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Development of a Biofilm Model for Evaluating Poultry Drinking Water Sanitation Procedures

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Development of a Biofilm Model for Evaluating Poultry Drinking Water Sanitation Procedures

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ABSTRACT

Enclosed water system has created a minimal sanitation focus leading to biofilm issues which are a source of flock health challenges. A series of *in vitro* tests were conducted to evaluate biofilm growth on polyvinylchloride (PVC) surfaces when exposed to treated/untreated water sources (test water) that are typically supplied in commercial barns. PVC test coupons (15.16 cm^2) were immersed in test water in beakers to grow biofilm. Test water supplies were characterized for microbial, mineral and pH content. Temperature of test water was set at 90 °F (32.2 °C) on d1 and then dropping 1°F each day over 7-day period (in °C, from d 2-d 6 - 31.6, 31.1, 30.5, 30, 29.4). Water inside beakers was gently agitated that bathed the coupons to mimic flowing water. Experiment 1 was conducted using low microbial content water (< 3 log_{10} APC cfu/ml); and experiments 2, 3 and 4 utilized higher bacteria content water (> 3 log_{10} APC cfu/ml) to produce biofilm in test coupons. Experiment 4 also included seeding the avian pathogenic *E. coli sero* group O2 strain in pathogen free water containing 7 d old biofilm test coupons and determining if it would incorporate into the biofilm community post 48- hour exposure. Sanitizers tested on the coupons included chlorine based product (CBP) (T1) and a hydrogen peroxide based product (HPBP) (T2) dosed to attain residuals in water of 3-5 ppm free chlorine and 25-50 ppm hydrogen peroxide. Control was untreated test water (T3). Results showed that biofilm can quickly (< 7d) develop on PVC surface even in minimally contaminated water (> 2 log_{10} cfu/cm^2 by day 7 in experiment 1); and the use of sanitizers was effective in limiting rapid biofilm formation (< 2 log_{10} APC cfu/cm^2 in treated test coupons vs. > 4 log_{10} cfu/cm^2 in untreated test coupons in experiment 2) or reducing bacterial load in already established biofilm (3.82 log_{10} cfu/cm^2 by day 7 in experiment 3), yet CBP proved more effective than HPBP tested

(3.82 vs 2.14 $\log_{10}$ cfu/cm^2 reduction). Experiment 4 demonstrated that treating water inhibited *E. coli* O2 from being incorporated into established biofilm.

Key Words: PVC, biofilm levels, sanitizers, *E. coli* sero group O2

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Lastly, I would also like to remember my family members: my father -Mattha Bahadur Maharjan, my mother -Lila Maharjan, and my brothers -Amir Maharjan and Prachanda Maharjan, on this occasion.

Pramir Maharjan

DEDICATION

This dissertation work is dedicated to all the poultry producers for their hard work to grow healthy chickens and contribution to feed people around the world.

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INTRODUCTION

In poultry production, water quality can be correlated with the body weight, feed conversion, livability and condemnation and thus it affects the overall performance of birds (Barton, 1996). Every farm should routinely test their water supplies for its microbial and mineral content to assure that these parameters are within the acceptable range of poultry drinking water standards. Water supplies are vulnerable to contamination to unacceptable microbial levels (> 3 $\log_{10}$ cfu/ml) even on farms with consistent water sanitation practices (Maharjan *et al.,* 2016), and thus water systems are prone to biofilm built up over time. Farms which practice regular water sanitation, and clean waterlines between flocks using concentrated solutions of disinfectants still develop > 4 $\log_{10}$ cfu/cm^2 by the end 6^{th} week of broiler grow-out period (Maharjan *et al.,* 2012). Poultry waterlines are major portion of poultry water system, and are generally constructed of polyvinylchloride (PVC) material. Several studies suggest that water systems with PVC pipe material can grow biofilm even when the water supply is clean, potable and treated (Van der Wende *et al.,* 1989; Pederson, 1990; Percival and Walker, 1999). Non sanitized water systems can harbor high levels of biofilm in water lines and foul the water supply (Flemming, 2002).

Biofilm are complex communities of a matrix of different species of enclosed microbial cells cooperating with one another for survival and are firmly attached to hydrated surfaces (Davey and O'toole, 2000; Xavier and Foster, 2007). Biofilm bacteria are different from their free-living planktonic counterparts in terms of growth rate and composition, and show increased level of resistance to disinfectants (Donlan and Donlan, 2002; Prakash *et al.,* 2003; Oliveira *etal.,*

2010; De Beer *et al.,* 1994). Water system biofilm can harbor pathogens such as *Campylobacter, Salmonella, Escherichia coli* including avian pathogenic (APEC) strains, *Pseudomonas*; including protozoans, and viruses. These organisms enter water system and incorporate into established biofilm, and thus enhance the risk of flock positivity to these pathogens (Humphrey *et al.,* 1993; Hanning *et al.,* 2008; Dou *et al.,* 2016). Birds, particularly chicks, remain vulnerable to microbial challenges from biofilm (Zimmer *et al.,* 2003). Further, biofilm clogs water pipes and filters, and thus, restrict water flow which can lead to poor flock performance (Fairchild and Ritz 2009; Watkins, 2006).

