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BIOLOGICAL CHARACTERISTICS OF SOME MICROBES CONTRIBUTING IN BIOFILM FORMATION

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In partial fulfillment of the requirements

For the award of the degree of

DOCTOR OF PHILOSOPHY IN Microbiology

BY

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Under The Guidance of

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DEPARTMENT OF AGRICULTURAL MICROBIOLOGY

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B.Sc. Agric. Sci. (Microbiology), Fac. Agric., Zagazig Univ., 2004

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2015

APPROVAL SHEET

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Date: / / 2015

SUPERVISION SHEET

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ABSTRACT

The aims of this study were to determine microbial population contributing in natural biofilm. In addition to, study the behavior and biological characteristics of two pathogenic bacteria including *L. monocytogenes* and *S.* Typhimurium in biofilm state. To achieve first aim, forty eight natural biofilm samples were collected from different types of microhabitats to determine the characteristics of the predominant microorganisms. The results of natural biofilm samples showed that the most frequent pathogens were *Staph. aureus*, *L. monocytogenes*, *Salmonella* spp and *Candida albicans* in all tested samples. While, to achieve second aim, water distribution system model was designed for testing of these bacteria. Six samples of synthetic biofilm were collected at three different biofilm ages (10, 40 and 90 days-old). The collected biofilm samples were exposed to different antimicrobial agents. Results cleared that the highest growth of *L. monocytogenes* and *S.* Typhimurium biofilm was reported in I pipe material. Results showed the log reduction of 90 days-old *L. monocytogenes* biofilm formation, when exposed to 3.0 mg/l of chlorine dose, on six pipe materials; PVC, PP, PE, I, Cu and R been 3.96, 4.16, 4.21, 4.17, 4.32 and 4.03 CFU/cm^2, respectively. While, the effective MICs of Ag ions were 500 mg at 5 min against 90 days-old *L. monocytogenes* and 300 at 15 min for *S.* Typhimurium biofilm. Additionally, the effective MICs of AgNPs were recorded 500 mg at 15 min for 90 days-old *L. monocytogenes* and 500 mg at 10 min for *S.* Typhimurium. On the other hand, the results of antibiotic susceptibility indicated the planktonic cells were more sensitive than the biofilm cells. Also, the longest survival time in tested water of *L. monocytogenes* and *S.* Typhimurium biofilm cells scraped from I pipe material was observed. In contrast, the shortest survival time of biofilm cells scraped from Cu pipe material was recorded.

Key words: Biofilm, pathogenic microbes, EPS, chlorine, AgNPs, antibiotics.

DEDICATION

I dedicate this work to whom my heartfelt thanks; to my mother, my wife and my son (Mazen), for their patience, help and for all the support they lovely offered along the period of my post graduation.

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*Praise and thanks be to **ALLAH**, for assisting and directing me to the right way.*

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CONTENTS

INTRODUCTION

Drinking water distribution systems (DWDS) are complicated engineering systems consisting of pipes, storage vessels, fittings, and valves. DWDS are made of a variety of different materials such as cast iron, polyvinyl chloride (PVC) and polyethylene (PE) that interact with the bulk water. The water that consumers drink at the tap has traveled potentially large distances taking significant durations through the distribution network and it is accepted that from leaving the treatment plant deterioration in quality might occur. This deterioration in water quality will be influenced by factors such as decay of disinfectant residual, temperature, hydraulic regime, water residence time and bacterial regrowth (Ramos *et al.,* 2010).

The supply of safe drinking water to the public is one of the great technological advancements of the 19th century, as well as a major technological challenge (Poitelon *et al.,* 2009). It is well known that microorganisms are widely present in the drinking water treatment systems especially, in the storage tanks, filter systems, and the interior of pipe walls (Bonadonna *et al.,* 2009).

Uncontrolled and excessive microbial growth not only leads to the deterioration of water quality and the associated undesirable tastes, odors, and visual turbidity, but can also cause process malfunctioning such as clogging of filters, biofouling and biocorrosion (Hammes *et al.,* 2008). Additionally, many problems in DWDS can be associated with biofilm formation, such as hosting opportunistic pathogens (Sun *et al.,* 2014), and promoting nitrification (Gomez-Alvarez *et al.,* 2009).

Therefore, a sound understanding of microbial community structure of DWDS biofilm and its influential factors is of great importance in designing effective control strategies and thus improving drinking water quality for the consumer (Liu *et al.,* 2013a). Many studies usually used to simulated DWDS or bioreactors to study biofilm microbial communities. These studies have been documented that the microbial community structure of DWDS biofilm can be affected by a variety of factors such as pipe materials, disinfectants, water age and biofilm age (Krishna *et al.,* 2013; Revetta *et al.,* 2013; Wang *et al.,* 2014).

Several years can be necessary for the achievement of the steady state of biofilm formation (Liu *et al.,* 2012), which limits the relevance of short-term model studies (Berry *et al.,* 2006). Due to limited access and high cost involved in sampling biofilm within real DWDS, so far, the composition and dynamics of bacterial communities in real

DWDS remain poorly understood. In addition, although it has been reported that different water sources (ground water and surface water) can result in a significant difference of bacterial community diversity and composition in real DWDS (Sun *et al.*, 2014), it remains unclear whether or not the application of different purification strategies for the same raw water can play an important role in shaping biofilm microbial community structure in real DWDS. Therefore, the objectives of the present study were determining the microbial population contributing in biofilm formation. In addition to, study the behavior and biological characteristics of some pathogenic bacteria in biofilm state. These aims were achieved by the follows;

1. Using rapid methods (enzymatic culture based method) for detection and determination of microbial communities in natural biofilm samples from microhabitats.
2. Determination impact of pipe materials on biofilm formation and growth rate.
3. Comparing the conventional disinfectant agent (chlorine) and alternative disinfectant agents (silver and silver nano-particles) against biofilm cells.
4. Comparing the effect of different disinfectants or antimicrobial agents on planktonic and biofilm cells.
5. Comparing the susceptibility of different ages of biofilm cells to disinfectant agents.
6. Determination the sensitivity of biofilm cells to different antibiotics.
7. Selection the proper plumping material to be used in the drinking water distribution system and households.

REVIEW OF LITERATURE

Water is the most essential substance for all life on earth and a precious resource for human civilization. Reliable access to clean and affordable water is considered one of the most basic humanitarian goals, and remains a major global challenge for the 21st century (WHO, 2012).

Drinking water (DW) is water of sufficient quality to ensure that it can be consumed or used without risk of immediate or long term harm. The provision of safe DW is considered a top priority in civilized societies. Microbiologically and chemically contaminated DW has been linked with several health problems (WHO, 2011). The consumption of contaminated DW can cause a wide range of diseases and health related problems in all people or in those more susceptible, like infants, young children, elderly or unwell people or those who are immunecompromised. So, the DW treatment is a necessary process to ensure the public health security. In addition, Deborde and von Gunten (2008) indicated the DW is one of the most closely monitored resources. It is subjected to several treatment processes until it is ready to be distributed and consumed. However, after the distribution starts the treatment continues; drinking water distribution systems (DWDS) has always a residual concentration of disinfectant in order to decrease the possible regrowth events.

Treated drinking water enters distribution systems with a physical load (particles), a microbial load (biomass) and a nutrient load (biomass and nutrients), as a result of biological and physiochemical processes during drinking water distribution.

In general, the water at consumers' taps has low quality than the final effluent from the drinking water treatment plant (Verberk *et al.*, 2007). These may be due to, the recognized harbors of pathogenic microbial contaminants in DWDS are biofilm that develop in the inner walls of pipes of distribution networks (Sharma, 2003). In these microbial consortia, pathogenic microorganisms are protected from stress conditions (chlorine, shear stress, temperature, antibiotics and other disinfectants), which allows them to remain viable. When, the detachment of portions of the biofilm occurs, they enter bulk water, which permits a possible outbreak of disease (Huq *et al.*, 2008). It is well known that biofilm constitute one of the major microbial problems in DWDS, which contributes to the deterioration of water quality (Momba *et al.*, 2000).

However, their elimination from these systems is almost impossible. But several aspects can be considered in order to prevent and control their growth, particularly the nutrient content of water, the concentration of residual disinfectant, the hydrodynamic conditions of the network, the pipe materials and their conservation conditions, the diversity of microorganisms present in DWDS and environmental factors, like pH and the temperature of water (Simões *et al.*, 2012)

12. Drinking water needs and concerns

WHO (2011) defines drinking water (DW) as water of enough quality to ensure that it can be used or consumed without risk of immediate or long-term harm. The consumption of water contaminated by chemical or biological can be responsible for the development of a range of health related problems in people, mainly those more susceptible (infants, children, elderly or immunocompromised people). Additionally, the DW concept can be different in developing and developed countries. In developing countries the main concern is about microbial contamination that is responsible for 45% of all deaths being the chemical contamination insignificant. While in developed countries both problems constitute a concern (Gilbert, 2012).

The risks and impacts of chemical contaminants are a concern in developed countries. However, the risks from microbial pathogens remains ever present and waterborne diseases are one of the most important water-associated health problems (Farkas *et al.*, 2013).

13. Drinking water quality assessment-microbiological aspects and biofilm

Tap water supposes not to be and is not sterile, the microbial load in bulk water consisting mainly in inoffensive heterotrophic microorganisms that most probably coming from the biofilm by detachment during dispersion. Routine monitoring of raw water sources, treated water, drinking water in pipe networks, service reservoirs and finally at the consumers must be done.

According to European and WHO regulations, the microbial indicators assessed by standardized conventional culturing techniques are: the colony counts at 37^{o}C and 22^{o}C, total coliform, *Escherichia coli*, intestinal enterococci and *Clostridium perfringens*. The greatest microbial risk being associated with ingestion of water contaminated with human or animal feces, thus the potential presence of pathogenic bacteria, viruses and cysts of protozoan parasites; fecal indices (*E. coli*, intestinal enterococci and *Cl. perfringens*) presence must be routinely investigated. The shortcomings of water quality monitoring based on fecal indicators and heterotrophic plate count, resulting in underestimation of drinking water microbial populations in numbers and composition are discussed worldwide considering the following;

a. Only a small volume approximated to represent from 2 x10^{7} to 5 x10^{7}CFU/ml of delivered drinking water is examined in routine monitoring (Allen, 2011);

b. In drinking water systems, the high majority of bacteria, estimated at 95%, are located attached at the surfaces (biofilm), while only 5% are found in water phase (planktonic cells) and detected by sampling as commonly used for quality control (Flemming *et al.*, 2002). Other studies are indicating bacterial numbers characterizing the biomass in pipe biofilm being 25 times more abundant than the suspended cells (Servais *et al.*, 2004). Although, the common idea that biofilm dominate the distribution systems has been proven to be not true under all conditions by Srinivasan (2008), whose findings suggest that bulk bacteria may dominate in network sections containing chlorine residuals lower than 0.1mg/l and having residence time longer than 12 hrs.

c. A significant percent of water and biofilm disburden may be in a viable but non cultivable state, unable to grow on artificial growth media but alive and capable of renewed activity and so hygienically relevant (Moritz *et al.*, 2010). A small fraction of waterborne microorganisms (0.01%) are estimated to be culturable heterotrophic bacteria (Watkins and Jian, 1997; Exner *et al.*, 2003);

d. Limitations of detection methods (Lehtola *et al.*, 2006; 2007; September *et al.*, 2007). The investigation of drinking water associated biofilm from four European countries namely France, great Britain, Portugal and Latvia confirmed *E. coli* presence by culturing techniques in one out of five pipes whereas all networks except one were positive for *E. coli* using the PNA FISH methods; their viability was also demonstrated. *E.coli* contributed with percents from 0.001% to 0.1% in the total bacterial numbers (Juhna *et al.*, 2007);

e. Fecal indicators are the best predictors of potential risks, but their concentrations rarely correlate perfectly with those of pathogens (Payment and Locas, 2011).

14. Biofilm in drinking water distribution systems

Biofilm are a set of microorganisms embedded in a matrix of extracellular polymeric substances (EPS), which are secreted by them, and attached to a surface. Biofilm are a protective niche for the microorganisms (Fang *et al.* 2010). As biofilm occur usually in wet surfaces, their presence in DWDS is unavoidable.

Biofilm formation, also known as biofouling, is a well recognized problem in the water industry and DWDS. Biofouling, in general, refers to the undesirable accumulation of biotic matter on a surface. It has been shown to be of considerable hygienic, operational and economical relevance, not only in DWDS but also in other purified water supply systems, such as dental unit waterlines, (Franco, *et al.*, 2005) dialysis units, (Pontoriero *et al.*, 2003) laboratories (McFeters *et al.*, 1993), reverse osmosis systems (Flemming, 1997), pharmaceutics (Riedewald, 1997), the semiconductor industry (Kim *et al.*, 1997). Biofilm formation depends on several biotic and abiotic factor, namely environmental factors (temperature and pH), concentration of residual disinfectants, nature and concentration of nutrients, hydrodynamic conditions (flow rate, design of network and presence of dead ends), type and conservation of pipe materials, type and diversity of microorganisms and sediment accumulation (Jang *et al.*, 2011).

Bacteria seem to initiate biofilm development in specific environmental conditions, such as nutrient availability (O'Toole *et al.*, 2000). For example, biofilm will be formed most rapidly on surfaces that are rougher, hydrophobic and coated by surface conditioning films (Donlan, 2002). Cell attachment can also be encouraged by an increase in flow velocity and by water temperature or nutrient concentration changes, if these factors do not exceed critical values (Simões *et al.*, 2007). So, the cell attachment to a surface and subsequent biofilm formation is a very complex process, with many variables affecting the process. This biological film is considered a stable point in a biological cycle that includes roughly four different steps: initiation, maturation, maintenance and dissolution (O'Toole *et al.*, 2000).

In drinking water systems, virtually any surface in contact with water will be colonized by microorganisms (Wingender and Flemming, 2004). The tendency of bacteria to attach to surfaces has been shown early in the twentieth century in studies performed by Henrici (1933) and ZoBell (1943) but the term biofilm was not established until the 1970s (Costerton *et al.*, 1987). Since that time the ubiquity and significance of biofilm in various sectors not only including almost any natural environment, but also household, medical, industrial and technical systems have become increasingly clear (Hall-Stoodley and Stoodley, 2009).

Despite the fact that drinking water systems provide relatively unfavorable conditions, including low nutrient levels, low temperatures, high shear forces and disinfectant residuals, materials exposed to drinking water systems are rapidly colonized reaching maximal cell counts within weeks. It has been estimated that 95 % of the overall bacterial biomass in drinking water distribution systems can be located in biofilm on surfaces, while only 5% occur in the water phase (Flemming *et al.*, 2002), but a domestic

hot water system, 72 % of the culturable bacteria were found to be surface-associated (Bagh *et al.*, 2004).

Colonization of the internal surfaces of drinking water systems mostly occurs thin and patchy in the form of single cells or micro-colonies (Wingender and Flemming, 2004), but occasionally dense multilayer biofilm have been detected (Kilb *et al.*, 2003). The extent of biofilm formation in drinking water systems can be assessed either directly by scanning electron microscopy (Lee *et al.*, 2011) or by determination of total cell counts and culturable microorganisms of biofilm using epi-fluorescence microcopy. In several months to years old biofilm on pipe surfaces or coupons exposed to drinking water, it was found the total cell counts ranged between 10^4 - 10^8 cells/cm^2 were detected (Wingender and Flemming, 2004; Långmark *et al.*, 2005).

Concentrations of culturable heterotrophic plate counts (HPC) bacteria in established biofilm (younger biofilm) were found to vary between approximately 10^1 - 10^6 colony forming units (CFU)/cm^2 (Långmark *et al.*, 2005). In oligotrophic water environments such as drinking water systems, the numbers of culturable bacteria can be several orders of magnitude lower than the total cell numbers. Usually, the fraction of culturable bacteria represent between approximately 0.01 % and a few percent of the total cell counts (Wingender and Flemming, 2004).

Biofilm is present on all surfaces exposed to water during the different steps in drinking water production and supply including abstraction from source water, treatment (coagulation, filtration), disinfection, distribution (water mains, plumbing systems in buildings, *etc.*) and drinking water storage, filters, distribution pipes, storage tanks, tap fittings and shower heads provide suitable surfaces for microbial colonization (Eboigbodin *et al.*, 2008).

By the time the water reaches the consumer's tap, its quality may differ from the quality at the time of treatment in the waterworks (Pepper *et al.*, 2004). Apart from aesthetic problems such as changes in odor, taste, color and turbidity of the water caused by drinking water biofilm (Kerr *et al.*, 2003), it has become obvious that microorganisms with pathogenic properties can persist and multiply in biofilm of drinking water systems. Biofilm have the ability to function as an environmental reservoir for pathogenic microorganisms and thus are potential sources of contamination. They present a health risk when pathogens are released from the biofilm and are transmitted to susceptible human hosts upon exposure to contaminated water. Several strict or opportunistic pathogens including bacteria, viruses and protozoa have been shown to survive in biofilm isolated from drinking water systems (Bonadonna *et al.*, 2009).

According to WHO and the European community directive, drinking water should meet the quality requirements at the point of consumption. A high water quality has to be maintained throughout the distribution system, including passage through domestic plumbing. Therefore it is important to investigate and understand biofilm in both distribution mains and domestic plumbing. Conditions prevailing in domestic plumbing systems might differ significantly from those in water mains. The inner diameter of pipes is much smaller resulting in a larger ratio of surface to water volume (WHO, 2008).

As a consequence of longer periods of stagnation and insufficient insulation of the pipes, the water temperature increases favoring microbial growth. In domestic plumbing, the choice of construction materials is less regulated than in the distribution system leading to a large variety of materials utilized. Some of these materials might leach nutrients and thus support massive biofilm growth (Flemming *et al.,* 2002; Bressler *et al.,* 2009).

15. Composition of extracellular polymeric substances (EPS) and their roles in biofilm formation

Drinking water (DW) biofilm are composed of complex microbial communities functionally organized and embedded in a gelatinous matrix of extracellular polymers excreted by microorganisms. Extracellular polymers, also known as extracellular polymeric substances (EPS) are the key substances keeping biofilm organisms together, gluing them to the surface and providing protection against agents of stress. Also, any inorganic particles passing nearby (corrosion products, clays, sand, *etc.*) may also be incorporated in the biofilm increasing its mechanical strength (Simões *et al.*, 2010).

The EPS consisting of polysaccharides, proteins, DNA and lipids in varying ratios contribute to the mechanical stability of microbial biofilm. So, they play an important role in the organization of the biofilm community (Flemming and Wingender, 2010). According to Tsuneda *et al.* (2003), the amounts of polysaccharides account for 75-89% of the biofilm EPS composition, indicating that they are the major components.

The EPS matrix provides the shelter from environmental stress such as a high salinity, extreme pH, UV radiation, chlorination and desiccation and thus permits survival under hostile conditions. One advantage is the ability of the extracellular polymeric matrix, which they excrete to capture and concentrate environmental nutrients, such as carbon, nitrogen and phosphate (Flemming and Wingender, 2010). Another advantage of the biofilm mode of growth is that it enables resistance to a number of removal strategies,

particularly antimicrobial and mechanical stresses (Bridier *et al.,* 2011). DWDS disinfection with chlorine dioxide and chlorine, for example, can reduce the concentration of planktonic bacteria, but have little to no effect on the concentration of biofilm bacteria (Gagnon *et al.,* 2005; Simões *et al.,* 2010).

Furthermore, biofilm act as a diffusion barrier to antimicrobial agents, slowing penetration (Simões *et al.,* 2007; Bridier *et al.,* 2011). Antimicrobial agents interact with EPS components, reducing the agent's concentration and their effectiveness (Stewart, 2003; Bridier *et al.,* 2011). Another advantage of living in a biofilm is the possibility of metabolic interactions between bacteria with different physiological requirements (Stewart, 2003; Stewart and Franklin, 2008). This will promote the formation of spatial niches in a biofilm in response to environmental conditions and the activity of their neighbors in order to optimize the nutritive resources (Stewart and Franklin, 2008).

16. Outbreaks of biofilm formation in drinking water distribution system

Several outbreaks with public health risks occurred due to malfunctioning DW treatment plants and distribution networks, which failed to maintain an adequate level of disinfectant to prevent the growth of pathogens and/or harbored the pathogens. Waterborne outbreaks have been due to *Escherichia coli* O157:H7, *Campylobacter* spp., *Helicobacter pylori, P. aeruginosa*, *Salmonella* Typhimurim, *Cryptosporidium parvum*, *Giardia* and some viruses (norovirus, calcivirus, enterovirus) in Canada, France, Italy, England, Finland, Switzerland, Northern Ireland, Norway, Belarus, New Zealand, Poland and the United States (Kvitsand and Fiksdal, 2010).

Thirty one events in 2000-2001 and 30 events in 2003–2004 in the USA, from 1920 to 2002, at least 1870 outbreaks were associated with drinking water in the United States, an average of 22.5 per year and 883 illnesses, which amounts to an average of 10-648 cases each year (Craun *et al.,* 2006; Liang *et al.,* 2006). These outbreaks were attributed to the microbial contamination with viruses (norovirus, hepatitis A virus), protozoa (*Cryptosporidium* spp., *Giardia* spp., *Naegleria fowleri*) and pathogenic bacteria (*Salmonella typhimurium*, *Vibrio cholerae*, *Legionella* spp., *Shigella* spp., *E. coli* O157:H7 and *Campylobacter jejuni*) or to chemical/toxins. According to WHO (2009) report between 2000 and 2007 in 14 European countries (Belgium, Czech Republic, Croatia, Estonia, Finland, Greece, Hungary, Italy, Lithuania, Norway, Slovakia, Spain, Sweden and the United Kingdom), there were 354 outbreaks of waterborne diseases related to drinking water, resulting in over 47, 617 episodes of illness. All the participating countries had a routine surveillance system for waterborne outbreaks, based on a legal framework.

The most common causative agents were bacteria (*Campylobacter*, *Aeromonas* spp. and *Shigella sonnei*) and were responsible for 163 (44.9%) outbreaks and 33.3% of disease cases. Viral agents were implicated in 136 outbreaks (37.5%) and 49.4% of cases of disease, while protozoa caused 17 outbreaks (4.7%) and 9.9% of disease cases. Ten cases were caused by chemical contamination (0.2%), while, in 37 cases (7.1%), an unknown microbial agent was implicated. At least 325 waterborne outbreaks worldwide were due to parasitic protozoan (Karanis *et al.*, 2007).

17. Stages of biofilm formation

The formation of biofilm can be divided into several distinct stages even though the particular development processes may vary depending on the microbial species involved and the prevailing environmental conditions (Fig. 1) (Stoodley *et al.*, 2002; Hall-Stoodley *et al.*, 2004).

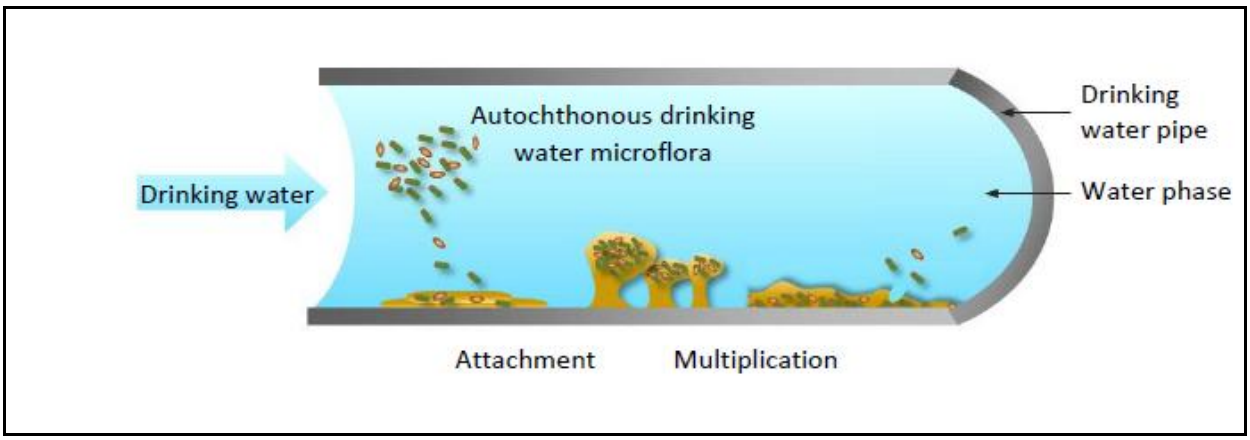


Fig. 1. Biofilm formation and development in a drinking water pipe (Hall-Stoodley *et al.*, 2004).

Quorum sensing allows bacterial cells to communicate resulting in a cohesiveness of function that benefits an entire population and allow the community to operate as a living system. The channels between cell clusters deliver water and nutrients to each cell and facilitate waste removal. These structures combined with strong adhesive properties and sophisticated cell-cell communication make biofilm highly resistant to conventional cleansing agents such as biocides and disinfectants (Smith and Chapman, 2010).

The steps of biofilm formation include the initial attachment of cells to a surface, subsequent multiplication of the cells resulting in microcolonies, followed by the continuous proliferation of attached bacteria leading to the establishment of a mature biofilm, and the passive detachment or active release of single cells or aggregates of cells into the surrounding environment (Fig. 2) (Stoodley *et al.*, 2002).

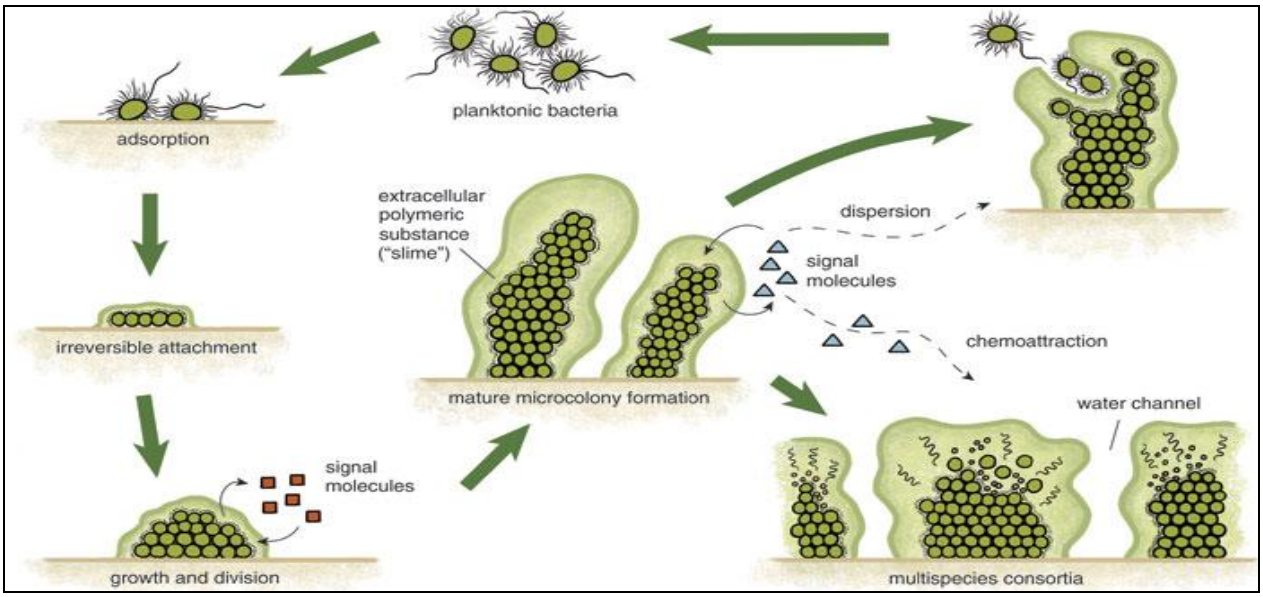


Fig. 2. Stages of biofilm formation and its lifecycle (Stoodley *et al.*, 2002).

a. Attachment/ adhesion

The primary adhesion stage constitutes the beneficial contact between a conditioned surface and planktonic microorganisms. During the process of attachment, the organism must be brought into close proximity of the surface, propelled either randomly or in a directed fashion via chemotaxis and mobility (Prakash *et al.*, 2003). This step is reversible and it is characterized by a number of physicochemical variables that defines the interaction between the microbial cell surface and the conditioned surface of interest (Liu *et al.*, 2004).

b. Irreversible adhesion/ colonization

The second step is the irreversible adhesion during which bacteria start to express adhesion protein such as curli or fimbriae to adhere to the surface. Microorganisms start to produce intercellular connections (intercellular curli for example) and a polymeric matrix, usually called extracellular polymeric substances (EPS). This matrix is a complex hydrogel embedding the bacteria community and building up in three dimensions. The backbone of this gel is mainly composed of polysaccharides produced by bacteria (such as colanic acid, chitosan, alginate), other components such as enzymes, DNA, RNA, nutrients, proteins, surfactants (Flemming *et al.*, 2007). The exact role of the matrix is not yet completely elucidated but it has been demonstrated that the matrix acts as a protective layer (Fux *et al.*, 2005) and is microenvironment-conservative (Beech, 2004).

After the adherence of microorganism to the inert surface, the association becomes stable for micro-colonies formation (Bechmann and Eduvean, 2006). The microorganism

begins to multiply while sending out chemical signals that intercommunicate among the bacterial cells. In this way, the bacteria multiply within the embedded exopolysaccharide matrix, thus giving rise to formation of micro-colonies (Prakash *et al.,* 2003).

c. Maturation

Maturation results in the formation of pillars and masses of tightly packed cells intermixed with fluid filled channels allowing for the exchange of nutrients, oxygen, and waste products between the biofilm and the surrounding liquid (Cloete *et al.,* 2009). Mature biofilm is complicated structures containing sectors with distinctive microenvironments that differ in cell densities, oxygen and nutrient levels, and pH ranges. As a result, the metabolic and reproductive functionality of the bacteria located in these distinct sectors are quite divergent (Kaplan, 2010).

The maturation of biofilm generates many processes already having taken place, such as quorum sensing (Nadell *et al.,* 2008), gene transfer (Molin, 2003), persisted development (Lewis, 2005). All of these processes contribute to the community life of the biofilm and play an important role in biofilm survival and biofilm spreading, since they allow also detachment of biofilm parts and release of free bacteria, which is the most common way for biofilm to spread (Kaplan *et al.,* 2003).

d. Detachment and dispersal

As the biofilm gets older, cells detach, disperse and colonize a new niche. This detachment can be due to various factors including, fluid dynamics and shear effects of the bulk fluid (Brugnoni *et al.,* 2007). At some point of biofilm may partially dissolve releasing cells that more away to other where a new cycle begins (Prakash *et al.,* 2003). Individual bacteria employ somewhat different methods of dispersal, which can be divided into three discrete stages: (a) detachment of cells from the colony; (b) relocation of cells to an alternative site; and (c) reattachment of the cells to a new substrate site (Kaplan, 2010). Rochex *et al.* (2009) found that one dominant species often comprises most of the weakly cohesive, thick top layer of the biofilm; while a more diverse population comprises the strongly cohesive, thin basal layer. These findings suggest that determining species diversity may be an important parameter in understanding detachment and dispersal.

18. The effects and problems of biofilm formation on the water quality

Biofilm or biofouling is suspected to be the primary source of microorganisms in DWDS that are fed with treated water and have no pipeline breaches and are of particular

concern in older DWDS (Wingender and Flemming, 2011). The microbial growth in biofilm is a highly relevant for water quality since they may directly affect cell density in the bulk phase. Moreover, many problems in DWDS are microbial in nature, including biofilm growth, nitrification, microbially mediated corrosion and the occurrence and persistence of pathogens (Emtiazi *et al.,* 2004). So, the main problems of biofilm as a follows,

a. Biofim act as a reservoir for pathogens

DWDS are known to harbor biofilms, even in the continuous presence of a disinfectant. These biofilm are a source of planktonic bacteria, which will remain present when the water is delivered through a consumer's tap. The presence of biofilm in DWDS constitutes one of the currently recognized hazards affecting the microbiological quality of the product and may lead to a number of unwanted effects on the organoleptic quality of the distributed water. Importantly, biofilm constitute a persistent reservoir of pathogenic microorganisms, which are responsible for several waterborne diseases and fed with treated water (Simões and Simões, 2013).

Also, the risk from microbial pathogens remains ever present in the developed world and a daily challenge for the water treatment engineers and scientists. Waterborne diseases are one of the most important water-associated health problems.

b. Drinking water aesthetic (taste, color and odor) problems

Aesthetic and organoleptic characteristics of water may be affected by a series of chemical substances, resulting in color, odor and taste degradation. The substances originate in microbial activity and decomposition in source waters and in distribution systems, disinfectants used in water treatment, materials used in pipes and joints in water networks.

The substances produced from microbial decompositions indole, skatole, putrescine, cadaverine, β-phenylethylamine butyric, propionic and stearic acids can cause many aesthetic effects such as fishy, grassy, woody tastes fecal, rotten, cheese, pungent and odor related to microbial activity and decay that may be produced in the journey of drinking water from drinking water sources to the tap, that may influence consumers perception. These chemical compounds are usually attributed to microbial biofilm associated to drinking water processing and distribution (UK Environment Agency, 2004).

c. Microorganisms-surface interactions and microbially influenced corrosion

Biofilm proved to be interdependent on surface characteristics, investigations of microbial reversible and irreversible attachment in primary or secondary colonization and in drinking water biofilm composition concluded as follows,

1. The hydrophobic and hydrophilic properties of the substrate are influencing biofilm formation. Exopolysacharides produced by some bacteria facilitate cell adhesion to hydrophilic surfaces, while exopolymers of other bacteria may show a preference for hydrophobic substrata (Beech *et al.*, 2005).
2. Pipe materials may be corroded, influencing disinfection effectiveness: corrosion products in iron pipes react with free chlorine and lead to residual disinfectants depletion.
3. Microbially influenced corrosion represents another undesirable impact of biofilm associated to drinking water treatment and distribution, involving metallic or non metallic materials deterioration as a result of pipes inner surface biofouling. Physiological groups of bacteria classified on account of the ability to use different substrates in their nutrition or in respiration such as ammonifying bacteria can produce ammonium and ammonia as metabolites (Muntean, 2009).

8. Factors affecting biofilm growth in DWDS

The attachment of microorganisms to surfaces and the subsequent biofilm development are very complex processes, affected by several factors, as previously stated. In DWDS these include the nature and concentration of nutrients, sediment accumulation, the type and diversity of microorganisms present and their microbial interactions, concentration of free residual disinfectants, environmental factors including pH and temperature, water residence time, hydrodynamics conditions, design of network including the presence of dead ends and diameter of pipes, characteristics of the material covering the distribution pipes and their ages.

However, in real systems all these factors work together to influence biofilm accumulation. Thus, the impact of some of them may be insignificant compared with the impact of others and must therefore be considered carefully for each system. During the last decades an extensive research has been done in this topic which resulted into several published reports on the effects of diverse factors in DW biofilm formation (Lehtola *et al.*, 2006). The main factors are briefly described as a follows;

a. Environmental factors (pH and temperature)

The pH and temperature are considered two important factors affecting life by modifying the electrostatic interactions between surfaces and microorganisms, microbial metabolism, enzymatic activity, kinetics and equilibrium of reactions, and other properties (e.g. diffusivity, solubility). Also, pH and temperature affect the effectiveness of disinfection. Chlorine residuals present in DWDS are drastically reduced when temperature increases and pH decreases (Simões *et al.*, 2012).

b. Disinfectants

Other important variable in biofilm formation is the concentration of disinfectant residual in DWDS. The most used disinfectants are chlorine, chloramines, chlorine dioxide, ozone and UV radiation (Lee and Nam, 2005). From all the disinfectants chlorine is by far the most widely used in DWDS. Chemical disinfection and maintenance of chlorine residual through the distribution systems are almost worldwide strategy to prevent bacterial regrowth during water transportation (Codony *et al.*, 2005). Even so, regrowth may occur when the chlorine residual decays further down in the distribution system (Hallam *et al.*, 2002).

c. Hydrodynamics

The hydrodynamic conditions in DWDS are variable. These conditions alternate from laminar to turbulent flow, but stagnant water also occur in places where the water consumption is low, as well as in reservoirs and buildings. The flow velocity may cause different effects on biofilm accumulation and detachment. Nutrient transport rates within the biofilm increase with the flow velocity until a maximum value is reached, and then decrease as the velocity is further increased. This transport rate promotes bacterial growth within the biofilm. On the other hand, the biofilm density and detachment increase with the flow velocity (Melo, 2003).

As result of wide research on effects of flow velocity on biofilm accumulation controversial results were obtained. Several authors observed that biofilm formation increases with flow velocity, while others achieved the opposite effect (Tsai, 2005; Paris *et al.*, 2007).

d. Nutrients

The DWDS are extreme environments where the availability of nutrients is very low and there is a constant residual concentration of disinfectant. (Armbruster *et al.*, 2012). Several studies from the DWDS around the world observed that the organic carbon content was the limiting nutrient. An increase in this nutrient promoted bacteria regrowth in Australia (Chandy and Angles, 2001), France (Servais *et al.*, 2004), USA

(Soini *et al.*, 2002), Singapore (Hu *et al.*, 2008), Spain (Frias, *et al.*, 2001) and China (Bai *et al.*, 2006).

Batté *et al.* (2003) observed that the addition of phosphorous did not affect the accumulation of biofilm although phosphorous was being incorporated in biofilm. Other researchers observed that the limiting nutrient was phosphorous in DWDS from Japan (Sathasivan and Ohgaki, 1999) and Finland (Lehtola *et al.*, 2002). The detection of the limiting nutrient in DWDS is very important since the addition of phosphate based compounds has been proposed to prevent pipe corrosion and the bacterial regrowth (Appenzeller *et al.*, 2001).

Hydrodynamic conditions and nutrients are the two main parameters that influence biofilm growth in particular the structure, density and thickness (Yu *et al.*, 2007). High shear stress and nutrient limitations led to thin and dense biofilm that will have reduced internal nutrient diffusion and increased resistance to removal and cohesion. Under low flow velocities and high nutrients content, the biofilm grow quickly with a low dense structure but with many pores, channels and protuberances (Paris *et al.*, 2007).

e. Materials

The variability of materials in DWDS is high. Formerly, the majority of pipelines in DW networks were made of iron-based or cement-based materials. More recently, polymeric materials have been preferred, mainly polyvinyl chloride (PVC), polyethylene (PE), because they are easier to handle and implement. In fact, it is possible to find all this types of materials in the same DWDS. The influence of support materials on biofilm growth is well documented in the literature (Momba *et al.*, 2000; Yu *et al.*, 2010). However, there is still controversy about the effects of surface materials on biofilm development when polymeric and metallic materials were compared.

Some reports demonstrated that DW biofilm grew less on polymeric materials than on iron matrices (Chang *et al.*, 2003). This fact was attributed to iron corrosion products that favor biofilm protection from mechanical and chemical stresses. Other studies reported higher biofilm formation on PVC and PE surfaces than on galvanized steel materials (Cloete *et al.*, 2003). While, other works concluded that there was no significant difference in the colonization of the investigated materials (stainless steel, PVC and PE) after decades of operation (Wingender and Flemming, 2004).

Lehtola *et al.* (2004) found that the biofilm grew faster on PE than on copper pipes, but such differences could not be detected in older piping systems. The main characteristics of materials that have been identified as important on biofilm formation

are the roughness and the surface physicochemical properties (chemical composition, solid surface tension, hydrophobicity and surface charge).

f. Microorganisms

The physiological state and the type of microorganisms present in the bulk water will affect the attachment process, since each microorganism has different surface properties, extracellular appendages and abilities to produce EPS. Cell surface hydrophobicity and the presence of extracellular filamentous appendages may influence the rate and the extent of microbial attachment. The hydrophobicity of the cell surface is important in adhesion because hydrophobic interactions tend to increase with the increase in the non-polar nature of one or both surfaces involved, i.e., the microbial cell and the adhesion surface (Donlan, 2002). According to Drenkard and Ausubel (2002), the ability of bacteria to attach to each other and to surfaces depends in part on the interaction of hydrophobic domains.

Many microorganisms produce extracellular filamentous appendages. These may, therefore, play a role in the attachment process. In fact, their radius of interaction with the surface is far lower than that of the cell itself. A number of such structures are known to exist - flagella, pili or fimbrae, prothecae, stalks and holdfast. These structures are responsible for motility, involved in the cell-surface interactions and adhesiveness (Simões *et al.*, 2010). EPS produced by microorganisms are responsible for binding cells and other particulate materials together (cohesion) and to the surface (adhesion) (Allison, 2003).

g. Sediment accumulation

Sediments can consist of either organic matter, including microorganisms, or insoluble material, mainly iron and manganese. Significant microbial activity may occur in accumulated sediments. Organic and inorganic particles can also accumulate in low-flow areas or dead-ends of the DWDS, and enhance microbial activity by providing protection and nutrients (EPA, 2002).

9. Biofilm prevention and control in drinking water distribution systems

Several studies have been performed to understand how it is possible to control biofilm development. The influence of chemical treatment with biocides and disinfectants, mechanical treatment or physical pretreatment and also manipulation of the operational conditions are strategies studied to control the biofilm development in DWDS (Douterelo *et al.*, 2013).

In addition to, Ollos *et al.* (2003) who evaluated several factors biodegradable organic matter concentration, monochloramine, chlorine disinfection, pipe material and temperature in biofilm development using an annular reactor as DWDS model. Temperature and flow velocity are factors studied using different DWDS models to understand how it can affect the biofilm formation in pipe walls. The nutrient concentration such as phosphorus in the DW also can affect the biofilm development (Douterelo *et al.,* 2013).

a. Pre-treatment

The maintenance of sufficient residual chlorine in the system is difficult when the water supplies have a high chlorine demand due to the presence of organic matter. Consequently, one strategy to optimize DW quality is to reduce the content of organic matter and nutrients by more effective pre-treatments (ion-exchange, activated carbon, reverse osmosis, nanofiltration, ultrafiltration, and microfiltration). However, to decrease the organic content would be a very expensive process and ineffective toward bacteria in DWDS, which are able to grow in oligotrophic environments (Zhou *et al.,* 2009).

Nevertheless, some developed countries, have taken the approach of distributing high quality DW without the use of residual chlorine. The control of microbial growth in these countries is obtained through limitation of the nutrients essential for growth by more appropriate DW treatments such as sedimentation, filtration, UV disinfection, ozone and hydrogen peroxide by the production of biologically stable DW. In general, microorganisms need a C: N: P (carbon, nitrogen and phosphorous) ratio of 100: 10: 1, where carbon is the growth-limiting nutrient. Thus, restricting the carbon concentration will decrease the propensity for microbial growth (Chandy and Angles, 2001).

b. Material selection

Other preventative strategies have attempted to identify materials that do not promote or can even suppress biofilm formation. The different materials such as ethylene-propylene, natural latex, stainless steel (SS), mild steel, polypropylene, polyethylene (PE), chlorinated polyvinyl chloride (PVC) and unplasticized (PVC) were ranked according to their biofilm growth propensity, which unfortunately led to the conclusion that there is hardly any material that does not allow biofilm formation (Rogers *et al.,* 1994). However, the copper pipe materials have inhibitory effect (respiratory chain inhibition) when compared to biofilm growth on other materials (high density PE, PVC, silicon, SS and glass) (Hem and Skjevrak, 2002). Concentrations of copper ions relevant to DWDS seem to induce a viable but non culturable state in some species accompanied by a loss of culturability and cytotoxicity (Dwidjosiswojo *et al.,* 2011).

The type and stability of the material used in DWDS is an important factor that can influence biofilm proliferation. There is a distinct development rate and microbial community structure of biofilm in different types of pipe (Simões *et al.,* 2006). Bacteria are able to leach nutrients from the materials (Rogers, *et al.,* 1994). In case of iron pipes, it can be more reactive with disinfectants and quench their antimicrobial effects (Kerr *et al.,* 2003). Additionally, Niquette *et al.* (2000) cleared that iron pipes can support the formation of biofilm from 10 to 45 times more than plastic pipes. Thus, the type of material can also affect the disinfectant efficiency of biofilm.

Biofilm grown on copper, PE, PVC and cement lined ductile iron were a much inactivated with lower amount of free chlorine or monochloramine than those grown on unlined iron surfaces (Hallam *et al.,* 2002). In cement-lined ductile iron, the cement provides a layer of protection for the iron against attack by chlorine. The pipe service age is another important factor influencing chlorine decay and this effect decreases in the following order: cast iron, steel, cement-lined cast iron = cement-lined ductile iron, PVC = PE (Al-Jasser, 2007).

The consumption of chlorine is caused by chemical reactions of the antimicrobial with water constituents and with both the biofilm and tubercles formed on the pipe wall, as well as a reaction with the pipe wall material itself. This means that older pipes have more impact on the decay of chlorine concentration than new ones and cast iron pipes are probably the worst choice for DWDS due to their oxidation susceptibility (Al-Jasser, 2007).

c. Hydrodynamics

The distribution network must be planned to avoid zones of water stagnation or high water residence times in pipes and sediment accumulation. Pipes with long water residence times and dead-ends are associated with zones of high organic material sedimentation and, consequently, abundant biofilm formation. High bacterial numbers are associated with periods of non-flow or the storage of water in household pipes or tanks (Ayoub and Malaeb, 2006). On the other hand, the biofilm grown in DWDS contributes to the accumulation of inorganic particles, like iron and manganese, in the distribution network, which are responsible for discolored water, which are a persistent cause of customer dissatisfaction. Moreover, the sediment accumulation in DWDS may also decrease the disinfectant concentration (Ginige *et al.,* 2011).

Moreover, Douterelo *et al*. (2013) used an experimental DWDS to study the hydrodynamic influence in biofilm structure and composition through flushing application. The flushing of DWDS can be altered the bacterial community structure of

pipe-wall, but didn't completely remove it from the surface, particularly under highly flow conditions (Douterelo *et al.*, 2013).

d. Chemical disinfection and alternative techniques

There is an urgent need to seek and develop new and alternative techniques for water disinfection to minimize the environmental and public health impacts of traditional techniques. WHO has stated that the risks to health from disinfection by-products are extremely small in comparison with inadequate disinfection. However, the development of safe and effective alternative disinfection methods is desirable. The main strategy to control biofilm accumulation in DWDS is chemical disinfection, particularly with chlorine, and an increase in its residual concentration through the network (WHO, 2011).

Water disinfection is a process used to kill or irreversibly inactivate microorganisms to ensure microbiologically safe water through DWDS. A chemical disinfection process that is based on unspecific oxidative processes to inactivate microorganisms is often used. The most used techniques to achieve an efficient disinfection of DWDS are chlorination, chloramination or chlorine dioxide addition, ozone treatment and UV treatment (Chowdhury, 2012).

1. Chlorine

In spite of, chlorination is a low cost process, easy to apply and able to inactivate a wide variety of pathogenic microorganisms. Therefore, this is the predominant disinfection method used in water treatment (Donnermair and Blatchley, 2003). Chlorine is a strong oxidizing agent and is the most commonly used disinfectant due to its effectiveness, high solubility, stability, ease of use and low cost. Furthermore, it can provide a disinfectant level in water that prevents or should prevent microbial regrowth (Simões *et al.*, 2010).

The residual concentration of free chlorine leaving the treatment plant should be less than 1.0 mg/l and nearer to 0.5 mg/l (WHO, 2011). The levels of disinfectants usually employed in DWDS are not sufficient to prevent the growth and development of microbial biofilm and once biofilm are established on pipe surfaces, their elimination is almost impossible. The application of 10 mg/l of sodium hypochlorite significantly reduced the number of viable cells in biofilm, but one day after disinfection the bacteria recovered their viability (Zhou *et al.*, 2009).

The chlorine action in cells viability was intensively studied, there are many theories for the chlorine action one of them, chlorine can be destroy the microorganisms by combining with proteins to form N-chloro compounds (LeChevallier and Au, 2004).

The chlorine has powerful effects on sulphydryl groups of proteins and converted several α-amino acids by oxidation. Chlorine can cause physiological damage to the bacterial cell membrane as cytochromes, iron-sulfur proteins and nucleotides are highly vulnerable to the chlorine oxidative effect. Therefore, respiration, glucose transport and ATP levels decrease in chlorine-treated bacteria (WHO, 2011).

Although being an efficient process, chlorination has some disadvantages, namely its dependency of water conditions, like pH and temperature. The concentration of chlorine used is also a critical aspect. High concentrations can cause organoleptic problems it means strong odor and taste), and increase the production of carcinogenic by-products compound that can be harmful to human health, like trihalomethanes and halogenic acids. The use of chlorine in excess can also be responsible for the development of chlorine resistant microorganisms (Nieuwenhuijsen *et al.,* 2000).

From the disinfectants other than chlorine can be used in DWDS is chloramines. Chloramines are less effective than free chlorine and produce the same by-products as chlorine, but in lower amounts. Their residual concentration is kept for longer periods and chloramines are not as reactive as chlorine with iron and corrosion products (LeChevallier *et al.,* 1998). Some reports suggest the combined use of chlorine and monochloramine in order to obtain higher disinfection and reduced by-products. However, combined residual chlorine requires a contact time of a hundred times longer than free residual chlorine to achieve the same degree of elimination of pathogens. Chlorine dioxide is another effective water disinfectant, but is not widely used. This chemical does not produce trihalomethanes nor react with ammonia. When applied in low amounts and concomitantly with chlorine it decreased significantly the formation of trihalomethanes (Charrois and Hrudey, 2007).

2. Silver ions (Ag^+)

Silver's antimicrobial effect has been demonstrated in numerous applications against different types of microorganisms (Hall-Stoodley and Stoodley, 2005). The bactericidal efficacy of silver is through its binding to disulfide or sulfhydryl groups in cell wall proteins (Beaudeau *et al.,* 2008). Silver also binds to DNA. Through these binding events, metabolic processes are disrupted, leading to cell death. Silver has been reported to delay or prevent the formation of biofilm in water filters, cooling towers and distribution systems (Hrudey and Hrudey, 2004; Shannon *et al.,* 2008).

Silver exerts its antimicrobial effect by progressive elution from the devices. Silver is effective against planktonic bacteria (Craun *et al.,* 2006) and has been used for water disinfection in Europe (Snyder *et al.,* 2007). In addition, silver, in combination with

copper, has proven effective against some pathogens in hospital distribution systems for more than a decade (Karanis *et al.*, 2007).

3. Silver nanoparticles (AgNPs)

Among metal nanoparticles with proven antimicrobial activity, those made of silver are particularly effective bactericidal agents (Seil and Webster, 2012). The antibacterial properties of silver have long been known and nanoparticles of this metal (AgNPs) are believed to be less toxic than silver ions. Additionally, the application of AgNPs in various fields has expanded considerably. AgNPs have been successfully used in medical and pharmaceutical and nano-engineering for the delivery of therapeutic agents, in chronic disease diagnostics, and as part of sensors (Thiwawong *et al.*, 2013).

The comparison of the various nano-silver activities that have been studied is difficult because of differences in the chemistry and physical properties of the particles employed. Furthermore the bactericidal effect of AgNPs is dependent on the size and shape of the particles. The specific surface area of a dose of AgNPs increases as the particle size decreases, allowing greater material interaction with the surrounding environment. In addition, triangular-shaped particles of silver display more bacterial killing activity than rods or spherical particles (Pal *et al.*, 2007). Other characteristics affecting the biological activity of nanoparticles are zeta potential and particle chemistry, with the former likely to play a significant role in the ability of particles to penetrate into the cell (Seil and Webster, 2012).

AgNPs probably have multiple mechanisms of antibacterial action, but due to the current dearth of knowledge on this subject, the exact basis for the activity of AgNPs is still uncharacterized. Some studies have shown that AgNPs release Ag^{+} ions in the presence of water (Damm and Münstedt, 2008). Accordingly, Lok *et al.* (2007) calculated that approximately 12% of the silver is present in the ionic form, tightly associated with the oxidation layer. However, their experimental design makes it difficult to distinguish between the mechanisms of action of AgNPs and dissolved Ag ions. Hence, it was suggested that nano-silver affects bacterial membrane permeability by attaching to the cell membrane surface and modifying the cell potential. Observation of large numbers of nanoparticles inside bacteria suggests that this is important to the antibacterial mechanism (Morones *et al.*, 2005).

Proteomic analysis (2-DE and MS identification) of *E. coli* cells revealed that short-exposure to AgNPs resulted in the accumulation of envelope precursors, which is indicative of the dissipation of the proton motive force. Proteins whose expression was found to be stimulated by AgNPs over 1.8-fold were the inclusion body binding proteins

A and B (IbpA and IbpB) (Lok *et al.,* 2006). Furthermore, AgNPs have been shown to interact with bacterial membrane proteins, intracellular proteins, phosphate residues in DNA, and to interfere with cell division, leading to bacterial cell death (Xu *et al.,* 2004). Presence of biocidal Ag^+ ions released from the nano-particle surfaces evokes bacterial DNA conglomeration defense mechanisms, which protect the cell from toxic effects, but simultaneously compromises its replication ability. Thus microbial responses to ionic silver and nanoparticles are different, and knowledge of both is required for a complete understanding of the antibacterial activity of AgNPs. Some studies have reported that nano-silver causes oxidative damage, leading to the production of reactive oxygen species like free-radicals (Wang *et al.,* 2008) and it has been suggested that the production of reactive oxygen species is one of the primary mechanisms of nanoparticle toxicity (Khan, 2012).

10. Behavior study of some bacterial strains for biofilm formation in DWDS model

a. Behavior of *Listeria monocytogenes* for biofilm formation in DWDS model

Discovery of *L. monocytogenes* goes back to 1924, when for the first time it was isolated as the etiological agent of a septicaemic disease (causing death of rabbits and guinea pigs) **(**Swaminathan and Gerner-Smidt, 2007**)**. *L. monocytogenes* is a Gram-positive, facultative anaerobic, non-spore-forming, rod-shaped bacterium with optimal growth temperature range of 30 - 37˚C. *L. monocytogenes* is a food and water-borne pathogen causing disease for human and animals (Pagadala *et al.,* 2012).

It is widely distributed in different environments and can be found in fresh water, soil, animal fecal matter and sewage **(**Vaid *et al.,* 2010). It is considered a serious bacterial pathogen in immunocompromised individuals such as cancer and HIV patients, elderly and pregnant women. Ingestion of *L. monocytogenes* can cause listeriosis in human and animals (Pagadala *et al.,* 2012).

Among eight species of *Listeria* genus, only two are considered pathogen (Barbuddhe *et al.,* 2012). *L. ivanoii* is pathogens for animals, when *L. monocytogenes* is pathogenic for both animals and humans (Brugère-Picoux, 2008). Listeriosis can lead to gastroenteritis, septicaemia, perinatal infections, stillbirth, abortion, meningitis and meningoencephalitis in immunocompromised individuals (Barbuddhe *et al.,* 2012).

Listeriosis affects a wide variety of mammals including monogastric, ruminants (mostly sheep) and human with morality rate of 20-30% (Chaturongkasumrit *et al.,* 2011).

L. monocytogenes cells can be fixed onto various surfaces, especially in inaccessible parts of industrial equipment due to biofilm formation (Pan *et al.,* 2006). This can occur rapidly and the substrate, once attached, is difficult to remove (Frank and Koffi, 1990). It has been found in biofilm on plastic surfaces, polypropylene, rubber, stainless steel and glass (Jeong and Frank, 1994).

b. Behavior of *Salmonella* Typhimurium for biofilm formation in DWDS model

Salmonella was named after Daniel Elmer Salmon who first isolated *S.* Chloeraesuis from pigs with swine cholera in 1884 (Humphrey, 2000). In the early 1980's, Le Minor *et al*. (1982) proposed that the genus *Salmonella* be reclassified into two species, *Salmonella enterica* and *Salmonella bongori*. Presently there are over 2500 serovars identified in *Salmonella enterica* by the Kauffmann-White Scheme based on their somatic (O) and flagella (H) antigens (Sonne-Hansen and Jenabian, 2005) with serovars Typhimurium and Enteritidis responsible for over 80% of all human cases of salmonellosis (Pelludat *et al.,* 2005).

S. Typhimurium is a Gram negative bacillus, motile through the presence of surface structures called flagella and a group of ubiquitous pathogens which can cause gastroenteritis, typhoid fever and septicaemia in humans and infections in animals. Although primarily an intestinal bacterium, *Salmonella enterica* are widespread in the environment and commonly found in farm effluents, human sewage and fecal contaminated materials. Despite being extensively studied and recognized as a main cause of gastrointestinal disease in humans for many decades, *Salmonella* continues to provide new challenges to scientists, particularly because of their rapid evolution (Davidson *et al.,* 2008) and acquisition of new multi-drug resistance genes (Lynne *et al.,* 2008).

Salmonella is a ubiquitous enteric pathogen with a worldwide distribution that comprises a large number of serovars characterized by different host specificity and distribution. This microorganism is one of the leading causes of intestinal illness through the world as well as the etiological agent of more severe systemic diseases such as typhoid and paratyphoid fever (Pond, 2005). Zoonotic salmonellae are commonly described as foodborne pathogens however; drinking water as well as natural water is known to be an important source for the transmission of these enteric microorganisms

(Ashbolt, 2004; El-Leithy, 2009). Thus, *Salmonella* spp. can also contaminate fresh fruit and vegetables through contaminated fecal content polluting freshwater and soil (Herman *et al.*, 2008).

Salmonella spp. is frequently found in environmental samples. They are usually present in large numbers in raw sewage (10^3-10^4 CFU/L) and can still be present in wastewater effluent after advanced secondary treatment including coagulation, filtration and disinfection (Wéry *et al.*, 2008). Soil and sediment were also found to harbor salmonellae (Gorski *et al.*, 2011) and sediment particles are believed to function as a micro ecological niche enhancing *Salmonella* spp survival in lakes (Chandran and Hatha, 2005). In the aquatic environment this pathogen has been repeatedly detected in various types of natural waters such as rivers, lakes, coastal waters, estuarine as well as contaminated ground water (Levantesi *et al.*, 2010).

Salmonella spp. posses a cell-surface appendage (SEF-17 *fimbriae*) that facilitates adhesion to inanimate surfaces, and provides cells resistance to mechanical forces. Some studies on the biofilm formation process have revealed that *Salmonella* and *E. coli*, as well as many other species of the enterobacteriaceae family, produce cellulose as a crucial component of the bacterial extracellular matrix and its formation is essential for the survival of the bacteria in the environment (Lasa *et al.*, 2005).

Salmonella spp. is environmentally persistent pathogens capable of forming biofilm on different surfaces under different environmental conditions and may act as continuous sources of food contamination. *Salmonella* can form biofilm on plastics (Momba and Kaleni, 2002), glass (Prouty and Gunn, 2003) and stainless steel (Joseph *et al.*, 2001). Moreover, it can be considered possible found in water supplies due to its ability to colonize surfaces and replicate in biofilm of distribution system pipes (Jones and Bradshaw, 1996). However, standard disinfection procedures used in drinking water treatment processes are active against *Salmonella* spp. (Smith *et al.*, 2006).

11. Quantification of biofilm in DWDS models

All the biofilm studies require the definition of an appropriate method to quantify biofilm formation and to provide information on its characteristics, particularly for the resident population. Biofilm can be quantified through the increase of biological activity or by the number of cells (Liu *et al.*, 2013b). A part from the quantification of cell numbers, it is also important to obtain information on other biofilm constituents, particularly the EPS. Most of these methods require the biofilm scraping from the substratum and its dispersion in an adequate solution, generally saline water (Park *et al.*, 2012) or an appropriate buffer. Moreover, to achieve efficient biofilm dispersion in the

selected solution it is necessary to use some physical treatment as vortex and/ or ultrasonication (Jang *et al.*, 2012).

The exceptions to the scraping requirement are some microscopic methods, as atomic force microscopy (AFM), scanning electron microscopy (SEM), transmission electron microscopy (TEM) and confocal scanning laser microscopy (CSLM), which can allow a direct analysis of biofilm adhered to a surface, if the sampling coupons are flat. However, even if the direct microscopic analysis of coupon surfaces is important to provide information on the biofilm structure, these methods cannot determine all relevant aspects involving the biofilm formation process. Therefore, the combination of information from different methods will provide a more detailed picture on DW biofilm formation and composition (Mathieu *et al.*, 2014).

a. Biofilm cells (sessile cells) enumeration

The biofilm cells quantification through cell enumeration is the mostly used method. The biofilm development and dynamics is commonly monitored through the enumeration of cultivable, metabolic active, viable and/or total cells (Jang *et al.*, 2012).

Heterotrophic plate count (HPC) methods are often used to determine the numbers of cultivable bacteria.These methods only enumerate a fraction of heterotrophic bacteria on an agar-based medium under defined incubation temperature and time. To quantify the HPC, it is necessary to scrape the biofilm from the reactor/coupon surface and dilute it to an adequate concentration, before plating. This is a method often used to evaluate biofilm cell numbers in several DWDS models (Gagnon *et al.*, 2005).

The microbial metabolic active and total cell numbers are assessed through microscopic analysis after a staining process and the results are represented in terms of numbers of cells per unit of surface area. 40, 6-diamidino-2-phenylindole (DAPI) or acridine orange are common dyes used for total cell counts (Park *et al.*, 2012). DAPI is a fluorescent stain that binds to A-Trichregions in DNA fluorescing blue, and since it is able to pass through the cell membrane it stains both live and dead cells. Acridine orange is a cell-permeable fluorescent stain that interacts with RNA and DNA fluorescing green to red, providing information on the numbers of total and viable cells (Yu *et al.*, 2007). The BacLight Live/Dead (L/D) stains provide the bacterial viability kit that allows the assessment of both viable and total bacterial cell counts. This kit is composed of two nucleic acid binding stains; SYTO 9 and propidium iodide (PI). SYTO9 penetrates all bacterial membranes and stains the cells green, while PI only penetrates cells with damaged membranes and the combination of the two stains produces red fluorescing cells (Simõs and Simõs, 2013). These stains interact with all the existing biofilm bacteria and

their quantification is processed by epiflourescence microscopy. Metabolic active bacteria are usually assessed after being stained by the redox dye 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC) which (Jungfer *et al.*, 2013).

b. Extracellular polymeric substances (EPS) quantification

EPS contribute to biofilm stability and adhesion properties. The EPS matrix might also be a site for free extracellular enzyme activity; however, little is known about participation of enzyme activity in EPS during biofilm formation. Also, they are composed of a variety of organic substances and carbohydrates are its predominant constituents, whereas proteins also exist in substantial quantities. Therefore, the EPS quantification methods are usually based on the analysis of carbohydrates and proteins. However, the reliability of the analysis is strongly dependent on the extraction methods used to separate the EPS from the biofilm cells (Romaní *et al.*, 2008).

To quantify the carbohydrates it is often used the modified phenol-sulfuric acid method (Fang *et al.*, 2010). The carbohydrates are broken down by the concentrated sulfuric acid to monosaccharides. These compounds react with phenol and produce a yellow gold color with a maximum absorption at 490 nm (Dubois *et al.*, 1956).

c. Microscopic analysis

Many microscopic analyses are non-destructive, which means that it allows the direct observation of biofilm without a scraping step. These methods can be advantageous not only the possibility of biofilm loss in the scraping process does not exist, but also it allows the study of the entire biofilm structure. Biofilm has a three-dimensional (3D) structured, heterogeneous community of microbial cells enclosed in an exopolysaccharide matrix (also called glycocalyx) that are irreversibly attached to an inert or living surface (Hall-Stoodley *et al.*, 2004).

Different microscopic techniques for biofilm monitoring including scanning electron microscopy (SEM) have been proved to be suitable tools in order to follow the study of adhesion stage and biofilm formation. SEM as a specialized field of science that employs the electron microscope as a tool and uses a beam of electrons to form an image of a specimen allowing imaging and quantification of surface topographic features (Garret *et al.*, 2008).

Moreover, transmission electron microscopy (TEM) also has been used to assess the structure of the biofilm (Kämper *et al.*, 2004). In addition to that, atomic forces microscopy (AFM) is one of these techniques and it provides topographic images from

the micro to nano-scale, providing qualitative and quantitative information on the physic chemical properties of biofilm substratum interactions (Beech *et al.*, 2005).

MATERIALS AND METHODS

1. Determination of the microbial populations in natural biofilm

a. Sources of natural biofilm samples

Forty eight natural biofilm samples were scraped from different drinking water distribution system (DWDS) and some types of microhabitats such as bathroom, kitchen and laboratory sink drainage pipes.

1. Drinking water distribution pipes

Twenty natural biofilm samples as a natural biofilm were collected from three different pipe materials (five samples from PP, five samples from PVC and ten samples from iron) (Table 1).

2. Samples collected from microinhabitants

a. Bathroom sinks drainage pipes

Ten natural biofilm samples as natural areas were collected from plastic materials of sink drainage pipes from five house bathrooms (Table 1).

b. kitchen sinks drainage pipes

Ten natural biofilm samples as natural areas were collected from plastic materials of kitchen drainage pipes from five house kitchens (Table 1).

c. Laboratory sinks drainage pipes

Eight natural biofilm samples as natural areas were collected from plastic materials of lab. sink drainage pipes from bacteriology and parasitology lab at Water Pollution Research Dept., National Research Centre (Table 1).

Table 1. The natural biofim samples collected from different sources.

Sources of biofilm samples	Types of pipe materials	Number of biofilm samples
Drinking water distribution pipes	PP	5
	PVC	5
	Iron	10
Laboratory (lab) sink drainage pipes	Plastic	8
Kitchen sink drainage pipes	Plastic	10
Bathroom sink drainage pipes	Plastic	10

PP=Polypropylene, PVC=Polyvinyl chloride

b. Collection and preparation of biofilm samples

All procedures in biofilm samples collecting and processing in microbiological analyses were performed under sterile conditions. The natural biofilm samples were harvested by scraping 10 cm^2 from inner surface of pipes using sterile cotton swabs. The swabs were submerged into tubes each one containing 10 ml sterile water and homogenized by using vortex agitator for 5 min (Zhou *et al.*, 2009). The natural biofilm samples were preserved in ice box and immediately transferred to the laboratory at Bacteriology Lab, Water Pollution Research Dept., National Research Centre (NRC) for microbiological examination according to APHA (2012).

c. Microbiological analyses of natural biofilm samples

Microbiological analyses were performed by culture-dependent techniques according to APHA (2012). Biofilm samples or their appropriate dilutions were inoculated in non selective media (plate count agar) and selective-differential growth media (enzymatic-based culture media). The plates were counted by using colony counter (Cook Electoromics LTD.). Results were reported as the number of CFU/cm^2.

The detached biomass (biofilm cells suspension) was diluted with sterile saline solution. The total viable bacterial counts (TVBC) at both 37 and 22^oC were enumerated using pour plate technique. Spread plate technique used to enumerate the total coliform, fecal coliform, *E.coli*, fecal streptococci. In addition to, some pathogenic microbes such

as *Pseudomonas aeruginosa, Salmonella* spp., *Staphylococcus aureus, Listeria monocytogenes, Bacillus cereus, Clostridium perfringens* and *Candida albicans*.

1. Enumeration of total viable bacterial counts in natural biofilm samples

The pour plate technique was used to enumerate the total viable bacterial counts involved in biofilm. One ml of appropriate dilution of biofilm cell suspension was transferred into sterile Petri dishes. Cooled melted agar (45^{o}C) was poured into Petri dishes. Two plates were incubated at 37±0.5°C for 24 hrs and one un-inoculated plate served as a control and other two plates were incubated at 22±0.5°C for 48 hrs. After the incubation period, all the inoculated plates were counted using the colony counter (Cook Electoromics LTD.).

2. Enumeration of coliform group

The spread plate technique was used to determine the presence of some microorganisms involved in biofilm. 0.1 ml of suitable dilution of biofilm samples was aseptically transferred onto the Rapid Hicoliform agar (HiMedia- India) for enumerating total and fecal coliform, and spread uniformly with a sterile glass rod. The inoculated agar plates were incubated at 37±0.5°C for 24 hrs. The presence of total coliform is indicated by a blue-green color of the colonies and fecal coliform indicated by a blue fluorescence under UV light. While, to enumerate *E. coli* 100 µl of suspended biofilm cells was transferred onto ECC agar medium (HiMedia-India). The inoculated plates were incubated at 37±0.5°C for 24 hrs. Colonies of *E. coli* appeared blue to purple color.

3. Enumeration of fecal streptococci

100 µl of suitable dilution of biofilm sample was aseptically transferred onto the Rapid HiEnterococci agar (HiMedia- India) for enumerating fecal streptococci and spread uniformly with a sterile glass rod. The inoculated agar plates were incubated at 37±0.5°C for 24 hrs. The typical enterococci colonies appeared blue green color.

4. Enumeration of some pathogenic microbes

For *Pseudomonas aeruginosa*, 100 µl of suitable dilution of biofilm samples was aseptically transferred onto the Hifluoro Pseudomonas agar (HiMedia- India). Inoculated plates were incubated at 37±0.5°C for 24 hrs. *P. aeruginosa* cleaves the fluorogenic

compound to release the fluorogen which produces a visible fluorescence under long wave UV light.

For *Salmonella* spp., 100 µl of suitable dilution of biofilm samples was aseptically spread onto the Hicrome Improved Salmonella agar (HiMedia- India). Inoculated plates were incubated at 37±0.5°C for 24 hrs. The typical colonies of *Salmonella* were light pink color.

For *Staphylococcus aureus*, 100 µl of suitable dilution of biofilm samples was aseptically spread onto the Hicrome Aureus agar. Inoculated plates were incubated 37±0.5°C for 24-48 hrs. *Staph. aureus* colonies were brown black, with a clear zone around the colony.

For *Listeria monocytogenes*, 100 µl of suitable dilution of biofilm samples was aseptically spread onto the Hicrom Listeria selective agar (HiMedia- India). Inoculated plates were incubated at 35°C for 24-48 hrs. The color of typical colonies was bluish green.

For *Bacillus cereus*, 100 µl of suitable dilution of biofilm samples was aseptically spread onto the Hicrome Bacillus agar (HiMedia- India). Inoculated plates were incubated 30±0.5°C for 24 hrs. *Bacillus cereus* colonies were blue colonies in color.

For *Clostridium perfringens*, 100 µl of suitable dilution of biofilm samples was aseptically spread onto the M-CP agar base (HiMedia- India). Yellow colonies appeared old rose to pink-red were considered to be *Cl. perfringens* after incubation at 44°C for 24-48 hrs under anaerobic conditions.

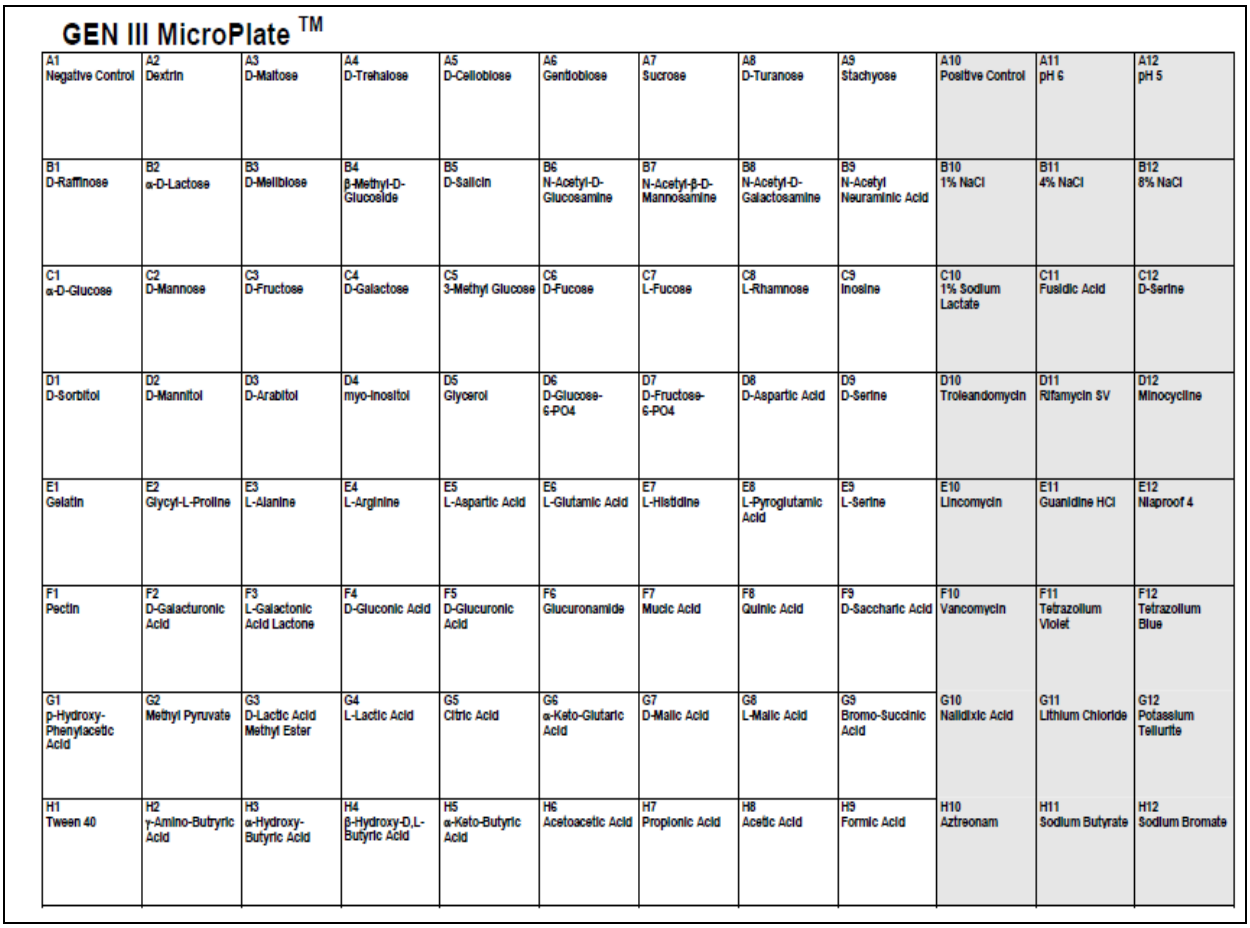
For enumeration of *Candida albicans*, 100 µl of suitable dilution of biofilm samples was aseptically transferred onto the Hicrome Candida agar (HiMedia- India). *Candida albicans* colonies were light green in color after incubation at 30°C for 48 hrs.

d. Confirmation and identification of microbial isolates using BIOLOG GN III.

Typical colonies of each bacterial species were picked up from the surface of the specific chromogenic media to identify by using BIOLOG GEN III system (BIOLOG, USA). The GEN III MicroPlate™ test panel provides a standardized micro-method using

94 biochemical tests to profile and identify a broad range of Gram-negative and Gram-positive bacteria. Biolog's microbial identification systems software is used to identify the bacterium from its phenotypic pattern in the GEN III MicroPlate (Fig. 3).

The suspected colony was streaked onto TSA plate and incubated at 37^{o}C for 24 hrs. The microplate was incubated at 37^{o}C for 24 hrs. The reading was carried out automatically by the computerized MicroStation™ system.



GEN III MicroPlate ™

A1 Negative Control	A2 Dextrin	A3 D-Maltose	A4 D-Trehalose	A5 D-Cellobiose	A6 Gentiobiose	A7 Sucrose	A8 D-Turanose	A9 Stachyose	A10 Positive Control	A11 pH 6	A12 pH 5
B1 D-Raffinose	B2 α-D-Lactose	B3 D-Melibiose	B4 β-Methyl-D-Glucoside	B5 D-Salicin	B6 N-Acetyl-D-Glucosamine	B7 N-Acetyl-β-D-Mannosamine	B8 N-Acetyl-D-Galactosamine	B9 N-Acetyl Neuraminic Acid	B10 1% NaCl	B11 4% NaCl	B12 8% NaCl
C1 α-D-Glucose	C2 D-Mannose	C3 D-Fructose	C4 D-Galactose	C5 3-Methyl Glucose	C6 D-Fucose	C7 L-Fucose	C8 L-Rhamnose	C9 Inosine	C10 1% Sodium Lactate	C11 Fusidic Acid	C12 D-Serine
D1 D-Sorbitol	D2 D-Mannitol	D3 D-Arabitol	D4 myo-Inositol	D5 Glycerol	D6 D-Glucose-6-PO4	D7 D-Fructose-6-PO4	D8 D-Aspartic Acid	D9 D-Serine	D10 Troleandomycin	D11 Rifamycin SV	D12 Minocycline
E1 Gelatin	E2 Glycyl-L-Proline	E3 L-Alanine	E4 L-Arginine	E5 L-Aspartic Acid	E6 L-Glutamic Acid	E7 L-Histidine	E8 L-Pyroglutamic Acid	E9 L-Serine	E10 Lincomycin	E11 Guanidine HCl	E12 Niaproof 4
F1 Pectin	F2 D-Galacturonic Acid	F3 L-Galactonic Acid Lactone	F4 D-Gluconic Acid	F5 D-Glucuronic Acid	F6 Glucuronamide	F7 Mucic Acid	F8 Quinic Acid	F9 D-Saccharic Acid	F10 Vancomycin	F11 Tetrazolium Violet	F12 Tetrazolium Blue
G1 p-Hydroxy-Phenylacetic Acid	G2 Methyl Pyruvate	G3 D-Lactic Acid Methyl Ester	G4 L-Lactic Acid	G5 Citric Acid	G6 α-Keto-Glutaric Acid	G7 D-Malic Acid	G8 L-Malic Acid	G9 Bromo-Succinic Acid	G10 Nalidixic Acid	G11 Lithium Chloride	G12 Potassium Tellurite
H1 Tween 40	H2 γ-Amino-Butyric Acid	H3 α-Hydroxy-Butyric Acid	H4 β-Hydroxy-D,L-Butyric Acid	H5 α-Keto-Butyric Acid	H6 Acetoacetic Acid	H7 Propionic Acid	H8 Acetic Acid	H9 Formic Acid	H10 Aztreonam	H11 Sodium Butyrate	H12 Sodium Bromate

Fig. 3. Layout of assays in the MicroPlate.

2. Behavior studies and biological characteristics of *L. monocytogenes* and *S.* Typhimurium which involved in biofilm formation

It is difficult to understand how biofilm are formed and preview their behavior and control inside of the drinking water distribution system (DWDS), so the development of appropriate devices to form biofilm studies is much important. Thus, this part of study

was performed using a laboratory-scale simulated drinking water distribution system (DWDS) which inoculated separately by *Listeria monocytogenes* as a Gram positive bacterium and as a Gram negative bacterium. *L. monocytogenes* and *S.* Typhimurium were selected as representative organisms due to their relative impacts on human disease associated with water and foodborne illnesses.

a. Microorganisms used and preparation

Two strains *L. monocytogenes* ATCC 25152 and *S.* Typhimurium ATCC 14028 as planktonic cells were cultured in brain-heart infusion broth (Oxoid-UK) and tryptic soya broth (TSB) (Oxoid-UK) and incubated at 37^{o}C during 24 hrs. After the incubation, the suspensions were homogenized by the vortex and centrifuged at 3000 rpm for 20 min and washed three times by adding sterile phosphate buffered solution. The density of the inocula (10^{6} CFU/ml) which used separately for biofilm formation was determined by pour plate method using plate count agar. The bacterial suspensions of *L. monocytogenes* and *S.* Typhimurium were inoculated separately in the container of DWDS model and pumped into the system to allow the formation of biofilm in different tested pipe materials.

b. *In vitro* laboratory-scale simulated drinking water distribution system (DWDS) model

As shown in Fig. (4), the designed system consisted of six different sets of identical pipes which used in the drinking water distribution till now in closed system. The most common of pipe materials which used in household were polyvinyl chloride (PVC), polypropylene (PP), polyethylene (PE), iron (I), copper (Cu) and rubber (R). The length and internal diameters of each distribution pipes were one meter and three cm. The inoculated water by bacterial suspension was pumped using a feeding pump (EMMA Pump, Italy) from the contact tank containing 30 liter. The flow rate of the used pump was 30 l/min into the designed DWDS (Hemdan *et al.*, 2015).

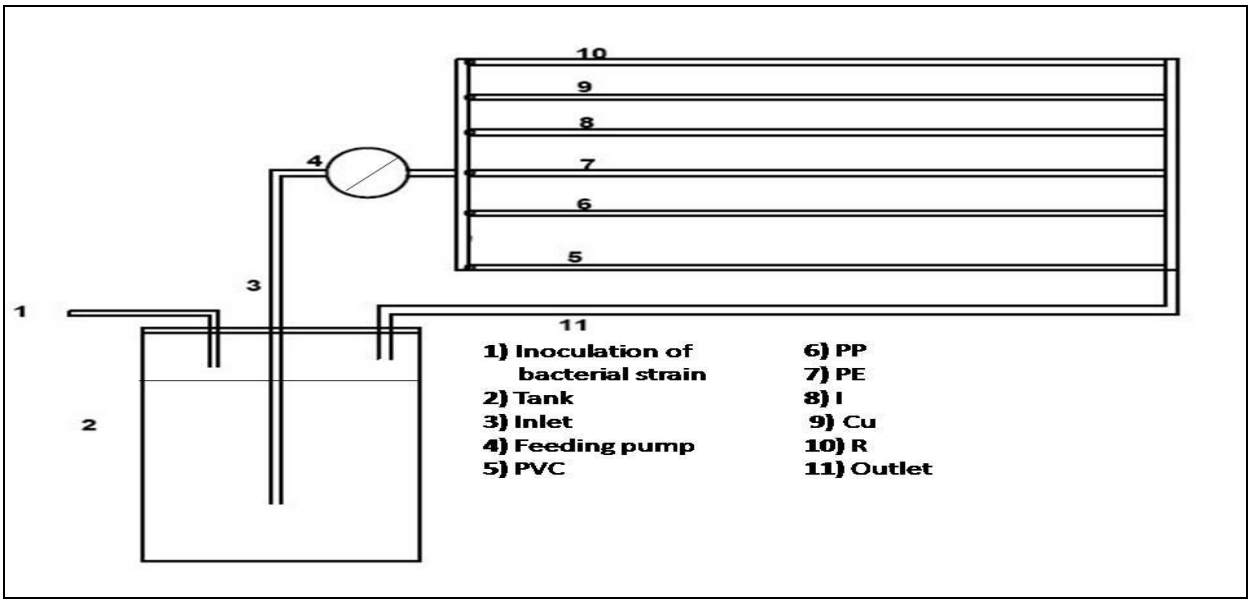


Fig. 4. Layout of *in vitro* simulated DWDS model (Hemdan *et al.*, 2015).

L. monocytogenes and *S.* Typhimurium biofilm formation were studied using two designed experimental models. The first model of DWDS injected by the bacterial suspension (10^6) of *L. monocytogenes*, and the second model was done by inoculating the bacterial suspension (10^6) of *S.* Typhimurium.

c. Biofilm sampling procedure

Six biofilm samples which formed in different types of pipe materials in two designed models of *L. monocytogenes* and *S.* Typhimurium were analyzed every 10 days to 90 days.

According to Marques *et al.* (2007) the biofilm samples were collected and scarped from the inner surface of pipe pieces (10 cm^2) of tested pipe materials using sterile cotton swabs. The scraped biofilm samples were suspended in sterile tubes containing 10 ml sterile water. The tubes were carefully well mixed using vortex shaken (Vortex-Genie 2®, model G56E) for 5 min. The counts of biofilm cells and amount of EPS were directly determined. After that, the biofilm cells in each tubes were enrichment to be (10^6 CFU/cm^2) and preserved for further experiments.

d. Enumeration of biofilm/sessile cells

The biofilm samples were diluted appropriately from tenfold serial dilutions depending on the cell density of biofilm samples. The biofilm cells were enumerated

using two techniques. The first technique was pour plate technique using non-selective media including plate count agar (PCA) and R2A agar. While the second technique used was spread plate technique using specific media for each tested pathogenic bacteria. *L. monocytogenes* was determined using Hicrome Listeria agar (HLSA) base and Listeria selective agar (LSA) (Oxoid-UK). While, *S.* Typhimurium was determined using Hicrome Improved Salmonella agar (HISA) (HiMedia, India) and Bismuth sulfite agar (BSA) (Oxoid-UK) media as specific media APHA (2012).

According to APHA (2012), the pour plate and spread plate technique were used to enumerate total counts of *L. monocytogenes* and *S.* Typhimurium biofilm cells. Replicate plates for biofilm samples from different pipe materials and different ages were analyzed for each dilution within the appropriate counts between 30 and 300 CFU selected for enumeration. In the pour plate technique, plate count agar and R2A agar media (Oxoid-UK) were used. While, a specific culture agar media were used in a spread plate technique. The density of cells in the biofilm was reported as CFU/cm^2.

e. Measurement of exopolysaccharides (EPS)

The EPS of six biofilm samples which formed in different types of pipe materials in two designed models of *L. monocytogenes* and *S.* Typhimurium were analyzed every 10 days to 90 days.

1. Extraction of EPS

The EPS was extracted from six biofilm samples using Cation exchange resin method according to Denkhaus *et al,* (2007); Michalowski *et al.* (2009) through the following steps;

a. In the tubes containing scraped biofilm formed on tested pipe materials, 1.5 ml of phosphate buffer was added in each tube. Phosphate buffer was used to counteract the decrease in pH value caused by treatment with a cation exchange resin Dowex Marathon C, Na^+ form, strongly acid (Sigma-Aldrich, Germany).

b. The resin was conditioned before application following the manufacturer's instructions. Conditioned resin was added to each screw tube (0.5 g per tube) and EPS extraction was performed for 1 hr on a shaker at 300 rpm and 4°C. The

extraction time and resin concentration used to minimize microbial cell disruption and maximize EPS extraction.

c. Crude EPS extracts were centrifuged at 10,000×rpm for 15 min to remove the solid parts of biofilm. The resulting clear EPS extracts (supernatant) were analyzed polysaccharide content.

2. Measurement of exopolysaccharide contents

The polysaccharide contents of crude EPS was determined using phenol-sulfuric acid method according to the protocol described by Dubois *et al.* (1956). Phenol solution (80%) was added immediately (12.5 µl) to 0.5 ml of EPS extract then 1.25 ml of concentrated sulfuric acid was pipetted rapidly into the tubes to ensure sufficient mixing. Samples were allowed to stand for 10 min before mixing and were later incubated in a water bath at 30°C for 20 min. The absorption was measured at 485 nm against a reagent blank using Spectrophotometer (UV-VIS recording Spectrophotometer- SHIMADZV).

According to Hofmann *et al.* (2009) the polysaccharide content was calculated using standard curve of glucose (0-200 µg/ml). Results are given as glucose equivalents per cm^2 of biofilm surface area.

f. Determination the effect of different antimicrobial agents against planktonic and biofilm cells

The planktonic cells (*L. monocytogenes* ATCC 25152 and *S.* Typhimurium ATCC 14028) were subcultured into 100 ml TSB and incubated at 37°C for 24 hrs then centrifuged at 3000 rpm for 20 min. The numbers of *L. monocytogenes* and *S.* Typhimurium planktonic cells (10^6 CFU/ml) of cells were obtained.

In case of preserved stocked tubes which previously mentioned (*L. monocytogenes* and *S.* Typhimurium biofilm), after biofilm formation, the pipe pieces were removed from tested pipe materials at different ages (10, 40 and 90 days-old). The biofilm cells adhered to the pieces were scraped using sterile swabs and suspended in sterile distilled water. Aliquots of the suspensions were centrifuged and re-suspended in sterile distilled water according to Rajasree *et al.* (2014). The initial bacterial populations of the biofilm cells were 10^6 CFU/cm^2.

1. Effect of chlorine concentrations

a. Preparation of chlorine water

Chlorine gas from Giza drinking water treatment plant was injected into one liter brown glass-stopper bottle containing 250 ml chlorine-free distilled water and stored in dark at 4^{o}C.

b. Determination of chlorine concentration.

