as shown in *figure 22*. However, the subsequent samples (Week 3, 4) were returned blank (negative) which indicates that either the water conditions are not favorable for *L. Pneumophila* proliferation or *L. Pneumophila* was released with emptied water before its duplication. *L. Pneumophila* concentration in the contaminated jar 30C experienced huge variations depending on the quality of 5% raw greywater added to the jars. This means that restrict monitoring procedure should be maintained to avoid any untreated bypass to the tank which contribute to high microbial contamination. The total results of microbial tests are shown in Appendix I.

	HSB tank	Jar 30	Jar 7	Jar 30C
W1	0	0	0	9.4
W2	2.3	0	0	149
W3	0	0	0	5.8
W4	0	0	0	154.7

Figure 22. Graphs showing L. Pneumophila concentrations in HSB tank, Jar 30, Jar 7, Jar 30C over four weeks.

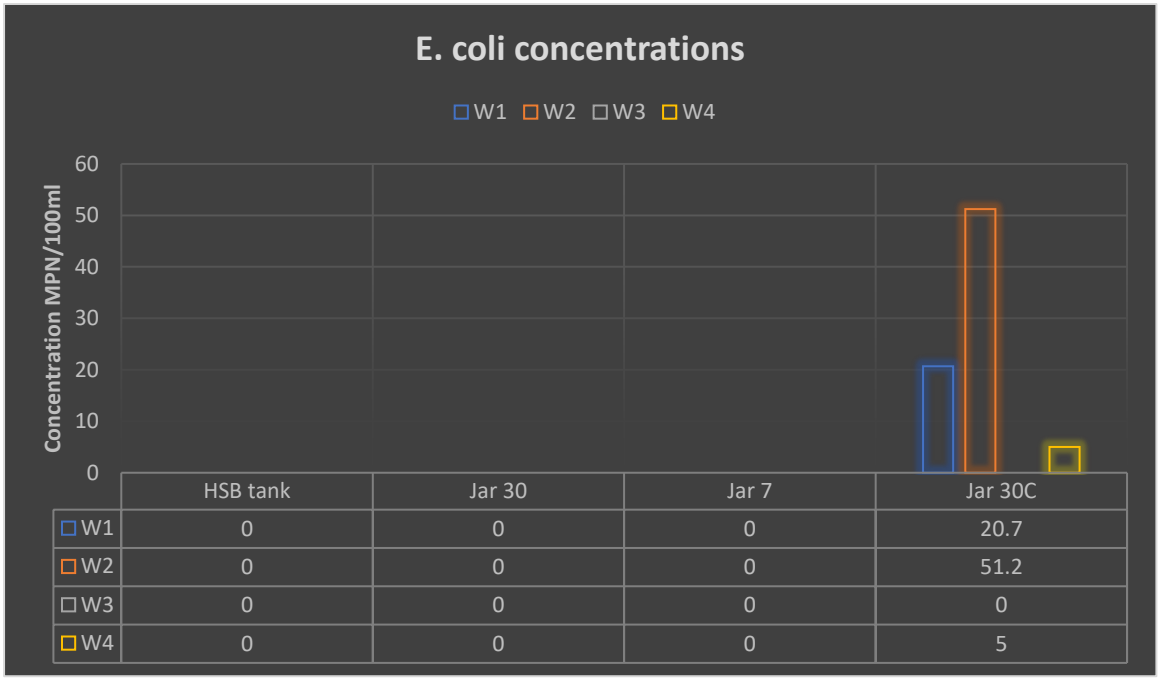
4.2 *Total coliform* and *E. coli* occurrence in water

No *coliform* or *E. coli* were detected in the treated HSB tank and Lab jars throughout the study except 2 MPN/100 ml *total coliforms* were recorded in the HSB clean tank in the first week as shown in *figure 23*. However, similar to *L. Pneumophila*, the subsequent tests were retuned blank. High variations were observed in the contaminated Jar 30C due to significant differences in raw greywater quality. For example, total coliform concentration jumped from 12MPN/100ml to 1122 MPN/100ml from week 1 to week 2. The week 2 was corresponded to Easter holidays so this high value could be attributed to users' behaviors during the holiday.



	HSB tank	Jar 30	Jar 7	Jar 30C
W1	2	0	0	12.1
W2	0	0	0	1011.2
W3	0	0	0	7.5
W4	0	0	0	22.7

Figure 23. Graphs showing total coliforms concentrations in HSB tank, Jar 30, Jar 7, Jar 30C over four weeks.



	HSB tank	Jar 30	Jar 7	Jar 30C
W1	0	0	0	20.7
W2	0	0	0	51.2
W3	0	0	0	0
W4	0	0	0	5

Figure 24. Graphs showing E. coli concentrations in HSB tank, Jar 30, Jar 7, Jar 30C over four weeks.

4.3 Conditions affecting bacterial growth

4.3.1 Organic nutrients

Measuring the concentration of organic matter in treated water is important for controlling the bacterial regrowth in water, but also specifying the nature of these compounds is necessary to study the growth potential of specific pathogen such as *L. Pneumophila*. Due to the difficulty of measuring AOC by simple chemical methods especially at low concentration (Kooij et al., 1982), COD and TOC were used throughout the experiment to estimate the organic nutrients

content. The growth of *L. Pneumophila* was reported to have correlation to the concentration of TOC in water (Leoni et al., 2005).

The treatment process effluent sample which was used as a reference recorded low COD and TOC values which are 1 and 1.52 mg/l, respectively. Additionally, COD results in the HSB clean tank and the Lab jars were found in the range from 0 to 2 mg/L. No big difference was experienced in the Jar 30C as the maximum COD value was 3 mg/l. Furthermore, the maximum TOC recorded throughout the study either in the HSB clean tank or in the Lab jars was 2.164 mg/L. This maximum value was tested in Jar 7 not in the Jar 30C which indicates that the added 5% of untreated greywater was not highly contaminated with TOC or COD. This was confirmed by (Knutson & Ericsson, 2019) report which stated that the average COD and TOC in HSB LL untreated greywater were 80 and 18 mg/L, respectively. The values of TOC and COD are close to the drinking water standards SLVFS 2001:30 which are 1.5 and 4 mg/L, respectively.