It was considered worthwhile to understand the nature of biofilm growth in waterlines, especially during the first week of brooding when water supplies are warmed and have very slow flow. In addition, water quality typically supplied in commercial poultry houses can also contain nutrients such as iron and manganese which are required for growth of some pathogenic microbes. Therefore, i*n vitro* experiments were designed to develop a model that would mimic the conditions of warm, slow moving water thus providing a way to monitor biofilm growth over time and to determine if this phase of poultry production would increase the susceptibility of water systems to biofilm development. PVC sections (internal surface area 15.16 cm^2) were utilized in the study to grow biofilm. Test water was characterized for mineral and microbial content for each experiment replication. Water was considered sub optimal microbial quality (unacceptable for poultry) if the microbial enumeration was > 3 $\log_{10}$ cfu/ml. A primary objective of the study was to understand the differences in the biofilm growth rate on PVC surface when exposed to microbiologically acceptable poultry drinking water (< 3 $\log_{10}$ cfu/ml) versus sub optimal microbial water under treated and untreated conditions. Another goal of this

study included understanding if avian pathogenic *E. coli*, sero group O2 when present in the water would incorporate into biofilm within the distribution pipes and if this incorporation into biofilm would be affected by treating water with a sanitizer.

Studies on water system biofilm mitigation intervention have implicated that treating water with sanitizers can significantly lower the flock probability of becoming positive for different food borne pathogens (Jeffrey *et al.*, 2001; Pearson *et al.*, 1993). Chlorine or hydrogen peroxide based disinfectants are two of the primary sanitizers utilized in poultry drinking water systems. This project evaluated these two classes of sanitizers to determine their efficacy in mitigating probable biofilm issues in poultry water and water system.

I. LITERATURE REVIEW

1. Water Needs and Poultry Water Consumption

Water is an essential component of all living forms and is a physiological requirement of all animals including poultry. Because water is such a critical nutrient, water consumption is a primary welfare indicator for commercial birds (Manning *et al.*, 2007). While the total content of water in a bird averages from 65-70% of its lean body mass (Ellis and Jehl, 1991; USDA, 2011), water consumed by birds is utilized for nutrient transportation, body temperature regulation, joint lubrication and various other intra and extracellular biochemical reactions. Providing good quality drinking water free of microbes and contaminants to poultry is an essential component of an optimal production system.

Water consumption in birds is influenced by several factors. Daily water intake is governed by housing environment such as ambient temperature (Watkins, 2009; May and Lott, 1992), and humidity and air velocity (May *et al.*, 2000). Feed intake (Lott, 1991) and dietary formulation (Radu *et al.*, 1987; Marks and Pesti., 1984) also influence daily water intake. Management factors such as drinking water presentation (May *et al.*, 1997; Feddes *et al.*, 2002; Quichimbo *et al.*, 2013); bird factors such age and sex (Pesti *et al.*, 1985), and genetics (Deeb and Cahaner, 2001) also influence water consumption. Water quality parameters such as water temperature (Xin *et al.*, 2002; Harris *et al*., 1975) and levels of minerals and contaminants (Vodela *et al.*, 1997; Damron and Flunker, 1995) also affect the consumption of water, and thus, the performance of birds. High water consumption has been correlated to optimal feed to gain ratio (Marks, 1981). Health and performance of birds is affected if the microbial/mineral contamination in water is beyond acceptable level (King, 1996).

Improved selection strategies within and between breeds result in enhanced production traits in birds such as growth rate (Thiruvenkadan *et al.,* 2011; Beiki *et al.,* 2013), feed efficiency (Willems *et al.,* 2013; Varkoohi *et al.,* 2010) and yield (Lalev *et al.,* 2012). Unfortunately, genetic selection has not always given positive production impacts. Repercussions of selection include physiological alterations (Crossley and Altimiras, 2012; Gosnak *et al.,* 2010) and complications (Huff *et al.,* 2006) in birds, and evolution of undesired traits such as a reduction in ability to manage stressors in the environment (Mashaly *et al.,* 2000). To minimize the unfavorable effects of selection pressures and to exploit the full genetic potential given by selection in modern breeds, husbandry practices need to be reviewed accordingly, as energy requirements and therefore the water requirements of birds change. Broiler chickens currently drink significantly more water than the commercial strains of birds reared 10 years ago. In a study conducted by Williams *et al.,* (2013), broiler birds reared in 2010-2011 drank 5.5 gallons more on day 7 and 13 gallons more on day 42 per 1000 birds as compared to birds that were reared a decade earlier. With the current bird drinking significantly more water, it is very important that water provided to birds is safe and free from pathogenic microbes and undesired contaminants.

2. Poultry Drinking Water Standards

The following table was adapted from Watkins (2008) and lists the poultry drinking water quality standards for microbes and minerals, as well as treatment options to corrections when the contaminant is out of compliance.