Chlorine concentration was determined by adding two ml of H_2SO_4 (conc.), one gm potassium iodide (KI) and 0.5 ml chlorine water were added into 100 ml free chlorine distilled water in 250 ml conical flask with quick fit stopper. The flask was kept in the dark for 5 min then titrated with sodium thiosulfate (titrant) until the pale yellow color appear, then 1 ml of starch solution was added and continues in titration until the blue color disappeared. Chlorine (mg per liter) was calculated from the following equation;

mg Cl_2/l = ml of titrant X 0.9 (MW of Cl x N of sodium thiosulfate titrant) / ml of sample.

The planktonic cells and biofilm/sessile cells of *L. monocytogenes* and *S.* Typhimurium were exposed to the different concentrations of chlorine water of 0.2, 0.6, 1.0, 1.4, 1.8, 2.2, 2.6 and 3.0 mg/l for 10 min**,** to determine the effective dose of chlorine able to reduce the counts of biofilm cells and study the effect of the biofilm age on chlorine.

c. Determination of chlorine breakpoint and residual chlorine

Nine flasks (250 ml conical flask with quick fit stopper) containing 100 ml sterile distilled water were inoculated by 500 µl of tested strains (planktonic cells and biofilm cells developed on different types of pipe materials at different ages). Then inoculated by different conc. of chlorine water of 0.2, 0.6, 1.0, 1.4, 1.8, 2.2, 2.6 and 3.0 mg/l and un-inoculated chlorine water flask was used as control. Flasks were incubated for 10 min in dark.

According to APHA (2012) one ml was transferred from each flask to determine total bacterial counts using plate count agar at each chlorine dose. The residual chlorine was determined by adding 5 ml phosphate buffer solution and 5 ml from N, N-diethyl-p-

phenylenediamine (DPD) indicator reagent to 100 ml of each inoculated flask and the absorbance was immediately measured using spectrophotometer (UV-VIS Recording spectrophotometer- SHIMADZV) at wavelength 515 nm. The breakpoint for each strain was determined by drawing the chlorine doses (mg/l) (X axis) versus residual chlorine (mg/l) (Y axis).

2. Determination of antibacterial effect and the minimal inhibitory concentration (MIC) of silver ions (Ag^+)

a. Preparation of working stock solution

Silver metal (Ag^+) with 99.99 % highly purified (Merck Germany) was used. Stock solution of silver ions was prepared by dissolving 1.6 g of $AgNO_3$ in 1000 ml of distilled water to get final concentration of 1g Ag^+ in 1000 ml of distilled water.

Five concentrations of Ag ions working stock solution (Ag^+) including 50, 100, 200, 300 and 500 mg/l at different contact times (5, 10 and 15 min) were used. The toxicity assay for five concentrations of silver ions which used as an antibacterial was measured using Microtox analyzer 500.

b. Determination of antibacterial effect and MIC of silver ions

The antibacterial effect and MIC (the minimum concentration able to complete removal of planktonic and biofilm cells in short time) of Ag ions were determined for the three ages of biofilm formed on each tested pipe material and compared with planktonic cells.

The stock biofilm and planktonic cells were previously prepared. Each one of stock suspension was exposed to five concentrations of Ag ions at three contact times. This was carried out by using six flasks (250 ml conical flask) containing 100 ml sterile distilled water were inoculated with 500 µl of planktonic cells and biofilm cells at different ages. Then the later flasks were inoculated by different concentrations of Ag ions stock solution from 50, 100, 200, 300 and 500 mg/l with un-inoculated of flask used as a control.

All flasks were incubated for different contact times (5, 10 and 15 min) in a dark to determine MIC. The recovery of viable cells which exposed to doses of Ag ions was

determined according to APHA (2012). Using pour plate technique, one ml from each flask was transferred to sterile Petri dishes to determine total cell counts.

3. Determination of antibacterial effect and MIC of silver nanoparticles (AgNPs)

a. Synthesis of silver nanoparticles in montmorillonite (MMT)

Synthesis of AgNPs was carried out according to Shameli *et al.* (2010). Ten g MMT (Kunipa-F Japan) was suspended into 100 ml solution containing 0.63 g silver nitrate ($AgNO_3$) and the suspension was kept under stirring for 24 hrs at ambient temperature. Afterwards, 00.56 g of $NaBH_4$ with purity 98.5%, (Sigma, Germany) was added to the suspension and stirring was continued for one hr. The suspension was finally centrifuged at 5,000 rpm for 20 min. The precipitates washed four times using double distilled water in order to remove the silver ion residue, and dried overnight at 40°C.

b. Characterization of the synthetic AgNPs

The examination of synthetic AgNPs was carried out by using transmission electron microscope analysis model JEM 2100-HRTEM. Microscope operated at 300 kV (LaB6 cathode, point resolution 0.17 nm) with an EDX (Energy Dispersive X-ray) detector attached. Powder samples were dispersed in ethanol and the suspension was treated with ultrasound for 10 min. A drop of a very dilute suspension was placed on a holey carbon-coated copper grid and allowed to dry by evaporation at ambient temperature. The images were recorded on a CCD camera with the resolution of 1024 × 1024 pixels using the Digital Micrograph software package.

c. Evaluation of antibacterial activity and MIC of AgNPs

The antibacterial effect and MIC of AgNPs were determined for the three ages of biofilm formed on each tested pipe material and compared with plankonic cells. The stock biofilm and planktonic cells were previously prepared.

The stock working solution of AgNPs was prepared by dissolving one g of AgNPs in 1000 ml of distilled water to get final concentration of 1g/1000 ml. Five different concentrations of AgNPs stock solution (50, 100, 200, 300 and 500 mg/l) at different contact times (5, 10 and 15 min) were used. The toxicity assay of different concentrations of AgNPs which used as an antibiofilm activity was measured using Microtox analyzer 500.

The stock biofilm and planktonic cells were previously prepared. Each one of stock suspension was exposed to five concentrations of AgNPs at three contact times. This was carried out by using six flasks (250 ml conical flask with) containing 100 ml sterile distilled water were inoculated with 500 µl of planktonic cells and biofilm cells at different ages. Then the later flasks were inoculated by different concentrations of AgNPs stock solution from 50, 100, 200, 300 and 500 mg/l with un-inoculated of flask used as a control.

All flasks were incubated for different contact times (5, 10 and 15 min) in a dark to determine MIC. The recovery of viable cells which exposed to doses of AgNPs was determined according to APHA (2012). Using pour plate technique, one ml from each flask was transferred to sterile Petri dishes to determine total cell counts.

4. Antibiotic susceptibility test

The susceptibility tests were performed by using the standard disk according to NCCLS (2012). As shown in Table (2), the six antibiotic groups were chosen.

Table 2. The suggested antibiotic groups of tested antibiotic

Antibiotic group	Antibiotic name	Antibiotic abbreviation	Disk content
Penicillin group	Amoxicillin	AML	10 µg
3rd generation Cephalosporin's group	Cefixime	CFM	5 µg
Quinolones group	Ciprofloxacin	CIP	5 µg
Tetracycline group	Tetracycline	TE	30 µg
Macrolides group	Clarithromycin	CLR	15 µg
Aminoglycosides group	Streptomycin	S	10 µg

a. Estimation of inhibition zone

The biofilm cells from six different pipe materials (PVC, PP, PE, I, Cu and R) at different ages (10, 40 and 90 days-old) and planktonic cells were examined for the

susceptibility for six different antibiotics by using the standard disk diffusion method as follows;

1. The appropriate volume (100 µl) of either biofilm cells or planktonic cells suspension inoculated onto the entire surface of a dried MHA plate by using a sterile cotton swab. The MHA plates were held at ambient temperature under a biological hood for 10 min to allow evaporation/adsorption of free surface liquid.
2. Antibiotic discs were placed on the surface of each inoculated MHA plate and incubated for 24 hrs at 37°C.
3. The diameter of the inhibition zone (in mm) around each disk was measured and interpreted according to NCCLS (2012) to classify the antibiotic sensitivity of each isolates (sensitive, intermediate or resistant).

b. Reporting results

Susceptible, intermediate and resistant interpretations of zone diameter measurements are reported and defined as a following:

1. **Susceptible (S)**

The susceptible category implies that isolates are inhibited by the usually achievable concentration of antimicrobial agent when the recommended dosage is used for the site of infection.

2. **Intermediate (I)**

The intermediate category implies that the response rate of isolates may be lower than for the susceptible isolates.

3. **Resistant (R)**

The resistant category implies that isolates are not inhibited by the usually achievable concentration of antimicrobial agent with normal dosage schedule and/or that demonstrate zone diameters.

g. Survival of *L. monocytogenes* and *S.* Typhimurium biofilm cells

The biofilm cells, which formed by *L. monocytogenes* (6 samples) and *S.* Typhimurium (6 samples) at 90 days-old which scraped from different types of pipe materials were examined to survive and persist in two types of drinking water (tap and

ground water). The physio-chemical characteristics of tap and ground water which used in the experiment were analyzed according to APHA (2012). Also, the planktonic cells (*L. monocytogenes* ATCC 25152 and *S.* Typhimurium ATCC 14028) were examined for their survival at the in the tested water.The inocula density (10^6 CFU/ml) of biofilm cells of *L. monocytogenes* and *S.* Typhimurium formed on tested pipe material and their planktonic cells, in tested water were determined by ten folds serial dilutions using both selective media and plate count agar weekly internal.

1. Inoculation of tap and groundwater by biofilm cell suspensions

Fourteen flasks (7 flask containing 200 ml of sterilized tap water and 7 flask containing 200 ml of sterilized tap water) were inoculated by one ml of each strain were incubated at 20±2°C (ambient temperature) (Fig. 5).

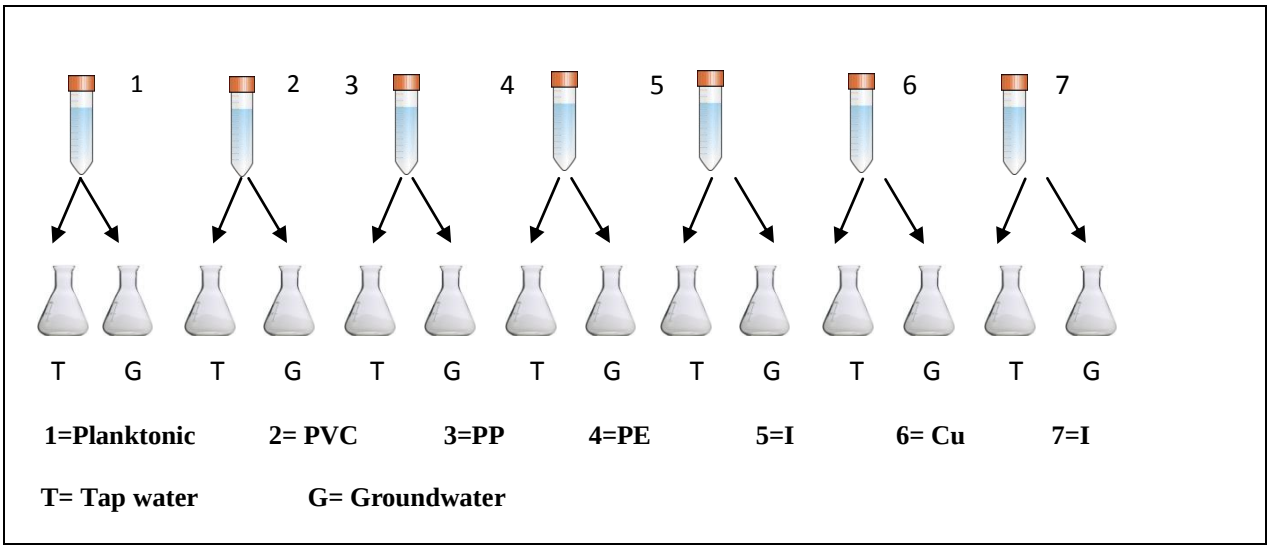


Fig. 5. Illustrated diagram for the experiment of 90 days-old biofilm survival.

2. Determination of total survived bacterial cells

Determination of the survived and persisted bacterial cells were counted in the first and second days, then counted at weekly intervals (7, 14, 21, and 28 for 105 days) using tenfold serial dilution from each flask (14 flasks). Listeria selective agar as a specific medium and plate count agar as a general medium were used to determine of *L. monocytogenes* counts, while Bismuth sulfite agar as a specific medium and plate count agar as a general medium were used to determine of *S.* Typhimurium.

h. Microscopic examination of *L. monocytogenes* and *S.* Typhimurium biofilm

1. Epi-fluorescence microscopic examination of biofilm

The enumeration of the suspended bacterial biofilm cells at different ages (10, 40 and 90 days-old) was performed by using 4, 6 Diamidino-2-Phenylindole (DAPI) (Sigma- UK) staining method as described by Brunk *et al*. (1979). The biofilm samples were gently homogenized in vortex for 2 min and a small volume (0.5-4 ml) was filtered through a 0.22 mm black polycarbonate membrane (chmlab group, Spain). After 10 min of incubation with 200 µl of 0.5 mg/ml DAPI solution in the dark, the polycarbonate membrane was washed and counted using non-fluorescent immersion oil on glass microscope slides. Then the membranes were examined using an epi-fluorescence microscope (Olympus BX43, Japan).

The biofilm cells were visualized under an epi-fluorescence microscope equipped with a filter sensitive to DAPI fluorescence (359 nm excitation filter in combination with a 461 nm emission filter). A total of 20 fields with 300 cells or a sufficient number of microscopic fields was counted and calculated per cm^2 (Simões *et al.,* 2012).

2. Transmission electron microscopy (TEM) examination of *L. monocytogenes*

Six biofilm samples of *L. monocytogenes* developed on different pipe materials after 90 days-old were examined by transmission electron microscopy (TEM) model JEM 2100-HRTEM. Biofilm samples were investigated by the negative contrast method and the method of ultrathin sections. For the investigation of total samples by the negative contrast method, the biofilm cells suspensions were applied to a Formvar coated grid stabilized with carbon. The samples were stained by a 1% water solution of the ammonium molybdate (Sigma, UK) for 30 Sec then examined under TEM (Didenko *et al.,* 2000; 2012).

3. Scanning electron microscopic (SEM) examination of *S.* Typhimurium

Six 90 days-old *S.* Typhimurium biofilm samples developed on different pipe materials (PVC, PP, PE, I, Cu and R) were examined by scanning electron microscopy (SEM) model JEOL JXA-840A, electron probe microanalyzer, Japan**.**

According to, Yu *et al.* (2010) 1x1cm^2 of pipe coupons was prepared for examination using scanning electron microscopy. The samples were prepared by fixation with 2.5% glutaradehyde for 1 hr and with 1% osmium tetroxide for 1 hr. And then they were dehydrated in 50, 70, 90 and 95% ethanol for 10 min per each step and two times in 100% ethanol for 10 min. They were dehydrated two times in HMDS (hexamethyldisilizane) for 10 min, air dried, and coated with gold. Their images were analyzed using SEM.

i. Toxicity assay for the tested plumping materials

Six samples of tested plumping materials (PVC, PP, PE, I, Cu and R) were prepared to measure and determine their toxicity.The determination of toxicity assay was carried out using Microtox analyzer 500. Microtox analyzer Model 500 is fully automated and temperature controlled and needs no daily adjustment or calibration. A marine luminescent bacterium *Vibrio fischeri* (earlier referred as *Photobacterium phosphoreum*) has been widely used for acute toxicity estimation with several commercial tests.

The bioassay is based on the detection of light output changes through the light production (which directly relative to the metabolic activity of the bacterial population) and any inhibition of enzymatic activity causes a corresponding decrease in bioluminescence. The luminescent bacteria are exposed to a range of concentrations of the testing substance. The light emitted from the bacteria is measured by a light reader (Microtox Model 500 Analyzer®) to set up a dose response relationship, (IC_{50}), a concentration which inhibits 50% of light output and then recorded as a toxicity degree (Farre and Barcelo, 2003).

j. Statistical analysis

The statistical analysis was carried out using SPSS version 14.0, computer application. All the data were transformed in decimal logarithms and processed. This is beside determination of the correlations between many dependant and independent factors as follows;

1. Bivariante person correlation between the biofilm ages and the counts of the biofilm cells.
2. Bivariante person correlation between the biofilm ages and the amounts of produced exopolysaccharide by biofilm cells.
3. The correlation between the residual chlorine doses and tested pathogenic bacteria counts at different chlorine doses and after contact time 10 min by using regression analysis (R^2).
4. The correlation between the doses of Ag ions, contact time and log counts using Homogeneity test of variances from one way *ANOVA*.
5. The correlation between the doses of AgNPs, contact time and log counts using Homogeneity test of variances from one way *ANOVA*.
6. Bivariante person correlation between the survival time and the log counts of biofilm and planktonic cells in different tested water.

RESULTS AND DISCUSSION

1. Enumeration of microbial populations in natural biofilm

Drinking water distribution systems (DWDS) contain a diverse microbial community of bacteria, protozoa, virus, algae and fungi (Armbruster *et al.,* 2012). Bacteria are generally dominant in biofilm due to their high growth rates, small size, adaptation capacities and the ability to produce EPS. However, viruses, protozoa, fungi and algae may also be present in DW biofilm. By adopting the sessile mode of life, biofilm embedded microorganisms enjoy a number of advantages over their planktonic counterparts (Snelling *et al.,* 2006).

In DWDS, under most operating conditions a large fraction of total biomass is found attached to the pipe walls (Srinivasan *et al.,* 2008). The presence of biofilm can cause serious problems for humans whereas, it can be formed in a different environment inhabited by microorganisms, so it has been shown as a reservoirs of pathogens and they only require the presence of hydrated environment and minimum amount of nutrients for their formation (McLean *et al.*, 2013).

Also, biofilm formation makes microorganisms more resistant to antibiotic and disinfectant agents, synergism between species and domination of the immediate environment. The impacts of biofilm formation include damage of industrial equipment, food contamination which can cause food borne infections, food poisoning and nosocomial infections. It is necessary to monitor of water distribution system pipes and other drainage accessories (*e.g.* kitchen, bathroom and laboratories sink drainage pipes) for biofilm formation (Elias and Banin, 2012).

There are a variety of environmental opportunistic human pathogens that can pass through drinking water treatment barriers in very low densities and take advantage off and colonize selected sites in the water supply systems. They are typical biofilm organisms that grow on the periphery of the distribution systems (long pipe runs into dead ends) and throughout the pipe network where the water can be stagnant. The most important organisms to consider are *Pseudomonas aeruginosa*, *Aeromonas* spp.,

Salmonella spp., *E. coli* and others that are widespread in surface water. Their presence in the water supply indicates biofilm development in sediment accumulations in the pipeline, especially iron pipes (WHO, 2008). Therefore, the main scope of this part of the present study aimed to investigate and understand structure and diversity of microbial community that involved in natural biofilm formation in different microinhabitants.

Total viable bacterial counts (TVBC) at 37and 22^{o}C were enumerated in all biofilm samples by using pour plate technique. The spread plate technique was used to determine of total coliform (TC), fecal coliform (FC), *E. coli* and fecal streptococci (FS). Some pathogenic microbes (*Staph. aurues, Bacillus cerues, L. monocytogenes, Salmonella* spp, *P. aeruginosa, Cl. perfringens* and *Candida albicans* were enumerated as well.

a. Microbiological analysis of the natural biofilm collected from three different materials of DWDS

The biofilm formation in DWDS has many adverse consequences. Knowledge of microbial community structure of DWDS biofilm can aid in the design of an effective control strategy. However, a biofilm bacterial community in real DWDS and the impact of the drinking water purification strategy remain unclear (Wu *et al.,* 2015).

In case of the biofilm formation on PP material, results in Table (3) and Fig. (6) showed that, the average counts of TVBC at 37and 22^{o}C were 1.3x10^{3} and 1.9x10^{3} CFU/cm^{2}, respectively. The obtained counts are lower than those of Rogers *et al.* (1994) who found that, the TVBC in PP materials were 2.1x10^{5}-5.5x10^{7} CFU/cm^{2}. The coliform group has been used extensively as the basis for regulating the microbial quality of drinking water. Good quality drinking water can suffer serious contamination in distribution systems because of breaches in the integrity of pipelines and storage reservoirs (Shrestha *et al.,* 2009). In the present study, the averages counts of TC, FC, *E.coli* and FS were at samples 2 and 5 were 1.6x10^{2}, 8.4x10, 6.6x10 and 1.3x10^{2} CFU/10cm^{2}, respectively. While, the biofilm samples nos. 1, 3 and 4 were free from TC, FC, *E. coli* and FS

All tested pathogenic microbes in all the biofilm samples were absent. It means that, these pathogens may be not found or their numbers are very low in drinking water, even though the TVBC was present in drinking water. Whereas, in drinking water and associated biofilm, the presence of heterotrophic and oligotrophic bacteria does not necessarily represent an infectious risk and a concern in terms of the consumer's health (Glasmacher *et al.*, 2003).

Table 3. Determination of the classical bacterial indicators in the natural biofilm collected from PP material of DWDS.

Biofilm samples	TVBC/ cm^2		Colony forming unit/$10cm^2$			
	37°C	22°C	TC	FC	*E. coli*	FS
1	$2.7x10^2$	$3.4x10^2$	ND	ND	ND	ND
2	$2.1x10^3$	$2.8x10^3$	$2.2x10^2$	$1.9x10^2$	$1.5x10^2$	$1.1x10^2$
3	$4.5x10^2$	$5.7x10^2$	ND	ND	ND	ND
4	$6.1x10^2$	$7.4x10^2$	ND	ND	ND	ND
5	$3.4x10^3$	$5.1x10^3$	$5.8x10^2$	$2.3x10^2$	$1.8x10^2$	$5.4x10^2$
Min.	$2.7x10^2$	$3.4x10^2$	$2.2x10^2$	$1.9x10^2$	$1.5x10^2$	$1.1x10^2$
Max.	$3.4x10^3$	$5.1x10^3$	$5.8x10^2$	$2.3x10^2$	$1.8x10^2$	$5.4x10^2$
Average	$1.3x10^3$	$1.9x10^3$	$1.6x10^2$	8.4x10	6.6x10	$1.3x10^2$

ND= Not detected TC: Total coliform, FC: Fecal coliform, FS: Fecal streptococci.

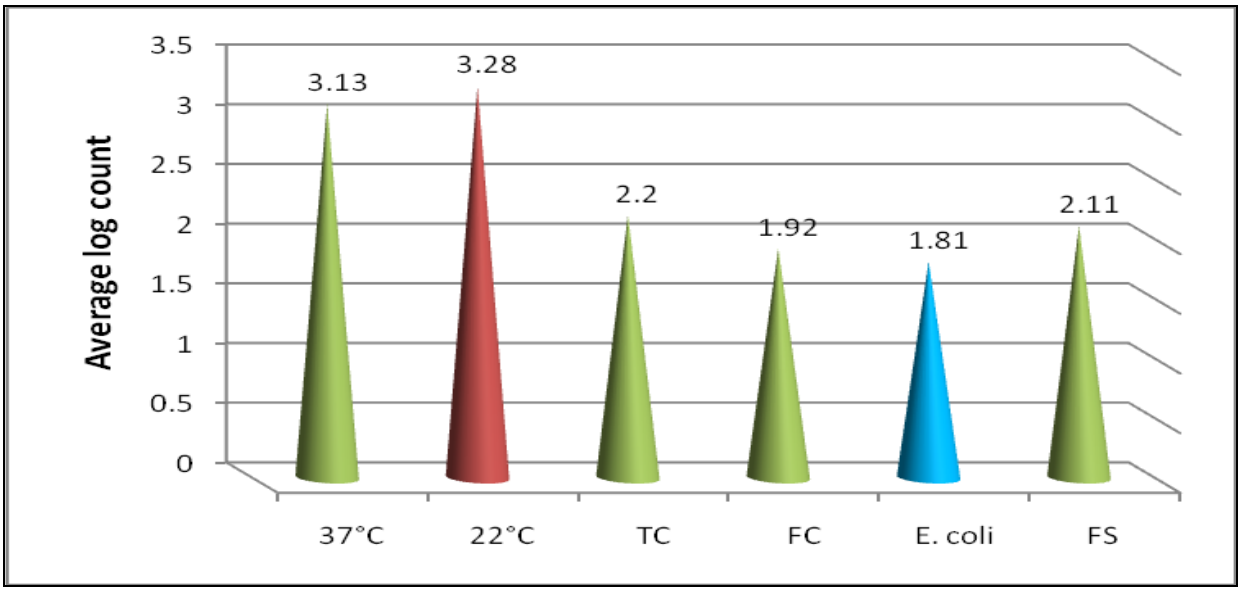


Fig. 6. Average log counts of the bacterial indicators in the natural biofilm collected from PP material of DWDS.

Regarding the biofilm from PVC pipe (Table 4; Fig. 7), results demonstrated that counts of TVBC at 37and 22°C were ranged between $3.6x10^2$-$6.1x10^3$ and $4.1x10^2$-$7.2x10^3$ CFU/cm^2, respectively. These results are compatible to the results of Shin *et al.* (2007) they found that, TVBC was ranged between $2.0x10^1$-$8.0x10^4$ CFU/cm^2.

In addition, TC, FC, *E. coli* and FS were present in all biofilm samples except sample no. 5. The counts of TC, FC, *E. coli* and FS were ranged between $1.9x10^2$-$6.4x10^2$, $1.2x10^2$-$4.5x10^2$, $1.2x10^2$-$3.4x10^2$ and $2.3x10^2$-$4.5x10^2$ CFU/10cm^2, respectively.

These results higher than those of Wingender and Flemming (2004) who found real counts of coliform in PVC pipes scraped from DWDS were 3 CFU/10cm^2. Also, coliform counts were 11 CFU/10cm^2 from PVC coupons exposed to by-pass of DWDS (Juhna *et al.*, 2007).

Concerning the results of tested pathogenic microbes, it was cleared that, all tested pathogenic microbes were absent in all PVC pipe. Although these samples were absent from pathogens, it must be negligible the detection of TVBC presence. These due to the non-pathogenic bacterial populations act as a primary role in biofilm formation (LeChevallier, 2003).

Table 4. Determination of the bacterial indicators in the natural biofilm collected from PVC material of DWDS.

Biofilm samples	TBC/cm^2		Colony forming unit/10cm^2			
	37°C	22°C	TC	FC	*E. coli*	FS
1	$1.8x10^3$	$2.7x10^3$	$4.1x10^2$	$3.7x10^2$	$2.8x10^2$	$2.7x10^2$
2	$5.2x10^3$	$6.1x10^3$	$1.9x10^2$	$1.2x10^2$	$1.2x10^2$	$4.5x10^2$
3	$6.1x10^3$	$7.2x10^3$	$6.4x10^2$	$4.5x10^2$	$3.4x10^2$	$2.3x10^2$
4	$1.3x10^3$	$2.1x10^3$	$3.8x10^2$	$2.3x10^2$	$1.8x10^2$	$3.6x10^2$
5	$3.6x10^2$	$4.1x10^2$	ND	ND	ND	ND
Min.	$3.6x10^2$	$4.1x10^2$	$1.9x10^2$	$1.2x10^2$	$1.2x10^2$	$2.3x10^2$
Max.	$6.1x10^3$	$7.2x10^3$	$6.4x10^2$	$4.5x10^2$	$3.4x10^2$	$4.5x10^2$
Average	$2.9x10^3$	$3.6x10^3$	$3.2x10^2$	$2.3x10^2$	$1.8x10^2$	$2.6x10^2$

ND= Not detected TC: Total coliform, FC: Fecal coliform, FS: Fecal streptococci.

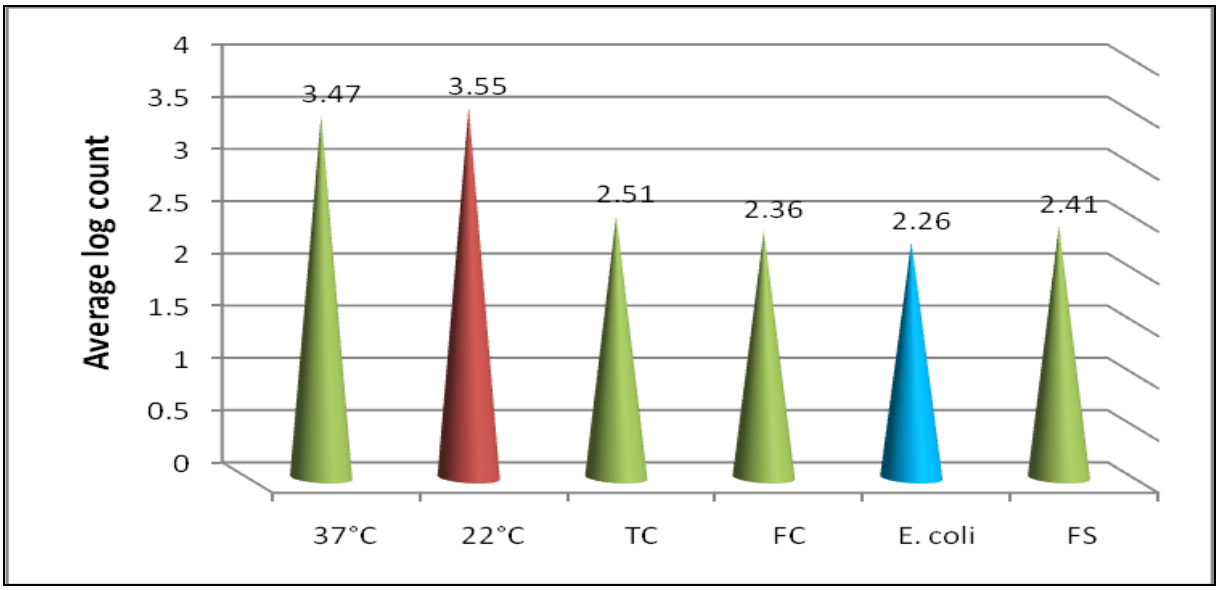


Fig. 7. Average log counts of the bacterial indicators in the natural biofilm collected from PVC pipe material of DWDS.

Iron and steel pipes have been used in water distribution systems in several centuries throughout the world and are subjected some problems such as corrosion, causing deterioration of potable water quality due to unwanted chemical and biochemical reactions (Husband and Boxall, 2011).

Concerning I pipe material, the results indicated that the lowest counts of TVBC at 37 and 22°C were $3.1x10^3$ and $2.8x10^3$ CFU/cm^2, respectively. While, the highest counts of TVBC at 37 and 22°C were $2.6x10^6$ and $4.7x10^6$ CFU/cm^2. In addition to that, the average counts of TVBC at 37 and 22°C were $2.8x10^5$ and $5.0x10^5$ CFU/cm^2, respectively (Table 5; Fig. 8). Also, September *et al.* (2007) found that, the heterotrophic plate counts were ranged between 1.0x10 - $1.9x10^9$ CFU/cm^2 in biofilm samples collected from I pipe material. While a variety of potential pathogens were isolated at various densities up to 10^4 CFU/cm^2 from some sampling points.

In case of TC, FC, *E. coli* and FS, the results cleared that the average counts were $2.6x10^4$, $7.8x10^3$, $6.2x10^3$ and $1.2x10^3$ CFU/10 cm^2, respectively. Also, counts of TC, FC, *E. coli* and FS ranged between $1.7x10^2$-$7.8x10^4$, $1.2x10^2$-$6.7x10^4$, $1.1x10^2$ -$5.3x10^4$ and $1.4x10^2$-$7.4x10^3$ CFU/10 cm^2, respectively. Friedman *et al.* (2003) found that, coliform bacteria present within corrosion tubercles. In addition to Emde *et al.* (1992) found that

total coliform group was present in greater numbers in corrosion deposits than in the untreated water supply. Therefore, Reynolds *et al.* (2005) suggested that the areas of greatest public health risk can be found through monitoring of indicator bacteria on environmental surfaces.

Table 5. Determination of the bacterial indicators in the natural biofilm collected from iron material of DWDS.

Biofilm samples	TVBC/cm^2		Colony forming unit/10cm^2			
	37^{o}C	22^{o}C	TC	FC	*E. coli*	FS
1	$4.4x10^3$	$5.2x10^3$	$3.2x10^2$	$2.4x10^2$	$2.1x10^2$	$1.8x10^2$
2	$2.6x10^4$	$3.1x10^4$	$4.1x10^3$	$3.1x10^3$	$2.9x10^3$	$2.7x10^2$
3	$1.5x10^4$	$2.4x10^4$	$2.9x10^3$	$1.9x10^3$	$1.4x10^3$	$4.3x10^2$
4	$2.6x10^6$	$4.7x10^6$	$1.8x10^5$	$6.7x10^4$	$5.3x10^4$	$7.4x10^3$
5	$6.1x10^3$	$7.3x10^3$	$4.9x10^2$	$3.9x10^2$	$3.6x10^2$	$2.4x10^2$
6	$2.9x10^4$	$3.5x10^4$	$6.2x10^2$	$5.7x10^2$	$5.7x10^2$	$3.9x10^2$
7	$1.2x10^5$	$1.9x10^5$	$7.8x10^4$	$4.8x10^3$	$3.1x10^3$	$2.5x10^3$
8	$4.2x10^3$	$6.9x10^3$	$2.7x10^2$	$1.2x10^2$	$1.2x10^2$	$3.2x10^2$
9	$3.1x10^3$	$2.8x10^3$	$5.2x10^2$	$3.2x10^2$	$2.5x10^2$	$1.4x10^2$
10	$5.7x10^3$	$6.4x10^3$	$1.7x10^2$	$1.3x10^2$	$1.1x10^2$	$2.3x10^2$
Min.	$3.1x10^3$	$2.8x10^3$	$1.7x10^2$	$1.2x10^2$	$1.1x10^2$	$1.4x10^2$
Max.	$2.6x10^6$	$4.7x10^6$	$7.8x10^4$	$6.7x10^4$	$5.3x10^4$	$7.4x10^3$
Average	$2.8x10^5$	$5.0x10^5$	$2.6x10^4$	$7.8x10^3$	$6.2x10^3$	$1.2x10^3$

TC: Total coliform, FC: Fecal coliform, FS: Fecal streptococci.

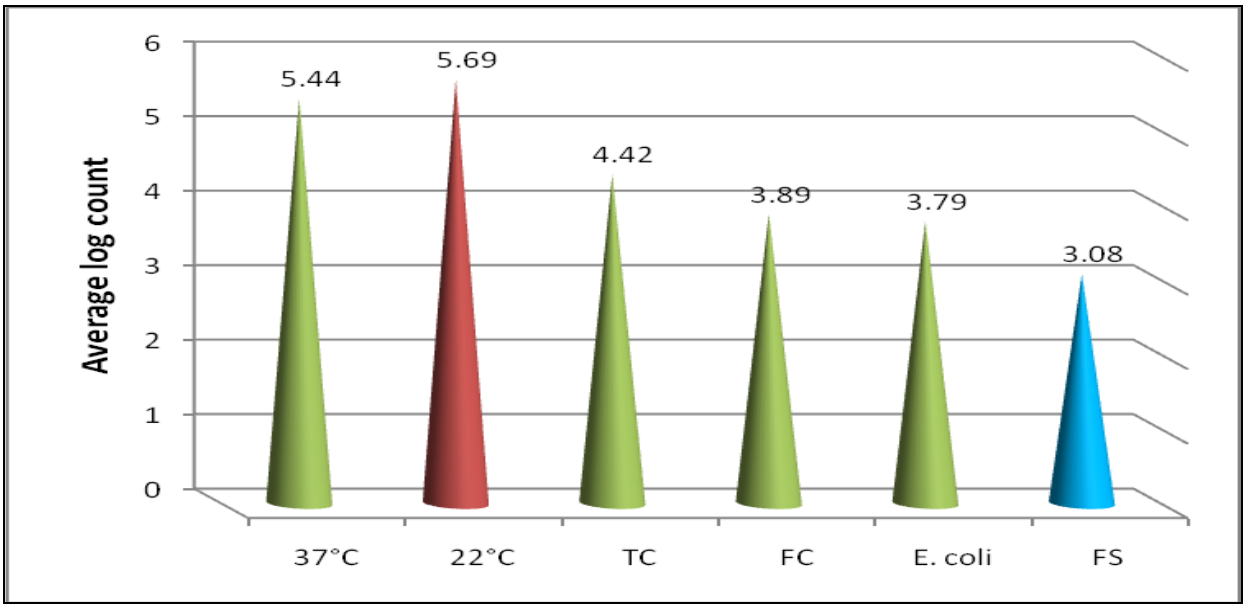


Fig. 8. Average log counts of the bacterial indicators in the natural biofilm collected from I pipe of DWDS.

In case of some pathogenic bacteria Table (6) and Fig. (9) showed that, the lowest average counts were recorded with *Staph. aureus* was $5.4x10^2$ CFU/$10cm^2$. While, the highest average counts were recorded with *Candida albicans* was $1.3x10^4$ CFU/$10cm^2$. In addition, the average counts of *Staph. aurues*, *Bacillus cerues*, *L. monocytogenes*, *Salmonella* spp., *P. aeruginosa*, *Cl. perfringens* and *Candida albicans* were $5.4x10^2$, $1.5x10^3$, $1.1x10^3$, $1.2x10^3$, $2.1x10^3$, $7.5x10^2$ and $1.3x10^4$ CFU/$10cm^2$, respectively. Also, September *et al.*, (2007) found that, the potential pathogens were isolated at various densities up to 10^4 CFU/cm^2 from some biofilm sampling points in I pipe material.

The results of the present study summarized that, the TVBC of biofilm scraped from iron pipe material was higher than PVC and PP pipe materials in DWDS. Moreover, the tested pathogenic bacteria were absent in all biofilm samples in PVC and PP pipe materials, while they were present in iron pipe material. This may be due to that I pipe material can allow microbial community form more than plastic based materials.

Also, Chang *et al.* (2003) found that biofilm formation on pipes made of rough surface materials such as iron, concrete-lined cast iron, and galvanized steel was greater than that on smooth-surface polyvinyl chloride (PVC) and polypropylene (PP) pipe. Furthermore, pipe materials may also affect the structure of microbial communities in biofilm and outlet water from the pipes.

Table 6. Enumeration of pathogenic microbes in the natural biofilm collected from iron material of DWDS.

Biofilm samples	Colony forming unit/10cm^2					
	Staph. aurues	***Bacillus cerues***	***L. monocytogenes***	***Salmonella* spp.**	***P. aeruginosa***	***perfr***
1	$2.5x10^2$	$2.4x10^2$	$2.6x10^2$	$4.3x10^2$	$4.9x10^2$	3.4
2	$3.8x10^3$	$3.9x10^2$	$5.3x10^3$	$2.8x10^2$	$3.8x10^3$	3.2
3	$4.7x10^2$	$5.2x10^2$	$4.2x10^2$	$3.2x10^3$	$2.7x10^3$	2.4
4	$2.3x10^3$	$1.3x10^4$	$3.7x10^3$	$5.3x10^3$	$1.3x10^4$	4.6
5	$5.2x10^2$	$4.7x10^2$	$1.2x10^2$	$3.7x10^2$	$3.9x10^2$	1.3
6	$3.1x10^2$	$2.3x10^3$	$3.0x10^2$	$1.3x10^3$	$4.8x10^2$	2.5
7	$2.9x10^3$	$3.1x10^3$	$2.4x10^2$	$4.5x10^2$	$1.7x10^2$	5.3
8	$3.7x10^2$	$2.4x10^2$	$3.1x10^2$	$2.8x10^2$	$2.4x10^2$	3.7
9	$4.2x10^2$	$1.9x10^2$	$1.5x10^2$	$6.4x10^2$	$4.5x10^2$	4.2
10	$1.3x10^2$	$3.0x10^2$	$4.2x10^2$	$3.2x10^2$	$3.2x10^2$	3.5
Min.	$1.3x10^2$	$1.9x10^2$	$1.2x10^2$	$2.8x10^2$	$1.7x10^2$	1.3
Max.	$3.8x10^3$	$1.3x10^4$	$5.3x10^3$	$5.3x10^3$	$1.3x10^4$	4.6
Average	$5.4x10^2$	$1.5x10^3$	$1.1x10^3$	$1.2x10^3$	$2.1x10^3$	7.5

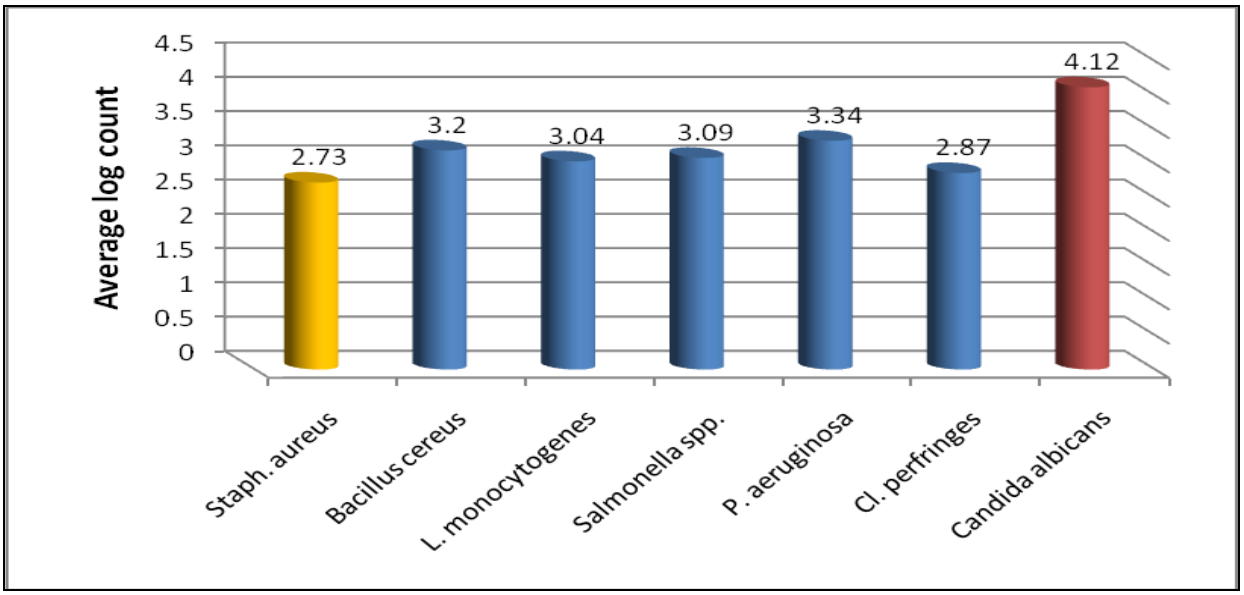


Fig. 9. Average log counts of the pathogenic microbes in the natural biofilm collected from I pipe of DWDS.

a. Microbiological analysis of the natural biofilm collected from microinhabitants

Fomites (bathroom and kitchen surfaces) in households have been found to contribute to the transmission of human pathogens **(**Reynolds *et al.,* 2005). Because many microbes are capable of causing infections at low doses (Sinclair *et al.,* 2008), these pathogens can survive for hours to weeks on the surfaces such as cutting boards and bathroom floors.

Besides, the fomites have been proposed as a significant transmission route for outbreaks (Duan *et al.,* 2003), or longer on surfaces with moisture and biological substances. They are also believed to play a significant role in the transmission of other respiratory and enteric infections (Boone and Gerba, 2007).

In case of bathroom sink drainage pipes, The results of this study showed that, the lowest counts of TVBC at 37 and 22^{o}C were 2.5x10^{3} and 3.1x10^{3} CFU/cm^{2}, while the highest counts of TVBC at 37 and 22^{o}C were 4.2x10^{6} and 5.6x10^{6} CFU/cm^{2}, respectively. On the other hand, the counts of TC, FC, and *E. coli* ranged between 4.2x10^{2} -7.2x10^{4}, 2.1x10^{2} -3.6x10^{4} and 1.6x10^{2} - 2.2x10^{4} CFU/10cm^{2}, respectively. Also, the counts of FS ranged between 1.5x10^{2} and 1.3x10^{3} CFU/10cm^{2} (Table 7; Fig. 10).

The tested pathogenic microorganisms were found in all tested biofilm samples. The results indicated that, the highest counts of tested pathogens were $5.4x10^3$ and $5.7x10^3$ CFU/10cm^2 for *Staph. aurues*, and *P. aeruginosa*, respectively. But, the lowest counts of *Salmonella* spp. were $1.4x10^2$ CFU/10cm^2 (Table 8; Fig. 11). Moreover, the average counts of *Staph. aurues, Bacillus cerues, L. monocytogenes, Salmonella* spp, *P. aeruginosa, Cl. perfringens* and *Candida albicans* were $1.6x10^3$, $6.8x10^2$, $3.7x10^2$, $7.3x10^2$, $1.6x10^3$, $8.5x10^2$ and $9.2x10^2$ CFU/10cm^2, respectively. Rusin *et al.* (1998) found that areas that were moist or frequently touched by human hands (kitchen faucet handles, and kitchen sink drains) had higher numbers of fecal coliform, coliform and heterotrophic bacteria than other areas in the kitchen.

Table 7. Enumeration of the bacterial indicators in the natural biofilm collected from bathroom sink drainage pipes.

Biofilm samples	TVBC/ cm^2		Colony forming unit/10cm^2			
	37^{o}C	22^{o}C	TC	FC	*E. coli*	FS
1	$1.2x10^4$	$3.4x10^4$	$4.6x10^3$	$2.9x10^3$	$1.3x10^3$	$5.2x10^3$
2	$3.2x10^4$	$4.6x10^4$	$1.6x10^3$	$7.3x10^2$	$5.8x10^2$	$2.6x10^2$
3	$5.2x10^4$	$5.7x10^4$	$2.3x10^3$	$6.2x10^2$	$3.4x10^3$	$3.8x10^2$
4	$2.5x10^3$	$3.1x10^3$	$4.2x10^2$	$2.1x10^2$	$1.6x10^2$	$7.4x10^2$
5	$4.2x10^6$	$5.6x10^6$	$2.5x10^5$	$8.3x10^4$	$6.3x10^4$	$3.4x10^3$
6	$1.6x10^5$	$2.7x10^5$	$7.2x10^3$	$3.4x10^3$	$2.1x10^3$	$6.5x10^2$
7	$3.2x10^4$	$5.0x10^4$	$3.1x10^3$	$1.8x10^3$	$1.1x10^3$	$1.5x10^2$
8	$5.7x10^4$	$6.2x10^4$	$3.5x10^3$	$2.3x10^3$	$1.6x10^2$	$2.7x10^2$
9	$4.9x10^3$	$5.3x10^3$	$1.4x10^3$	$6.4x10^2$	$2.3x10^2$	$3.4x10^2$
10	$1.1x10^6$	$2.4x10^6$	$7.2x10^4$	$3.6x10^4$	$2.2x10^4$	$1.3x10^3$
Min.	$2.5x10^3$	$3.1x10^3$	$4.2x10^2$	$2.1x10^2$	$1.6x10^2$	$1.5x10^2$
Max.	$4.2x10^6$	$5.6x10^6$	$7.2x10^4$	$3.6x10^4$	$2.2x10^4$	$1.3x10^3$
Average	$5.6x10^5$	$8.5x10^5$	$3.4x10^4$	$1.3x10^4$	$9.4x10^3$	$1.2x10^3$

TC: Total coliform, FC: Fecal coliform, FS: Fecal streptococci.

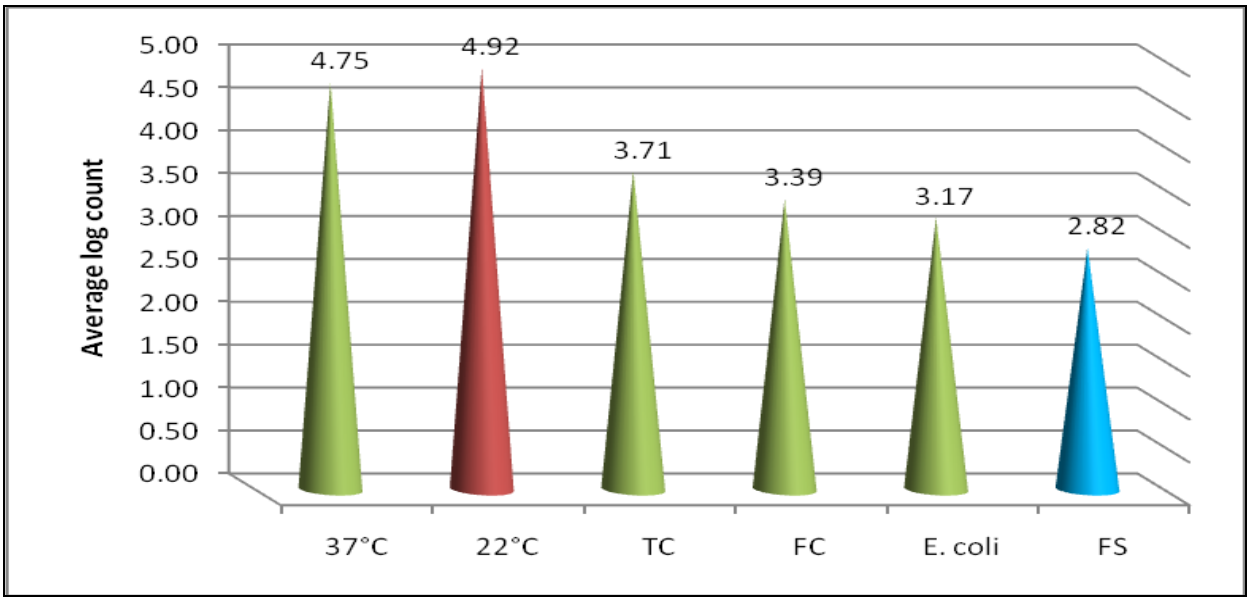


Fig. 10. Average log counts of the bacterial indicators in the natural biofilm collected from bathroom sink drainage pip.

Table 8. Enumeration of pathogenic microbes in the natural biofilm collected from plastic material of bathroom sink drainage pipes.

Biofilm samples	Colony forming unit/10cm^2						
	Staph. aurues	***Bacillus cerues***	***L. monocytogenes***	***Salmonella* spp**	***P. aeruginosa***	***Cl. perfringens***	***Candida albicans***
1	$4.6x10^3$	$1.2x10^3$	$3.2x10^2$	$4.2x10^2$	$1.2x10^3$	$3.2x10^3$	$2.3x10^3$
2	$2.5x10^2$	$5.1x10^2$	$4.2x10^2$	$3.6x10^2$	$4.8x10^2$	$5.2x10^2$	$1.5x10^2$
3	$4.1x10^2$	$3.5x10^2$	$2.9x10^2$	$2.9x10^2$	$1.7x10^2$	$2.4x10^2$	$2.4x10^2$
4	$1.5x10^2$	$4.3x10^2$	$4.8x10^2$	$1.8x10^2$	$2.4x10^2$	$3.9x10^2$	$3.6x10^2$
5	$2.4x10^3$	$1.6x10^3$	$3.9x10^2$	$4.5x10^2$	$4.2x10^3$	$1.5x10^3$	$4.2x10^2$
6	$4.3x10^2$	$2.2x10^2$	$3.5x10^2$	$2.3x10^2$	$3.7x10^3$	$4.1x10^2$	$2.7x10^2$
7	$3.7x10^2$	$2.6x10^2$	$4.7x10^2$	$5.8x10^2$	$2.9x10^2$	$2.2x10^2$	$2.8x10^3$
8	$2.6x10^3$	$4.7x10^2$	$2.6x10^2$	$3.2x10^2$	$3.2x10^2$	$3.5x10^2$	$5.2x10^2$
9	$1.9x10^2$	$3.4x10^2$	$3.4x10^2$	$1.4x10^2$	$1.1x10^2$	$1.8x10^2$	$3.8x10^2$
10	$5.4x10^3$	$1.5x10^3$	$4.2x10^2$	$2.8x10^2$	$5.7x10^3$	$2.9x10^3$	$1.8x10^3$
Min.	$1.5x10^2$	$2.2x10^2$	$2.6x10^2$	$1.4x10^2$	$5.8x10^2$	$1.8x10^2$	$1.5x10^2$
Max.	$5.4x10^3$	$1.6x10^3$	$4.8x10^2$	$5.8x10^2$	$5.7x10^3$	$2.9x10^3$	$2.8x10^3$
Average	$1.6x10^3$	$6.8x10^2$	$3.7x10^2$	$7.3x10^2$	$1.6x10^3$	$8.5x10^2$	$9.2x10^2$

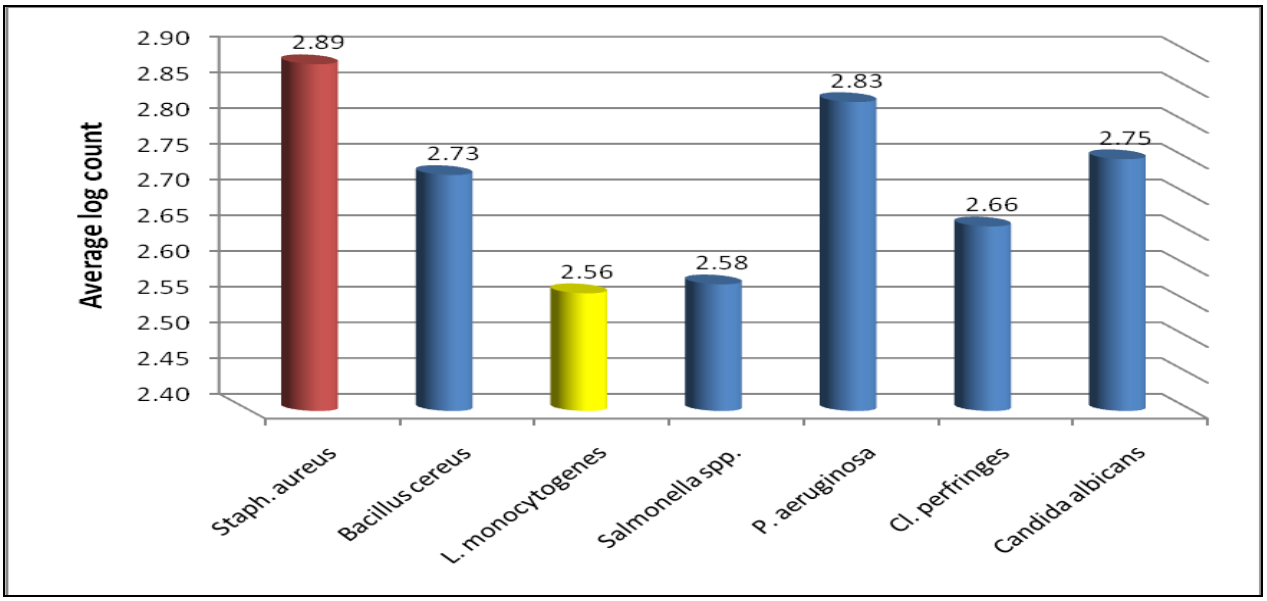


Fig. 11. Average log counts of the pathogenic microbes in the natural biofilm collected from bathroom sink drainage pipe.

Flores *et al*., (2013) indicated that, the microbes especially, bacteria have been able to colonize and form on the kitchen surfaces and the exchange of these microbes between humans and the kitchen environment can impact human health by direct way. However, the understanding of the overall diversity of these communities have limited and the across surfaces and sources of bacteria to kitchen surfaces. In addition, the full extent of bacterial diversity in kitchens remains largely unknown as most previous studies of kitchen microbes focused on pathogen detection and relied upon cultivation dependent techniques that preclude in-depth community characterization (Sinclair and Gerba, 2011).

In the present study, results of natural biofilm collected from kitchen sink drainage pipes were clearly indicated that, the average counts of TVBC at 37 and 22^{o}C were $6.5x10^{6}$ and $1.0x10^{7}$ CFU/cm^{2}, respectively (Table 9 and Fig. 12). Also, Sinclair and Gerba, (2011) indicated that, TVBC was very high and was found on all the kitchens surfaces with range of $5.0x10^{3}$-$1.6x10^{4}$ CFU/cm^{2}.

Moreover, the average counts of TC, FC, *E. coli* and FS were $7.2x10^{5}$, $2.8x10^{5}$, $8.8x10^{4}$ and $2.3x10^{4}$ CFU/10 cm^{2}, respectively. Also, the counts of TC, FC, *E. coli* and FS ranged between $2.8x10^{3}$-$3.1x10^{6}$, $7.2x10^{2}$-$1.6x10^{6}$, $4.2x10^{2}$- $4.6x10^{5}$ and $1.2x10^{2}$-$1.0x10^{5}$ CFU/10cm^{2}, respectively. The obtained results showed that, high counts of fecal

bacterial indicators. These may be due to the washing of contaminated vegetables and fruits which irrigated by insufficient treated wastewater that can a source of non pathogenic and pathogenic microbes. Also, Berger *et al.* (2010) cleared that the kitchen as a well known that food items have been harbor pathogenic bacteria.

In case of results of pathogenic microbes, it is cleared that, the highest average counts were recorded with *L. monocytogenes*, *Salmonella* spp. and *Candida albicans* as $2.5x10^4$, $1.0x10^4$ and $5.1x10^4$ CFU/10 cm^2, respectively. While, the lowest average counts was found with *Cl. perfringens* as $7.7x10^2$ CFU/$10cm^2$. Furthermore, the average counts of *Staph. aurues*, *Bacillus cerues*, *L. monocytogenes*, *Salmonella* spp., *P. aeruginosa*, *Cl. perfringens* and *Candida albicans* were $5.2x10^3$, $1.5x10^3$, $2.5x10^4$, $1.0x10^4$, $1.1x10^3$, $7.7x10^2$ and $5.1x10^4$ CFU/10 cm^2, respectively (Table 10; Fig. 13). The obtained results confirmed that, Gram positive bacterial pathogens have been more frequently isolated from dry surfaces in households than Gram negative bacteria (Beumer and Kusumaningrum, 2003).

The most common culprits of bacterial food-borne illnesses in industrialized nations are *Campylobacter* spp., *Salmonella* spp., *Clostridium perfringens* and various strains of *E. coli* (Mead *et al*., 1999). Moreover, Donlan (2002) reported that some pathogens are associated with and grow in kitchen sink drainage pipe biofilm. These organisms are *L. monocytogenes, Campylobacter* spp., *E. coli* O157:H7, *Salmonella typhimurium* and *Vibrio cholerae*.

The results can be found that both Gram-negative and Gram-positive bacteria can be readily cultivated from a variety of kitchen surfaces, with moist surfaces typically yielding the greatest number of colony forming units/cm^2. The vast majority of bacteria on kitchen surfaces are likely harmful (Sinclair and Gerba, 2011). The exposures of these microbes arise both directly, from handling, preparing and eating food, and indirectly, from contact with surfaces that harbor microbes derived from a range of potential sources, including humans, food and aerosolized water (Medrano-Felix *et al.*, 2011).

Generally, human skin was found to be the primary source of bacteria in each of these locations, whereas other environmental sources such as soil and outdoor air were

much less important. In the kitchen environment, both humans and raw foods brought into the kitchen are likely major contributors of bacteria, although their relative importance as sources has not previously been studied (Hewitt *et al.*, 2012).

Table 9. Determination of the bacterial indicators in the natural biofilm collected from kitchen sink drainage pipes.

Biofilm samples	TBC/cm^2		Colony forming unit/10cm^2			
	37°C	22°C	TC	FC	*E. coli*	FS
1	$1.2x10^7$	$3.4x10^7$	$3.1x10^6$	$1.6x10^6$	$4.6x10^5$	$5.2x10^4$
2	$4.8x10^5$	$6.2x10^5$	$2.9x10^4$	$3.7x10^3$	$2.4x10^3$	$7.2x10^2$
3	$2.9x10^6$	$3.1x10^6$	$4.2x10^5$	$2.7x10^5$	$5.2x10^4$	$2.6x10^4$
4	$3.6x10^4$	$6.2x10^4$	$2.8x10^3$	$7.2x10^2$	$4.2x10^2$	$1.2x10^2$
5	$2.3x10^5$	$4.6x10^5$	$1.2x10^4$	$5.3x10^3$	$1.9x10^3$	$3.9x10^2$
6	$8.4x10^6$	$1.2x10^7$	$5.7x10^5$	$3.1x10^5$	$1.2x10^5$	$5.3x10^4$
7	$3.5x10^5$	$4.9x10^5$	$1.8x10^4$	$4.9x10^3$	$2.6x10^3$	$1.2x10^3$
8	$7.1x10^4$	$8.5x10^4$	$4.3x10^3$	$2.6x10^3$	$9.8x10^2$	$3.5x10^2$
9	$3.9x10^7$	$5.2x10^7$	$2.6x10^6$	$5.8x10^5$	$2.3x10^5$	$1.0x10^5$
10	$1.5x10^6$	$3.6x10^6$	$4.7x10^5$	$2.7x10^4$	$1.3x10^4$	$4.5x10^3$
Min.	$3.6x10^4$	$6.2x10^4$	$2.8x10^3$	$7.2x10^2$	$4.2x10^2$	$1.2x10^2$
Max.	$3.9x10^7$	$5.2x10^7$	$3.1x10^6$	$1.6x10^6$	$4.6x10^5$	$1.0x10^5$
Average	$6.5x10^6$	$1.0x10^7$	$7.2x10^5$	$2.8x10^5$	$8.8x10^4$	$2.3x10^4$

TC: Total coliform, FC: Fecal coliform, FS: Fecal streptococci.

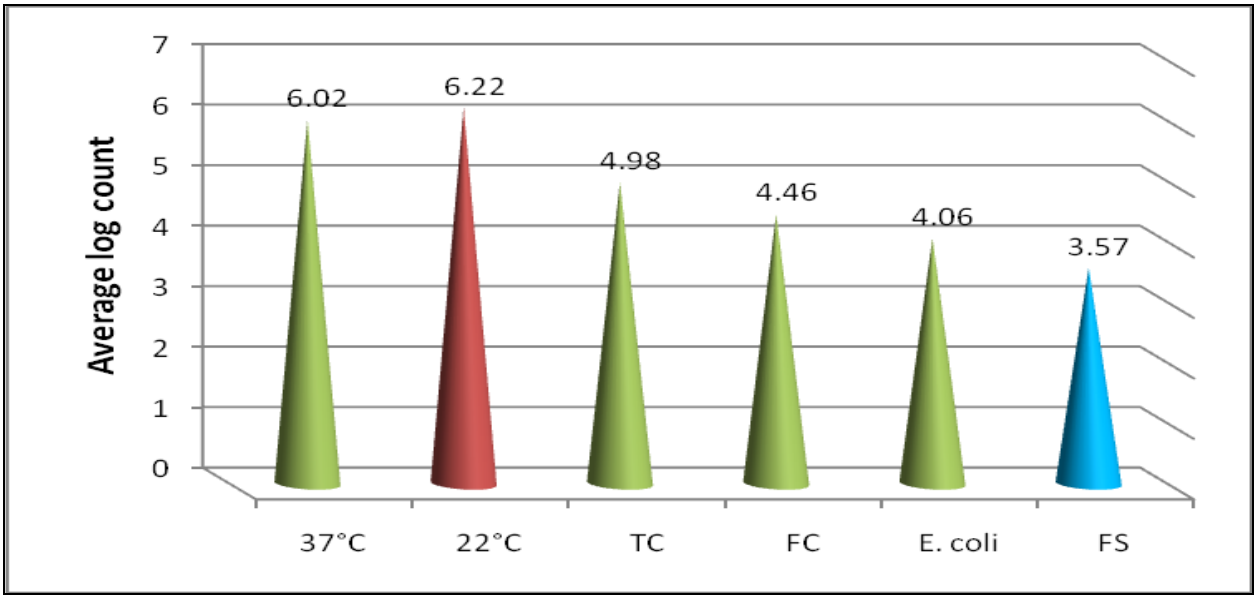


Fig. 12. Average log counts of the bacterial indicators in the natural biofilm collected kitchen sink drainage pipes.

Table 10. Enumeration of pathogenic microbes in the natural biofilm collected from plastic material of kitchen sink drainage pipes.

Biofilm samples	Colony forming unit/10cm^2						
	Staph. aurues	***Bacillus cerues***	***L. monocytogenes***	***Salmonella* spp**	***P. aeruginosa***	***Cl. perfringens***	***Candida albicans***
1	$4.2x10^4$	$6.2x10^3$	$1.9x10^5$	$3.5x10^4$	$2.7x10^3$	$3.1x10^2$	$4.1x10^5$
2	$3.7x10^2$	$2.4x10^2$	$4.2x10^3$	$1.5x10^3$	$3.2x10^3$	$1.6x10^2$	$2.7x10^3$
3	$2.1x10^3$	$3.6x10^3$	$2.9x10^4$	$5.1x10^4$	$2.4x10^3$	$2.3x10^2$	$1.9x10^3$
4	$1.1x10^2$	$5.4x10^2$	$1.2x10^2$	$8.9x10^2$	$2.5x10^2$	$4.6x10^2$	$2.1x10^2$
5	$3.6x10^2$	$2.1x10^2$	$4.2x10^3$	$1.4x10^3$	$7.9x10^2$	$2.8x10^2$	$3.8x10^2$
6	$2.4x10^3$	$1.7x10^2$	$2.6x10^3$	$3.7x10^3$	$1.4x10^2$	$5.3x10^2$	$5.6x10^4$
7	$5.3x10^2$	$7.4x10^2$	$3.1x10^3$	$2.8x10^3$	$6.3x10^2$	$3.2x10^2$	$3.2x10^3$
8	$6.4x10^2$	$4.3x10^2$	$2.3x10^3$	$9.3x10^2$	$1.6x10^2$	$6.1x10^2$	$2.6x10^2$
9	$4.2x10^3$	$2.5x10^3$	$1.6x10^4$	$3.2x10^3$	$7.4x10^2$	$2.7x10^2$	$4.3x10^4$
10	$1.5x10^2$	$8.5x10^2$	$3.7x10^3$	$4.3x10^3$	$2.6x10^2$	$4.6x10^3$	$9.1x10^2$
Min.	$1.1x10^2$	$1.7x10^2$	$1.2x10^2$	$8.9x10^2$	$1.4x10^2$	$1.6x10^2$	$2.1x10^2$
Max.	$4.2x10^4$	$6.2x10^3$	$1.9x10^5$	$5.1x10^4$	$3.2x10^3$	$4.6x10^3$	$4.1x10^5$
Average	$5.2x10^3$	$1.5x10^3$	$2.5x10^4$	$1.0x10^4$	$1.1x10^3$	$7.7x10^2$	$5.1x10^4$

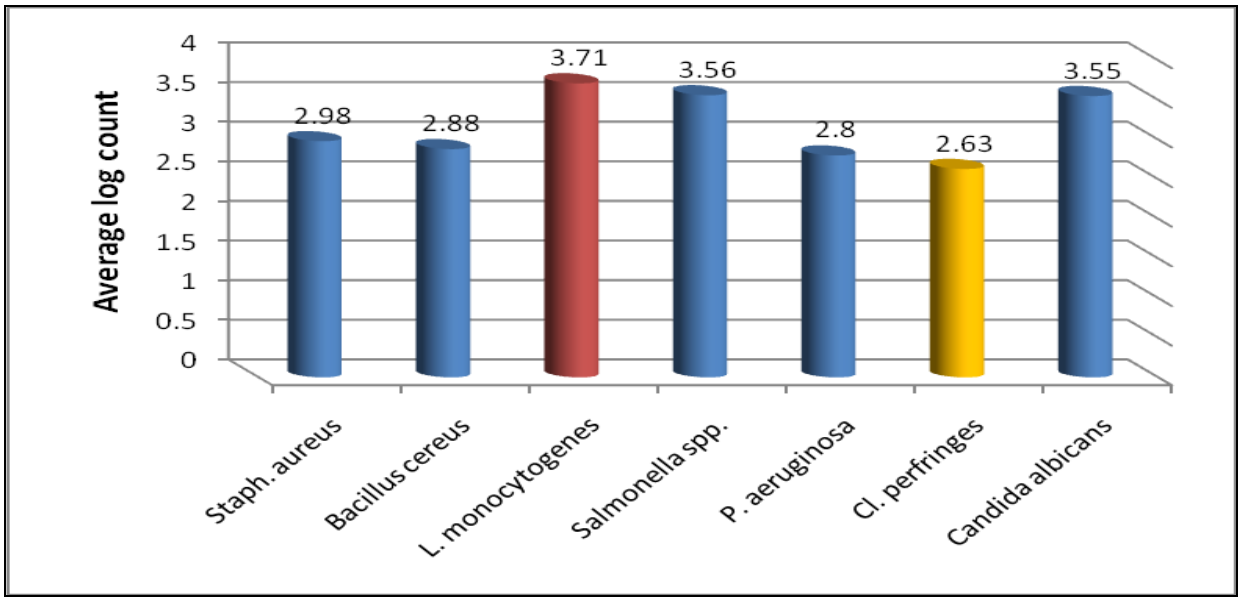


Fig. 13. Average log counts of the pathogenic microbes in the natural biofilm collected from kitchen sink drainage pipe.

Concerning lab sink drainage pipes, the average counts of TVBC at 37 and 22^{o}C were 3.9x10^{4} and 6.7x10^{4} CFU/cm^{2}, respectively. While, the average counts of TC, FC, *E. coli* and FS were 1.5x10^{3}, 6.1x10^{2}, 2.8x10^{2} and 7.9x10^{2} CFU/10cm^{2}, respectively (Table 11; Fig. 14).

The results of pathogenic microbes showed that, average counts of *Staph. aurues*, *Bacillus cerues*, *L. monocytogenes*, *Salmonella* spp, *P. aeruginosa*, *Cl. Perfringens* and *Candida albicans* were 1.2x10^{3}, 4.8x10^{2}, 3.0x10^{2}, 1.1x10^{3}, 7.7x10^{2}, 1.3x10^{3} and 9.1x10^{2} CFU/10cm^{2}, respectively. **(**Table 12; Fig. 15**)**.

In the present study, it can be cleared that, the biofilm samples collected from biological lab. sink drainage pipes have numerous of pathogenic and non pathogenic microorganisms. Hung and Henderson (2009) observed that biofilm associated with medical and laboratories surfaces are often derived from the sink flora. Also, Mahami and Adu-Gyamfi, (2011) they noted that, the predominant organisms include *Staph. aureus*, *Pseudomonas*, *Enterococcus*, *E. coli* and *Candida albicans*.

Table 11. Determination of the classical bacterial indicators in the natural biofilm collected from lab. sink drainage pipes.

Biofilm samples	TBC/cm^2		Colony forming unit/10cm^2			
	37^{o}C	22^{o}C	TC	FC	*E. coli*	FS
1	$2.7x10^3$	$3.1x10^3$	$2.1x10^2$	$1.7x10^2$	$1.0x10^2$	$3.4x10^2$
2	$3.1x10^4$	$4.2x10^4$	$2.9x10^3$	$7.9x10^2$	$5.3x10^2$	$3.2x10^3$
3	$2.9x10^3$	$3.8x10^3$	$3.9x10^2$	$2.5x10^2$	$1.7x10^2$	$3.1x10^2$
4	$3.6x10^4$	$5.4x10^4$	$4.8x10^3$	$2.1x10^3$	$7.8x10^2$	$2.9x10^2$
5	$4.2x10^3$	$7.4x10^3$	$1.2x10^3$	$5.3x10^2$	$1.9x10^2$	$3.9x10^2$
6	$5.1x10^3$	$6.3x10^3$	$5.7x10^2$	$3.1x10^2$	$1.2x10^2$	$5.3x10^2$
7	$2.6x10^5$	$4.2x10^5$	$1.8x10^3$	$5.4x10^2$	$2.3x10^2$	$4.9x10^2$
8	$4.8x10^3$	$6.4x10^3$	$3.7x10^2$	$2.2x10^2$	$1.8x10^2$	$7.9x10^2$
Min.	$2.7x10^3$	$3.1x10^3$	$2.1x10^2$	$1.7x10^2$	$1.0x10^2$	$2.9x10^2$
Max.	$2.6x10^5$	$4.2x10^5$	$4.8x10^3$	$2.1x10^3$	$7.8x10^2$	$3.2x10^3$
Average	$3.9x10^4$	$6.7x10^4$	$1.5x10^3$	$6.1x10^2$	$2.8x10^2$	$7.9x10^2$

TC: Total coliform, FC: Fecal coliform, FS: Fecal streptococci.

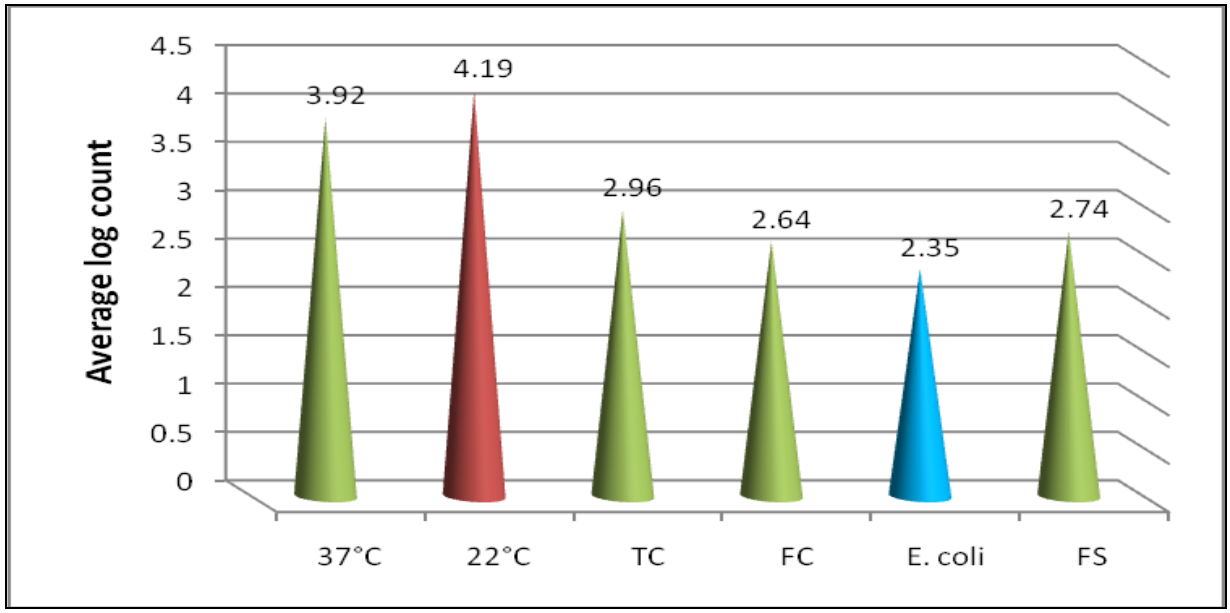


Fig. 14. Average log counts of the bacterial indicators in the natural biofilm collected from lab. sink drainage pipes.

Table 12. Enumeration of pathogenic microbes in the natural biofilm collected from plastic material of lab. sink drainage pipes.

Biofilm samples	Colony forming unit/10cm^2						
	Staph. aurues	***Bacillus cerues***	***L. monocytogenes***	***Salmonella* spp**	***P. aeruginosa***	***Cl. perfringens***	***Candida albicans***
1	$6.3x10^2$	$1.1x10^2$	$5.1x10^2$	$4.2x10^3$	$2.2x10^2$	$1.1x10^2$	$4.5x10^2$
2	$8.5x10^2$	$2.7x10^2$	$1.3x10^2$	$2.7x10^2$	$4.2x10^3$	$3.6x10^2$	$3.9x10^2$
3	$4.2x10^2$	$3.1x10^2$	$2.9x10^2$	$1.5x10^2$	$7.9x10^2$	$5.2x10^3$	$3.4x10^2$
4	$1.8x10^3$	$2.2x10^3$	$1.6x10^2$	$1.9x10^2$	$3.1x10^2$	$1.3x10^2$	$4.2x10^2$
5	$2.8x10^2$	$3.1x10^2$	$3.5x10^2$	$2.7x10^2$	$1.3x10^2$	$2.4x10^3$	$2.8x10^2$
6	$1.4x10^2$	$2.9x10^2$	$5.1x10^2$	$3.9x10^2$	$2.4x10^2$	$7.5x10^2$	$1.3x10^3$
7	$5.7x10^3$	$3.4x10^2$	$2.7x10^2$	$3.5x10^3$	$1.2x10^2$	$4.7x10^3$	$3.9x10^3$
8	$2.1x10^2$	$1.2x10^2$	$1.8x10^2$	$2.6x10^2$	$1.8x10^2$	$3.4x10^2$	$2.4x10^2$
Min.	$1.4x10^2$	$1.1x10^2$	$1.3x10^2$	$1.5x10^2$	$1.2x10^2$	$1.1x10^2$	$2.4x10^2$
Max.	$5.7x10^3$	$2.2x10^3$	$5.1x10^2$	$4.2x10^3$	$4.2x10^3$	$5.2x10^3$	$3.9x10^3$
Average	$1.2x10^3$	$4.8x10^2$	$3.0x10^2$	$1.1x10^3$	$7.7x10^2$	$1.3x10^3$	$9.1x10^2$

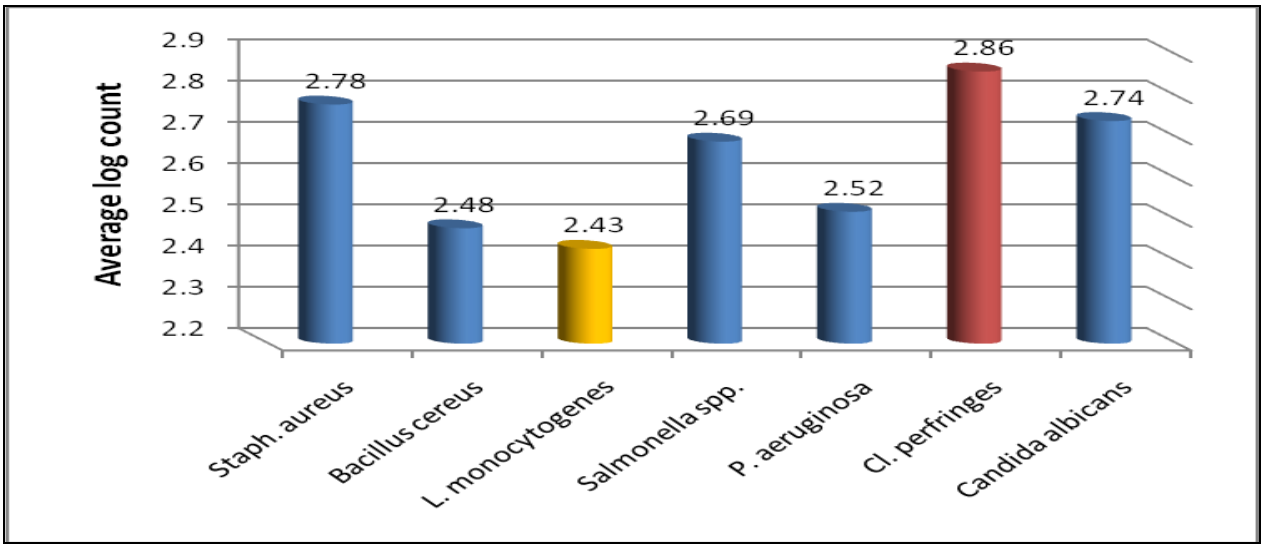


Fig. 15. Average log counts of the pathogenic microbes in the natural biofilm collected from plastic material of lab. sink drainage pipes.

a. Diversity of microbial populations in the tested biofilm samples

As shown in Fig. (16) TVBC at 37 and 22^{o}C, TC, FC, *E. coli* and FS were more widespread in DWDS (iron) than others. While, lower bacterial indicator counts were observed in the biofilm samples collected for lab. sink drainage pipes**.**

By regarding, the diversity of the pathogenic microbes, the highest average log counts of *Staph. aureus* recorded in natural biofilm samples of bathroom sink drainage pipes, while the lowest value recorded for *L. monocytogenes*. From the previous results of DWDS (iron pipes), it can be concluded that, *Candida albicans* was the most dominant, while the lowest dominant was *Staph. aureus*.

Furthermore, the highst counts of *L. monocytogenes* and *Salmonella* spp. were recorded in biofilm samples collected from kitchen sink drainage pipes, whereas *Cl. perfringens* was the lowest value. The findings of the current study showed that, the most prevalent of *Cl. perfringens* was found in the biofilm samples collected for lab. sink drainage pipes. But, *L. monocytogenes* was less prevalent (Fig. 17).

It could be summarized that the biofilm can be formed in any environment inhabited by microorganisms. The biofilm formation can either be beneficial or detrimental to the health of individuals who are exposed to the microhabitats (bathroom, kitchen and laboratories sink drainage pipes) where there are formed (Chikere and

Azubuike, 2014). Therefore, in any hygiene and sanitation improvement program, an effort should be made to include as many pathogen control points as possible. What is often left out of public health interventions is an assessment of fomite contamination as an indicator of improved household sanitation (Sinclair and Gerba, 2011).

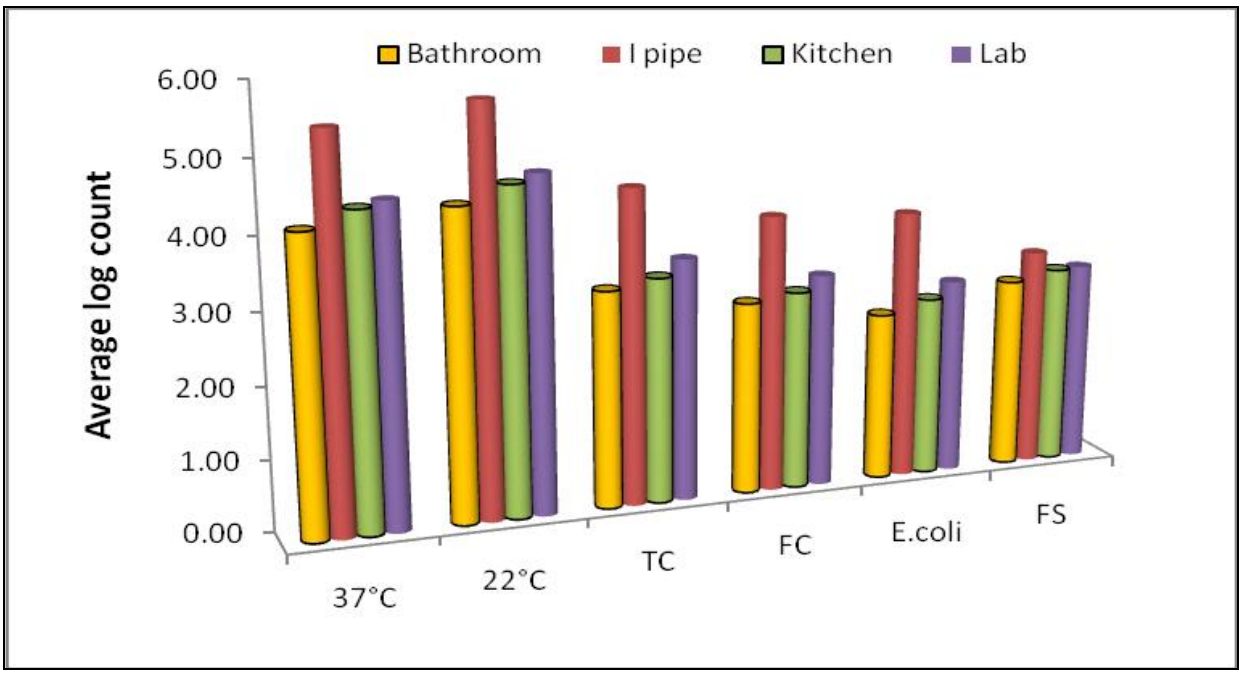


Fig. 16. Comparison diversity of the bacterial indicators in different biofilm samples which collected from I pipe, bathroom, kitchen and lab. sink drainage pipes.

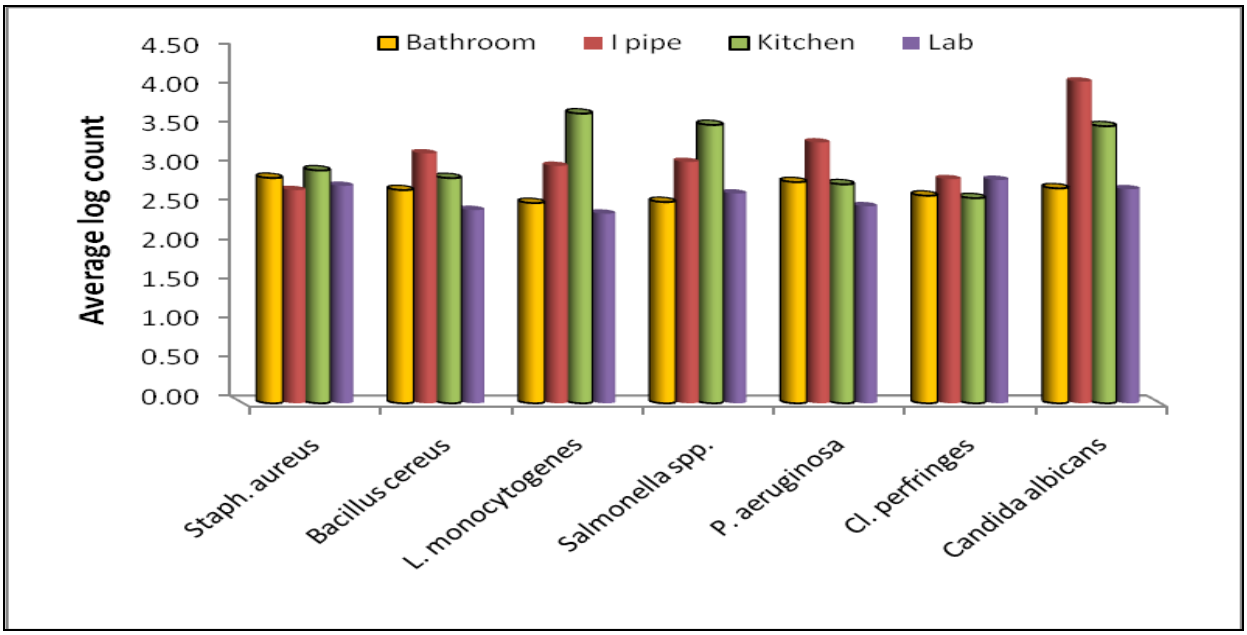


Fig. 17. Comparison diversity of the pathogenic microbes in different biofilm samples which collected from I pipe, bathroom, kitchen and lab. sink drainage pipes.

b. Confirmation and identification of bacterial isolates by using BIOLOG GN III

After preservation of typical isolates of some tested bacteria, these isolates were identified using Biolog GN III (Fig. 18). The results showed that, the confirmed bacterial isolates were *E.coli*, *Salmonella* spp., *Staph. aureus*, *L. monocytogenes* and *Bacillus cereus*.