This indicates that the average COD and TOC reduction achieved by the installed treatment processes were around 98% and 90% respectively. High COD and TOC were accidently recorded in the first week of the experiment. This is probably attributed to an error done before starting the experiment by sterilizing the jars with 75% ethanol which left ethanol on the container's interior surfaces.

Assimilable organic carbon (AOC) is usually used as an indicator to evaluate the microbial stability in water (Escobar & Randall, 2001; Kooij, 1992; Charnock & KJØNNØ, 2000). AOC typically represents 0.1% to 9% of TOC (Escobar & Randall, 2001) and it can reach to 13% (Zacheus et al., 2000). It has been forementioned that using oxidative water treatments such as ozonation, UV radiation or combination of UV and H_2O_2 change the organic matter structure in water by increasing the biodegradability of organic carbon (AOC) (Zacheus et al., 2000). For example, Zacheus et al., (2000) observed that AOC increased from 2% to 13% of TOC after ozonation.

The range of AOC in water could reach to 150 µg C/l depending on the percentage of AOC in TOC. The Advanced oxidation process (AOP) which were installed to remove organic contaminants in HSB greywater is expected to increase the fraction of AOC in the treated water. The prevention of bacterial regrowth in unchlorinated system is achieved by ensuring the level of AOC to be lower than 10 µg acetate-c eq/L (Kooij, 1992). This means that the current concentration of biodegradable matters could have a potential for bacterial growth in water. Kooij et al., (1982) concluded that the microbial growth can be inhibited by either maintaining free chlorine residuals or by controlling bacterial growth favorable conditions such as nutrients availability, temperature, biodegradable matters release from surface materials, and hydraulic conditions. Due to the difficulty of controlling water temperature, and hydraulic conditions, maintaining the nutrient availability at low levels as well as choosing materials that do not promote bacterial growth are the possible measures to avoid bacterial growth. Furthermore, despite maintaining disinfectant residuals in the tank is considered effective against bacterial growth as shown in *figure 7*, the formation of toxic by-products makes it less desired option.

Furthermore, since *L. Pneumophila* is considered a picky microorganism which utilizes a limited range of organic nutrients (Bitton, 2014), the available AOC could be consumed first by other types of microorganisms which prevents the growth of *L. Pneumophila*. For example, bacteria such as Mycobacteria and P. aeruginosa prefer to grow at very low AOC concentration because they are able to consume carbons more than others oligotrophic stagnant environments (Bartram et al., 2003). As shown in *figure 12*, different speciation of bacteria was detected in

the HSB treated greywater such as slow growing bacteria. An example of slow growing bacteria is mycobacteria that can survive relying on low level of AOC (Bartram et al., 2003).

Table 11 showing the organic nutrients values (COD, TOC) over 4 weeks in HSB clean tank and WET jars.

S.No.	Sample	Week Number	COD mg/L	TOC mg/L	Total carbon mg/L	Inorganic carbon mg/L
1	Treated water (Reference)	Week 0 (Reference)	1	1.52	1.71	0.19
S.No.	Sample	Week Number	COD mg/L	TOC mg/L	Total carbon mg/L	Inorganic carbon mg/L
1	HSB LL clean tank	Week 1	2	1.630	1.914	0.2835
2		Week 2	0	1.174	1.513	0.3386
3		Week 3	1	1.006	1.363	0.3572
4		Week 4	0	1.310	1.678	0.3678
1	Jar 30	Week 1	104	31.8	32.24	0.4407
2		Week 2	2	1.639	2.014	0.3750
3		Week 3	1	1.130	1.466	0.3362
4		Week 4	1	0.7201	1.086	0.3656
1	Jar 30C	Week 1	30	23.02	23.46	0.4377
2		Week 2	3	1.504	1.840	0.3363
3		Week 3	3	0.7398	2.382	1.642
4		Week 4	2	0.4043	1.851	1.447
1	Jar 7	Week 1	24	9.590	10.10	0.51142
2		Week 2	2	2.164	2.558	0.3939
3		Week 3	0	1.300	1.784	0.4483
4		Week 4	1	0.6347	1.035	0.4001

4.3.2 Inorganic nutrients

As shown in *figure 25 and 26*, Anions and cations detected in the HSB tank and Lab jars throughout the study lie too far below the drinking water limits even for the contaminated Jar 30C. Nevertheless, different studies have set lower standards than the drinking water standards based on experimental experiences. As shown in *figure 3*, the inorganic nutrients concentration is low even in HSB untreated greywater. The anions detected in the HSB tank and Lab jars were acetate, chloride, nitrate, phosphate, and sulfate. Acetate and phosphate were rarely detected throughout the experiment while chloride, nitrate, and sulfate were frequently recorded throughout the experiment. Nitrate, and sulfate values were recorded with maximum value of 0.025 mg/l and 0.056 mg/L, respectively. Cations including sodium, potassium, ammonium, magnesium, and calcium were detected in the HSB tank and the jars. Sodium was detected in all water samples with a highest value 0.0577 mg/L recorded in the Jar 30C. Ammonium was mainly detected in the Jar 30C but not for treated jars. Hardness cations (Mg^{+2}, Ca^{+2}) was recorded in all water samples with highest values of 0.005 mg Mg^{+2}/l and 0.0463 mg Ca^{+2}/l, respectively recorded in the Jar 30C. The total results of inorganic nutrients are shown in Appendix I, II.

These values indicate that it does not seem to encourage bacterial growth in the water. The absence of manganese Mn^{+2} is considered a good indicator to inhibit the *L. Pneumophila* growth since the concentration of Mn^{+2} below 3 µg/l is considered safe based on Borella et al., (2003) study. The impact of Ca^{+2}, Mg^{+2} on microbial growth is not confirmed yet. Based on (Leoni et al., 2005), the presence of Ca^{+2} and Mg^{+2} was not reported to have any effect on *L. Pneumophila* growth even at high concentration ranging from 58mg/l to 83 mg/l and 11mg/l to 16 mg/l, respectively. On the other hand, the positive *L. Pneumophila* samples were correlated to high magnesium and calcium concentrations based on Vickers et al., (1987) study. The nitrate and ammonium concentrations in water seem to have negligible effects on microbial growth based on Chu et al., (2005) which stated that nitrate /ammonium concentration below 0.1 mg/L is considered safe. No phosphate was detected in water which considered a good indication as phosphate is considered significant for microbial growth (Liu et al., 2016). Iron and Zinc which are significant nutrients for *L. Pneumophila* growth were not detected by IC (States et al.,1985).