Table 1. Water Quality Standards and Treatment Options				
Water Quality Indicator	**Levels considered average**	**Maximum Acceptable Level**	**Maximum Acceptable Levels Indicate**	**Treatment Options/Comments**
Total Bacteria (TPC) **Total Coliforms** **Fecal Coliforms**	0 CFU/ml 0 CFU/ml 0 CFU/ml	1000 CFU/ml 50 CFU/ml 0 CFU/ml	Dirty system, may taste bad and COULD have pathogens in the water system Water with >50 total coliforms or any faecal coliform has been in contact with human or animal faeces	Clean the system between flocks with approved sanitizing cleaners and establish a daily water sanitation system when birds are present Shock chlorinate as well
pH	6.5 - 7.8	5-8	Below 5 - metal corrosion Above 8 - Water sanitizers work poorly, “bitter” taste	Raise pH with soda ash (Na_2CO_3), lime Ca $(OH)_2$ or sodium hydroxide (NaOH) Lower pH-phosphoric acid, sulphuric acid and hydrochloric acid for strong alkalinity, citric acid and vinegar for weak alkalinity
Alkalinity	100 mg/l	300 mg/l	Associated with bicarbonate, sulphates and calcium carbonate • Can give water a bitter taste which makes it undesirable to the birds High levels can make it difficult to lower the pH Can be corrosive to cool cell pads	Acidification Anion Exchange de-alkalizer Can be reduced by removing free CO_2 (carbon dioxide) through aeration
Total Hardness	Soft 0 - 75mg/l as $CaCO_2$ Somewhat hard 76 to 150 Hard 151 to 300 Very Hard >300		Hardness causes scale which reduces pipe volume and drinkers hard are to trigger or leak (main factors are calcium and magnesium, but iron and manganese contribute small amount)	Do not use water softener if water already high in sodium unless using potassium chloride instead of sodium chloride (salt) Polyphosphates will sequester or tie-up hardness and keep in solution Acidification to below pH of 6.5
Calcium (Ca)	60 mg/l		No upper limit for calcium, but if values are above 110 mg/l may cause scaling	Treatment same for hardness
Iron (Fe)	0.2 mg/l	0.3 mg/l	Birds tolerant of metallic taste Iron deposits in drinkers may cause leaking Can promote growth of bacteria such as *E. coli* and Pseudomonas	Treatment includes addition of one of the following:chlorine, chlorine dioxide or ozone then filtration removal with proper sized mechanical filtration

Table 1. Water Quality Standards and Treatment Options (Cont.)				
Water Quality Indicator	**Levels considered average**	**Maximum Acceptable Level**	**Maximum Acceptable Levels Indicate**	**Treatment Options/Comments**
Sodium (Na)	50 mg/l	150 mg/l	□ With high Cl levels can cause flushing □ Can promote Enterococcus bacterial growth	□ Reverse Osmosis □ Blend with non-saline water □ Keep water clean and use daily sanitizers such as hydrogen peroxide or iodine to prevent microbial growth
Sulphates	15 - 40 mg/l	200 mg/l	□Sulphates can cause flushing in birds □ Rotten egg smell is hydrogen sulphide, by-product of sulphur-loving bacteria growth - this can cause air locks in water system as well as flushing in birds □ Since sulphides can gas off, test results may underestimate actual level present	□ Aerate water into a holding tank to gas off sulphur □ Anion exchange (chloride based) □ Treatment with oxidizing sanitizers then filtration □ If a rotten egg odour is present, shock chlorination of well is recommended plus a good daily water sanitation program while birds are present
Nitrates	1 - 5 mg/l	25 mg/l	□ Poor growth and feed conversions □ May indicate fecal contamination, test for coliform bacteria	□ Reverse osmosis □ Anion exchange
Lead	0 mg/l	0.05 mg/l	□ Can cause weak bones and fertility problems in broiler or turkey breeders	□ Lead is not naturally occurring. Look for pipes, fittings or solder that contain lead □ Water softeners and activated carbon can reduce lead
Zinc		1.5 mg/l	□ Higher levels may reduce growth rates	□ Look for locations where water may have come in contact with galvanized containers □ Water softener and activated carbon will reduce adsorption

Adapted from Watkins, 2008.

Health and Performance of birds are affected if the microbial/mineral contamination in water is beyond acceptable level (King, 1996).

The US poultry industry has adopted an enclosed drinking water system which is less vulnerable to microbial contamination than the open type bell or trough drinker. Salmonellosis has been detected in several farms in other parts of the world that do not use an enclosed system (Amaral, 2004; Poppe *et al.,* 1991). Furthermore, an enclosed system has an advantage of holding higher disinfectant residuals (Poppe *et al.,* 1986). However, the use of an enclosed system has its own drawbacks, since this type of system removed water supplies from being visually inspected and created a sense of "out of sight, out of mind" mentality. Since then more has been learned about biofilm and their role in creating microbial populations which survive and thrive within water lines and drinker systems and create health challenges that are not easily addressed. Several studies have shown that microbes are capable of forming biofilm in poultry water systems (Buswell *et al.,* 1998, Marin *et al,* 2009; Trachoo *et al*., 2002). Microbial biofilm, are formed in drinker lines in poultry houses over time due to gradual accumulation of various minerals, dirt, rust and algae. While providing a clean source of water is important, water can become contaminated within the poultry house due to microbial biofilm associated with water system. The results of a field evaluation shown in Table 2 shows how much microbial levels can change from source to end of the drinker lines, particularly if the drinker system is unhygienic (Watkins, 2008).

Table 2. Aerobic Bacteria Levels in Drip Samples (cfu/ml)

farms	**at source**	**at end of lines**
A	2,700	26,600
B	600	282,000
C	0	4,775,000

Distance between the source and end of the lines < 125m.