1

ID Result Species ID: Escherichia coli
ID Comment
ID Notice

	PROB	SIM	DIST	Organism Type	Species
==>1	0.996	0.724	5.201	GN-ENT	Escherichia coli
2	0.004	0.002	7.215	GN-ENT	Escherichia coli inactive
3	0.000	0.000	8.458	GN-ENT	Escherichia coli O157:H7
4	0.000	0.000	8.709	GN-ENT	Escherichia albertii

2

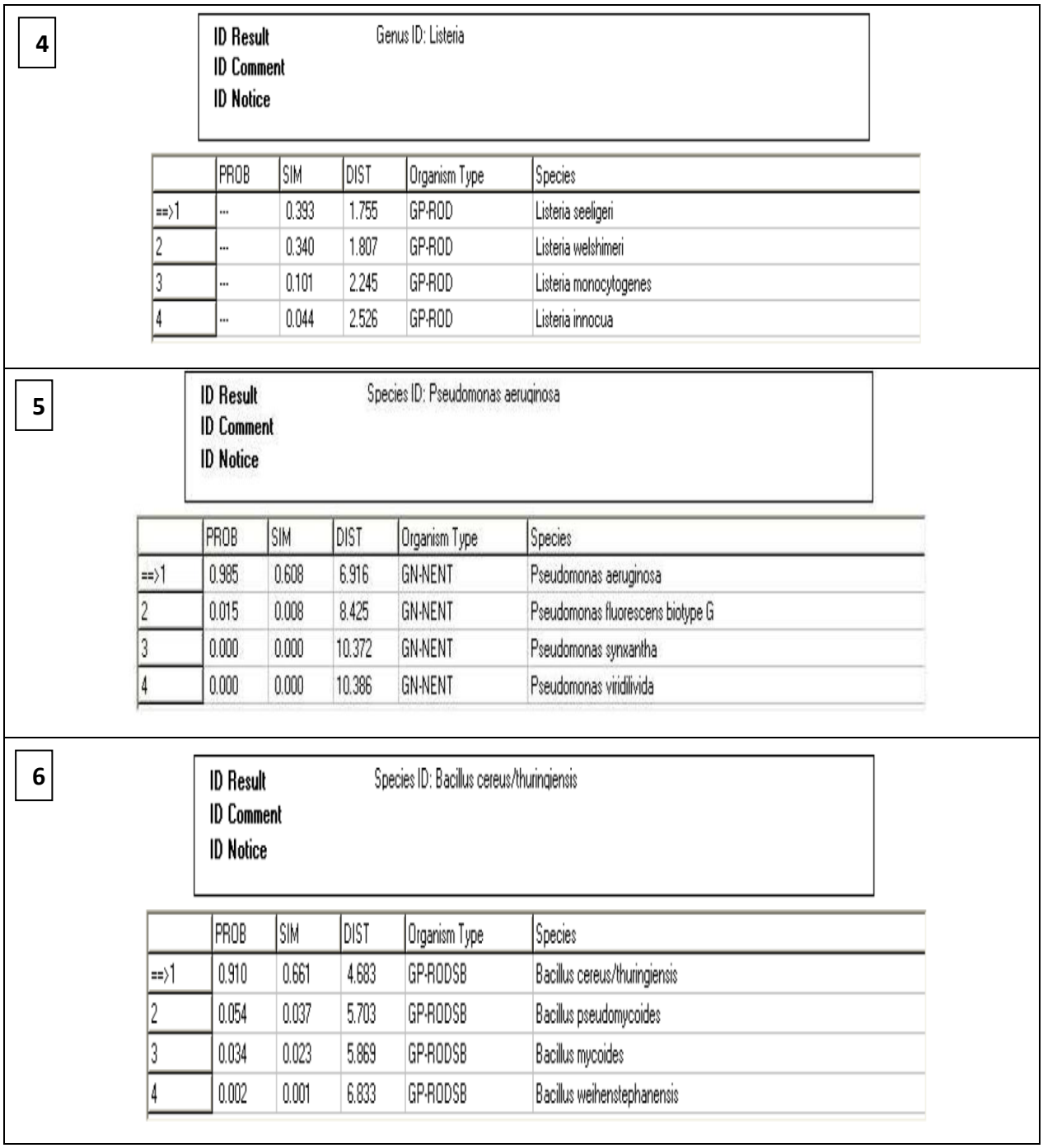
ID Result Genus ID: Salmonella
ID Comment Confirm by Serology
ID Notice

	PROB	SIM	DIST	Organism Type	Species
==>1	---	0.375	4.175	GN-ENT	Salmonella enterica (gp1) ST typhimurium
2	---	0.223	4.363	GN-ENT	Salmonella enterica (gp1)
3	---	0.111	4.596	GN-ENT	Salmonella enterica (gp1) ST paratyphi B
4	---	0.047	4.910	GN-ENT	Salmonella enterica (gp1) ST paratyphi A

3

ID Result Species ID: Staphylococcus aureus ss aureus
ID Comment
ID Notice

	PROB	SIM	DIST	Organism Type	Species
==>1	0.998	0.801	3.657	GP-COCCUS	Staphylococcus aureus ss aureus
2	0.001	0.001	6.198	GP-COCCUS	Staphylococcus epidermidis
3	0.001	0.000	6.252	GP-COCCUS	Staphylococcus caprae
4	0.001	0.000	6.352	GP-COCCUS	Staphylococcus saprophyticus ss saprophyticus



4

ID Result Genus ID: Listeria
ID Comment
ID Notice

	PROB	SIM	DIST	Organism Type	Species
==>1	---	0.393	1.755	GP-ROD	Listeria seeligeri
2	---	0.340	1.807	GP-ROD	Listeria welshimeri
3	---	0.101	2.245	GP-ROD	Listeria monocytogenes
4	---	0.044	2.526	GP-ROD	Listeria innocua

5

ID Result Species ID: Pseudomonas aeruginosa
ID Comment
ID Notice

	PROB	SIM	DIST	Organism Type	Species
==>1	0.985	0.608	6.916	GN-NENT	Pseudomonas aeruginosa
2	0.015	0.008	8.425	GN-NENT	Pseudomonas fluorescens biotype G
3	0.000	0.000	10.372	GN-NENT	Pseudomonas synxantha
4	0.000	0.000	10.386	GN-NENT	Pseudomonas viridilivida

6

ID Result Species ID: Bacillus cereus/thuringiensis
ID Comment
ID Notice

	PROB	SIM	DIST	Organism Type	Species
==>1	0.910	0.661	4.683	GP-RODSB	Bacillus cereus/thuringiensis
2	0.054	0.037	5.703	GP-RODSB	Bacillus pseudomycoides
3	0.034	0.023	5.869	GP-RODSB	Bacillus mycoides
4	0.002	0.001	6.833	GP-RODSB	Bacillus weihenstephanensis

Fig. 18. Biolog GN III profile for confirmed bacterial isolates; (1) *E. coli,* (2) *Salmonella* spp. (3) *Staph. aureus* (4) *L. monocytogenes* (5) *P. aeruginosa* and (6) *Bacillus cereus.*

2. Behavior studies and biological characteristics of *L. monocytogenes* and *S.* Typhimurium which involved in biofilm formation

The occurrence of outbreaks caused by water transmitted pathogens is not limited in many developing countries even affluent countries are affected. Most disease outbreaks in the several part of the world can cause by the consumption of contaminated DW. The most known disease associated with waterborne outbreaks in developed countries is gastroenteritis, there are many others*: e.g.,* cholera, typhoid fever, meningitis, encephalitis, dysentery, hepatitis, legionellosis, pulmonary illness, poliomyelitis, listeriosis, giardiasis and salmonellosis (WHO, 2011).

Water distribution systems are composed of a variety of materials and may harbor potential pathogens within surface-attached microbial biofilm (Waines *et al.,* 2011). Additionally, the biofilm formed in WDS are transported with the flow and this eventually deteriorates the water quality (Jefferson *et al.,* 2004). Biofilm formed by the microbes in WDS causes various problems such as obnoxious taste and odor, increased turbidity, reduced water pressure and flow, promote microbiologically influenced corrosion and release pathogenic bacteria, which is a major public health concern (Berry *et al.,* 2006). It has been reported that some of the biofilm forming microorganisms corrode plumbing materials made up of copper which affects the water quality by imparting unwanted color, odor, taste and turbidity (Hallam *et al.,* 2001).

Many researches on biofilm in water systems has focused on materials of public drinking water distribution systems, not including domestic plumbing system in public and private buildings. Indeed, the choice of domestic plumping materials systems is less regulated, which lead to a wide variety of materials used. Moreover, some of these materials might strongly enhance biofilm accumulation (Schönen, 1986; Eboigbodin *et al.,* 2008).

Additionally, most biofilm formation in WDS occurs on the pipes, because they constitute the greatest surface area available for contamination, and no single material has been developed for use in plumbing systems which is resistant to biofilm formation (Camper *et al.,* 1991). In fact, pipe material characteristics have been shown to have a

direct and profound influence on the amount, rate and type of biofilm formation, the subsequent prevalence of potential pathogens in the water, and the effectiveness of disinfection regimes such as UV treatment, ozonation and chlorination in their control (Lehtola *et al.*, 2005).

In DWDS, about 95% of total microbial cells are present in biofilm on pipe surfaces (Moritz *et al.*, 2010). Furthermore, microbial regrowth of biofilm formation may have adverse effects on DWDS such as pipe corrosion, declining quality of water taste and odor, disinfectant decay and spread of pathogenic diseases (Rubulis and Juhna, 2007). The survival and regrowth of microorganisms in DWDS can be affected by not only biological factors, but also interaction of various physico-chemical factors such as pipe material, water temperature, hydraulic conditions, nutrients, disinfectant residual type, and concentration in DWDS (Srinivasan *et al.*, 2008).

According to, the WHO, the drinking water should meet the quality requirements at the point of consumption (WHO, 2008). A high water quality has to be maintained throughout the distribution, including passage through domestic plumbing. Therefore it is important to investigate and understand biofilm in the drinking water distribution system (DWDS).

L. monocytogenes and *Salmonella* spp. are important pathogenic bacteria, which can be transmitted by food and water (Casarin *et al.*, 2014). Numerous studies have shown that these bacteria are capable of adhering and form biofilm on metal, glass, polyethylene and rubber surfaces (Morita *et al.*, 2011). Moreover, *L. monocytogenes* is one of the most important causative agent of the serious foodborne disease listeriosis, a sporadic bacterial infection that affects a wide range of animals including humans (Scallan *et al.*, 2011). Also, El-liethy (2009) reported that, *Salmonella* spp. and *Listeria* spp are considered as waterborne pathogens. Also, these pathogenic bacteria were detected in fresh and wastewaters.

Additionally, the enteric bacterial pathogen *Salmonella enterica* has been associated with numerous cases of foodborne infections worldwide, with its control constituting an ongoing challenge for food safety and public health authorities (Scallan *et*

al., 2011; EFSA-ECDC, 2012). *S. enterica* is capable of forming biofilm on various inert (*e.g.*, plastic, rubber, glass, stainless steel) or living (*e.g.*, plants, animal epithelial cells, gallstones) surfaces, an ability that contributes to its resistance and persistence in both host and non-host environments (Steenackers *et al.,* 2012).

For these reasons, in the preset study two designed model system were carried out to study the behavior of two bacterial pathogens (*L. monocytogenes* and *S.* Typhimurium) for biofilm formation in different pipe materials (PVC, PP, PE, I, Cu and R). And the ability of *L. monocytogenes* and *S.* Typhimurium to form biofilm in the inner surface of tested pipe materials in designed DWDS model was investigated.

a. Microbiological characterization of *L. monocytogenes* and *S.* Typhimurium biofilm grown on six different domestic plumbing materials

During the biofilm colonization and growth period (10-90 days), total counts of *L. monocytogenes* biofilm cells grown on six tested domestic plumping materials was determined. Four different culture media; plate count agar (PCA), R2A agar (R2A), Listeria selective agar (LSA) and HiCrome Listeria selective agar (HLSA) were used to enumerate the biofilm cells.

Results in Table (13) noticed that, the counts of *L. monocytogenes* biofilm cells were increased gradually with the increase of biofilm age. The counts of biofilm cells formed on PVC, PP, PE and R using PCA medium were (10^7 CFU/cm^2 at 60 days). While, in case of I pipe material, the counts (10^8 CFU/cm^2) were higher than other tested materials. The highest counts of *L. monocytogenes* biofilm formed on Cu pipe materials were 10^6 CFU/cm^2 at 60 days, which gradually decreased to reach 10^5 at 80 days.

Data shown in Fig. (19) showed that the growth curve of *L. monocytogenes* biofilm in I pipe material was higher than in PVC, PP, PE Cu and R. On the contrary, in Cu pipe the lowest growth curve of *L. monocytogenes* biofilm was lowest Form these results, it can be concluded that, the biofilm grown on I pipe was more enhanced than the others while in Cu pipe was less.

The pipe material used in water distribution systems is another important factor that influences the proliferation of the distribution system biofilm. It has been found that

some pipes frequently experience problems with coliform, taste and odor complaints. Also, there is a distinct development rate and microbe community structure of biofilm in different types of pipes (Lehtola *et al.*, 2005). Accordingly, some reports even suggested that the choice of most approved pipe material had only little impact on the ultimate biofilm density in the long-term studies (Bachmann and Edyvean 2005).

Therefore, to understand the impacts of different plumbing materials on biofilm formation in water supply system, the current research examined the bacterial community compositions of *L. monocytogenes* and *S.* Typhimurium biofilm grown on different plumping materials.

Table 13. Estimation of growth rate of *L. monocytogenes* biofilm grown on six different domestic plumping materials using PCA medium.

Biofilm age (days-old)	Colony forming unit (CFU/cm^2) using PCA Types of tested domestic plumping materials					
	PVC	**PP**	**PE**	**I**	**Cu**	**R**
10	$3.4x10^2$	$2.3x10^2$	$3.8x10^2$	$6.3x10^3$	$2.9x10^2$	$3.0x10^2$
20	$5.1x10^3$	$4.1x10^3$	$3.8x10^3$	$4.8x10^4$	$7.4x10^2$	$2.4x10^3$
30	$5.2x10^4$	$3.9x10^4$	$2.3x10^4$	$3.2x10^5$	$5.6x10^3$	$4.8x10^4$
40	$4.1x10^5$	$4.8x10^5$	$6.7x10^5$	$9.5x10^6$	$2.8x10^4$	$1.5x10^5$
50	$2.6x10^6$	$3.2x10^6$	$4.5x10^6$	$5.2x10^7$	$3.9x10^5$	$5.3x10^6$
60	$5.4x10^7$	$2.6x10^7$	$3.2x10^7$	$1.4x10^8$	$6.2x10^6$	$2.4x10^7$
70	$5.2x10^7$	$4.5x10^7$	$4.9x10^7$	$2.7x10^8$	$3.4x10^6$	$3.8x10^7$
80	$7.3x10^7$	$5.2x10^7$	$5.6x10^7$	$3.8x10^8$	$5.1x10^5$	$5.2x10^7$
90	$3.6x10^7$	$6.9x10^7$	$7.2x10^7$	$5.7x10^8$	$3.2x10^5$	$7.8x10^7$

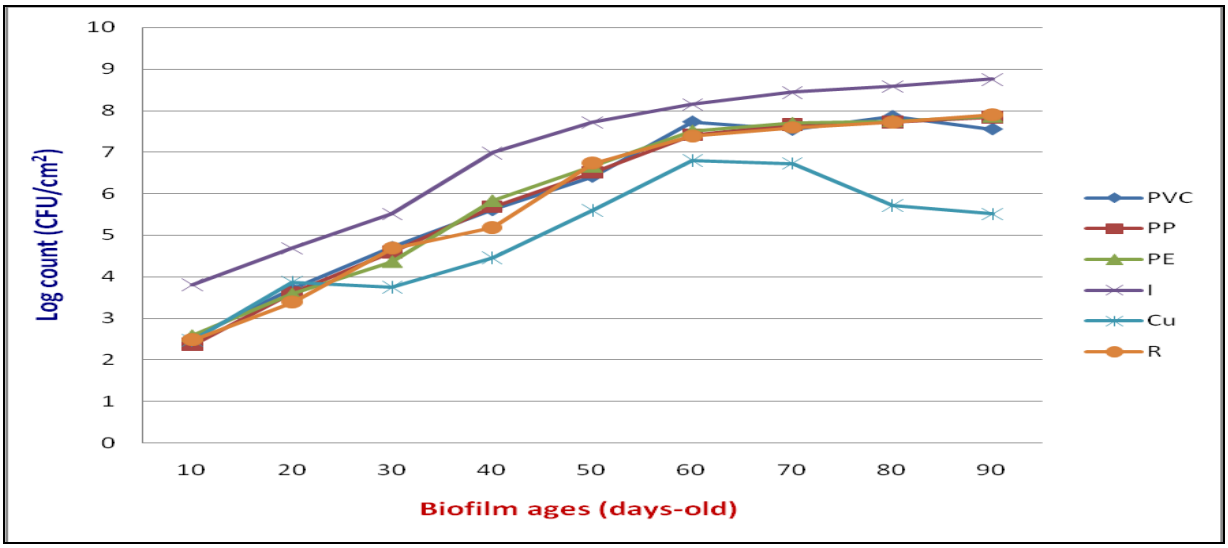


Fig. 19. Growth curve of *L. monocytogenes* biofilm grown on six different domestic plumping materials using PCA medium.

By regarding, the counts of biofilm cells which recovered using R2A medium, the results showed that the total counts of biofilm cells grown on I pipe material reached up to 10^8 CFU/cm^2 at 60 days. While, the total counts of biofilm cells grown on the other pipe materials were 10^7 CFU/cm^2 except in Cu pipe material was 10^6 CFU/ cm^2 at the same days-old (Table 14). Additionally, the growth curve of *L. monocytogenes* biofilm formed on Cu confirmed that the counts slightly decreased after 70 days-old. While, the highly growth curve recorded in I pipe materials (Fig. 20).

Table 14. Estimation of growth rate of *L. monocytogenes* biofilm grown on six different domestic plumping materials using R2A medium.

Biofilm age (days-old)	Colony forming unit (CFU/cm^2) using R2A Types of tested domestic plumping materials					
	PVC	**PP**	**PE**	**I**	**Cu**	**R**
10	$3.6x10^2$	$4.2x10^2$	$3.2x10^2$	$1.1x10^4$	$4.3x10^2$	$5.9x10^2$
20	$5.4x10^3$	$3.4x10^3$	$6.3x10^3$	$4.5x10^5$	$7.7x10^2$	$6.1x10^3$
30	$6.2x10^4$	$4.9x10^4$	$5.4x10^4$	$7.2x10^6$	$4.2x10^3$	$4.5x10^4$
40	$4.3x10^5$	$3.5x10^5$	$3.8x10^5$	$3.8x10^7$	$5.8x10^4$	$6.4x10^5$
50	$5.2x10^6$	$5.4x10^6$	$7.4x10^6$	$8.6x10^7$	$7.2x10^5$	$5.9x10^6$
60	$2.8x10^7$	$2.9x10^7$	$1.8x10^7$	$3.4x10^8$	$3.2x10^6$	$1.2x10^7$
70	$4.1x10^7$	$3.8x10^7$	$4.2x10^7$	$5.2x10^8$	$6.0x10^6$	$4.2x10^7$
80	$5.6x10^7$	$4.1x10^7$	$5.9x10^7$	$6.3x10^8$	$8.2x10^5$	$6.7x10^7$
90	$7.3x10^7$	$6.8x10^7$	$8.1x10^7$	$7.9x10^8$	$5.7x10^5$	$3.8x10^7$

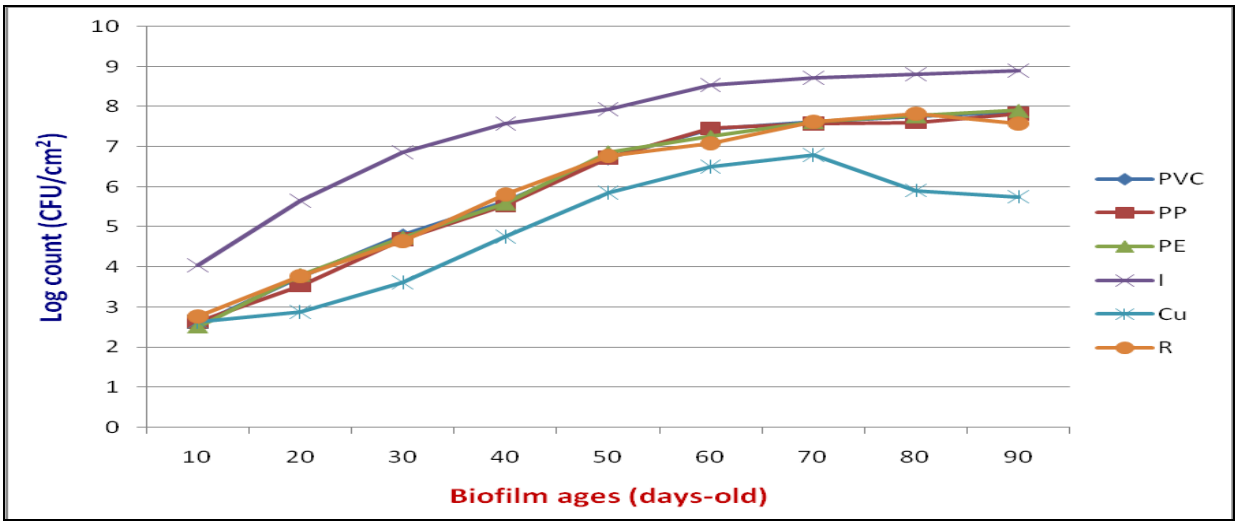


Fig. 20. Growth curve of *L. monocytogenes* biofilm grown on six different domestic plumping materials using R2A medium.

As shown in Table (15) and Fig. (21), the results found that, the lower growth rate (10^6 CFU/cm^2 at 70 days) of *L. monocytogenes* was shown in Cu pipe material using LSA medium. Of the contrary, the highest growth rate (10^8 CFU/cm^2 at 70 days) was found in biofilm grown on I pipe material. Also, results showed the highest growth rate of *L. monocytogenes* biofilm formed on PVC, PE and R pipe materials being 10^7 CFU/cm^2 at 60 days, while it was (10^6 CFU/cm^2) in PP and R pipes.

In addition, the highest growth curve of *L. monocytogenes* biofilm was reported in I pipe, while it was lowest in Cu pipe. In case of plastic-based materials (PVC, PP, PE and R) no differences between them.

Table 15. Estimation of *L. monocytogenes* biofilm growth rate on six different domestic plumping materials by using LSA medium.

Biofilm age (days-old)	Colony forming unit (CFU/cm^2) by using LSA Types of domestic plumping materials					
	PVC	**PP**	**PE**	**I**	**Cu**	**R**
10	$1.3x10^2$	$2.1x10^2$	$4.7x10^2$	$2.8x10^3$	$1.4x10^2$	$2.5x10^2$
20	$2.5x10^3$	$5.9x10^2$	$8.3x10^2$	$3.4x10^4$	$5.6x10^2$	$4.8x10^2$
30	$4.7x10^4$	$3.4x10^4$	$1.1x10^4$	$2.9x10^5$	$2.7x10^3$	$5.4x10^3$
40	$3.3x10^5$	$2.8x10^5$	$2.5x10^5$	$4.2x10^6$	$7.2x10^3$	$2.9x10^4$
50	$2.9x10^6$	$7.4x10^5$	$8.4x10^5$	$5.2x10^7$	$2.3x10^4$	$3.7x10^5$
60	$1.2x10^7$	$4.9x10^6$	$1.2x10^7$	$9.5x10^7$	$5.4x10^5$	$6.5x10^6$
70	$2.4x10^7$	$1.8x10^7$	$3.5x10^7$	$1.4x10^8$	$3.4x10^6$	$2.1x10^7$
80	$3.7x10^7$	$3.6x10^7$	$5.7x10^7$	$3.5x10^8$	$1.2x10^5$	$3.5x10^7$
90	$2.8x10^7$	$5.2x10^7$	$4.2x10^7$	$5.7x10^8$	$7.6x10^4$	$4.9x10^7$

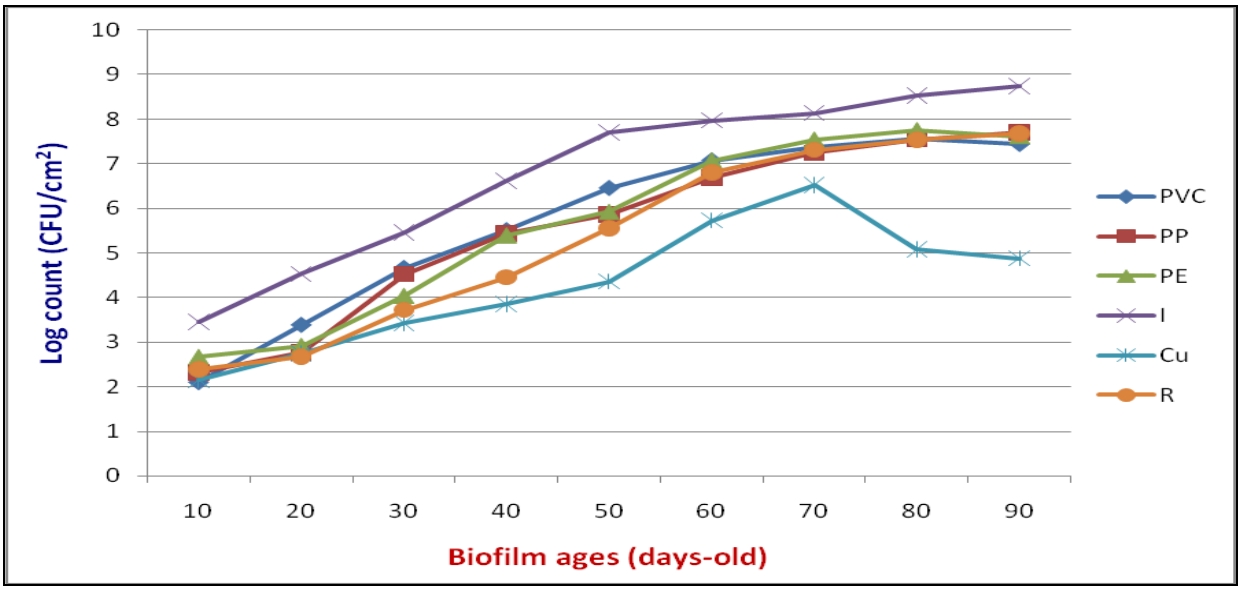


Fig. 21. Growth curve of *L. monocytogenes* biofilm grown on six different domestic plumping materials using LSA medium.

From Table (16), it could be clearly seen that, counts of *L. monocytogenes* biofilm cells at 90 days by using HLSA medium which grown on PVC, PP, PE, I, Cu and R were $3.1x10^7$, $7.4x10^6$, $5.3x10^7$, $5.7x10^8$, $2.1x10^4$ and $3.7x10^7$ CFU/cm^2, respectively. Moreover, the highest growth rate was reported in I pipe material. While, in Cu pipe material the lowest growth rate was reported. As shown in Fig. (22), the growth curve of

L. monocytogenes biofilm formed on I pipe material was greater than others. But, the lowest growth curve was shown in Cu pipe.

Copper is used for household drinking water distribution systems given its physical and chemical properties that make it resistant to corrosion. However, there is evidence that, under certain conditions, it can corrode and release unsafe concentrations of copper to the water. In addition to, research on drinking water copper pipes has developed conceptual models that include several physical–chemical mechanisms (Pizarro *et al.*, 2014).

Table 16. Estimation of *L. monocytogenes* biofilm growth rate on six different domestic plumping materials using HLSA medium.

Biofilm age (days-old)	Colony forming unit (CFU/cm^2) using HLSA Types of tested domestic plumping materials					
	PVC	PP	PE	I	Cu	R
10	$3.9x10^2$	$4.1x10^2$	$1.8x10^2$	$3.1x10^3$	$2.4x10^2$	$4.5x10^2$
20	$3.1x10^3$	$3.6x10^3$	$1.2x10^3$	$2.9x10^4$	$4.8x10^2$	$6.2x10^2$
30	$2.8x10^4$	$7.1x10^4$	$1.9x10^4$	$6.2x10^5$	$3.7x10^3$	$5.4x10^3$
40	$2.9x10^5$	$3.5x10^5$	$2.4x10^5$	$8.4x10^6$	$4.6x10^3$	$6.5x10^4$
50	$5.7x10^6$	$1.8x10^6$	$7.2x10^5$	$5.2x10^7$	$1.5x10^4$	$3.7x10^5$
60	$2.1x10^7$	$6.3x10^6$	$1.8x10^7$	$1.8x10^8$	$6.4x10^5$	$1.2x10^7$
70	$3.4x10^7$	$2.1x10^7$	$2.9x10^7$	$2.3x10^8$	$3.8x10^6$	$2.6x10^7$
80	$4.2x10^7$	$4.3x10^7$	$3.4x10^7$	$4.1x10^8$	$4.4x10^5$	$3.1x10^7$
90	$3.1x10^7$	$7.4x10^6$	$5.3x10^7$	$5.7x10^8$	$2.1x10^4$	$3.7x10^7$

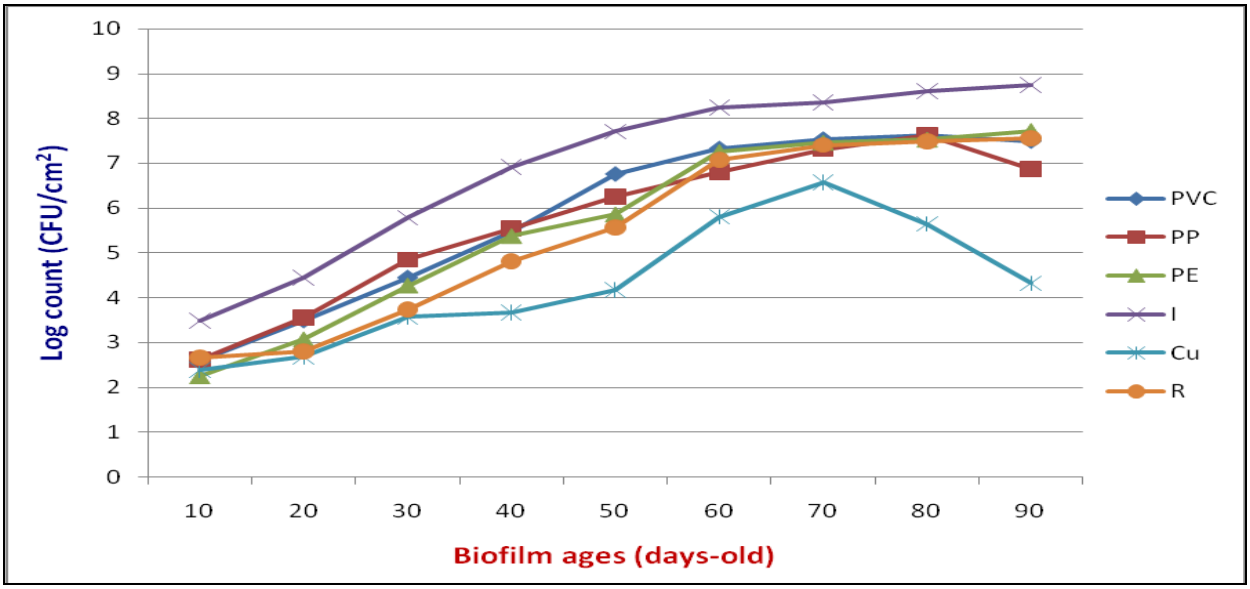


Fig. 22. Growth curve of *L. monocytogenes* biofilm grown on six different domestic plumping materials using HLSA medium.

In this study, during the growth period (10-90 days), the total counts of *S.* Typhimurium biofilm grown on six tested domestic plumping materials were determined. Four different culture media including plate count agar (PCA), R2A agar (R2A), Bismuth sulfite agar (BSA) and HiCrome Improved Salmonella agar (HISA) were used to enumerate biofilm cells.

The results represented in Table (17) demonstrated that, the highest counts of *S.* Typhimurium biofilm ($5.2x10^9$ CFU/cm^2 at 80 days) were recorded in I pipe material using PCA medium. In contrast, the lowest counts (10^6 CFU/cm^2 at 80 days) were reported on Cu pipe materials, and gradually decreased to reach 10^5 CFU/cm^2 at 90 days. Also, counts of biofilm grown on PVC, PP, PE and R were 10^7 CFU/cm^2 at 60 days. The lowest growth curve of *S.* Typhimurium biofilm was found on Cu pipe material (Fig. 23). On the contrary, the highest growth curve was formed on I pipe material. While, the growth curve of bacterial biofilm was similar to PVC, PP, PE and R.

By concerning the counts of *S.* Typhimurium biofilm cells formed on PVC, PP, PE and R using R2A medium were 10^7 CFU/cm^2 at 60 days. Also, the lowest value was reported in Cu pipe. On the contrary, in I pipe material the highest value was determined (Table 18). Data represented graphically in Fig. (24) indicated that, the lowest growth curve of *S.* Typhimurium biofilm was found in Cu pipe material. In contrast, the highest growth curve of *S.* Typhimurium biofilm was recorded in I pipe material. While, the growth curve results indicated that no differences in PVC, PP, PE and R pipe materials.

Table 17. Estimation of growth rate of *S.* Typhimurium biofilm on six different domestic plumping materials by using PCA medium.

Biofilm age (days-old)	Colony forming unit (CFU/cm^2) by using PCA Types of tested domestic plumping materials					
	PVC	PP	PE	I	Cu	R
10	$3.6x10^3$	$2.5x10^3$	$4.6x10^3$	$3.8x10^4$	$5.7x10^2$	$2.9x10^3$
20	$2.4x10^4$	$4.1x10^4$	$6.2x10^4$	$4.2x10^5$	$3.5x10^3$	$3.1x10^4$
30	$2.8x10^5$	$3.7x10^5$	$3.4x10^5$	$2.7x10^6$	$1.8x10^4$	$4.2x10^5$
40	$3.7x10^6$	$6.2x10^6$	$2.7x10^6$	$3.8x10^7$	$2.7x10^5$	$2.6x10^6$
50	$5.2x10^6$	$4.3x10^6$	$8.2x10^6$	$4.9x10^7$	$5.2x10^5$	$6.4x10^6$
60	$1.8x10^7$	$3.6x10^7$	$3.7x10^7$	$1.5x10^8$	$6.3x10^6$	$3.9x10^7$
70	$4.6x10^8$	$2.8x10^8$	$2.1x10^8$	$3.2x10^9$	$2.8x10^7$	$5.2x10^7$
80	$5.3x10^8$	$7.1x10^8$	$5.6x10^8$	$5.2x10^9$	$4.2x10^6$	$2.7x10^8$
90	$3.2x10^8$	$4.6x10^8$	$1.9x10^8$	$3.8x10^9$	$2.3x10^5$	$4.5x10^8$

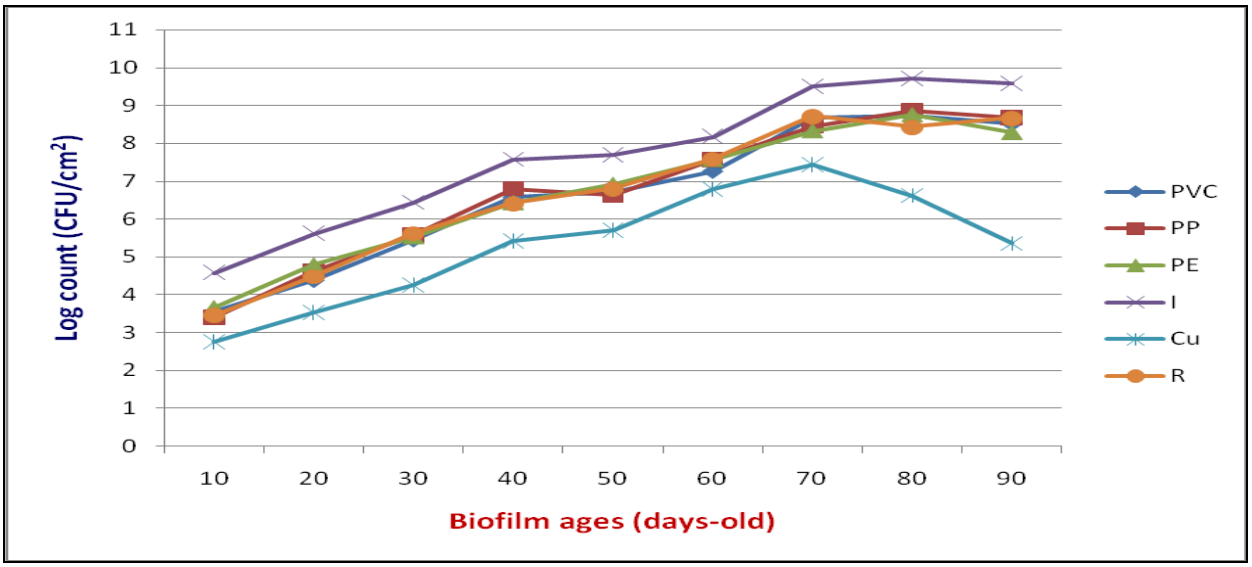


Fig. 23. Growth curve for *S.* Typhimurium biofilm grown on six different domestic plumping materials by using PCA medium.

Table 18. Estimation of growth rate of *S.* Typhimurium biofilm on six different domestic plumping materials by using PCA.

Biofilm age (days-old)	Colony forming unit (CFU/cm^2) using R2A agarTypes of tested domestic plumping materials					
	PVC	**PP**	**PE**	**I**	**Cu**	**R**
10	$2.5x10^3$	$4.3x10^3$	$3.1x10^3$	$6.2x10^4$	$4.1x10^2$	$3.7x10^3$
20	$3.8x10^4$	$3.7x10^4$	$4.6x10^4$	$2.9x10^5$	$6.2x10^3$	$5.4x10^4$
30	$4.2x10^5$	$2.4x10^5$	$6.3x10^5$	$4.2x10^6$	$4.5x10^4$	$6.3x10^5$
40	$5.3x10^6$	$3.2x10^6$	$5.6x10^6$	$2.4x10^7$	$3.2x10^5$	$4.4x10^6$
50	$3.7x10^6$	$6.5x10^6$	$3.7x10^6$	$4.1x10^7$	$2.9x10^5$	$3.8x10^6$
60	$4.3x10^7$	$4.3x10^7$	$5.2x10^7$	$3.5x10^8$	$7.5x10^6$	$2.6x10^7$
70	$2.5x10^8$	$2.6x10^8$	$3.9x10^8$	$1.8x10^9$	$4.8x10^7$	$3.2x10^7$
80	$2.7x10^8$	$3.5x10^8$	$5.3x10^8$	$3.7x10^9$	$2.6x10^6$	$5.4x10^8$
90	$6.3x10^8$	$7.3x10^8$	$2.5x10^8$	$4.8x10^9$	$3.2x10^5$	$6.2x10^8$

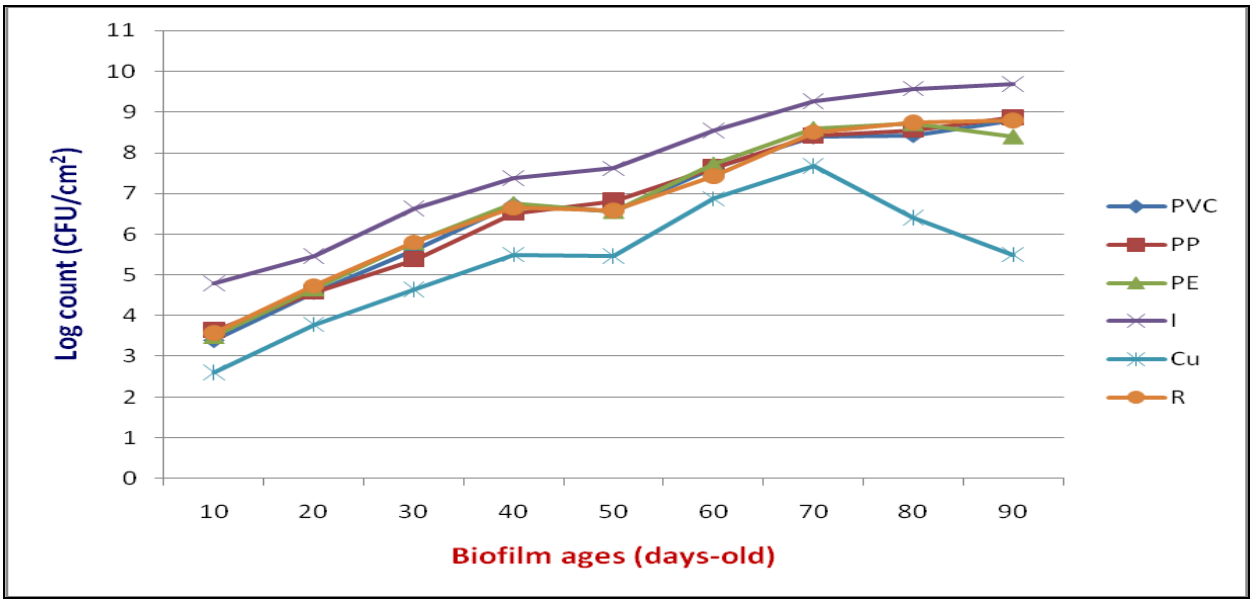


Fig. 24. Growth curve of *S.* Typhimurium biofilm grown on six different domestic plumping materials using R2A medium.

As shown in Table (19) the lowest growth was recorded in Cu pipe. While, in I pipe material, the highest value was determined. Counts of *S.* Typhimurium biofilm grown on PVC, PP, PE and R were 10^7 CFU/cm^2 at 60 days using BSA medium. The results illustrated in Fig. (25) indicated no difference in PVC, PP, PE and R pipe materials. While, the lowest growth curve recorded in Cu pipe material. Whereas, the highest growth curve of bacterial biofilm was recorded in I pipe material.

The lowest counts was recorded in Cu pipe and regularly decreased to reach 10^5 CFU/cm^2 at 90 days. On the contrary, in I pipe material the highest value (10^9 CFU/cm^2 at 70 days) was determined. While, the counts of *S.* Typhimurium biofilm grown on PVC, PP, PE and R was 10^7 CFU/cm^2 at 60 days by using HISA medium (Table 20).

Accordingly, the results presented in Fig. 26 indicated the lowest growth curve of *S.* Typhimurium biofilm was recorded in Cu pipe material. Whereas, the highest growth curve of was recorded in I pipe material. In the case of PVC, PP, PE and R pipe materials growth curve was similar.

Results of this study demonstrated that, the biofilm formation by both *L. monocytogenes* and *S.* Typhimurium on I pipe material was better than other pipe materials. While, in case of the biofilm formation on plastic based materials (PVC, PP, PE and R), it was less than I pipe material and more than Cu pipe materials. This may be due to the formation of biofilm in plastic-based materials is lower than metals-based materials especially iron pipe. These findings are in good agreement with Liu *et al.* (2014) who reported that PVC seems more suitable as a plumbing material in drinking water distribution systems, given that the cast iron faucet supports more complex bacterial diversity than the PVC faucet. Besides, some previous studies showed that plastic-based materials support less biofilm biomass than metal materials (Niquette *et al.*, 2000).

In the past, the majority of pipelines in drinking water networks were made of iron-based or cement-based materials. While, polymeric materials have been preferred, mainly polyvinyl chloride (PVC) and PE, because they are easier to handle and implement (Momba and Kaleni, 2002).

Table 19. Estimation of growth rate of *S.* Typhimurium biofilm on six different domestic plumping materials by using BSA medium.

Biofilm age (days-old)	Colony forming unit (CFU/cm^2) using BSA Types of tested domestic plumping materials					
	PVC	**PP**	**PE**	**I**	**Cu**	**R**
10	$2.3x10^3$	$1.7x10^3$	$5.2x10^3$	$3.5x10^4$	$2.4x10^2$	$4.3x10^3$
20	$1.5x10^4$	$3.3x10^4$	$7.3x10^4$	$6.2x10^5$	$5.2x10^3$	$2.7x10^4$
30	$2.7x10^5$	$4.5x10^5$	$2.9x10^5$	$1.8x10^6$	$4.1x10^4$	$3.5x10^5$
40	$3.5x10^6$	$2.4x10^6$	$1.2x10^6$	$2.3x10^7$	$2.8x10^5$	$2.1x10^6$
50	$2.8x10^6$	$7.5x10^6$	$3.6x10^6$	$4.1x10^7$	$3.5x10^5$	$3.4x10^6$
60	$3.1x10^7$	$5.4x10^7$	$4.7x10^7$	$2.3x10^8$	$4.4x10^6$	$2.9x10^7$
70	$1.8x10^8$	$2.2x10^8$	$3.8x10^8$	$1.9x10^9$	$3.6x10^7$	$1.3x10^7$
80	$3.4x10^8$	$4.3x10^8$	$6.2x10^8$	$4.2x10^9$	$2.8x10^6$	$2.6x10^8$
90	$5.1x10^8$	$3.6x10^8$	$4.8x10^8$	$3.7x10^9$	$1.9x10^5$	$3.8x10^8$

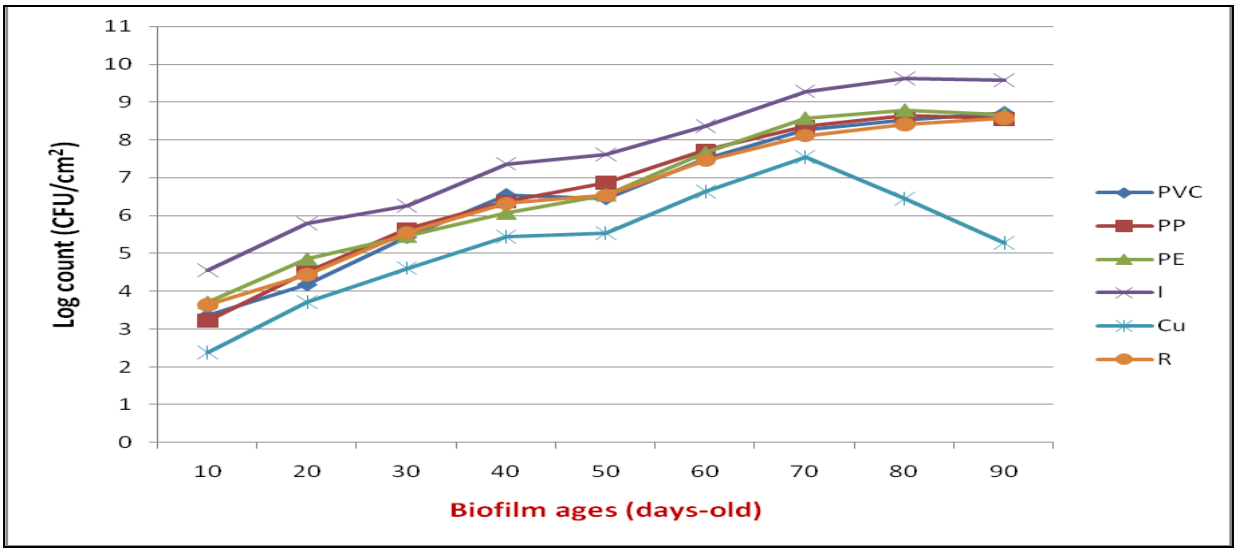


Fig. 25. Growth curve of *S.* Typhimurium biofilm grown on six different domestic plumping materials using BSA medium.

Table 20. Estimation of growth rate of *S.* Typhimurium biofilm on six different domestic plumping materials by using HISA medium.

Biofilm age (days-old)	Colony forming unit (CFU/cm^2) by using HISA Types of tested domestic plumping materials					
	PVC	PP	PE	I	Cu	R
10	$1.4x10^3$	$3.2x10^3$	$2.7x10^3$	$3.4x10^4$	$2.3x10^2$	$3.8x10^3$
20	$2.3x10^4$	$2.4x10^4$	$3.4x10^4$	$2.1x10^5$	$1.5x10^3$	$4.5x10^4$
30	$3.4x10^5$	$4.3x10^5$	$4.3x10^5$	$3.2x10^6$	$4.2x10^4$	$2.3x10^5$
40	$2.7x10^6$	$3.1x10^6$	$3.6x10^6$	$2.8x10^7$	$5.1x10^5$	$4.7x10^6$
50	$2.3x10^6$	$2.6x10^6$	$2.4x10^6$	$6.5x10^7$	$3.4x10^5$	$3.2x10^6$
60	$4.5x10^7$	$1.8x10^7$	$5.2x10^7$	$3.6x10^8$	$2.7x10^6$	$2.7x10^7$
70	$5.2x10^8$	$2.7x10^8$	$1.3x10^8$	$2.2x10^9$	$4.3x10^7$	$5.7x10^7$
80	$2.7x10^8$	$2.2x10^8$	$3.6x10^8$	$4.7x10^9$	$2.9x10^6$	$3.8x10^8$
90	$4.2x10^8$	$1.5x10^8$	$2.2x10^8$	$5.2x10^9$	$1.7x10^5$	$3.2x10^8$

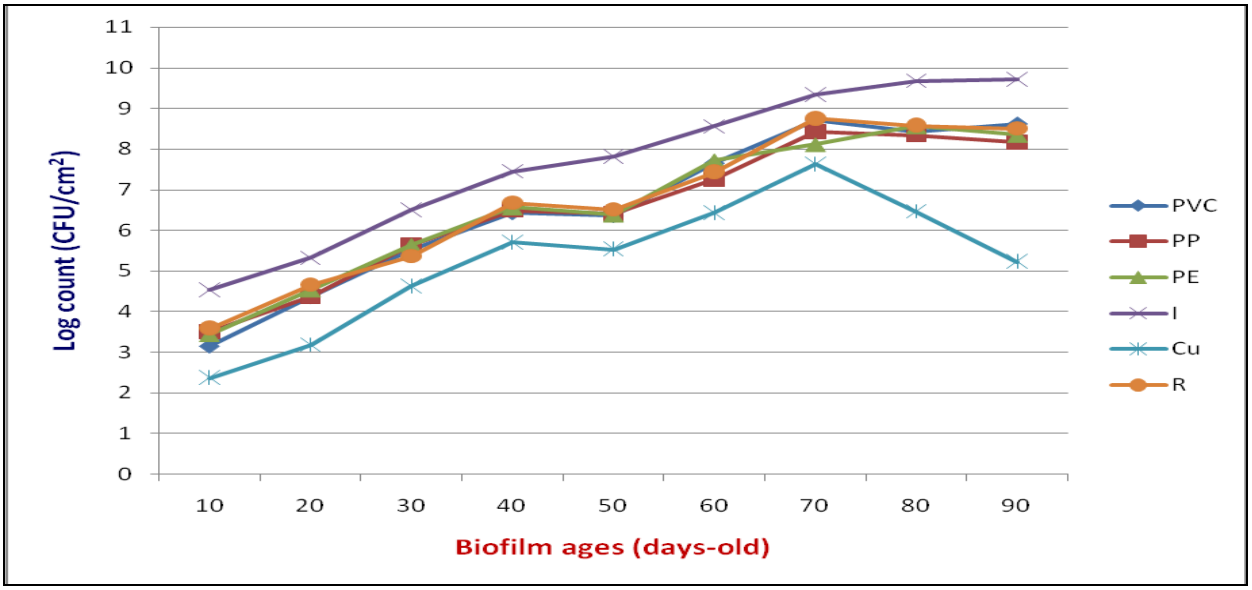


Fig. 26. Growth curve for *S.* Typhimurium biofilm grown on six different domestic plumping materials by using HISA medium.

Some researchers demonstrated that drinking water biofilm grew less on polymeric materials (PE, PVC, and Teflon) than on iron matrices (grey iron, cast iron, galvanized steel, cemented steel, asbestos–cement and cemented cast iron). This fact was attributed to iron corrosion products that favor biofilm protection from the effect of flow rate and disinfectants. Also, the accumulation of corrosion products and dissolved substances in the older pipes can increase the roughness of the pipe (Christensen, 2009),

thus favoring the development of biofilm. In addition, older deposits may have greater biomass and contain more bacteria (Chowdhury, 2011).

Also, van der Kooij and Veenendaal (2001) observed that PE supports biofilm formation in a higher degree than PVC. On the other hand, Cloete *et al.* (2003) and Bachmann and Edyvean (2005) demonstrated that the biofilm formation was higher on the PVC surfaces than on galvanized steel piping.

Accordingly, Lehtol *et al.* (2004) concluded that, the main characteristics of materials that have been identified as important on biofilm formation are the roughness and the surface physicochemical properties (chemical composition, solid surface tension, hydrophobicity and surface charge). Another aspect is the leaching of volatile components from pipe materials that can be metabolized by biofilm microorganisms. Also, van der Kooij *et al*. (1995) observed that the polymeric materials in contact with DW could release biodegradable compounds, thus enhancing biofilm formation. Moreover, corrosion resistance of the materials may be another important factor when choosing the material for the DWDS.

In contrast, the obtained results of this study proved real that, the biofilm formation in Cu pipe materials was lower than other tested materials. Also, the growth rate of biofilm in Cu pipe was slower than others. Additionally, the biofilm formation rate in the first stage was lower which concomitant than other tested materials. In spite of the Cu pipe is considered as a metal-based material, the results recorded that, the growth rate of biofilm was lower than tested plastic based materials.

Actually explanation may be revealed the formation of biofilm on of Cu pipe material may cause corrosion for it, and the released copper ions that can inhibit the bacterial communities (biofilm). Also, copper ions act as antibacterial agent. Consequently, copper pipes are known as one of the most resistant to pollution materials with a property of the toxicity of copper ions to microorganisms, especially for bacteria in biofilm (Santo-Domingo *et al.,* 2003). Moreover, The results also in agreement with the results of Lehtola *et al.* (2004, 2005) they found that biofilm grew faster in PE than in copper pipes, but such differences could not be detected in older piping systems; these

authors also studied the release of nutrients from the surface materials to the bulk water and the deleterious effects that this may cause on the water quality and on the efficacy of chlorine disinfection. Additionally, Wingender and Flemming (2004) and Zacheus *et al.* (2000) concluded that there was no significant difference in the colonization of the investigated materials (stainless steel, PVC and PE), in some cases after decades of operation.

Besides, van der Kooij *et al.* (2005) demonstrated the high bacterial concentration with PEX (cross-linked polyethylene), but no significant difference was observed in bacterial concentrations of biofilm for copper and stainless steel pipe materials. When, Schwartz *et al.* (1998) explained the bacterial population in biofilm on different pipe materials, the highest population was recorded in PE pipe, followed by stainless steel and PVC pipes, while the lowest population was found in copper pipe.

Also, copper corrodes producing structural failure of water distribution systems and increasing the copper concentration of bulk water to health threatening levels (Dietrich *et al.*, 2004). Thus, for pipes with the presence of biofilm the complication of copper with the biomass and the hydrodynamics are the main mechanisms for copper release (Pizarro *et al.*, 2014).

On the other hand, the obtained results in the present study indicated that, no differences between the culture medium used for enumerate the biofilm cells. Also, most previous studies on biofilm of distribution systems have used only culture-dependent methods to determine the bacteria present (Schwartz *et al.*, 1998). Also, Hyun-Jung *et al.* (2010) investigated the concentration, species diversity, and community structure of the bacteria in biofilm formed in four different pipe materials (steel, copper, stainless steel, and polyvinyl chloride), and analyzed the impact of bacteria on drinking water quality by using both culture-dependent and culture-independent methods. Despite, many culture-independent molecular methods such as in situ bacterial 16S rRNA gene profiles (Burtscher *et al.*, 2009), flow-cytometric total cell counts or adenosine tri-phosphate (ATP) analysis (Berney *et al.*, 2008) have been used to study the presence of microorganisms in drinking water. Nonetheless, heterotrophic plate counts (HPC) and

selective plating for pathogens are still commonly used to monitor the bacterial presence in drinking water. Additionally, Ramos-Martínez *et al.* (2012) reported that, the heterotrophic plate counts (HPC) has been chosen as a method of biofilm quantification.

In addition, comparative evaluation of classical techniques involving bacterial growth on specific selective medium and molecular methods based on 16s rDNA sequence identity reveals a high discrepancy between what was expected to grow and the species isolated from specific selective growth medium. Bacterial analyses of water based on selective isolation and culturing approach is recommended to be interpreted with caution (September *et al.*, 2007).

From statistical analysis, the obtained results recorded the strong correlation ($p \leq 0.01$) between the counts of biofilm cells harvested from tested pipe materials and biofilm ages all biofilm cells. These results were similar to those of Zhu *et al.* (2014) they demonstrated the HPC attached to pipe surfaces exhibited significant ($p \leq 0.01$) variations throughout the experiment.

Commonly, the findings of the present study revealed this *L. monocytogenes* and *S.* Typhimurium real own a high ability to form biofilm on plastic and metal-based materials. These results confirm previous findings, which showed that *Salmonella* spp. and *L. monocytogenes* are remarkably able to form biofilm on plastic surfaces (Stepanovic´*et al.*, 2004). Also, the bacterial communities or biofilm were affected by the different pipe materials (Liu *et al.*, 2014).

In the last part, by comparison between the growth rate of biofilm both *L. monocytogenes* and *S.* Typhimurium, the findings showed that, the growth rate of *S.* Typhimurium biofilm was higher than *L. monocytogenes* biofilm. These results verified the results of Stepanovic´ *et al.* (2004) they recorded that, the quantities of biofilm produced by *Salmonella* spp. were greater than those produced by tested *L. monocytogenes*. Also, the greater biofilm production by *Salmonella* spp. than that by *L. monocytogenes* is in agreement with the published superiority of Gram negative bacteria to form biofilm on inert surfaces (Pompermayer and Gaylarde 2000).

From the obtained results, it could be concluded that the highest biofilm accumulation by *L. monocytogenes* on the six tested pipe materials was recorded in I followed by tested plastic based materials (PVC > PP > R >PE) > Cu. While, the growth rate of *S.* Typhimurium biofilm formation in I pipe was more than tested plastic based materials (PP > R > PVC >PE) and Cu. From these results, it is cleared that, the differences between the growth rate of *L. monocytogenes* and *S.* Typhimurium. The results were compatible with the results of Zhu *et al.,* (2014) they investigated the five experimental pipe materials were ranked in the order: concrete-coated polycarbonate (CP) > cast iron (CI) > polyethylene (PE) > stainless steel (STS) > copper (Cu).

b. Estimation of the exopolysaccharide (EPS) amounts produced by *L. monocytogenes* and *S.* Typhimurium biofilm

In this part of the present study, to evaluate the role of polymers in biofilm formation, the extracellular polymeric substance that binds bacteria together in the biofilm community was characterized. The amounts of exopolysaccharide were determined in *L. monocytogenes* and *S.* Typhimurium biofilm grown on six different domestic plumping materials during experimental periods (10 to 90 days) each 10 days-old.

Extracellular polymeric substances (EPS) synthesized by microbes vary greatly in composition depending upon their environment (Sutherland, 2001). And the production of EPS is common in most of the pathogenic organisms and biofilm forming microbes. EPS is responsible for the structural and functional integrity of the biofilm. These EPS determine the physicochemical and biological parameters of the biofilm. Also, it has a significant effect on the development of biofilm by providing a structure for microbial cells and their products. (Wimpenny *et al.,* 2000).

The EPS are containing of polysaccharides, proteins, DNA and lipids with different percentages. These components involve to the mechanical stability of microbial biofilm. EPS are rich of polysaccharides and other non-sugar compounds such as proteins (Bayles, 2007). Also, Flemming and Wingender (2010) reported that, the EPS play an important role in the biofilm accumulation. Also, Tsuneda *et al.* (2003) recorded that the

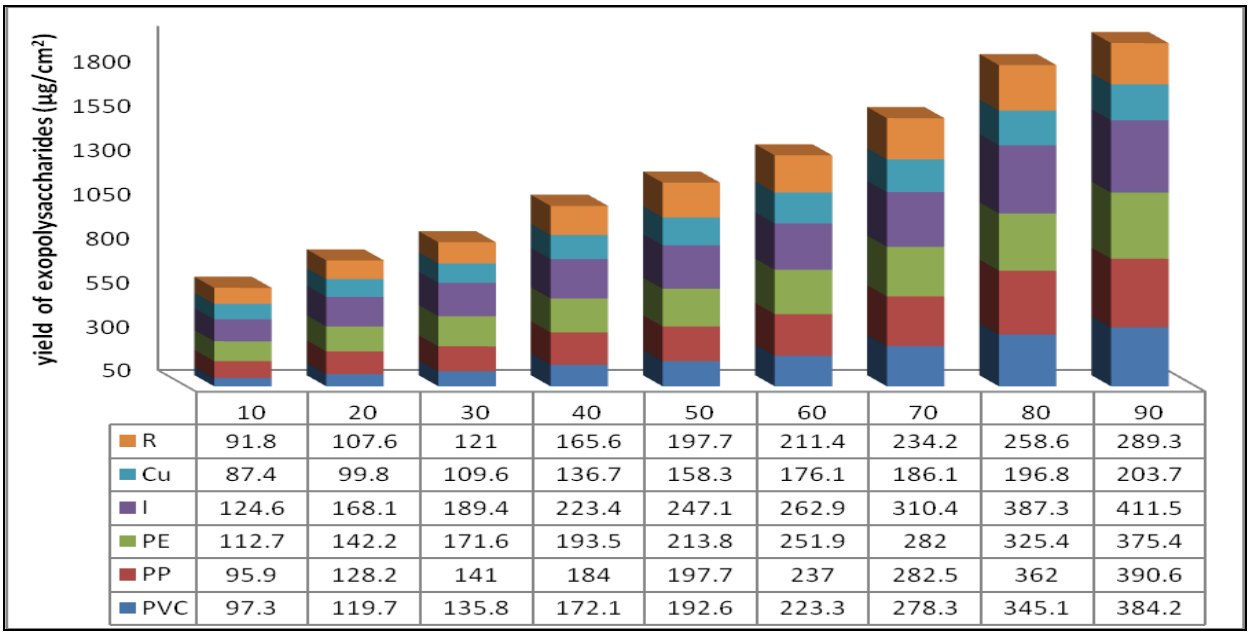
polysaccharides and proteins accounts for 75-89% of the biofilm EPS composition, indicating that they are the major components.

Data represented in Table (21) and Fig. (27) showed that the highest amounts of exopolysaccharide of *L. monocytogenes* biofilm were recorded in I pipe. While, the lowest amounts of exopolysaccharide were in Cu pipe material in all biofilm ages from 10 to 90 days-old. The amounts of exopolysaccharide of PVC, PP, PE, I, Cu and R pipe materials ranged between 97.3-384.2, 95.9-390.6, 112.7-375.4, 124.6-411.5, 87.4-203.7 and 91.8-289.3 µg/cm^2 of biofilm, respectively.

Statistically, data represented in Table (22) reported, there was a significant positive correlation between the quantities of exopolysaccharide and biofilm ages ($P \leq 0.01$).

Table 21. Exopolysaccharide (EPS) yield in *L. monocytogenes* biofilm grown on six different domestic plumping materials.

Biofilm age (days-old)	Yield of exopolysaccharide (EPS) (µg/cm^2) Types of tested domestic plumping materials					
	PVC	**PP**	**PE**	**I**	**Cu**	**R**
10	97.3	95.9	112.7	124.6	87.4	91.8
20	119.7	128.2	142.2	168.1	99.8	107.6
30	135.8	141.0	171.6	189.4	109.6	121.0
40	172.1	184.0	193.5	223.4	136.7	165.6
50	192.6	197.7	213.8	247.1	158.3	197.7
60	223.3	237.0	251.9	262.9	176.1	211.4
70	278.3	282.5	282.0	310.4	186.1	234.2
80	345.1	362.0	325.4	387.3	196.8	258.6
90	384.2	390.6	375.4	411.5	203.7	289.3



	10	20	30	40	50	60	70	80	90
R	91.8	107.6	121	165.6	197.7	211.4	234.2	258.6	289.3
Cu	87.4	99.8	109.6	136.7	158.3	176.1	186.1	196.8	203.7
I	124.6	168.1	189.4	223.4	247.1	262.9	310.4	387.3	411.5
PE	112.7	142.2	171.6	193.5	213.8	251.9	282	325.4	375.4
PP	95.9	128.2	141	184	197.7	237	282.5	362	390.6
PVC	97.3	119.7	135.8	172.1	192.6	223.3	278.3	345.1	384.2

Fig. 27. The amounts of exopolysaccharide extracted from *L. monocytogenes* biofilm grown on six different domestic plumping materials.

Table 22. Bivariate correlation between amounts of exopolysaccharide, the ages of *L. monocytogenes* biofilm and the types of tested domestic plumping materials.

Person correlation		Types of tested domestic plumping materials					
		PVC	PP	PE	I	Cu	R
Age	r	.979**	.980**	.991**	.984**	.988**	.994**
	P-value	.000	.000	.000	.000	.000	.000
PVC	r	1	.999**	.993**	.995**	.946**	.972**
	P-value		.000	.000	.000	.000	.000
PP	r	.999**	1	.993**	.996**	.949**	.973**
	P-value	.000		.000	.000	.000	.000
PE	r	.993**	.993**	1	.992**	.962**	.982**
	P-value	.000	.000		.000	.000	.000
I	r	.995**	.996**	.992**	1	.952**	.976**
	P-value	.000	.000	.000		.000	.000
Cu	r	.946**	.949**	.962**	.952**	1	.991**
	P-value	.000	.000	.000	.000		.000
R	r	.972**	.973**	.982**	.976**	.991**	1
	P-value	.000	.000	.000	.000	.000	

**** Statistically highly significant (P≤ 0.01)** **r. correlation coefficient**

Concerning the exopolysaccharide yield produced by *S.* Typhimurium biofilm, the results revealed real, the highest yield of exopolysaccharide was presented in iron (I) pipe. In contrast, the lowest yield was reported in Cu pipe material in all biofilm ages

from 10 to 90 days-old. Furthermore, the amounts of exopolysaccharide of PVC, PP, PE, I, Cu and R pipe materials were ranged between 62.8-206.5, 66.4-213.6, 63.6-219.0, 78.9-274.9, 59.3-142.4 and 61.3-198.5 µg/cm^2 of *L. monocytogenes* biofilm, respectively (Table 23; Fig. 28) .

As shown in Table (24), a significant positive correlation between the quantities of exopolysaccharide and biofilm ages ($P \leq 0.01$).

Table 23. Exopolysaccharide (EPS) yield in *S.* Typhimurium biofilm grown on six different domestic plumping materials.

Biofilm age (days-old)	Yield of exopolysaccharide (EPS) (µg/cm^2) Types of tested domestic plumping materials					
	PVC	**PP**	**PE**	**I**	**Cu**	**R**
10	62.8	66.4	63.6	78.9	59.3	61.3
20	73.4	76.9	71.6	91.4	67.5	70.2
30	89.4	90.3	87.3	112.5	79.5	84.6
40	96.2	103.5	99.2	132.7	86.2	97.4
50	113.5	121.3	127.6	164.6	98.4	117.3
60	135.8	139.4	141.4	185.2	116.2	131.3
70	163.5	172.7	169.6	203.8	124.2	152.3
80	182.3	194.2	193.2	237.5	133.6	173.4
90	206.5	213.6	219.0	274.9	142.4	198.5

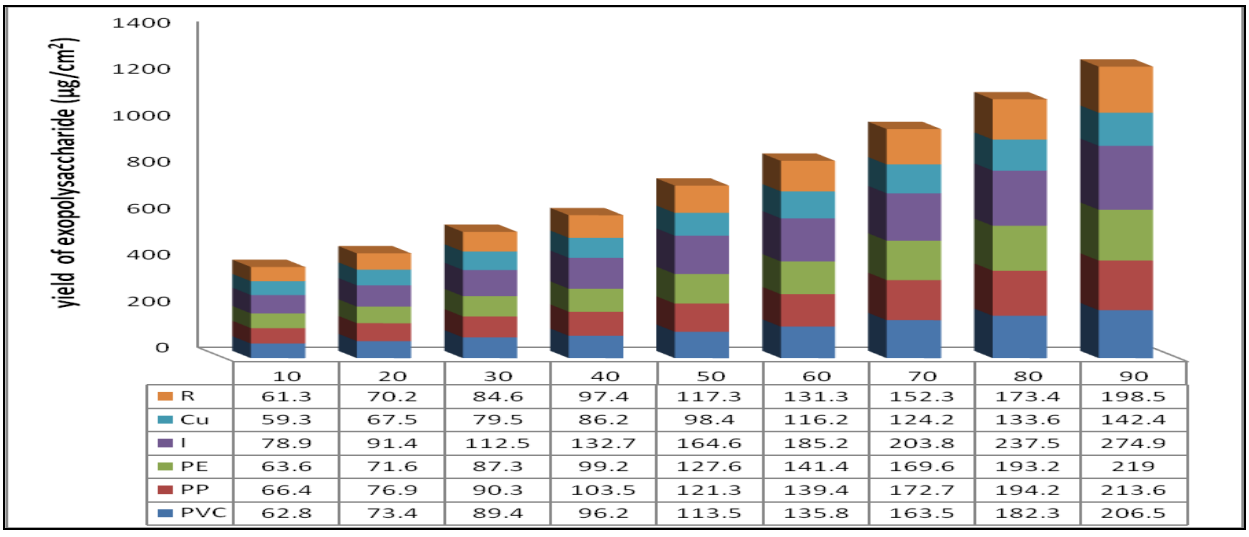


Fig. 28. The amounts of exopolysaccharide extracted from *S.* Typhimurium biofilm grown on six different domestic plumping materials.

Table 24. Bivariate correlation between amounts of exopolysaccharide , the ages of *S.* Typhimurium biofilm and the types of tested domestic plumping materials.

Person correlation		Types of tested domestic plumping materials					
		PVC	PP	PE	I	Cu	R
Age	r	.987**	.988**	.990**	.993**	.996**	.992**
	P-value	.000	.000	.000	.000	.000	.000
PVC	r	1	.999**	.997**	.993**	.987**	.997**
	P-value		.000	.000	.000	.000	.000
PP	r	.999**	1	.998**	.992**	.986**	.997**
	P-value	.000		.000	.000	.000	.000
PE	r	.997**	.998**	1	.997**	.988**	.999**
	P-value	.000	.000		.000	.000	.000
I	r	.993**	.992**	.997**	1	.989**	.999**
	P-value	.000	.000	.000		.000	.000
Cu	r	.987**	.986**	.988**	.989**	1	.988**
	P-value	.000	.000	.000	.000		.000
R	r	.997**	.997**	.999**	.999**	.988**	1
	P-value	.000	.000	.000	.000	.000	

**** Statistically highly significant (P≤0.01) r. correlation coefficient**

Results showed the reverse relationship between increase of biofilm growth rate and the production of exopolysaccharide amounts was reported. This is due to the exopolysaccharide act as a source of nutrients for bacterial proliferation to biofilm formation. Also, Bayles (2007) demonstrated the bacterial cell death is one of the important factors that can promote biofilm formation because of the release of cell contents.

Consequently, the obtained results noticed, real the amounts of exopolysaccharide (EPS) in biofilm produced by *L. monocytogenes* (Gram positive bacterium) were greater than *S.* Typhimurium (Gram negative bacterium). In spite of, the biomass growth rate of *S.* Typhimurium biofilm was more than *L. monocytogenes* biofilm grown on all tested plumping materials. It is mean the presence of inverse proportion between the biomass growth rate and the amount of exopolysaccharide (EPS).

These findings are in agreement with those of Evans *et al*. (1994) they reported the microbes produced low amount of EPS when they are rapidly growing and consuming substrate. Also, Turakhia and Characklis (1988) reported that, the relationship between EPS production and biomass growth rate (or substrate consumption rate) seems to depend

on the kind of microorganisms involved and the system conditions. Additionally, Olivera *et al.* (1993) explained the production of EPS was increasing without a drop in synthesis even when cell density started to decrease. Consequently, the high amount of EPS production led to decrease biofilm cell densities. This is due to the death of those cells that have spent more energy on EPS synthesis than they can increase at the low oxygen tensions in the depth of the biofilm (Kreft and Wimpenny, 2001).

Additionally, Sutherland (2001) suggested that the amount of EPS synthesis within the biofilm would depend greatly on the availability of carbon substrates (both inside and outside of the cells) and on the balance between carbon and other limiting nutrients. And the presence of excess available carbon substrate and limitations in other nutrients, such as nitrogen, potassium or phosphate, will promote the synthesis of EPS. Also, the relationship of the EPS production rate to the substrate-consumption rate is subject to significant controversy.

c. Microscopic examination of *L. monocytogenes* and *S.* Typhimurium grown on six different domestic plumping materials

There are several studies of the biofilm colonization recommended both the metabolic measurements, including total extracellular enzyme activities and structures in addition to structural parameters by using microscopic examination such as TEM and SEM (Romaní and Sabater, 1999). Microbes frequently live within multicellular, solid surface-attached assemblages termed biofilm. These microbial communities have architectural features that contribute to population heterogeneity and consequently to emergent cell functions. Therefore, three-dimensional (3D) features of biofilm structure are important for understanding the physiology and ecology of these microbial systems (Harrison *et al.,* 2006). Thus, the microscopic examination of biofilm is made by structure using three dimensional (3-D) data, such as Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) (Bandara *et al.*, 2010).

In this study, three microscopic methods (Epi-fluorescence, transmission and scanning electron microscope) were used to investigate biofilm formation . Direct counting of both microorganisms biofilm was carried out using epi-fluorescence

microscopy. In addition the structure of *L. monocytogenes* biofilm was investigated by using transmission electron microscopy (TEM). While, scanning electron microscopy (SEM) was used to examine the structure of *S.* Typhimurium biofilm.

1. Epi-fluorescence microscopic examination of *L. monocytogenes* and *S.* Typhimurium biofilm

The total cell counts of three ages of *L. monocytogenes* and *S.* Typhimurium biofilm were determined by using epi-fluorescence microscopy. As shown in Table (25) and Fig. (29), the highest total cell counts of *L. monocytogenes* biofilm were recorded in I pipe. On the contrary, the lowest counts were found in Cu pipe materials. Additionally, the total counts of PVC, PP, PE, I, Cu and R in three ages ranged between $2.9x10^3$-$4.8x10^9$, $4.3x10^3$- $6.3x10^8$, $3.5x10^3$-$3.7x10^9$, $2.8x10^4$-$4.5x10^{10}$, $5.4x10^3$-$2.8x10^6$ and $4.7x10^3$-$3.3x10^8$ cells/cm^2, respectively. It means that, the total cells densities increasing by increase biofilm age.

Regarding, *S.* Typhimurium biofilm, results were found, real the highest counts was observed in I pipe. The lowest total cells counts were formed in Cu pipe materials. Moreover, the total cells counts of PVC, PP, PE, I, Cu and R in three ages ranged between $4.2x10^4$- $7.6x10^9$, $7.6x10^3$- $9.4x10^8$, $2.4x10^4$-$2.6x10^9$, $3.7x10^4$-$7.2x10^{10}$, $6.2x10^3$-$4.5x10^7$ and $1.3x10^4$-$5.3x10^9$ cells/cm^2, respectively (Tabel 26, Fig. 30).

The results of the present investigation indicated real the direct method is unsuitable for enumerating the total biofilm cells. In spite of the epi-fluorescence microscopy is interesting to assess the overall cell population, but does not provide information on the bacteria that survived the DW disinfection process and that are able to multiply (Banning *et al.*, 2002). When, comparing between the culture-based method and direct microscopic examination method by using epi-fluorescence microscope, the obtained results showed the counts of biofilm cells by using cultivation method was lower than the counts by using epi-fluorescence microscopy. These results was in agreement of results of Simões *et al.* (2012) they explained number of total bacteria was mostly higher than those cultivable. Additionally, the number of total cells was 1 to 2 log units higher than the number of cultivable cells as adjudged by HPC (Lautenschlager *et*

al., 2010). Furthermore, Zhu *et al.*, (2014) observed that the cultivability in the biofilm was significantly ($p \leq 0.001$) different for different pipe materials but no significant with visualized by fluorescent dyes.

The microscopic examination by using epi-fluorescence microscopy not recommended to enumerate biofilm cells. Additionally, the cultivation technique is really promising since it is a rapid and inexpensive way to quantify fixed biomass without disrupting the biofilm. This method can also be used when inhibitory compounds or compounds that attenuated bacterial ability to be cultivated are present (Chang *et al.*, 2003).

Table 25. Total cells counts of *L. monocytogenes* biofilm grown on six domestic plumping materials in three ages.

Types of tested pipe materials	Total cell numbers (cells/cm^2) Biofilm ages		
	10 days-old	**40 days-old**	**90 days-old**
PVC	$2.9x10^3$	$5.9x10^7$	$4.8x10^9$
PP	$4.3x10^3$	$3.6x10^6$	$6.3x10^8$
PE	$3.5x10^3$	$4.3x10^7$	$3.7x10^9$
I	$2.8x10^4$	$3.8x10^8$	$4.5x10^{10}$
Cu	$5.4x10^3$	$6.2x10^5$	$2.8x10^6$
R	$4.7x10^3$	$5.3x10^6$	$3.3x10^8$

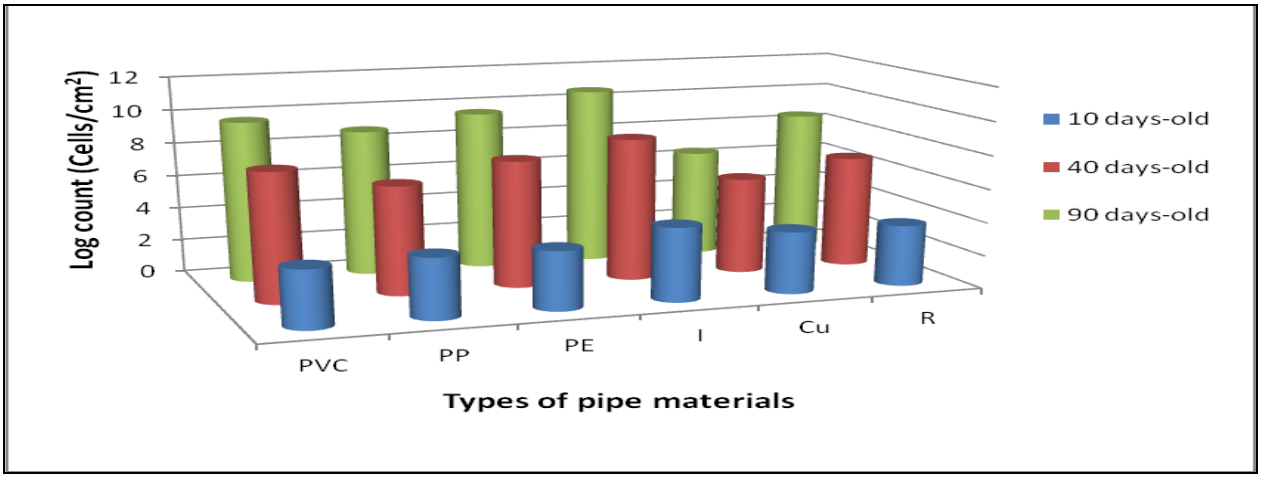


Fig. 29. Total cells densities of *L. monocytogenes* biofilm grown on six domestic plumping materials at three biofilm ages.

Table 26. Total cells counts of *S.* Typhimurium biofilm grown on six domestic plumping materials in three ages.

Types of tested pipe materials	Total cell numbers (cells/cm^2) Biofilm ages		
	10 days-old	40 days-old	90 days-old
PVC	$4.2x10^4$	$8.4x10^7$	$7.6x10^9$
PP	$7.6x10^3$	$1.4x10^7$	$9.4x10^8$
PE	$2.4x10^4$	$3.5x10^7$	$2.6x10^9$
I	$3.7x10^4$	$5.6x10^8$	$7.2x10^{10}$
Cu	$6.2x10^3$	$1.3x10^6$	$4.5x10^7$
R	$1.3x10^4$	$3.2x10^6$	$5.3x10^9$

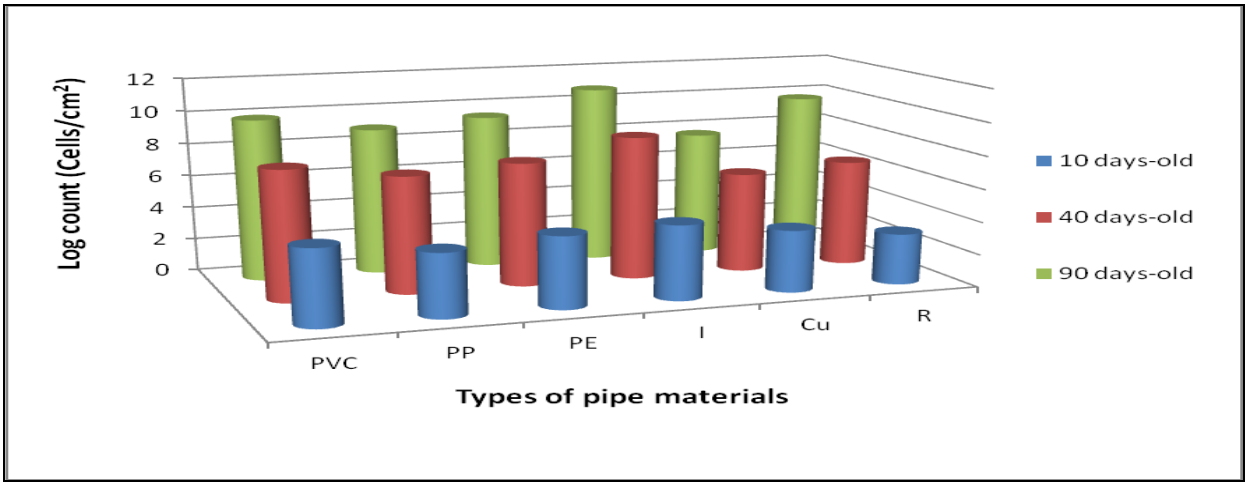


Fig. 30. Total cells densities of *S.* Typhimurium biofilm grown on six domestic plumping materials at three biofilm ages.

b. Transmission electron microscopic (TEM) examination of 90 days-old *L. monocytogenes* biofilm grown on six domestic plumping materials

In the present study, the kinetics of the formation of *L. monocytogenes* biofilm on six plumping materials was explained using the transmission electron photomicrograph. The photomicrograph indicated that the cells were embedded in a polymer matrix and exopolysaccharide. Also, it was produced from the biofilm cells in different shapes. As shown in Fig. 31, it was found that the pipe materials were affected by the quantity and shape of the exopolysaccharide produced from biofilm cells. Also, the differences in the density of the extracellular matrix and the sizes of cell aggregates were established (Smirnova *et al.*, 2010).

In addition to, *L. monocytogenes* biofilm grow on copper pipe produced lower amounts of exopolysaccharide than others. Therefore, EPS plays a vital role in the build-up of biofilm (O'Toole *et al.*, 2000). In comparison to many other biofilm studies, they showed cells surrounded in a heavy, slimy polymeric matrix layers (Flemming *et al.*, 2002).

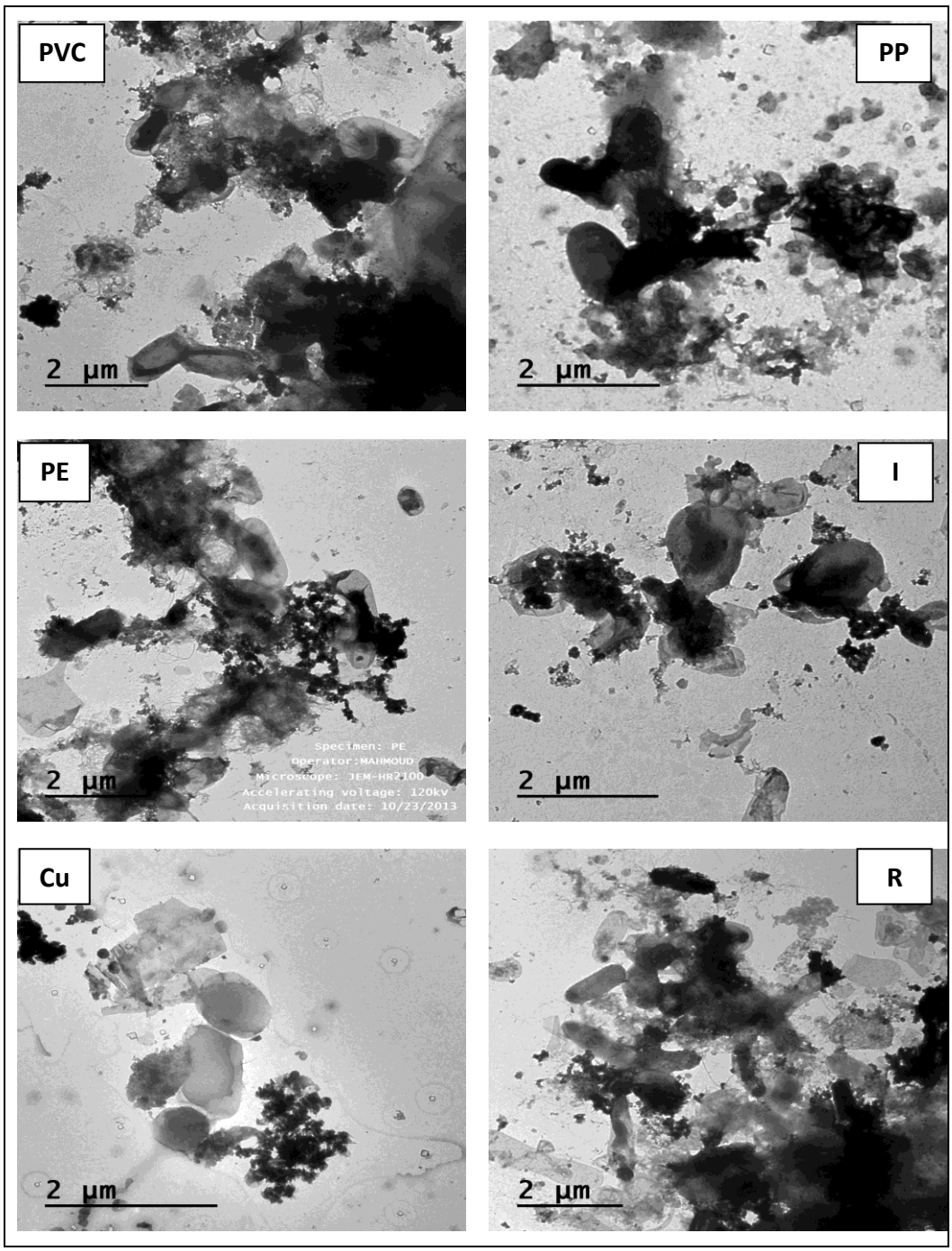
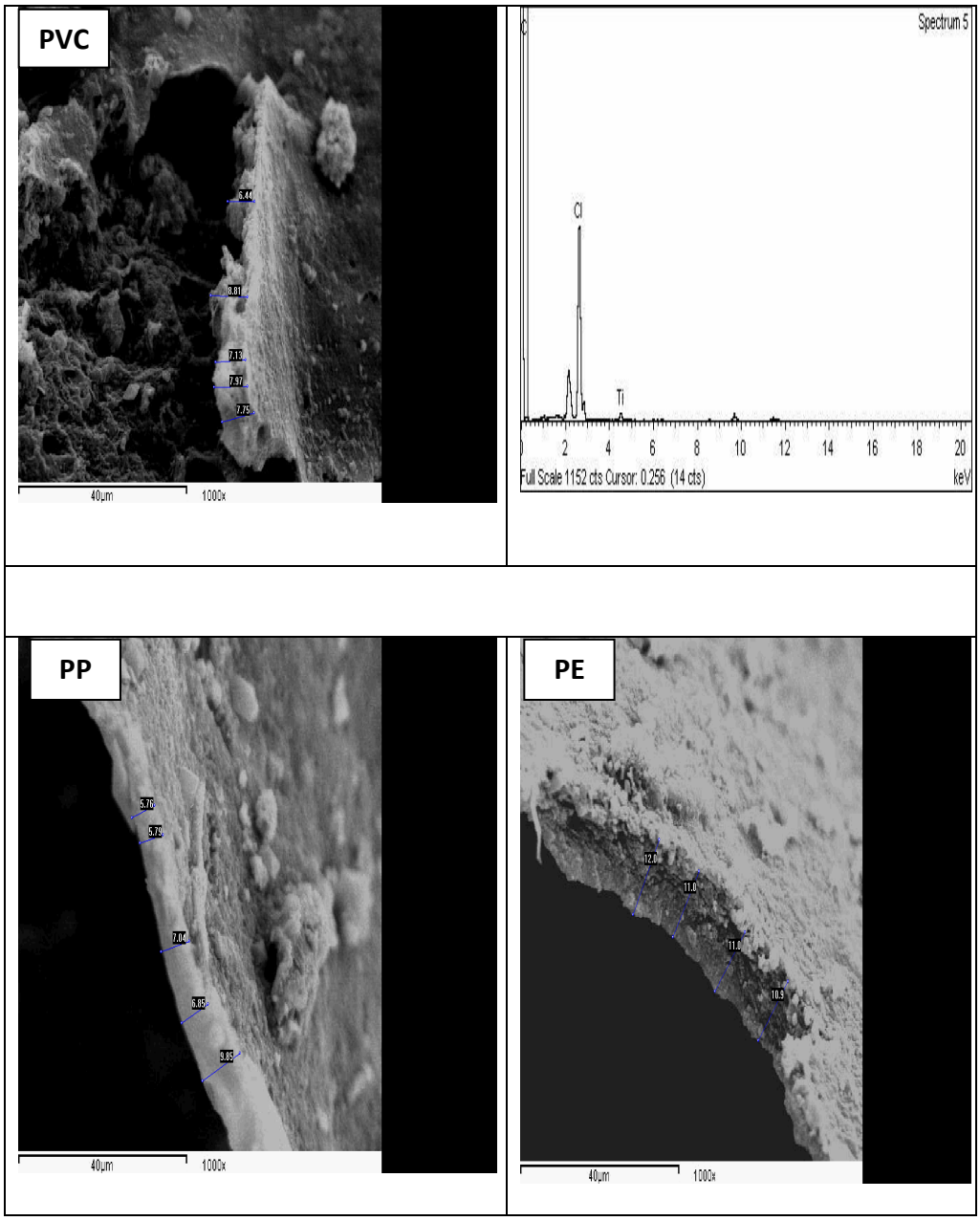


Fig. 31. TEM photomicrographs of *L. monocytogenes* biofilm grown on six different domestic plumping materials.