4.3.3 Other conditions

In addition to the nutrient's availability in water, other external conditions which enhancing the microbial growth were tested. Firstly, the HSB clean tank, Jar 30, and Jar S4 were placed in favorable temperature conditions for microbial growth ranging from 20 to 35 °C which is considered enhancing for *L. Pneumophila* and *coliforms* growth (Leoni et al., 2005; Bartram et al., 2003). Secondly, another condition is water stagnation which is used to examine the effect of intermittent use of water in tanks on the microbial quality. In Jar S4, despite combination of growth enhancing conditions including high TOC which in turn high AOC concentration (Kooij, 1992), water stagnation conditions (Leoni et al., 2005) as well as *L. Pneumophila* preferred temperature (35 °C) (Leoni et al., 2005) were tested, no coliforms, *E. coli* and *L. Pneumophila* were detected. This indicates that none of these bacteria was discharged in the treatment effluent to the tank and jars. Thirdly, some physical parameters such as suspended solids affect the microbial quality in water were tested. Turbidity was found around 0.5 NTU during the study which fulfill the drinking water limit (1.5 NTU). This indicates that the protection which SS provide to pathogens as stated by (Gross et al., 2015) is low. Finally, no disinfectant residuals were maintained in the system which increase the microbial growth potential in the tanks. Consequently, despite of the increased number of microbial growth boosters, no microbial growth was observed throughout the study.

This biological stability can be attributed to two main hypotheses: Firstly, there were no bacteria in the treatment effluent throughout the experiment which is supported by the claim of the treatment system designer that the treatment processes were designed to provide a better than drinking water quality. Secondly, the water characteristics and the ambient condition in the tank do not seems to enhance the microbial growth even at presence of pathogens to the tanks.

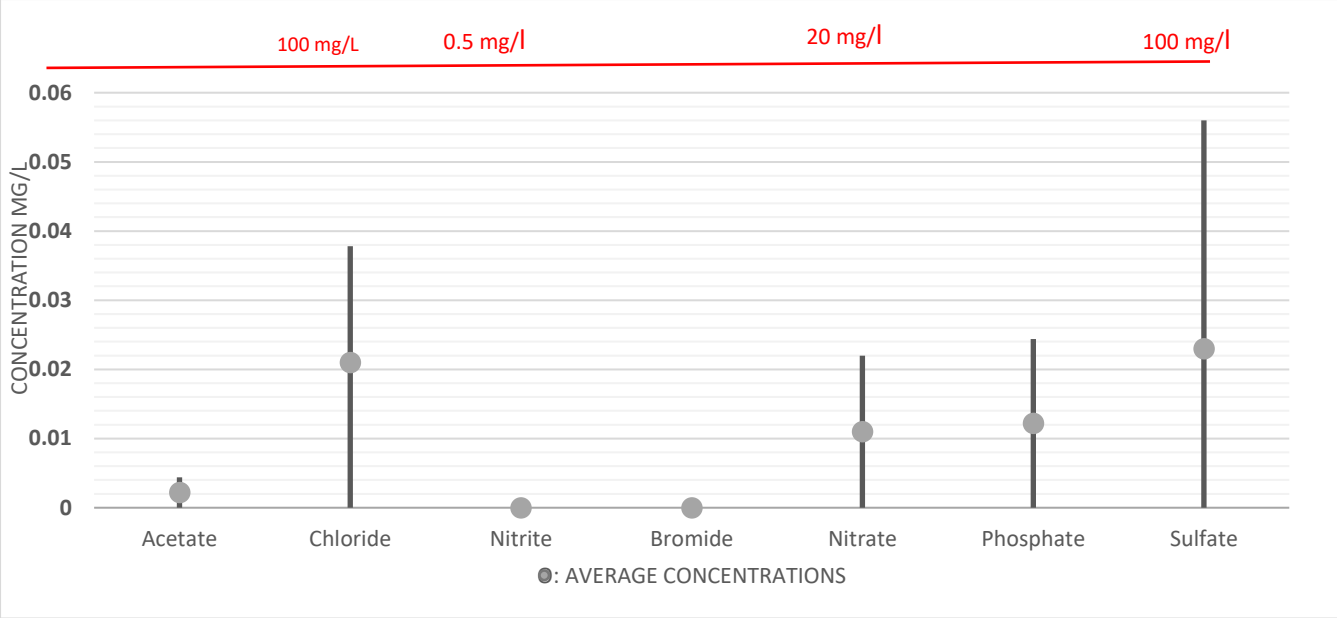


Figure 25 showing the lowest-average-highest concentrations of anions in the HSB tank, Jar 30, Jar 7, Jar 30C over four weeks.