3. Water and Water Systems Are Vulnerable to Contamination and Biofilm Buildup

Water is susceptible to microbial contamination regardless of how good the farm management system is. Both top and bottom producing farms have been reported to experience *E. coli* and *Pseudomonas* in water supplies (Barton, 1996). Poultry specific endemic pathogens like *Campylobacter* easily thrive in poultry drinking water (Cools *et al.,* 2003). Coliforms like *E. coli* are readily found in fecal contaminated well water (Jafari *et al.,* 2006) and are associated with the cases of colibacillosis in chickens. *Salmonella* infections in chickens have been traced from various water sources (Waage *et al.,* 1999; Johnson *et al.,* 2003). Avian influenza strains that cause high mortality in poultry and are capable of causing flu pandemics in humans can persist for long period of time in water (Brown *et al.,* 2007). Similarly, water contamination through viruses in feces can lead to viral diseases such as infectious bursal disease and avian encephalomyelitis. Protozoal diseases like histomoniasis and coccidiosis can also be transmitted by contaminated water (Amaral, 2004). Reduced broiler performance was recorded when birds received water contaminated with coliforms and *Enterobacter,* and flocks experienced more aggravated conditions when these microbes were accompanied by elevated nitrate-nitrogen contamination (Grizzle *et al.,* 1997 a, b). Testing and treating water can help reduce potential microbial contamination issues related to water and the water system.

Water system biofilm and associated pathogens

Several epidemiological studies have revealed that the water source and water systems in broiler houses have been implicated for the horizontal transmission of several microbial pathogens to birds. Studies have cited that the water supply could act both as low and high risk factors for flocks testing positive for microbial pathogens (Humphrey *et al.,* 1993; Pearson *et al.,*

1993). Many strains of *Salmonella* are able to produce biofilm and thus Salmonellosis in chickens has been traced from biofilm associated with water tanks and drinker lines (Marin *et al.,* 2009). *Listeria monocytogenes* were found to form biofilm in PVC microtiter well plates and the growth media types (at 32 °C) were shown to influence the amount of deposition in biofilm (Moltz, 2005). *Campylobacter jejuni*, thermophilic and microaerophilic enteric pathogens associated with poultry (Reeser *et al.,* 2007) can be tracked from poultry water system and drinker line biofilm (Pearson *et al.,* 1993; Gregory *et al.,* 1997; Sparks, 2009). A study reported that two-day old biofilm on PVC from gram positive chicken house isolates and *Psudomonas* spp. facilitated the *C. jejuni* attachment and viability (Trachoo *et al.,* 2002). Similarly, Hanning *et al.,* (2008), found that *C. jejuni'*s attachment to surfaces is facilitated by pre-established biofilm.

Biofilm forming ability of microbes is affected by temperature and nutrient availability (Sanders et al., 2008). Besides, it is also affected by the genes in bacteria that encode for adherence. Nemati *et al.,* 2009 studied 171 *Staphyloccocus* isolates from poultry for biofilm forming genes that encode for microbial surface components recognizing adhesive matrix molecule and were found to be positive for genes such as clfA, clfB, eno and fnbA. There are evidences of *E. coli*, including avian pathogenic strains (APEC), forming biofilm both in broiler and layer farm water systems (Ahmad *et al.,* 2008; Dou *et al.,* 2016). The biofilm forming ability of APEC strains has been shown to be variable (Skyberg *et al.,* 2007); and studies have reported that many strains of APEC exhibit strong and moderate biofilm forming ability (Dou *et al.,* 2016). *E. coli* attachment to established biofilm on PVC could be affected by age and physical properties of biofilm, and also the physico-chemical properties of water (Janjaroen *et*

al., 2013). Genes such as *E. coli* common pilus (ECP) and an invasion protein, ibeA, in APEC strains have been reported to have a role in biofilm forming ability and thus the virulence of the strains (Stacy *et al.,* 2014; Wang *et al.,* 2011).

When disinfectants act on biofilm, their efficacy against the microbial species in biofilms is greatly reduced as compared to their efficacy against planktonic counterparts due to their limited penetrability into the biofilm matrix (De Beer *et al.,* 1994; Oliveira *et al.,* 2010). Biofilm mitigation intervention studies have depicted a clear implication that treating water with sanitizers can significantly lower the flock probability of becoming positive for different food borne pathogens (Jeffrey *et al.,* 2001; Pearson *et al.,* 1993). Chlorine based and hydrogen peroxide based disinfectants are two primary disinfectants in poultry drinking water sanitation practices. Their efficacy in inactivating microbial biofilm such as *Pseudomonas aeroginosa* (Wirtanen *et al.,* 2001) and *L. monocytogenes* (Robbins *et al.,* 2005) biofilm has been well tested. In this dissertation work, the efficacy of these sanitizers in treating sub optimal microbial water for microbial control; in inhibiting suboptimal microbial water for their biofilm forming ability on PVC surface; and in removing biofilm on PVC surface derived from sub optimal microbial water; were studied. The results indicated that treating water with these sanitizers helped mitigate microbial issues in water and water systems.

4. Disinfectants for Water System Sanitation

Disinfection is the main part of an effective biosecurity program in poultry operations to prevent entry of disease agents and foodborne pathogens in birds (Dorea *et al.,* 2010; Newell *et al.,* 2011). Ideal disinfectants used as a drinking water sanitizer should create disinfectant

residuals throughout the distribution system and should inactivate microbes, control biofilm or neutralize undesired contaminants. The Environmental Protection Agency (EPA, 2013), has listed the following characteristics (Table 3) in disinfectant residuals as ideal in drinking water for humans. These also hold true for drinking water disinfection/sanitation in animals as well.