1. **Scanning electron microscopic (SEM) examinations of 90 days-old *S.* Typhimurium biofilm grown on six domestic plumping materials**

The structure of a biofilm formed by bacteria differs depending upon the environmental conditions. The biofilm structures are irregular, mushroom, flat, streamers, filamentous and mounds shaped. The biofilm formed in high flow velocity water are streamers regardless of the content of water, whereas biofilm formed in low flow or stagnant water are mushroom like structure or mound shape. The biofilm structure also differs with the availability of nutrients in the given environment and also genetic aspects (Hall-Stoodley *et al.*, 2004).

The structures of six biofilm grown on PVC, PP, PE, I, Cu and R were examined by using SEM. As shown in Fig. (32), the photomicrographs observed the thick biofilm wrapped by EPS. The highest thickness was shown in I and R pipe materials. In contrast, the lowest thickness was cleared in Cu and PP pipe materials. Additionally, the obtained results strongly imply that there are substantial differences among 90 days-old *L. monocytogenes* biofim grown on tested pipe materials. The results of present study were compatible with the results of with Feazel *et al.* (2009); Hong *et al.* (2010), they explained the inner surface of PVC faucet was covered with a layer of extracellular matrix material, where microbes were clumped and embedded. Matrix material was also observed previously on the surface of plastic substratum in water meters and showerheads. SEM analysis revealed that the visibly different biofilm patterns on the two faucets with different materials. The observation was comparable with that of corroded cast iron coupons under chlorine residue in water (Wang *et al.*, 2012).


PVC
Spectrum 5
Cl
Ti
0 2 4 6 8 10 12 14 16 18 20
Full Scale 1152 cts Cursor: 0.256 (14 cts)
keV
40µm
1000x
PP
5.76
5.79
7.04
6.85
9.89
40µm
1000x
PE
12.0
11.0
11.8
10.9
40µm
1000x

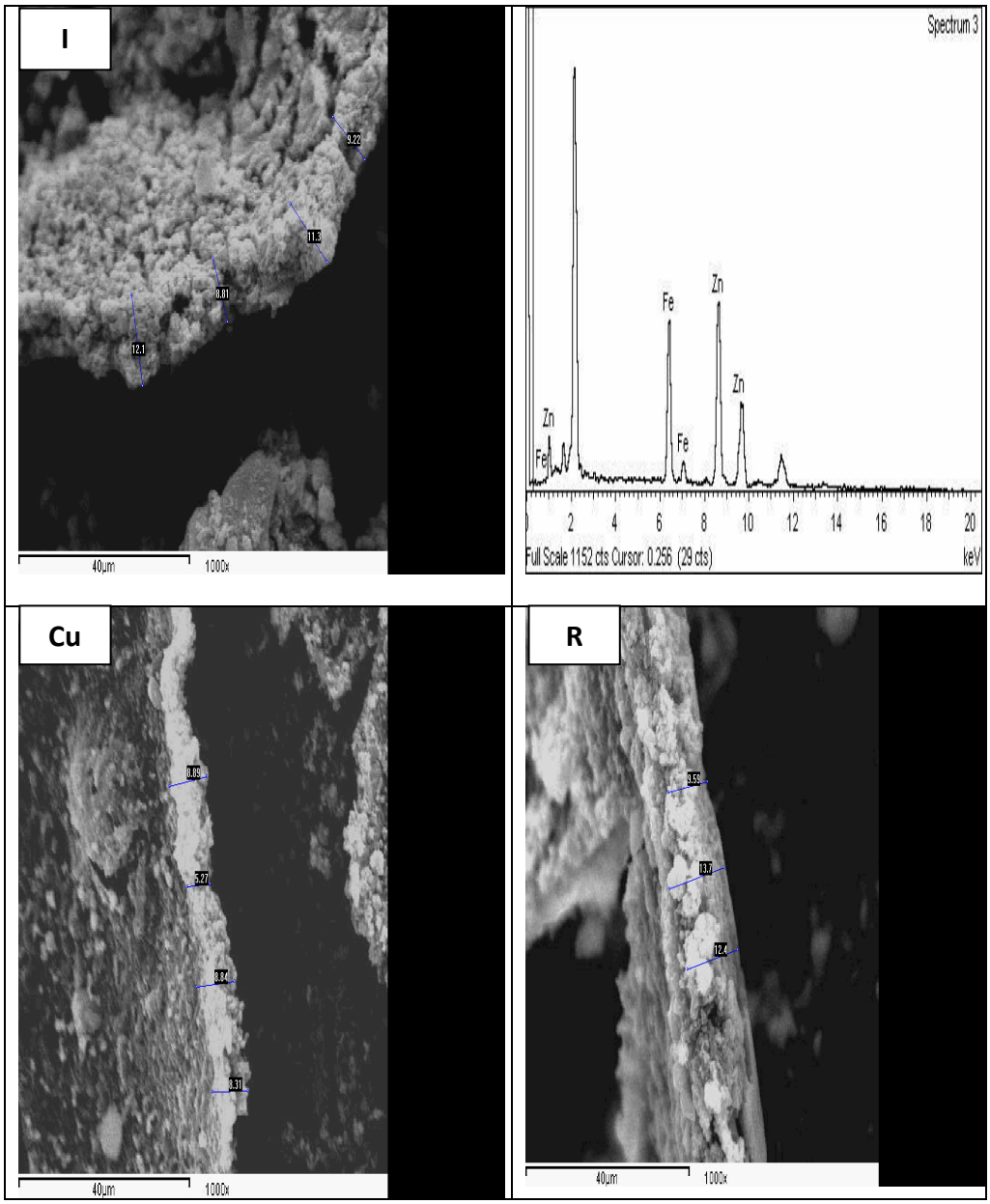


Fig. 32. SEM images of *S.* Typhimurium biofilm grown on six different domestic plumping materials.

d. Control and prevention of biofilm formation using different antimicrobial agents against planktoinc and biofilm cells of *L. monocytogenes* and *S.* Typhimurium

Bacterial communication through excreted signaling molecules is another advantage of living in biofilm communities (Nadell *et al.*, 2008). A significant advantage of the biofilm mode of growth is the potential for dispersion via detachment (Stewart, 2012). Under the direction of fluid flow, detached microorganisms travel to other regions to attach and promote biofilm formation on clean areas. Therefore, this advantage allows a persistent bacterial source population, usually resistant to antimicrobial agents, while at the same time enabling the continuous shedding to promote bacterial spread (Codony *et al.*, 2005).

1. Efficacy of chlorine against *L. monocytogenes* and *S.* Typhimurium biofilm grown on domestic plumping materials

The prevention of biofilm formation partly relies on drinking water nutrient reduction strategies, but the best available technology such as nanofiltration, which significantly lowers the organic matter level, is not able to radically reduce microbial cells or their cultivability within the biofilm (Liu *et al.*, 2013a). Biofilm prevention is also related to disinfection practices, but again a number of studies have demonstrated their limited efficiency. Indeed, most traditional disinfectants (chlorine, chloramines) are consumed by reactions with corrosion products and deposits (Wang *et al.*, 2012), pipe materials (Hubbard *et al.*, 2009) and exopolymeric substances (Xue *et al.*, 2012). Such restricted efficiency due to reaction-diffusion limited penetration has been previously reported on pure strain biofilm: *Pseudomonas aeruginosa* in alginate beads, *Staphylococcus epidermidis* in capillary flow cells, as well as binary populations of *P. aeruginosa* and *Klebsiella pneumoniae* on stainless steel coupons (Lee *et al.*, 2011). As a result, even in continuously chlorinated drinking water distribution systems (0.1- 0.4 mg Cl_2/l), biofilm grow and harbor active bacteria whose species composition varies depending on the disinfectant concentration (Mathieu *et al.*, 2009). Complementary

curative strategies to remove biofilm by water flushing are hardly effective due in particular to the viscoelastic properties of the systems (Paul *et al.*, 2012).

Drinking water biofilm represent a complex biophysical world embedded in an exopolymeric matrix (EPS) (Chen and Stewart, 2002; Aldeek *et al.*, 2013). As the biofilm EPS matrix plays a key role in the resistance of biofilm to disinfectants (Xue *et al.*, 2012), effective cleaning procedures should break it up in order to disperse the biofilm and allow disinfectants to diffuse rapidly.

Therefore, the purpose of this part was to evaluate the behavior and biological characteristics for *L. monocytogenes* and *S.* Typhimurium biofilm. For this purpose, the influence of different disinfectants and antimicrobial agents against *L. monocytogenes* and *S.* Typhimurium biofilm was e investigated. The biofilm samples were collected from designed DWDS model. Then, they were exposed to four different disinfectant and antimicrobial agents as a following: chlorine, silver ions (Ag ions), silver nanoparticles (Ag NPs) and antibiotics.

a. Comparative susceptibility of planktonic cells and three different ages of *L. monocytogenes* biofilm to chlorine

The effect of chlorine doses against the planktonic and biofilm cell in three different ages (10, 40 and 90 days-old) of *L. monocytogenes* was examined. They were exposed to eight different chlorine doses. Also, the residual or free chlorine was determined using DPD methods.

1. Chlorine demand for inactivation of *L. monocytogenes* planktonic cells

In this study, *L. monocytogenes* ATCC 25152 was used as a reference strain (planktonic cells). The initial counts of planktonic cells of *L. monocytogenes* ($1.6x10^6$ CFU/ml) were exposed to eight chlorine doses including 0.2, 0.6, 1.0, 1.4, 1.8, 2.2, 2.6 and 3.0 mg/l. The results showed that, the most effective chlorine dose was 3.0 mg/l, which led to complete inactivation (100% removal) with 6 log reduction. Additionally, the breakpoint was recorded at 0.6 mg/l chlorine (Table 27; Fig. 33).

The statistical analysis showed that, the coefficient of determination (R^2) between the chlorine dose and the residual chlorine was 0.975. Also, the R^2 between the residual chlorine and log reduction of bacterial counts was 0.942.

Table 27. Determination of effective chlorine dose and its residual against *L. monocytogenes* planktonic cells.

Chlorine (mg/l)		Bacterial counts (CFU/ml)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$1.6x10^6$	6.204	0
0.2	0.11	$3.7x10^4$	4.568	1.635
0.6	0.21	$1.2x10^4$	4.079	2.124
1.0	0.55	$2.8x10^3$	3.447	2.756
1.4	0.62	$4.6x10^2$	2.662	3.541
1.8	0.97	$2.1x10^2$	2.322	3.881
2.2	0.92	7.2x10	1.857	4.346
2.6	1.2	1.3x10	1.113	5.090
3.0	1.3	ND	ND	6.204

ND= Not detected

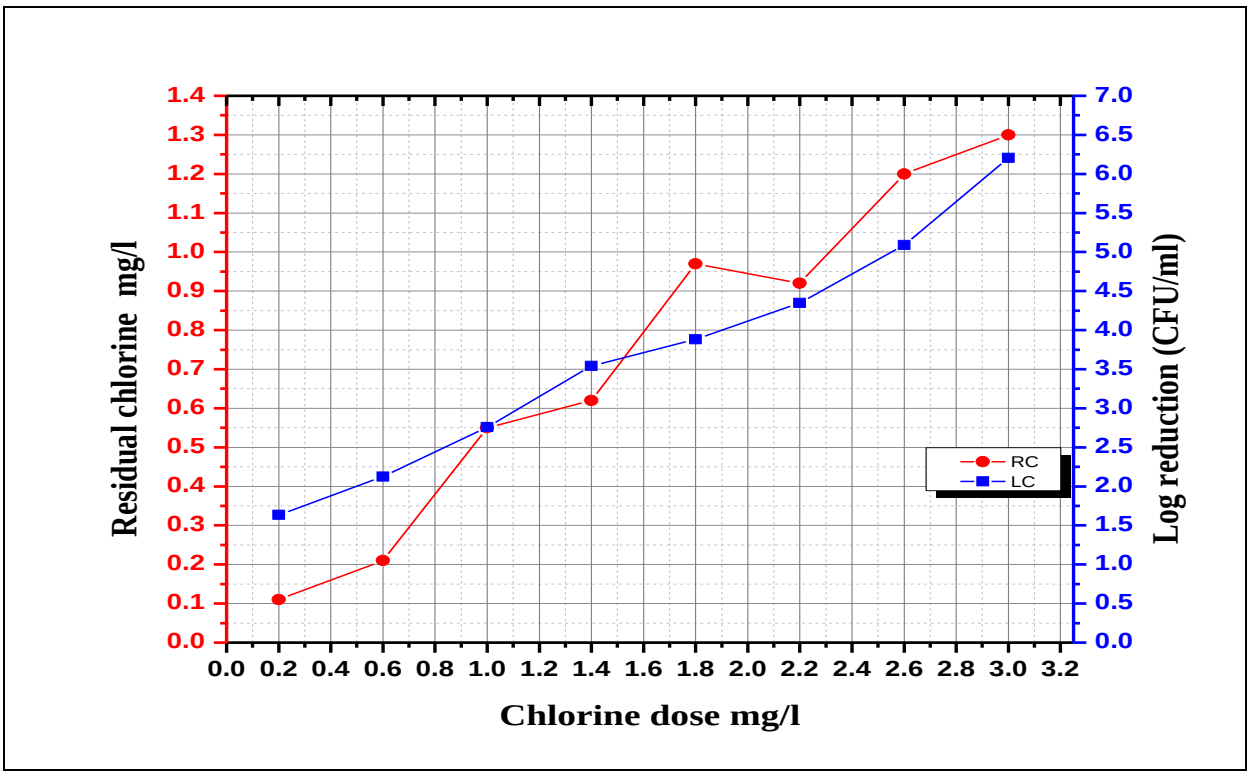


Fig. 33. The relationship between the chlorine doses, residual chlorine and log reduction of *L. monocytogenes* planktonic cells.

2. Chlorine demand for inactivation of *L. monocytogenes* biofilm grown on PVC pipe material

The six samples of *L. monocytogenes* biofilm cells, were scraped from six different pipeline materials (PVC, PP, PE, I, Cu and R) in three different ages (10, 40, and 90 days-old) and were exposed to the same eight chlorine doses.

As shown in Tables (28, 29, 30) and Figs. (34, 35, 36) the results of *L. monocytogenes* biofilm cells, which collected from PVC pipeline in different ages (10, 40, and 90 days-old), it is clear that, the initial log counts of 10 days-old biofilm cells were 6.6 CFU/cm^2. In this age, 3.0 mg/l of chlorine dose was able to complete the removal of cells. While, the initial log counts at 40 and 90 days-old of biofilm cells were 6.6 and 6.3 CFU/cm^2. The log reduction reached 4.7 and 3.9 CFU/cm^2. Moreover, the breakpoints of chlorine dose for 10, 40 and 90 days-old of biofilm cells were 0.6, 0.6 and 1.4 mg/l, respectively.

Statistically the coefficient of determination between the chlorine dose and the residual chlorine of 10, 40 and 90 days-old biofilm cells was 0.987, 0.983 and 0.898, respectively. While, the R^2 between the residual chlorine and log reduction of bacterial counts of 10, 40 and 90 days-old of biofilm cells were 0.922, 0.904 and 0.761, respectively.

Table 28. Determination of effective chlorine dose and its residual against 10 days-old *L. monocytogenes* biofilm grown on PVC pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	4.8x10^6	6.681	0
0.2	0.07	2.1x10^4	4.322	2.359
0.6	0.12	4.3x10^3	3.633	3.047
1.0	0.26	1.6x10^3	3.204	3.477
1.4	0.32	3.4x10^2	2.531	4.149
1.8	0.43	1.1x10^2	2.041	4.639
2.2	0.57	8.2x10	1.913	4.767
2.6	0.72	2.5x10	1.397	5.283
3.0	0.77	ND	ND	6.681

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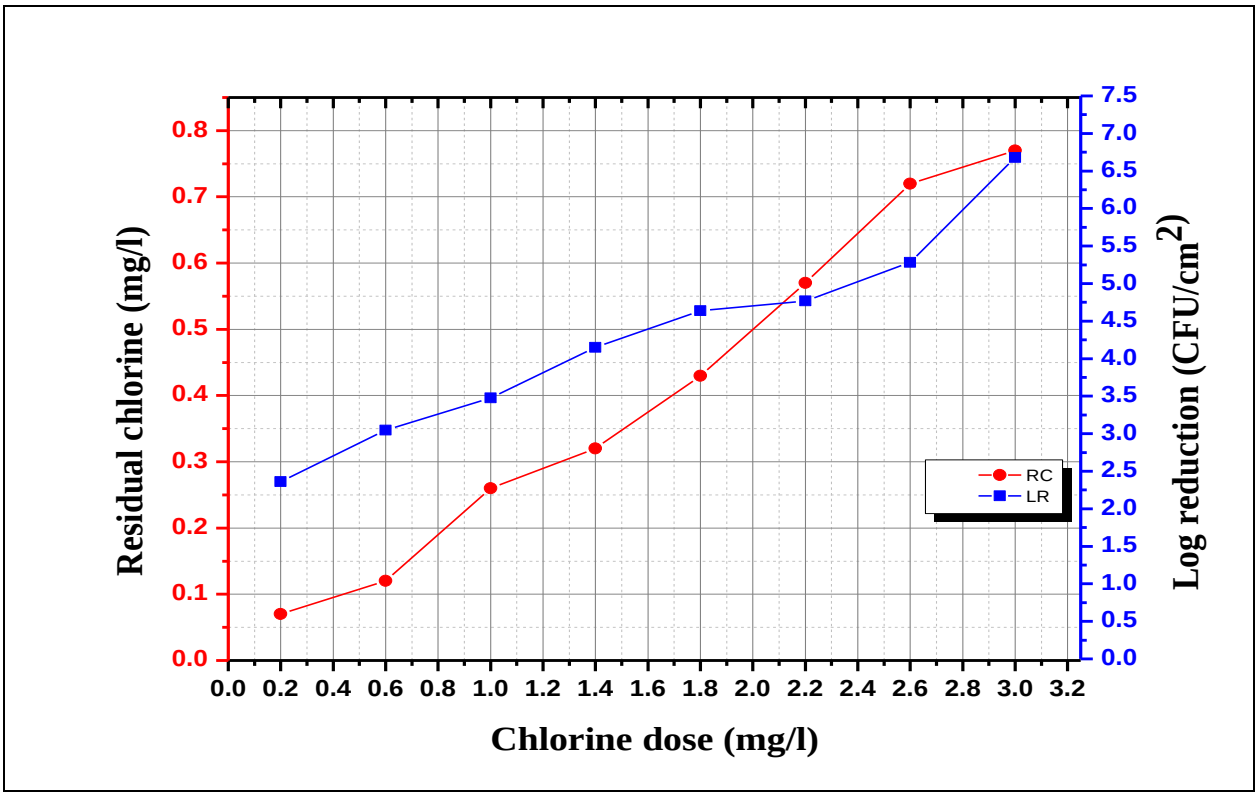

Fig. 34. The relationship between the chlorine doses, residual chlorine and log reduction of 10 days-old *L. monocytogenes* biofilm grown on PVC.

Table 29. Determination of effective chlorine dose and its residual against 40 days-old *L. monocytogenes* biofilm grown on PVC pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$4.7x10^6$	6.672	0
0.2	0.07	$5.3x10^4$	4.724	1.947
0.6	0.1	$2.6x10^4$	4.414	2.257
1.0	0.21	$7.1x10^3$	3.851	2.820
1.4	0.28	$4.5x10^3$	3.653	3.018
1.8	0.33	$3.4x10^2$	2.531	4.140
2.2	0.39	$2.6x10^2$	2.414	4.257
2.6	0.52	$1.7x10^2$	2.230	4.441
3.0	0.61	9.3x10	1.968	4.703

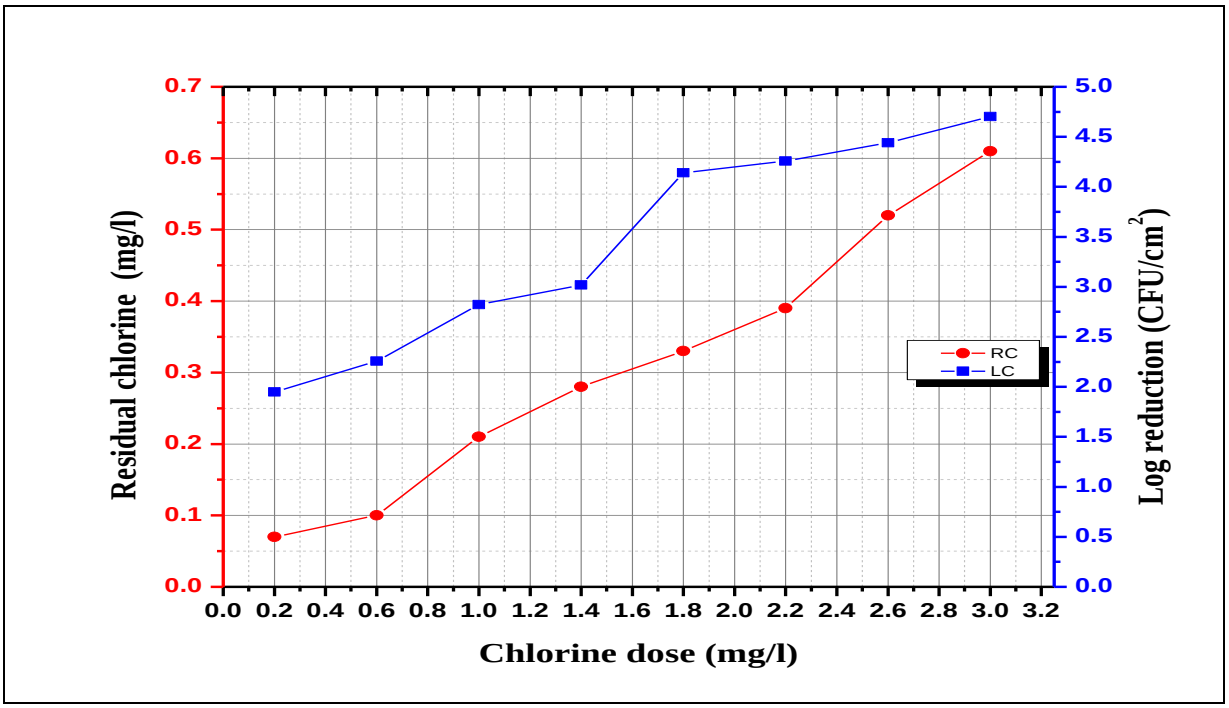

Fig. 35. The relationship between the chlorine doses, residual chlorine and log reduction of 40 days-old *L. monocytogenes* biofilm grown on PVC.

Table 30. Determination of effective chlorine dose and its residual against 90 days-old *L. monocytogenes* biofilm grown on PVC pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$2.4x10^6$	6.380	0
0.2	0.03	$2.8x10^5$	5.447	0.933
0.6	0.16	$1.2x10^4$	4.079	2.301
1.0	0.19	$9.7x10^3$	3.986	2.393
1.4	0.18	$6.3x10^3$	3.799	2.580
1.8	0.63	$4.8x10^3$	3.681	2.698
2.2	0.55	$7.6x10^2$	2.880	3.499
2.6	0.66	$4.1x10^2$	2.612	3.767
3.0	0.9	$2.6x10^2$	2.414	3.965

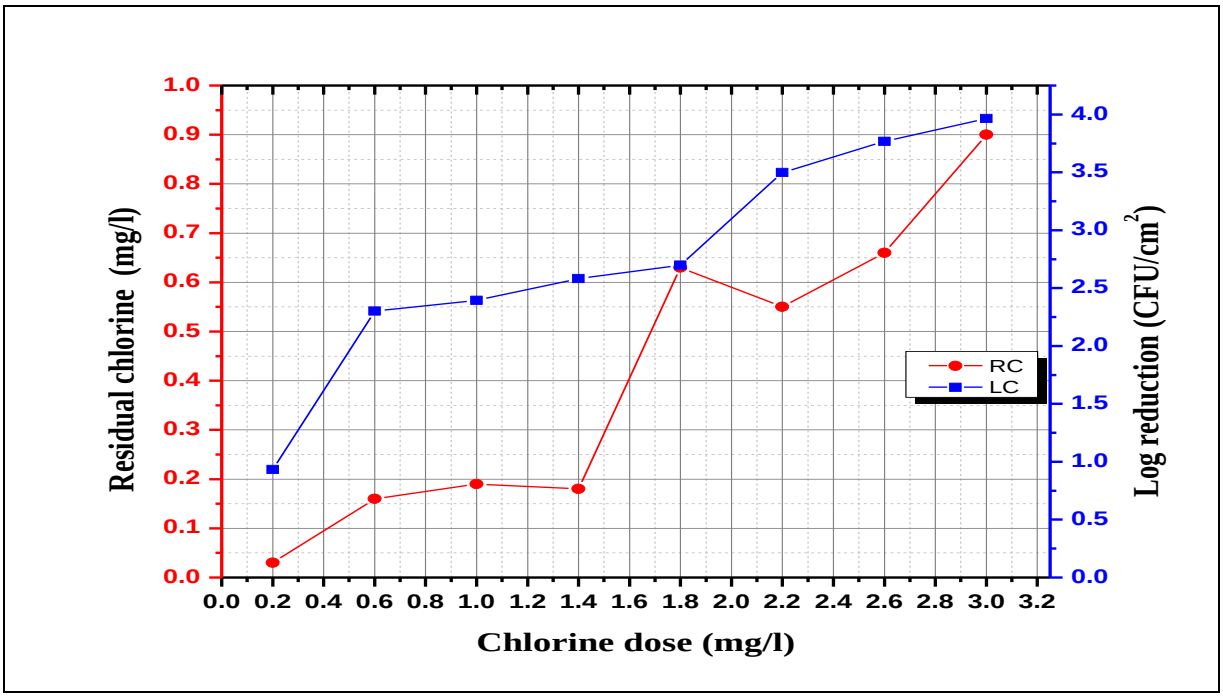

Fig. 36. The relationship between the chlorine doses, residual chlorine and log reduction of 90 days-old *L. monocytogenes* biofilm grown on PVC.

3. Chlorine demand for inactivation of *L. monocytogenes* biofilm grown on PP pipe material

In the case of PP pipeline, the initial counts of biofilm formation of *L. monocytogenes* at 10 days-old were 6.5 CFU/cm^2. The results demonstrated that, 3 mg/l of chlorine dose was able to completely remove the log counts. While, 40 and 90 days-old biofilm cells (the initial log counts 6.6 and 6.5 CFU/cm^2) the log reduction were 4.7 and 4.16 CFU/cm^2. Additionally, the breakpoints of chlorine dose for 10, 40 and 90 days-old of biofilm cells were 0.6, 1.4 and 2.2 mg/l, respectively (Tables 31, 32, 33; Figs. 37, 38, 39).

Statistically, the coefficient of determination (R^2) between the chlorine dose and the residual chlorine of 10, 40 and 90 days-old of biofilm cells was 0.990, 0.984 and 0.927, respectively. While, the R^2 between the residual chlorine and log reduction of bacterial counts of 10, 40 and 90 days-old of biofilm cells were 0.922, 0.904 and 0.761, respectively.

Table 31. Determination of effective chlorine dose and its residual against 10 days-old *L. monocytogenes* biofilm grown on PP pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$3.9x10^6$	6.591	0
0.2	0.21	$5.2x10^4$	4.716	1.875
0.6	0.31	$4.1x10^3$	3.612	2.978
1.0	0.54	$2.8x10^2$	2.447	4.143
1.4	0.64	$2.2x10^2$	2.342	4.248
1.8	0.85	$1.4x10^2$	2.146	4.444
2.2	1.02	$7.3x1^0$	1.863	4.727
2.6	1.3	1.6x10	1.204	5.386
3.0	1.45	ND	ND	6.591

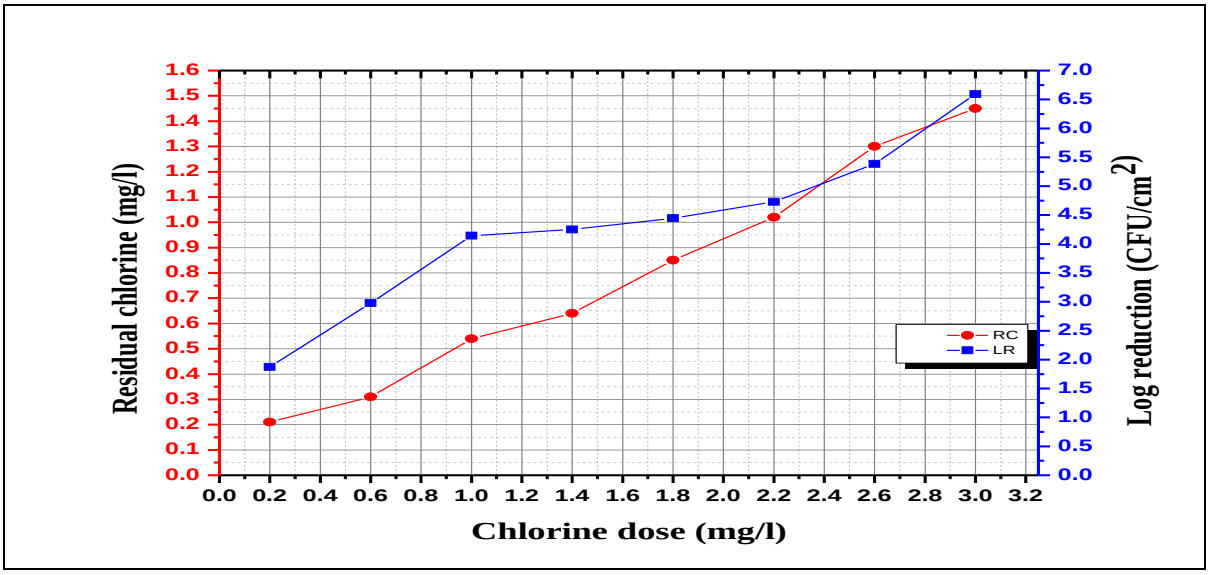

Fig. 37. The relationship between the chlorine doses, residual chlorine and log reduction of 10 days-old *L. monocytogenes* biofilm grown on PP.

Table 32. Determination of effective chlorine dose and its residual against 40 days-old *L. monocytogenes* biofilm grown on PP pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0	$4.5x10^6$	6.653	0
0.2	0.09	$6.4x10^4$	4.806	1.847
0.6	0.19	$2.6x10^4$	4.414	2.238
1.0	0.31	$3.3x10^3$	3.518	3.134
1.4	0.35	$1.2x10^3$	3.079	3.574
1.8	0.54	$4.7x10^2$	2.672	3.981
2.2	0.69	$2.3x10^2$	2.361	4.291
2.6	0.73	$1.6x10^2$	2.204	4.449
3.0	0.92	8.7x10	1.939	4.713

ND= Not detected

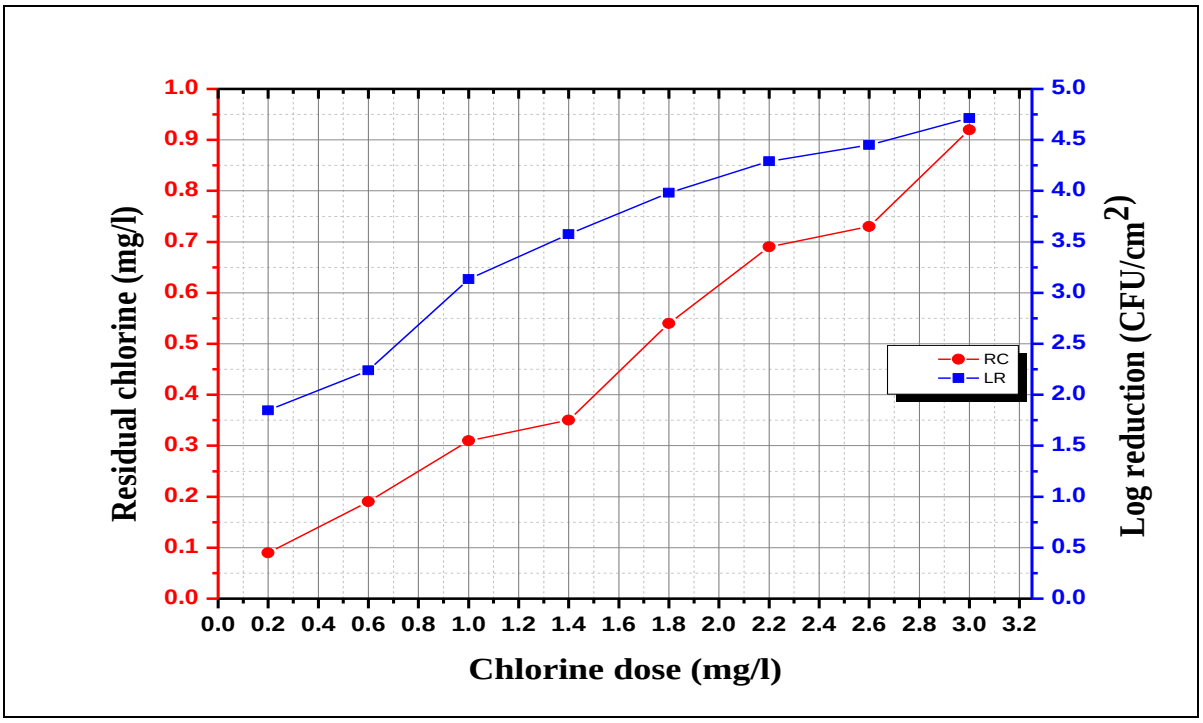

Fig. 38. The relationship between the chlorine doses, residual chlorine and log reduction of 40 days-old *L. monocytogenes* biofilm grown on PP.

Table 33. Determination of effective chlorine dose and its residual against 90 days-old *L. monocytogenes* biofilm grown on PP pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$3.5x10^6$	6.544	0
0.2	0.11	$4.5x10^5$	5.653	0.890
0.6	0.19	$2.9x10^4$	4.462	2.081
1.0	0.25	$5.4x10^3$	3.732	2.811
1.4	0.30	$3.6x10^3$	3.556	2.987
1.8	0.37	$2.8x10^3$	3.447	3.096
2.2	0.35	$9.7x10^2$	2.986	3.557
2.6	0.39	$4.5x10^2$	2.653	3.890
3.0	0.55	$2.4x10^2$	2.380	4.163

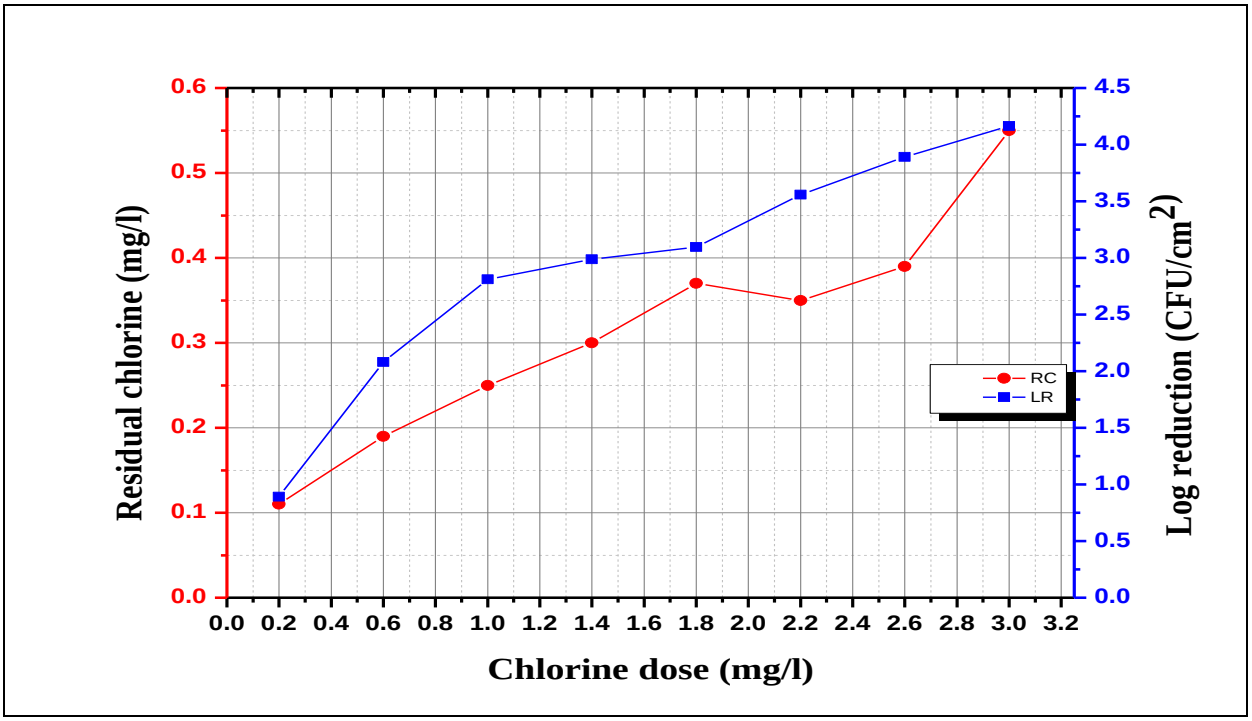

Fig. 39. The relationship between the chlorine doses, residual chlorine and log reduction of 90 days-old *L. monocytogenes* biofilm grown on PP.

4. Chlorine demand for inactivation of *L. monocytogenes* biofilm grown on PE pipe material

Regarding the biofilm cells of *L. monocytogenes* growing on the PE pipe, the initial log counts of different ages were 6.5, 6.3 and 6.7 CFU/cm^2 at 10, 40 and 90 days-old, respectively. The obtained results indicated that, respectively: 3 mg/l of chlorine dose was able to reduce the biofilm cells (4.21 and 5.50 log) for 90 and 40 days-old. While, the biofilm cells of 10 days-old *L. monocytogenes* were completely removed (6.54 log CFU/cm^2). Furthermore, the breakpoints of chlorine dose for 10, 40 and 90 days-old of biofilm cells were 0.6, 1.0 and 1.4 mg/l, respectively (Tables 34, 35, 36; Figs. 40, 41, 42).

From statistical analysis results, the coefficient of determination (R^2) between the chlorine dose and the residual chlorine of 10, 40 and 90 days-old of biofilm cells was 0.986, 0.984 and 0.975, respectively. While, the R^2 between the residual chlorine and log reduction of bacterial counts of 10, 40 and 90 days-old of biofilm cells were 0.965, 0.958 and 0.960 respectively.

Table 34. Determination of effective Chlorine dose and its residual against 10 days-old *L. monocytogenes* biofilm grown on PE pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$3.5x10^6$	6.544	0
0.2	0.21	$2.6x10^4$	4.414	2.129
0.6	0.26	$4.5x10^3$	3.653	2.890
1.0	0.48	$2.0x10^3$	3.301	3.243
1.4	0.78	$1.2x10^2$	2.079	4.464
1.8	0.95	7.4x10	1.869	4.674
2.2	1.2	3.3x10	1.518	5.025
2.6	1.4	ND	ND	6.544
3.0	1.5	ND	ND	6.544

ND= Not detected

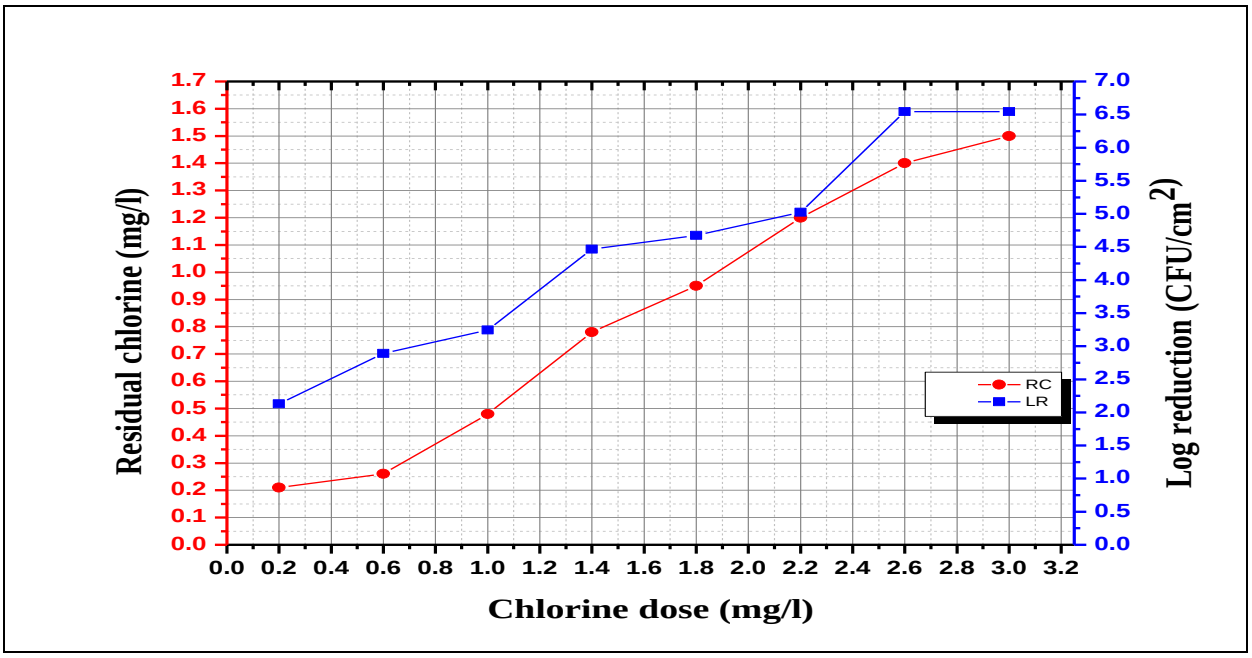

Fig. 40. The relationship between the chlorine doses, residual chlorine and log reduction of 10 days-old *L. monocytogenes* biofilm grown on PE.

Table 35. Determination of effective chlorine dose and its residual against 40 days-old *L. monocytogenes* biofilm grown on PE pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	Residual chlorine	Counts	Log counts	Log reduction
0.0	0.00	$2.0x10^6$	6.301	0
0.2	0.09	$4.3x10^4$	4.633	1.667
0.6	0.21	$1.9x10^4$	4.278	2.022
1.0	0.28	$5.6x10^3$	3.748	2.552
1.4	0.55	$3.4x10^3$	3.531	2.769
1.8	0.61	$2.9x10^2$	2.462	3.838
2.2	0.79	$1.4x10^2$	2.146	4.154
2.6	0.87	9.5x10	1.977	4.323
3.0	1.1	6.2x10	0.792	5.508

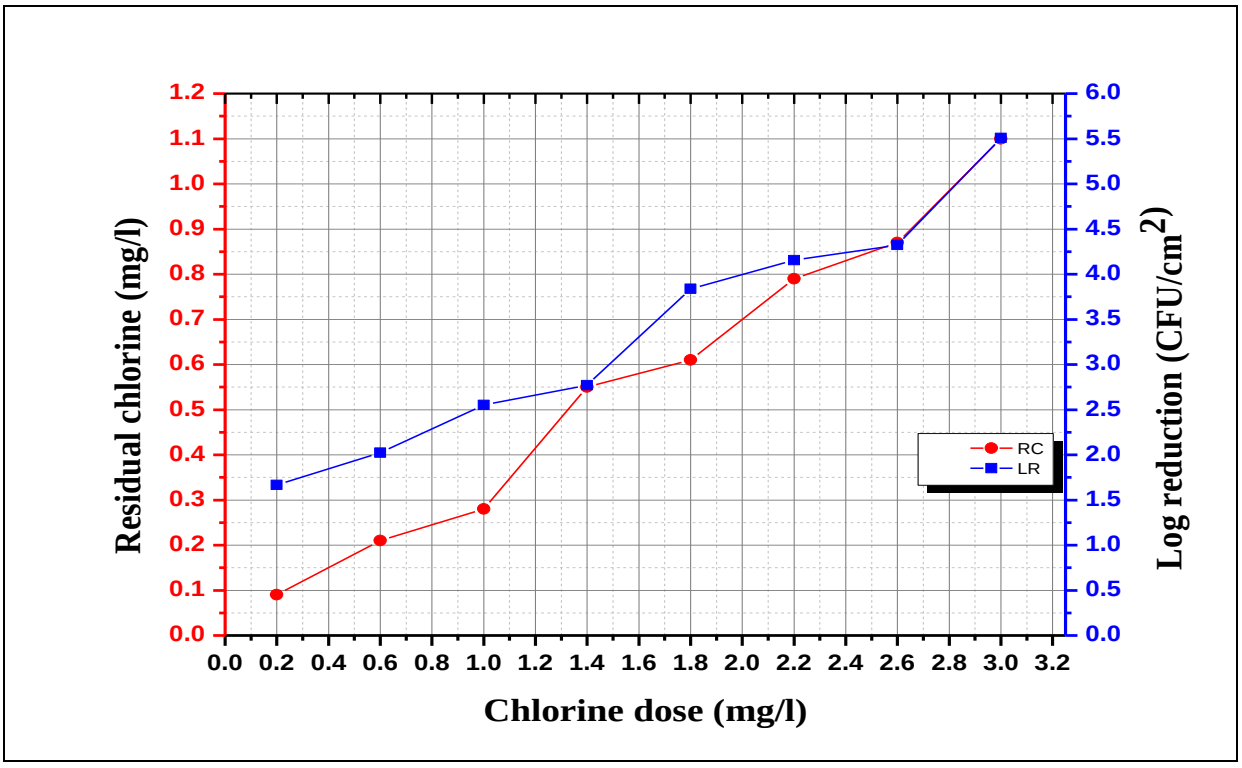

Fig. 41. The relationship between the chlorine doses, residual chlorine and log reduction of 40 days-old *L. monocytogenes* biofilm grown on PE.

Table 36. Determination of effective chlorine dose and its residual against 90 days-old *L. monocytogenes* biofilm grown on PE pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$6.2x10^6$	6.792	0
0.2	0.1	$4.2x10^5$	5.623	1.169
0.6	0.18	$1.8x10^5$	5.255	1.537
1.0	0.24	$5.2x10^4$	4.716	2.076
1.4	0.27	$2.3x10^4$	4.361	2.430
1.8	0.38	$4.1x10^3$	3.612	3.179
2.2	0.53	$1.6x10^3$	3.204	3.588
2.6	0.59	$7.3x10^2$	2.863	3.929
3.0	0.71	$3.8x10^2$	2.579	4.212

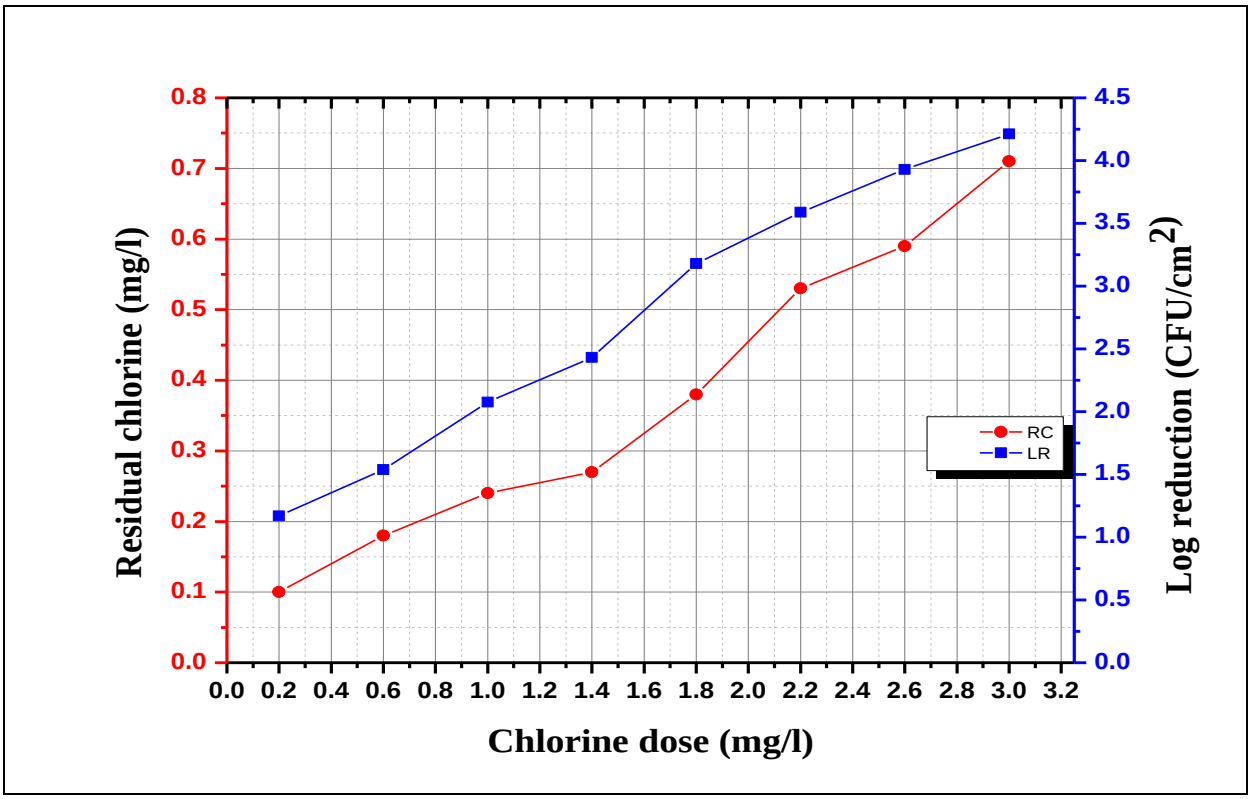

Fig. 42. The relationship between the chlorine doses, residual chlorine and log reduction of 90 days-old *L. monocytogenes* biofilm grown on PE.

5. Chlorine demand for inactivation of *L. monocytogenes* biofilm grown on I pipe material

The results in Tables (37, 38, 39) and Figs. (43, 44, 45), it is explained that, the biofilm formation of *L. monocytogenes* on the iron pipes revealed more resistant to different chlorine doses in all ages. Whereas, the initial log counts of the obtained ages were 6.5, 6.7 and 6.8 CFU/cm^2 at 10, 40 and 90 days-old, respectively. On the other hand, the high level from chlorine dose (3 mg/l) was not able to complete removal of biofilm cells not only for 90 days-old biofilm (older biofilm), but also for 10 days-old biofilm (younger biofilm). Besides, the breakpoints of chlorine dose at 10, 40 and 90 days-old of biofilm cells were 0.6, 1.8 and 2.2 mg/l, respectively.

Consequently, the statistical analysis results indicated the coefficient of determination (R^2) between the chlorine dose and the residual chlorine of 10, 40 and 90 days-old of biofilm cells was 0.993, 0.982 and 0.958, respectively. While, the R^2

between the residual chlorine and log reduction of bacterial counts of 10, 40 and 90 days-old of biofilm cells was 0.888, 0.939 and 0.953, respectively.

Table 37. Determination of effective chlorine dose and its residual against 10 days-old *L. monocytogenes* biofilm grown on I pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$3.4x10^6$	6.531	0
0.2	0.1	$1.6x10^4$	4.204	2.327
0.6	0.19	$3.7x10^3$	3.568	2.963
1.0	0.42	$1.9x10^3$	3.278	3.252
1.4	0.58	$2.5x10^2$	2.397	4.133
1.8	0.72	$1.4x10^2$	2.146	4.385
2.2	0.93	$1.1x10^2$	2.041	4.490
2.6	1.0	9.0x10	1.954	4.577
3.0	1.2	7.5x10	1.875	4.656

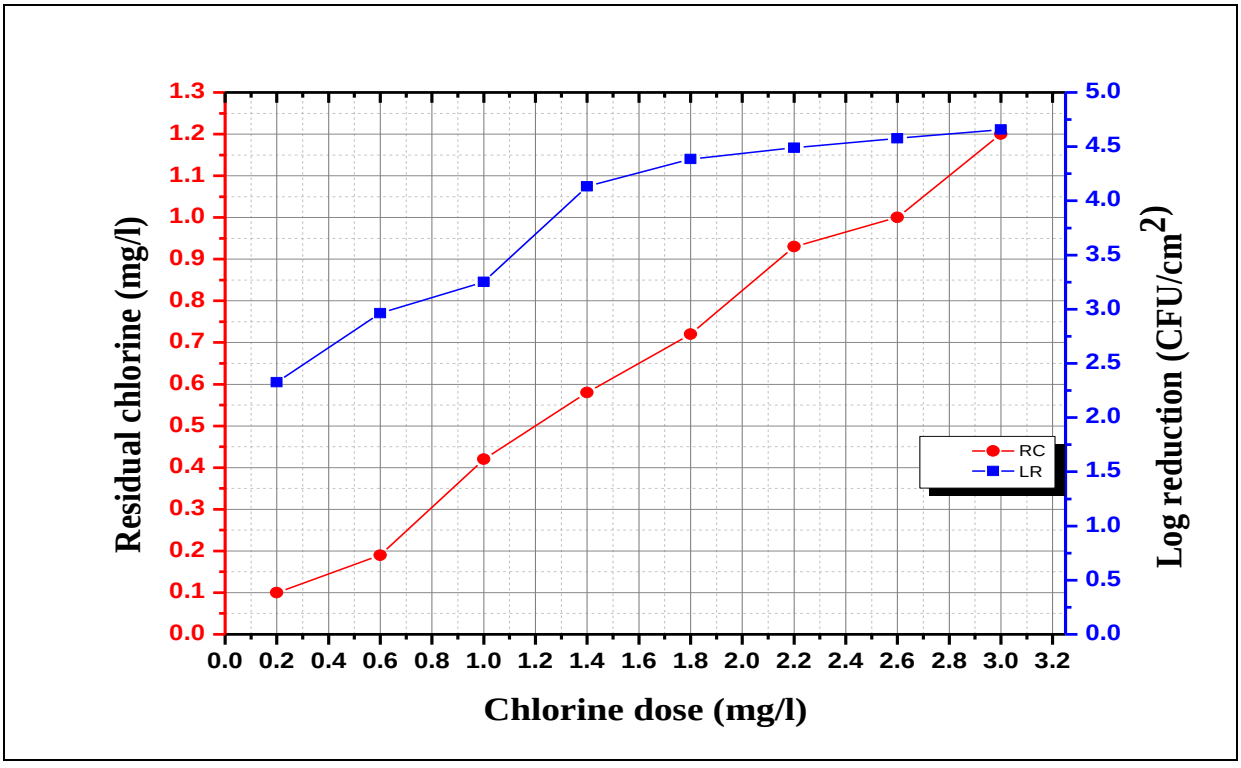

Fig. 43. The relationship between the chlorine doses, residual chlorine and log reduction of 10 days-old *L. monocytogenes* biofilm grown on I pipe material.

Table 38. Determination of effective chlorine dose and its residual against 40 days-old *L. monocytogenes* biofilm grown on I pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$5.4x10^6$	6.732	0
0.2	0.1	$3.7x10^4$	4.568	2.164
0.6	0.25	$1.4x10^4$	4.146	2.586
1.0	0.45	$4.5x10^3$	3.653	3.079
1.4	0.66	$1.2x10^3$	3.079	3.653
1.8	0.72	$5.2x10^2$	2.716	4.016
2.2	1.1	$3.2x10^2$	2.505	4.227
2.6	1.15	$2.4x10^2$	2.380	4.352
3.0	1.3	$1.6x10^2$	2.204	4.528

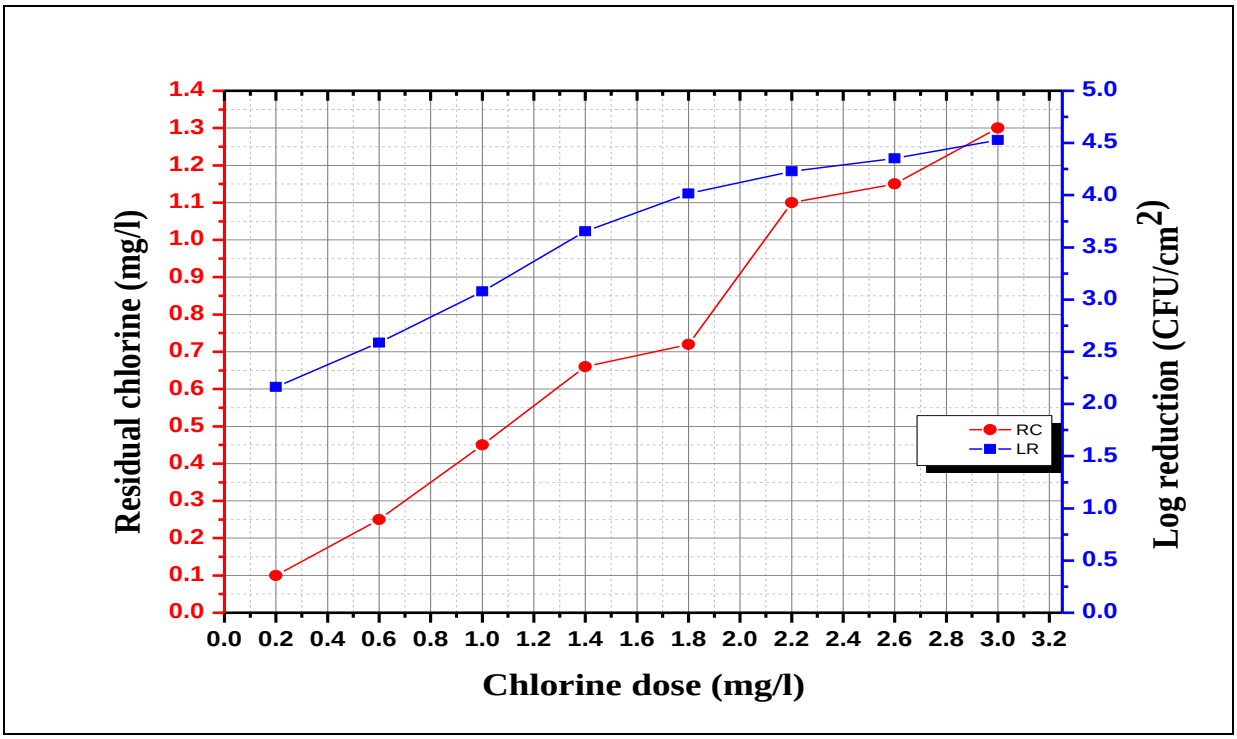

Fig. 44. The relationship between the chlorine doses, residual chlorine and log reduction of 40 days-old *L. monocytogenes* biofilm grown on I pipe material.

Table 39. Determination of effective chlorine dose and its residual against 90 days-old *L. monocytogenes* biofilm grown on I pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$6.8x10^6$	6.832	0
0.2	0.08	$1.4x10^5$	5.146	1.686
0.6	0.19	$5.6x10^4$	4.748	2.084
1.0	0.29	$3.1x10^4$	4.491	2.341
1.4	0.43	$1.1x10^4$	4.041	2.791
1.8	0.65	$6.2x10^3$	3.792	3.040
2.2	0.58	$3.9x10^3$	3.591	3.241
2.6	0.75	$7.1x10^2$	2.851	3.981
3.0	1	$4.5x10^2$	2.653	4.179

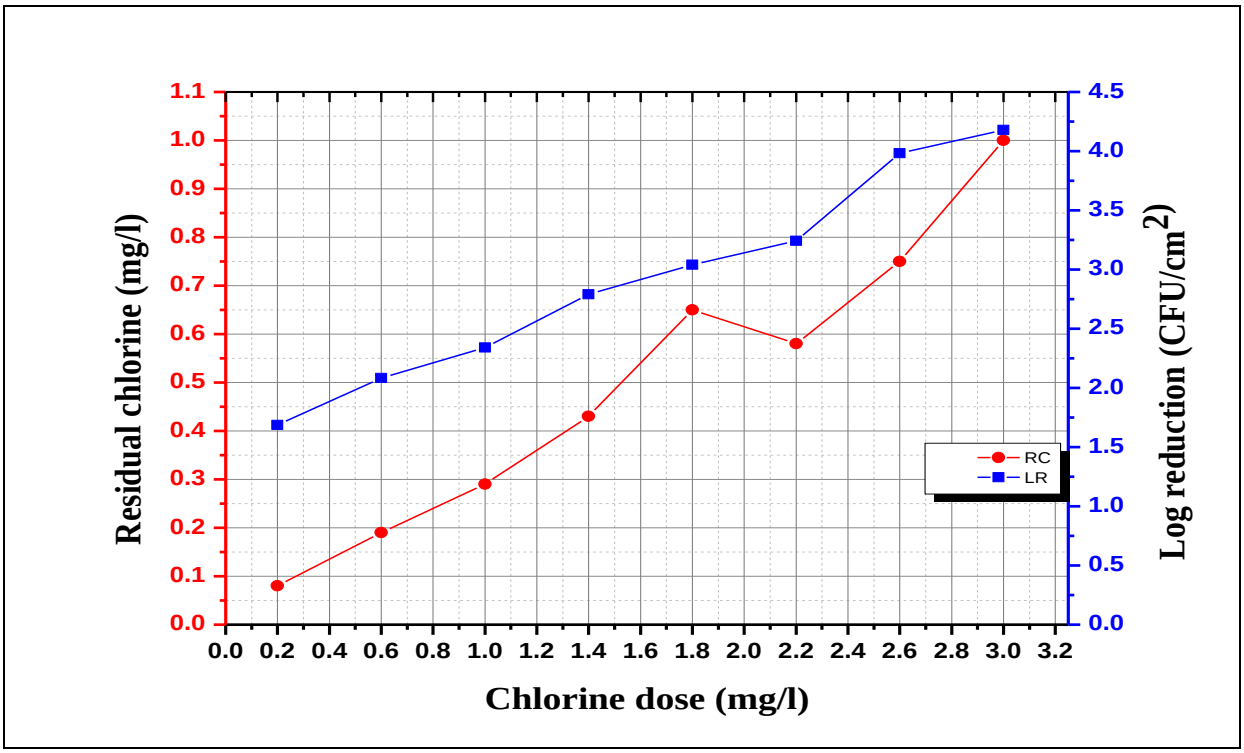

Fig. 45. The relationship between the chlorine doses, residual chlorine and log reduction of 90 days-old *L. monocytogenes* biofilm grown on I pipe material.

6. Chlorine demand for inactivation of *L. monocytogenes* biofilm grown on Cu pipe material

In case of biofilm formation by *L. monocytogenes* on Cu pipe materials, the results revealed that 90 days-old biofilm cells were completely removed (6.3 log reduction) at 2.2 mg/l of chlorine doses. While, 3.0 mg/l of chlorine dose was able to reduce initial log counts (6.5 CFU/cm^2) of 40 days-old of biofilm. Also, 3.0 mg/l of chlorine dose was able to reduce 4.3 log count of 10 days-old biofilm cells. As well, the breakpoints of chlorine dose for 10, 40 and 90 days-old of biofilm cells were 0.6, 0.6 and 1.4 mg/l, respectively (Tables 40, 41, 42; Figs. 46, 47, 48).

As a result of the statistical analysis reported the coefficient of determination (R^2) between the chlorine dose and the residual chlorine of 10, 40 and 90 days-old of biofilm cells was 0.948, 0.986 and 0.979, respectively. While, the R^2 between the residual chlorine and log reduction of bacterial counts of 10, 40 and 90 days-old of biofilm cells were 0.940, 0.683 and 0.885, respectively.

Table 40. Determination of effective chlorine dose and its residual against 10 days-old *L. monocytogenes* biofilm grown on Cu pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$1.5x10^6$	6.176	0
0.2	0.08	$4.6x10^4$	4.662	1.513
0.6	0.13	$7.3x10^3$	3.863	2.312
1.0	0.34	$5.8x10^3$	3.763	2.412
1.4	0.48	$2.4x10^3$	3.380	2.795
1.8	0.54	$6.3x10^2$	2.799	3.376
2.2	0.73	$4.2x10^2$	2.623	3.552
2.6	1.0	$1.1x10^2$	2.041	4.134
3.0	1.1	7.1x10	1.851	4.324

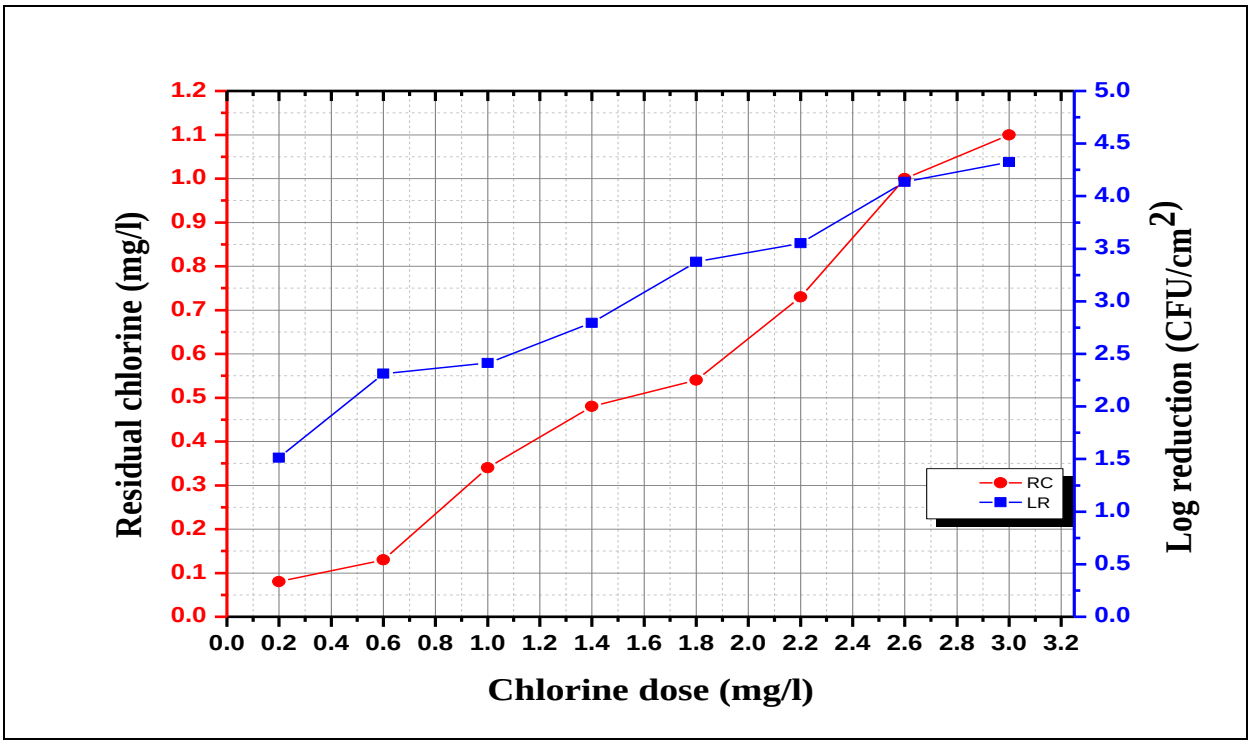

Fig. 46. The relationship between the chlorine doses, residual chlorine and log reduction of 10 days-old *L. monocytogenes* biofilm grown on Cu pipe material.

Table 41. Determination of effective chlorine dose and its residual against 40 days-old *L. monocytogenes* biofilm grown on Cu pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$3.4x10^6$	6.531	0
0.2	0.16	$7.6x10^3$	3.880	2.650
0.6	0.22	$2.8x10^3$	3.447	3.084
1.0	0.36	$8.5x10^2$	2.707	3.823
1.4	0.45	$5.1x10^2$	2.380	4.151
1.8	0.57	$3.4x10^2$	2.113	4.417
2.2	0.70	$2.4x10^2$	2.929	3.602
2.6	0.75	$1.3x10^2$	2.531	4.000
3.0	0.97	ND	ND	6.531

ND= Not detected

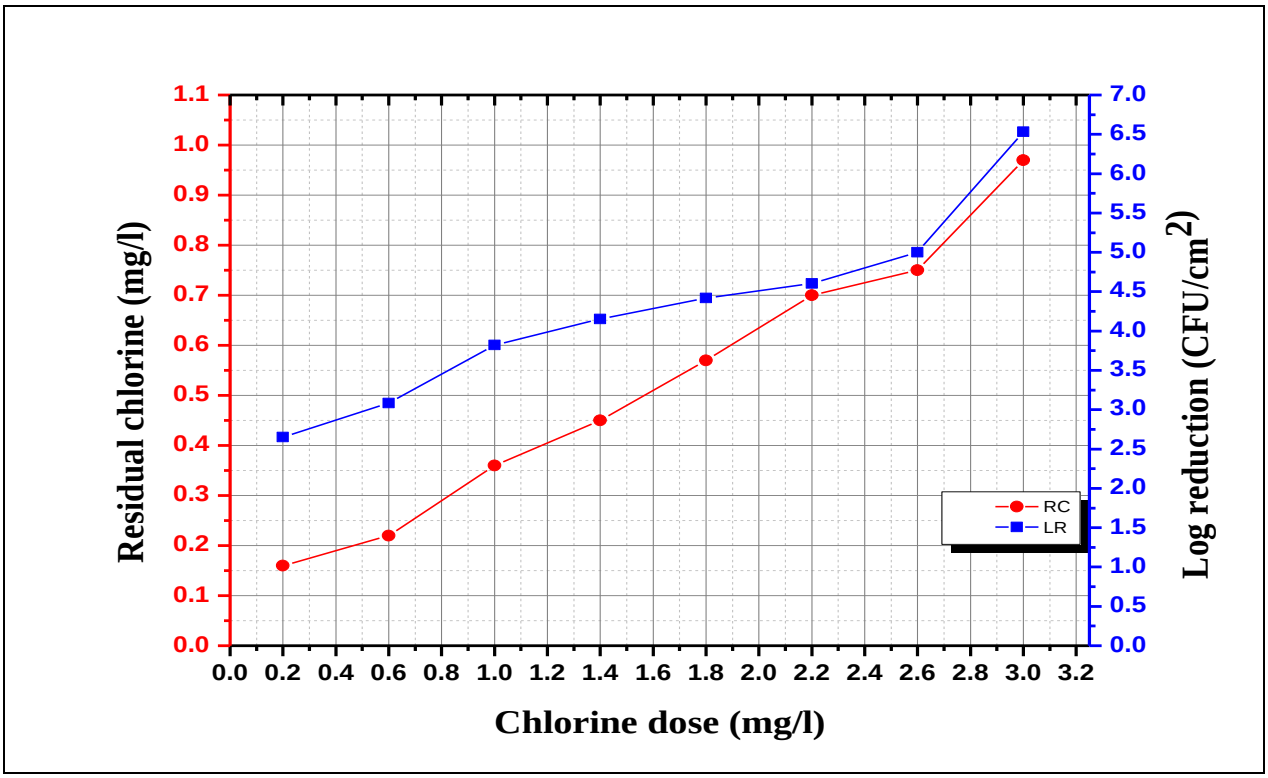

Fig. 47. The relationship between the chlorine doses, residual chlorine and log reduction of 40 days-old *L. monocytogenes* biofilm grown on Cu pipe material.

Table 42. Determination of effective chlorine dose and its residual against 90 days-old *L. monocytogenes* biofilm grown on Cu pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$2.4x10^6$	6.380	0
0.2	0.09	$2.1x10^3$	3.322	3.057
0.6	0.17	$4.2x10^2$	2.623	3.756
1.0	0.21	$1.6x10^2$	2.204	4.176
1.4	0.25	9.5x10	1.973	4.407
1.8	0.45	4.3x10	1.633	4.746
2.2	0.60	ND	ND	6.380
2.6	0.66	ND	ND	6.380
3.0	0.90	ND	ND	6.380

ND= Not detected

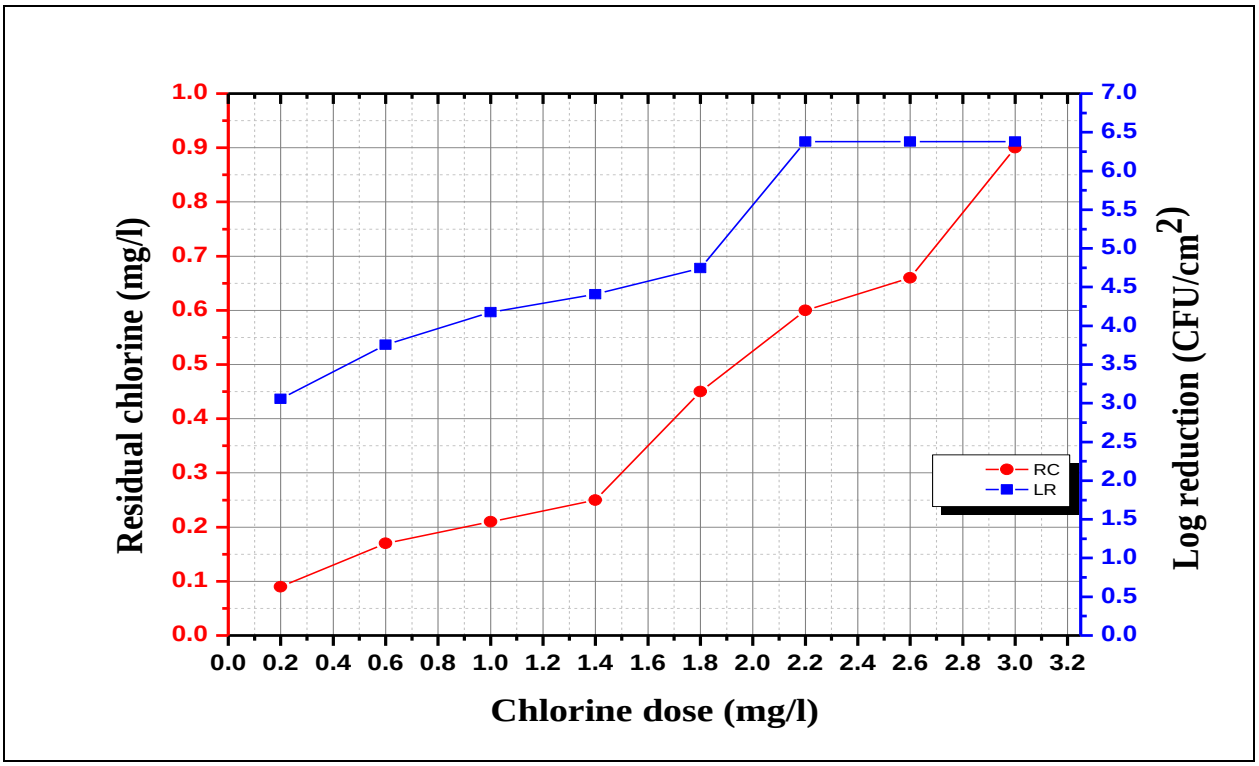

Fig. 48. The relationship between the chlorine doses, residual chlorine and log reduction of 90 days-old *L. monocytogenes* biofilm grown on Cu pipe material.

7. Chlorine demand for inactivation of *L. monocytogenes* biofilm grown on R pipe material

Regarding, the biofilm cells of *L. monocytogenes* grown on the inner surface of R materials, the initial log counts of three different ages were 6.4, 6.6 and 6.6 CFU/cm^2, respectively. The results of biofilm cells, which scraped after 10 days-old, were completely reduced, when exposed to 3 mg/l of chlorine dose. On the other hand, the log reduction after 90 and 40days-old of biofilm cells were respectively: 4.0, 4.6 CFU/cm^2. Additionally, the breakpoints of chlorine dose for 10, 40 and 90 days-old of biofilm cells were respectively: 0.6, 1.0 and 1.4 mg/l (Tables 43, 44, 45; Figs. 49, 50, 51).

As a result of the statistical analysis reported, the coefficient of determination (R^2) between the chlorine dose and the residual chlorine of 10, 40 and 90 days-old of biofilm cells was 0.986, 0.986 and 0.970, respectively. While, the R^2 between the residual chlorine and log reduction of bacterial counts of 10, 40 and 90 days-old of biofilm cells were 0.850, 0.970 and 0.978, respectively.

Table 43. Determination of effective chlorine dose and its residual against 10 days-old *L. monocytogenes* biofilm grown on R pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$3.1x10^6$	6.491	0
0.2	0.1	$4.5x10^4$	4.653	1.838
0.6	0.18	$3.5x10^3$	3.544	2.947
1.0	0.41	$1.2x10^3$	3.079	3.412
1.4	0.58	$4.2x10^2$	2.623	3.868
1.8	0.69	$2.7x10^2$	2.431	4.059
2.2	0.92	$1.2x10^2$	2.079	4.412
2.6	1.0	7.5x10	1.875	4.616
3.0	1.1	ND	ND	6.491

ND= Not detected

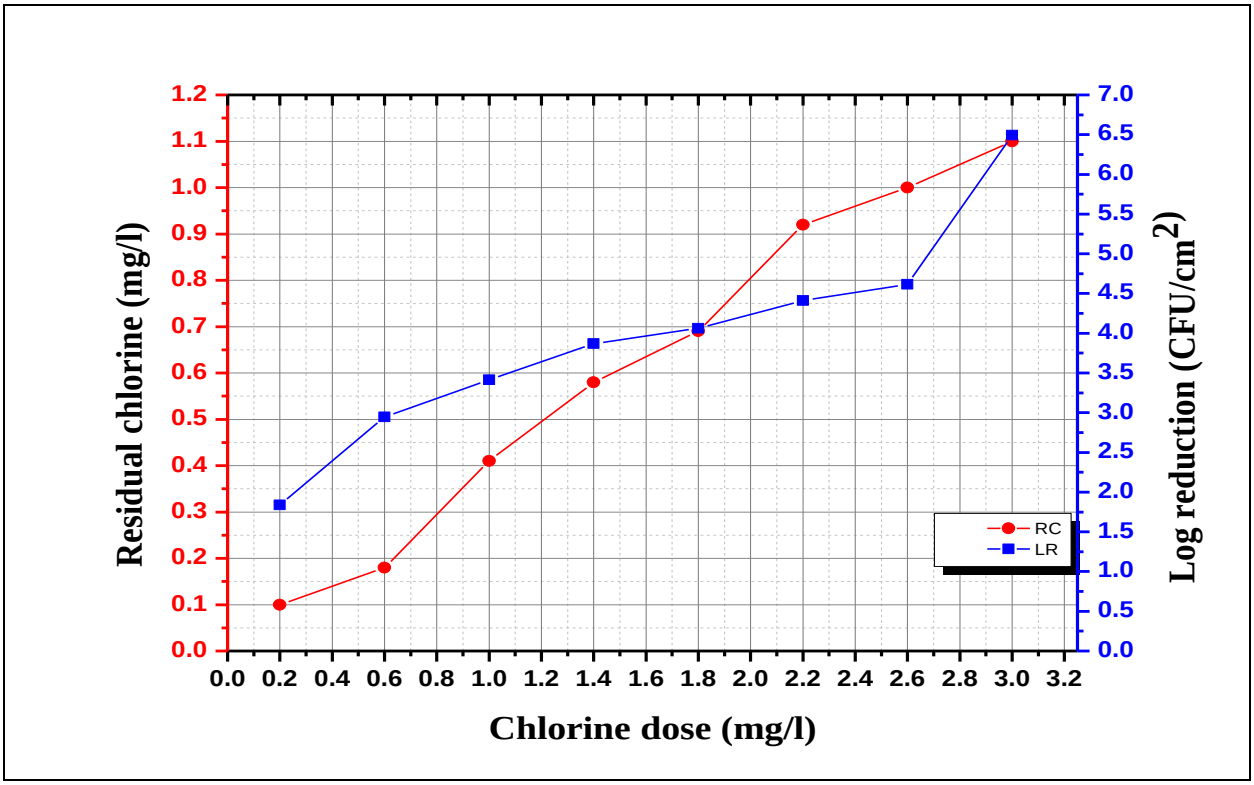

Fig. 49. The relationship between the chlorine doses, residual chlorine and log reduction of 10 days-old *L. monocytogenes* biofilm grown on R pipe material.

Table 44. Determination of effective chlorine dose and its residual against 40 days-old *L. monocytogenes* biofilm grown on R pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$4.8x10^6$	6.681	0
0.2	0.09	$2.4x10^5$	5.380	1.301
0.6	0.26	$4.5x10^4$	4.653	2.028
1.0	0.34	$1.3x10^4$	4.113	2.567
1.4	0.57	$3.2x10^3$	3.505	3.176
1.8	0.68	$1.7x10^3$	3.230	3.450
2.2	0.75	$4.9x10^2$	2.690	3.991
2.6	0.88	$2.5x10^2$	2.397	4.283
3.0	1.1	$1.2x10^2$	2.079	4.602

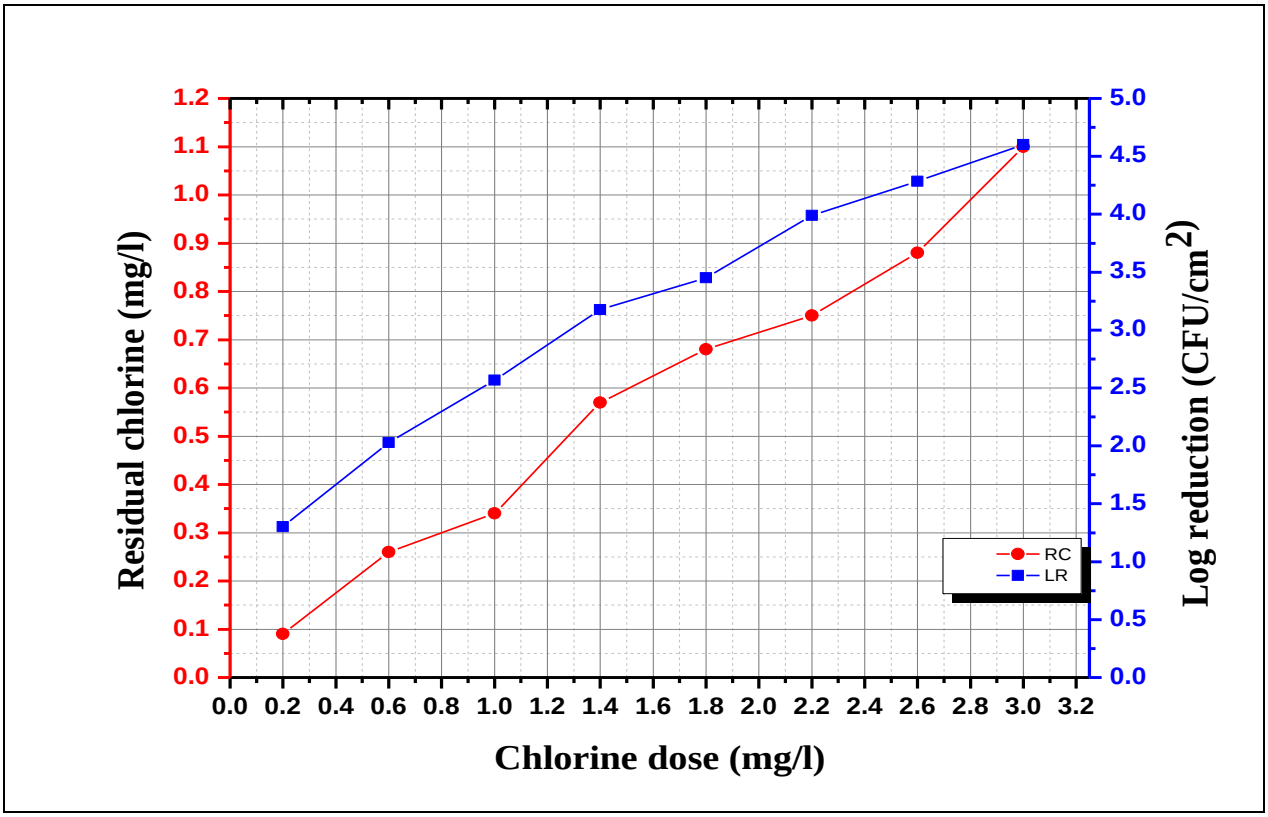

Fig. 50. The relationship between the chlorine doses, residual chlorine and log reduction of 40 days-old *L. monocytogenes* biofilm grown on R pipe material.

Table 45. Determination of effective chlorine dose and its residual against 90 days-old *L. monocytogenes* biofilm grown on R pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	Residual chlorine	Counts	Log counts	Log reduction
0.0	0.00	$4.6x10^6$	6.662	0
0.2	0.08	$1.4x10^5$	5.146	1.516
0.6	0.18	$7.6x10^4$	4.880	1.781
1.0	0.25	$4.3x10^4$	4.633	2.029
1.4	0.29	$2.6x10^4$	4.414	2.247
1.8	0.45	$9.6x10^3$	3.982	2.680
2.2	0.49	$5.3x10^3$	3.724	3.938
2.6	0.67	$4.9x10^2$	2.690	3.972
3.0	0.81	$3.7x10^2$	2.568	4.094

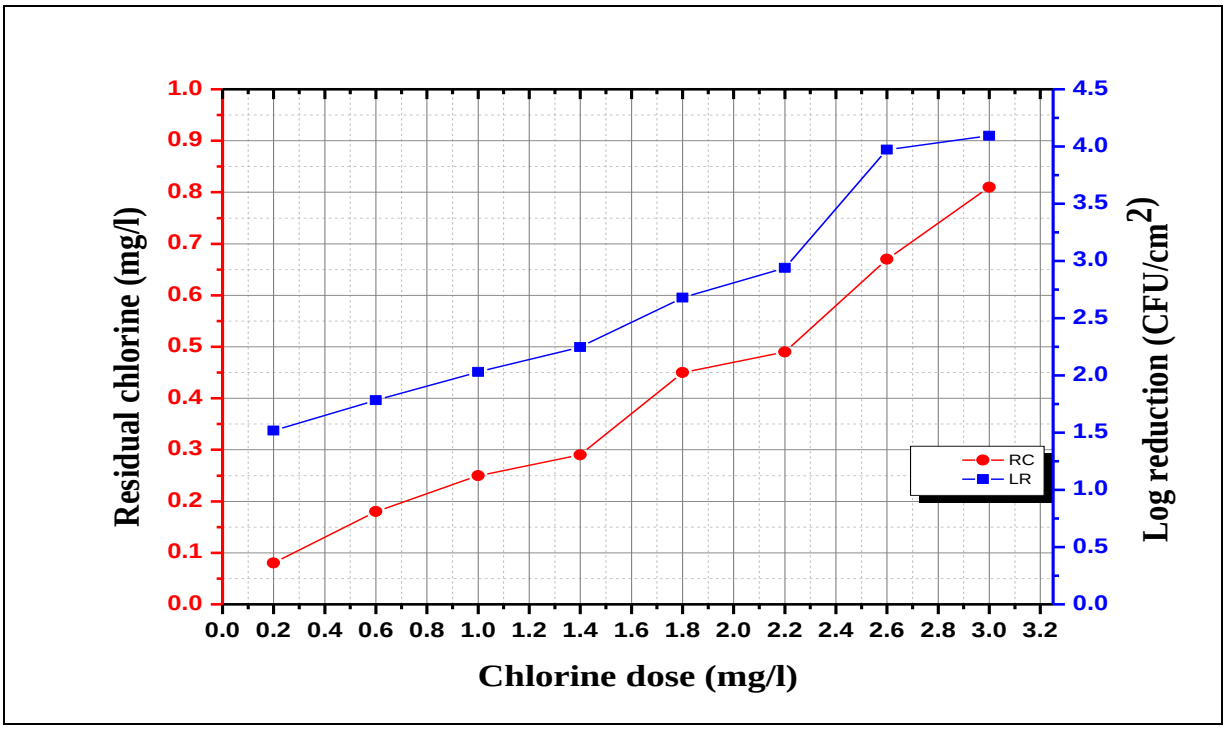

Fig. 51. The relationship between the chlorine doses, residual chlorine and log reduction of 90 days-old *L. monocytogenes* biofilm grown on R pipe material.

b. Comparative susceptibility of planktonic cells and three different ages of *S.* Typhimurium biofilm to chlorine

The effect of chlorine doses against the planktonic and biofilm cell at three different ages (10, 40 and 90 days-old) of *S.* Typhimurium was examined. They were exposed to eight different chlorine doses. Also, the residual or free chlorine was determined using DPD methods.