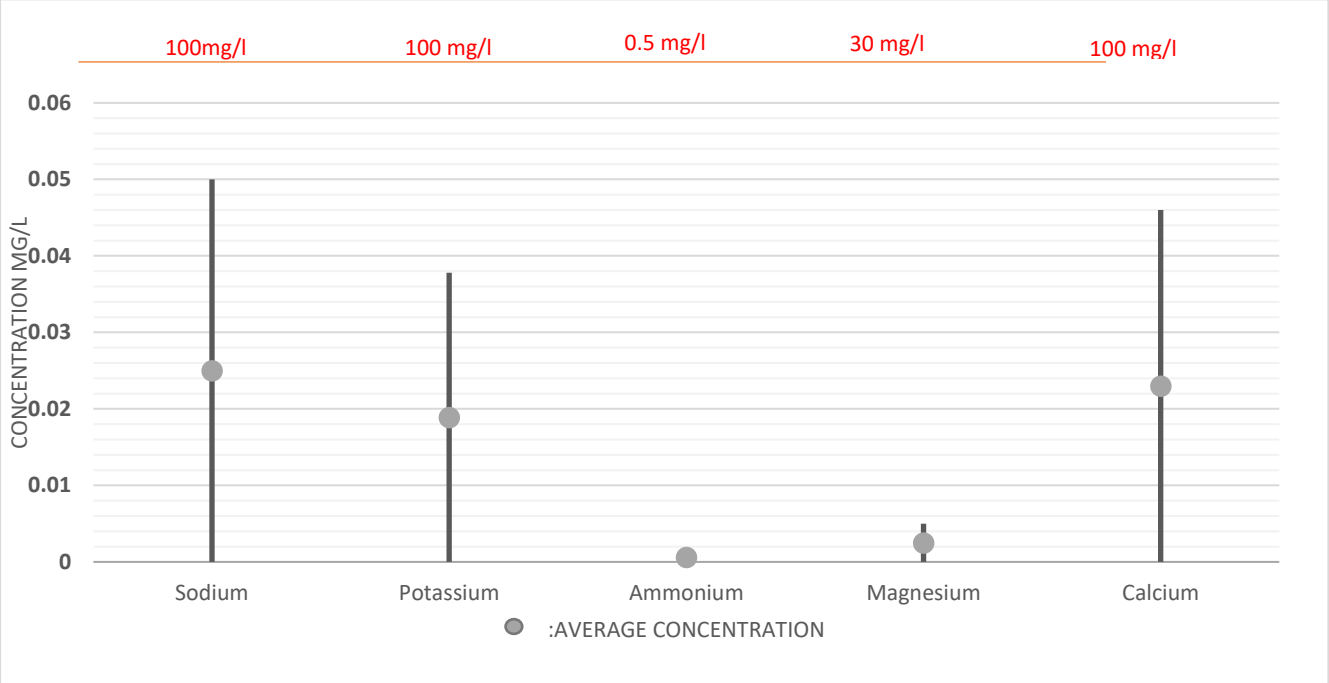
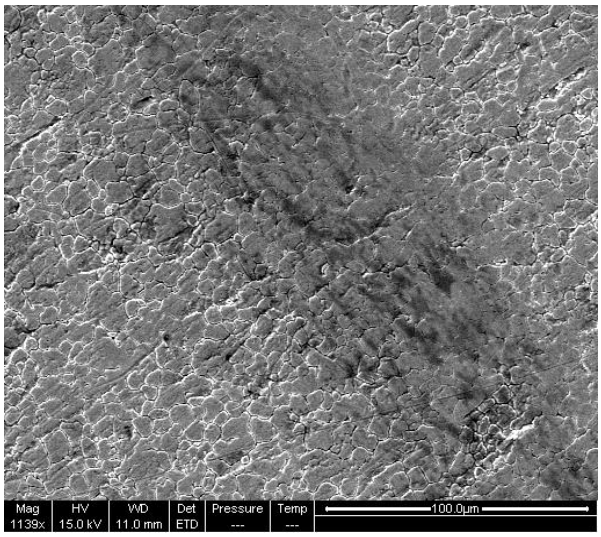


Figure 26 Graph showing the lowest-average-highest concentrations of cations in the HSB tank, Jar 30, Jar 7, Jar 30C over four weeks.

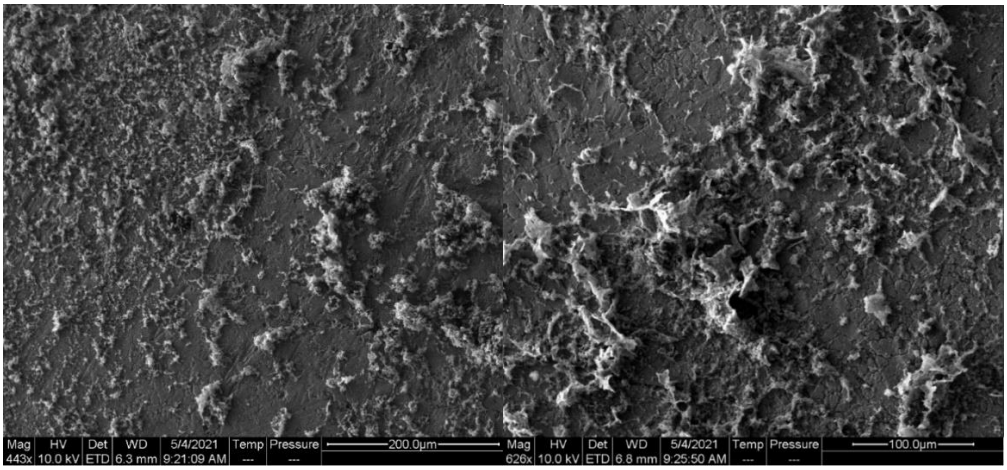
4.4 Biofilm formation

ESEM showed that there was not any biofilm formed on the neither HSB clean tank coupons nor the Jar 30, Jar S4, Jar S7 coupons throughout the study. No difference was observed on different materials coupons in the HSB clean tank. The biofilm started forming on coupons in Jar 30C which had the highest bacterial concentration after two weeks as shown in *figure 27*. In week 2, small and loose biofilm was observed but it was getting thicker and covering more

space over time. A biofilm formation was observed also on Jar 7 coupons at week 3 as shown in *figure 28*. The biofilms covered only small areas of the surface.