Table 3. Water Treatment Desired Characteristics

Chemical
Easily measured on-site under field conditions
Minimal to no interferences with common constituents in drinking water
Generates minimal to no disinfection by-products
Long-lasting
Selectively reactive (minimal to no corrosion/reaction with dissolved metals, pipe materials, linings, etc.)
Operational/Physical
Highly soluble in water
Safely generated, transported, stored, and fed
Cost-effective relative to the application (large- or small-scale)
Inactivation Capabilities
Effectively and efficiently inactivates wide range of organisms (bacteria, viruses, protozoa, algae, fungi)
Effectively inactivates microorganisms present in the bulk water and those associated with particles/biofilm
Achieves desired level of organism inactivation at doses that are safe for human consumption

Table 3. Water Treatment Desired Characteristics (Cont.)
Aesthetic
Achieves desired level of organism inactivation without creating tastes and odors
Overfeed can be detected by taste, odor, and/or color

Even though each class of disinfectant acts specifically against microbes, their general biocidal activity can be explained by their ability to oxidize or rupture the cell wall of microorganisms or to diffuse into cells and interfere with the cellular metabolism (Cho *et al.,* 2010; Denyer and Stewart, 1998). In the case of viral agents, permanent disruption in capsular proteins or nucleic acids occurs (Thurman and Gerba, 1988).

Oxidation Reduction Potential (ORP) values measure the oxidizing ability of the disinfectants in water to oxidize/kill microbes. The ORP values are affected by concentration of oxidizing residuals and are pH dependent (Park *et al.*, 2004; Aziz, 2005; Yang *et al.*, 2003) and 650 mV or above in water is considered enough to destroy most bacteria and viruses within a few seconds (Yang *et al.,* 2003). Secondary oxidant functions of disinfectants in water include oxidation of iron and manganese (Aieta and Berg, 1986) which helps to minimize drinker coagulation (Watkins, 2007), and maintaining a biologically safe and stable environment in water thereby preventing the regrowth of microbes, algal blooms and biofilm formation in the water distribution systems (Lund and Ormerod, 1995).

Increased efficacy is also attained by cleaning away organic matter and then applying the disinfectant (Stringfellow *et al.,* 2009). At higher concentrations, most disinfectants act in random and non-specific ways against microbes (Maillard, 2002).

5. Chlorine and Hydrogen Peroxide as Poultry Water System Sanitizers

In poultry operations, the most commonly used chlorine based disinfectants for drinking water sanitation are sodium hypochlorite, chlorine gas and calcium hypochlorite (Anonymous, 2009) which when present in the optimal pH range will create hypochlorous acid (HOCl) on hydrolysis (EPA. 1999).

$Cl_{2\,(g)} + H_2O \Rightarrow HOCl + H^+Cl^-$

$NaOCl + H_2O \Rightarrow HOCl + Na^+ + OH^-$

$Ca(OCl)_2 + H_2O \Rightarrow Ca(OH)_2 + 2HOCl$

Hypochlorous acid has a strong germicidal action. However, in high pH conditions (>8.5 pH), it dissociates completely into hypochlorite ions which has a less germicidal action than the hypochlorous acid. The pH range between 6.5 and 8 .5 has incomplete dissociation, while pH below 6.5 has no or a negligible dissociation of the hypochlorous form (EPA, 1999; Galal-Gorchev, 1996).

$HOCl \Leftrightarrow H^+ + O\,Cl^-$

Chlorination is more effective at lower pH levels (Park *et al.,* 2004) and often drinking water is acidified to support chlorine disinfectant efficacy for improved sanitizing residual which supports better bird performance (Philipsen, 2006). However, careful selection among various

acid products available is necessary to avoid water consumption impacts (Hughes *et al.,* 2009). When using chlorine and acidifiers together in water, they should be mixed and injected separately to avoid poisonous chlorine gas formation (Ziggity Systems, Inc., 2005).

When drinking water has 2-5 ppm free chlorine residual, it is effective against most microbial growth in water (Watkins, 2007). Adding chlorine in drinking water showed increased livability in birds (Ono *et al.,* 2007). Chlorine levels below 50 ppm in drinking water are well tolerated by birds; above 50 ppm, impacts on water intake and production performances are detected with toxic level developing at 200 ppm (Khan *et al.,* 2010; Hulan and Prooudfoot, 1982).

When chlorine sanitizers are used in high pH water (Galel-Gorchev, 1996; Park, 2004), or in weaker concentrations (Payment, 1999; Stern *et al.,* 2002), or in water systems with well-established biofilm (De Beer *et al.,* 1994), the sanitizing value of chlorine can be compromised. Therefore, it becomes equally important for the poultry industry to identify alternative disinfectants to chlorine as well as their optimal usage levels.

Another effectively used water sanitizer is hydrogen peroxide. Maintaining 25-50 ppm of hydrogen peroxide residuals in the water is considered the Effective Residual Concentration (ERC) (Watkins, 2009) against most microbes. Stabilized hydrogen peroxide products hold higher concentrations of residuals for a longer time than non-stabilized (Clark *et al.,* 2009). Heavy metal ions like silver and copper, and organic acids like peracetic and ascorbic acid blended with hydrogen peroxide synergize the disinfecting property of hydrogen peroxide

(Alasri *et al,* 1992; Pedahzur *et al.,* 1997; Ragab-Depre, 1982; de Velasquez *et al.,* 2008) particularly in heavily contaminated water (Tofant *et al.,* 2006).

Hydrogen peroxide inactivates microbes through oxidative stress by forming very strong oxidizing agents, hydroxyl (OH^-) radicals, from superoxide ($O_2^{\cdot -}$) radicals (Linley *et al.,* 2012), and readily oxidizing the proteins and microbial enzymes; however, efficacy differs between liquid and gaseous forms (Finnegan *et al.,* 2010).

$$O_2^{\cdot -} + H_2O_2 \Rightarrow O_2 + OH^- + OH^{\cdot}$$

This disinfectant at 3% has a rapid bactericidal effect and is effective against a wide range of viruses, yeast, and fungi (Block, 1999). Successful cleaning of poultry waterlines with hydrogen peroxide products with minimal equipment damage can also be done (Watkins and Scantling, 2011), which depicts its ability to act against biofilm.