1. Chlorine demand for inactivation of *S.* Typhimurium planktonic cells

In this study, *S.* Typhimurium ATCC 14028 was used as a reference strain (planktonic cells). The initial counts of planktonic cells of *S.* Typhimurium ($2.5x10^6$ CFU/ml) was exposed to eight chlorine doses (0.2, 0.6, 1.0, 1.4, 1.8, 2.2, 2.6 and 3.0 mg/l). The results showed that, the most effective chlorine dose was 0.6 mg/L, which led to complete inactivation (100% removal). Additionally, the breakpoint recorded at 0.6 mg/l chlorine **(**Table 46; Fig. 52).

The statistical analysis showed that, the coefficient of determination (R^2) between the chlorine dose and the residual chlorine was 0.988. Also, the R^2 between the residual chlorine and log reduction of bacterial counts was 0.351.

Table 46. Determination of effective chlorine dose and its residual against *S.* Typhimurium planktonic cells.

Chlorine (mg/l)		Bacterial counts (CFU/ml)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$2.5x10^6$	6.397	0
0.2	0.15	$1.4x10^2$	2.146	4.251
0.6	0.26	ND	ND	6.397
1.0	0.52	ND	ND	6.397
1.4	0.76	ND	ND	6.397
1.8	0.91	ND	ND	6.397
2.2	1.15	ND	ND	6.397
2.6	1.23	ND	ND	6.397
3.0	1.35	ND	ND	6.397

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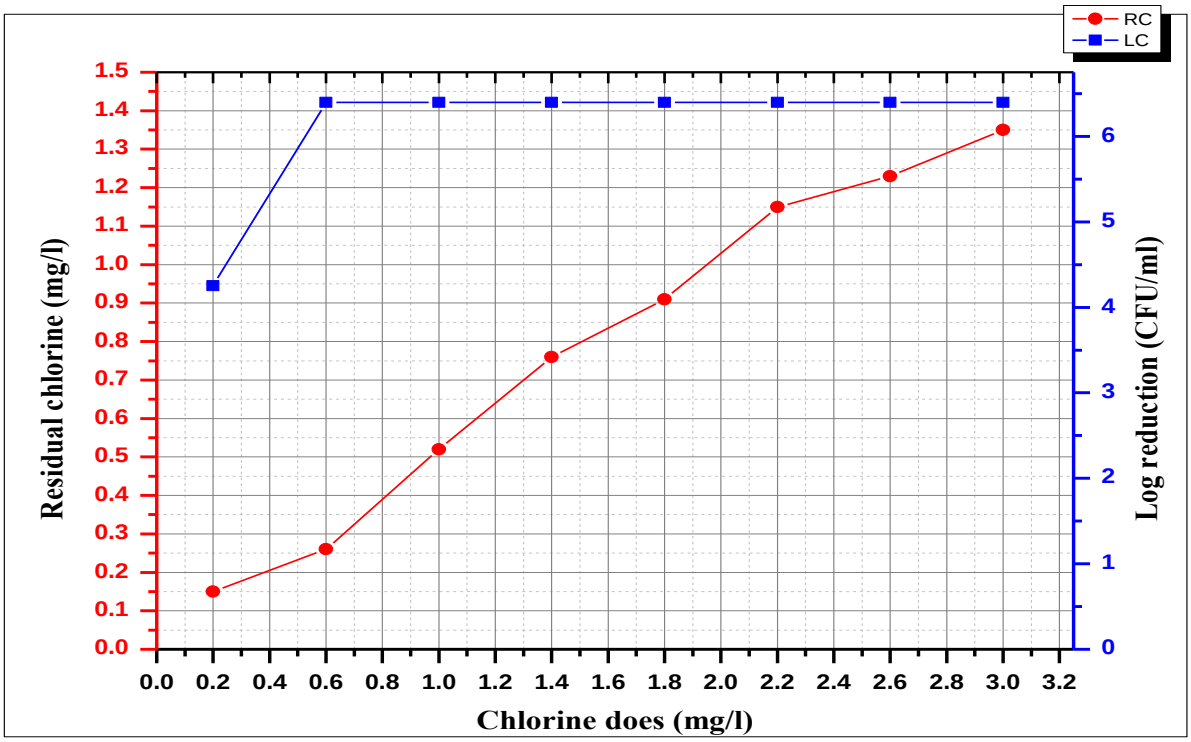

Fig. 52. The relationship between the chlorine doses, residual chlorine and log reduction of *S.* Typhimurium planktonic cells.

2. Chlorine demand for inactivation of *S.* Typhimurium biofilm grown on PVC pipe material

The six samples of *S.* Typhimurium biofilm cells, were scraped from six different pipeline materials (PVC, PP, PE, I, Cu and R) in three different ages (10, 40, and 90 days-old) and were exposed to the same eight chlorine doses.

As shown in Tables (47, 48, 49) and Figs. (53, 54, 55) *S.* Typhimurium biofilm cells, which collected from PVC pipeline in different ages (10, 40, and 90 days-old), cleared that, the initial log counts of 10 days-old biofilm cells were 6.4 CFU/cm^2. In this age, 1.0 mg/l of chlorine dose was able to complete removal of cells While, the initial logs counts of 40 and 90 days-old of biofilm cells were 6.7 and 6.2 CFU/cm^2, respectively. The complete log reduction was recorded at 1.4 and 1.8 mg/l. Moreover, the breakpoints of chlorine dose for 10, 40 and 90 days-old of biofilm cells were 1.0, 1.4 and 1.4 mg/l, respectively.

Statistically, the coefficient of determination (R^2) between the chlorine dose and the residual chlorine of 10, 40 and 90 days-old of biofilm cells was 0.988, 0.960 and 0.960, respectively. While, the R^2 between the residual chlorine and log reduction of bacterial counts of 10, 40 and 90 days-old of biofilm cells was 0.484, 0.564 and 0.874, respectively.

Table 47. Determination of effective chlorine dose and its residual against 10 days-old *S.* Typhimurium biofilm grown on PVC pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$2.7x10^6$	6.431	0
0.2	0.11	$3.9x10^4$	4.591	1.840
0.6	0.26	$1.5x10^2$	2.176	4.255
1.0	0.29	ND	ND	6.431
1.4	0.60	ND	ND	6.431
1.8	0.70	ND	ND	6.431
2.2	0.81	ND	ND	6.431
2.6	1.0	ND	ND	6.431
3.0	1.17	ND	ND	6.431

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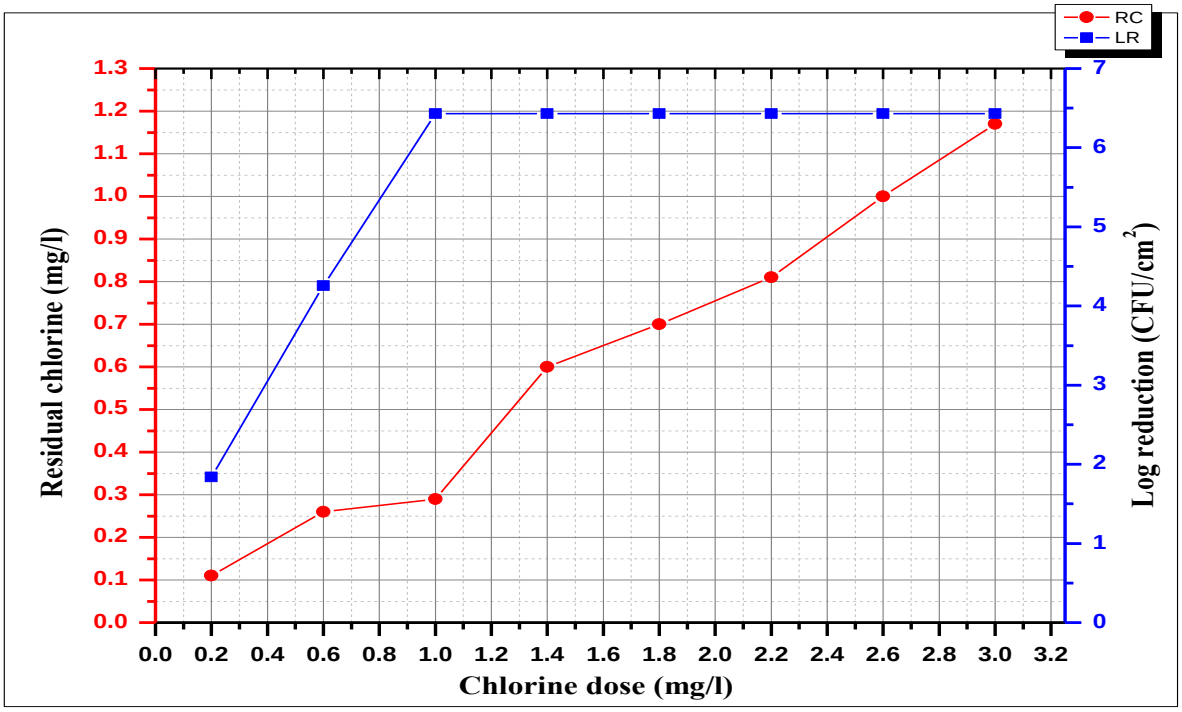

Fig. 53. The relationship between the chlorine doses, residual chlorine and log reduction of 10 days-old *S.* Typhimurium biofilm grown on PVC pipe material.

Table 48. Determination of effective chlorine dose and its residual against 40 days-old *S.* Typhimurium biofilm grown on PVC pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$5.3x10^6$	6.724	0
0.2	0.1	$6.2x10^4$	4.792	1.931
0.6	0.19	$4.9x10^3$	3.690	3.034
1.0	0.28	$1.1x10^2$	2.041	4.682
1.4	0.30	ND	ND	6.724
1.8	0.46	ND	ND	6.724
2.2	0.54	ND	ND	6.724
2.6	0.75	ND	ND	6.724
3.0	0.96	ND	ND	6.724

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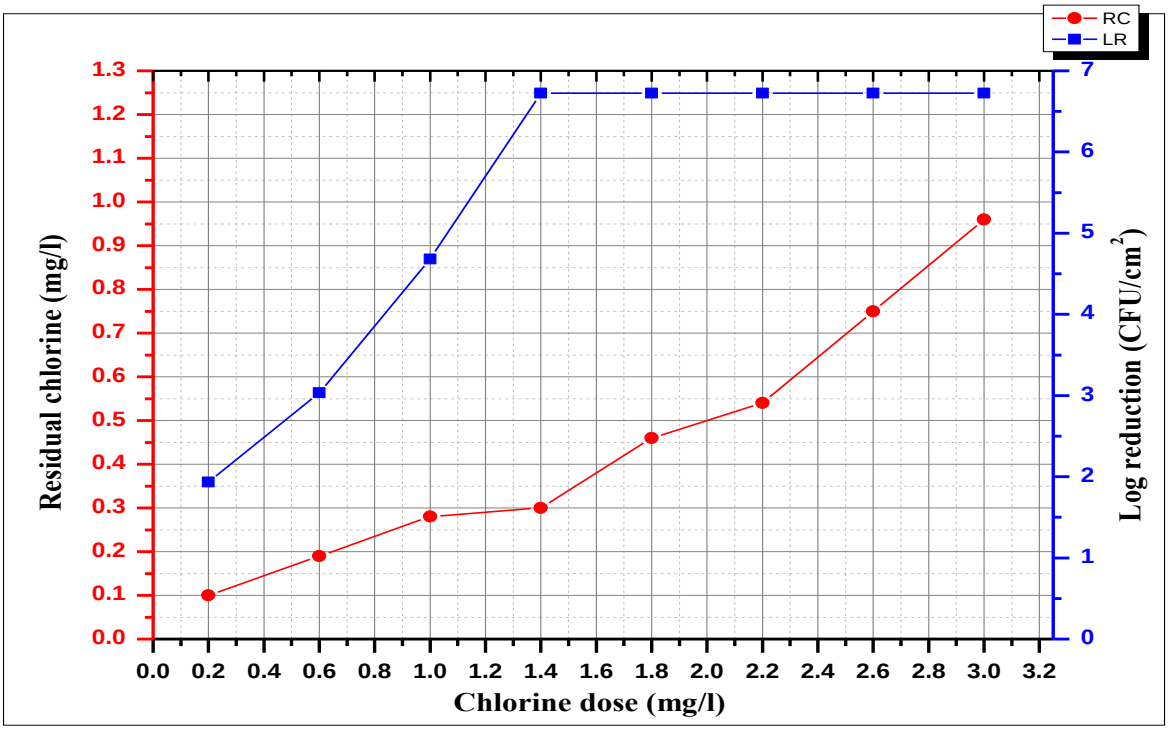

Fig. 54. The relationship between the chlorine doses, residual chlorine and log reduction of 40 days-old *S.* Typhimurium biofilm grown on PVC pipe material.

Table 49. Determination of effective chlorine dose and its residual against 90 days-old *S.* Typhimurium biofilm grown on PVC pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$1.8x10^6$	6.255	0
0.2	0.11	$7.3x10^4$	4.863	1.391
0.6	0.17	$1.4x10^4$	4.146	2.109
1.0	0.26	$4.6x10^3$	3.662	2.592
1.4	0.24	$2.1x10^2$	2.322	3.933
1.8	0.35	ND	ND	6.255
2.2	0.48	ND	ND	6.255
2.6	0.50	ND	ND	6.255
3.0	0.52	ND	ND	6.255

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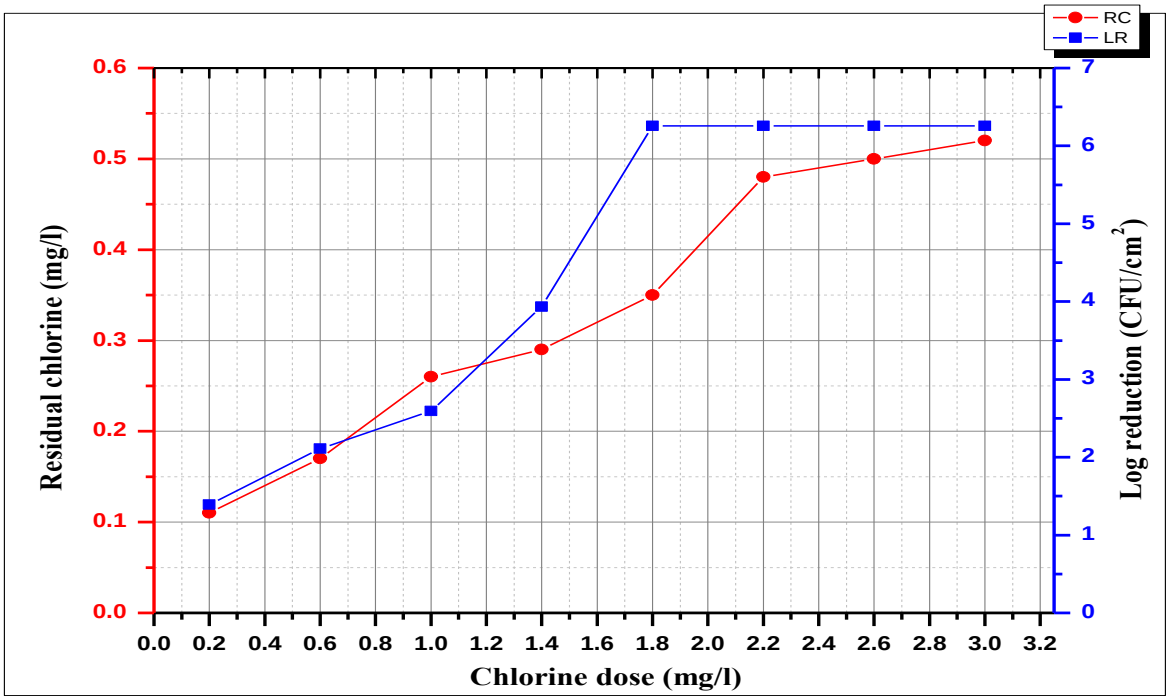

Fig. 55. The relationship between the chlorine doses, residual chlorine and the log reduction of 90 days-old *S.* Typhimurium biofilm grown on PVC pipe material.

3. Chlorine demand for inactivation of *S.* Typhimurium biofilm grown on PP pipe material

In case of biofilm formation of *S.* Typhimurium on PP pipe materials, the results revealed the 10 days-old biofilm cells were completely removed at 0.6 mg/l of chlorine doses. While, 40 and 90 days-old of biofilm cells were completely inactivated at 1.0 and 1.4 mg/l. While, the initial log counts were 6.4, 6.6 and 6.7 CFU/cm^2 in different ages, respectively. Also, the breakpoints of chlorine dose for 10, 40 and 90 days-old of biofilm cells were 0.6, 1.0 and 1.0 mg/l, respectively (Tables 50, 51, 52; Figs. 56, 57, 58).

The result of the statistical analysis reported the coefficient of determination (R^2) between the chlorine dose and the residual chlorine of 10, 40 and 90 days-old of biofilm cells was 0.991, 0.994 and 0.945, respectively. While, the R^2 between the residual chlorine and log reduction of bacterial counts of 10, 40 and 90 days-old of biofilm cells were 0.367, 0.495 and 0.518, respectively.

Table 50. Determination of effective Chlorine dose and its residual against 10 days-old *S.* Typhimurium biofilm grown on PP pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$3.1x10^6$	6.491	0
0.2	0.1	$2.5x10^2$	2.397	4.093
0.6	0.23	ND	ND	6.491
1.0	0.46	ND	ND	6.491
1.4	0.57	ND	ND	6.491
1.8	0.65	ND	ND	6.491
2.2	0.89	ND	ND	6.491
2.6	0.98	ND	ND	6.491
3.0	1.1	ND	ND	6.491

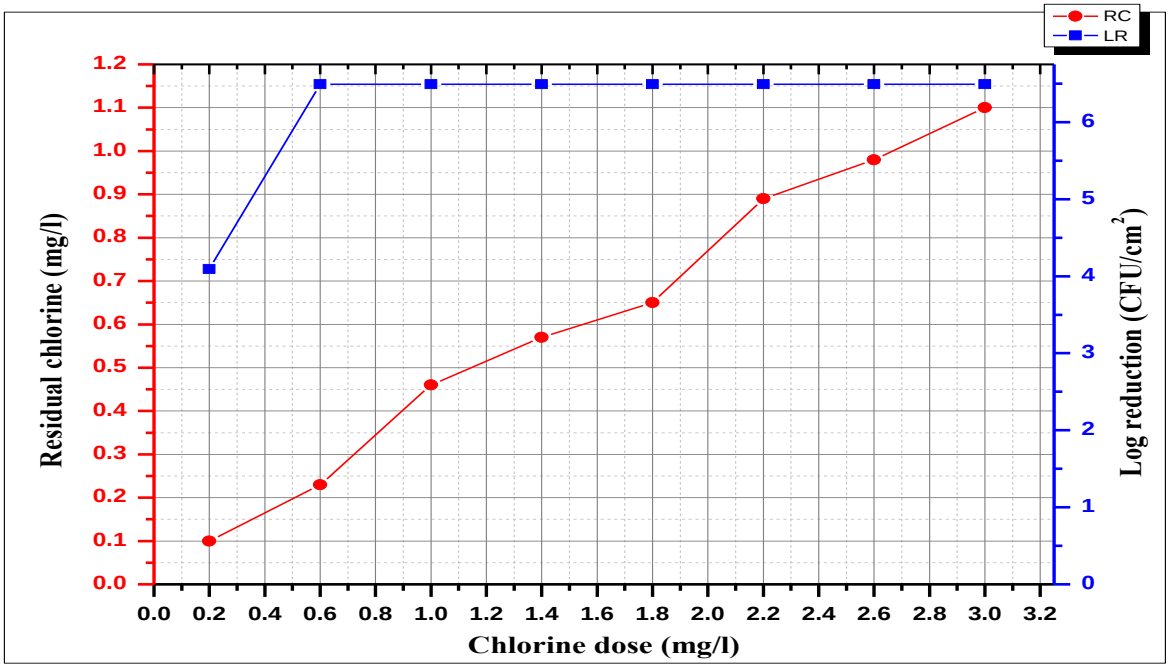

Fig. 56. The relationship between the chlorine doses, residual chlorine and the log reduction of 10 days-old *S.* Typhimurium biofilm grown on PP pipe material.

Table 51. Determination of effective chlorine dose and its residual against 40 days-old *S.* Typhimurium biofilm grown on PP pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$4.7x10^6$	6.672	0
0.2	0.1	$7.9x10^3$	3.897	2.774
0.6	0.23	$1.3x10^2$	2.113	4.558
1.0	0.29	ND	ND	6.672
1.4	0.42	ND	ND	6.672
1.8	0.54	ND	ND	6.672
2.2	0.64	ND	ND	6.672
2.6	0.80	ND	ND	6.672
3.0	0.94	ND	ND	6.672

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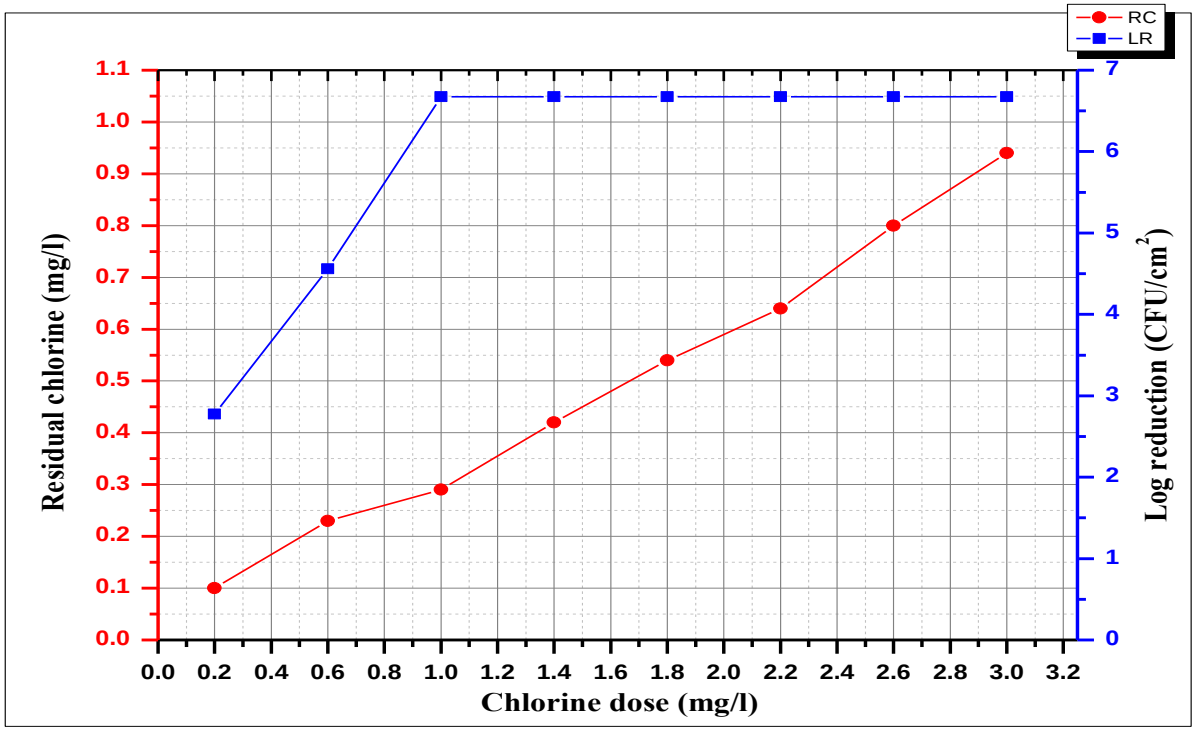

Fig. 57. The relationship between the chlorine doses, residual chlorine and the log reduction of 40 days-old *S.* Typhimurium biofilm grown on PP pipe material.

Table 52. Determination of effective chlorine dose and its residual against 90 days-old *S.* Typhimurium biofilm grown on PP pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$6.3x10^6$	6.799	0
0.2	0.09	$2.5x10^4$	4.397	2.401
0.6	0.12	$4.1x10^2$	2.612	4.186
1.0	0.14	8.4x10	1.924	4.875
1.4	0.24	ND	ND	6.799
1.8	0.27	ND	ND	6.799
2.2	0.29	ND	ND	6.799
2.6	0.44	ND	ND	6.799
3.0	0.54	ND	ND	6.799

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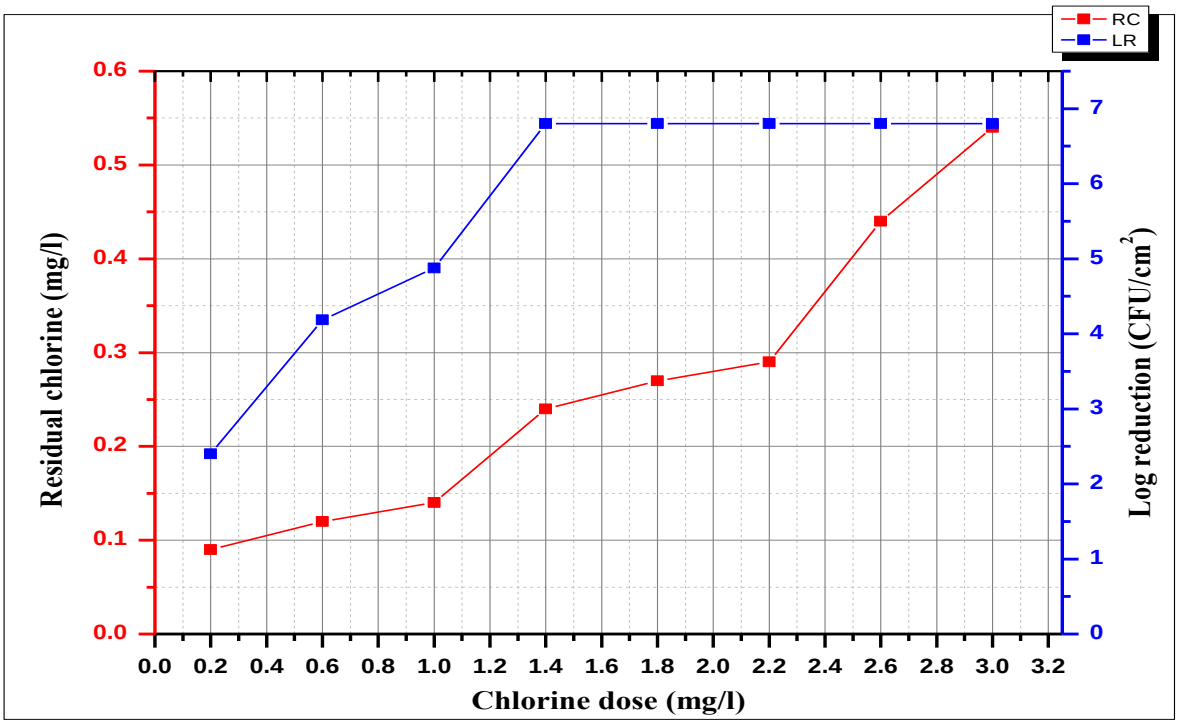

Fig. 58. The relationship between the chlorine doses, residual chlorine and the log reduction of 90 days-old *S.* Typhimurium biofilm grown on PP pipe material.

4. Chlorine demand for inactivation of S. Typhimurium biofilm grown on PE pipe material

Concerning the biofilm cells of *S.* Typhimurium growing on the PE pipe, the initial log counts of different ages were 6.6, 6.5 and 6.8 CFU/cm^2, respectively at 10, 40 and 90 days-old. The obtained results showed that, 1.0 mg/l of chlorine dose was able to complete reduction of the10 day-old biofilm cells. While, 40 and 90 days-old biofilm cells were completely removed at 1.4 and 1.8 mg/l. Furthermore, the breakpoints of chlorine dose for 10, 40 and 90 days-old of biofilm cells were 0.6, 1.0 and 1.0 mg/l, respectively (Tables 53, 54 55; Figs. 59, 60, 61).

From statistical analysis results, the coefficient of determination (R^2) between the chlorine dose and the residual chlorine of 10, 40 and 90 days-old of biofilm cells was 0.968, 0.969 and 0.973, respectively. While, the R^2 between the residual chlorine and log reduction of bacterial counts of 10, 40 and 90 days-old of biofilm cells were 0.595, 0.622 and 0.763, respectively.

5. Chlorine demand for inactivation of *S.* Typhimurium biofilm grown on I pipe material

In case of I pipe material, the initial counts of biofilm formation of 10 days-old *S.* Typhimurium was 6.7 CFU/cm^2. The results showed that, 1.4 mg/l of chlorine dose were able to completely remove the initial log counts. While, the initial counts (6.4 and 6.5 CFU/cm^2) of 40 and 90 days-old biofilm cells were completely remove using 1.8 and 2.2 mg/l chlorine dose.

Additionally, the breakpoints of chlorine dose for 10, 40 and 90 days-old of biofilm cells were 1.8, 2.2 and 1.8 mg/l, respectively (Tables 56, 57, 58; Figs. 62, 63, 64).

From statistical analysis results, the coefficient of determination (R^2) between the chlorine dose and the residual chlorine of 10, 40 and 90 days-old of biofilm cells was 0.988, 0.989 and 0.955, respectively. While, the R^2 between the residual chlorine and log reduction of bacterial counts of 10, 40 and 90 days-old of biofilm cells were 0.725, 0.813 and 0.826, respectively.

Table 53. Determination of effective chlorine dose and its residual against 10 days-old *S.* Typhimurium biofilm grown on PE pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	Residual chlorine	Counts	Log counts	Log reduction
0.0	0.00	$4.9x10^6$	6.690	0
0.2	0.11	$3.4x10^4$	4.531	2.158
0.6	0.20	$1.7x10^2$	2.230	4.459
1.0	0.42	ND	ND	6.690
1.4	0.69	ND	ND	6.690
1.8	0.98	ND	ND	6.690
2.2	1.10	ND	ND	6.690
2.6	1.16	ND	ND	6.690
3.0	1.23	ND	ND	6.690

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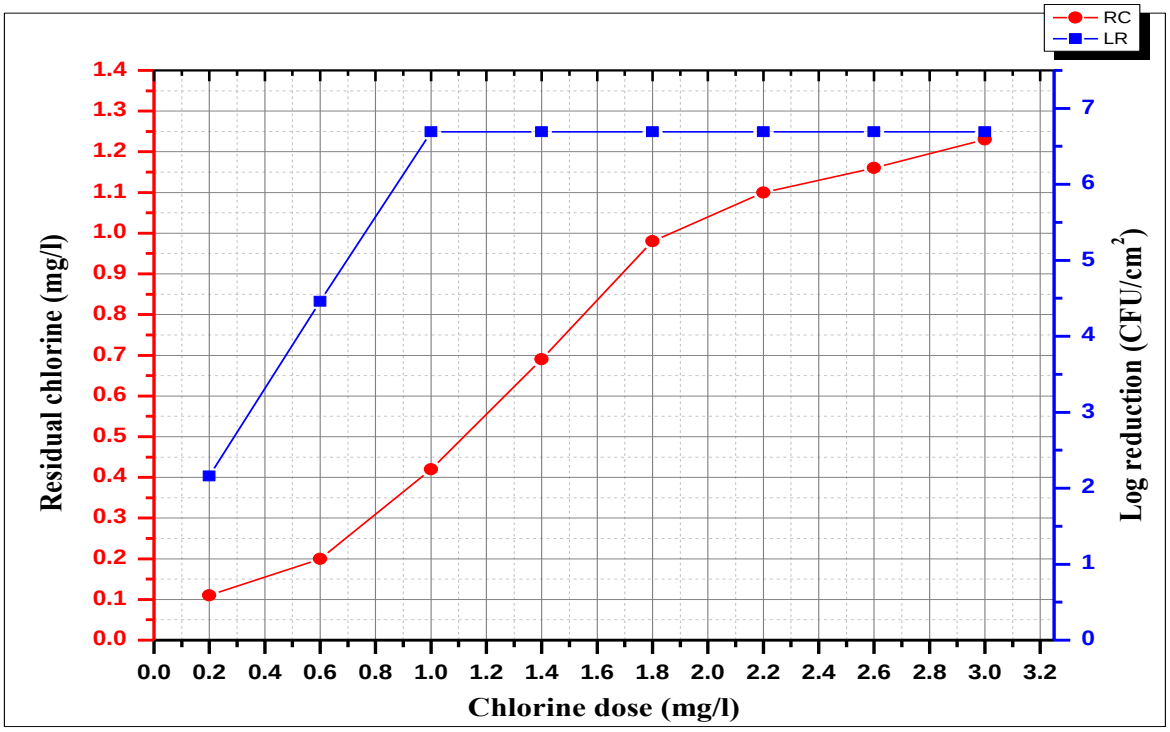

Fig. 59. The relationship between the chlorine doses, residual chlorine and the log reduction of 10 days-old *S.* Typhimurium biofilm grown on PE pipe material.

Table 54. Determination of effective chlorine dose and its residual against 40 days-old *S.* Typhimurium biofilm grown on PE pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$3.7x10^6$	6.568	0
0.2	0.1	$2.6x10^4$	4.414	2.153
0.6	0.16	$1.3x10^3$	3.113	3.454
1.0	0.17	9.7x10	1.986	4.581
1.4	0.29	ND	ND	6.568
1.8	0.34	ND	ND	6.568
2.2	0.44	ND	ND	6.568
2.6	0.49	ND	ND	6.568
3.0	0.67	ND	ND	6.568

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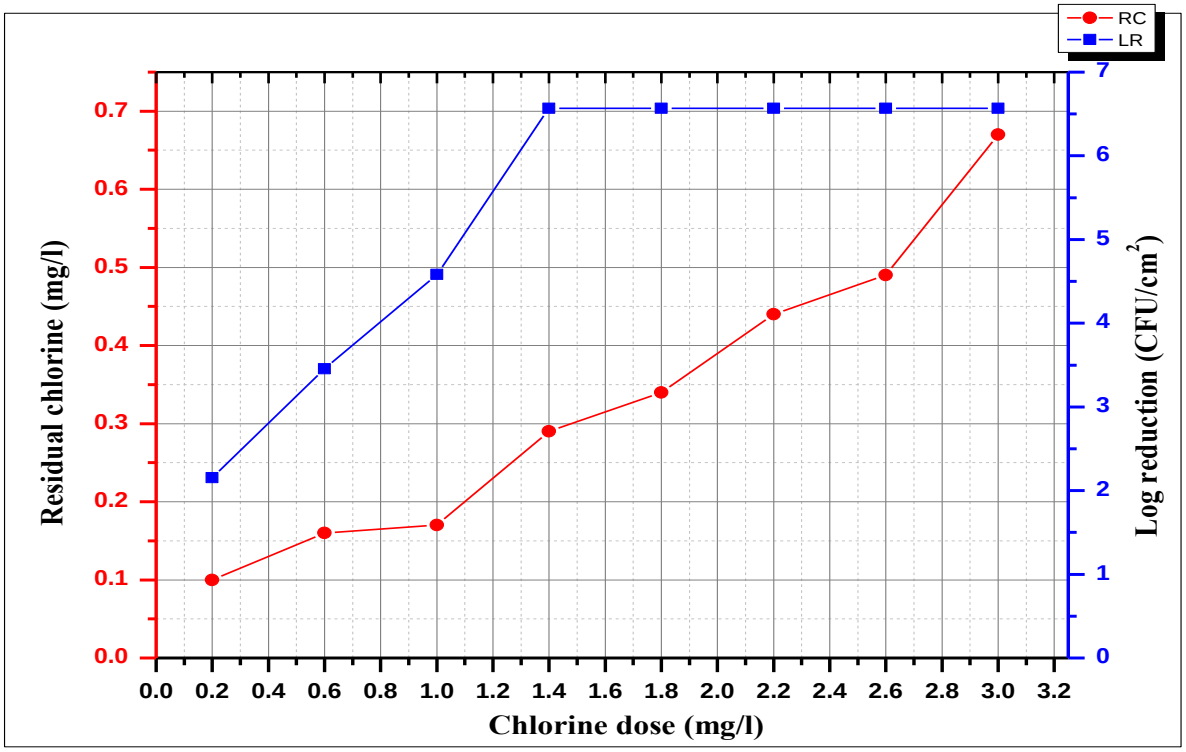

Fig. 60. The relationship between the chlorine doses, residual chlorine and the log reduction of 40 days-old *S.* Typhimurium biofilm grown on PE pipe material.

Table 55. Determination of effective chlorine dose and its residual against 90 days-old *S.* Typhimurium biofilm grown on PE pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$7.2x10^6$	6.857	0
0.2	0.1	$5.3x10^4$	4.724	2.133
0.6	0.13	$6.7x10^3$	3.826	3.031
1.0	0.15	$2.0x10^2$	2.301	4.556
1.4	0.26	7.9x10	1.897	4.959
1.8	0.28	ND	ND	6.857
2.2	0.42	ND	ND	6.857
2.6	0.45	ND	ND	6.857
3.0	0.56	ND	ND	6.857

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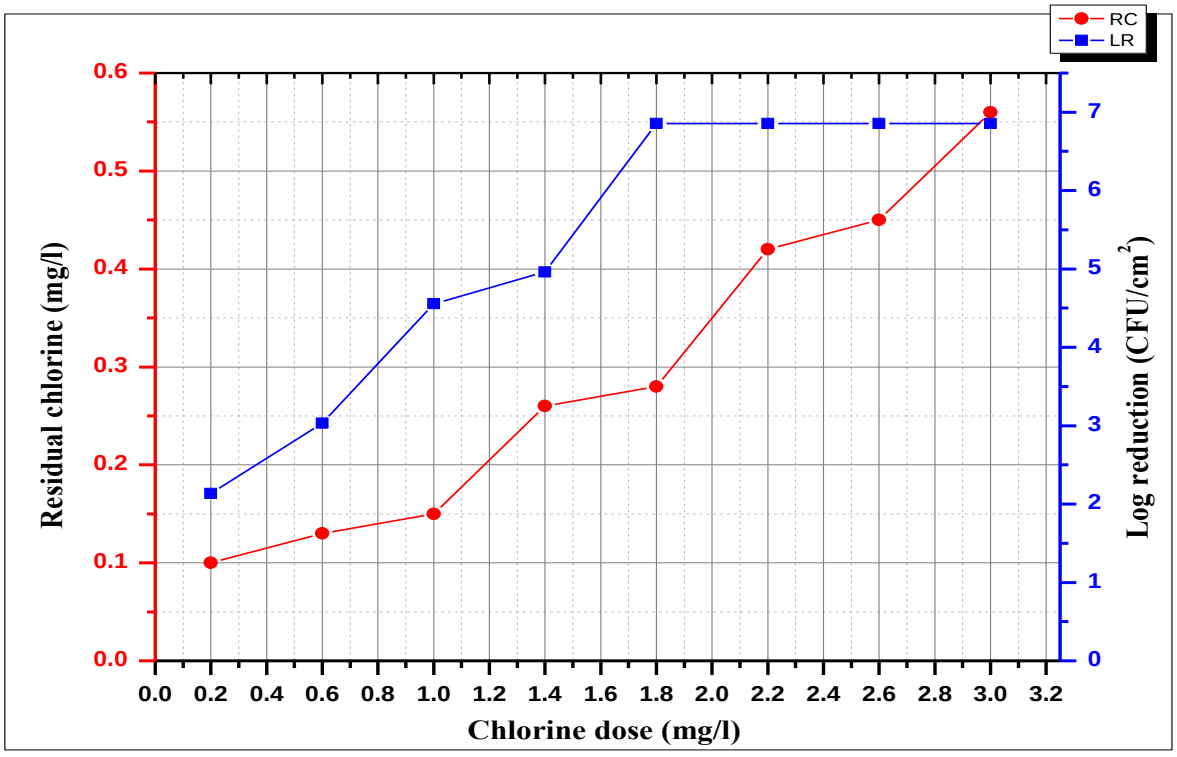

Fig. 61. The relationship between the chlorine doses, residual chlorine and the log reduction of 90 days-old *S.* Typhimurium biofilm grown on PE pipe material.

Table 56. Determination of effective chlorine dose and its residual against 10 days-old *S.* Typhimurium biofilm grown on I pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$5.2x10^6$	6.716	0
0.2	0.1	$1.3x10^4$	4.113	2.602
0.6	0.19	$3.6x10^3$	3.556	3.159
1.0	0.30	$1.4x10^2$	2.146	4.569
1.4	0.41	ND	ND	6.716
1.8	0.51	ND	ND	6.716
2.2	0.74	ND	ND	6.716
2.6	0.80	ND	ND	6.716
3.0	0.86	ND	ND	6.716

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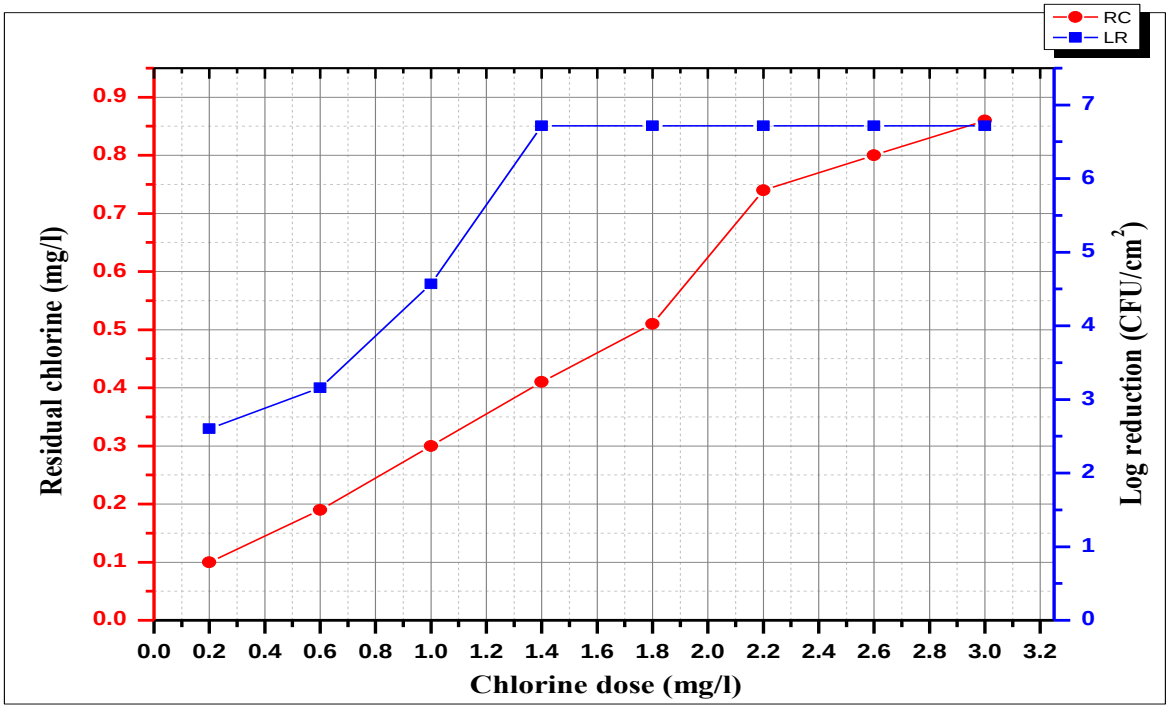

Fig. 62. The relationship between the chlorine doses, residual chlorine and the log reduction of 10 days-old *S.* Typhimurium biofilm grown on I pipe material.

Table 57. Determination of effective chlorine dose and its residual against 40 days-old *S.* Typhimurium biofilm grown on I pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$2.9x10^6$	6.462	0
0.2	0.1	$2.6x10^4$	4.414	2.047
0.6	0.16	$6.8x10^3$	3.832	2.629
1.0	0.24	$2.7x10^2$	2.431	4.031
1.4	0.33	8.5x10	1.929	4.532
1.8	0.43	ND	ND	6.462
2.2	0.49	ND	ND	6.462
2.6	0.70	ND	ND	6.462
3.0	0.77	ND	ND	6.462

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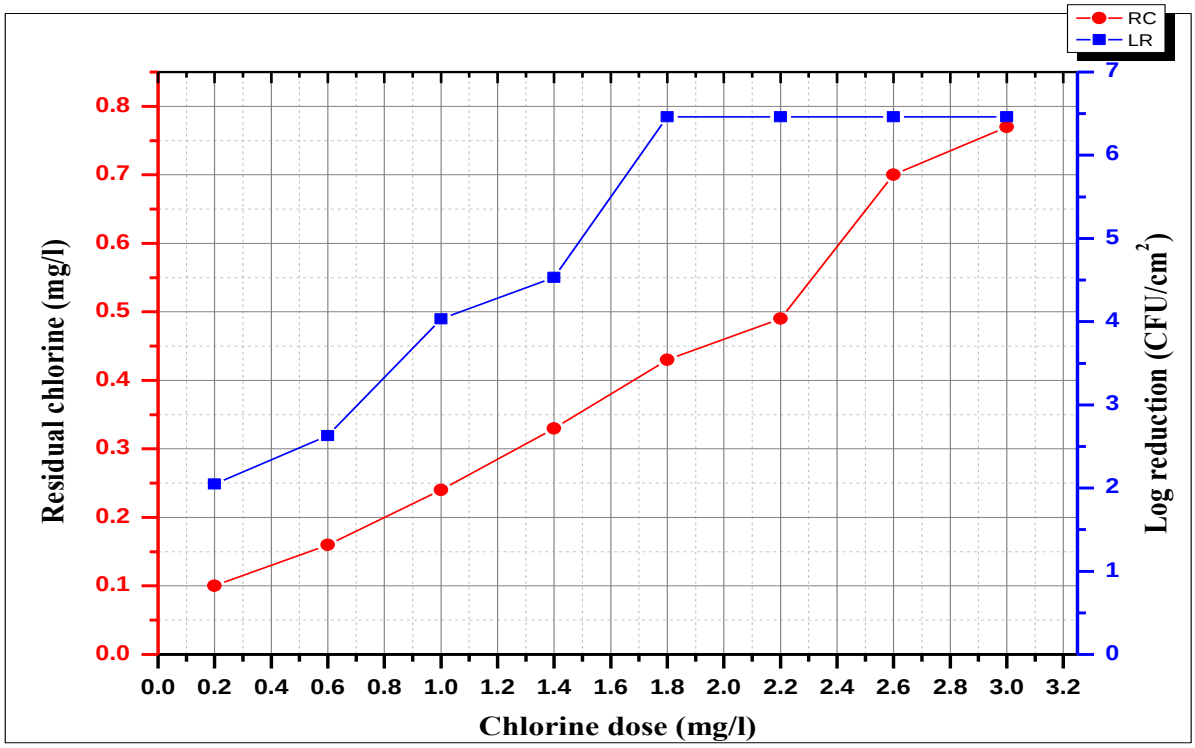

Fig. 63. The relationship between the chlorine doses, residual chlorine and the log reduction of 40 days-old *S.* Typhimurium biofilm grown on I pipe material.

Table 58. Determination of effective chlorine dose and its residual against 90 days-old *S.* Typhimurium biofilm grown on I pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$3.7x10^6$	6.568	0
0.2	0.1	$3.8x10^5$	5.579 4	0.988
0.6	0.14	$5.2x10^4$	4.716	1.852
1.0	0.17	$3.6x10^3$	3.556	3.011
1.4	0.20	$1.5x10^2$	2.176 1	4.392
1.8	0.24	4.8x10	1.681	4.886
2.2	0.38	ND	ND	6.568
2.6	0.45	ND	ND	6.568
3.0	0.56	ND	ND	6.568

ND= Not detected

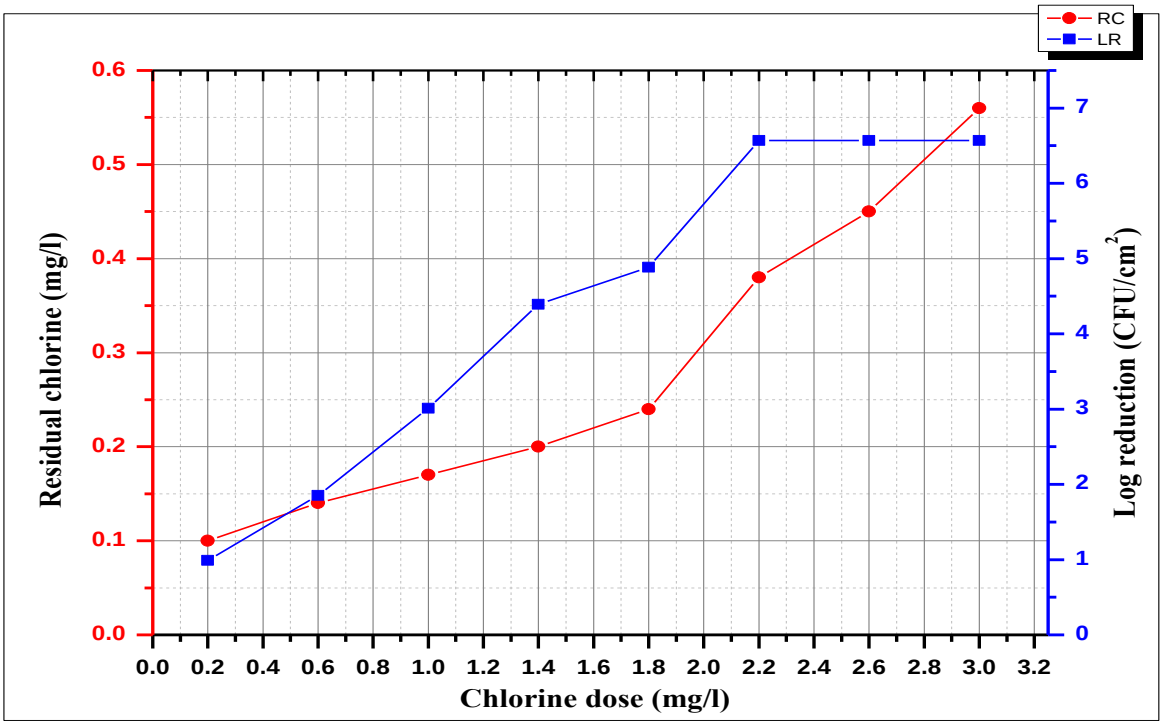

Fig. 64. The relationship between the chlorine doses, residual chlorine and the log reduction of 90 days-old *S.* Typhimurium biofilm grown on I pipe material.

6. Chlorine demand for inactivation of *S.* Typhimurium biofilm grown on Cu pipe material

The results presented in Tables (59, 60, 61) and Figs. (65, 66, 67), explained that 1.0 mg/l of chlorine dose was able to complete inactivation (6.6 CFU/cm^2) of 10 days-old *S.* Typhimurium biofilm formed on the Cu pipe. Also, the breakpoints were at 1.0 mg/l. Moreover, the results of 40 and 90 days-old biofilm (6.5 and 6.4 CFU/cm^2) revealed the effective chlorine doses were 1.0 and 0.6 mg/l. On the other hand, the breakpoints of chlorine dose for 40 and 90 days-old of biofilm cells were 1.4 mg/l.

Statisically, results indicated the coefficient of determination (R^2) between the chlorine dose and the residual chlorine of three ages of biofilm cells was 0.984, 0.985 and 0.990, respectively. While, the R^2 between the residual chlorine and log reduction of bacterial counts of three ages of biofilm cells were 0.625, 0.640 and 0.328, respectively.

7. Chlorine demand for inactivation of *S.* Typhimurium biofilm grown on R pipe material

As shown in Tables (62, 63, 64) and Figs. (68, 69, 70) the biofilm formation by *S.* Typhimurium on the R material, revealed the effective chlorine dose against 10 days-old biofilm (at 1.0 mg/l). While, 1.4 and 1.8 mg/l of chlorine dose showed complete reduction (6.6 and 6.4 CFU/cm^2) of 40 and 90 days-old of biofilm. The breakpoints of chlorine dose for 10, 40 and 90 days-old of biofilm cells were 0.6, 1.0 and 1.8 mg/l, respectively. The statistical analysis results indicated the coefficient of determination (R^2) between the chlorine dose and the residual chlorine of three ages of biofilm cells was 0.989, 0.969 and 0.961, respectively. While, the R^2 between the residual chlorine and log reduction of bacterial counts of three ages of biofilm cells were 0.603, 0.741 and 0.711, respectively.

Table 59. Determination of effective chlorine dose and its residual against 10 days-old *S.* Typhimurium biofilm grown on Cu pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$4.7x10^6$	6.672	0
0.2	0.10	$3.5x10^2$	2.544	4.128
0.6	0.21	8.9x10	1.949	4.722
1.0	0.31	ND	ND	6.672
1.4	0.56	ND	ND	6.672
1.8	0.69	ND	ND	6.672
2.2	0.8	ND	ND	6.672
2.6	1.1	ND	ND	6.672
3.0	1.3	ND	ND	6.672

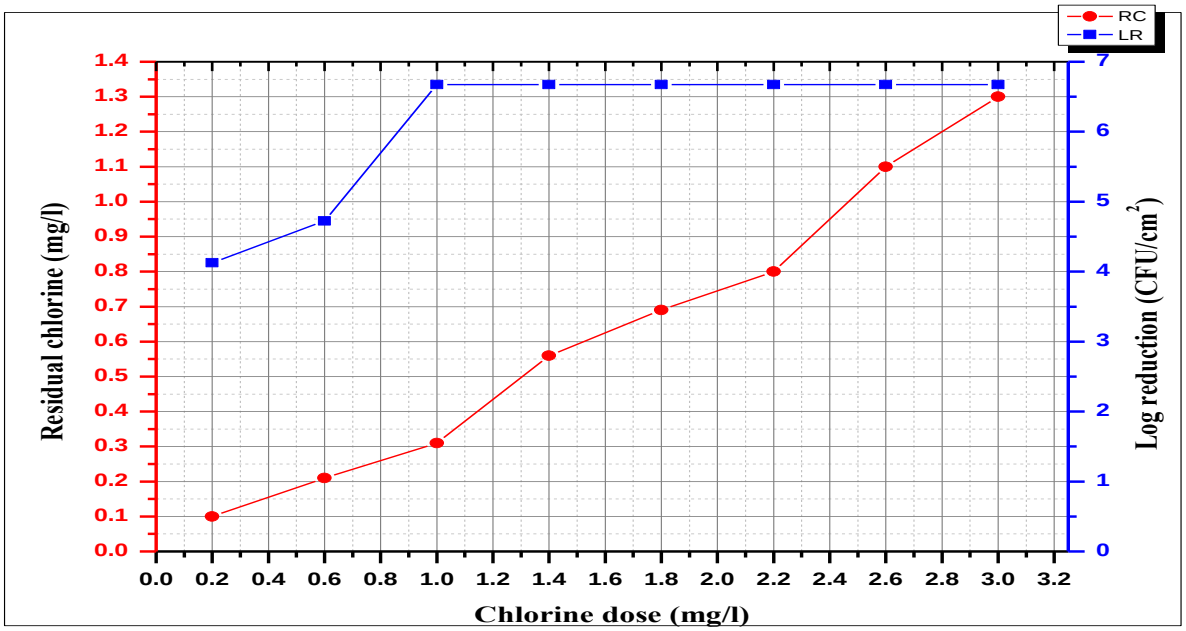

Fig. 65. The relationship between the chlorine doses, residual chlorine and the log reduction of 10 days-old *S.* Typhimurium biofilm grown on Cu pipe material.

Table 60. Determination of effective chlorine dose and its residual against 40 days-old *S.* Typhimurium biofilm grown on Cu pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$3.4x10^6$	6.531	0
0.2	0.15	$6.2x10^3$	3.792	2.739
0.6	0.24	9.1x10	1.959	4.572
1.0	0.33	ND	ND	6.531
1.4	0.36	ND	ND	6.531
1.8	0.46	ND	ND	6.531
2.2	0.54	ND	ND	6.531
2.6	0.58	ND	ND	6.531
3.0	0.62	ND	ND	6.531

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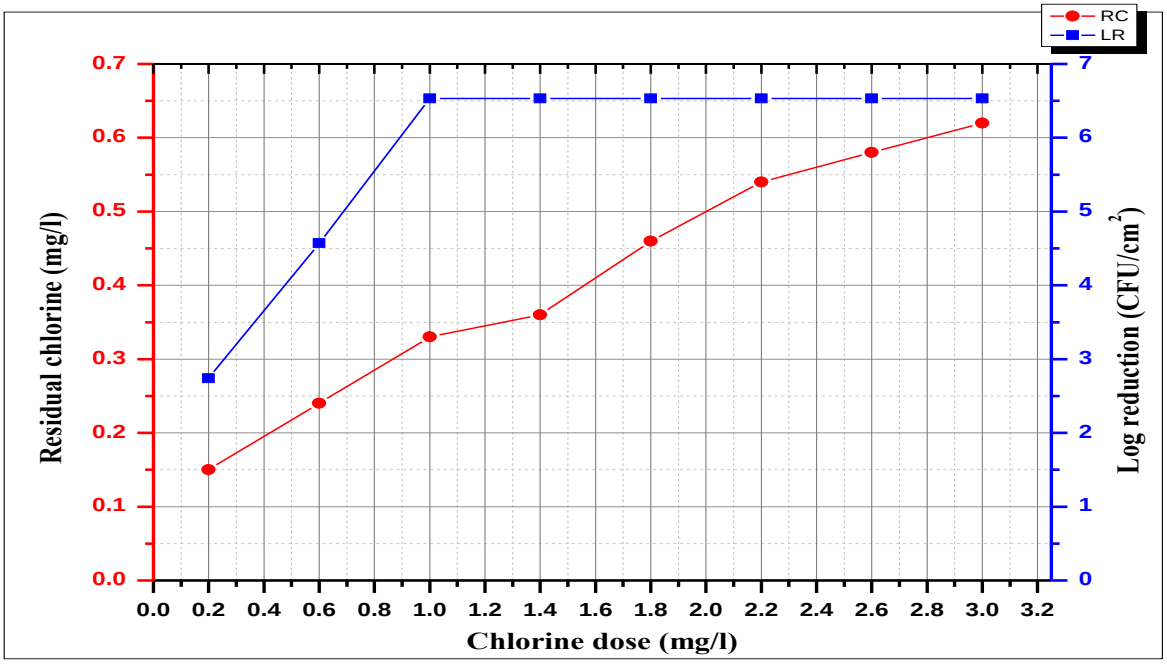

Fig. 66. The relationship between the chlorine doses, residual chlorine and the log reduction of 40 days-old *S.* Typhimurium biofilm grown on Cu pipe material.

Table 61. Determination of effective chlorine dose and its residual against 90 days-old *S.* Typhimurium biofilm grown on Cu pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$2.6x10^6$	6.414	0
0.2	0.1	$1.2x10^2$	2.079	4.335
0.6	0.2	ND	ND	6.414
1.0	0.33	ND	ND	6.414
1.4	0.38	ND	ND	6.414
1.8	0.52	ND	ND	6.414
2.2	0.68	ND	ND	6.414
2.6	0.70	ND	ND	6.414
3.0	0.84	ND	ND	6.414

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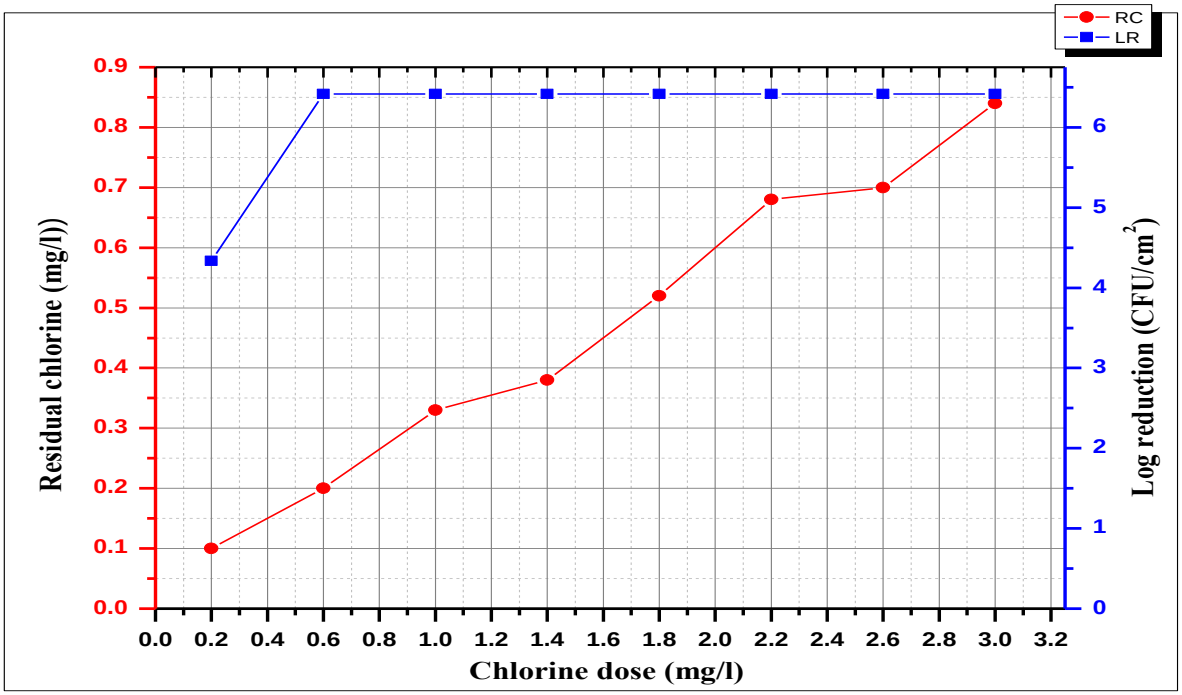

Fig. 67. The relationship between the chlorine doses, residual chlorine and the log reduction of 90 days-old *S.* Typhimurium biofilm grown on Cu pipe material.

Table 62. Determination of effective chlorine dose and its residual against 10 days-old *S.* Typhimurium biofilm grown on R pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$6.1x10^6$	6.785	0
0.2	0.12	$3.9x10^4$	4.591	2.194
0.6	0.23	$1.4x10^2$	2.146	4.639
1.0	0.47	ND	ND	6.785
1.4	0.67	ND	ND	6.785
1.8	0.83	ND	ND	6.785
2.2	0.91	ND	ND	6.785
2.6	1.1	ND	ND	6.785
3.0	1.2	ND	ND	6.785

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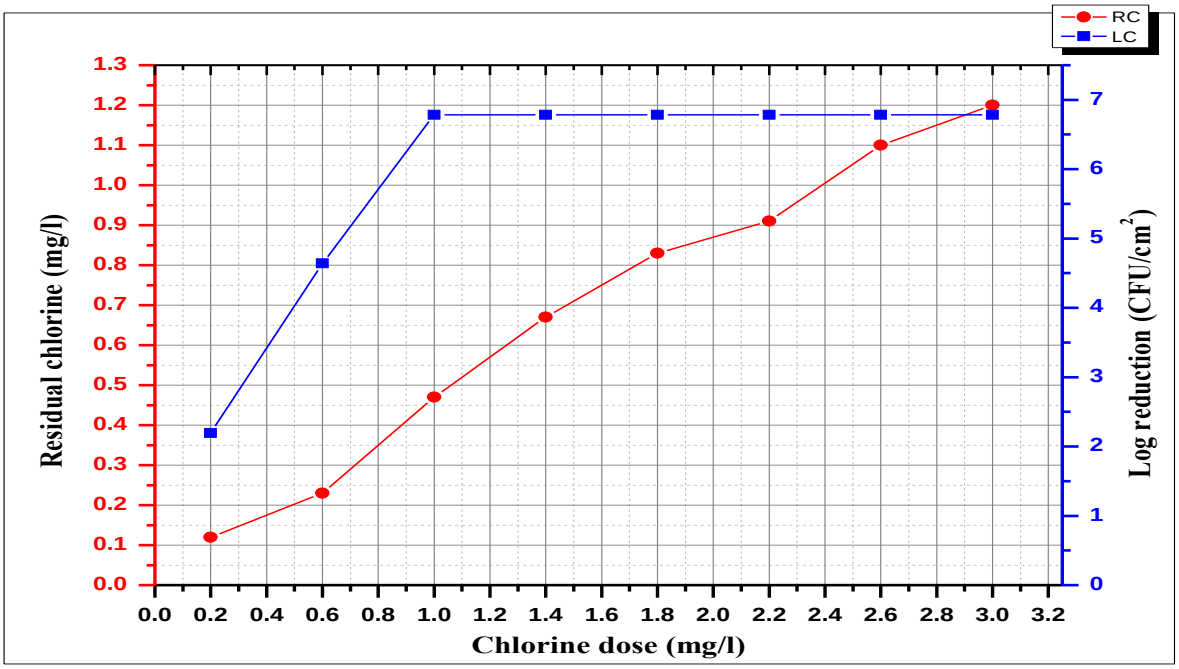

Fig. 68. The relationship between the chlorine doses, residual chlorine and the log reduction of 10 days-old *S.* Typhimurium biofilm grown on R pipe material.

Table 63. Determination of effective chlorine dose and its residual against 40 days-old *S.* Typhimurium biofilm grown on R pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$4.9x10^6$	6.690	0
0.2	0.1	$8.3x10^4$	4.919	1.771
0.6	0.17	$2.1x10^3$	3.322	3.367
1.0	0.20	7.3x10	1.863	4.826
1.4	0.31	ND	ND	6.690
1.8	0.40	ND	ND	6.690
2.2	0.53	ND	ND	6.690
2.6	0.55	ND	ND	6.690
3.0	0.56	ND	ND	6.690

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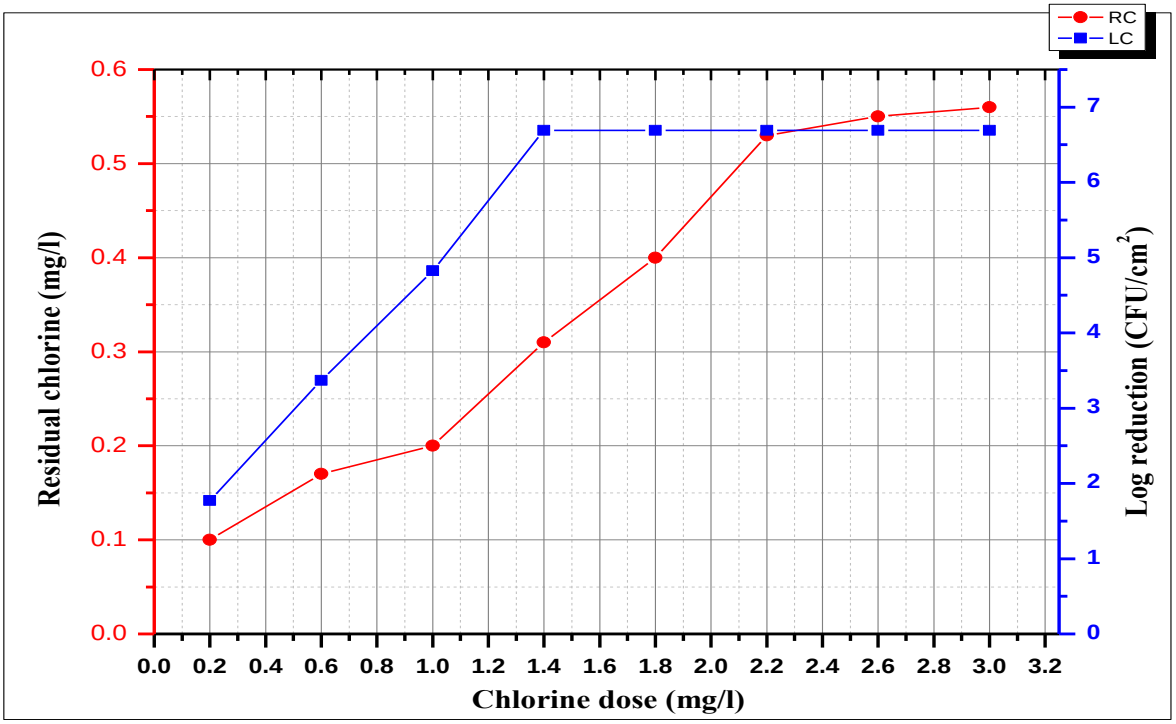

Fig. 69. The relationship between the chlorine doses, residual chlorine and the log reduction of 40 days-old *S.* Typhimurium biofilm grown on R pipe material.

Table 64. Determination of effective chlorine dose and its residual against 90 days-old *S.* Typhimurium biofilm grown on R pipe material.

Chlorine (mg/l)		Bacterial counts (CFU/cm^2)		
Chlorine dose	**Residual chlorine**	**Counts**	**Log counts**	**Log reduction**
0.0	0.00	$3.1x10^6$	6.491	0
0.2	0.1	$1.7x10^5$	5.230	1.260
0.6	0.14	$5.3x10^3$	3.724	2.767
1.0	0.17	$2.4x10^2$	2.380	4.111
1.4	0.21	9.2x10	1.963	4.527
1.8	0.23	ND	ND	6.491
2.2	0.32	ND	ND	6.491
2.6	0.36	ND	ND	6.491
3.0	0.45	ND	ND	6.491

ND= Not detected

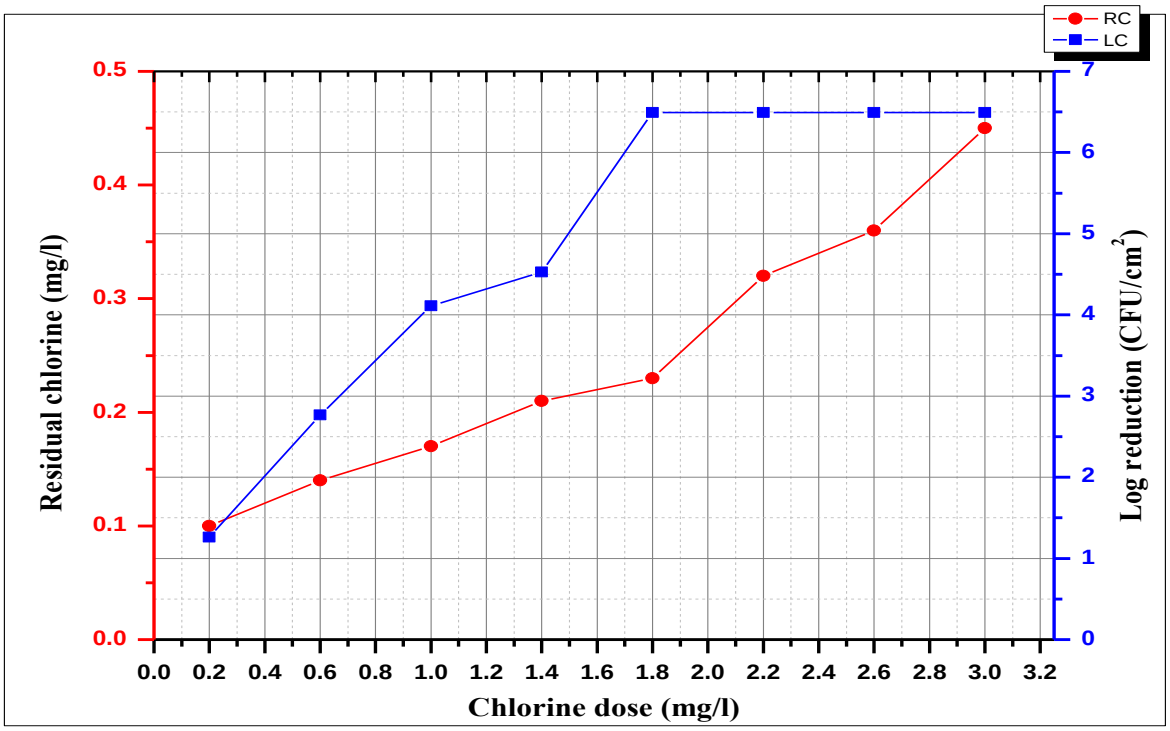

Fig. 70. The relationship between the chlorine doses, residual chlorine and the log reduction of 90 days-old *S.* Typhimurium biofilm grown on R pipe material.

Attempts at removal of drinking water biofilm rely on various preventive and curative strategies such as nutrient reduction in drinking water, disinfection, or water flushing, have demonstrated limited efficiency. The main reason for these failures is the cohesiveness of the biofilm driven by the physico-chemical properties of its exopolymeric matrix (EPS). Effective cleaning procedures should break up the matrix and/or change the elastic properties of bacterial biofilm (Mathieu *et al.*, 2014).

The chemical disinfection, especially chlorine, is considered as the most approach to prevent the development of biofilm in the water distribution system. The microbes, which have passed through the treatment stage, can be destroyed or irreversibly inactivate by chlorination, so as to ensure microbiologically potable water (WHO, 2011). Most of the previous studies have provided sufficient information regarding the effects of pipe materials on biofilm biomass and microbial diversity at low chlorine levels (<1.0 mg/l free chlorine) (Lin *et al.,* 2013). However, municipal water supplies are usually disinfected with 0.5- 3.0 mg/L chlorine to control bacterial growth. The average free chlorine concentrations are usually more than 1.0 mg/l free chlorine at the outlet of the treatment plant (Zhang and Andrews, 2012). Consequently, there is a pressing need to continually improve the understanding of the effect of pipe materials on chlorine-resistant biofilm formation (biomass and microbial diversity) near the treatment plant pipelines (>1.0 mg/l free chlorine) (Simões and Simões, 2013).

The maintenance of chlorine residue is needed at all points in distribution system supplied with chlorine as a disinfectant. Chlorine residuals of drinking water have long been recognized as an excellent indicator for studying water quality in the distribution network. In the absence of a disinfectant residual, microorganisms in the distribution network will be recovered at high levels (Kitazawa, 2006). In addition to, the presence of any disinfectant residual reduces the microorganism level and frequency of occurrence at the consumer's tap. Keeping residual chlorine at a certain level in tap water is effective not only in improving sanitary conditions but also in suppressing the re-growth of microorganisms and preventing the formation of biofilm on the internal surface of distribution pipelines (Unnisa and Hassan, 2013). Moreover, the effectiveness of

disinfectant residual depends on the concentration, the contact time and the presence of microorganisms (Besner *et al.*, 2002). Also, there are various investigations showing that, the adequate disinfectant residuals can be control of the biofilm accumulation (Zhang *et al.*, 2008).

Some studies have demonstrated that chlorine is able to control biofilm formation by reducing the rate of biofilm growth, promoting the biofilm detachment and decreasing the activity of microorganisms (Codony *et al.*, 2005). However, the presence of residual chlorine is also one of the stress factors that lead to biofilm formation (Kokare *et al.*, 2008). Nevertheless, some European countries notably the Netherlands, Germany, Austria and Switzerland have taken the approach of distributing high quality DW without the use of residual chlorine. The control of microbial growth in these countries is obtained through limitation of the nutrients essential for growth by more appropriate DW treatments (sedimentation, filtration, UV disinfection, ozone, peroxide) by the production of biologically stable DW (Simões and Simões, 2013).

From the obtained results, it was recorded real the effective doses of chlorine capable of cause the complete removal of *L. monocytogenes* and *S.* Typhimurium planktonic cells were 3.0 and 0.6 mg/l. Additionally, chlorine effectively inactivates the majority of organisms that cause diseases in humans like at 0.2 mg/l chlorine concentration for 3 minutes cause 99.99% reduction of *Escherichia coli* and at 0.5 mg/l chlorine for 6 min reduces 99.9% *Salmonella Typhimurium* (Hashmi *et al*., 2009).The bacterial regrowth to form biofilm in DWDS can occur even in the presence of disinfectant in bulk water (Zhang and DiGiano, 2002). Additionally, El-Liethy (2013) found that 1.4 mg/l of chlorine is enough to inactivate 10^6 CFU/ml of *E.coli* O157

The results of this study showed, the biofilm formed by *L. monocytogenes* and *S.* Typhimurium grown on iron (I) pipe material in all ages were more resistant to different chlorine doses than the biofilm cells formed on other pipe material. This may be due to the iron pipe corrosion that influences the efficiency of chlorine for the eradication of bacterial biofilm. Also, the biofilm formed on iron presented the highest biomass and the second highest cultivability throughout the experiment. In addition to, disinfectants could

substantially affect cast iron corrosion (Bachmann and Edyvean, 2005). Also, Appenzeller *et al.* (2005) demonstrated that iron in drinking water may promote both growth and cultivability of the most of bacteria. Iron rust not only increases pipe surface porosity and roughness for microbial attachment, but also can serve as a nutrient and the rust scavenges free residual chlorine, resulting in its removal before it can reach and react with the biofilm microorganisms (Wang *et al.*, 2012; Simões and Simões, 2013). Also, iron pipes are more reactive with disinfectants and quench their antimicrobial effects (Simões and Simões, 2013). Therefore, bacteria that colonized on iron were more protected from chlorine residual than those colonized on other materials.

In this study, all the biofilm ages including 10, 40 and 90 days-old of *L. monocytogenes* and *S.* Typhimurium formed on Cu pipe material were completely inactivated using 3.0 mg/l of chlorine dose except 10 days-old *L. monocytogenes* biofilm (younger age). These may be due to the release of copper residuals that have antibacterial properties for long run, which can cause damage of cell membranes and nucleic acid structure (Bruins *et al.*, 2000). So, the copper plumbing material is widely used due to the ease fittings with different pipes varieties (Zhang *et al.*, 2008). Also, the lowest microbial numbers in Cu pipes during the early stage is attributed to the release of copper ions from Cu pipe with these metal ions known to be growth-inhibiting to bacteria (Bachmann and Edyvean, 2005). Additionally, Nguyen *et al.*(2011) demonstrated that, the metal-based materials can form the corrosion products on pipe surfaces and release metals into water as a result of chemical or biological reactions.

Vaz-Moreira *et al.* (2013) concluded that the chlorination step in water treatment can promote a reduction on bacterial diversity and cultivability but it is not suitable for all pipe materials. The difference of biofilm cultivability on different materials was most probably due to different cultivable chlorine-resistant bacteria species from various pipe materials. Additionally, the most complicated effects on microbial regrowth and biofilm formation come from pipe materials. Certain pipe materials can modify and decay disinfectant residuals, leading to increased microbial regrowth in DWDS (Lehtola *et al.*, 2004).

In this study, when comparing the ages of different pipe materials, the results showed that, after 90 days-old, more resistance to the dose of chlorine was revealed. The presence of EPS that coated biofilm cells, which the protected the cells from any disinfectant or antimicrobial agents. The EPS matrix is prevented by the penetration of antimicrobial agents, for reaching the microorganisms within the biofilm by the diffusion limitation and/or the chemical interaction with the extracellular proteins and polysaccharides (Sutherland, 2001). Whereas, the biofilm EPS play a vital role to protect the biofilm cells towards antimicrobial agents (Suzuki, 2001).

The results indicated real the pipe material plays a vital role in the resistance of biofilm to chlorine. Also, Zhu *et al.* (2014) observed the pipe materials have a strong influence on chlorine-resistant microbial densities and cultivability in biofilm formation. Accordingly, the pipe material has strong effects on speed, biomass, and community of chlorine-resistant biofilm stabilization in high chlorinated water distribution system biofilm.

In this study, the biofilm cells scraped from all tested pipe materials were more resistant to chlorine than planktonic cells. Also, the young biofilm age (10 days) was more sensitive to chlorine than the old biofilm ages (40 and 90 days). Also, Schwartz *et al.* (2003) found the biofilm cells are more resistant than planktonic populations, as they are including chlorine and antibiotics. Additionally, regular testing is essential to ensure that adequate free residual chlorine is still present in the treated water. The maintenance of chlorine residue is needed at all points in distribution system supplied with chlorine as a disinfectant. Keeping residual chlorine at a certain level in tap water is effective not only in improving sanitary conditions but also in suppressing the re-growth of microorganisms and preventing the formation of biofilm on the internal surface of distribution pipelines (Unnisa and Hassan, 2013).

As a final point, the results summarized the real alternative disinfectant must be used for control and preventing the unwanted biofilm which formed in the inner surface of pipe material. The WHO mentioned the risks from disinfection by-products to health

are very small, in comparison with insufficient disinfection. Therefore, the improvement of safe and effective alternative disinfection methods is highly desirable (WHO, 2011).

2. Efficacy of Ag ions against *L. monocytogenes* and *S.* Typhimurium biofilm grown on domestic plumping materials

This section of the present study was carried out to determine the antibiofilm activity for five doses (50, 100, 200, 300 and 500 mg/l) of Ag ions at three different contact times (5, 10 and 15 min). Six biofilm samples and planktonic cells of *L. monocytogenes* and *S.* Typhimurium were exposed to above doses.

a. Comparative susceptibility of planktonic cells and three different ages of *L. monocytogenes* biofilm to silver ions (Ag ions)

1. Antibiofilm activity and MIC of Ag ions against *L. monocytogenes* planktonic cells

The data presented in Table (65) showed the Ag ion doses which able to complete removal of planktonic cells of *L. monocytogenes* were 100 mg at 15 min but 200, 300 and 500 mg/l able to complete removal. While, 50 mg can't cause completely inhibit the palnktonic cells. So, the proper effective dose at low contact time was recorded in100 mg at 15 min. The MIC of Ag ions of planktonic cell was 100 mg at 15 min. The MIC of Ag ions of planktonic cell was 100 mg at 15 min. Also, from toxicity assay, the results indicated that all used Ag ions doses were toxic.

The suggested statistical model (one way *ANOVA*- Homogeneity test of variances) indicated, there was a highly significant ($P \leq 0.05$) between the different doses of Ag ions and the counts of planktonic cells.

Table 65. Determination of antibiofilm effect and MIC for different doses of Ag ions against *L. monocytogenes* planktonic cells

Contact time (min)	Doses of Ag ions (mg/l)				
	50	**100**	**200**	**300**	**500**
Zero	$3.7x10^6$	$3.7x10^6$	$3.7x10^6$	$3.7x10^6$	$3.7x10^6$
5	$5.9x10^2$	$1.2x10^2$	ND	ND	ND
10	$2.1x10^2$	4.6x10	ND	ND	ND
15	9.3x10	ND	ND	ND	ND

ND= Not detected

2. Antibiofilm activity and MIC of Ag ions against *L. monocytogenes* biofilm grown on PVC pipe material

Table (66) and Fig. (71) represented that, the MIC of Ag ions for inactivation of 10, 40 and 90 days-old *L. monocytogenes* biofilm grown on PVC pipe material.

The results showed that MIC for 10 days-old was 200 mg at contact time 10 min, while after 40 days were 200 mg at 15 min and 300 mg at 10 min for 90 days-old of biofilm, respectively.

From the results of statistical analysis (Homogeneity test of variances from one way *ANOVA*), it was found there was a highly significant correlation ($P \leq 0.05$) between the different doses of Ag ions and *L. monocytogenes* biofilm ages grown on PVC pipe material.

Table 66. Determination of antibiofilm effect and MIC for different doses of Ag ions against three ages of *L. monocytogenes* biofilm grown on PVC pipe material.

Ag ions doses (mg/l)	Contact time (min)	CFU/cm^2 The ages *L. monocytogenes* biofilm grown on PVC pipe material								
		10 days-old			40 days-old			90 days-old		
		Count	Log count	Log reduction	Count	Log count	Log reduction	Count	Log count	Log reduction
Control		$2.5x10^6$	6.397	0	$4.6x10^6$	6.662	0	$5.2x10^6$	6.716	0
50	5	$5.3x10^2$	2.724	3.673	$3.9x10^3$	3.591	3.071	$4.2x10^4$	4.623	2.092
	10	$1.3x10^2$	2.113	4.283	$4.2x10^2$	2.623	4.039	$2.1x10^4$	4.322	2.393
	15	9.8x10	1.991	4.406	$2.3x10^2$	2.361	4.301	$6.2x10^3$	3.792	2.923
100	5	5.1x10	1.707	4.690	$1.1x10^2$	2.041	4.621	$4.0x10^3$	3.602	3.113
	10	3.7x10	1.568	4.829	8.9x10	1.949	4.7133	$1.1x10^3$	3.041	3.674
	15	1.9x10	1.278	5.119	5.7x10	1.755	4.906	$7.6x10^2$	2.880	3.835
200	5	7	0.845	5.552	2.3x10	1.361	5.301	$4.1x10^2$	2.612	4.103
	10	ND	ND	6.397	9	0.954	5.708	$1.6x10^2$	2.204	4.511
	15	ND	ND	6.397	ND	ND	6.662	8.3x10	1.919	4.796
300	5	ND	ND	6.397	ND	ND	6.662	1.4x10	1.146	5.569
	10	ND	ND	6.397	ND	ND	6.662	ND	ND	6.716
	15	ND	ND	6.397	ND	ND	6.662	ND	ND	6.716
500	5	ND	ND	6.397	ND	ND	6.662	ND	ND	6.716
	10	ND	ND	6.397	ND	ND	6.662	ND	ND	6.716
	15	ND	ND	6.397	ND	ND	6.662	ND	ND	6.716

ND: Not Detected.

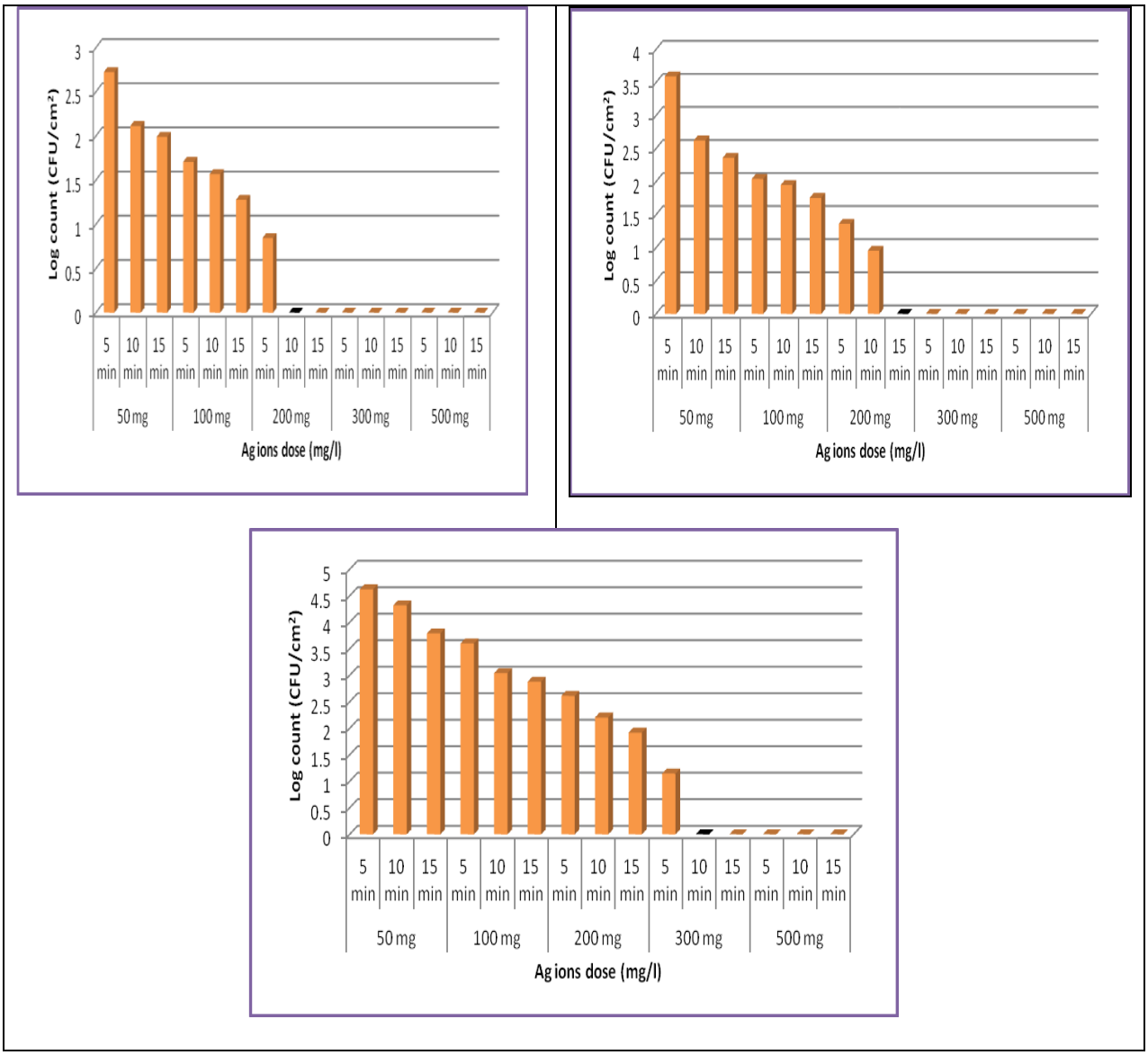

Fig. 71. Antibiofilm effect and MIC of different doses of Ag ions against 10, 40 and 90 days-old *L. monocytogenes* biofilm grown on PVC pipe material (left- right).