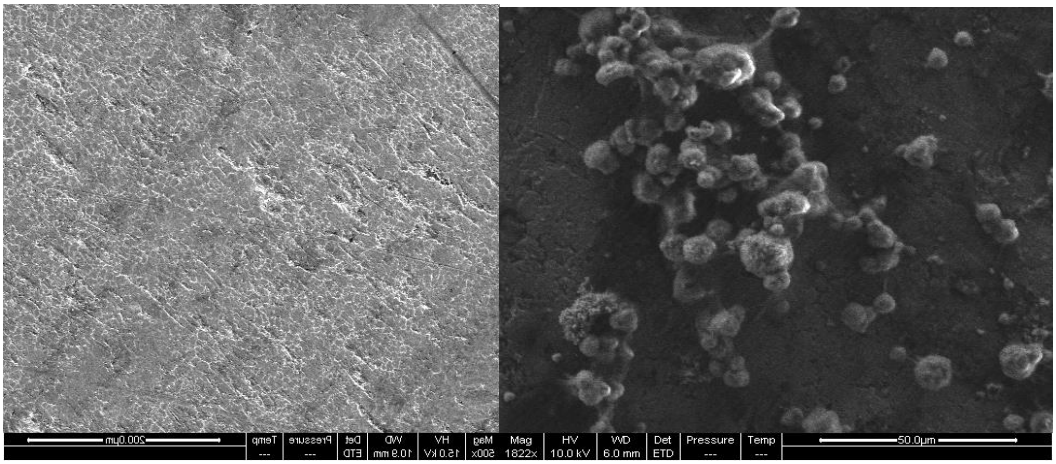


(a)

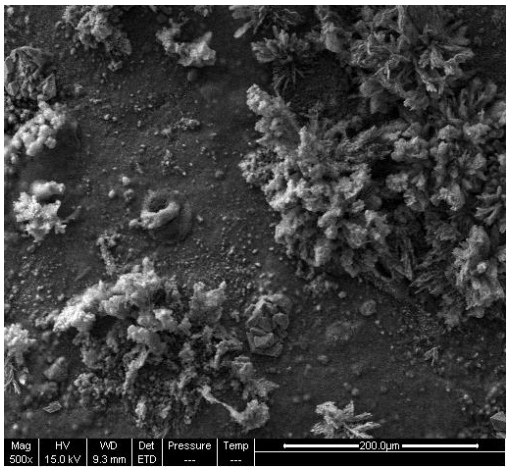


(b) *(c)*

Figure 27 Scanning electron microscopy of microorganisms attached on stainless steel coupons extracted from the Jar 30C at intervals a) reference b) 2weeks c) 3 weeks.



(a) (b)



(c)

Figure 28 Scanning electron microscopy of microorganisms attached on stainless steel coupons extracted from the Jar 7 at intervals a) reference b) 3weeks c) 4 weeks.

4.5 Biofilm quantification

Table 12 and *figure 29* show the biofilm formed on each of the coupons, which was determined by calculating the difference between the initial weight of the coupons after extracting from water after 4 weeks and then drying at 105°C and the final weight after burning at 550°C. Only differences in weights were recorded in Jar 7 and Jar 30C which were 2.7 and 5.5 mg, respectively. This was confirmed by the results of ESEM as only biofilm was noticed only on Jar 7 and Jar 30C coupons as shown in *Figures 27* and *28*. As discussed before, *coliform*, *E. coli* or *L. Pneumophila* were not detected in bulk water in Jar 7 throughout the study, therefore, it is probably other bacteria speciation which are out of interest in this study formed these biofilms.

Table 12. Biofilm quantification on the coupons inserted in HSB tank and Jar 30, Jar 7, Jar 30C.

Coupon name	Weight after drying at 105 °C (g)	Weight after burn at 550°C (g)	Weight difference (mg)	Weight difference/area (mg/cm^2)
HSB clean tank	1.4641	1.4641	0	0
Jar 30	2.4081	2.4081	0	0
Jar 7	4.8928	4.8901	2.7	0.675