6. Objectives

Microbial hygiene of water and water systems in poultry operations is one prime requirement for ensuring bird health and optimizing performance, especially when birds are young. Treating water supplies in the first week of the bird grow out period is a mandatory practice to keep water within an acceptable microbial population, and to mitigate early biofilm issues in water systems. Based on this assumption, in vitro experiments were conducted to understand what differences it would make in terms of waterline hygiene, measured by biofilm load, in the first week of brooding if the waterlines were supplied with different types of water and under sanitizer treated/untreated conditions. Therefore, the goal of this experiment was to develop a bacterial biofilm model mimicking the conditions in waterlines during a typical first week of brooding in a commercial poultry house, and at the same time evaluate the efficacy of two commonly utilized poultry drinking water sanitizer products, chlorine and hydrogen peroxide based, for mitigating the biofilm issues.

References

Ahmad, S., M. Ashraf, M. Siddique, F. Mehmood, M. Arshad, and H. Khan. 2008. Biofilm formation and drinking water quality in relation to Escherichia coli at commercial poultry farms. J of Agri. Soc. Sci. 4(2): 77-80.

Aieta, E. M., and J. D. Berg. 1986. A review of chlorine dioxide in drinking water treatment. Journal (Am. Water Works Assoc.) 78:62-72.

Alasri, A., C. Roques, G. Michel, C. Cabassud, and P. Aptel. 1992. Bactericidal properties of peracetic acid and hydrogen peroxide, alone and in combination, and chlorine and formaldehyde against bacterial water strains. Can. J. Microbiol. 38:635-642.

Amaral, LA do. 2004. "Drinking Water as a Risk Factor to Poultry Health. Revista Brasileira De Ciência Avícola 6 (4): 191-199.

Anonymous, 2009. B and B Chlorination. Chlorination for Poultry Water Supplies. Albert City, Iowa Greensboro, North Carolina. http://www.bbchlor.com/PDF%20Files/Chlorination-For-Poultry-Water-Supplies-09.pdf

Aziz. T. 2005. Chlorinating Drinking Water on Poultry Farms. World Poult. 21(5):24-25.
Barton, T. L. 1996. Relevance of water quality to broiler and turkey performance. Poult. Sci. 75:854-856.

Beiki, H., A. Pakdel, M. Moradi-Shahrbabak, and H. Mehrban. 2013. Evaluation of growth functions on Japanese quail lines. J Poult. Sci. 50:20-27.

Block, SS. 1999. "Disinfection, Sterilization, and Preservation." Lea & Febiger, Philadelphia: 818-819.

Brown, J. D., D. E. Swayne, R. J. Cooper, R. E. Burns, and D. E. Stallknecht. 2007. Persistence of H5 and H7 avian influenza viruses in water. Avian Dis. 51:285-289.

Buswell, C. M., Y. M. Herlihy, L. M. Lawrence, J. T. McGuiggan, P. D. Marsh, C. W. Keevil, and S. A. Leach. 1998. Extended survival and persistence of Campylobacter spp. in water and aquatic biofilms and their detection by immunofluorescent-antibody and -rRNA staining. Appl. Environ. Microbiol. 64:733-741.

Cho, M., J. Kim, J. Y. Kim, J. Yoon, and J. Kim. 2010. Mechanisms of Escherichia coli inactivation by several disinfectants. Water Res. 44:3410-3418.

Clark, T, B. Dean and S.Watkins. 2009. Evaluation of Different Hydrogen Peroxide Products for Maintaining Adequate Sanitizing Residual in Water. Avian Advice 11(1):1-3.

Cools, Ils, M. Uyttendaele, C. Caro, E.D'Haese, HJ Nelis, and Johan Debevere. 2003. "Survival of *Campylobacter jejuni* Strains of Different Origin in Drinking Water." *J . Appl. Microbiol.* 94 (5): 886-892.

Crossley, D. A., and J. Altimiras. 2012. Effect of selection for commercially productive traits on the plasticity of cardiovascular regulation in chicken breeds during embryonic development. Poult. Sci. 91:2628-2636. doi:91/10/2628 [pii].

Damron, B. L., and L. K. Flunker. 1995. Calcium supplementation of hen drinking water. Poult. Sci. 74:784-787.

Davey, M. E., and G. A. O'toole. 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev. 64:847-867.

De Beer, D., R. Srinivasan, and P. S. Stewart. 1994. Direct measurement of chlorine penetration into biofilms during disinfection. Appl. Environ. Microbiol. 60:4339-4344.

de Velasquez, MA T Orta, I. Yáñez-noguez, B. Jiménez-cisneros, and V. Luna Pabello. 2008. Adding silver and copper to hydrogen peroxide and peracetic acid in the disinfection of an advanced primary treatment effluent. Environ. Technol. 29:1209-1217.

Deeb, N., and A. Cahaner. 2001. Genotype-by-environment interaction with broiler genotypes differing in growth rate. 1. The effects of high ambient temperature and naked-neck genotype on lines differing in genetic background. Poult. Sci. 80:695-702.