3. Antibiofilm activity and MIC of Ag ions against *L. monocytogenes* biofilm grown on PP pipe material

As shown in Table (67) and Fig. (72), the MIC of Ag ions for 10, 40 and 90 days-old *L. monocytogenes* biofilm grown on PP pipe material were 200 mg at 5 min, 200 mg at 10 min and 300 mg at 5 min, respectively.

Statistically, there was a greatly significant correlation ($P \leq 0.05$) between the different doses of Ag ions and *L. monocytogenes* biofilm ages grown on PP pipe material.

4. Antibiofilm activity and MIC of Ag ions against *L. monocytogenes* biofilm grown on PE pipe material

By regarding the results of 10, 40 and 90 days-old *L. monocytogenes* biofilm grown on PE pipe material, it was recorded real the MIC of Ag ions for removal of biofilm were 200 mg at 10 min, 200 mg at 10 and 300 mg at 10 min, respectively (Table 68; Fig. 73).

The results of statistical analysis (Homogeneity test of variances from one way *ANOVA*), showed that it was a highly significant correlation ($P \leq 0.05$) between the different doses of Ag ions and *L. monocytogenes* biofilm ages grown on PE pipe material.

Table 67. Determination of antibiofilm effect and MIC for different doses of Ag ions against three ages of *L. monocytogenes* biofilm grown on PP pipe material.

Ag ions doses (mg/l)	Contact time (min)	CFU/cm^2 The ages of *L. monocytogenes* biofilm on PP pipe material								
		10 days-old			40 days-old			90 days-old		
		Count	Log count	Log reduction	Count	Log count	Log reduction	Count	Log count	Log reduction
Control		$1.9x10^6$	6.278	0	$7.4x10^6$	6.869	0	$3.8x10^6$	6.579	0
50	5	$2.8x10^2$	2.447	3.831	$1.6x10^3$	3.204	3.665	$2.5x10^4$	4.397	2.181
	10	$1.0x10^2$	2	4.278	$3.4x10^2$	2.531	4.337	$4.8x10^3$	3.681	2.898
	15	8.3x10	1.919	4.359	$1.2x10^2$	2.079	4.790	$1.6x10^3$	3.204	3.375
100	5	3.2x10	1.176	5.102	7.5x10	1.875	4.994	$6.1x10^2$	2.785	3.794
	10	1.5x10	0.477	5.8016	3.2x10	1.505	5.364	$2.7x10^2$	2.431	4.148
	15	ND	ND	6.278	1.2x10	1.079	5.790	$1.1x10^2$	2.041	4.538
200	5	ND	ND	6.278	4	0.602	6.267	7.3x10	1.863	4.716
	10	ND	ND	6.278	ND	ND	6.267	5.8x10	1.763	4.816
	15	ND	ND	6.278	ND	ND	6.267	1.3x10	1.113	5.465
300	5	ND	ND	6.278	ND	ND	6.267	ND	ND	6.579
	10	ND	ND	6.278	ND	ND	6.267	ND	ND	6.579
	15	ND	ND	6.278	ND	ND	6.267	ND	ND	6.579
500	5	ND	ND	6.278	ND	ND	6.267	ND	ND	6.579
	10	ND	ND	6.278	ND	ND	6.267	ND	ND	6.579
	15	ND	ND	6.278	ND	ND	6.267	ND	ND	6.579

ND: Not Detected.

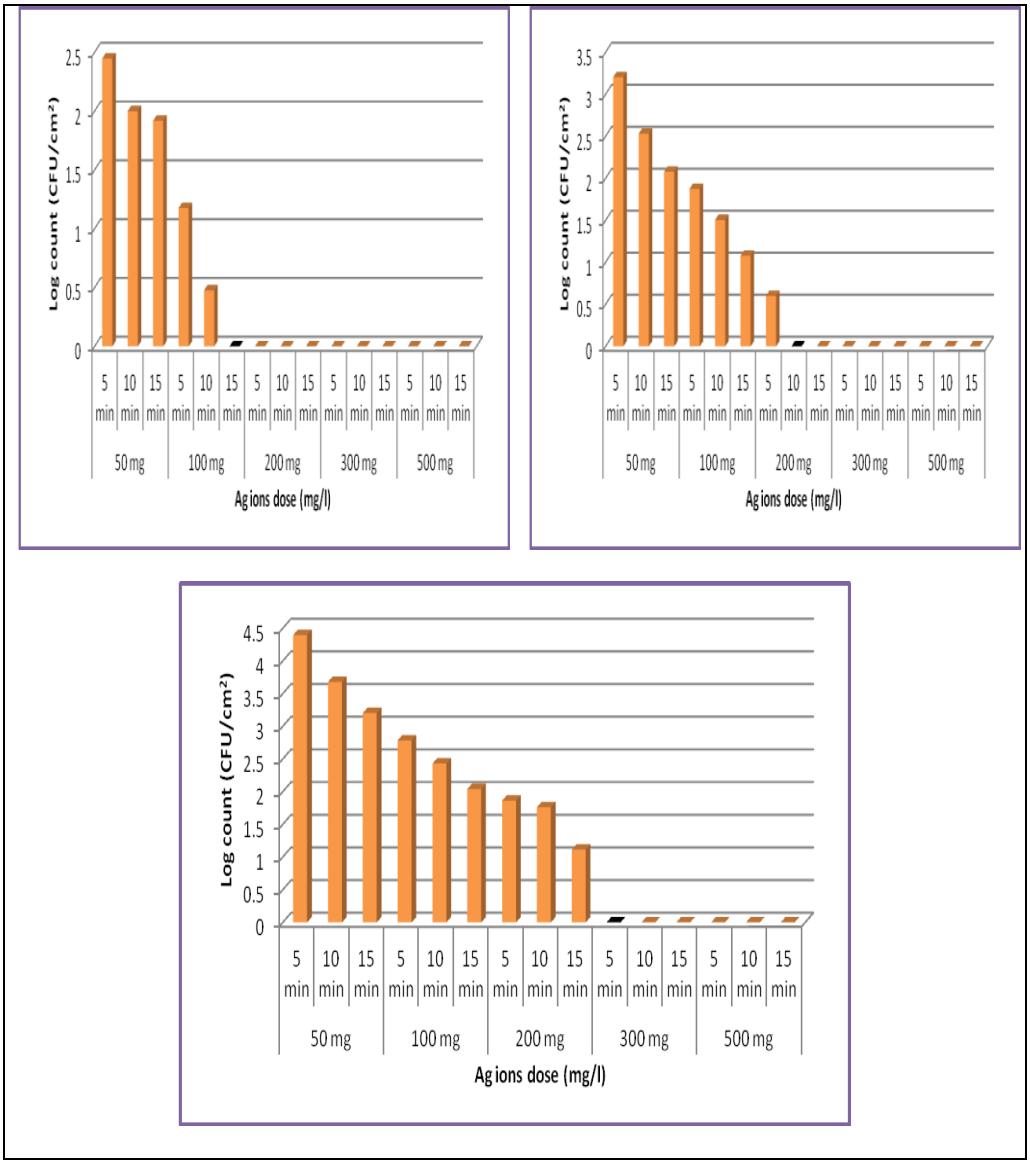

Fig. 72. Antibiofilm effect and MIC of different doses of Ag ions against 10, 40 and 90 days-old *L. monocytogenes* biofilm grown on PP pipe material (left- right).

Table 68. Determination of antibiofilm effect and MIC for different doses of Ag ions against three ages of *L. monocytogenes* biofilm grown on PE pipe material.

Ag ions doses (mg/l)	Contact time (min)	CFU/cm^2 The ages of *L. monocytogenes* biofilm grown on PE pipe material								
		10 days-old			40 days-old			90 days-old		
		Count	Log count	Log reduction	Count	Log count	Log reduction	Count	Log count	Log reduction
Control		$4.2x10^6$	6.623	0	$5.1x10^6$	6.707	0	$7.8x10^6$	6.892	**0**
50	**5**	$4.1x10^2$	2.612	4.010	$3.4x10^3$	3.531	3.176	$5.3x10^4$	4.724	2.167
	10	$2.3x10^2$	2.361	4.2615	$2.6x10^2$	2.414	4.292	$6.8x10^3$	3.832	3.059
	15	$1.2x10^2$	2.079	4.544	$1.1x10^2$	2.041	4.666	$2.1x10^3$	3.322	3.569
100	**5**	9.5x10	1.977	4.6455	8.5x10	1.929	4.778	$1.1x10^3$	3.041	3.850
	10	4.9x10	1.690 1	4.933	5.1x10	1.707	5	$4.2x10^2$	2.623	4.268
	15	1.3x10	1.113	5.509	2.0x10	1.301	5.406	$1.7x10^2$	2.230	4.661
200	**5**	9	0.954	5.669	7	0.845	5.862	9.7x10	1.986	4.905
	10	ND	ND	6.623	ND	ND	6.707	6.5x10	1.812	5.079
	15	ND	ND	6.623	ND	ND	6.707	2.9x10	1.462	5.429
300	**5**	ND	ND	6.623	ND	ND	6.707	9	0.954	5.937
	10	ND	ND	6.623	ND	ND	6.707	ND	ND	6.892
	15	ND	ND	6.623	ND	ND	6.707	ND	ND	6.892
500	**5**	ND	ND	6.623	ND	ND	6.707	ND	ND	6.892
	10	ND	ND	6.623	ND	ND	6.707	ND	ND	6.892
	15	ND	ND	6.623	ND	ND	6.707	ND	ND	6.892

ND: Not Detected.

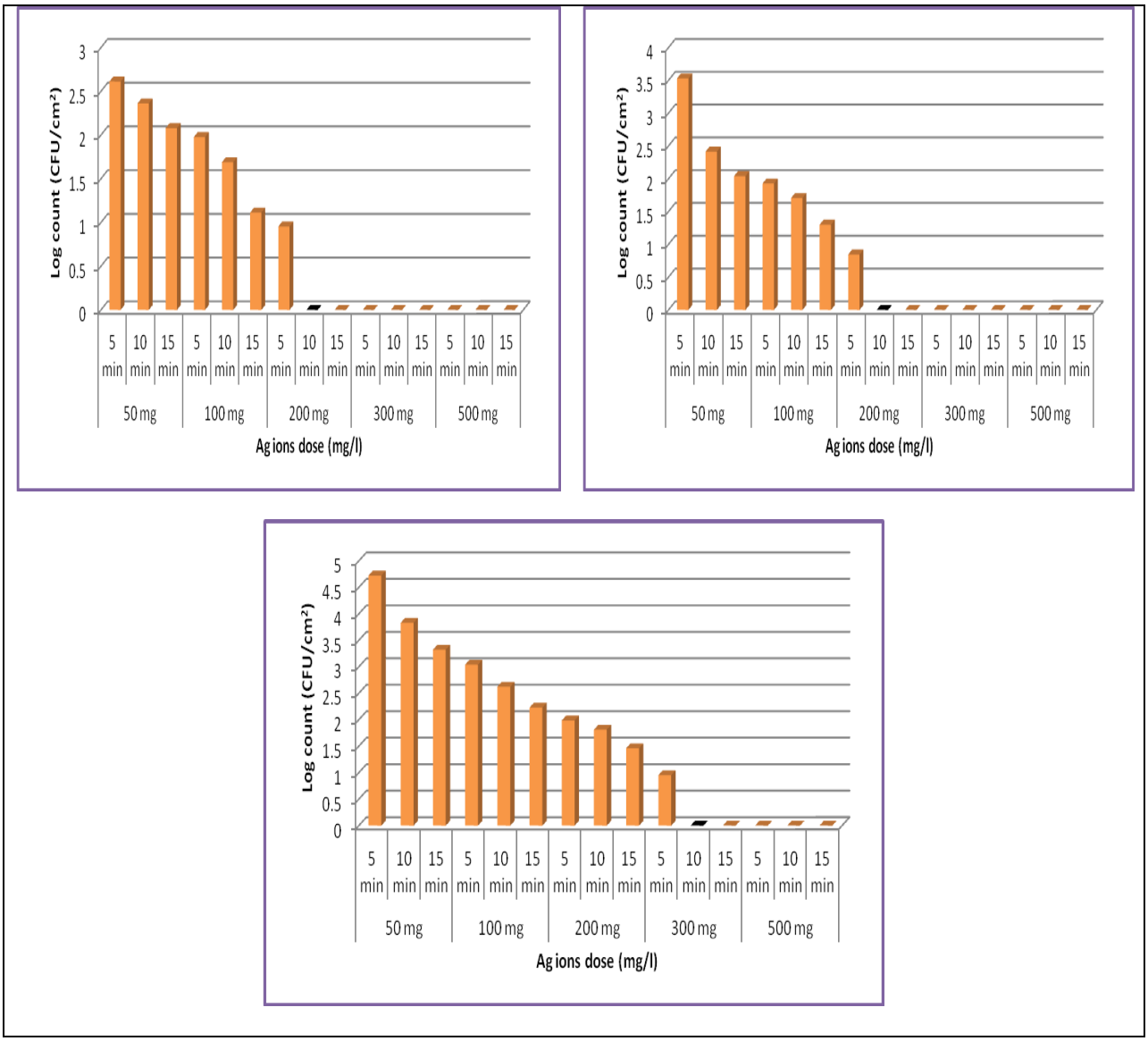

Fig. 73. Antibiofilm effect and MIC of different doses of Ag ions against 10, 40 and 90 days-old *L. monocytogenes* biofilm grown on PE pipe material (left- right).

5. Antibiofilm activity and MIC of Ag ions against *L. monocytogenes* biofilm grown on I pipe material

The data shown in Table (69) and Fig. (74) indicated, the antibiofilm activity and MIC (300 mg at 5 min, 300 mg at 15 min and 500 mg at 5 min) of Ag ions were recorded against10, 40 and 90 days-old *L. monocytogenes* biofilm grown on I pipe material.

Statistically, (Homogeneity test of variances from one way *ANOVA*), the results was investigated, there was a deeply significant correlation ($P \leq 0.05$) between the different doses of Ag ions and 10, 40 and 90 days *L. monocytogenes* biofilm grown on I pipe material.

6. Antibiofilm activity and MIC of Ag ions against *L. monocytogenes* biofilm grown on Cu pipe material

Concerning the results of *L. monocytogenes* biofilm grown on Cu pipe material, it was demonstrated the MIC for 10, 40 and 90 days-old of biofilm were 200 mg at 5 min, 200 mg at 5 and 100 mg at 15 min, respectively (Table 70; Fig. 75).

The results of statistical analysis found a deeply significant correlation ($P \leq 0.05$) between the different doses of Ag ions and *L. monocytogenes* biofilm ages grown on Cu pipe material.

7. Antibiofilm activity and MIC of Ag ions against *L. monocytogenes* biofilm grown on R pipe material

The obtained results in Table (71) and Fig. (76) showed real the MIC of Ag ions which can be completely removed of 10, 40 and 90 days-old *L. monocytogenes* biofilm grown on R pipe material were 200 mg at 10 min, 200 mg at 15 min and 300 mg at 5 min, respectively.

Table 69. Determination of antibiofilm effect and MIC for different doses of Ag ions against three ages of *L. monocytogenes* biofilm grown on I pipe material.

Ag ions doses (mg/l)	Contact time (min)	CFU/cm^2 The ages of *L. monocytogenes* biofilm grown on I pipe material								
		10 days-old			40 days-old			90 days-old		
		Count	Log count	Log reduction	Count	Log count	Log reduction	Count	Log count	Log reduction
Control		$7.4x10^6$	6.869	0	$2.3x10^6$	6.361	0	$6.3x10^6$	6.799	0
50	5	$5.6x10^3$	3.748	2.167	$7.3x10^4$	4.863	1.498	$7.3x10^5$	5.863	0.936
	10	$1.4x10^3$	3.146	3.059	$2.4x10^4$	4.380	1.981	$3.3x10^5$	5.518	1.280
	15	$6.4x10^2$	2.806	3.569	$5.7x10^3$	3.755	2.605	$4.9x10^4$	4.690	2.109
100	5	$3.1x10^2$	2.491	3.850	$3.4x10^3$	3.531	1.331	$1.3x10^4$	4.113	2.685
	10	$1.5x10^2$	2.176	4.268	$7.3x10^2$	2.863	3.498	$8.1x10^3$	3.908	2.890
	15	8.6x10	1.934	4.661	$4.1x10^2$	2.612	3.748	$5.2x10^3$	3.716	3.083
200	5	4.2x10	1.623	4.905	$2.8x10^2$	2.447	3.914	$2.1x10^3$	3.322	3.477
	10	1.2x10	1.079	5.079	$1.6x10^2$	2.204	4.157	$7.5x10^2$	2.875	3.924
	15	2	0.301	5.429	9.7x10	1.986	4.374	$4.6x10^2$	2.662	4.136
300	5	ND	ND	5.937	6.9x10	1.838	4.522	$2.3x10^2$	2.361	4.437
	10	ND	ND	6.892	1.2x10	1.079	5.282	8.9x10	1.949	4.849
	15	ND	ND	6.892	ND	ND	6.361	2.6x10	1.414	5.384
500	5	ND	ND	6.892	ND	ND	6.361	ND	ND	6.799
	10	ND	ND	6.892	ND	ND	6.361	ND	ND	6.799
	15	ND	ND	6.892	ND	ND	6.361	ND	ND	6.799

ND: Not Detected.

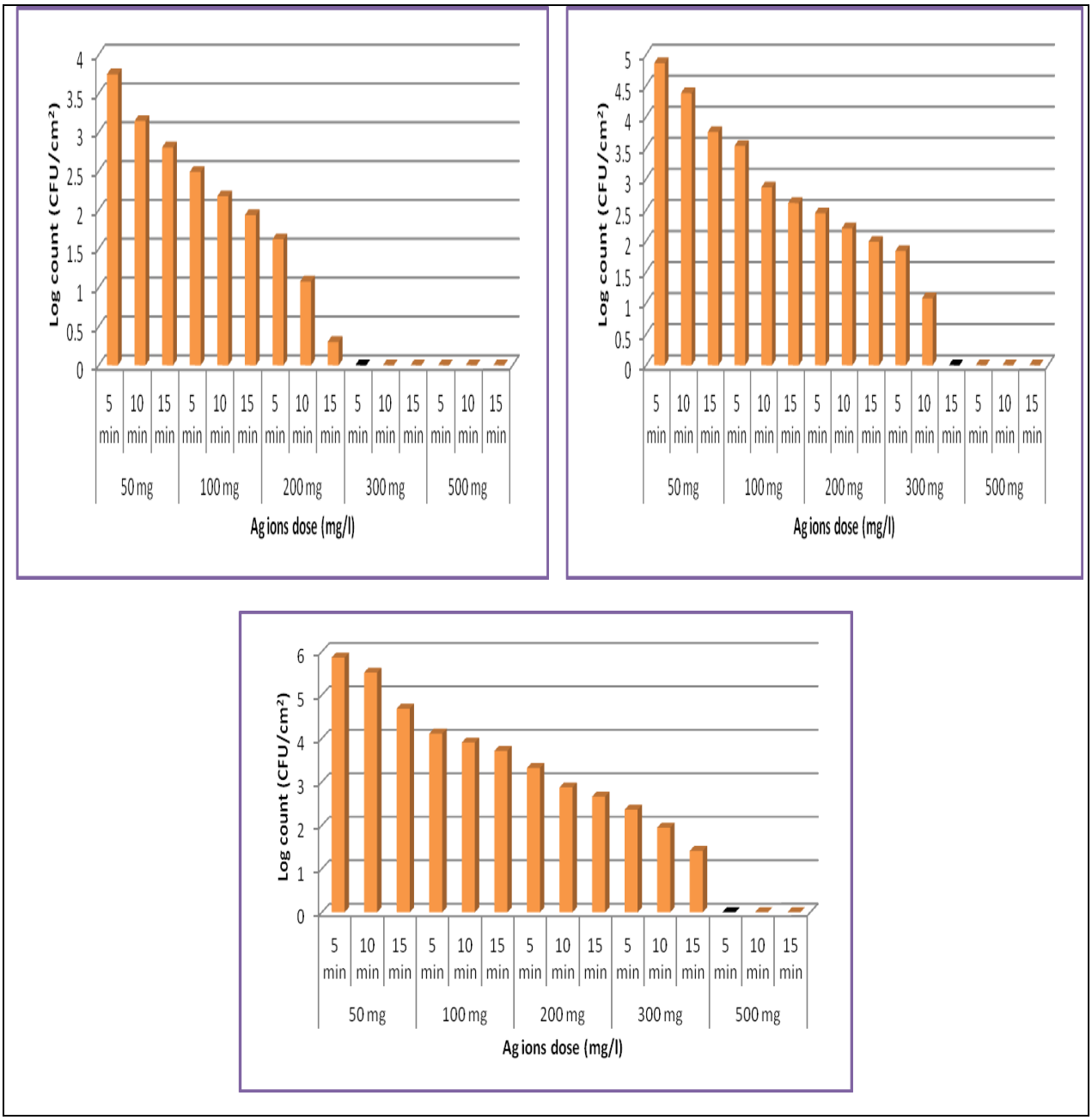

Fig. 74. Antibiofilm effect and MIC of different doses of Ag ions against 10, 40 and 90 days-old *L. monocytogenes* biofilm grown on I pipe material (left- right).

Table 70. Determination of antibiofilm effect and MIC for different doses of Ag ions against three ages of *L. monocytogenes* biofilm grown on Cu pipe material.

Ag ions doses (mg/l)	Contact time (min)	CFU/cm^2 The ages of *L. monocytogenes* biofilm grown on Cu pipe material								
		10 days-old			40 days-old			90 days-old		
		Count	Log count	Log reduction	Count	Log count	Log reduction	Count	Log count	Log reduction
Control		$3.1x10^6$	6.612	0	$2.7x10^6$	6.431	0	$2.1x10^6$	6.322	0
50	5	$3.2x10^2$	2.505	4.107	$4.6x10^3$	3.662	2.768	$4.3x10^2$	2.633	3.688
	10	$1.6x10^2$	2.204	4.408	$1.6x10^2$	2.204	4.227	$1.9x10^2$	2.278	4.043
	15	9.1x10	1.959	4.653	9.1x10	1.959	4.472	8.6x10	1.934	4.387
100	5	6.3x10	1.799	4.813	6.3x10	1.799	4.632	3.7x10	1.568	4.754
	10	2.3x10	1.361	5.251	2.3x10	1.361	5.069	1.2x10	1.079	5.243
	15	1.1x10	1.041	5.571	1.1x10	1.041	5.389	ND	ND	6.322
200	5	ND	ND	6.612	ND	ND	6.431	ND	ND	6.322
	10	ND	ND	6.612	ND	ND	6.431	ND	ND	6.322
	15	ND	ND	6.612	ND	ND	6.431	ND	ND	6.322
300	5	ND	ND	6.612	ND	ND	6.431	ND	ND	6.322
	10	ND	ND	6.612	ND	ND	6.431	ND	ND	6.322
	15	ND	ND	6.612	ND	ND	6.431	ND	ND	6.322
500	5	ND	ND	6.612	ND	ND	6.431	ND	ND	6.322
	10	ND	ND	6.612	ND	ND	6.431	ND	ND	6.322
	15	ND	ND	6.612	ND	ND	6.431	ND	ND	6.322

ND: Not Detected.

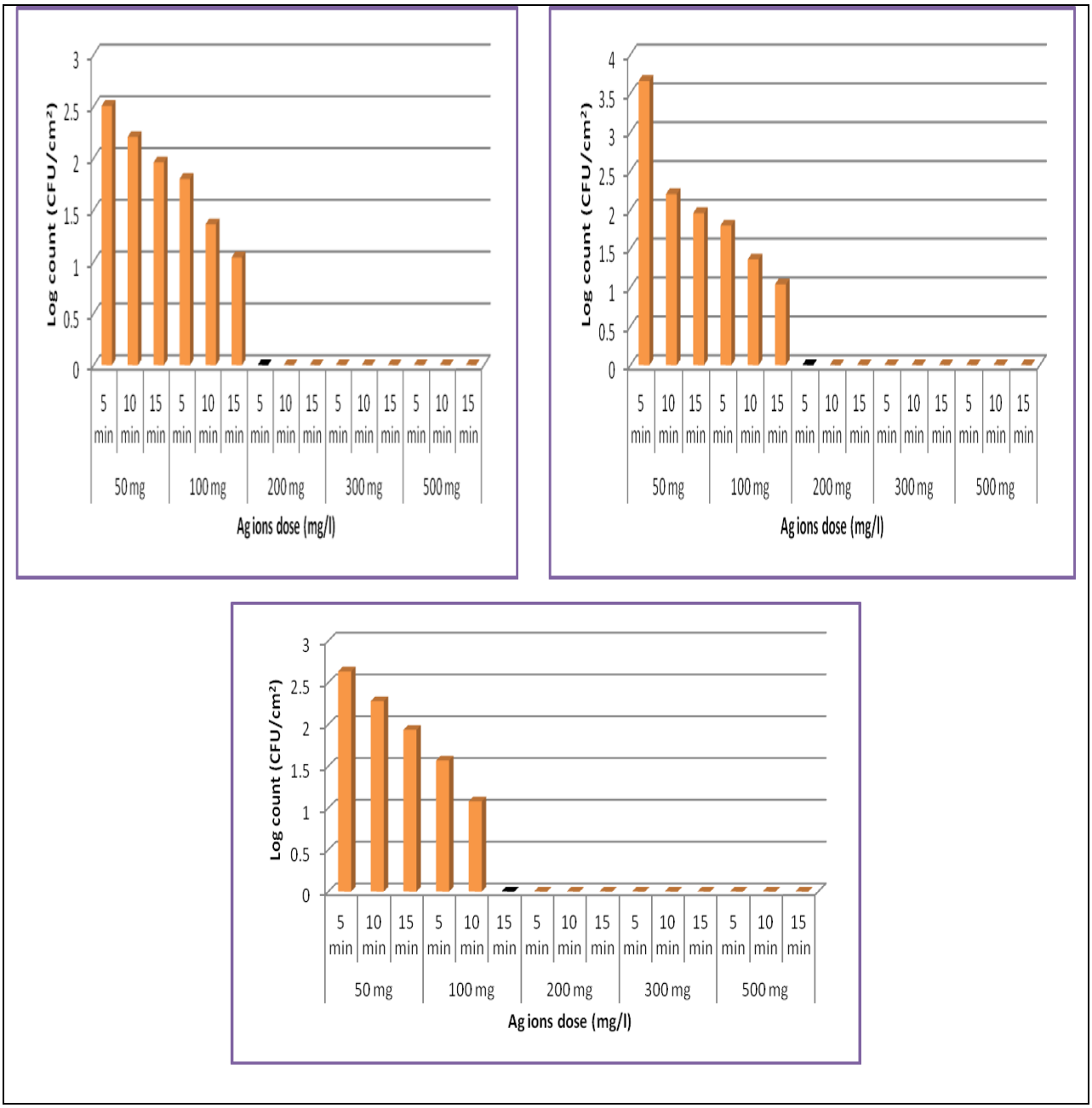

Fig. 75. Antibiofilm effect and MIC of different doses of Ag ions against 10, 40 and 90 days-old *L. monocytogenes* biofilm grown on Cu pipe material (left- right).

Table 71. Determination of antibiofilm effect and MIC for different doses of Ag ions against three ages of *L. monocytogenes* biofilm grown on R pipe material.

Ag ions doses (mg/l)	Contact time (min)	CFU/cm^2 The ages of *L. monocytogenes* biofilm grown on R pipe material								
		10 days-old			40 days-old			90 days-old		
		Count	Log count	Log reduction	Count	Log count	Log reduction	Count	Log count	Log reduction
Control		$4.9x10^6$	6.690	0	$6.4x10^6$	6.806	0	$3.7x10^6$	6.568	0
50 mg	5	$2.7x10^2$	2.431	4.258	$2.5x10^3$	3.397	3.408	$6.7x10^4$	4.826	1.742
	10	$1.3x10^2$	2.113	4.576	$4.6x10^2$	2.662	4.143	$2.1x10^4$	4.322	2.245
	15	8.3x10	1.919	4.771	$2.3x10^2$	2.361	4.444	$4.6x10^3$	3.662	2.905
100	5	4.7x10	1.672	5.018	1.1x10	2.041	4.764	$5.4x10^2$	2.732	3.835
	10	2.9x10	1.462	5.227	8.4x10	1.924	4.881	$2.9x10^2$	2.462	4.105
	15	1.7x10	1.230	5.459	5.1x10	1.707	5.098	$1.7x10^2$	2.230	4.337
200	5	9	0.954	5.735	3.2x10	1.505	5.301	9.1x10	1.959	4.609
	10	ND	ND	6.690	1.1x10	1.041	5.764	6.5x10	1.812	4.755
	15	ND	ND	6.690	ND	ND	6.806	1.4x10	1.146	5.422
300	5	ND	ND	6.690	ND	ND	6.806	ND	ND	6.568
	10	ND	ND	6.690	ND	ND	6.806	ND	ND	6.568
	15	ND	ND	6.690	ND	ND	6.806	ND	ND	6.568
500	5	ND	ND	6.690	ND	ND	6.806	ND	ND	6.568
	10	ND	ND	6.690	ND	ND	6.806	ND	ND	6.568
	15	ND	ND	6.690	ND	ND	6.806	ND	ND	6.568

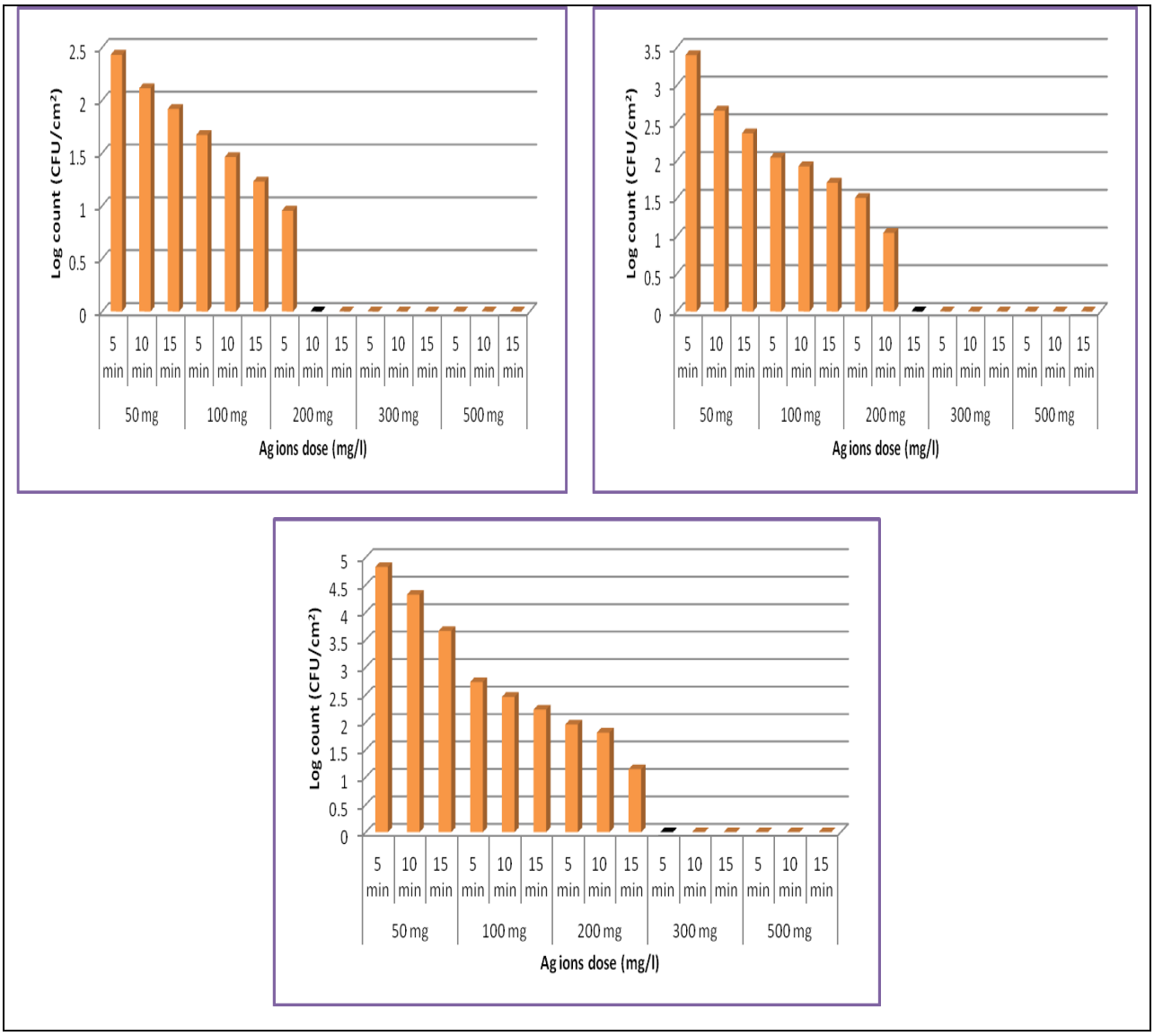

Fig. 76. Antibiofilm effect and MIC of different doses of Ag ions against 10, 40 and 90 days-old *L. monocytogenes* biofilm grown on Cu pipe material (left- right).

b. Comparative susceptibility of planktonic cells and three different ages of *S.* Typhimurium biofilm to silver ions (Ag ions)

1. Antibiofilm activity and MIC of Ag ions against *S.* Typhimurium planktonic cells

As shown in Table (72) the Ag ions dose which able to complete removal of planktonic cells of *S.* Typhimurium was 100 mg at 5 min and 200, 300 and 500 mg/l. While, the concentration of Ag ions (50 mg) was not able to completely reduce of palnktonic cells. So, the suitable effective dose at low contact time was recorded in100 mg at 5 min.

The statistical analysis results found that an extremely significant ($P \leq 0.05$) between the different doses of Ag ions and the counts of planktonic cells.

Table 72. Determination of antibiofilm effect and MIC for different doses of Ag ions against *S.* Typhimurium planktonic cells.

Contact time (min)	Doses of Ag ions (mg/l)				
	50	**100**	**200**	**300**	**500**
Zero time	$2.8x10^6$	$2.8x10^6$	$2.8x10^6$	$2.8x10^6$	$2.8x10^6$
5	$1.5x10^2$	ND	ND	ND	ND
10	2.8x10	ND	ND	ND	ND
15	6	ND	ND	ND	ND

ND= Not detected

2. Antibiofilm activity and MIC of Ag ions against *S.* Typhimurium biofilm grown on PVC pipe material

The results shown in Table (73) and Fig. (77) explained, the MIC of Ag ions for 10, 40 and 90 days-old *S.* Typhimurium biofilm grown on PVC pipe material was 200 mg at 5 min, 200 mg at 15 and 300 mg at 5 min, respectively.

The results of statistical analysis (Homogeneity test of variances from one way *ANOVA*), it was found a deeply significant correlation ($P \leq 0.05$) between the different doses of Ag ions and biofilm ages grown on PVC pipe material.

3. Antibiofilm activity and MIC of Ag ions against *S.* Typhimurium biofilm grown on PP pipe material

The data represented in Table (74) and Fig. (78) found, the MIC of Ag ions for 10, 40 and 90 days-old *S.* Typhimurium biofilm grown on PP pipe material were 100 mg at 15 min, 200 mg at 10 min and 200 mg at 15 min, respectively.

The results of statistical analysis, explained that, there was a greatly significant correlation ($P \leq 0.05$) between the different doses of Ag ions and biofilm ages grown on PP pipe material.

4. Antibiofilm activity and MIC of Ag ions against *S.* Typhimurium biofilm grown on PE pipe material

In case of *S.* Typhimurium biofilm grown on PE pipe material, data presented in Table (75) and Fig. (79) demonstrated, the MIC of Ag ions for 10, 40 and 90 days-old of biofilm were 200 mg at 5 min, 200 mg at 15 and 300 mg at 5 min, respectively.

From statistical analysis results, it was revealed that, there was a greatly significant correlation ($P \leq 0.05$) between the different doses of Ag ions and biofilm ages grown on PE pipe material.

Table 73. Determination of antibiofilm effect and MIC for different doses of Ag ions against three ages of *S.* Typhimurium biofilm grown on PVC pipe material.

Ag ions doses (mg/l)	Contact time (min)	CFU/cm^2 The ages of *S.* Typhimurium biofilm grown on PVC pipe material								
		10 days-old			40 days-old			90 days-old		
		Count	Log count	Log reduction	Count	Log count	Log reduction	Count	Log count	Log reduction
Control		$4.6x10^6$	6.662	0	$2.6x10^6$	6.414	0	$3.4x10^6$	6.531	0
50	5	$4.6x10^2$	4	4	$4.1x10^3$	3.612	2.802	$4.9x10^4$	4.690	1.841
	10	$1.2x10^2$	4.583	4.583	$1.8x10^3$	3.255	3.159	$1.4x10^4$	4.146	2.385
	15	8.1x10	4.754	4.754	$7.4x10^2$	2.869	3.545	$7.3x10^3$	3.863	2.668
100	5	4.5x10	5.009	5.009	$3.4x10^2$	2.531	3.883	$2.4x10^3$	3.380	3.151
	10	2.3x10	5.301	5.301	$1.1x10^2$	2.041	4.373	$8.2x10^2$	2.913	3.617
	15	9	5.708	5.708	8.3x10	1.919	4.495	$5.6x10^2$	2.748	3.783
200	5	ND	6.662	6.662	4.5x10	1.653	4.761	$2.4x10^2$	2.380	4.151
	10	ND	6.662	6.662	1.9x10	1.278	5.136	8.6x10	1.934	4.596
	15	ND	6.662	6.662	ND	ND	6.414	3.2x10	1.505	5.026
300	5	ND	6.662	6.662	ND	ND	6.414	ND	ND	5.026
	10	ND	6.662	6.662	ND	ND	6.414	ND	ND	5.026
	15	ND	6.662	6.662	ND	ND	6.414	ND	ND	5.026
500	5	ND	6.662	6.662	ND	ND	6.414	ND	ND	5.026
	10	ND	6.662	6.662	ND	ND	6.414	ND	ND	5.026
	15	ND	6.662	6.662	ND	ND	6.414	ND	ND	5.026

ND: Not Detected.

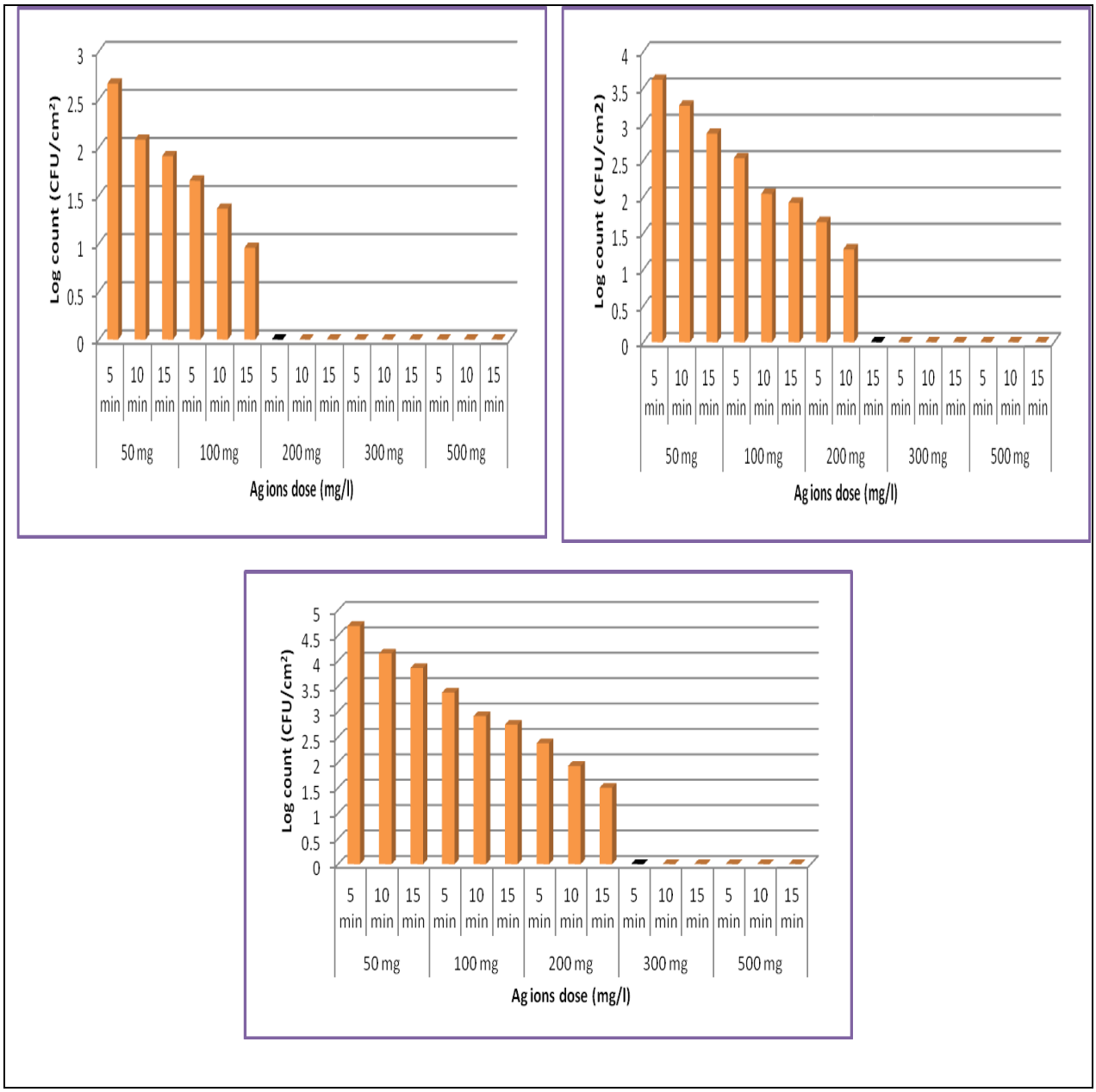

Fig. 77. Antibiofilm effect and MIC of different doses of Ag ions against 10, 40 and 90 days-old *S.* Typhimurium biofilm grown on PVC pipe material (left- right).

Table 74. Determination of antibiofilm effect and MIC for different doses of Ag ions against three ages of *S.* Typhimurium biofilm grown on PP pipe material.

Ag ions doses (mg/l)	Contact time (min)	CFU/cm^2 The ages of *S.* Typhimurium biofilm grown on PP pipe material								
		10 days-old			40 days-old			90 days-old		
		Count	Log count	Log reduction	Count	Log count	Log reduction	Count	Log count	Log reduction
Control		$3.2x10^6$	6.505	0	$3.8x10^6$	6.579	0	$2.9x10^6$	6.462	0
50	**5**	$1.8x10^2$	2.255	4.249	$3.5x10^3$	3.544	3.035	$7.5x10^3$	3.875	2.587
	10	9.6x10	1.982	4.522	$9.4x10^2$	2.973	3.606	$4.8x10^3$	3.681	2.781
	15	6.1x10	1.785	4.719	$5.3x10^2$	2.724	3.855	$8.1x10^2$	2.908	3.553
100	**5**	2.9x10	1.462	5.042	$2.4x10^2$	2.380	4.199	$5.4x10^2$	2.732	3.730
	10	1.2x10	1.079	5.425	9.5x10	1.977	4.602	$2.9x10^2$	2.462	4
	15	ND	ND	6.505	5.2x10	1.716	4.863	$1.5x10^2$	2.176	4.286
200	**5**	ND	ND	6.505	2.7x10	1.431	5.148	8.6x10	1.934	4.527
	10	ND	ND	6.505	ND	ND	6.579	2.4x10	1.380	5.082
	15	ND	ND	6.505	ND	ND	6.579	ND	ND	6.462
300	**5**	ND	ND	6.505	ND	ND	6.579	ND	ND	6.462
	10	ND	ND	6.505	ND	ND	6.579	ND	ND	6.462
	15	ND	ND	6.505	ND	ND	6.579	ND	ND	6.462
500	**5**	ND	ND	6.505	ND	ND	6.579	ND	ND	6.462
	10	ND	ND	6.505	ND	ND	6.579	ND	ND	6.462
	15	ND	ND	6.505	ND	ND	6.579	ND	ND	6.462

ND: Not Detected.

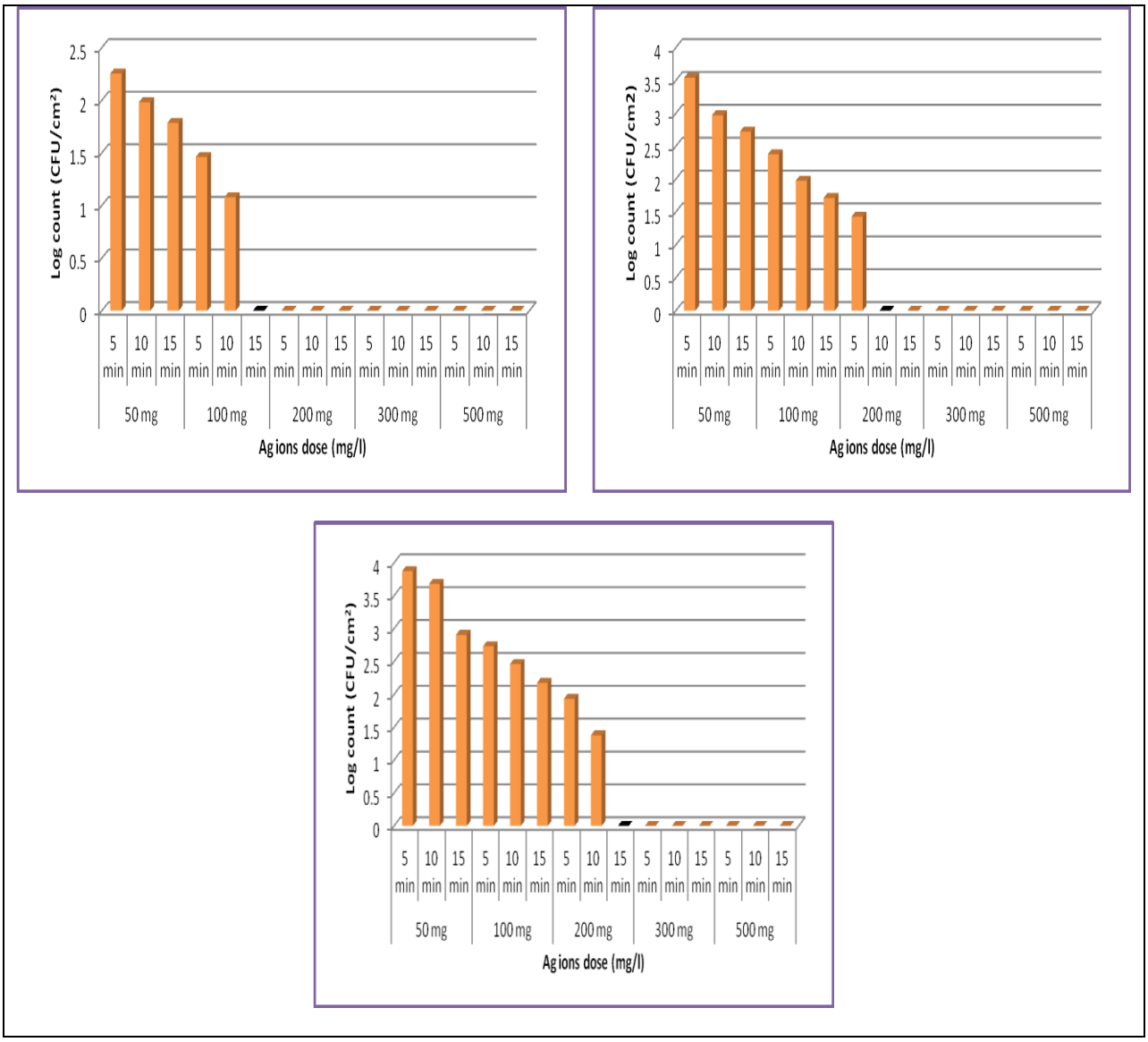

Fig. 78. Antibiofilm effect and MIC of different doses of Ag ions against 10, 40 and 90 days-old *S.* Typhimurium biofilm grown on PP pipe material (left- right).

5. Antibiofilm activity and MIC of Ag ions against *S.* Typhimurium biofilm grown on I pipe material

Concerning, *S.* Typhimurium biofilm grown on I pipe material, data presented in Table (76) and Fig. (80) demonstrated this, the MIC of Ag ions for 10, 40 and 90 days-old of biofilm were 200 mg at 10 min, 300 mg at 5 and 300 mg at 15 min, respectively. Statistically, the obtained results demonstrated, there was a deeply significant correlation between ($P \leq 0.05$) the different doses of Ag ions and biofilm ages grown on I pipe material.

6. Antibiofilm activity and MIC of Ag ions against *S.* Typhimurium biofilm grown on Cu pipe material

The results of *S.* Typhimurium biofilm grown on Cu pipe material, it was revealed real the MIC of Ag ions for 10, 40 and 90 days-old of biofilm were 100 mg at 15 min, 200 mg at 10 and 200 mg at 5 min, respectively (Table 77; Fig. 81).

The results of statistical analysis, it was found that, there was a greatly significant correlation between ($P \leq 0.05$) the different doses of Ag ions and biofilm ages grown on Cu pipe material.

7. Antibiofilm activity and MIC of Ag ions against *S.* Typhimurium biofilm grown on R pipe material

As shown in Table (78) and Fig. (82), The obtained results of *S.* Typhimurium biofilm grown on R pipe material, it was found the MIC of Ag ions for 10, 40 and 90 days-old of biofilm were 200 mg at 5 min, 200 mg at 15 and 300 mg at 5 min, respectively.

From the obtained results represented in this study, it was found that, all used Ag ions doses (50, 100, 200, 300 and 500 mg/l) were toxic. In addition to, 50 mg/l cannot cause any inhibition for both planktonic and biofilm cells.

The antimicrobial properties of silver compounds and silver in its ionic form (Ag^{+}) have been applied in a wide range of applications including water systems (Silvestry-Rodriguez *et al.*, 2007). Exposure to silver is considered safe for humans; yet, a standard of 0.1 mg/l of soluble silver is allowed in drinking water (WHO, 2004). Moreover, most strains of bacteria have not yet developed resistance to Ag ions (Silver, 2003). Also (Harrison *et al.*, 2004) found that, Ag ions to be the most toxic of 17 different metals tested for the eradication of biofilm while screening various microorganisms, among them, *Pseudomonas aeruginosa*. Many studies have demonstrated superior performance of silver nanoparticles (AgNPs) over Ag ions in controlling the growth and activity of various microorganisms (Lok *et al.*, 2006) and AgNPs have been shown to inactivate planktonic *Escherichia coli* in aqueous suspensions (Dror-Ehre *et al.*, 2009).

Table 75. Determination of antibiofilm effect and MIC for different doses of Ag ions against three ages of *S.* Typhimurium biofilm grown on PE pipe material.

Ag ions doses (mg/l)	Contact time (min)	CFU/cm^2 The ages of *S.* Typhimurium biofilm grown on PE pipe material								
		10 days-old			40 days-old			90 days-old		
		Count	Log count	Log reduction	Count	Log count	Log reduction	Count	Log count	Log reduction
Control		$1.5x10^6$	6.176	0	$1.4x10^6$	6.146	0	$4.2x10^6$	6.623	0
50	5	$3.4x10^2$	2.531	3.644	$2.9x10^3$	3.462	2.683	$1.7x10^4$	4.230	2.392
	10	$1.0x10^2$	2	4.176	$1.1x10^3$	3.041	3.104	$7.2x10^3$	3.857	2.765
	15	7.8x10	1.892	4.283	$5.2x10^2$	2.716	3.430	$4.9x10^3$	3.690	2.933
100	5	3.2x10	1.505	4.670	$1.6x10^2$	2.204	3.942	$1.2x10^3$	3.079	3.544
	10	1.7x10	1.230	4.945	8.7x10	1.939	4.206	$8.5x10^2$	2.929	3.693
	15	3	0.477	5.698	4.9x10	1.690	4.455	$5.3x10^2$	2.724	3.898
200	5	ND	ND	6.176	2.6x10	1.414	4.731	$1.2x10^2$	2.079	4.544
	10	ND	ND	6.176	1.3x10	1.113	5.032	7.3x10	1.863	4.759
	15	ND	ND	6.176	ND	ND	6.146	2.8x10	1.447	5.176
300	5	ND	ND	6.176	ND	ND	6.146	ND	ND	6.623
	10	ND	ND	6.176	ND	ND	6.146	ND	ND	6.623
	15	ND	ND	6.176	ND	ND	6.146	ND	ND	6.623
500	5	ND	ND	6.176	ND	ND	6.146	ND	ND	6.623
	10	ND	ND	6.176	ND	ND	6.146	ND	ND	6.623
	15	ND	ND	6.176	ND	ND	6.146	ND	ND	6.623

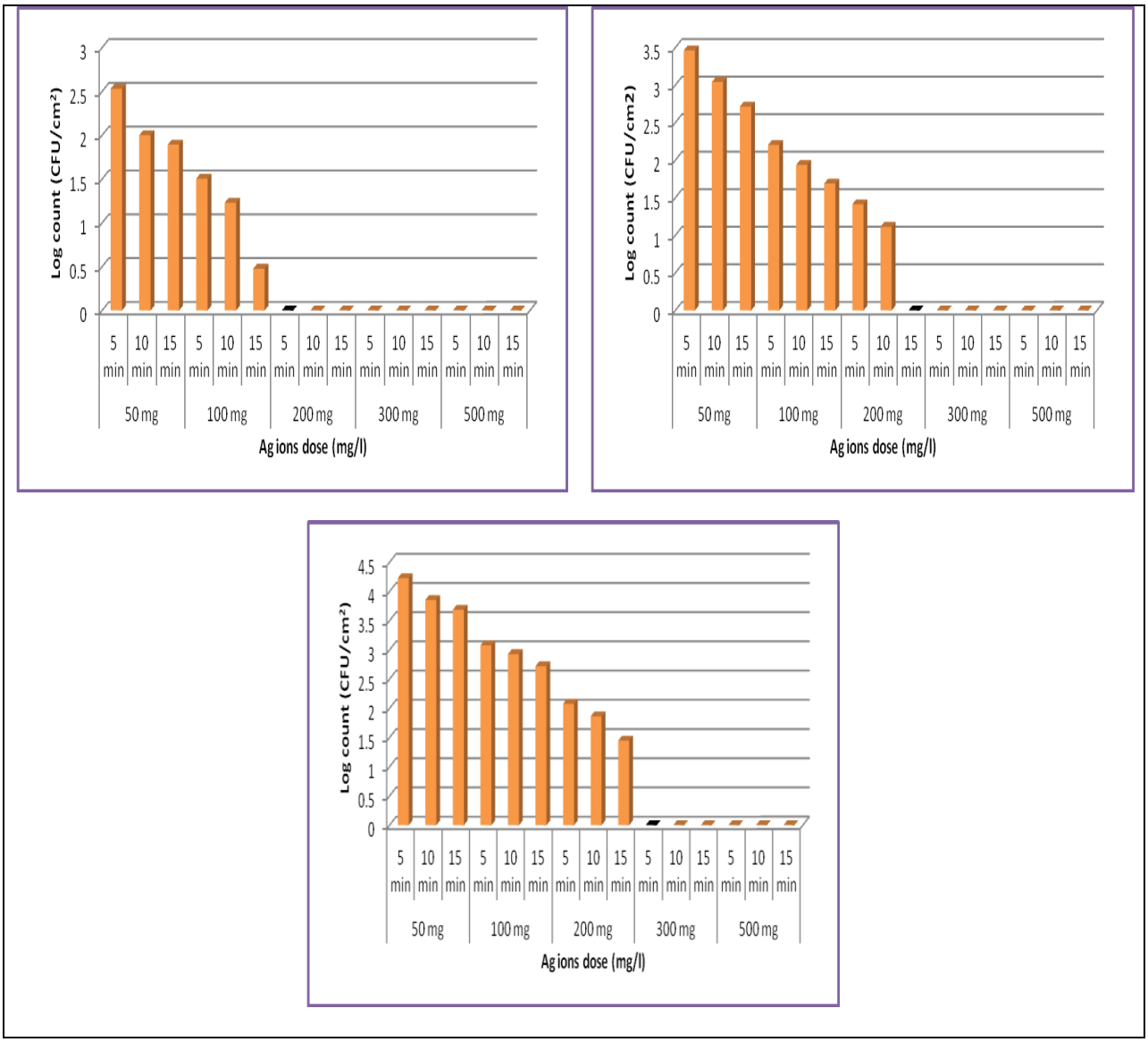

Fig. 79. Antibiofilm effect and MIC of different doses of Ag ions against 10, 40 and 90 days-old *S.* Typhimurium biofilm grown on PE pipe material (left- right).

Table 76. Determination of antibiofilm effect and MIC for different doses of Ag ions against three ages of *S.* Typhimurium biofilm grown on I pipe material.

Ag ions doses (mg/l)	Contact time (min)	CFU/cm^2 The ages of *S.* Typhimurium biofilm grown on I pipe material								
		10 days-old			40 days-old			90 days-old		
		Count	Log count	Log reduction	Count	Log count	Log reduction	Count	Log count	Log reduction
Control		5.2x10^6	6.716	0	5.2x10^6	6.716	0	3.1x10^6	6.491	0
50	5	3.5x10^3	3.544	3.171	3.1x10^4	4.491	2.224	2.0x10^5	5.301	1.190
	10	7.2x10^2	2.857	3.858	1.7x10^4	4.230	2.485	6.2x10^4	4.792	1.698
	15	4.6x10^2	2.662	4.053	5.7x10^3	3.755	2.960	2.9x10^4	4.462	2.028
100	5	2.3x10^2	2.361	4.354	2.3x10^3	3.361	3.354	8.1x10^3	3.908	2.582
	10	1.4x10^2	2.146	4.569	7.1x10^2	2.851	3.864	4.3x10^3	3.633	2.857
	15	7.9x10	1.897	4.818	4.3x10^2	2.633	4.082	1.8x10^3	3.255	3.236
200	5	2.5x10	1.397	5.318	2.1x10^2	2.322	4.393	6.5x10^2	2.812	3.678
	10	ND	ND	6.716	9.3x10	1.968	4.747	2.7x10^2	2.431	4.059
	15	ND	ND	6.716	3.2x10	1.505	5.210	1.3x10^2	2.113	4.377
300	5	ND	ND	6.716	ND	ND	6.716	5.8x10	1.763	4.727
	10	ND	ND	6.716	ND	ND	6.716	7	0.845	5.646
	15	ND	ND	6.716	ND	ND	6.716	ND	ND	6.491
500	5	ND	ND	6.716	ND	ND	6.716	ND	ND	6.491
	10	ND	ND	6.716	ND	ND	6.716	ND	ND	6.491
	15	ND	ND	6.716	ND	ND	6.716	ND	ND	6.491

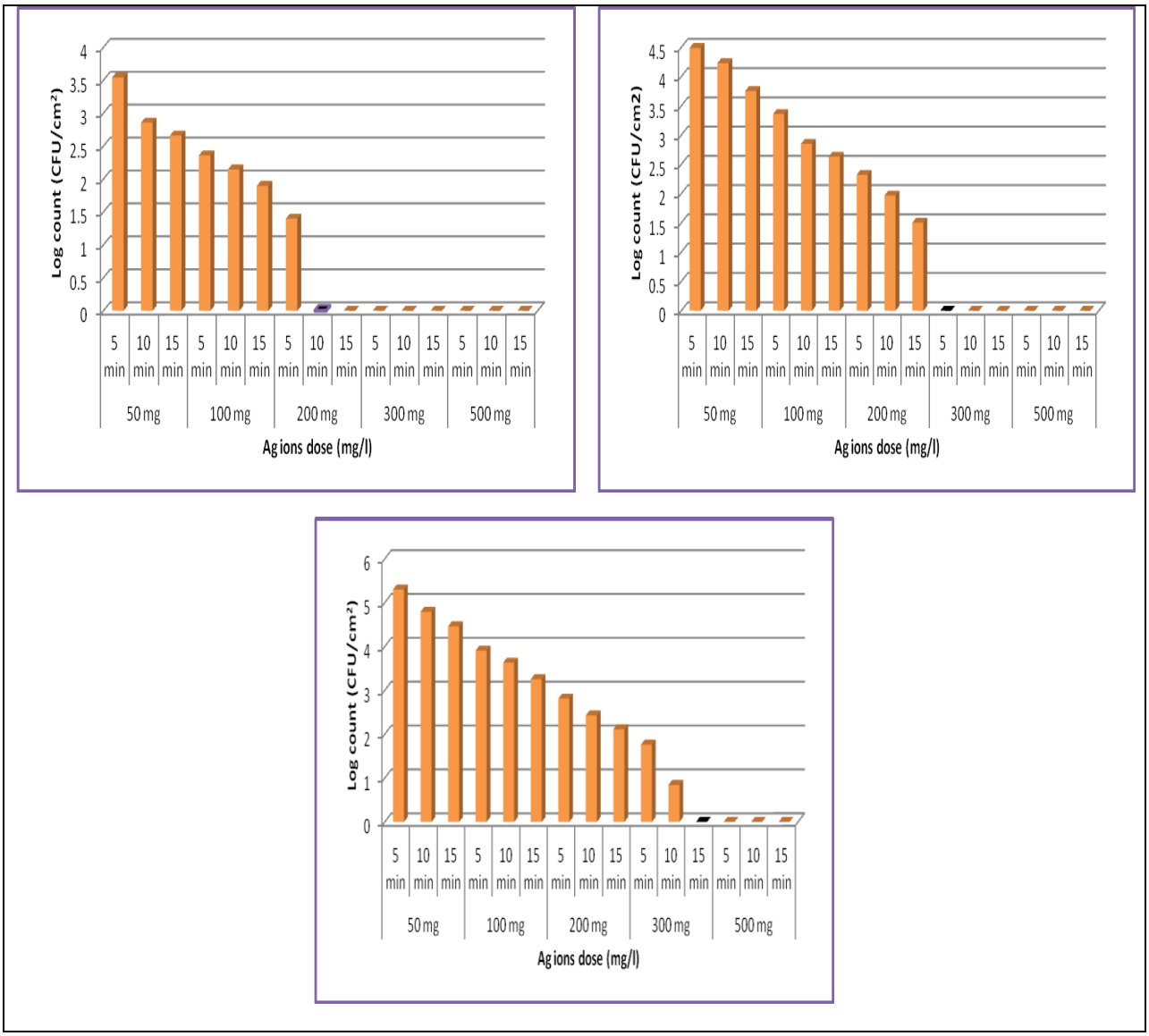

Fig. 80. Antibiofilm effect and MIC of different doses of Ag ions against 10, 40 and 90 days-old *S.* Typhimurium biofilm grown on I pipe material (left- right).

Table 77. Determination of antibiofilm effect and MIC for different doses of Ag ions against three ages of *S.* Typhimurium biofilm grown on Cu pipe material.

Ag ions doses (mg/l)	Contact time (min)	CFU/cm^2 The ages of *S.* Typhimurium biofilm grown on Cu pipe material								
		10 days-old			40 days-old			90 days-old		
		Count	Log count	Log reduction	Count	Log count	Log reduction	Count	Log count	Log reduction
Control		$2.9x10^6$	6.462	0	$4.3x10^6$	6.633	0	$4.6x10^6$	6.662	0
50	5	$2.1x10^2$	2.322	4.140	$1.6x10^3$	3.204	3.429	$6.2x10^2$	2.792	3.870
	10	9.8x10	1.991	4.471	$5.2x10^2$	2.716	3.917	$2.9x10^2$	2.462	4.200
	15	7.5x10	1.875	4.587	$1.1x10^2$	2.041	4.592	$1.2x10^2$	2.079	4.583
100	5	4.2x10	1.623	4.839	7.2x10	1.857	4.776	7.4x10	1.869	4.793
	10	1.9x10	1.278	5.183	4.3x10	1.633	5	3.5x10	1.724	4.938
	15	ND	ND	6.462	2.6x10	1.414	5.218	1.4x10	1.146	5.516
200	5	ND	ND	6.462	8	0.903	5.730	ND	ND	6.662
	10	ND	ND	6.462	ND	ND	6.633	ND	ND	6.662
	15	ND	ND	6.462	ND	ND	6.633	ND	ND	6.662
300	5	ND	ND	6.462	ND	ND	6.633	ND	ND	6.662
	10	ND	ND	6.462	ND	ND	6.633	ND	ND	6.662
	15	ND	ND	6.462	ND	ND	6.633	ND	ND	6.662
500	5	ND	ND	6.462	ND	ND	6.633	ND	ND	6.662
	10	ND	ND	6.462	ND	ND	6.633	ND	ND	6.662
	15	ND	ND	6.462	ND	ND	6.633	ND	ND	6.662

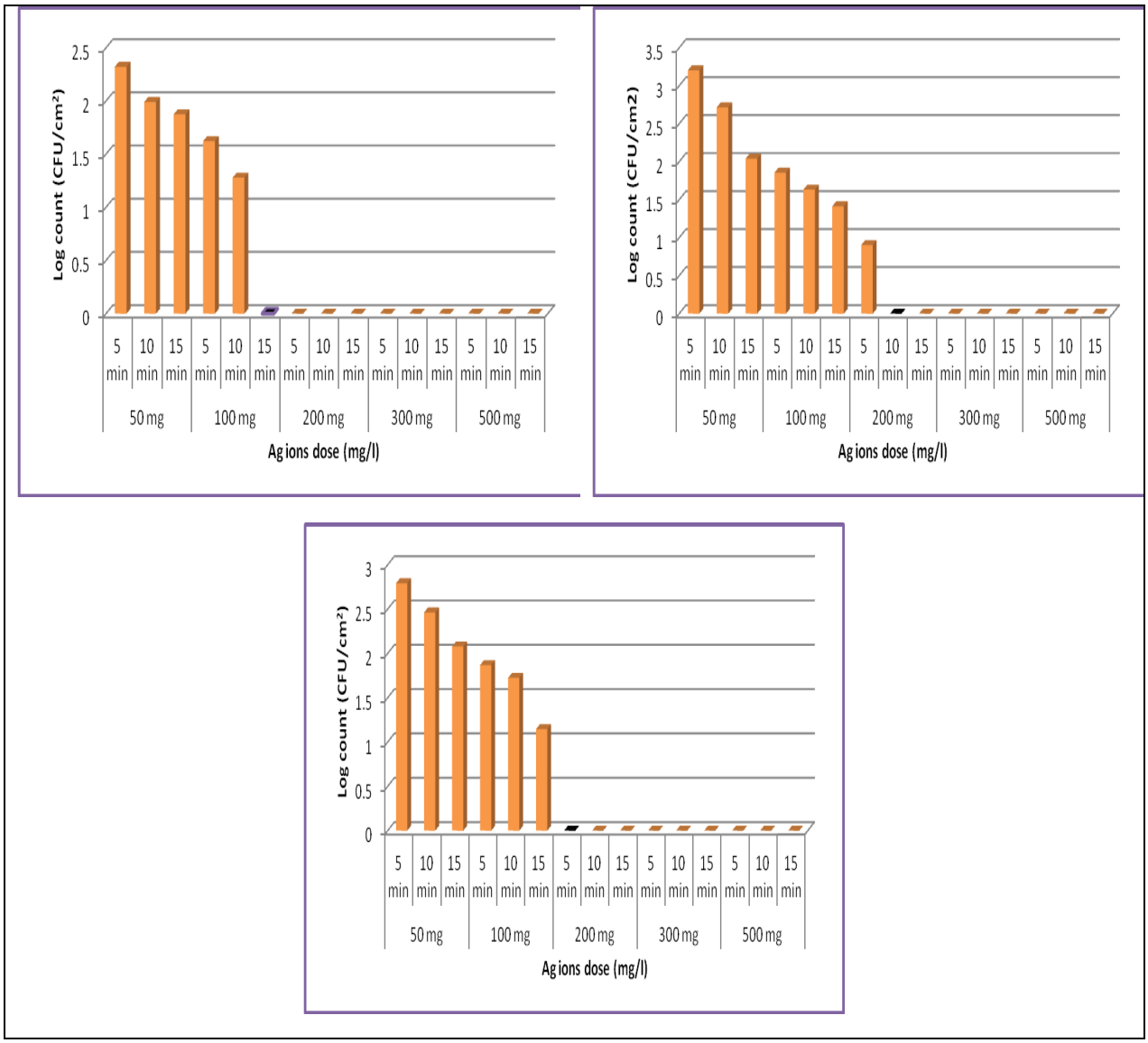

Fig. 81. Antibiofilm effect and MIC of different doses of Ag ions against 10, 40 and 90 days-old *S.* Typhimurium biofilm grown on Cu pipe material (left- right).

Table 78. Determination of antibiofilm effect and MIC for different doses of Ag ions against three ages of *S.* Typhimurium biofilm grown on R pipe material.

Ag ions doses (mg/l)	Contact time (min)	CFU/cm^2 The ages of *S.* Typhimurium biofilm grown on R pipe material								
		10 days-old			40 days-old			90 days-old		
		Count	Log count	Log reduction	Count	Log count	Log reduction	Count	Log count	Log reduction
Control		$3.8x10^6$	6.579	0	$2.9x10^6$	6.462	0	$5.3x10^6$	6.724	0
50	5	$4.7x10^2$	2.672	3.907	$3.2x10^3$	3.505	2.957	$2.8x10^4$	4.447	2.277
	10	$1.1x10^2$	2.041	4.538	$1.0x10^3$	3	3.462	$4.6x10^3$	3.662	3.061
	15	8.3x10	1.919	4.660	$6.4x10^2$	2.806	3.656	$1.7x10^3$	3.230	3.493
100	5	5.6x10	1.748	4.831	$2.7x10^2$	2.431	4.031	$9.1x10^2$	2.959	3.765
	10	2.3x10	1.361	5.218	1.5x10	2.176	4.286	$6.5x10^2$	2.812	3.911
	15	8	0.903	5.676	9.6x10	1.982	4.480	$2.3x10^2$	2.361	4.362
200	5	ND	ND	6.579	7.3x10	1.863	4.599	8.5x10	1.929	4.794
	10	ND	ND	6.579	2.8x10	1.447	5.015	5.1x10	1.707	5.016
	15	ND	ND	6.579	ND	ND	6.462	1.9x10	1.278	5.445
300	5	ND	ND	6.579	ND	ND	6.462	ND	ND	6.724
	10	ND	ND	6.579	ND	ND	6.462	ND	ND	6.724
	15	ND	ND	6.579	ND	ND	6.462	ND	ND	6.724
500	5	ND	ND	6.579	ND	ND	6.462	ND	ND	6.724
	10	ND	ND	6.579	ND	ND	6.462	ND	ND	6.724
	15	ND	ND	6.579	ND	ND	6.462	ND	ND	6.724

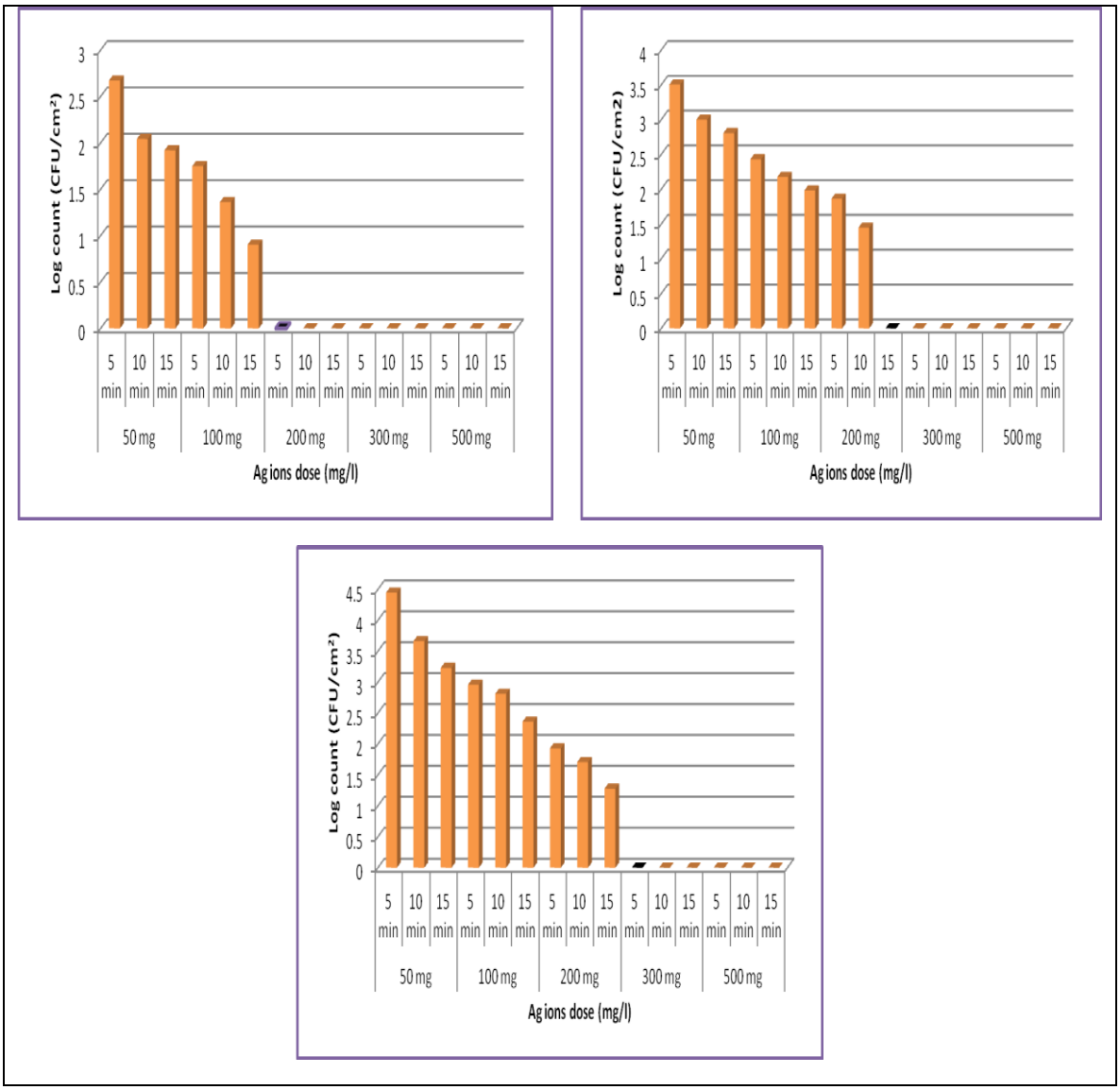

Fig. 82. Antibiofilm effect and MIC of different doses of Ag ions against 10, 40 and 90 days-old *S.* Typhimurium biofilm grown on R pipe material (left- right).

The inhibitory effect of silver is probably the sum of distinct mechanisms of action. A number of studies suggest that silver ions react with sulfhydryl (SH) groups of proteins (Feng *et al.,* 2000) and play an essential role in bacterial inactivation (Morones *et al.,*2005). Micromolar levels of silver ions have been reported to uncouple respiratory electron transport from oxidative phosphorylation, which inhibits respiratory chain enzymes or interferes with membrane permeability to protons and phosphate. Additionally, several studies conducted by Feng *et al.*(2000) and by Jung *et al.*(2008) have shown the activity of silver ions on *Escherichia coli* (Gram-negative) and

Staphylococcus aureus (Gram-positive). Also, Feng *et al.* (2000) found the treated these bacteria with $AgNO_3$ and studied the effects on cell morphology using combined electron microscopy (TEM and SEM) and X-ray microanalyses. *E. coli* and *S. aureus* underwent similar morphological changes after silver ion treatment characterized by a cytoplasm membrane detachment from cell walls and the appearance of an electron-light region in the center of the cells, which contained condensed deoxyribonucleic acid (DNA) molecules probably formed to protect DNA from injuries mediated by the silver ions. Small electron-dense granules either surrounding the cell wall or deposited inside the cells were also present (Feng *et al.*, 2000).

Accordingly, Jung *et al.* (2008) reported results corroborating the morphological changes described by Feng *et al.*(2000) and also suggested that in the presence of silver ions, bacterial cells reach an active but non-culturable state and eventually die. Also, Jung *et al.* (2008) suggested that the thickness of the peptidoglycan layer of gram-positive bacteria may prevent to some extent, the action of the silver ions, since they found a higher inhibitory activity of silver ion solution against *E. coli* than against *Staph. aureus.* In addition to this, silver ions and silver nanoparticles also have inhibitory and lethal effects on bacterial species such as *E. coli*, *Staph. aureus* and even yeast (Kim *et al.*, 2007).

3. Efficacy of silver nanoparticles (AgNPs) against *L. monocytogenes* and *S.* Typhimurium biofilm grown on domestic plumping materials

Another preventative and promising strategy suggests the control of biofilm with an aqueous suspension of silver nanoparticles as a pretreatment in water systems prior to the main treatment units, such as membrane filtration, It was not meant as a treatment for the eradication of existing or mature biofilm or as a disinfection process. The pretreatment with molecularly-capped silver nanoparticles was able to control or retard biofilm formation on pipe surfaces (Dror-Ehre *et al.*, 2010).

Silver-ions have been reported to possess strong biocidal effects (Singh *et al.*, 2008). The silver-compounds are used as disinfection agents from the ancient time. The silver in the form of nanoparticles (AgNPs) is effective against bacteria resistant to

antibiotics, as well as against fungi and viruses. The antibacterial mechanisms of silver ion action have been studied for a long time; however, they are only partially understood. Mechanisms of the AgNPs action on bacteria are even less clear. Silver ions can interact with the bases in DNA, rather than with the phosphate groups, and affect the DNA ability to replicate (Feng *et al.*, 2000). It was assumed that AgNPs can release silver-ions and this mechanism plays a significant role in AgNPs antimicrobial effects. It was reported that AgNPs possess antimicrobial effect without ions release (Lubick *et al.*, 2008).

AgNPs can penetrate the cell membrane as reported by electron microscopy (Gogoi *et al.*, 2006). It was recently suggested that AgNPs have a pleiotropic action on bacterial cells. They attach to the cell membrane surface and disturb its permeability by modifying the cell potential and inhibiting cell respiration. AgNPs/silver cations bind to thiol groups of bacteria proteins disturbing their activity and leading to cell death (Nadtochenko *et al.*, 2010).

Therefore, this section of the present study was carried out to determine the antibiofilm activity to five doses (50, 100, 200, 300 and 500 mg/l) of AgNPs at three different contact times (5, 10 and 15 min). Six biofilm samples and planktonic cells of tested microorganisms were exposed to these doses. As shown in Fig. (83), TEM micrographs showed that, the diameter of the particles which containing of silver ions.

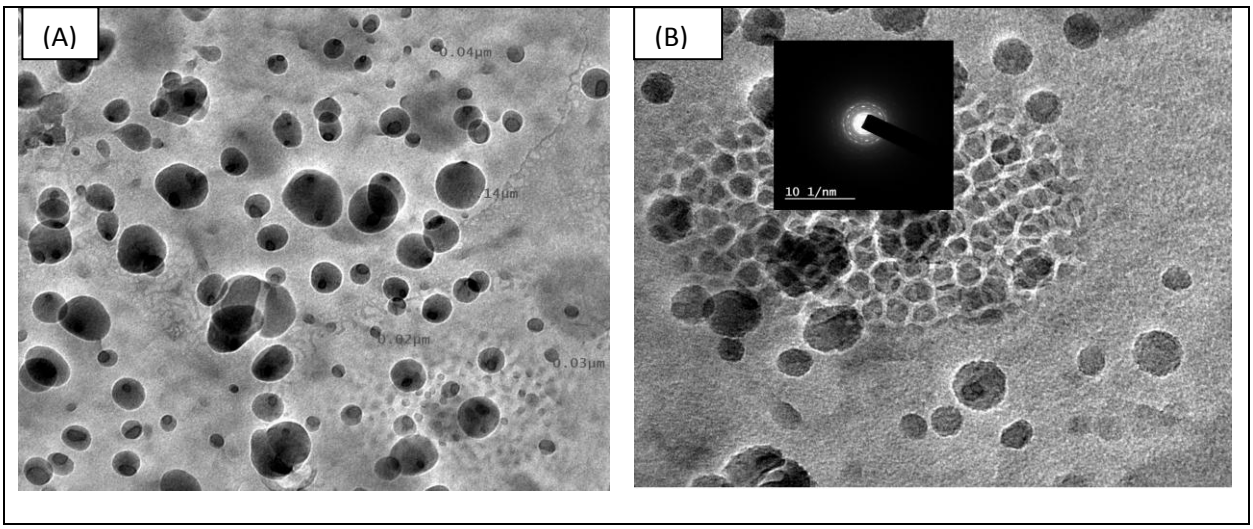

Fig. 83. TEM micrograph at 10 nm of (A) synthetic AgNPs characteristics and (B) with the corresponding selected-area electron diffraction (SEAD) pattern.

a. Comparative susceptibility of planktonic cells and three different ages of *L. monocytogenes* biofilm to AgNPs

1. Antibiofilm activity and MIC of AgNPs against *L. monocytogenes* planktonic cells

Data presented in Table (79) showed real the AgNPs dose which able to complete removal of planktonic cells of *L. monocytogenes* was 300 mg at 5 min. While, 50, 100 and 200 mg didn't able to totally remove the palnktonic cells. Therefore, the suitable effective dose at low contact time (MIC) was recorded in 300 mg at 5 min. Also, from toxicity assay, the results indicated that all used AgNPS doses were non toxic.

The suggested statistical model (one way *ANOVA*- Homogeneity test of variances) indicated, there was a highly significant ($P \leq 0.05$) between the different doses of AgNPs and the counts of planktonic cells.

Table 79. Determination of antibiofilm effect and MIC for different doses of AgNPs against *L. monocytogenes* planktonic cells

Contact time (min)	Doses of AgNPs (mg/l)				
	50	**100**	**200**	**300**	**500**
Zero	$4.5x10^6$	$4.5x10^6$	$4.5x10^6$	$4.5x10^6$	$4.5x10^6$
5	$1.9x10^3$	$2.4x10^2$	$1.1x10^2$	ND	ND
10	$5.1x10^2$	$1.3x10^2$	6.3x10	ND	ND
15	$2.8x10^2$	8.7x10	2.7x10	ND	ND

ND= Not detected

2. Antibiofilm activity and MIC of AgNPs against *L. monocytogenes* biofilm grown on PVC pipe material

Results represented in Table (80) and Fig. (84) showed that, the MIC of AgNPs for 10days-old *L. monocytogenes* biofilm grown on PVC pipe material were 300 mg at 10 min, while , 40 and 90 days-old were 300 mg at 15 min and 500 mg at 15 min.

From the results of statistical analysis (Homogeneity test of variances from one way *ANOVA*), it was found that a deeply significant correlation between ($P \leq 0.05$) the different doses of AgNPs and biofilm ages grown on PVC pipe material.

3. Antibiofilm activity and MIC of AgNPs against *L. monocytogenes* biofilm grown on PP pipe material

As shown in Table (81) and Fig. (85), the MIC of AgNPs for 10, 40 and 90 days-old *L. monocytogenes* biofilm grown on PP pipe material were 300 mg at 5 min, 300 mg at 15 and 500 mg at 15 min, respectively.

Statistically, the results found that, there was a greatly significant correlation ($P \leq 0.05$) between the different doses of AgNPs and biofilm ages grown on PP pipe material.

4. Antibiofilm activity of and MIC of AgNPs against *L. monocytogenes* biofilm grown on PE pipe material

Concerning the results of 10, 40 and 90 days-old *L. monocytogenes* biofilm grown on PE pipe material, the MIC of AgNPs for was 300 mg at 10 min, 500 mg at 15 and 500 mg at 15 min, respectively (Table 82; Fig. 86).

The results of statistical analysis (Homogeneity test of variances from one way *ANOVA*), it was found there was a deeply significant correlation ($P \leq 0.05$) the different doses of AgNPs and biofilm ages grown on PE pipe material.

5. Antibiofilm activity of and MIC of AgNPs against *L. monocytogenes* biofilm grown on I pipe material

Data shown in Table (83) and Fig. (87) presented, the antibiofilm activity and MIC (500 mg at 10 min and 500 mg at 15 min) of AgNPs recorded against10 and 40 days-old *L. monocytogenes* biofilm grown on I pipe material. The 90 days-old *L. monocytogenes* biofilm grown on I pipe material was more resistant to highest doses (500 mg) of AgNPs and contact time (15 min).

From results of statistical analysis by using Homogeneity test of variances from one way *ANOVA*, it was presented, there was a deeply significant correlation between ($P \leq 0.05$) the different doses of AgNPs and biofilm ages grown on I pipe material.

Table 80. Determination of antibiofilm effect and MIC for different doses of AgNPs against *L. monocytogenes* biofilm grown on PVC pipe materials.