Jar 30C	5.6421	5.6366	5.5	1.375

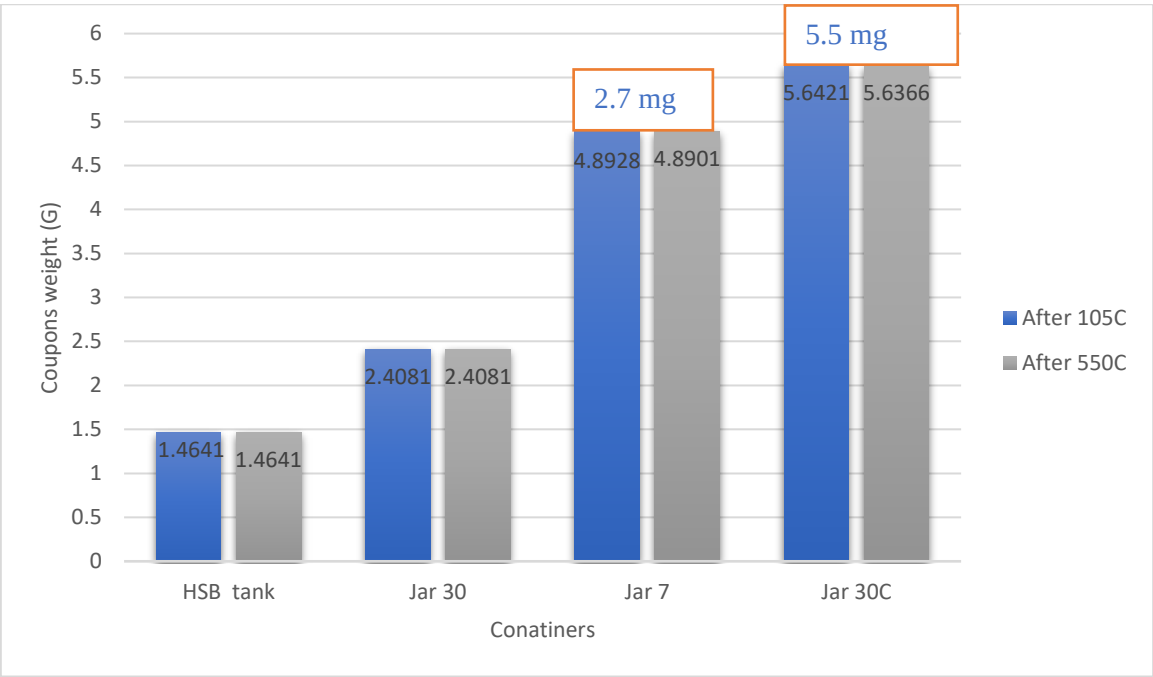


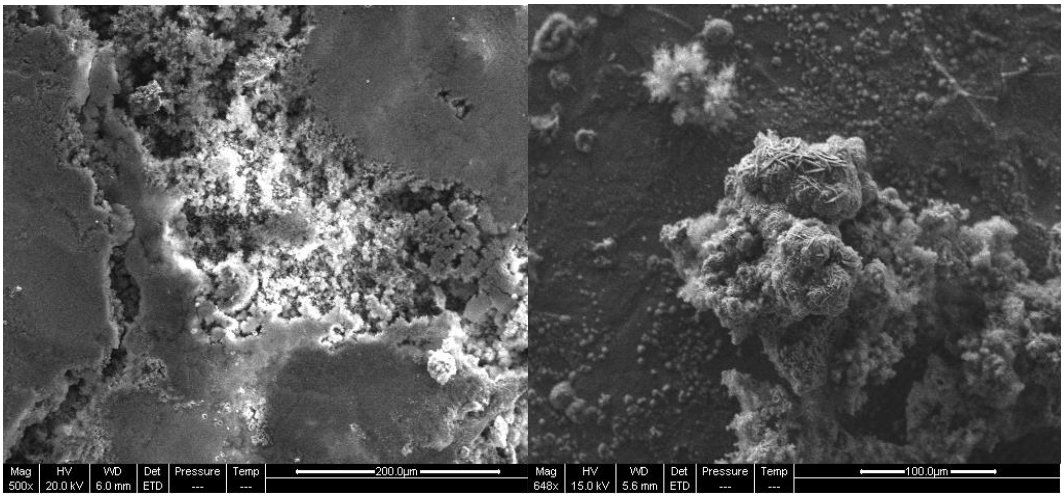
Figure 29 showing the results of biofilm quantification of HSB tank and Jar 30, Jar 7, Jar 30C.

4.6 The effect of storage on bacterial occurrences and biofilm formation

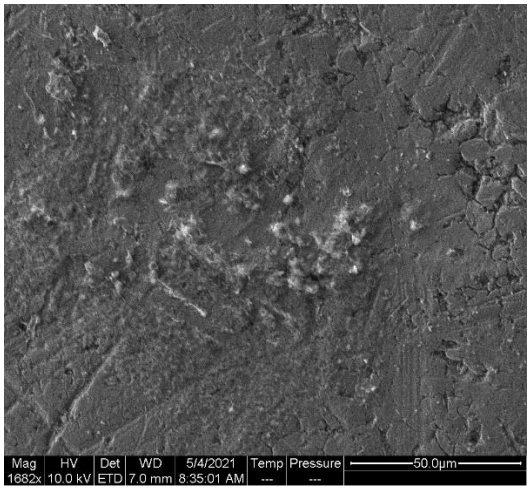
There has not been noticed any storage influence on the water microbial quality under different conditions. Despite of the high TOC measured in storage water because of ethanol, no *Coliform*, *E. coli* and *L. Pneumophila* were detected either in Jar S7 after 7 weeks or Jar S4 after 4 weeks of storage. No biofilms were formed in JS4 throughout the study while loose biofilms were observed on Jar S7 coupon after 7 weeks. *Figure 30* shows comparison between the biofilm formation on coupons in JS4 and JS7 and untreated greywater in a jar at 35°C. This confirms the results of ESEM and biofilm quantification method that some biofilms were formed at low temperature. Surface damages and high corrosion were noticed in the coupons in Jar S4 as shown in *figure 30* (a), *figure 31*. This corrosion was only noticed on steel coupons but not on stainless steel coupons. Therefore, to reduce the potential of microbial growth and biofilm formation in water, non-corrosive materials should be used in the system.

Table 13.. Concentration of total coliforms, E. coli, L. Pneumophila in Jar S4 and Jar S7 after 4 weeks, 7 weeks.

S.No.	Sample	Week Number	Total Coliform (MPN/100ml)	E. coli MPN/100ml)	L. Pneumophila MPN/100ml)	COD Mg/l	TOC Mg/l
1	JS4	Week 0	0	0	0	2	1.52
2		Week 4	0	0	0	1120	438.6
1	JS7	Week 0(source)	0	0	0	2	0.8726
2		Week 8	0	0	0	34	5.690



(a) (b)



(c)

Figure 30 Scanning electron microscopy of microorganisms attached on stainless steel coupons extracted from the intervals a) Jar S4 after 4 weeks b) coupon in untreated greywater after 4 weeks c) Jar S7 after 7 weeks.

(a) (b)

Figure 31 showing a) damage occurred to steel coupon inserted in Jar S4 b) The water in Jar S4 after 4 weeks.

4.7 Biofilm growth dependency on water characteristics and operational conditions

The results of biofilm formation can be interpreted through several factors as following:

4.7.1 Nutrient availability

The biofilms on surfaces inserted in ozonated water was reported to form faster due to the increase of biodegradability of organic matter (Zacheus et al., 2000). To reduce biofilm formation, the AOC concentration should be maintained below 10µg of acetate carbon equivalent per liter (Kooij et al., 1992). Furthermore, the microbial occurrence in water is the main factor contributing to biofilm formation (Zacheus et al., 2000). In general, no *coliform*, *E. coli* or *L. Pneumophila* detected in the Jar 30, Jar 7, Jar S4, Jar S7 and the HSB clean tank throughout the study is the main reason for not forming any biofilms on the coupons. On the other hand, the characteristics of water in the Jar 30C was able to form biofilm at week 2 because of the availability of bacteria in water.

Regarding the inorganic nutrients, the phosphate concentration was recorded below the biofilm enhancing concentration suggested by (Chu et al., 2005; Lehtola et al., 2002) which starting from 1 µg/l. It is noteworthy to mention that the inorganic phosphorus and nitrogen is low even in untreated greywater as shown in *figure 3*. Moreover, nitrogen concentrations, in the forms of nitrate or ammonium, was recorded too far from the biofilm growth enhancing concentration suggested by Chu et al., (2005) which equal to 0.1 N mg/L even in the contaminated jar since the ammonium or nitrate concentration was low even in untreated greywater as shown in *figure 3*. No manganese was detected in the HSB tank and the lab jars which indicate that manganese cannot enhance the biofilm formation in water as Borella et al., (2003) stated that manganese at low concentration (> 3 µg/L) could enhance the *L. Pneumophila* colonization. Ca^{+2}, Mg^{+2} which their significance to *L. Pneumophila* adherence was reported by (Koubar et al., 2013)

lie too far from the drinking water standards which indicates that these elements don't play a role in biofilm formation.