Denyer, S. P., and G. Stewart. 1998. Mechanisms of action of disinfectants. Int. Biodeterior. Biodegrad. 41:261-268.

Donlan, RM. and RM Donlan. 2002. "Biofilms: Microbial Life on Surfaces." Emerg. Infect. Dis. 8 (9): 881.

Dorea, F., R. Berghaus, C. Hofacre, and D. Cole. 2010. Survey of biosecurity protocols and practices adopted by growers on commercial poultry farms in Georgia, US A. Avian Dis. 54:1007-1015.

Dou, X., J. Gong, X. Han, M. Xu, H. Shen, D. Zhang, L. Zhuang, J. Liu, and J. Zou. 2016. Characterization of avian pathogenic Escherichia coli isolated in eastern China. Gene 576:244-248.

Ellis, H. I., and J. R. Jehl Jr. 1991. Total body water and body composition in phalaropes and other birds. Physiol. Zool.64 (4):973-984.

EPA, 2016. Revised Total Coliform Rule And Total Coliform Rule. https://www.epa.gov/dwreginfo/revised-total-coliform-rule-and-total-coliform-rule

Fairchild, B. D., and C. W. Ritz. 2009. Poultry drinking water primer. http://extension.uga.edu/publications/detail.cfm?number=B1301

Feddes, J. J., E. J. Emmanuel, and M. J. Zuidhoft. 2002. Broiler performance, body weight variance, feed and water intake, and carcass quality at different stocking densities. Poult. Sci. 81:774-779.

Finnegan, M., E. Linley, S. P. Denyer, G. McDonnell, C. Simons, and J. Y. Maillard. 2010. Mode of action of hydrogen peroxide and other oxidizing agents: differences between liquid and gas forms. J. Antimicrob. Chemother. 65:2108-2115. doi:10.1093/jac/dkq308 [doi].

Flemming, H. 2002. Biofouling in water systems–cases, causes and countermeasures. Appl. Microbiol. Biotechnol. 59:629-640.

FSIS, 2011. Water in Meat and Poultry. http://www.fsis.usda.gov/wps/wcm/connect/42a903e2-451d-40ea-897a-22dc74ef6e1c/Water_in_Meats.pdf?MOD=AJPERES

Galal-Gorchev, H. 1996. Chlorine in water disinfection. Pure and Applied Chemistry 68:1731-1735.

Gošnak, R. Dahmane, I. Eržen, A. Holcman, and D. Škorjanc. 2010. "Effects of Divergent Selection for 8-Week Body Weight on Postnatal Enzyme Activity Pattern of 3 Fiber Types in Fast Muscles of Male Broilers (Gallus gallusdomesticus)." Poult. Sci. 89 (12): 2651-2659.

Gregory, E., H. Barnhart, D. W. Dreesen, N. J. Stern, and J. L. Corn. 1997. Epidemiological study of Campylobacter spp. in broilers: source, time of colonization, and prevalence. Avian Dis. 41(4):890-898.

Grizzle, J., T. Armbrust, M. Bryan, and A. Saxton. 1997a. Water quality II: The effect of water nitrate and bacteria on broiler growth performance. The Journal of Applied Poultry Research 6:48-55.

Grizzle, J., T. Armbrust, M. Bryan, and A. Saxton. 1997b. Water quality III: the effect of water nitrate and bacteria on broiler breeder performance. J. Appl. Poult. Res. 6:56-63.

Hanning, I., R. Jarquin, and M. Slavik. 2008. Campylobacter jejuni as a secondary colonizer of poultry biofilms. J. Appl. Microbiol. 105:1199-1208.

Harris, G. C.,Jr, G. S. Nelson, R. L. Seay, and W. H. Dodgen. 1975. Effect of drinking water temperature on broiler performance. Poult. Sci. 54:775-779.

Hughes, J, B. Dean, T. Clark and S. Watkins. 2009. Chlorination/Acidification Affects Salmonella Contamination. Avian Advice 9(2): 1-3.

Huff, G., W. Huff, N. Rath, J. Balog, N. B. Anthony, and K. Nestor. 2006. Stress-induced colibacillosis and turkey osteomyelitis complex in turkeys selected for increased body weight. Poult. Sci. 85:266-272.

Hulan, H. W., and F. G. Proudfoot. 1982. Effect of sodium hypochlorite (Javex) on the performance of broiler chickens. Am. J. Vet. Res. 43:1804-1806.

Humphery, T., A. Henley, and D. Lanning. 1993. The colonization of broiler chickens with Campylobacter jejuni: some epidemiological investigations. Epidemiol. Infect. 110:601-607.

Jafari, R., A. Fazlara, and M. Govahi. 2006. An investigation into Salmonella and fecal coliform contamination of drinking water in broiler farms in Iran. Int. J. Poult. Sci. 5:491-493.

Janjaroen, Dao, Fangqiong Ling, Guillermo Monroy, Nicolas Derlon, Eberhard Mogenroth, Stephen A. Boppart, Wen-Tso Liu, and Thanh H. Nguyen. "Roles of ionic strength and biofilm roughness on adhesion kinetics of Escherichia coli onto groundwater biofilm grown on PVC surfaces." Water Res. 47, no. 7 (2013): 2531-2542.

Jeffrey, J. S., E. R. Atwill, and A. Hunter. 2001. Farm and management variables linked to fecal shedding of Campylobacter and Salmonella in commercial squab production. Poult. Sci. 80:66-70.