AgNPs doses (mg/l)	Contact time (min)	CFU/cm^2 The ages *L. monocytogenes* biofilm grown on PVC pipe material								
		10 days-old			40 days-old			90 days-old		
		Count	Log count	Log reduction	Count	Log count	Log reduction	Count	Log count	Log reduction
Control		$2.5x10^6$	6.397	0	$4.6x10^6$	6.662	0	$5.2x10^6$	6.716	0
50	5	$3.7x10^3$	3.568	2.829	$5.3x10^4$	4.724	1.938	$2.8x10^5$	5.447	1.268
	10	$6.4x10^2$	2.806	3.591	$6.2x10^3$	3.792	2.870	$9.5x10^4$	4.977	1.738
	15	$2.1x10^2$	2.322	4.075	$7.3x10^2$	2.863	3.799	$4.9x10^4$	4.690	2.025
100	5	$5.3x10^2$	2.724	3.673	$2.4x10^3$	3.380	3.282	$3.7x10^4$	4.568	2.147
	10	$1.6x10^2$	2.204	4.193	$5.2x10^2$	2.716	3.946	$5.9x10^3$	3.770	2.945
	15	9.4x10	1.973	4.424	$2.9x10^2$	2.462	4.200	$2.8x10^3$	3.447	3.268
200	5	$1.5x10^2$	2.176	4.221	$1.4x10^2$	2.146	4.516	$4.3x10^3$	3.633	3.082
	10	7.8x10	1.892	4.505	$1.0x10^2$	2	4.662	$1.5x10^3$	3.176	3.539
	15	3.5x10	1.544	4.853	8.9x10	1.949	4.713	$4.1x10^2$	2.612	4.103
300	5	1.1x10	1.041	5.356	7.2x10	1.857	4.805	$6.2x10^2$	2.792	3.923
	10	ND	ND	6.397	2.9x10	1.462	5.200	$3.5x10^2$	2.544	4.171
	15	ND	ND	6.397	ND	ND	6.662	$1.3x10^2$	2.113	4.602
500	5	ND	ND	6.397	ND	ND	6.662	9.5x10	1.977	4.738
	10	ND	ND	6.397	ND	ND	6.662	1.9x10	1.278	5.437
	15	ND	ND	6.397	ND	ND	6.662	ND	ND	6.716

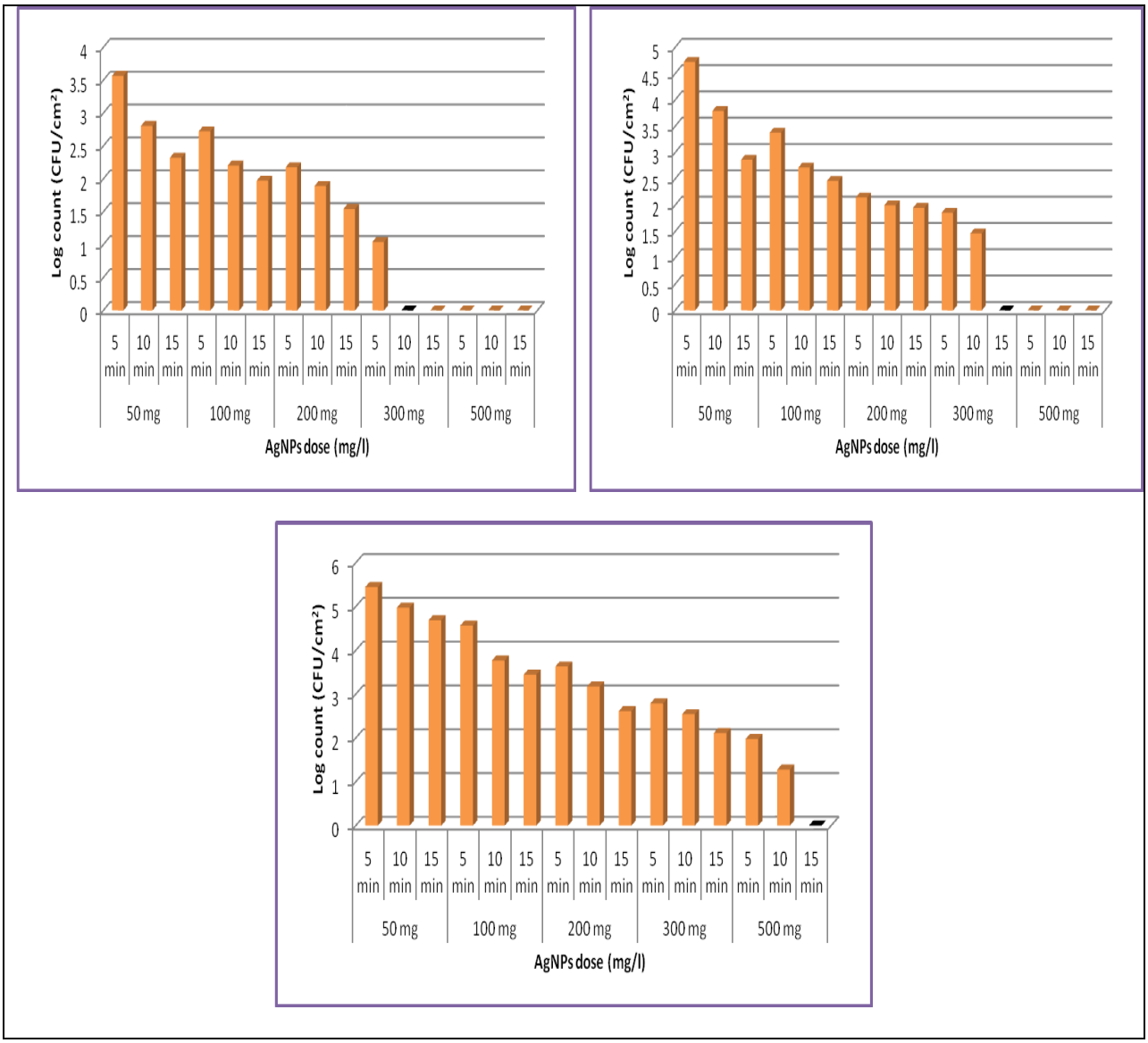

Fig. 84. Antibiofilm effect and MIC of different doses of AgNPs against 10, 40 and 90 days-old *L. monocytogenes* biofilm grown on PVC pipe material (left- right).

Table 81. Determination of antibiofilm effect and MIC for different doses of AgNPs against *L. monocytogenes* biofilm grown on PP pipe materials.

AgNPs doses (mg/l)	Contact time (min)	CFU/cm^2 The ages *L. monocytogenes* biofilm grown on PP pipe material								
		10 days-old			40 days-old			90 days-old		
		Count	Log count	Log reduction	Count	Log count	Log reduction	Count	Log count	Log reduction
Control		$1.9x10^6$	6.278	0	$7.4x10^6$	6.869	0	$3.8x10^6$	6.579	0
50	5	$5.2x10^3$	3.716	2.562	$3.7x10^4$	4.568	2.301	$5.3x10^5$	5.724	0.855
	10	$8.1x10^2$	2.908	3.370	$2.7x10^3$	3.431	3.437	$6.3x10^4$	4.799	1.780
	15	$3.3x10^2$	2.518	3.760	$6.7x10^2$	2.826	4.043	$2.1x10^4$	4.322	2.257
100	5	$3.0x10^2$	2.477	3.801	$7.2x10^2$	2.857	4.011	$2.4x10^4$	4.380	2.199
	10	9.8x10	1.991	4.287	$3.6x10^2$	2.556	4.312	$6.1x10^3$	3.785	2.794
	15	7.2x10	1.857	4.421	$1.7x10^2$	2.230	4.638	$3.2x10^3$	3.505	3.074
200	5	9.8x10	1.991	4.287	9.8x10	1.991	4.878	$5.4x10^3$	3.732	2.847
	10	6.2x10	1.792	4.486	7.3x10	1.863	5.005	$2.4x10^3$	3.380	3.199
	15	2.7x10	1.431	4.847	6.3x10	1.799	5.069	$3.7x10^2$	2.568	4.011
300	5	ND	ND	6.278	4.3x10	1.633	5.235	$4.9x10^2$	2.690	3.889
	10	ND	ND	6.278	ND	ND	6.869	$2.3x10^2$	2.361	4.218
	15	ND	ND	6.278	ND	ND	6.869	9.7x10	1.986	4.593
500	5	ND	ND	6.278	ND	ND	6.869	3.4x10	1.531	5.048
	10	ND	ND	6.278	ND	ND	6.869	8	0.903	5.676
	15	ND	ND	6.278	ND	ND	6.869	ND	ND	6.579

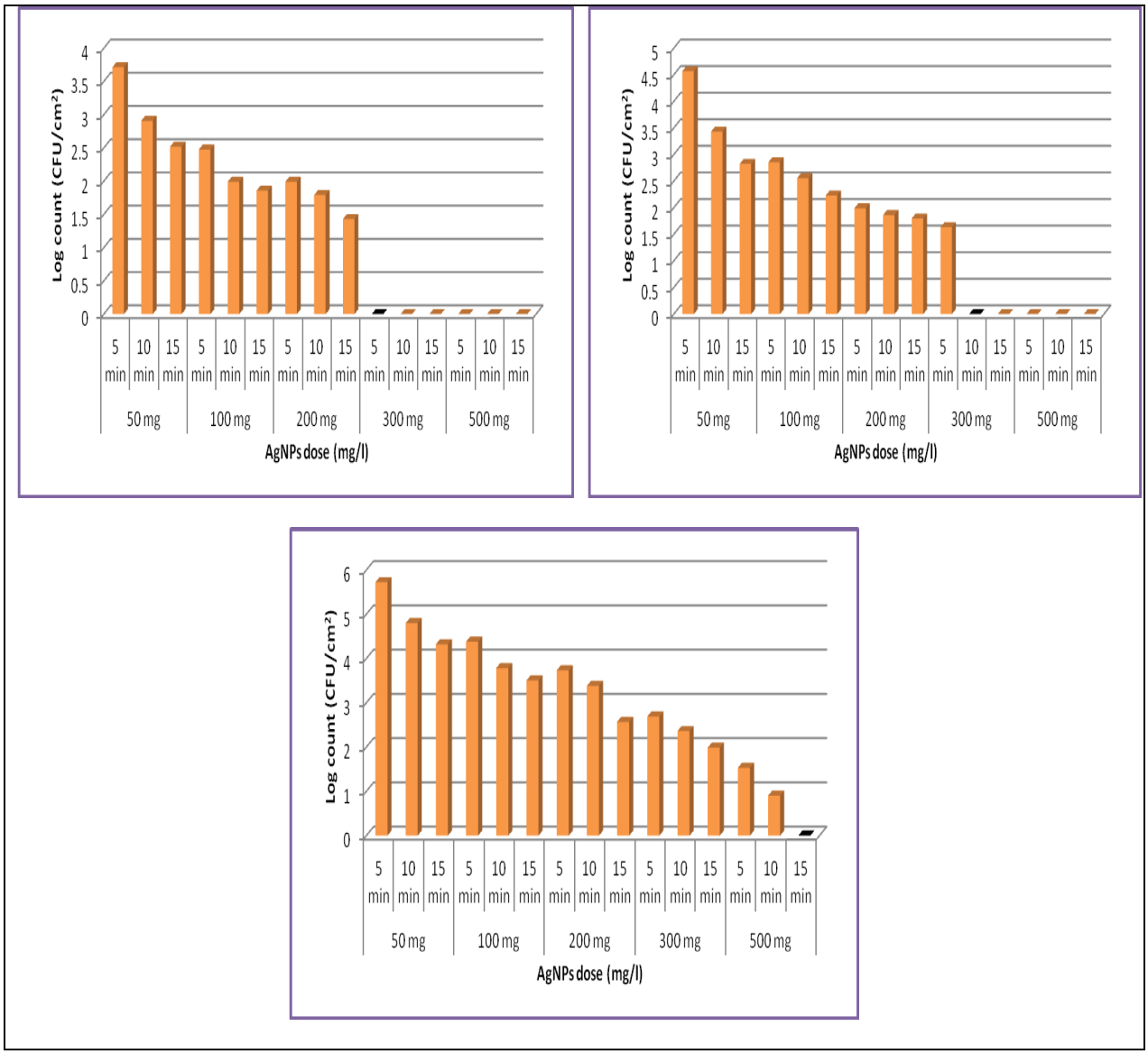

Fig. 85. Antibiofilm effect and MIC of different doses of AgNPs against 10, 40 and 90 days-old *L. monocytogenes* biofilm grown on PP pipe material (left- right).

Table 83. Determination of antibiofilm effect and MIC for different doses of AgNPs against *L. monocytogenes* biofilm grown on I pipe materials.

AgNPs doses (mg/l)	Contact time (min)	CFU/cm^2 The ages *L. monocytogenes* biofilm grown on I pipe material								
		10 days-old			40 days-old			90 days-old		
		Count	Log count	Log reduction	Count	Log count	Log reduction	Count	Log count	Log reduction
Control		$7.4x10^6$	6.869	0	$2.3x10^6$	6.361	0	$6.3x10^6$	6.799	0
50	5	$4.8x10^4$	4.681	2.187	$3.8x10^5$	5.579	0.781	$3.1x10^6$	6.491	0.307
	10	$2.3x10^4$	4.361	2.507	$7.5x10^4$	4.875	1.486	$1.4x10^6$	6.146	0.653
	15	$7.2x10^3$	3.857	3.011	$3.4x10^4$	4.531	1.830	$7.6x10^5$	5.880	0.918
100	5	$5.7x10^3$	3.755	3.113	$4.8x10^4$	4.681	1.680	$4.7x10^5$	5.672	1.127
	10	$1.4x10^3$	3.146	3.723	$2.6x10^4$	4.414	1.946	$2.3x10^5$	5.361	1.437
	15	$7.5x10^2$	2.875	3.994	$6.7x10^3$	3.826	2.535	$8.3x10^4$	4.919	1.880
200	5	$6.9x10^2$	2.838	4.0303	$3.9x10^3$	3.591	2.770	$1.6x10^5$	5.204	1.595
	10	$3.9x10^2$	2.591	4.278	$2.0x10^3$	3.301	3.060	$6.4x10^4$	4.806	1.993
	15	$1.2x10^2$	2.079	4.790	$1.2x10^3$	2.079	4.282	$1.8x10^4$	4.255	2.544
300	5	9.2x10	1.963	4.905	$7.4x10^2$	2.869	3.492	$2.6x10^4$	4.414	2.384
	10	ND	ND	6.869	$2.6x10^2$	2.414	3.946	$6.7x10^3$	3.826	2.973
	15	ND	ND	6.869	9.1x10	1.959	4.402	$2.9x10^3$	3.462	3.336
500	5	ND	ND	6.869	5.3x10	1.724	4.637	$3.1x10^3$	3.491	3.307
	10	ND	ND	6.869	1.9x10	1.278	5.082	$5.1x10^2$	2.707	4.091
	15	ND	ND	6.869	ND	ND	6.361	$1.1x10^2$	2.041	4.757

ND: Not Detected.

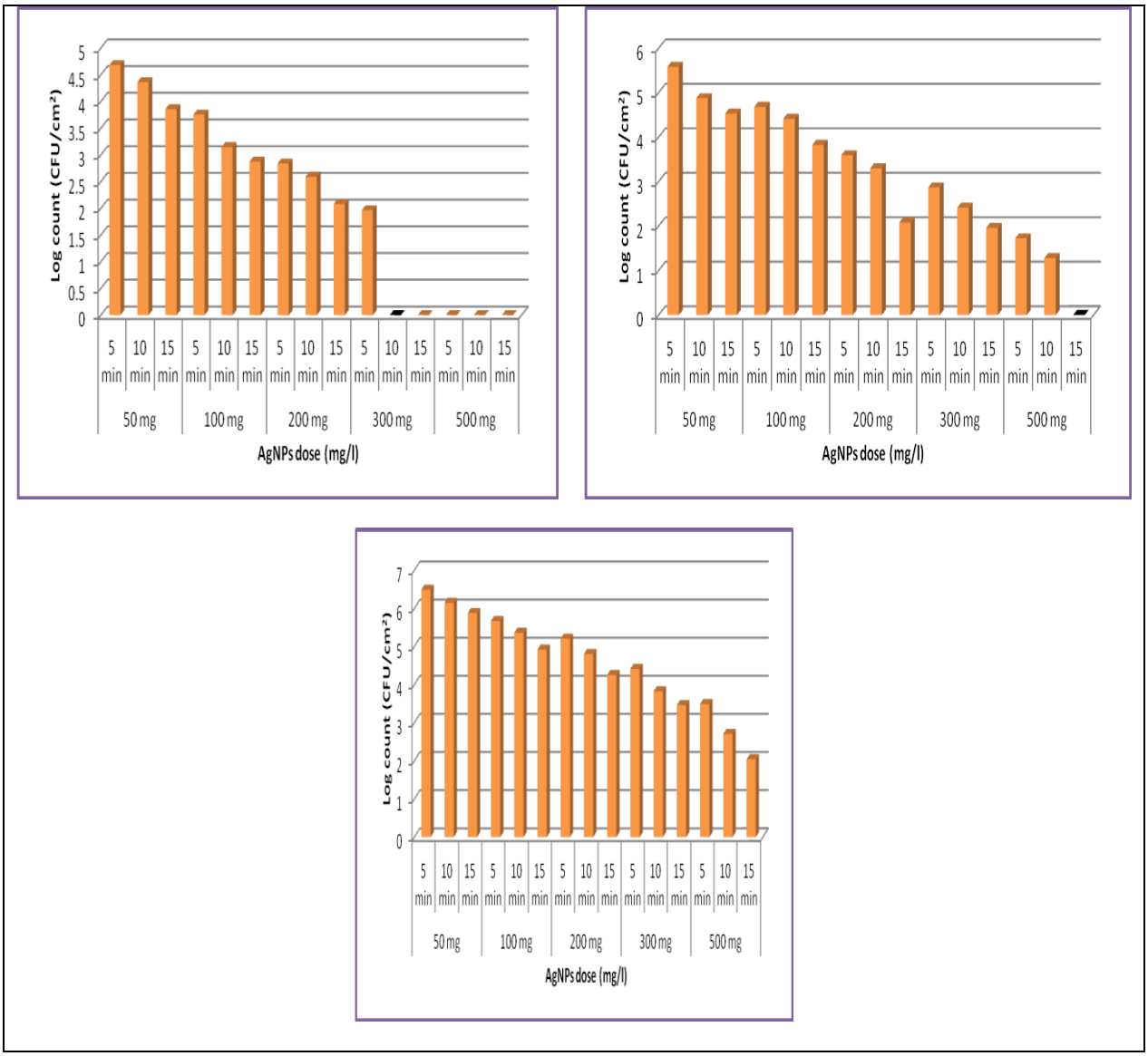

Fig. 87. Antibiofilm effect and MIC of different doses of AgNPs against 10, 40 and 90 days-old *L. monocytogenes* biofilm grown on I pipe material (left- right).

1. **Antibiofilm activity of and MIC of AgNPs against *L. monocytogenes* biofilm grown on Cu pipe material**

Regarding the results of *L. monocytogenes* biofilm grown on Cu pipe material, it was established the MIC of AgNPs for 10, 40 and 90 days-old of biofilm were 300 mg at 5 min, 200 mg at 15 and 200 mg at 10 min, respectively (Table 84; Fig. 88).

The results of statistical analysis (Homogeneity test of variances from one way *ANOVA*), it was found, there was a deeply significant correlation ($P \leq 0.05$) between the different doses of AgNPs and biofilm ages grown on Cu pipe material.

2. **Antibiofilm activity of and MIC of AgNPs against *L. monocytogenes* biofilm grown on R pipe material**

Results in Table (85) and Fig. (89), real the MIC of AgNPs which can be completely removed of 10, 40 and 90 days-old *L. monocytogenes* biofilm grown on R pipe material were 300 mg at 5 min, 300 mg at 15 min and 500 mg at 15 min, respectively.

From statistical analysis results, it was revealed that, there was a significantly correlation ($P \leq 0.05$) between the different doses of AgNPs and biofilm ages grown on R pipematerial.

Table 84. Determination of antibiofilm effect and MIC for different doses of AgNPs against *L. monocytogenes* biofilm grown on Cu pipe materials.

AgNPs doses (mg/l)	Contact time (min)	CFU/cm^2 The ages *L. monocytogenes* biofilm grown on Cu pipe material								
		10 days-old			40 days-old			90 days-old		
		Count	Log count	Log reduction	Count	Log count	Log reduction	Count	Log count	Log reduction
Control		$3.1x10^6$	6.491	0	$2.7x10^6$	6.431	0	$4.1x10^6$	6.612	0
50	5	$5.3x10^3$	3.724	2.767	$8.3x10^3$	3.919	2.512	$7.4x10^3$	3.869	2.743
	10	$7.1x10^2$	2.851	3.640	$2.6x10^3$	3.414	3.016	$2.6x10^3$	3.414	3.197
	15	$3.6x10^2$	2.556	3.935	$5.3x10^2$	2.724	3.707	$4.4x10^2$	2.643	3.969
100	5	$7.4x10^2$	2.869	3.622	$7.9x10^2$	2.897	3.533	$3.8x10^2$	2.579	4.033
	10	$2.0x10^2$	2.301	4.190	$2.6x10^2$	2.414	4.016	$1.1x10^2$	2.041	4.571
	15	8.9x10	1.949	4.541	9.1x10	1.959	4.472	5.3x10	1.724	4.888
200	5	9.0x10	1.954	4.537	$1.1x10^2$	2.041	4.389	2.1x10	1.322	5.290
	10	7.4x10	1.869	4.622	5.3x10	1.724	4.707	ND	ND	6.612
	15	2.8x10	1.447	5.044	ND	ND	6.431	ND	ND	6.612
300	5	ND	ND	6.491	ND	ND	6.431	ND	ND	6.612
	10	ND	ND	6.491	ND	ND	6.431	ND	ND	6.612
	15	ND	ND	6.491	ND	ND	6.431	ND	ND	6.612
500	5	ND	ND	6.491	ND	ND	6.431	ND	ND	6.612
	10	ND	ND	6.491	ND	ND	6.431	ND	ND	6.612
	15	ND	ND	6.491	ND	ND	6.431	ND	ND	6.612

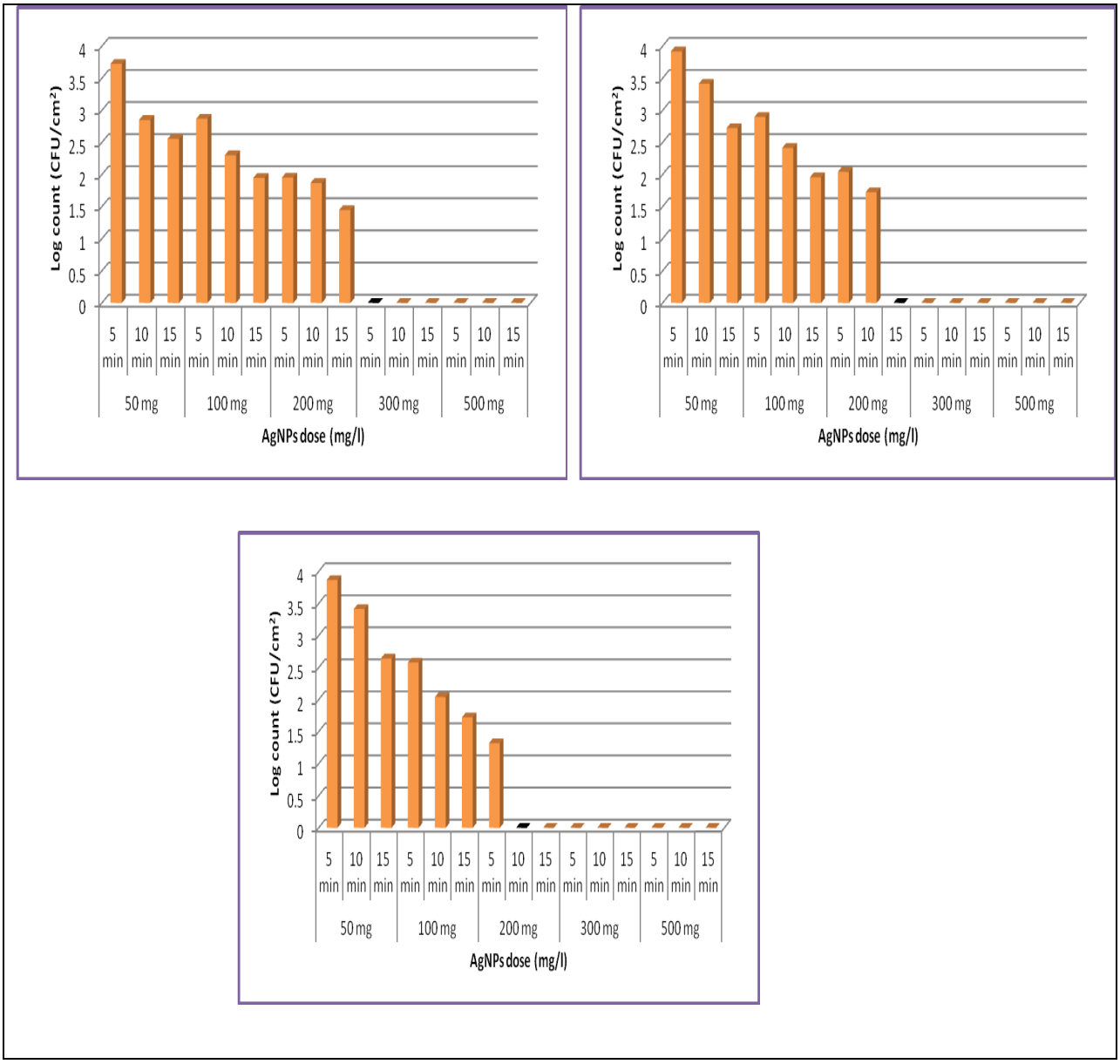

Fig. 88. Antibiofilm effect and MIC of different doses of AgNPs against 10, 40 and 90 days-old *L. monocytogenes* biofilm grown on Cu pipe material (left- right).

Table 85. Determination of antibiofilm effect and MIC for different doses of Ag NPs against *L. monocytogenes* biofilm grown on R pipe materials.

AgNPs doses (mg/l)	Contact time (min)	CFU/cm^2 The ages *L. monocytogenes* biofilm grown on R pipe material								
		10 days-old			40 days-old			90 days-old		
		Count	Log count	Log reduction	Count	Log count	Log reduction	Count	Log count	Log reduction
Control		$4.9x10^6$	6.690	0	$6.4x10^6$	6.806	0	$3.9x10^6$	6.591	0
50	5	$4.0x10^3$	3.602	3.088	$4.2x10^4$	4.623	2.182	$2.7x10^5$	5.431	1.1597
	10	$5.2x10^2$	2.716	3.974	$5.1x10^3$	3.707	3.098	$7.3x10^4$	4.863	1.727
	15	$3.6x10^2$	2.556	4.133	$8.4x10^2$	2.924	3.881	$3.2x10^4$	4.505	2.085
100	5	$4.6x10^2$	2.662	4.027	$3.6x10^3$	3.556	3.249	$8.3x10^3$	3.9190	2.671
	10	$1.9x10^2$	2.278	4.411	$7.3x10^2$	2.863	3.942	$5.2x10^3$	3.716	2.875
	15	9.1x10	1.959	4.731	$5.4x10^2$	2.732	4.073	$2.4x10^3$	3.380	3.210
200	5	8.7x10	1.939	4.750	$2.7x10^2$	2.431	4.374	$5.4x10^3$	2.732	3.858
	10	5.8x10	1.763	4.926	$1.4x10^2$	2.146	4.660	$2.1x10^3$	3.322	3.268
	15	1.4x10	1.146	5.544	9.4x10	1.973	4.833	$5.3x10^2$	2.724	3.866
300	5	ND	ND	6.690	4.2x10	1.623	5.182 7	$4.3x10^2$	2.633	3.957
	10	ND	ND	6.690	1.4x10	1.146	5.660	$2.2x10^2$	2.342	4.248
	15	ND	ND	6.690	ND	ND	6.806	$1.1x10^2$	2.041	4.549
500	5	ND	ND	6.690	ND	ND	6.806	8.1x10	1.908	4.682
	10	ND	ND	6.690	ND	ND	6.806	1.2x10	1.079	5.511
	15	ND	ND	6.690	ND	ND	6.806	ND	ND	6.591

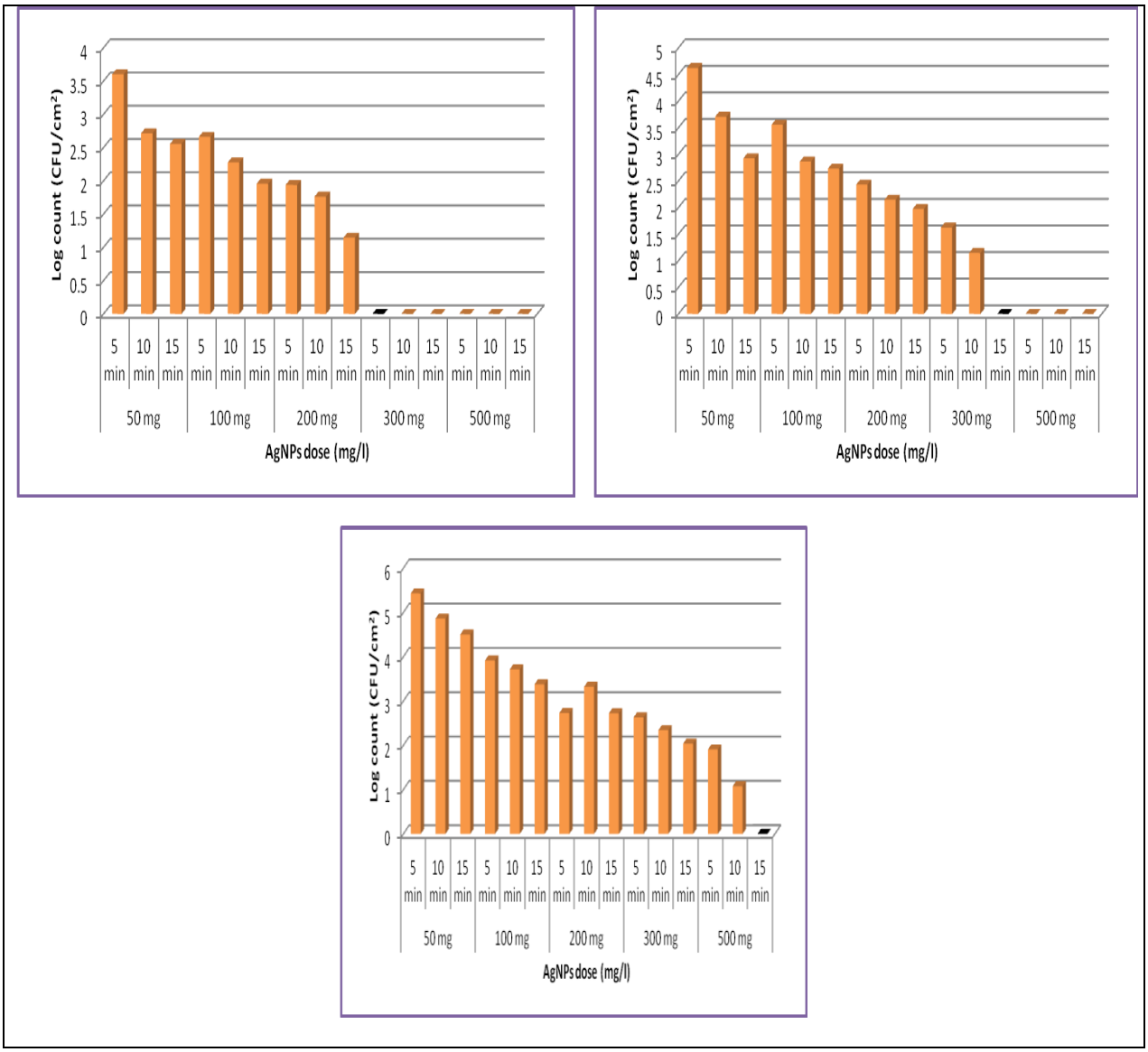

Fig. 89. Antibiofilm effect and MIC of different doses of AgNPs against 10, 40 and 90 days-old *L. monocytogenes* biofilm grown on R pipe material (left- right).

b. Comparative susceptibility of planktonic cells and three different ages of *S.* Typhimurium biofilm to AgNPs

1. Antibiofilm activity and MIC of AgNPs against *S.* Typhimurium planktonic cells

As shown in Table (86) the results showed the AgNPs dose which able to complete removal of planktonic cells of *S.* Typhimurium was 200 mg at 15 min. So, the suitable effective dose at low contact time (MIC) was recorded in200 mg at 15 min.

The statistical model (one way ANOVA- Homogeneity test of variances) recorded this, there was an extremely significant ($P \leq 0.05$) between the different doses of AgNPs and the counts of planktonic cells.

Table 86. Determination of antibiofilm effect and MIC for different doses of AgNPs against *S.* Typhimurium planktonic cells.

Contact time (min)	Doses of AgNPs (mg/l)				
	50	100	200	300	500
Zero	$3.6x10^6$	$3.6x10^6$	$3.6x10^6$	$3.6x10^6$	$3.6x10^6$
5	$5.1x10^2$	$1.2x10^2$	1.8x10	ND	ND
10	$1.8x10^2$	7.4x10	6	ND	ND
15	9.8x10	2.9x10	ND	ND	ND

ND= Not detected

2. Antibiofilm activity and MIC of AgNPs against *S.* Typhimurium biofilm grown on PVC pipe material

Results shown in Table (87) and Fig. (90) explained, real the MIC of AgNPs for 10, 40 and 90 days-old *S.* Typhimurium biofilm grown on PVC pipe material were 200 mg at 15 min, 300 mg at 10 min and 300 mg at 15 min, respectively.

The results of statistical analysis (Homogeneity test of variances from one way *ANOVA*), indicated the strong significant correlation ($P \leq 0.05$) between the different doses of AgNPs and biofilm ages grown on PVC pipe material.

2. Antibiofilm activity and MIC of AgNPs against *S.* Typhimurium biofilm grown on PP pipe material

The data represented in Table (88) Fig. (91) found, the MIC of AgNPs for 10, 40 and 90 days-old *S.* Typhimurium biofilm grown on PP pipe material were 200 mg at 5 min, 300 mg at 10 and 300 mg at 15 min, respectively.

The results of statistical analysis, explained that, there was a greatly significant correlation ($P \leq 0.05$) between the different doses of AgNPs and biofilm ages grown on PP pipe material.

3. Antibiofilm activity and MIC of AgNPs against *S.* Typhimurium biofilm grown on PE pipe material

In case of *S.* Typhimurium biofilm grown on PE pipe material, The data represented in Table (89) and Fig. (92) confirmed this, the MIC of AgNPs for 10, 40 and 90 days-old of biofilm were 200 mg at 15 min, 300 mg at 10 min and 300 mg at 15 min, respectively.

From statistical analysis results, it was revealed that, there was a greatly significant correlation ($P \leq 0.05$) between the different doses of AgNPs and biofilm ages grown on PE pipe material.

Table 87. Determination of antibiofilm effect and MIC for different doses of AgNPs against *S.* Typhimurium biofilm grown on PVC pipe materials.

AgNPs doses (mg/l)	Contact time (min)	CFU/cm^2 The ages *S.* Typhimurium biofilm grown on PVC pipe material								
		10 days-old			40 days-old			90 days-old		
		Count	Log count	Log reduction	Count	Log count	Log reduction	Count	Log count	Log reduction
Control		$2.6x10^6$	6.414	0	$4.5x10^6$	6.653	0	$3.7x10^6$	6.568	0
50	5	$7.3x10^2$	2.863	3.551	$4.7x10^3$	3.672	2.981	$5.6x10^4$	4.748	1.820
	10	$3.1x10^2$	2.491	3.923	$1.4x10^3$	3.146	3.507	$7.8x10^3$	3.892	2.676
	15	$1.2x10^2$	2.079	4.335	$6.2x10^2$	2.792	3.860	$3.4x10^3$	3.531	3.036
100	5	8.3x10	1.919	4.495	$4.6x10^2$	2.662	3.990	$8.5x10^2$	2.929	3.638
	10	4.7x10	1.672	4.742	$1.9x10^2$	2.278	4.374	$5.2x10^2$	2.716	3.852
	15	2.8x10	1.447	4.967	9.7x10	1.986	4.666	$2.6x10^2$	2.414	4.153
200	5	1.5x10	1.176	5.238	8.4x10	1.924	4.728	$1.4x10^2$	2.146	4.422
	10	8	0.903	5.511	5.3x10	1.724	4.928	9.3x10	1.968	4.599
	15	ND	ND	6.414	2.9x10	1.462	5.190	7.4x10	1.869	4.698
300	5	ND	ND	6.414	1.0x10	1	5.653	4.5x10	1.653	4.914
	10	ND	ND	6.414	ND	ND	5.653	2.1x10	1.322	5.245
	15	ND	ND	6.414	ND	ND	5.653	ND	ND	6.568
500	5	ND	ND	6.414	ND	ND	5.653	ND	ND	6.568
	10	ND	ND	6.414	ND	ND	5.653	ND	ND	6.568
	15	ND	ND	6.414	ND	ND	5.653	ND	ND	6.568

ND: Not Detected.

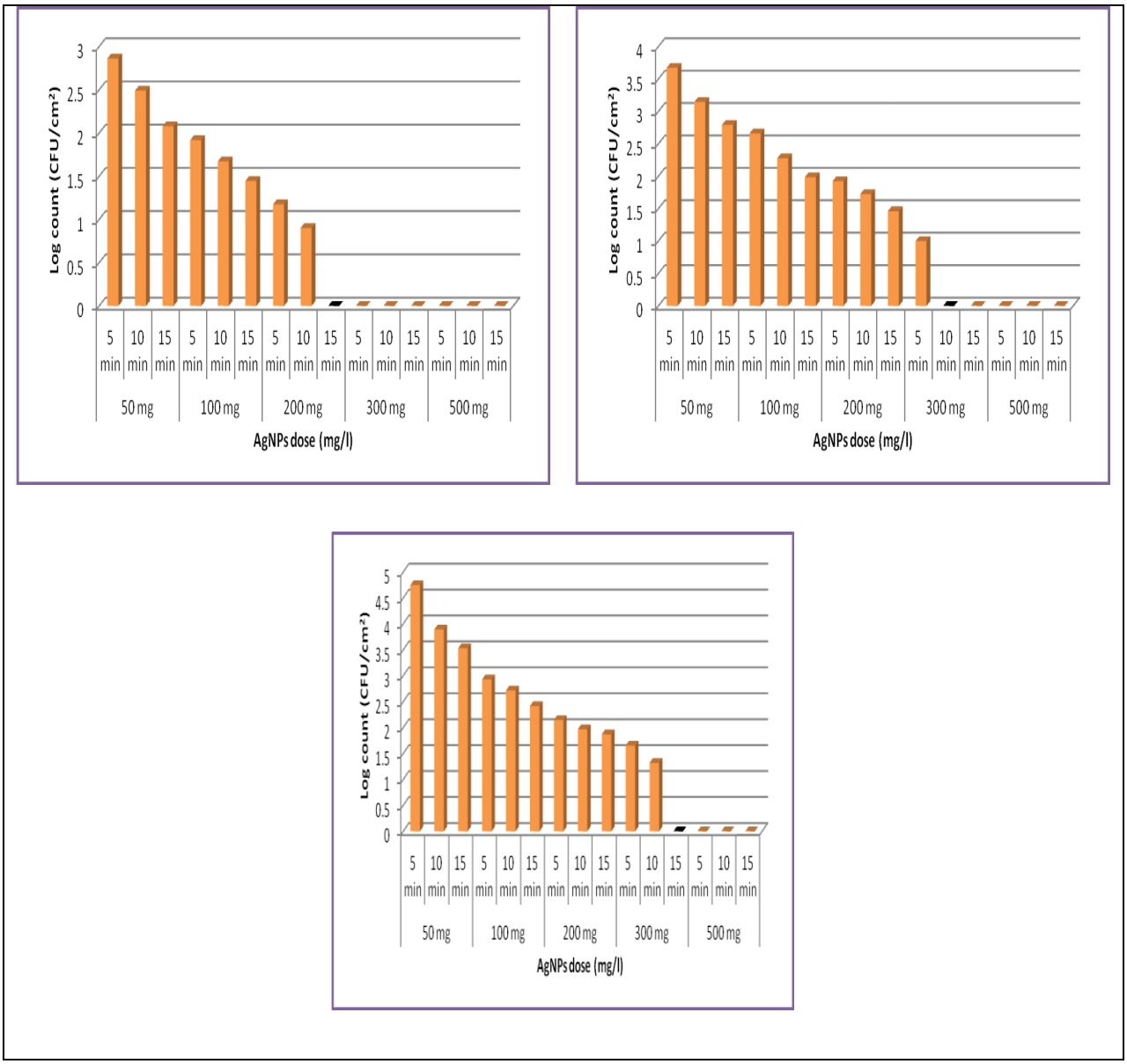

Fig. 90. Antibiofilm effect and MIC of different doses of AgNPs against 10, 40 and 90 days-old *S.* Typhimurium biofilm grown on PVC pipe material (left- right).

Table 88. Determination of antibiofilm effect and MIC for different doses of Ag NPs against *S.* Typhimurium biofilm grown on PP pipe materials.

AgNPs doses (mg/l)	Contact time (min)	CFU/cm^2 The ages *S.* Typhimurium biofilm grown on PP pipe material								
		10 days-old			40 days-old			90 days-old		
		Count	Log count	Log reduction	Count	Log count	Log reduction	Count	Log count	Log reduction
Control		$4.3x10^6$	6.633	0	$3.8x10^6$	6.579	0	$2.6x10^6$	6.414	0
50	5	$4.9x10^2$	2.690	3.943	$3.2x10^3$	3.505	3.074	$4.3x10^4$	4.633	1.781
	10	$2.5x10^2$	2.397	4.235	$2.1x10^3$	3.322	3.257	$6.3x10^3$	3.799	2.615
	15	8.5x10	1.929	4.704	$9.2x10^2$	2.963	3.615	$2.7x10^3$	3.431	2.983
100	5	9.1x10	1.959	4.674	$7.3x10^2$	2.863	3.716	$7.4x10^2$	2.869	3.545
	10	6.4x10	1.806	4.827	$4.5x10^2$	2.653	3.926	$4.6x10^2$	2.662	3.752
	15	2.3x10	1.361	5.271	$1.4x10^2$	2.146	4.433	$2.9x10^2$	2.462	3.952
200	5	ND	ND	6.633	9.5x10	1.977	4.602	$1.1x10^2$	2.041	4.373
	10	ND	ND	6.633	7.3x10	1.863	4.716	8.7x10	1.939	4.475
	15	ND	ND	6.633	3.2x10	1.505	5.074	6.4x10	1.806	4.608
300	5	ND	ND	6.633	7	0.845	5.734	3.1x10	1.491	4.923
	10	ND	ND	6.633	ND	ND	6.579	1.1x10	1.041	5.373
	15	ND	ND	6.633	ND	ND	6.579	ND	ND	6.414
500	5	ND	ND	6.633	ND	ND	6.579	ND	ND	6.414
	10	ND	ND	6.633	ND	ND	6.579	ND	ND	6.414
	15	ND	ND	6.633	ND	ND	6.579	ND	ND	6.414

ND: Not Detected.

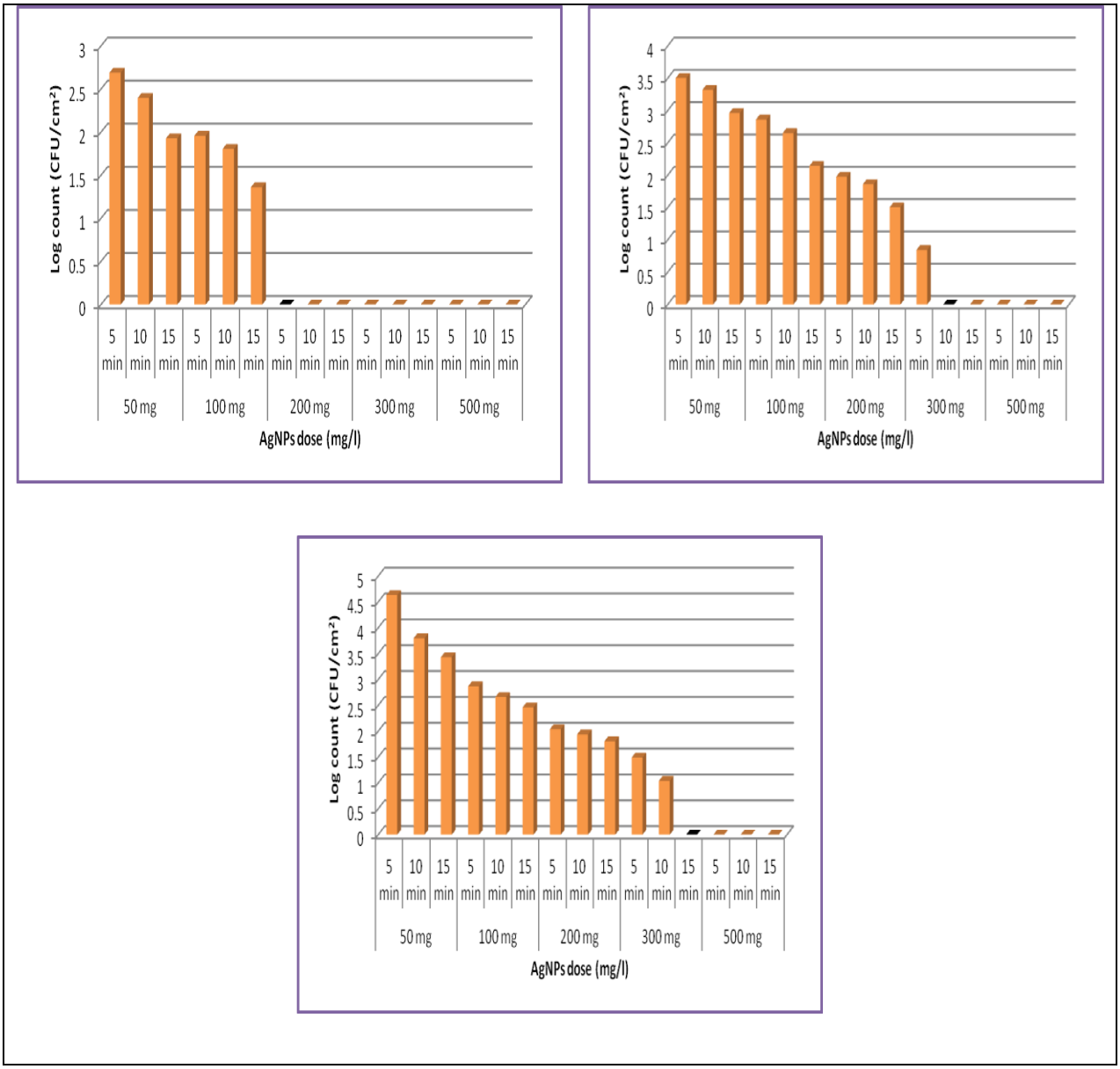

Fig. 91. Antibiofilm effect and MIC of different doses of AgNPs against 10, 40 and 90 days-old *S.* Typhimurium biofilm grown on PP pipe material (left- right).

Table 89. Determination of antibiofilm effect and MIC for different doses of Ag NPs against *S.* Typhimurium biofilm grown on PE pipe materials.

AgNPs doses (mg/l)	Contact time (min)	CFU/cm^2 The ages *S.* Typhimurium biofilm grown on PE pipe material								
		10 days-old			40 days-old			90 days-old		
		Count	Log count	Log reduction	Count	Log count	Log reduction	Count	Log count	Log reduction
Control		$1.5x10^6$	6.176	0	$2.7x10^6$	6.431	0	$4.2x10^6$	6.623	0
50	5	$5.4x10^2$	2.732	3.443	$5.3x10^3$	3.724	2.707	$3.8x10^4$	4.579	2.043
	10	$3.8x10^2$	2.579	3.596	$3.4x10^3$	3.531	2.899	$5.4x10^3$	3.732	2.890
	15	$2.1x10^2$	2.322	3.853	$1.2x10^3$	3.079	3.352	$2.6x10^3$	3.414	3.208
100	5	$1.6x10^2$	2.204	3.971	$6.5x10^2$	2.812	3.618	$9.1x10^2$	2.959	3.664
	10	9.7x10	1.986	4.189	$3.6x10^2$	2.556	3.875	$7.3x10^2$	2.863	3.759
	15	6.4x10	1.806	4.369	$1.5x10^2$	2.176	4.255	$4.1x10^2$	2.612	4.010
200	5	2.3x10	1.361	4.814	8.9x10	1.949	4.481	$2.7x10^2$	2.431	4.191
	10	1.1x10	1.041	5.134	5.7x10	1.755	4.675	$1.3x10^2$	2.113	4.509
	15	ND	ND	6.176	2.3x10	1.361	5.069	8.5x10	1.929	4.693
300	5	ND	ND	6.176	9	0.954	5.477	5.4x10	1.732	4.890
	10	ND	ND	6.176	ND	ND	6.431	1.7x10	1.230	5.392
	15	ND	ND	6.176	ND	ND	6.431	ND	ND	6.623
500	5	ND	ND	6.176	ND	ND	6.431	ND	ND	6.623
	10	ND	ND	6.176	ND	ND	6.431	ND	ND	6.623
	15	ND	ND	6.176	ND	ND	6.431	ND	ND	6.623

ND: Not Detected.

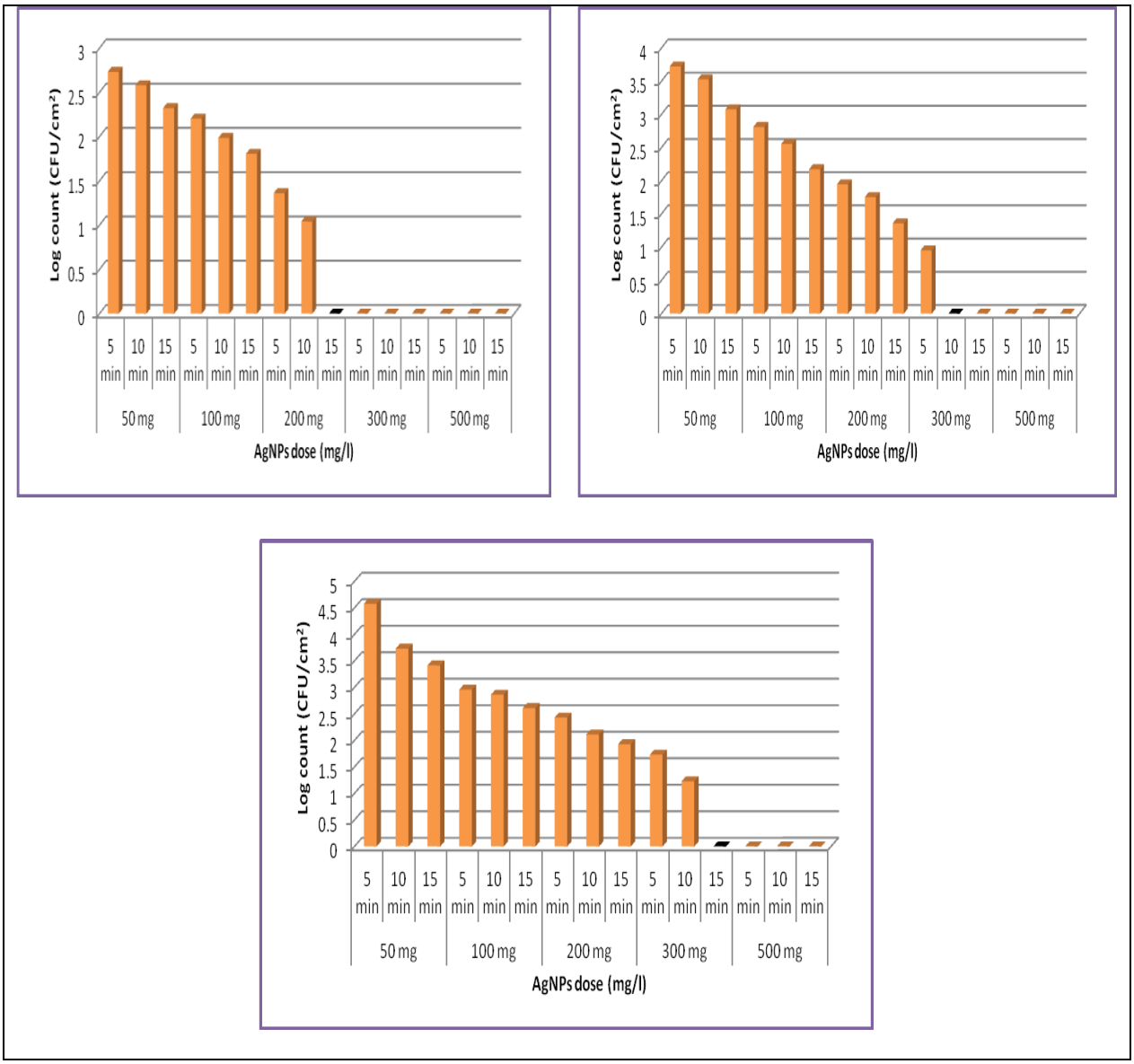

Fig. 92. Antibiofilm effect and MIC of different doses of AgNPs against 10, 40 and 90 days-old *S.* Typhimurium biofilm grown on PE pipe material (left- right).

4. Antibiofilm activity and MIC of AgNPs against *S.* Typhimurium biofilm grown on I pipe material

Concerning, *S.* Typhimurium biofilm grown on I pipe material, data presented in Table (90) and Fig. (93) demonstrated the MIC of AgNPs for 10, 40 and 90 days-old of biofilm was 300 mg at 15 min, 500 mg at 5 and 500 mg at 10 min, respectively.

Statistically, the obtained results demonstrated, there was a deeply significant correlation ($P \leq 0.05$) between the different doses of AgNPs and biofilm ages grown on I pipe material.

5. Antibiofilm activity and MIC of AgNPs against *S.* Typhimurium biofilm grown on Cu pipe material

Results of *S.* Typhimurium biofilm grown on Cu pipe material, it was revealed that the MIC of AgNPs for 10, 40 and 90 days-old of biofilm was 200 mg at 15 min, 200 mg at 15 min and 200 mg at 10 min, respectively (Table 91; Fig. 94).

The results of statistical analysis, it was found that, there was a greatly significant correlation ($P \leq 0.05$) between the different doses of AgNPs and biofilm ages grown on Cu pipe material.

f. Antibiofilm activity and MIC of AgNPs against *S.* Typhimurium biofilm grown on R pipe material

As shown in Table (92) and Fig. (95), for *S.* Typhimurium biofilm grown on R pipe material, the MIC of AgNPs for 10, 40 and 90 days-old of biofilm were 200 mg at 15 min, 300 mg at 10 and 300 mg at 15 min, respectively.

Table 90. Determination of antibiofilm effect and MIC for different doses of AgNPs against *S.* Typhimurium biofilm grown on I pipe materials.

AgNPs doses (mg/l)	Contact time (min)	CFU/cm^2 The ages *S.* Typhimurium biofilm grown on I pipe material								
		10 days-old			40 days-old			90 days-old		
		Count	Log count	Log reduction	Count	Log count	Log reduction	Count	Log count	Log reduction
Control		$5.2x10^6$	6.716	0	$4.2x10^6$	6.623	0	$3.7x10^6$	6.568	0
50	5	$4.3x10^3$	3.633	3.082	$6.2x10^4$	4.792	1.830	$5.4x10^5$	5.732	0.835
	10	$2.5x10^3$	3.39	3.318	$2.3x10^4$	4.361	2.261	$1.1x10^5$	5.041	1.526
	15	$1.1x10^3$	3.041	3.674	$1.0x10^4$	4	2.623	$6.1x10^4$	4.785	1.782
100	5	$8.3x10^2$	2.919	3.796	$6.3x10^3$	3.799	2.823	$2.7x10^4$	4.431	2.136
	10	$5.8x10^2$	2.763	3.952	$2.9x10^3$	3.462	3.160	$8.4x10^3$	3.924	2.643
	15	$2.4x10^2$	2.380	4.335	$8.2x10^2$	2.913	3.709	$5.3x10^3$	3.724	2.843
200	5	$1.3x10^2$	2.113	4.602	$5.6x10^2$	2.748	3.875	$1.8x10^3$	3.255	3.312
	10	9.7x10	1.986	4.729	$2.2x10^2$	2.342	4.280	$7.5x10^2$	2.875	3.693
	15	6.8x10	1.832	4.883	$1.4x10^2$	2.146	4.477	$4.3x10^2$	2.633	3.934
300	5	3.1x10	1.491	5.224	8.3x10	1.919	4.704	$2.5x10^2$	2.397	4.170
	10	5	0.698	6.017	5.8x10	1.763	4.859	$1.2x10^2$	2.0791	4.489
	15	ND	ND	6.716	1.8	1.255	5.367	6.4x10	1.806	4.762
500	5	ND	ND	6.716	ND	ND	6.623	2.1x10	1.322	5.245
	10	ND	ND	6.716	ND	ND	6.623	ND	ND	6.568
	15	ND	ND	6.716	ND	ND	6.623	ND	ND	6.568

ND: Not Detected.

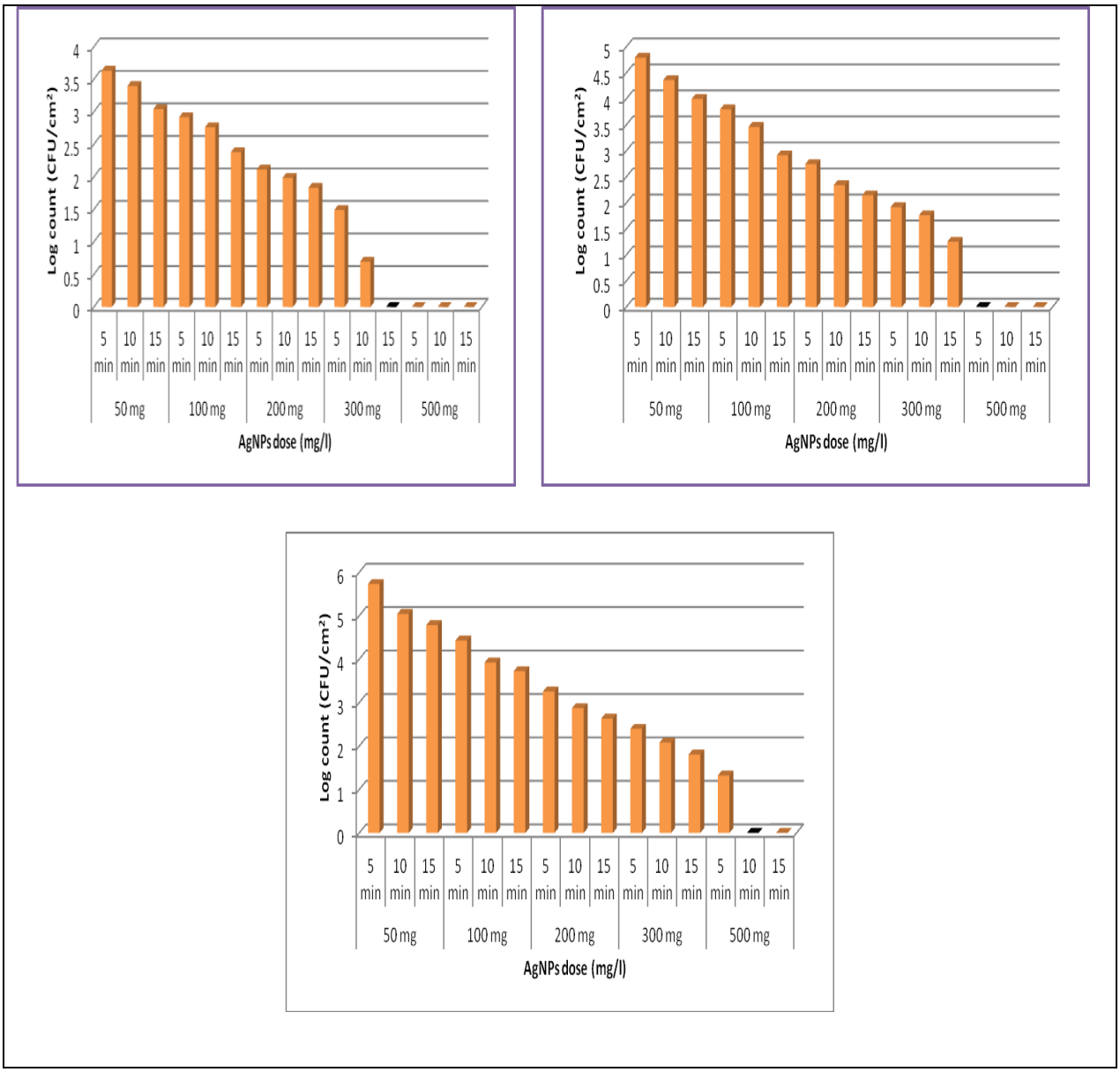

Fig. 93. Antibiofilm effect and MIC of different doses of AgNPs against 10, 40 and 90 days-old *S.* Typhimurium biofilm grown on I pipe material (left- right).

Table 91. Determination of antibiofilm effect and MIC for different doses of AgNPs against *S.* Typhimurium biofilm grown on Cu pipe materials.

AgNPs doses (mg/l)	Contact time (min)	CFU/cm^2 The ages *S.* Typhimurium biofilm grown on Cu pipe material								
		10 days-old			40 days-old			90 days-old		
		Count	Log count	Log reduction	Count	Log count	Log reduction	Count	Log count	Log reduction
Control		$4.1x10^6$	6.612	0	$3.4x10^6$	6.531	0	$1.6x10^6$	6.204	0
50	5	$3.9x10^2$	2.591	4.021	$1.8x10^3$	3.255	3.276	$4.7x10^2$	2.672	3.532
	10	$1.7x10^2$	2.230	4.382	$6.3x10^2$	2.799	3.732	$2.3x10^2$	2.361	3.842
	15	$1.0x10^2$	2	4.612	$3.5x10^2$	2.544	3.987	$1.0x10^2$	2	4.204
100	5	9.1x10	1.959	4.653	$1.4x10^2$	2.146	4.385	8.7x10	1.939	4.264
	10	7.4x10	1.869	4.743	9.3x10	1.968	4.562	6.5x10	1.812	4.391
	15	3.8x10	1.579	5.033	6.2x10	1.792	4.739 2	3.4x10	1.531	4.672
200	5	1.2x10	1.079	5.533	4.6x10	1.662	4.868	1.2x10	1.079	5.124
	10	3	0.477	6.135	1.9x10	1.278	5.252	ND	ND	6.204
	15	ND	ND	6.612	ND	ND	6.531	ND	ND	6.204
300	5	ND	ND	6.612	ND	ND	6.531	ND	ND	6.204
	10	ND	ND	6.612	ND	ND	6.531	ND	ND	6.204
	15	ND	ND	6.612	ND	ND	6.531	ND	ND	6.204
500	5	ND	ND	6.612	ND	ND	6.531	ND	ND	6.204
	10	ND	ND	6.612	ND	ND	6.531	ND	ND	6.204
	15	ND	ND	6.612	ND	ND	6.531	ND	ND	6.204

ND: Not Detected.

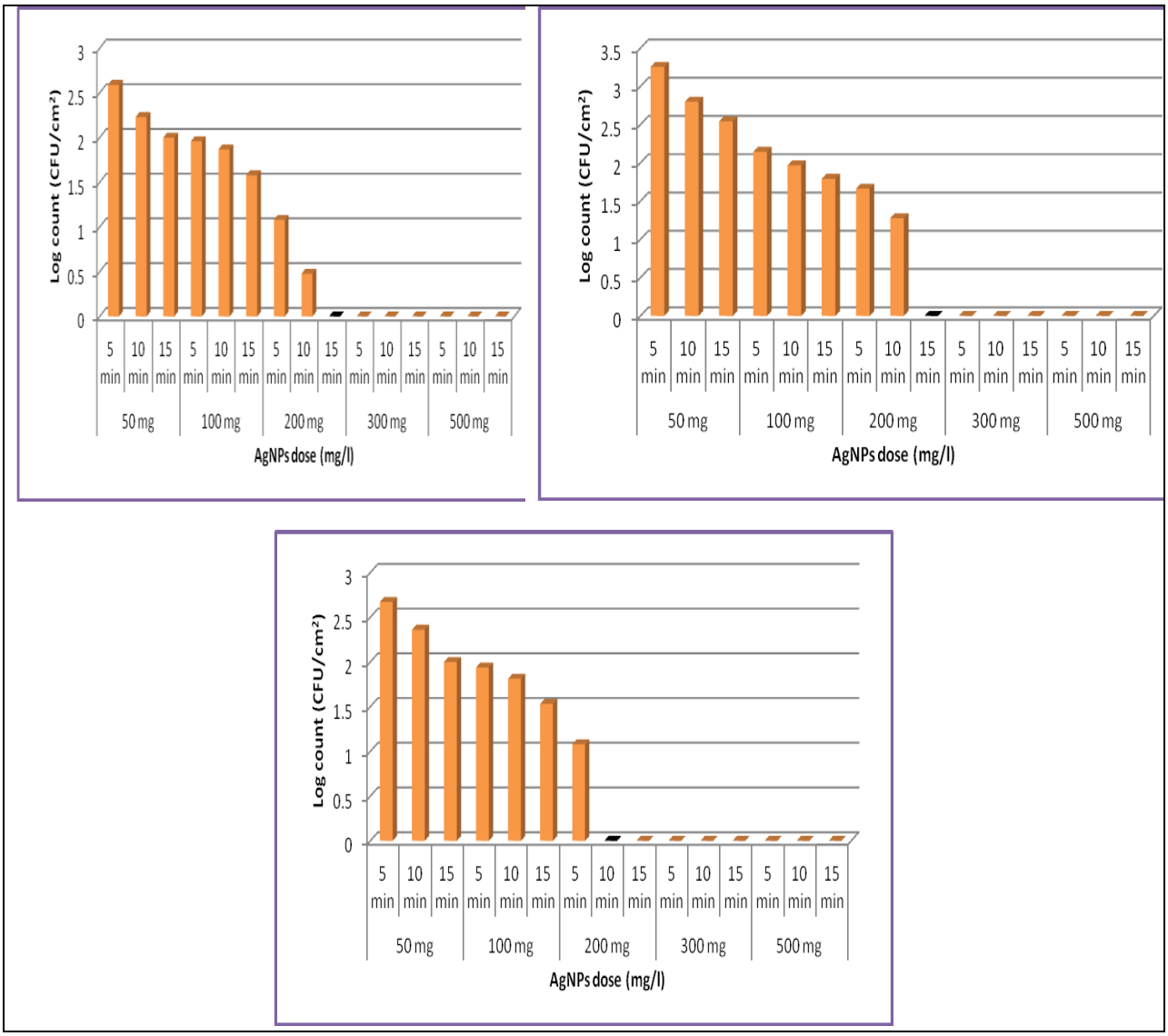

Fig. 94. Antibiofilm effect and MIC of different doses of AgNPs against 10, 40 and 90 days-old *S.* Typhimurium biofilm grown on Cu pipe material (left- right).

Table 92. Determination of antibiofilm effect and MIC for different doses of AgNPs against *S.* Typhimurium biofilm grown on R pipe materials.

AgNPs doses (mg/l)	Contact time (min)	CFU/cm^2 The ages *S.* Typhimurium biofilm grown on R pipe material								
		10 days-old			40 days-old			90 days-old		
		Count	Log count	Log reduction	Count	Log count	Log reduction	Count	Log count	Log reduction
Control		3.8x10^6	6.579	0	6.2x10^6	6.792	0	2.4x10^6	6.380	0
50	5	5.7x10^2	2.755	3.823	4.7x10^3	3.672	3.120	2.7x10^4	4.431	1.948
	10	2.9x10^2	2.462	4.117	2.4x10^3	3.380	3.412	8.4x10^3	3.924	2.455
	15	1.4x10^2	2.146	4.433	6.2x10^2	2.792	4	3.5x10^3	3.544	2.836
100	5	8.4x10	1.924	4.655	4.6x10^2	2.662	4.129	1.6x10^3	3.204	3.176
	10	6.2x10	1.792	4.787	2.7x10^2	2.431	4.361	6.8x10^2	2.832	3.547
	15	3.9x10	1.591	4.988	1.2x10^2	2.079	4.713	3.1x10^2	2.491	3.888
200	5	1.4x10	1.146	5.433	8.3x10	1.919	4.873	2.4x10^2	2.380	4
	10	7	0.845	5.734	5.2x10	1.716	5.076	1.2x10^2	2.079	4.301
	15	ND	ND	6.579	2.7x10	1.431	5.361	8.4x10	1.924	4.455
300	5	ND	ND	6.579	4	0.602	6.190	3.2x10	1.505	4.875
	10	ND	ND	6.579	ND	ND	6.792	1.4x10	1.146	5.234
	15	ND	ND	6.579	ND	ND	6.792	ND	ND	6.380
500	5	ND	ND	6.579	ND	ND	6.792	ND	ND	6.380
	10	ND	ND	6.579	ND	ND	6.792	ND	ND	6.380
	15	ND	ND	6.579	ND	ND	6.792	ND	ND	6.380

ND: Not Detected.

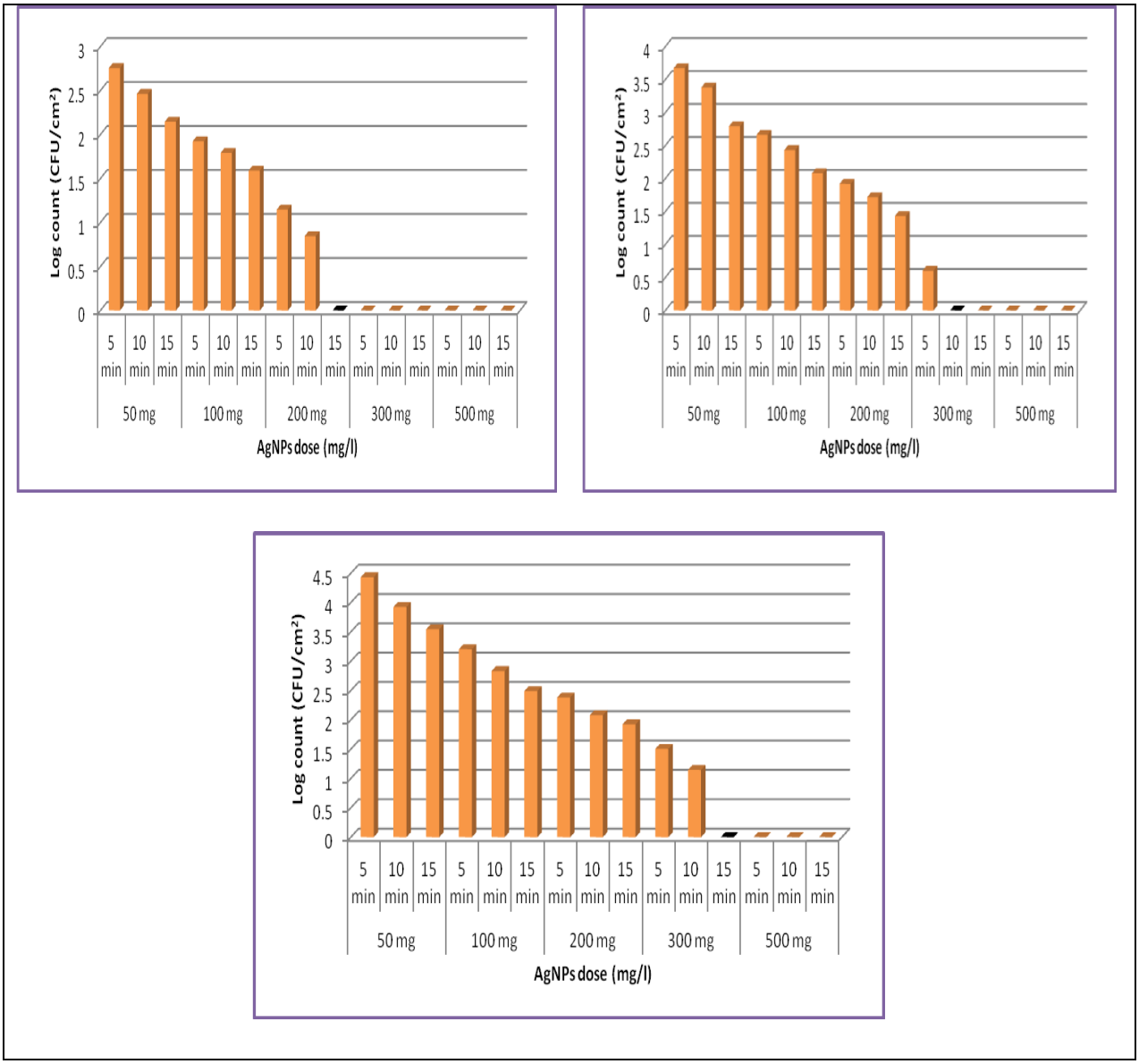

Fig. 95. Antibiofilm effect and MIC of different doses of AgNPs against 10, 40 and 90 days-old *S.* Typhimurium biofilm grown on R pipe material (left- right).

Nanotechnology represents a modern and innovative approach to develop and test new formulations based on metallic nanoparticles with antimicrobial properties. Silver nanoparticles represent a prominent nanoproduct with potential application in medicine and hygiene. Characteristics of silver nanoparticles such as shape and size are important not only for augmenting the antimicrobial activity, but also for reducing tissue and eukaryotic cell toxicities. The possible risks to human health posed by silver nanoparticles and the increased entry into the environment, with subsequent spread of microbial resistance, are of increasing concern given the rise of silver-containing products on the market. Therefore, further studies are needed to fully characterize the toxicity and the mechanisms involved with the antimicrobial activity of these particles. Additionally, this is an important area of research that deserves all our attention owing to its potential application in the fight against multi-drug resistant microorganisms (Durán *et al.*, 2010).

Silver nanoparticles (AgNPs) are antibacterial and multi-functional displaying low toxicity to human cells (Dastjerdi *et al.*, 2009). Its antimicrobial effect at low concentrations is therapeutic against over 650 disease causing organisms in the body (Lok *et al.*, 2006). The ability of silver to prevent the biofilm formation has also been demonstrated (Stobie *et al.*, 2008). The most common synthesis of silver nanoparticles is the chemical reduction of a silver salt solution by a reducing agent such as $NaBH_4$, citrate, or ascorbate (Sondi *et al.*, 2003). Additionally, AgNPs are efficient non-specific antimicrobial agents against the growth of a broad spectrum of bacterial and fungal species in planktonic form (Martínez-Gutierrez *et al.*, 2010). Their antimicrobial activities are attributed to the unique physico-chemical characteristics of AgNPs, such as the high surface area, mass ratio, high reactivity, and sizes in the nanometer range, which confer on them a major advantage for the development of alternative products against multi-drug resistant microorganisms (Martínez-Gutierrez *et al.*, 2012).

Although the cytotoxic mechanism(s) of AgNPs have not yet been elucidated fully, one of the main mechanisms may be the release of Ag^+ ions from the NPs as a result of their exposure to reactive entities generated intracellularly. These reactive

entities include highly reactive oxygen and nitrogen species that can corrode, degrade, or dissolve the NPs into their constituent's atoms. Ag^+ ions are detrimental to biomolecules, interfering with normal metabolic reactions and potentially destabilizing the membranes necessary for maintaining cellular integrity (Park *et al.*, 2011). The diffusivity of NPs is a parameter which controls the mobility, aggregation, and toxicity of these composites. The diffusion of NPs may be hindered by: (1) the porous structure of the biofilm; (2) the local accumulation of NPs by cells, non diffusing macromolecules, or the polysaccharide matrix; and (3) the adsorption of the solute to freely diffusing species, abiotic particles, or gas bubbles (Stewart, 2003).

There are several studies on the antimicrobial activity of silver nanoparticles (Li *et al.*, 2010), the mechanism of inhibitory effects of Ag ions on microorganisms is not yet fully elucidated. Some studies reported that the positive charge on the Ag ion would be crucial for antimicrobial activity (Dibrov *et al.*, 2002). Additionally, (Sondi and Salopek-Sondi, 2004) found silver nanoparticles have the ability to attach to the bacterial cell wall and subsequently penetrate it, thereby causing structural changes in the cell membrane like the permeability of the cell membrane and death of the cell. There is formation of 'pits' on the cell surface, and there is accumulation of the nanoparticles on the cell surface. However, negatively charged silver nanoparticles also killed Gram-negative bacteria in a nanoparticle concentration-dependent manner. Also, the activity was closely associated with the formation of "pits" in the cell wall of bacteria plus nanoparticles incorporation, accumulation and permeability increase of the bacterial cell membrane (Sondi and Salopek- Sondi, 2004).

In addition, several studies also suggested that the generation of free-radicals is involved in some way with the antimicrobial activity of silver nanoparticles. In fact, oxidative stress was observed in cells after silver nanoparticles interaction (Hussain, *et al.*, 2006). However, results described by Lok *et al.* (2007) differ in some way with those obtained by Kim *et al.* (2007) regarding the involvement of free-radicals in the antimicrobial activity of silver nanoparticles. The formation of free radicals by the silver nanoparticles may be considered to be another mechanism by which the cells die. There

have been electron spin resonance spectroscopy studies that suggested that there is formation of free radicals by the silver nanoparticles when in contact with the bacteria, and these free radicals have the ability to damage the cell membrane and make it porous which can ultimately lead to cell death. It has also been proposed that there can be release of silver ions by the nanoparticles and these ions can interact with the thiol groups of many vital enzymes and inactivate those (Feng *et al.*, 2008).

The damage of bacterial cell membrane with observations of pits and gaps on the cells was related to reduction of activity of some enzymes, leakage of sugars and proteins and cell death (Li *et al.*, 2010). Silver nanoparticle shape also affected antibacterial effect against *E. coli*: truncated triangular silver nanoplates displayed stronger biocidal effect than spherical or rod-shaped nanoparticles (Pal *et al.*, 2007).

The results of the present study showed real the doses of AgNPs between 400-500 mg/l were able to reduce the biofilm cell of *L. monocytogenes* and *Salmonella enteric* serovar Typhi. Also, AgNPs of 8.3 nm in diameter stabilized by hydrolyzed casein peptides strongly inhibited biofilm formation of *Escherichia coli* AB1157, *Pseudomonas aeruginosa* PAO1 and *Serratia proteamaculans* 94 in concentrations of 400–500 mg/l, 100 mg/l and 100–200 mg/l, respectively. The viability of *E. coli* AB1157 cells in biofilm was considerably reduced by AgNPs concentrations above 1000-150 mg/ml (Radzig *et al.*, 2013).

Generally, the comparison of the antimicrobial effect of silver ions and silver nanoparticles is an interesting field of research and some studies were performed in this direction. The obtained results were in agreement with the results of Morones *et al.* (2005) they showed that the overall effect of the silver nanoparticles was different from the effect of only silver ions.

In addition results of the present study concluded the MIC of AgNPs for reducing the numbers of biofilm cells was lower than MIC of Ag ions. Moreover, MICs values of $AgNO_3$ in terms of silver ions concentration (Ag^+) are lower than MICs values of AgNPs (Radzig *et al.*, 2013).

The antimicrobial action of AgNPs occurs due to penetration of nanoparticle inside bacterial cell membrane, thereby suppressing respiratory enzymes. Moreover, they interfere with DNA components inside the bacterial cells resulting in the prevention of its replication and transcription (Li *et al.,* 2010). Consequently, from obtained results, it was indicated the biofilm formation by *S.* Typhimurium (Gram-negative) was more susceptible to Ag ions and AgNPs used concentrations than *L. monocytogenes* (Gram-positive) biofilm. These result were concomitant with the results of Chatterjee *et al.,* (2015) they demonstrated *E. coli* was found to be more susceptible to AgNPs as compared to *Staph. aureus*.

On the other hand, Feng *et al.* (2000) and Durán *et al.* (2010) found when nanoparticles were used, no evidence was found for the formation of a low density region as reported previously by for silver ions. Instead, a large number of small silver nanoparticles were observed inside the bacteria. The results of Morones *et al.* (2005) also indicate that silver ions present in the nanoparticle solution contributed but it is not the sole mechanism of antimicrobial activity.

4. Efficacy of antibiotics against *L. monocytogenes* and *S.* Typhimurium biofilm formed on domestic plumping materials

a. Comparative susceptibility of planktonic cells and three different ages of *L. monocytogenes* biofilm to antibiotics

L. monocytogenes has long been recognized as a significant human and animal pathogen (Nightingale *et al.,* 2004) and it is often implicated as sources of human listeriosis cases and outbreaks (De Valk *et al.,* 2001). The outcome of listeriosis depends on the early administration of antibiotics which possessing rapid and bactericidal activity against *L. monocytogenes* (Goulet and Marchetti, 1996; Hof, 2003). Also, *L. monocytogenes* is widely susceptible to clinically relevant classes of antibiotics active against Gram-positive bacteria (Troxler *et al.,* 2000).

Therefore, this part aimed to evaluate antibiotic susceptibility of *L. monocytogenes* planktonic and biofilm cells. Also, data presented in Table (93) reported, the results were interpreted in according to NCCLS (2012).

Table 93. Antimicrobial susceptibility standard for *Staphylococcus aureus* according to NCCLS (2012).

Antibiotics	Abbreviation	Zone diameter (mm)		
		R	I	S
Amoxicillin 10 µg	(AML 10)	≤ 11	12- 14	≥15
Cefixime 5 µg	(CFM 5)	≤ 16	17- 19	≥ 20
Ciprofloxacin 5 µg	(CIP 5)	≤ 15	16- 20	≥ 21
Tetracycline 30 µg	(TE 30)	≤ 14	15-18	≥ 19
Clarithromycin 15 µg	(CLR 15)	≤ 13	14- 17	≥ 18
Streptomycin 10 µg	(S 10)	≤ 12	13-14	≥ 15

R: Resistant; I: Intermediate; S: Susceptible

Data presented in Table (94) and Fig. (96) showed that, *L. monocytogenes* planktonic cells were susceptible to all the tested antibiotics except cefixim which was less sensitive. The obtained results were in agreement with Altuntas *et al.* (2012) who reported real *L. monocytogenes* strains were susceptible to the antibiotics, including penicillin G, vancomycin, tetracycline, chloramphenicol, rifampicin, erythromycin, gentamicin and trimethoprim. Moreover, Ennaji *et al.* (2008) demonstrated that, all strains of *L. monocytogenes* isolated from poultry and meat were susceptible to a wide range of antibiotics effective against Gram-positive bacteria. In addition, Pesavento *et al.*, (2010) demonstrated the *L. monocytogenes* is slowly becoming resistant because of the uptake of resistance genes from other Gram-positive bacteria such as *Listeria* spp., *Staphylococcus* spp. and *Enterococcus* spp. A continued surveillance of emerging antimicrobial resistance of this pathogen is therefore important to ensure an effective treatment of human listeriosis.

It well known that, all *Listeria* spp. were naturally susceptible or intermediate to tetracyclines, aminoglycosides, penicillins (except oxacillin), loracarbef, cefazoline, cefaclor, cefotiam, cefoperazone, carbapenems, macrolides, lincosamides, glycopeptides, dalfopristin/quinupristin, chloramphenicol and rifampicin. While, *Listeria* spp. were naturally resistant or intermediate to the most 'modern' cephalosporins (cefetamet, cefixime, ceftibuten) (Troxler *et al.*, 2000).

Table 94. Antibiotic susceptibility of *L. monocytogenes* ATCC 25152 (Planktonic cells).

Tested strain	Inhibition zone diameter (mm)					
	AML 10	CFM 5	CIP 5	TE 30	CLR 15	S 10
Planktonic cells	25	17	32	28	22	28
Resistant pattern	S	I	S	S	S	S

R: Resistant; I: Intermediate; S: Susceptible

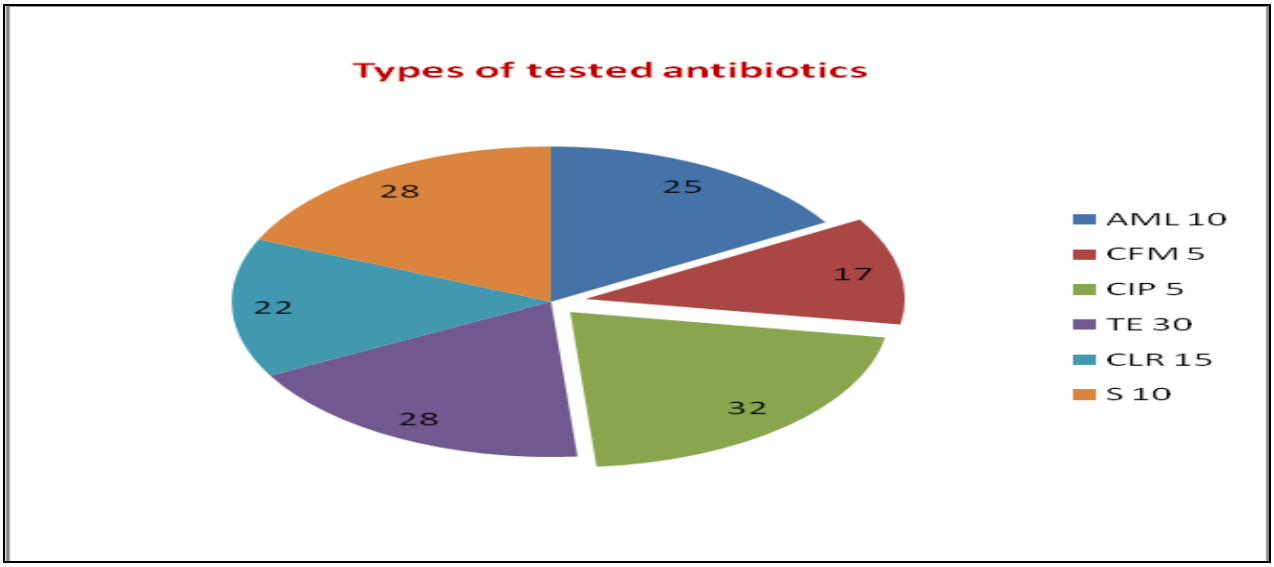

Fig. 96. Antibiotic susceptibility of *L. monocytogenes* planktonic cells.

The attached bacterial cells (biofilm cells) on the surface and grow as a biofilm are protected by EPS from the killing by disinfectant (Stewart, 2002). The planktonic cells of *L. monocytogenes* are slowly becoming antibiotic resistant, continued surveillance of emerging of antimicrobial resistance of these pathogen is important to ensure effective treatment of human listeriosis (Mauro *et al.*, 2007).

In the present study, 10 days-old *L. monocytogenes* biofilm grown on all the tested pipe materials except I pipe were susceptible to all tested antibiotics except to cefixime was resistant. While, the biofilm cells scraped from I pipe were intermediate to clarithromycin (Table 95; Fig. 97).

The results of antibiotic susceptibility against 40 days-old *L. monocytogenes* biofilm presented in Table (96) and Fig. (98). All biofilm cells were resistant to cefixime and susceptible to amoxicillin and streptomycin. Biofilm grown on I pipe material was intermediate to ciprofloxacin, clarithromycin and tetracycline. While, biofilm cells grown

on Cu pipe material was sensitive to amoxicillin, ciprofloxacin and clarithromycin and streptomycin then intermediate to tetracycline and resistant to cefixime.

The PP and PE biofilm cells were sensitive to ciprofloxacin and tetracycline and streptomycin and intermediate to clarithromycin. While in PVC, the biofilm cells were sensitive to all tested antibiotics except cefixime was resistant. Whereas, the biofilm cells grown on R were sensitive to ciprofloxacin and clarithromycin, amoxicillin and streptomycin then intermediate to tetracycline and resistant to cefixime.

Regarding of 90 days-old *L. monocytogenes* biofilm grown on all tested pipe materials were resistant to cefixime and sensitive to amoxicillin except I and PP pipes were intermediate. While, in Cu pipe material, the biofilm cells were sensitive to all tested antibiotics except cefixime was resistant (Table 97; Fig. 99).

Table 95. Antibiotic susceptibility of 10 days-old *L. monocytogenes* biofilm grown on six tested plumping materials.

Tested pipe materials	Inhibition zone diameter (mm)					
	AML 10	CFM 5	CIP 5	TE 30	CLR 15	S 10
PVC	22	11	29	23	21	25
Resistant pattern	S	R	S	S	S	S
PP	20	16	25	24	20	26
Resistant pattern	S	R	S	S	S	S
PE	24	13	27	25	19	23
Resistant pattern	S	R	S	S	S	S
I	18	9	21	19	17	21
Resistant pattern	S	R	S	S	I	S
Cu	25	14	31	23	22	27
Resistant pattern	S	R	S	S	S	S
R	21	15	29	22	20	25
Resistant pattern	S	R	S	S	S	S

R: Resistant; I: Intermediate; S: Susceptible

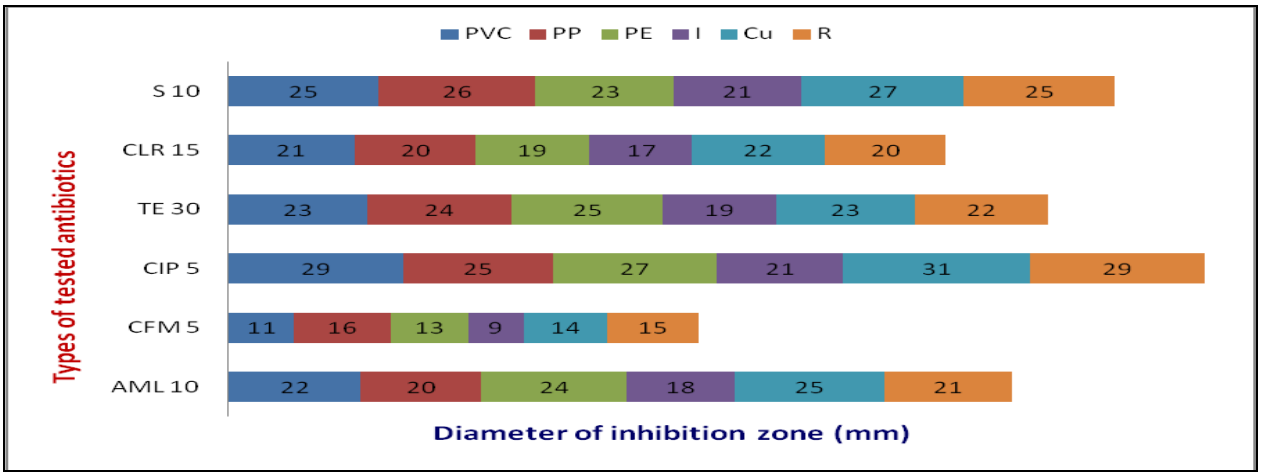

Fig. 97. Antibiotic susceptibility of 10 days-old *L. monocytogenes* biofilm harvested from six different pipe materials.