4.7.2 Effect of temperature

Despite the tendency of biofilm formation is likely to occur more at high temperatures than at low temperature (Else et al., 2003), suspected biofilms were formed in Jar 7 while no biofilm was observed in the treated jars at 30℃ and 35°C. This indicates that there was a growth of other speciation of bacteria that enhanced by the lower temperature. This may attribute to other bacteria speciation such as slow growing bacteria that were detected in the results of (Knutsson & Ericsson, 2019) as shown in *figure 11*. For example, mycobacteria are a group of slow growing bacteria that can survive relying on low level of AOC (Bartram et al., 2003). Furthermore, the loss of cultivable mycobacteria was minimal at low 2-4°C temperature compared to high temperature 30 to 37 °C (Traore & Slosarek, 1981).

4.7.3 Surface material

No biofilm formation difference between the two surfaces was recorded but pitted, damaged and corroded surfaces were noticed on the steel coupons in Jar S4. Furthermore, less amount of corrosion was noticed on the stainless-steel and zinc galvanized coupons. As forementioned, the accumulation of biofilms on corroding iron surfaces is much higher than those on noncorrosive materials such as PVC-U, PE, stainless steel (Ninquette et al., 2001; Ollos et al., 2003). Therefore, the potential of biofilm formation increases when corrosive material used in the system especially if other growth favorable conditions are presented. Additionally, it was noticed that corrosion materials were released into water especially in Jar S4 as shown in figure 25. These inorganic matters (mainly iron) can enhance the bacterial growth and biofilm formation (Van der Kooij et al., 2013). Therefore, avoiding use of corrosive materials in the system is preferred.

4.7.4 Effect of flow rate variations

Throughout the study, water stagnation was one of the main features of our experiment in the HSB tank and the lab jars as there was not continuous using for water. 50% of the water in the HSB clean tank and Lab jars was replaced to create water recirculation to build up new organic matters and feed the tank with contamination if presented. No biofilm was formed in Jar S4 throughout the study despite of the availability of several conditions which promoting biofilm formation such as water stagnation, temperature, and high TOC. This can be attributed to the high-water quality because if any of these bacteria are presented in water, it is highly expected to grow under these favorable conditions. For example, Leoni et al., (2005) stated that higher level of *L. Pneumophila* in centralized systems can be attributed to the stagnation of water in storage tanks which enhances to biofilm formation (Leoni et al., 2005).

4.7.5 Effect of pH on biofilm formation

Throughout the study pH was recorded in the range from 5 to 6.5. pH drops to isoelectric pH (e.g., isoelectric pH for PVC = 5.4) is able to reduce the electrostatic repulsion status between the bacteria and the surface which in turn enhances the attachment of the cells to surfaces (Liu et al., 2016). The isoelectric pH of the stainless-steel coupons is 2.4-3 which is not reached during the experiment which in turn does not seem to encourage any attachment between the cells and the surfaces. Therefore, it is recommended to use materials with isoelectric pH not close to water pH.

4.7.6 Disinfectant residuals

No disinfectant residuals were maintained in the tank and the jars which give a chance to biofilm formation if other conditions provide favorable environment to bacterial growth.

It can be concluded that the current treatment processes producing a high-quality water which have not experienced any *coliform*, *E. coli* or *L. Pneumophila* deterioration throughout the study despite the presence of combination of bacterial growth motivators (High temperature, water stagnation, surface materials, and no disinfectant residuals). Despite of the microbial stability observed under different conditions, the treated water is still not sterile and there is always a possibility for microbial deterioration in the tanks. Therefore, controlling growth favorable conditions is important to reduce microbial deterioration potentials. While some of these conditions including temperature, flow variations and stagnation is difficult to be controlled, the concentration and nature of biodegradable organic matter, water pH, surface materials are possible to be controlled to reduce the potential of biofilm formation as forementioned.

4.8 Limitations during monitoring treated greywater microbial stability.

Throughout the study, several challenges were faced that might affect the results. The monitoring program should be conducted over longer time to ensure that there will not be any microbial deterioration. The intermittent use of treated greywater during the experiment is considered another study limitation. To be more realistic, continuous use of treated greywater should occur throughout the experiment. Due to limited budget of the study, it was not possible to conduct different tests that are significant to obtain more precise results. Measuring AOC concentration in water is important to estimate the potential of a water for supporting microbial growth. A microbiological method (bioassay) that used to measure AOC in water requires professional persons as well as additional costs. Further studies are needed in the future to test the presence and growth of other speciation of bacteria not only *coliform*, *E. coli* or *L. Pneumophila*. Measuring heavy metals required for microbial growth is required to estimate the potential growth. These metals include iron, copper, Zinc etc. Furthermore, to obtain more representative, nondestructive, and in situ biofilm formation monitoring, biofilm sensors can be used in water. Molecular biological methods are preferred to be used for determination of specific microorganisms in water. This also could not be performed because of the limited budget as well as the need of professional persons. Moreover, to get better-quality images from ESEM, sputtering golden coat on biofilm samples is required.

Finally, the absence of specific standards for greywater reuse for showering has increased the uncertainties regarding the validity of the current quality for showering. Furthermore, the standards for showering reuse may be at lower quality than those for drinking water. This in turn leads to increase the cost of treatment and monitoring to provide a considerable safety factor.

5 Conclusion and suggestions

This study provides evidence that the HSB greywater treatment system installed in HSB LL produces high quality water which have not experienced any microbial regrowth in tanks since no *coliform, E. coli* or *L. Pneumophila* and biofilm formation were detected throughout the study. Therefore, freshly produced water quality which considered appropriate for showering reuse application According to drinking water quality standards were at the same quality through four weeks of monitoring despite of testing several favorable bacterial growth conditions such as temperature, water stagnation surface materials etc. This microbial stability is attributed to two main reasons: Firstly, none, or very few *coliforms, E. coli* or *L. Pneumophila* were initially presented in the treated water. Secondly, the produced water characteristics such as nutrient availability were not found to be sufficient for bacterial growth, if presented.

On the other hand, the formation of biofilm on coupons at low temperature indicated that there is possibility of bacteria specifications other than *coliforms* and *L. pneumophila* which were not tested throughout the experiment. Therefore, testing for other bacteria speciation is necessary to avoid posing any health risks to the users.

Despite the microbial stability which was experienced during the study, additional measures can be done to reduce the microbial deterioration potential the future. In general, to maintain water stability in tanks, several conditions should be fulfilled. These conditions include the nature and concentration of biodegradable compounds available in freshly treated water, the released of biodegradable compounds from construction materials, the water temperature, the hydraulic and stagnation conditions as well as the maintaining of disinfectant residuals in the tank. Throughout this study, despite of testing the favorable situation of these conditions, *no coliform, E. coli* or *L. Pneumophila* growth was observed.

Some conditions such as temperature and hydraulic and stagnation conditions are difficult to control while maintaining disinfectant residuals in tanks are considered unfavorable because of the potential of toxic by-products. The biodegradable matter and the materials used in construction can be controlled to prevent any microbial growth. Therefore, measuring AOC is significant throughout the monitoring process to estimate bacterial growth potential since TOC cannot provides a guarantee for preventing microbial growth. Furthermore, using non-corrosive materials which do not release biodegradable compounds such as stainless steel is important.

Finally, the results of this study should be viewed as a start point for a long-term monitoring to ensure continuous providing of safe water for showering.

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7 Appendix

Appendix I

S.No.	Sample	Week Number	*Total Coliform* (MPN/100ml)	*E. coli* MPN/100ml	*L. Pneumophila* MPN/100
1	Treated water (Reference)	Week 0 (Reference)	0	0	0
1	HSB LL clean tank	Week 1	2	0	0
2		Week 2	0	0	2.3
3		Week 3	0	0	
4		Week 4	0	0	0
1	Jar 30	Week 1	0	0	0
2		Week 2	0	0	0
3		Week 3	0	0	0
4		Week 4	0	0	0
1	Jar 30C	Week 1	12.1	20.7	9.4
2		Week 2	1011.2	51.2	149
3		Week 3	7.5	0	5.8
4		Week 4	22.7	5	154.7
1	Jar 7	Week 1	0	0	0
2		Week 2	0	0	0
3		Week 3	0	0	0
4		Week 4	0	0	0

Appendix II

Sample	Week No.	Litium	Sodium	Ammonium	Potassium	Magnesium	Manganese	Calcium
HSB clean tank	W 1	n.a.	0.001	n.a.	n.a.	0.001	n.a.	0.0187
	W 2	n.a.	0.0014	n.a.	0.003	0	n.a.	0.0097
	W3	n.a.	0.0046	0.0004	0.0021	0.001	n.a.	0.0188
	W 4	n.a.	0.0003	n.a.	0.0024	0	n.a.	0.0094
Jar 30	W 1	n.a.	0.0289	n.a.	0.0021	0.001	n.a.	0.0191
	W 2	n.a.	0.0031	n.a.	n.a.	0.001	n.a.	0.0116
	W 3	n.a.	0.003	n.a.	n.a.	0.001	n.a.	0.0283
	W 4	n.a.	0.0031	n.a.	n.a.	0.001	n.a.	0.0135
Jar 30C	W 1	n.a.	0.0408	0.0012	0.0023	0.003	n.a.	0.0308
	W 2	n.a.	0.0406	n.a.	0.0028	0.004	n.a.	0.0421
	W3	n.a.	0.0577	0.0003	0.0038	0.005	n.a.	0.0463
	W4	n.a.	0.0444	n.a.	0.0028	0.004	n.a.	0.0398

Jar 7	W 1	n.a.	0.0051	n.a.	0.001	0	n.a.	0.0145
	W 2	n.a.	0.0046	n.a.	0.0014	0.001	n.a.	0.0153
	W 3	n.a.	0.0025	n.a.	n.a.	0.001	n.a.	0.018
	W 4	n.a.	0.0015	n.a.	n.a.	0	n.a.	0.0104
Jar S4	W 0	n.a.	0.0029	n.a.	0.0015	0.001	n.a.	0.0146
	W 4	n.a.	0	n.a.	n.a.	0.001	n.a.	0.0107
Jar S7	W0	n.a.	0.0019	n.a.	n.a.	n.a.	n.a.	0.0007
	W 7	n.a.	0.0052	n.a.	n.a.	0.001	n.a.	0.0132

Appendix III

Sample	Week No.	Acetate	Chloride	Nitrite	Bromide	Nitrate	Phosphate	Sulfate
HSB clean tank	W 1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	W 2	n.a.	0.0217	n.a.	n.a.	n.a.	n.a.	0.0249
	W 3	n.a.	0.0222	n.a.	n.a.	n.a.	n.a.	0.0248
	W 4	n.a.	0.0212	n.a.	n.a.	n.a.	n.a.	0.024
Jar 30	W 1	n.a.	0.0254	n.a.	n.a.	n.a.	n.a.	0.056
	W 2	n.a.	0.021	n.a.	n.a.	n.a.	n.a.	n.a.
	W 3	n.a.	0.0203	n.a.	n.a.	n.a.	n.a.	n.a.
	W 4	n.a.	0.0203	n.a.	n.a.	n.a.	n.a.	n.a.
Jar 30C	W 1	n.a.	0.0281	n.a.	n.a.	n.a.	n.a.	0.0282
	W 2	n.a.	0.0325	n.a.	n.a.	0.022	n.a.	0.0319
	W 3	n.a.	0.0378	n.a.	n.a.	n.a.	n.a.	0.0327
	W 4	n.a.	0.0348	n.a.	n.a.	n.a.	n.a.	0.0317
Jar 7	W 1	0.0044	0.021	n.a.	n.a.	0.02	0.0244	0.0243
	W 2	n.a.	0.0211	n.a.	n.a.	0.021	n.a.	n.a.
	W 3	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	W 4	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Jar S4	W 0	n.a.	0.0205	n.a.	n.a.	n.a.	n.a.	n.a.
	W 4	0.1406	0.0289	n.a.	n.a.	0.025	n.a.	0.0619
Jar S7	W 0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.2064
	W 7	n.a.	0.0209	n.a.	n.a.	n.a.	n.a.	n.a.