Table 96. Antibiotic susceptibility of 40 days-old *L. monocytogenes* biofilm grown on six tested plumping materials.

Tested pipe materials	Inhibition zone diameter (mm)					
	AML 10	CFM 5	CIP 5	TE 30	CLR 15	S 10
PVC	20	9	25	19	18	22
Resistant pattern	S	R	S	S	S	S
PP	17	11	22	22	17	23
Resistant pattern	S	R	S	S	I	S
PE	21	8	25	21	17	21
Resistant pattern	S	R	S	S	I	S
I	15	5	17	16	14	18
Resistant pattern	S	R	I	I	I	S
Cu	22	16	27	20	20	23
Resistant pattern	S	R	S	I	S	S
R	19	13	24	18	19	20
Resistant pattern	S	R	S	I	S	S

R: Resistant; I: Intermediate; S: Susceptible

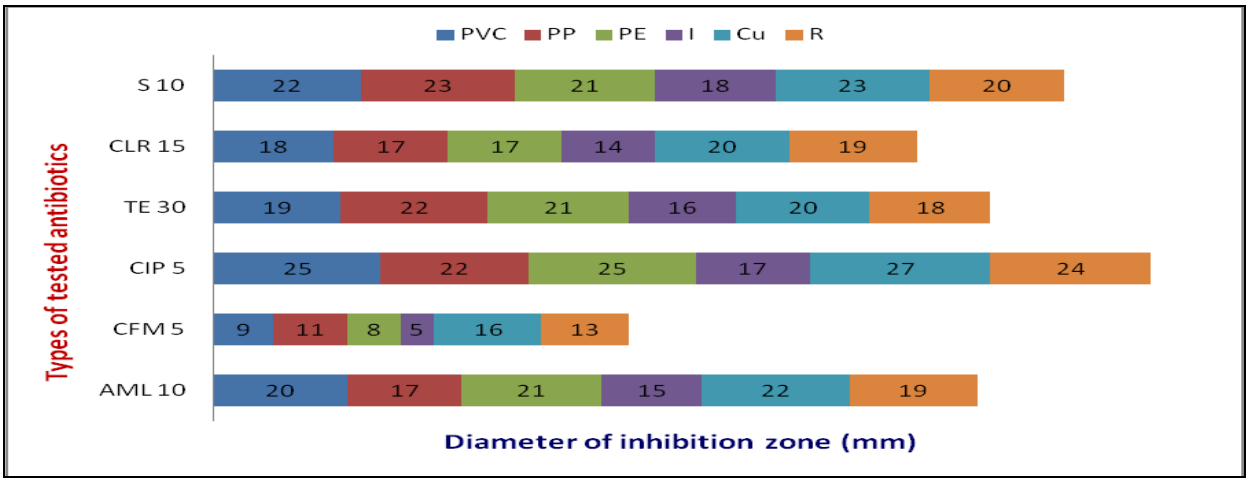

Fig. 98. Antibiotic susceptibility of 40 days-old *L. monocytogenes* biofilm harvested from six different pipe materials.

Table 97. Antibiotic susceptibility of 90 days-old *L. monocytogenes* biofilm grown on six tested plumping materials.

Tested pipe materials	Inhibition zone diameter (mm)					
	AML 10	CFM 5	CIP 5	TE 30	CLR 15	S 10
PVC	16	6	20	17	15	19
Resistant pattern	S	R	I	I	I	S
PP	14	8	18	19	13	20
Resistant pattern	I	R	I	S	R	S
PE	18	5	22	16	14	17
Resistant pattern	S	R	S	I	I	S
I	12	2	13	10	9	11
Resistant pattern	I	R	R	R	R	R
Cu	26	19	29	24	24	25
Resistant pattern	S	I	S	S	S	S
R	15	7	21	15	17	18
Resistant pattern	S	R	S	I	I	S

R: Resistant; I: Intermediate; S: Susceptible

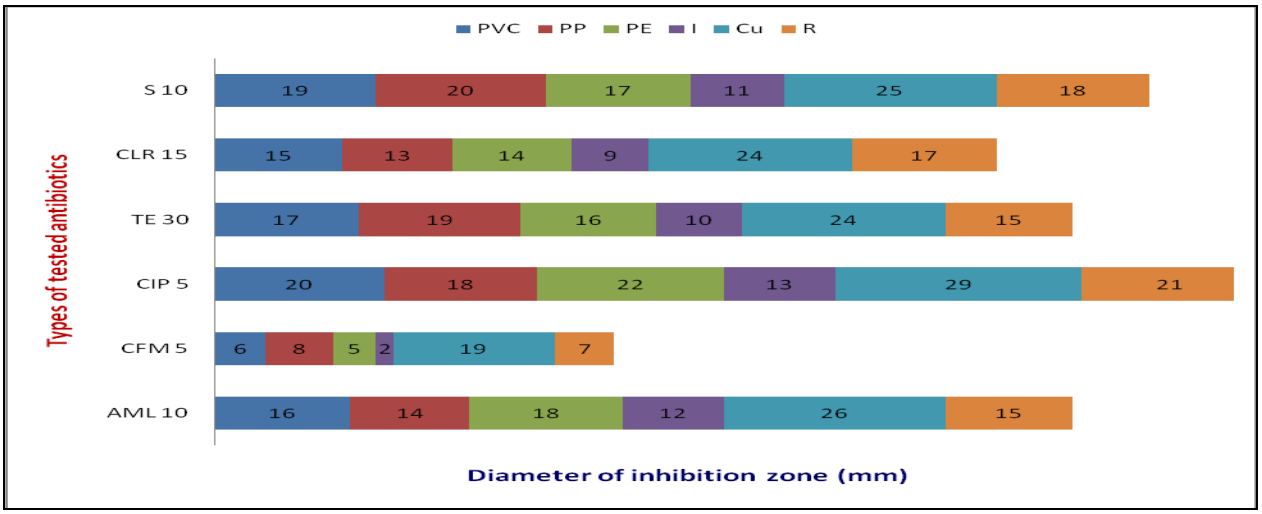

Fig. 99. Antibiotic susceptibility of 90 days-old *L. monocytogenes* biofilm harvested from six different pipe materials.

From the findings of the present research, it can be mentioned real *L. monocytogenes* planktonic cells were more susceptible to antibiotics than *L. monocytogenes* biofilm especially which formed in iron (I) pipe. This may be attributed to, the biofilm cells are embedded in polymer matrix which protected biofilm cells against antimicrobial agents and harsh environmental factors. These similar to (Simões and Vieira, 2009) who demonstrated the biofilm cells were more resistant to antimicrobials compared to planktonic cells. Bacteria living in biofilm can be up to 1000 times more resistant to antibacterial compounds (such as disinfectants, antibiotics and surfactants) than planktonic cells (Davey and O'Toole, 2000).

Also, the present study found the old age of biofilm cells were more reistant to the tested antibiotics than the young age of biofilm cells. This may be due to the production of large amounts of exopolysaccharides in 90 days-old than 10 days-old *L. monocytogenes* biofilm. Additionally, Mah and O'Toole, (2001) demonstrated the mechanism for enhanced antimicrobial resistance is believed to involve alterations in gene expression leading to a phenotype difference between the planktonic and sessile forms. The sessile forms are more resistant as they produce exopolysaccharides, have different growth characteristics and take up nutrients and drugs differently from their planktonic counterpart.

Stewart (2002) suggested that, the EPS matrix which surrounding the attached cells produces a potent barrier that restricts the penetration of chemically reactive biocides inside the biofilm. Both structure and properties of extracellular compounds associated with solid surface cells differ from those synthesized by planktonic bacteria (Drenkard and Ausubel, 2002). These differences refer mostly to polysaccharide components of the EPS layer. Additionally, many researches cleared evidences that the high range of polysaccharide components increased the amount of functional groups in the EPS matrix. It determines a lower susceptibility of biofilm populations to biocides, antibiotics and antimicrobial peptides. The functional groups of exopolysaccharides react with antimicrobial agents (Drenkard and Ausubel, 2002).

b. Comparative susceptibility of planktonic cells and three different ages of *S.* Typhimurium biofilm to antibiotics

Typhoid fever caused by the bacterium *S.* Typhimurium is an endemic disease in the tropic and subtropic. The disease is systemic and is often contracted by ingestion of contaminated food or water with the pathogen usually from a feco-oral source. The occurrence of typhoid fever is an indicator of poor personal and environmental hygiene. The illness may be mild or severe but sometimes fatal. It is encountered worldwide but is primarily found in developing countries where sanitary conditions are poor (WHO, 2001).

Also, *Salmonella* resistance against the optimal antimicrobials used to treat invasive salmonellosis is increasing, including resistance to extended-spectrum cephalosporins (ESCs) such as first-, second- and third-generation cephalosporins (Arlet *et al.*, 2006). Thus, the purpose of this section was comparing the antibiotic susceptibility between planktonic *S.* Typhimurium and biofilm cells. The antibiotic susceptibility test was carried out for planktonic *S.* Typhimurium cells and six biofilm samples in three ages (10, 40 and 90 days) using disc diffusion method.

Data presented in Table (98) reported, the results were interpreted in according to NCCLS (2012).

Table 98. Antimicrobial susceptibility standard for *Salmonella* spp according to NCCLS (2012).

Antibiotics	Abbreviation	Zone diameter (mm)		
		R	I	S
Amoxicillin 10 µg	(AML 10)	≤ 11	12- 14	≥15
Cefixime 5 µg	(CFM 5)	≤ 15	16- 18	≥ 19
Ciprofloxacin 5 µg	(CIP 5)	≤ 15	16- 20	≥ 21
Tetracycline 30 µg	(TE 30)	≤ 11	12- 14	≥ 15
Clarithromycin 15 µg	(CLR 15)	≤ 11	12- 14	≥ 15
Streptomycin 10 µg	(S 10)	≤ 11	12-14	≥ 15

R: Resistant; I: Intermediate; S: Susceptible

S. Typhimurium , particularly the multidrug resistant (MDR) strain is relatively ubiquitous and is the cause of many community endemic and epidemic typhoid fever infections. MDR strain of *S.* Typhimurium *is* of concern not only because of its resistance to available antibiotics resulting in high death rate but also because of its potential for epidemic outbreaks, which may be difficult to manage. The consequence of such outbreak will no doubt be devastating especially in developing countries where health facilities are often inadequate (Adabara *et al.*, 2012).

Moreover, *Salmonella* serovar Newport MDR-AmpC is resistant to ampicillin, chloramphenicol, streptomycin, tetracycline, amoxicillin-clavulanic acid, cephalothin, cefoxitin, ceftiofur and ceftriaxone (Zhao *et al.*, 2003). This resistance has severe implications for both humans and animals as ceftriaxone is primarily used to treat salmonellosis in children (Hohmann, 2001).

As shown in Table (99) and Fig. (100) the results indicated that *S.* Typhimurium planktonic cell was resistant to all tested antibiotics. Data presented in Table (100) and Fig. (101) found 10 days-old *S.* Typhimurium biofilm grown on all tested pipe materials resistant to all tested antibiotics.

Table 99. Antibiotic susceptibility *S.* Typhimurium ATCC 14028 (Planktonic cells).

Tested strain	Inhibition zone diameter (mm)					
	AML 10	CFM 5	CIP 5	TE 30	CLR 15	S 10
Planktonic cells	9	13	12	10	8	11
Resistant pattern	R	R	R	R	R	R

R: Resistant

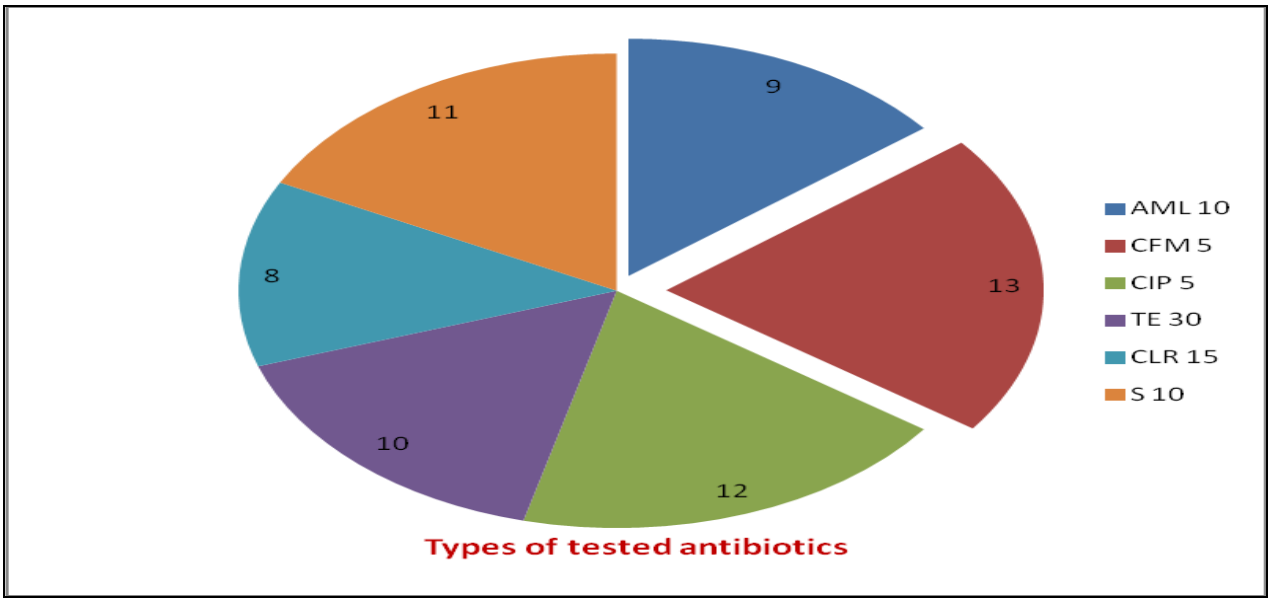

Fig. 100. Antibiotic susceptibility of *S.* Typhimurium planktonic cells.

Table 100. Antibiotic susceptibility of 10 days-old *S.* Typhimurium biofilm grown on six tested plumping materials.

Tested pipe materials	Inhibition zone diameter (mm)					
	AML 10	CFM 5	CIP 5	TE 30	CLR 15	S 10
PVC	6	11	8	7	5	6
Resistant pattern	R	R	R	R	R	R
PP	4	8	11	4	7	7
Resistant pattern	R	R	R	R	R	R
PE	8	7	10	7	6	9
Resistant pattern	R	R	R	R	R	R
I	3	5	7	3	3	5
Resistant pattern	R	R	R	R	R	R
Cu	9	13	12	10	8	4
Resistant pattern	R	R	R	R	R	R
R	9	11	11	8	8	7
Resistant pattern	R	R	R	R	R	R

R: Resistant; I: Intermediate; S: Susceptible

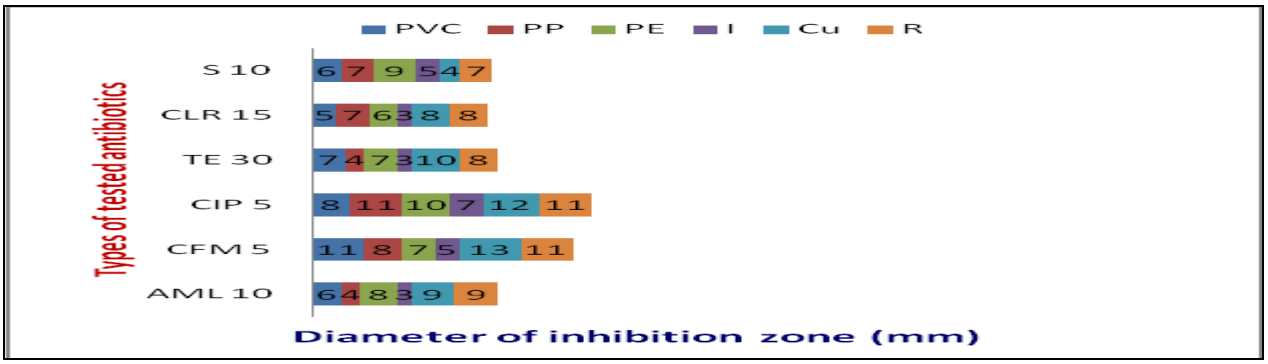

Fig. 101. Antibiotic susceptibility of 10 days-old *S.* Typhimurium biofilm harvested from six different pipe materials.

Regarding, 40 days-old *S.* Typhimurium biofilm, Results indicated the biofilm grown on all tested pipe materials were resistant to all tested antibiotics, except in Cu pipe the biofilm cells were intermediate to amoxicillin, ciprofloxacin, tetracycline, clarithromycin and streptomycin (Table 101; Fig. 102).

As shown in Table (102) and Fig. (103) the 90 days-old *S.* Typhimurium biofilm grown on Cu pipe material were sensitive to amoxicillin, tetracycline and streptomycin, while intermediate to cefixime, ciprofloxacin and clarithromycin. The biofilm grown on other tested materials were resistant to all tested antibiotics.

Table 101. Antibiotic susceptibility of 40 days-old *S. Typhimurium* biofilm grown on six tested plumping materials.

Tested pipe materials	Inhibition zone diameter (mm)					
	AML 10	CFM 5	CIP 5	TE 30	CLR 15	S 10
PVC	4	8	7	5	4	6
Resistant pattern	R	R	R	R	R	R
PP	3	5	9	2	1	5
Resistant pattern	R	R	R	R	R	R
PE	7	6	8	4	4	7
Resistant pattern	R	R	R	R	R	R
I	0	0	0	0	0	0
Resistant pattern	R	R	R	R	R	R
Cu	13	12	16	12	11	14
Resistant pattern	I	R	I	I	I	I
R	7	10	8	7	5	3
Resistant pattern	R	R	R	R	R	R

R: Resistant; I: Intermediate

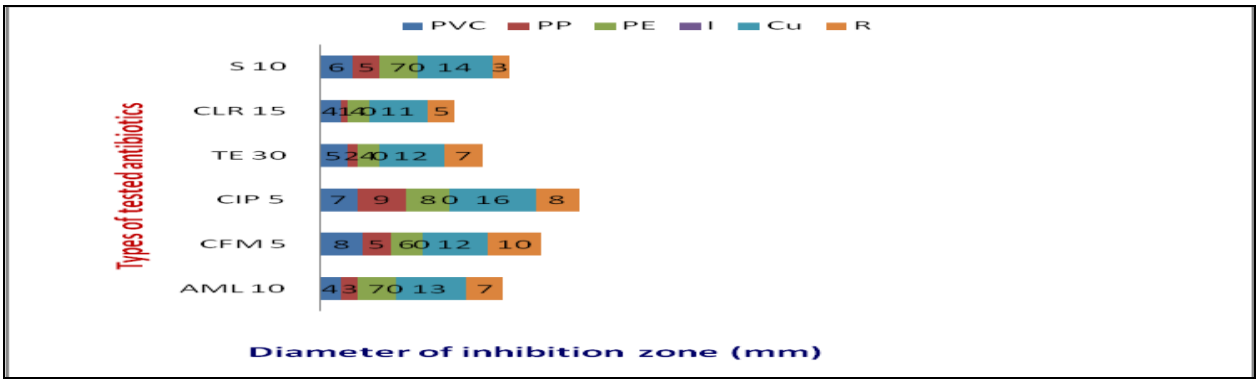

Fig. 102. Antibiotic susceptibility of 40 days-old *S.* Typhimurium biofilm harvested from six different pipe materials.

Table 102. Antibiotic susceptibility of 90 days-old *S.* Typhimurium biofilm grown on six tested plumping materials.

Tested pipe materials	Inhibition zone diameter (mm)					
	AML 10	CFM 5	CIP 5	TE 30	CLR 15	S 10
PVC	4	8	7	5	4	6
Resistant pattern	R	R	R	R	R	R
PP	3	5	9	2	1	5
Resistant pattern	R	R	R	R	R	R
PE	7	6	8	4	4	7
Resistant pattern	R	R	R	R	R	R
I	0	0	0	0	0	0
Resistant pattern	R	R	R	R	R	R
Cu	16	17	20	15	14	18
Resistant pattern	S	I	I	S	I	S
R	3	4	3	5	3	1
Resistant pattern	R	R	R	R	R	R

R: Resistant; I: Intermediate; S: Susceptible

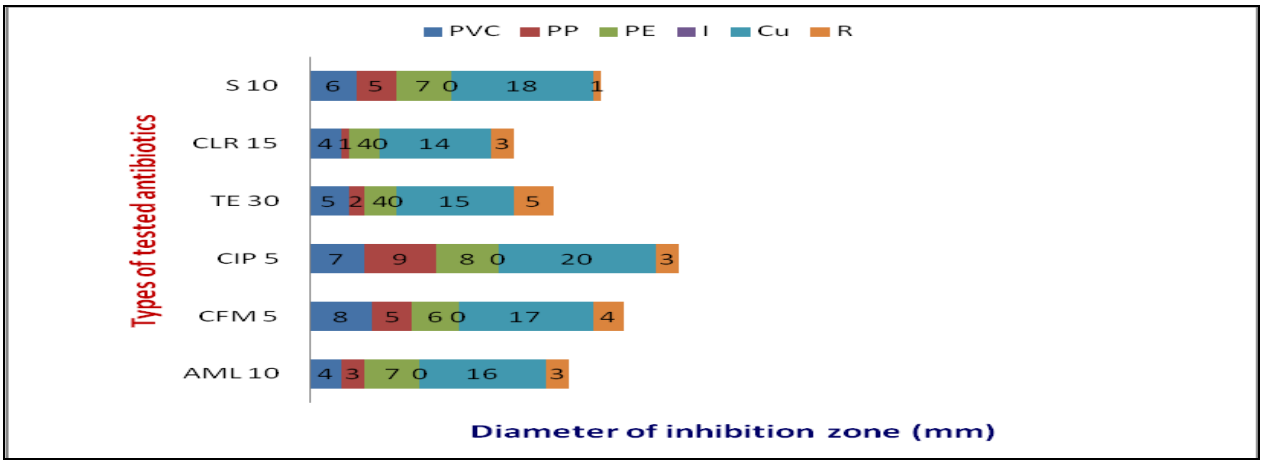

Fig. 103. Antibiotic susceptibility of 90 days-old *S.* Typhimurium biofilm harvested from six different pipe materials.

The obtained results of planktoinc cells are in good agreement with the results of Butt *et al.* (2003) they indicated *S.* Typhimurium has rapidly gained resistance to antibiotics like ampicillin, ceftriaxone, and cotrimoxazole, and also to previously effective drugs like ciprofloxacin. Additionally, *S.* Typhimurium were generally resistant to ceftriaxone, cefuroxime, amoxicillin, ampicillin, ciprofloxacin, and augmentin which are the drugs of choice routinely used in the study area for the treatment of typhoid fever (Adabara *et al.*, 2012). The emergence of antimicrobial resistance, especially the multidrug resistance to ampicillin, chloramphenicol, and cotrimoxazole, has further complicated the treatment and management of enteric fever. The resistance to well-known and trusted antimicrobial agents is widely recognized as one of the greatest challenges that physicians face in the management of adult and pediatric infections (Dajani, 2002).

From the all previous results, it can be summrized the biofilm provide the protection of bacteria cells against tested antimicrobial agents. also, the biofilm cells are more resistant to theses agents than planktonic cells. Also, Van Houdt and Michiels (2010) found biofilm formation provide protection to bacterial cells against a wide range of environmental challenges including UV light radiation, metal toxicity, pH and osmotic changes, dehydration, host immune responses, antimicrobial agents and disinfectants).

Also, Frank *et al.* (2007) found the biofilm cells are more resistant to antimicrobials compared to their planktonic counterparts. Reduced diffusion, neutralizing

mechanisms, persisted cells, resistant genes and stress responses are factors linked to biofilm-associated resistance (Parsek and Fuqua, 2004).

e. Survival and persistence of 90 days-old of biofilm of *L. monocytogenes* and *S.* Typhimurium

As shown in Table (103), the Physical, chemical and bacteriological of tested water.

Survival of 90 days-old *L. monocytogenes* biofilm cells which formed on different types of pipe materials in two different water types (groundwater and tap water) was mentioned. The biofilm samples were collected from six different pipe materials, in addition the planktonic cells of *L. monocytogenes*. The survival time of *L. monocytogenes* planktonic cell and 90 days-old *L. monocytogenes* biofilm cells scratched from different types of pipe materials (PVC, PP, PE, I, CU and R) in groundwater recovered using two media like plate count agar and Listeria selective agar (LSA).

Table 103. Physical, chemical and bacteriological characteristics of tested tap and groundwater.

Parameters	Unit	Tap water	Ground water	Egyptian Standards 2007
pH		7.1	7.2	6.5-8.5
Turbidity	NTU	0.5	0.4	1
Odor		Odorless	Odorless	Odorless
Color	Co/Pt Unite	Colorless	Colorless	Colorless
Electric Conductivity	µmohs/Cm	462	523	
Total Dissolved Solids	mg/L	255	266	1000
Total Hardness (as $CaCO_3$)	mg/L	150	148	500
Calcium Hardness (as $CaCO_3$)	mg/L	90	100	350
Ammonia	mgNH_3/L	0.0	68	0.5
Nitrite	mg NO_2/L	0.0	1.3	0.2
Nitrate	mg NO_3/L	0.3	14.8	45
Iron	mg/L	0.2	0.17	0.3
Manganese	mg/L	0.03	0.0	0.4
TVBC at 37°C	CFU/ml	3	5	≤50
TVBC at 22°C	CFU/ml	4	5	≤50
Total coliform	MPN-index/100ml	ND	ND	Free

1. Survival and persistence of 90 days-old *L. monocytogenes* biofilm

The data illustrated graphically in Fig. (104) showed, real the longest survival time in groundwater of *L. monocytogenes* biofilm cells scratched from I pipe material was observed up to 191 days. In contrast, the shortest survival time of biofilm cells scratched from Cu pipe material was up to 105 days using PCA media.

While, the scratched biofilm cells from PVC, PE and R were survived up to 158 days followed by the strain scratched from pp was survived up to 144 days. The reference strain (planktonic cell) was survived up to 116 days.

Regarding *L. monocytogenes* survival in groundwater results, the longest survival time in groundwater of *L. monocytogenes* biofilm cells scratched from I pipe material was observed up to 191 days. In contrast, the shortest survival time of biofilm cells scratched from Cu pipe material was up to 105 days using LSA media. While, the scraped biofilm cells from PVC, PE and R were survived up to 158 days followed by the strain scratched from pp was survived up to 144 days. The reference strain (planktonic cell) survived up to 116 days (Fig. 105).

As shown in Fig. (106), results indicated the longest survival time in tap water of *L. monocytogenes* biofilm cells scratched from I pipe material was observed up to 191 days. The shortest survival time of biofilm cells scratched from Cu pipe material was 105 days using PCA media. The scratched biofilm cells from PVC, PE and R were survived up to 158 days followed by the strain scratched from pp 144 days. The reference strain (planktonic cell) survived up to 116 days. Furthermore, the statistical analysis results showed, the presence a strong relationship with significance ($P \leq 0.05$) between the survival time and log counts.

Results presented graphically in Fig. (107) showed, the longest survival time in tap water of *L. monocytogenes* biofilm cells scratched from I pipe material was 191 days. In contrast, the shortest survival time of biofilm cells scratched from Cu pipe material was 105 days using LSA mediium. While, the scraped biofilm cells from PVC, PE and R survived up to 158 days followed by the strain scratched from (144 days). The reference strain (planktonic cell) survived 116 days.

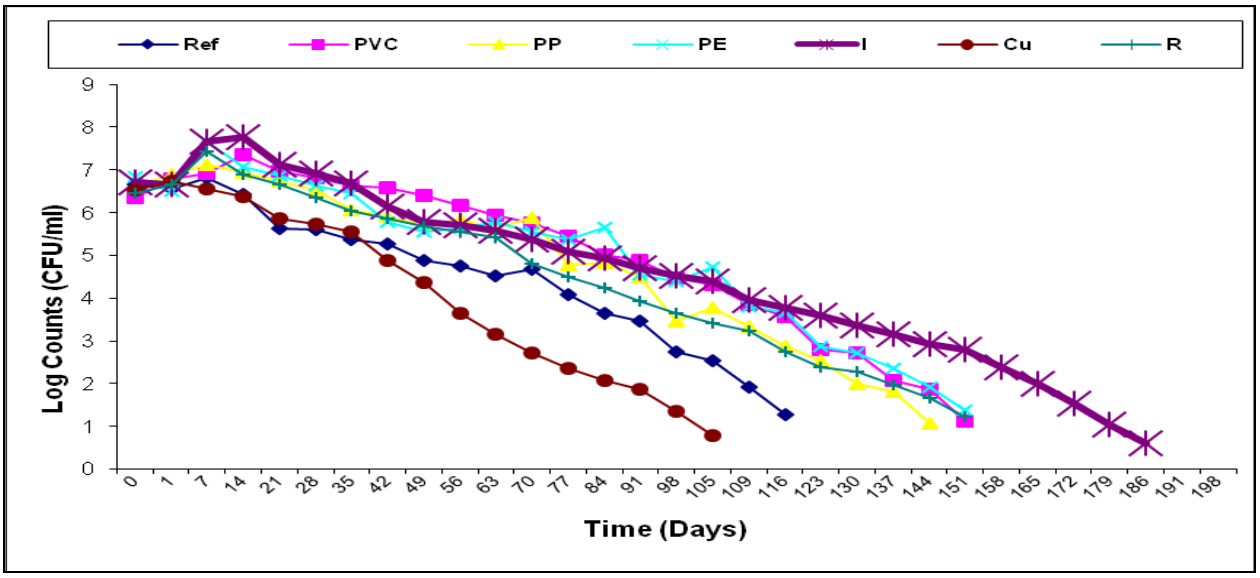

Fig. 104. Survival and persistence of *L. monocytogenes* planktonic and biofilm cells on groundwater and recovery using PCA.

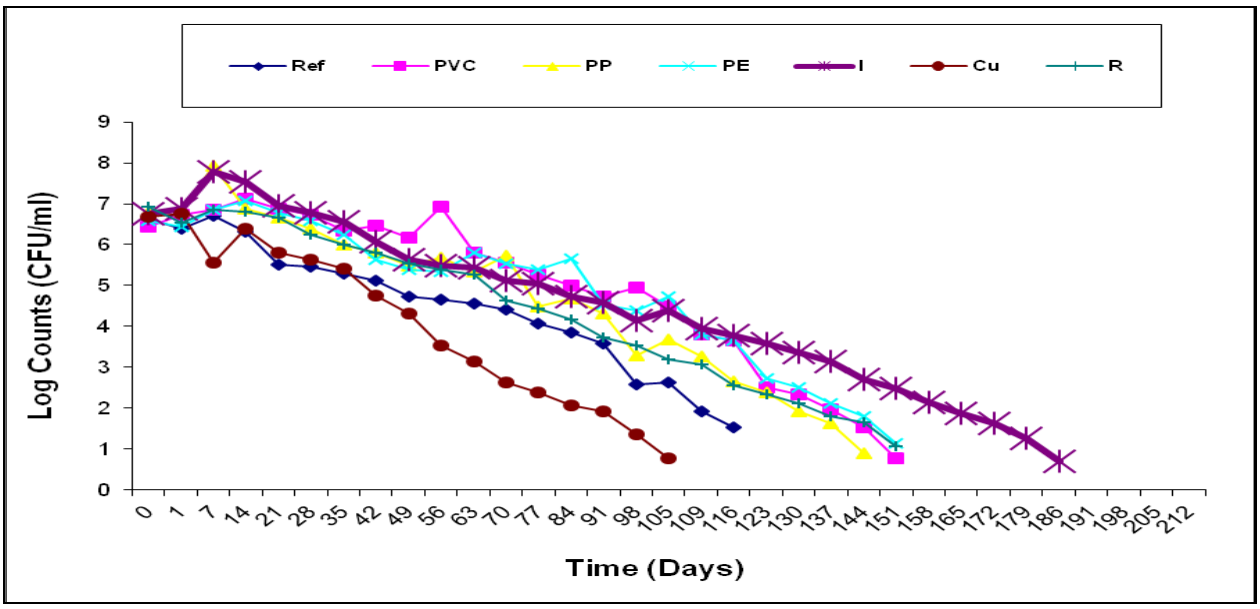

Fig. 105. Survival and persistence of *L. monocytogenes* planktonic and biofilm cells on groundwater and recovery using LSA.

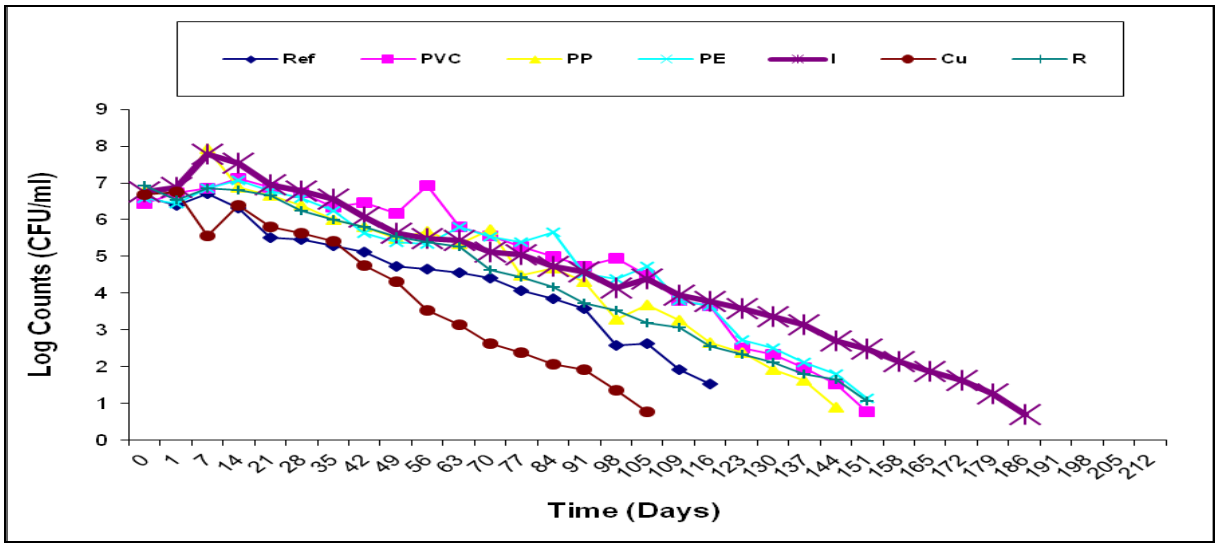

Fig. 106. Survival and persistence of *L. monocytogenes* planktonic and biofilm cells on tap water and recovery using PCA.

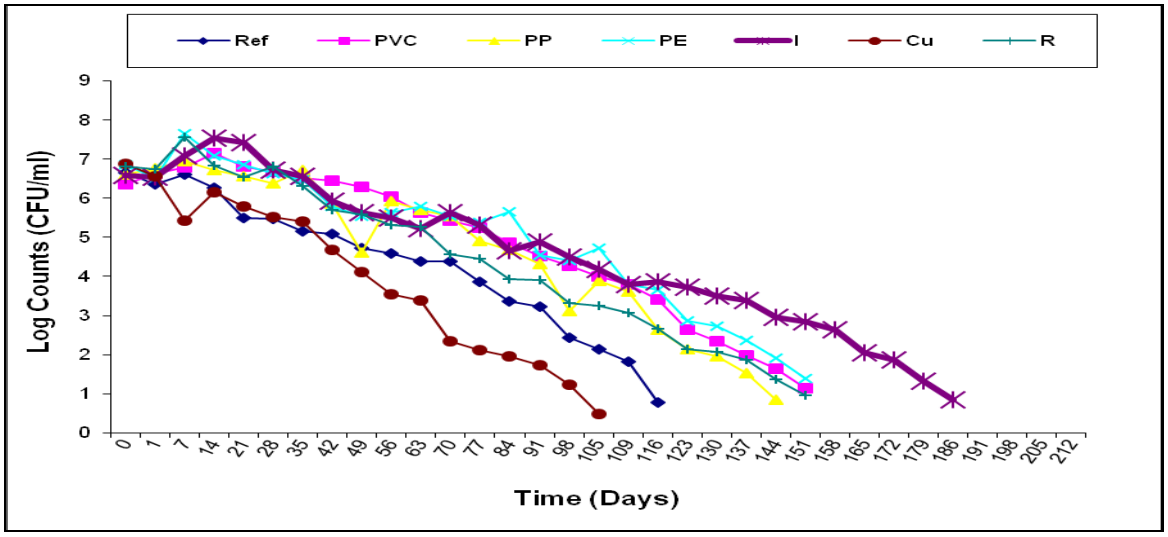

Fig. 107. Survival and persistence of *L. monocytogenes* planktonic and biofilm cells on tap water and recovery using LSA.

The long survival time of *L. monocytogenes* in water environments is of great importance from an epidemiological point of view. This is related to an ability of this pathogen to multiply and grow under unfavorable conditions for other microorganisms, especially at low temperature and within the wide range of pH (Vasseur *et al.*, 1999). Owing to this *L. monocytogenes* is able to survive in water even for 300 days, which can, consequently, cause a sporadic or epidemic incidence of listeriosis among people (Karatzas *et al.*, 2000). Mainly elderly people, pregnant women, neonates, people with

lowered immunity, suffering from AIDS and neoplasms and patients after organ transplantations are susceptible to disease (Ramaswamy *et al.,* 2007). The infection usually leads to meningitis, encephalitis, gastroenteritis, septicemia, as well as abortions, stillbirths, or neonate infections (Capita *et al.,* 2001). Mortality rates in the course of listeriosis are high and amounts to approximately 30% (Gasanov *et al.,* 2005).

2. Survival and persistence of 90 days-old *S.* Typhimurium biofilm

Survival of 90 days-old *S.* Typhimurium biofilm cells which formed on different types of pipe materials in two different water types (groundwater and tap water) were carried. The biofilm samples collected from six different pipe materials, in addition the planktonic cells of *S.* Typhimurium also were tested at the same conditions and water (Table 103).

As shown in Figs. (108,109,110,111), the survival time of *S.* Typhimurium planktonic cells and 90 days-old of biofilm cells scratched from different types of pipe materials (PVC, PP, PE, I, CU and R) in groundwater recovered by using two media like plate count agar and Bismuth sulfite agar (BSA).

The longest survival time of biofilm strain scratched from iron (I) pipe material was up to 198 days. The shortest survival time of biofilm strain scratched from Cu pipe was 109 days. The scratched strains from PVC, PE and R were survived up to 191 days followed by the strain scratched from pp survived up to 186 days. The reference strain (planktonic cell) was survived up to 151 days (Fig. 108).

As shown in Fig. (109), the longest survival time of *S.* Typhimurium planktonic cells and 90 days-old of biofilm cells scratched from different types of pipe materials (PVC, PP, PE, I, Cu and R) in groundwater recovered by BSA. The longest survival time of biofilm strain scratched from iron pipe was observed up to 205 days. In contrast, the shortest survival time of biofilm strain scratched from Cu pipe was up to 98 days. The scratched strains from PVC, PP, PE and R survived up to 186 days. On the other hand, the planktonic cell survived up to 144 days.

Data illustrated in Fig. (110) shows the longest survival time of *S.* Typhimurium planktonic cells and 90 days-old of biofilm cells scratched from different types of pipe materials (PVC, PP, PE, I, CU and R) in tap water recovered by PCA. The longest survival time of biofilm strain scratched from iron pipe was observed up to 212 days followed by strain scratched from R up to 191 days. The shortest survival time of biofilm strain scratched from Cu pipe was up to 109 days. The scratched strains from PVC, PP and PE were survived up to 186 days. The planktonic cell was survived up to 151 days.

The longest survival time of *S.* Typhimurium planktonic cells and 90 days-old biofilm cells scratched from different types of pipe materials (PVC, PP, PE, I, CU and R) in tap water recovered by bismuth sulfite agar. The longest survival time of biofilm strain scratched from iron pipe was observed up to 212 days followed by strain scratched from PE and R up to 191 days. In contrast, the shortest survival time of biofilm strain scratched from Cu pipe was up to 109 days. While the scratched strains from PVC and PP were survived up to 179 days. On the other hand, the planktonic cell was survived up to 144 days (Fig. 111).

Generally, in the present study, it was found, the presence a highly significant correlation ($P \leq 0.05$) between the survival times and log counts of all tested microorganisms. Vestby *et al.* (2009) showed a correlation between persistence and biofilm establishment of *Salmonella* thus this may be an important factor for its longevity in the factory environment. These Salmonella strains appear to be a greater risk to human health via food contamination by surviving for longer periods (Iibuchi *et al.*, 2010).

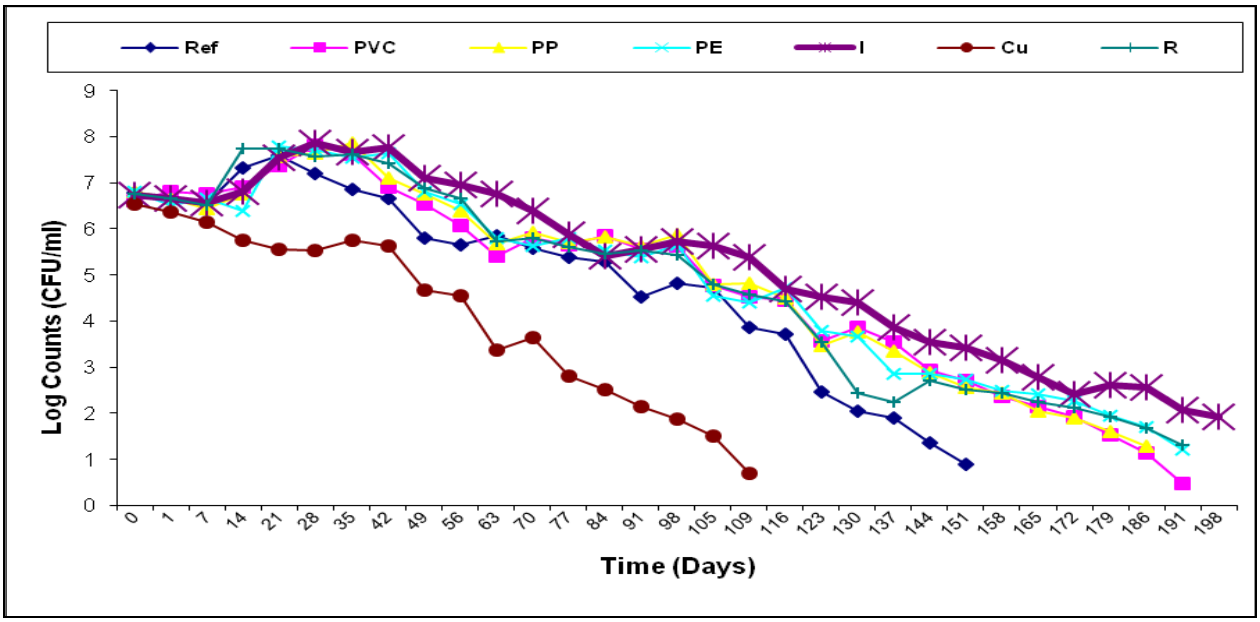

Fig. 108. Survival and persistence of *S.* Typhimurium planktonic and biofilm cells on groundwater and recovery using PCA.

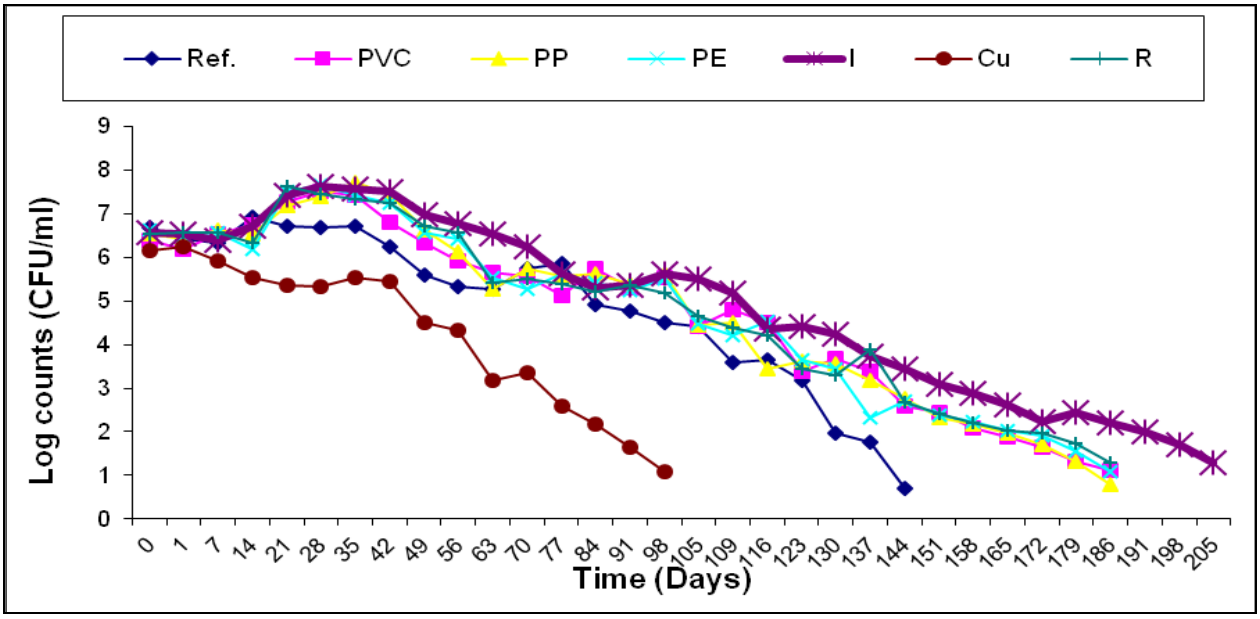

Fig. 109. Survival and persistence of *S.* Typhimurium planktonic and biofilm cells on groundwater and recovery using BSA.

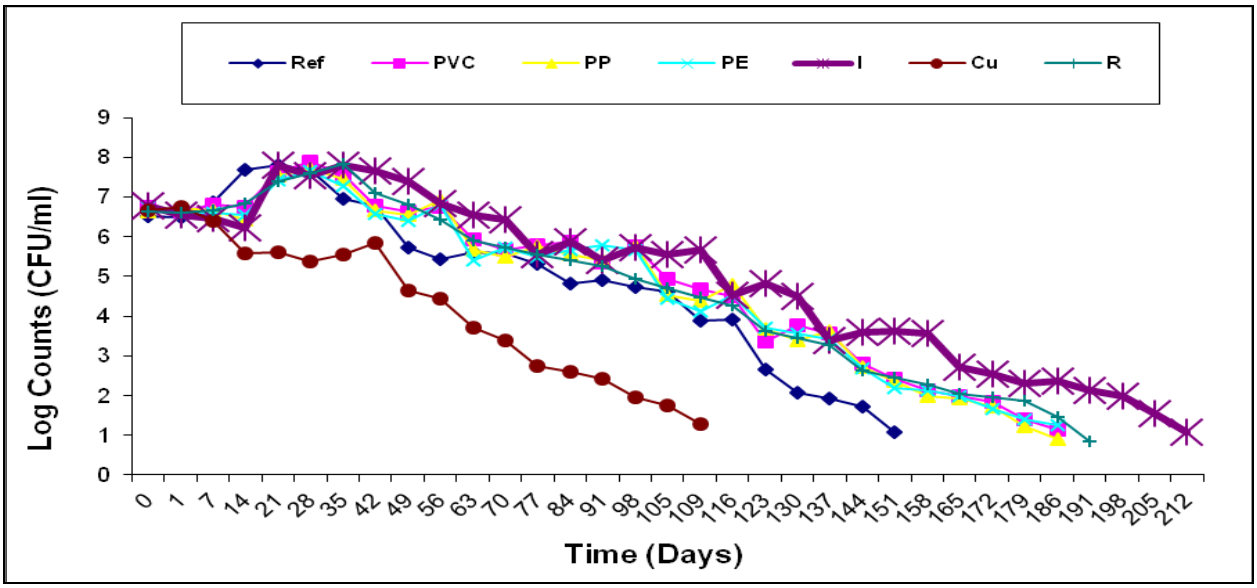

Fig. 110. Survival and persistence of *S.* Typhimurium planktonic and biofilm cells on tap water and recovery using PCA.

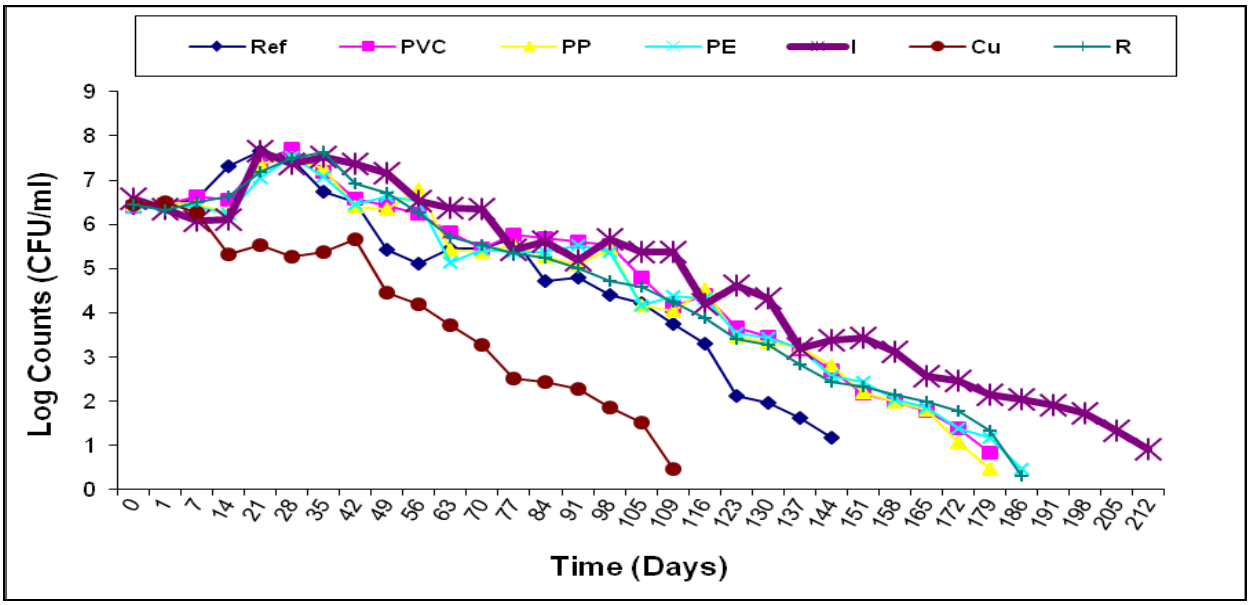

Fig. 111. Survival and persistence of *S.* Typhimurium planktonic and biofilm cells on tap water and recovery using BSA.

Contamination of environmental water by pathogenic microorganisms and subsequent infections originated from such sources during different contact and non-contact recreational activities are a major public health problem worldwide particularly in developing countries. The main pathogens frequently associated with enteric infection in developing countries are *S.* Typhimurium and paraTyphimurium. Although the natural

habitat of *Salmonella* is the gastrointestinal tract of animals and it find its way into natural water through faecal contamination are frequently identified from various aquatic environments (Abhirosh *et al*., 2008). Typhoid fever caused by *S.* Typhimurium is a common infectious disease occurring in all the parts of the world with it's the highest endemicity in certain parts of Asia, Africa, Latin America and in the Indian subcontinent with an estimated incidence of 33 million cases each year with significant morbidity and mortality (Threlfall, 2002). In most cases of the disease are transmitted by polluted water because of the poor hygienic conditions, inadequate clean water supplies and sewage treatment facilities (Girard *et al*., 2006).

Although, *Salmonella* spp. has been isolated from fresh, estuarine and marine waters, they showed differential survival response to those aquatic environments and the results were sometimes contradictory in relation to salinity. For instance, it has been reported that *Salmonella* showed very low survival in sea water (Lee *et al*., 2010). On the contrary Sugumar and Mariappan (2003) found that they exhibited very long survival up to 16 to 48 week in sea water. But, it is also documented that it survived for 54 days (Moore *et al*., 2003) and 58 days in freshwater (Sugumar and Mariappan, 2003).

f. Toxicity test for selected domestic pluming materials using Microtox analyser 500

Results of this study in Table (104) indicated that, all the tested pipe materials were non toxic. It refers no released toxic substances in the early stages of the tested pipes to the water. Also, these pipes did not cause any corrosion. In addition, EPA (2012) suggested the pipes for drinking water distribution should be suitable for the transport of water. In many countries norms have been established on the minimal required quality of the pipes. When in contact with water or soil, the material should be resistant to possible chemical reactions and the material should not allow toxic substances to be released into the water. Furthermore, the pipes have to be resistant against a specified internal and external press.

Table 104 . The toxicity degree for the tested pluming materials.

Tested pipe materials samples	EC $_{50\%}$	Toxicity unit	Toxicity degree
PVC	≥100	-	Non toxic
PP	≥100	-	Non toxic
PE	≥100	-	Non toxic
I	≥100	-	Non toxic
Cu	≥100	-	Non toxic
R	≥100	-	Non toxic

CONCLUSIONS

1. From this study, it was found that, the microbial population in the natural biofilm collected from sinks drainage pipes were varied and high in kitchen and bathroom sinks. Thus, it is recommended that, the microbial population in natural biofilm should be monitored from time to time in addition to this, washing these sinks each time interval by using disinfectants.
2. From the results of designed DWDS, It was showed that, the iron pipe materials were encouraged the biofilm formation. While, the plastic-based materials less encouraged the formation of biofilm. Also, the biofilm formation on copper pipes was inhibited. Thus, the iron pipes are not recommended for carrying water in DWDS. Thus the plastic-based materials are the most appropriate to be used for carrying the drinking water.
3. From the results of present study, it can be concluded that, *L. monocytogenes* and *S.* Typhimurium were able to form the biofilm on designed DWDS model. Also, the numbers of *S.* Typhimurium biofilm cells were greater than *L. monocytogenes* biofilm cells.
4. The present study found that, the culture based methods were more accurate than direct count methods by using epi-fluorescence microscope. So, the culture based methods were recommended for seriously determination of the real numbers of biofilm cells in addition to molecular approaches in the further studies.
5. The results showed, the amounts of exopolysaccharide produced from *L. monocytogenes* biofilm were more than *S.* Typhimurium biofilm.
6. From the results, it was found the planktonic cells were more sensitive to antimicrobial agents than the biofilm cells. Also, the young biofilm cells were more sensitive than the old biofilm cells. So, the biofilm formation in young ages must be prevented by using antimicrobial agents.
7. The results concluded that, the *L. monocytogenes* biofilm cells were more resistant to chlorine, Ag ions and AgNPs than *S.* Typhimurium biofilm cells. While in case

of antibiotics sensitivity test, the results showed *S.* Typhimurium biofilm cells were more resistant than *L. monocytogenes* biofilm cells. Thus, in situ clean up and flushing of DWDS must be carried out with definite intervals times with strong antimicrobial agents.

8. The survival and regrowth of microorganisms in DWDS can be affected by not only biological factors but also interaction of various physico-chemical factors such as pipe materials and disinfectant types, residuals and concentrations.
9. Finally, many studies for how to prevent and control the biofilm formation are recommended in the future.

SUMMARY

The objectives of the present study were determining the microbial population contributing in biofilm formation. In addition to, study the behavior and biological characteristics of some pathogenic bacteria in biofilm state.

To achieve the first aim forty eight natural biofilm samples were collected from different types of microhabitats (drinking water distribution system (DWDS), kitchen sink drainage pipes, bathroom sink drainage pipes and lab sink drainage pipes) to determine the characteristics of the predominant microorganisms. Spread plate method was used to determine the pathogenic microbes by using enzymatic cultures.

The results of microbiological characterization of natural biofilm collected from three different materials of the DWDS (PP, PVC and iron pipes) showed that, the heterotrophic plate count (TVBC) of biofilm scraped from iron pipe material was higher than PVC and PP pipe materials in DWDS. Moreover, the tested pathogenic bacteria were absent in all biofilm samples in PVC and PP pipe materials, while there were present in iron pipe material.

Regarding to the results of the microbiological examination of natural biofilm samples which collected from kitchen sink drainage pipes, bathroom sink drainage pipes and lab sink drainage pipes it can be observed that the average counts of bacterial indicators and tested pathogens except *Cl. perfringens* were high in natural biofilm samples collected from kitchen sink drainage than others.

To achieve the second aim the behaviour of two pathogenic bacteria (*L. monocytogenes* and *S.* Typhimurium) for biofilm formation were studied by using desigened DWDS model. The results recorded that, the highest growth curve of *L. monocytogenes* and *S.* Typhimurium biofilm were reported in I pipe, while the lowest was recorded in Cu pipe. In the case of plastic-based materials (PVC, PP, PE and R) the results were observed that no differences between them in the growth curve.

The amounts of exopolysaccharide were determined in *L. monocytogenes* and *S.* Typhimurium biofilm grown on six different domestic plumping materials during

experiments periods (10 to 90 days). After estimation the amounts of exopolysaccharide produced by *L. monocytogenes* and *S.* Typhimurium biofilm, the results indicated that there was a positive correlation with significant between the quantities of and the biofilm ages ($P \leq 0.01$). Additionally, the results indicated that, the highest amount of exopolysaccharide of *L. monocytogenes* biofilm was recorded in iron (I) pipe. Whereas, the lowest amount of exopolysaccharide was observed in Cu pipe material in all biofilm ages from 10 to 90 days. In addition to, the amount of exopolysaccharide (EPS) in biofilm produced by *L. monocytogenes* (Gram positive bacterium) was greater than *S.* Typhimurium (Gram negative bacterium). In spite of, the biomass growth rate of *S.* Typhimurium biofilm was more than *L. monocytogenes* biofilm grown on all tested plumping materials.

Three microscopic examination methods (epi-fluorescence, transmission and scanning electron microscope) were used to investigate biofilm formation of *L. monocytogenes* and *S.* Typhimurium biofilm grown on six different domestic plumping materials. When the direct counting of biofilm cells using epi-flourescence microscopy the results demonstrated that, highest total cells counts of either *L. monocytogenes* or *S.* Typhimurium biofilm were recorded in I pipe. On the contrary, the lowest total cells count was found in Cu pipe materials. By using TEM for investiagtion of 90 days-old *L. monocytogenes* biofilm , the photomicrograph results indicated that the biofilm cells were embedded in a polymer matrix and different shapes of exopolysaccharide. Regarding to results of SEM, the structure of 90 days-old *S.* Typhimurium biofilm grown on PVC, PP, PE, I, Cu and R observed the thick biofilm wrapped by EPS and the highest thickness was shown in I pipe materials. While, the lowest thickness in Cu pipe material was showed.

To control and prevent of biofilm formation by using different antimicrobial agents against planktoinc and biofilm cells of *L. monocytogenes* and *S.* Typhimurium, the results concluded that *L. monocytogenes* and *S.* Typhimurium biofilm grown on all tested pipe materials was more resistant to chlorine, Ag ions, AgNPs and antibiotics than

planktonic cells. Also younger biofilm age (10 days) was more sensitive to these antimicrobial agents than older ages (40 and 90 days-old).

The findings of this study, it can be summerized, the *L. monocytogenes* biofilm was more resistant to chlorine, Ag ions and AgNPs than *S.* Typhimurium biofilm. While in case of antibiotic, results showed *S.* Typhimurium biofilm was more resistant than *L. monocytogenes* biofilm.

From the previous results of the present study cleared that 90 days-old of biofilm was more resistant to antimicrobial agents and environmental factors. Thus, the survival and persistence of 90 days-old *L. monocytogenes* and *S.* Typhimurium biofilm were determined in two types of water (tap and ground water).

The results showed that, the longest survival time in groundwater of *L. monocytogenes* and *S.* Typhimurium biofilm cells scratched from I pipe material was observed. In contrast, the shortest survival time of biofilm cells scratched from Cu pipe material was recorded.

Finally, the toxicity test was carried out using Microtox 500 analyser to evaluate all tested plumping materials. The results found that all tested materials were nontoxic.

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APPENDIX

1. Media used

Plate Count Agar (OXOID, UK).

Plate count agar medium was used for the detection and enumeration of total viable bacterial counts in biofilm samples. 17.5 grams were suspended in 1000 ml distilled water. The medium boiled to dissolve the medium completely, then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min.

Ingredients	gm/l
Casein enzymic hydrolysate	5.0
Yeast extract	2.5
Dextrose	1.0
Agar	15.0

Final pH (at 25°C) was 7.0±0.2.

R2A agar (OXOID, UK).

R2A agar used for heterotrophic plate count of treated potable water using longer incubation periods, in accordance with European Pharmacopoeia. 18.12 grams were suspended in 1000 ml distilled water. Heated to boiling to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min. Overheating was avoided.

Ingredientes	gm/l
Casein hydrolysate	0.5
Yeast extract	0.5
Proteose peptone	0.5
Glucose	0.5
Starch	0.5
Dipotassium hydrogen phosphate	0.3
Magnesium sulphate anhydrous	0.24
Sodium pyruvate	0.3
Agar	15.0

Final pH (at 25°C) was 7.2±0.2.

Tryptone Soya broth (TSB) (OXOID, UK).

Tryptone Soya broth is a general purpose medium used for cultivation of a wide variety of microorganisms and recommended for sterility testing of moulds and lower bacteria. 30 grams were suspended in 1000 ml distilled water. Heated if necessary to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min. Mixed well and dispensed as desired.

Ingredientes	gm/l
Pancreatic digest of casein	17.0
Papaic digest of soyabean meal	3.0
Sodium chloride	5.0
Dextrose	2.5
Dibasic potassium phosphate	2.5

Final pH (at 25°C) was 7.3±0.2.

Brain-Heart Infusion broth (Oxoid-UK)

Ingredientes	gm/l
Brain infusion solids	12.5
Beef heart infusion solids	5.0
Proteose peptone	10.0
Glucose	2.0
Sodium chloride	5.0
Disodium phosphate	2.5

Final pH (at 25°C) was 7.4±0.2.

Rapid Hicoliform agar (HiMedia, India).

Rapid HiColiform Agar is used for detection and confirmation of total coliform and fecal coliform on the basis of enzyme substrate reaction from water samples, using a combination of chromogenic and fluorogenic substrates. Suspend 31.03 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 min. The presence of total coliform is indicated by a blue-green color of the colonies and fecal coliform indicated by a blue fluorescence under UV light after incubation at 35-37°C for 18-24 hrs.

Ingredientes	gm/l
Peptone special	5.0
Sodium chloride	5.0
Sorbitol	1.0
Dipotassium hydrogen Phosphate	2.7

Potassium dihydrogen Phosphate	2.0
Sodium lauryl sulphate	0.10
Chromogenic mixture	0.08
Fluorogenic mixture	0.05
Agar	15.0

Final pH (at 25°C) was 6.8±0.2.

HiCrome ECC Agar (HiMedia, India).

HiCrome ECC Agar is a differential medium recommended for presumptive identification of *Escherichia coli* and other coliform in environmental samples. Suspend 55.83 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 min. Cool to 50°C. Mix well and pour into sterile Petri plates.

The colonies of *E. coli* appeared blue to purple color after incubation at 35-37°C for 18-24 hrs

Ingredientes	**gm/l**
Peptone special	5.0
Sodium chloride	5.0
Yeast extract	3.0
Disodium hydrogen Phosphate	3.5
MonoPotassium hydrogen Phosphate	1.5
Chromogenic mixture	0.03
Neutral red	20. 3
Agar	15.0

Final pH (at 25°C) was 6.8±0.2.

Rapid HiEnterococci agar (HiMedia, India).

Rapid HiEnterococci agar was used for the selective isolation and enumeration of enterococci from environmental samples. Suspend 33.61 gm in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 min. Cool to 50°C and pour into sterile Petri plates. **Warning**: Sodium azide has a tendency to form explosive metal azides with plumbing materials. Positive enterococci colonies appeared blue green color after an incubation at 35-37°C for 18-24 hrs.

Ingredientes	gm/l
Peptone special	10.0
Sodium chloride	5.0
Disodium hydrogen Phosphate	1.25
Sodium Azide	5.0
Polysorbate 80	2.0
Chromogenic mixture	0.06
Agar	15.0

Final pH (at 25°C) was 7.2±0.2.

Hifluoro Pseudomonas Agar Base (HiMedia, India)

HiFluoro Pseudomonas Agar Base is recommended for selective isolation of *Pseudomonas aeruginosa* from clinical and non-clinical specimens by fluorogenic method. Suspend 46.75 grams in 1000 ml distilled water containing 10ml glycerol. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 min. Mix well and pour into sterile Petri plates. *P.aeruginosa* cleaves the fluorogenic compound to release the fluorogen which produces a visible fluorescence under long wave UV light after incubation at 35-37°C for 24- 48 hrs.

Ingredients	gm/l
Pancreatic digest of gelatin	18.0
Magnesium chloride	1.4
Potassium sulphate	10.0
Cetrimide	0.3
Fluorogenic mixture	2.05
Agar	15.0

Final pH (at 25°C) was 7.2±0.2.

HiCrome Improved Salmonella Agar (HiMedia, India)

HiCrome Improved Salmonella Agar (HiMedia, India) used for detection of *Salmonella spp.* in water samples. 26.25 grams of the medium were suspended in 1000 ml distilled water. Boiled gently to dissolve the medium completely. The medium was not autoclaved. Cooled to 50°C. Mixed well and poured into sterile Petri dishes. *Salmonella typhi* were light pink colonies after incubation at 35°C for 24-48 hrs.

Ingredients	gm/l
Peptone special	8.0
Yeast extract	2.0
Sodium deoxycholate	1.0
Chromogenic mixture	3.25
Agar	15.0

Final pH (at 25°C) was 7.3±0.2.

HiCrome Aureus Agar Base (HiMedia, India)

HiCrome Aureus Agar Base (HiMedia, India) used for isolation and identification of *Staphylococcus aureus* from water samples. 63.1 grams were suspended in 950 ml distilled water. Boiled to dissolve the medium completely. Sterilized by autoclaving at 15lbs pressure (121°C) for 15 min. Cooled to 50°C and aseptically 50 ml concentrated Egg yolk Tellurite Emulsion was added. Mixed well and poured into sterile Petri dishes. Lithium chloride is harmful. Avoid bodily contact and inhalation of vapors. On contact with skin, wash with plenty of water immediately. *S. aureus* colonies were brown black with clear zone around the colony.

Ingredientes	gm/l
Casein enzymatic hydrolysate	12.0
Pancreatic digest of gelatin	3.0
Beef extract	6.0
Yeast extract	5.0
Sodium pyruvate	10.0
Lithium chloride	5.0
Chromogenic mixture	2.1
Agar	15.0

Final pH (at 25°C) was 7.0±0.2.

HiCrome Listeria Agar Base (HiMedia, India)

HiCrome Listeria Agar Base (HiMedia, India) used for rapid and direct identification of *Listeria monocytogenes* in water samples. 33.62 grams were suspended in 500 ml distilled water. Boiled with stirring to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min. Cooled to 50°C. The rehydrated contents of 1 vial of HiCrome Listeria Selective Supplement (HiMedia, India) added aseptically. Mixed well and poured into sterile Petri dishes. *Listeria*

monocytogenes colonies were bluish green in color with positive rhamnose fermentation reaction after incubation at 35°C for 24-48 hrs.

Ingredients	gm/l
Peptone, special	23.0
Sodium chloride	5.0
Yeast extract	1.0
Meat extract	5.0
Lithium chloride	5.0
Rhamnose	10.0
Phenol red	0.12
Chromogenic mixture	5.13
Agar	15.0

Final pH (at 25°C) was 7.3±0.2.

HiCrome Bacillus Agar (Sigma)

HiCrome Bacillus Agar is recommended for detection and isolation of *Bacillus* from a mixed culture by chromogenic method. Suspend 49.2 g in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. DO NOT AUTOCLAVE. Cool to 45-50°C and aseptically add the rehydrated contents of 1 vial of Polymyxin B Selektiv Supplement (Fluka P9602) if desired. Mix well and pour into sterile petri plates. The chromogenic mixture present in the medium is cleaved by the enzyme beta-glucosidase found in *B.cereus* resulting in the formation of blue colonies after incubation at 30°C for 24-48 hrs.

Ingredientes	gm/l
Peptic digest of animal tissue	10.0
Meat extract	1.0
D-Mannitol	10.0
Sodium chloride	10.0
Chromogenic mixture	3.2
Phenol Red	0.025
Agar	15.0

Final pH (at 25°C) was 7.1±0.2.

M-CP Agar Base (HiMedia, India)

M-CP Agar Base with selective supplements used for isolation and enumeration of *Clostridium perfringens* from water sample. 35.6 grams of dehydrated powder were

suspended in 485 ml distilled water. Boiled to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min. Cooled to 50°C. Aseptically the rehydrated contents of 1 vial of M-CP Selective Supplement I (HiMedia, India) and 1 vial of M-CP Selective Supplement II (HiMedia, India) were added. Mixed well and poured into sterile Petri dishes. Yellow colonies becoming old rose to pink-red upon exposure to ammonia fumes for 30 seconds were considered to be *Clostridium perfringens* after incubation at 44°C for 24-48 hrs under anaerobic conditions.

Ingredients	gm/l
Tryptose	30.0
Yeast extract	20.0
Sucrose	5.0
L-Cysteine hydrochloride	1.0
Magnesium sulphate, $7H_2O$	0.1
Bromocresol purple	0.04
Ferric chloride, $6H_2O$	0.09
Indoxyl ß-D-glucoside	0.06
Agar	15.0

Final pH (at 25°C) was 7.6±0.2.

HiCrome Candida Differential Agar (HiMedia, India)

HiCrome Candida Differential Agar (HiMedia, India) used for isolation and identification of *Candida* species from water samples. 42.72 g were suspended in 1000 ml distilled water. Boiled to dissolve the medium completely. The medium was not autoclaved. Cooled to 50°C and poured into sterile Petri dishes. *Candida albicans* colonies were light green in color after incubation at 30°C for 48hrs.

Ingredients	gm/l
Peptone, special	15.0
Yeast extract	4.0
Dipotassium hydrogen phosphate	1.0
Chromogenic mixture	7.22
Chloramphenicol	0.5
Agar	15.0

Final pH (at 25°C) was 6.3±0.2.

Bismuth sulfite agar (Oxoid–UK)

Bismuth sulfite agar was used for isolation, enumeration and selection of *salmonella* from environmental samples. Suspend 52.33 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. DO NOT STERILIZE IN AUTOCLAVE or by fractional sterilization since overheating may destroy the selectivity of the medium. The sensitivity of the medium depends largely upon uniform dispersion of precipitated bismuth sulphite in the final gel, which should be dispersed before pouring into sterile Petri plates. The positive colonies were Olive-black to black colonies with metallic sheen color after incubation at 35-37°C for 48 hrs.

Ingredients	gm/l
Enzymatic Digest of Casein	5.0
Enzymatic Digest of Animal Tissue	5.0
Beef extract	5.0
Dextrose	5.0
Disodium phosphate	4.0
Ferrous sulphate	0.3
Bismuth sulphite indicator	8.0
Brilliantgreen	0.025
Agar	20.0

Final pH (at 25°C) was 7.7±0.2.

Listeria Selective agar (Oxoid–UK)

Listeria selective agar was used for isolation, enumeration and selection of *Listeria monocytogenes* from environmental samples. Suspend 57.0 grams in 1000 ml distilled water. Heat and boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 min. Cool to 50°C and hold at this temperature in a water bath Using sterile forceps, add one Listeria Select tab (MS33) per 500ml medium.

Warning: Listeria Selectatab (MS33) contains cycloheximide and therefore care should be taken at all times to avoid skin contact. *Listeria* monocytogenes colonies appeared black color approximately 1 mm in diameter that is surrounded by black halos.

Ingredients	gm/l
Peptone mixture	15.2
Lithium chloride	15.0
Sodium chloride	5.0
Glucose	0.5
Yeast extract	2.0
Dipotassium hydrogen phosphate	0.8
Starch	1.0
Enzymic casein	4.0
Asculin	1.0
Ferric ammonium citrate	0.5
Agar	12.0

Final pH (at 25°C) was 7.0±0.2.

Muller Hinton (MHA) agar (Difco, Franc)

Mueller Hinton Agar is used in antimicrobial susceptibility testing by the disk diffusion method. This formula conforms to Clinical and Laboratory Standard Institute (CLSI), formerly National Committee for Clinical Laboratory Standards (NCCLS). Suspend 38.0 gm in 1000 ml distilled water. Heat and boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 min. Cool to 50°C and pour into sterile Petri plates. The results refer to appropriate documents for correct zone sizes.

Ingredients	gm/l
Beef Extract	2.0
Acid Hydrolysate of Casein	17.5
Starch	1.5
Agar	17.0

Final pH (at 25°C) was 7.0±0.2.

2. Reagents used

a. Reagents of chlorine requirement

1. Preparation of starch indicator

5 g starch was added into a little cold water, and then poured into one liter of boiling distilled water, after settled for overnight. Clear supernatant was used.

2. Preparation of potassium dichromate solution

1.225 g potassium dichromate ($K_2Cr_2O_7$) **(**0.025 N) was dissolved in one liter distilled water.

3. Preparation of standard sodium thiosulfate solution

6.205 g sodium thiosulfate ($Na_2S_2O_3.5H_2O$) (0.025 N) was dissolved in one liter freshly boiled distilled water and standardized against potassium dichromate.

4. Preparation of N, N-diethyl-p-phenylenediamine (DPD) indicator solution

One g DPD oxalate, or 1.5 g DPD sulfate pentahydrate, or 1.1 g anhydrous DPD sulfate was dissolved in chlorine- free distilled water containing 8 ml (1+3) H_2SO_4 and 200 mg EDTA-disodium salt. Then maked up to one liter, and stored in a brown glass-Stoppard bottle in the dark and discarded when colored.

b. Reagents of EPS extraction

1. Preparation phosphate buffer solution

Dissolve 1.7 g/l KH_2PO_4 and 4.5 g/l $Na_2HPO_4 \cdot 12\ H_2O$ in 1000 ml distilled water and heat to boiling to complete dissolve. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 min. Final pH was adjusted to 7.0.

2. Phenol solution 80% (w/v)

Dissolve 80 g phenol crystals in 100 ml distilled water, the solution is yellow. Phenol solution should be protected from light. it may congeal or deposit crystals if stored at a temperature below 4°. It should be completely melted before use.

c. Reagents of Microtox analyzer 500

- Microtox Reagent2: freeze dried (lyophilized) luminescent bacterium *Vibrio fischerii* .
- Microtox Reconstitution Solution: specially purified distilled water free of organics/toxic material (storage 4^{o}C, 1 year maximum).
- Microtox Diluent: specially purified distilled water containing 2% NaCl to provide osmotic protection for the marine luminescent bacteria (storage 4^{o}C, 1 year maximum).

الملخص العربي

الخصائص البيولوجية لبعض الميكروبات المشاركة في تكوين الفيلم الحيوي

تهدف الدراسة الحالية إلي تحديد وتوصيف الميكروبات المشاركة في تكوين الفيلم الحيوي الطبيعي بالإضافة إلي دراسة سلوك والخصائص البيولوجية لبعض البكتيريا الممرضة كنموزج لتكوين الفيلم الحيوي.

ولتحقيق الهدف الأول تم تجميع 48 عينة من الفيلم الحيوي الطبيعي من مصادر بيئية مختلفة (شبكات توزيع مياه الشرب, مواسير صرف أحواض المطابخ, مواسير صرف أحواض الحمامات و مواسير صرف أحواض المعامل) وذلك لتحديد أعداد وأنواع الميكروبات بها وكذلك ماهو أكثرها انتشارا في مثل هذه الأوساط البيئية المختلفة. وتم تقدير أعداد هذه الميكروبات باستخدام طريقة الأطباق المصبوبة علي بيئات غير متخصصة و الفرد علي الأطباق باستخدام البيئات الإنزيمية السريعة.

وقد أظهرت نتائج التحليل الميكروبيولوجي لعينات الفيلم الحيوي الطبيعي المجمعة من شبكات توزيع مياه الشرب ذات مواسير (الحديد, PVC, PP) أن العد الكلي للبكتيريا الموجودة في عينات الفيلم الحيوي المتكون علي المواسير الحديد كانت اعلي من المجمعة من مواسير PVC, PP, بالإضافة إلي تواجد الميكروبات الممرضة أيضا في العينات المجمعة من المواسير الحديد وغيابها في الأنواع الأخري

علي الجانب الأخر, أظهرت نتائج الفحص الميكروبيولوجي لعينات الفيلم الحيوي المجمعة من مواسير صرف أحواض المطابخ, مواسير صرف أحواض الحمامات و مواسير صرف أحواض المعامل أن أعداد الدلائل البكتيرية والميكروبات الممرضة فيما عدا (*Cl. Perfringens*) كانت متواجدة بأعداد اعلي في عينات الفيلم الحيوي المجمعة من مواسير المطابخ عن غيرها. بالإضافة إلي ذلك, كانت نتائج التحليل الميكروبيولوجي للميكروبات الممرضة المختبرة أظهرت أن *Staph. aureus* أكثر انتشارا في عينات الفيلم الحيوي المجمعة من مواسير صرف أحواض الحمامات بينما كانت *L. monocytogenes* أقل تواجدا.

ولتحقيق الهدف الثاني لهذه الدراسة هو دراسة سلوك والخصائص البيولوجية لبعض البكتيريا المرضية التي تشارك في تكوين الفيلم الحيوي لذلك من خلال تصميم نظام يحاكي شبكة توزيع مياه الشرب لدراسة سلوك وخصائص نوعين من البكتيريا الممرضة وهما *L. monocytogenes, S.* Typhimurium

أوضحت النتائج المتحصل عليها أن أعلي معدل لمنحني النمو والزيادة في تكوين الفيلم الحيوي لكلا من بكتيريا ال *L. monocytogenes*, *S.* Typhimurium ظهر تأثيره علي المواسير المصنوعة من الحديد في حين أقلها علي المواسير المصنوعة من النحاس. بينما في حالة الفيلم الحيوي المتكون علي المواسير المصنوعة من

البلاستيك (PVC, PP, PE, R), أظهرت النتائج أنه لا يوجد فرق ملحوظ في معدل النمو للفيلم الحيوي حيث أنة يتكون بمعدل متقارب بين هذه الأنواع من المواسير. بالإضافة إلي ذلك أثبتت النتائج ان معدل النمو للفيلم الحيوي كان أقل في الفيلم الحيوي بكتيريا ال *L. monocytogenes* عن الفيلم الحيوي لبكتيريا ال *S.* Typhimurium .

ولدراسة العلاقة بين كمية السكريات العديدة وعمر الفيلم الحيوي تم تقدير كمية السكريات العديدة التي ينتجها الفيلم الحيوي ل *L. monocytogenes*, *S.* Typhimurium المتكون علي 6 أنواع من المواسير المختبرة خلال فترة التجربة من عمر 10 أيام وحتى عمر 90 يوم. وأظهرت النتائج وجود علاقة طردية بين كمية السكريات العديدة وعمر الفيلم الحيوي. كما أوضحت النتائج أيضا أن كمية السكريات العديدة المنتجة من الفيلم الحيوي المتكون علي مواسير الحديد أعلي من تلك المتكونة علي المواسير النحاس. وأظهرت النتائج أن كمية السكريات العديدة المنتجة من الفيلم الحيوي ل *L. monocytogenes* كانت أعلي من الكمية المنتجة من الفيلم الحيوي ل *S.* Typhimurium.

بالإضافة إلي ما سبق تم فحص الفيلم الحيوي ل *L. monocytogenes*, *S.* Typhimurium بأستخدام 3 طرق فحص ميكروسكوبي وهما الميكروسكوب الفلوروسينتي, الميكروسكوب الالكتروني النافذ والماسح وذلك للحصول إلي أعداد وتركيب خلايا الفيلم الحيوي.

فقد أظهرت نتائج العد المباشر لخلايا الفيلم الحيوي بأستخدام الميكروسكوب الفوروسينتي أن أعداد خلايا الفيلم الحيوي المجمعة من مواسير الحديد كانت أعلي عن غيرها بينما أقلها كانت للخلايا المكونة علي المواسير النحاس.

أوضحت النتائج المتحصل عليها للفيلم الحيوي لبكتيريا *L. monocytogenes* عمر 90 يوم باستخدام الميكروسكوب الإلكتروني النافذ, كانت خلايا الفيلم الحيوي منغمسة في السكريات العديدة وكذلك لوحظ أن خلايا الفيلم الحيوي المتكونة علي مواسير الحديد كانت تفرز كمية كبيرة من السكريات العديدة عن غيرها. وباستخدام الميكروسكوب الإلكتروني الماسح لفحص عمر 90 يوم من الفيلم الحيوي ل *S.* Typhimurium, أوضحت النتائج أن سمك طبقة الفيلم الحيوي المتكونة علي مواسير الحديد كانت أكبر عن غيرها وأقلها في النحاس. في حين أن طبقة الفيلم الحيوي المتكون المواسير المصنعة من البلاستيك كانت متقاربة في السمك.

نتيجة لما سبق من النتائج كان لابد من التحكم ومنع تكوين الفيلم الحيوي علي الأسطح الداخلية للمواسير, وذلك عن طريق تعريض خلايا الفيلم الحيوي لأنواع مختلفة من مضادات الميكروبات وكان منها الكلور و أيونات الفضة وجزيئات النانو من الفضة والمضادات الحيوية. حيث أظهرت النتائج أن خلايا الفيلم الحيوي كانت أكثر مقاومة لمضادات الميكروبات عن الخلايا حرة المعيشة. وكذلك عمر الفيلم الحيوي الصغير (10 أيام) كان أقل مقاومة لهذه المواد من الأعمار الكبيرة (40و 90 يوم). كذلك أظهرت النتائج أن الفيلم الحيوي ل *L. monocytogenes* أكثر مقاومة لمضادات الميكروبات المستخدمة في الدراسة عن الفيلم الحيوي ل *S.*

Typhimurium فيما عدا في حالة أستخدام المضادات الحيوية كانت خلايا الفيلم الحيوي ل *S.* Typhimurium أكثر مقاومة.

وللوصول إلي معرفة مدي قدرة خلايا الفيلم الحيوي علي المعيشة والبقاء ومقارنتها بالخلايا حرة المعيشة. تم اختبار قدرة المعيشة لعمر 90 يوم لخلايا الفيلم الحيوي لبكتيريا ال *L. monocytogenes*, *S.* Typhimurium علي نوعين من المياه هما مياه الحنفية والمياه الجوفية, حيث أظهرت النتائج أن خلايا الفيلم الحيوي المتكون علي المواسير الحديد كانت أكثر قدرة علي المعيشة لمدة أطول من غيرها بينما أقلها معيشه كانت لخلايا الفيلم الحيوي المكونة علي مواسير النحاس. بالأضافة الي ذلك كانت الخلايا حرة المعيشة لها القدرة علي البقاء أكثر من خلايا الفيلم الحيوي المتكونة علي المواسير النحاس.

وللوصول الي مياه آمنه صحيا خالية من أي مواد سامة بعد مرورها علي أنواع مختلفة من المواسير (المواسير المختبرة بالدراسة الحالية) فقد أظهرت نتائج اختبار السمي ة أن جميع أنواع المواسير المستخدمة في الدراسة الحالية كانت غير سامة.

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قسم: الميكروبيولوجيا الزراعيه **فرع:** **تاريخ منح الدرجة**: / /**2015**

المستخلص العربي

تهدف الدراسة الحالية إلي معرفة التوزيع الميكروبي الموجود بالفيلم الحيوي الطبيعي وكذلك إلي تحديد سلوك وخصائص نوعين من البكتيريا الممرضة ((*L. monocytogenes*, *S.* Typhimurium) في حالة الفيلم الحيوي. ولتحقيق ذلك تم تصميم نظام يحاكي شبكة توزيع المياه مكونة من 6 أنواع من المواسير. تم تجميع 6 عينات من الفيلم الحيوي علي 3 أعمار مختلفة (10, 40, 90 يوم). وتم تعريض خلايا الفيلم الحيوي المجمعة إلي 4 أنواع مختلفة من مضادات الميكروبات وهي الكلور وايونات الفضة وجزيئات النانو الفضة والمضادات الحيوية.حيث اظهرت نتائج عينات الفيلم الحيوي الطبيعي ان اكثر انواع الممرضات انتشارا في العينات المجمعة كانت (*Staph. aureus, L. monocytogenes, Salmonella, Clostridium perfiengens*). كما أوضحت النتائج أن اعلي معدل نمو لخلايا الفيلم الحيوي كانت من العينات المجمعة من المواسير الحديد بينما اقلها كانت من العينات المجمعة من المواسير النحاس. كذلك من نتائج التحليل الإحصائي والتي أظهرت وجود علاقة قوية ومعنوية بين كميات السكريات المتعددة وعمر الفيلم الحيوي. كما أظهرت النتائج أن خلايا الفيلم الحيوي *L. monocytogenes* بعمر 90 يوم النامية علي (PVC, PP, PE, I, Cu ,R) وجد أن عند تعريضها لجرعة كلور بتركيز 3ملجم/لتر لمدة 10دقائق كانت أعداد الخلايا التي تم تثبيطها علي النحو التالي 3.96, 4.16, 4.21, 4.17, 4.32, 4.03 مستعمرة /سم2. علي الجانب الأخر, أوضحت النتائج إن أكثر جرعة فعالة من ايونات لها القدرة علي قتل خلايا الفيلم الحيوي من البكتيريا المختبرة النامية علي 6 أنواع المواسير كانت 500 ملجم لمدة 15 دقيقة بالنسبة *L. monocytogenes* بينما في حين كانت *Salmonella* 500 ملجم لمدة 10 دقائق, بينما جزيئات النانو من الفضة كانت 500 ملجم لمدة 15 دقيقة و 50 ملجم لمدة 10 دقائق. من ناحية أخري أظهرت النتائج أن اختبار الحساسية للمضادات الحيوية كانت الخلايا حرة المعيشة أكثر حساسية من خلايا الفيلم الحيوي والتي أظهرت مقاومة عالية للمضادات الحيوية المختبرة. علي الجانب الأخر, أظهرت نتائج قدرة خلايا الفيلم الحيوي علي المعيشة في أوساط مائية مختلفة أن خلايا الفيلم الحيوي عمر 90 يوم المجمعة من مواسير الحديد كانت أكثر معيشة من النامية علي المواسير النحاس كذلك من نتائج التحليل الإحصائي أن هناك علاقة قوية و لها معنوية بين مدة المعيشة وأعداد خلايا الفيلم الحيوي.

الكلمات الدالة: الفيلم الحيوي, الميكروبات الممرضة, المواد البوليمرية ,الكلور, جزيئات النانو من الفضة, المضادات الحيوية.

الخصائص البيولوجية لبعض الميكروبات المشاركة في تكوين الفيلم الحيوي

رسالة دكتوراه

في العلوم الزراعية

(ميكروبيولوجيا زراعية)

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رسالة دكتوراه الفلسفة

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دكتوراه الفلسفة

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