Johnson, J. Y., Thomas, J. E., Graham, T. A., Townshend, I., Byrne, J., Selinger, L. B., & Gannon, V. P. (2003). Prevalence of Escherichia coli O157: H7 and Salmonella spp. in surface waters of southern Alberta and its relation to manure sources. Can. J. Microbiol. 49(5), 326-335.

Khan, MZ, A. Khan, and I. Javed. 2010. "Toxico-pathological effects of sodium hypochlorite administration through drinking water in female Japanese quail (*Coturnix japonica*)." Human & Exp. Toxicol. 29 (9): 779-788.

King, A. J. 1996. Water quality and poultry production. Poult. Sci. 75:852-853.

Lalev, M., M. Oblakova, N. Mincheva, P. Hristakieva, and I. Ivanova. 2012. Evaluation of productive traits of chicken lines from the national gene pool. Tarkia J.Sci 10:38-42.

Linley, E., S. P. Denyer, G. McDonnell, C. Simons, and J. Y. Maillard. 2012. Use of hydrogen peroxide as a biocide: new consideration of its mechanisms of biocidal action. J. Antimicrob. Chemother. 67:1589-1596. doi:10.1093/jac/dks129 [doi].

Lott, B. D. 1991. The effect of feed intake on body temperature and water consumption of male broilers during heat exposure. Poult. Sci. 70:756-759.

Lund, V., and K. Ormerod. 1995. The influence of disinfection processes on biofilm formation in water distribution systems. Water Res. 29:1013-1021.

Maharjan, P., T. Clark, C. Kuenzel, M. Foy, and S. Watkins. 2016. On farm monitoring of the impact of water system sanitation on microbial levels in broiler house water supplies. J. of Appl. Poult. Res. 2016: pfw010v1-pfw010.

Maharjan, P, M. K. Foy and S. Watkins. 2012. Field evaluations day 42 swab samples in commercial broiler houses that practice regular water sanitation. Unpublished data. Watkins Water Quality Lab. U of Arkansas.

Maillard, J. 2002. Bacterial target sites for biocide action. J. Appl. Microbiol. 92 (1): 16-27.

Manning, L., S. Chadd, and R. Baines. 2007. Key health and welfare indicators for broiler production. World's Poult. Sci. J. 63:46-62.

Marin, C., A. Hernandiz, and M. Lainez. 2009. Biofilm development capacity of Salmonella strains isolated in poultry risk factors and their resistance against disinfectants. Poult. Sci. 88:424-431. doi:10.3382/ps.2008-00241 [doi].

Marks, HL. 1981. "Role of Water in Regulating Feed Intake and Feed Efficiency of Broilers." *Poultry Science* 60 (4): 698-707.

Marks, H. L., and G. M. Pesti. 1984. The roles of protein level and diet form in water consumption and abdominal fat pad deposition of broilers. Poult. Sci. 63:1617-1625.

Mashaly, MM, MJ Heetkamp, HK Parmentier, and JW Schrama. 2000. "Influence of Genetic Selection for Antibody Production against Sheep Blood Cells on Energy Metabolism in Laying Hens." Poult. Sci. 79 (4): 519-524.

May, J. D., B. D. Lott, and J. D. Simmons. 2000. The effect of air velocity on broiler performance and feed and water consumption. Poult. Sci. 79:1396-1400.

May, J. D., B. D. Lott, and J. D. Simmons. 1997. Water consumption by broilers in high cyclic temperatures: bell versus nipple waterers. Poult. Sci. 76:944-947.

May, J. D., and B. D. Lott. 1992. Feed and water consumption patterns of broilers at high environmental temperatures. Poult. Sci. 71:331-336.

Moltz, A. G. 2005. Formation of biofilms by Listeria monocytogenes under various growth conditions. J. of Food Prot. 68:92-97.

Nemati, M., K. Hermans, L. A. Devriese, D. Maes, and F. Haesebrouck. 2009. Screening of genes encoding adhesion factors and biofilm formation in Staphylococcus aureus isolates from poultry. Avian Pathol. 38:513-517.

Newell, D. G., K. T. Elvers, D. Dopfer, I. Hansson, P. Jones, S. James, J. Gittins, N. J. Stern, R. Davies, I. Connerton, D. Pearson, G. Salvat, and V. M. Allen. 2011. Biosecurity-based

interventions and strategies to reduce Campylobacter spp. on poultry farms. Appl. Environ. Microbiol. 77:8605-8614. doi:10.1128/AEM.01090-10 [doi].

Oliveira, M., V. Santos, A. Fernandes, F. Bernardo, and C. L. Vilela. 2010. Antimicrobial resistance and in vitro biofilm-forming ability of enterococci from intensive and extensive farming broilers. Poult. Sci. 89:1065-1069. doi:10.3382/ps.2008-00436 [doi].

Ono, T., M. Miyake, R. Yashumoto, K. Yamashita, H. Fujii, E. Nakayama, I. Naito, and K. Sato. 2007. "Disinfection of drinking water and evaluation of viability and egg production in the broiler hens using the weak acid hypochlorous solution." Japanese J. of Poult. Sci. 44 (4): 148-143.

Park, H., Y. Hung, and D. Chung. 2004. Effects of chlorine and pH on efficacy of electrolyzed water for inactivating Escherichia coli O157: H7 and Listeria monocytogenes. Int. J. Food Microbiol. 91:13-18.

Payment, P. 1999. Poor efficacy of residual chlorine disinfectant in drinking water to inactivate waterborne pathogens in distribution systems. Canadian J. Microbial. 8: 709-715.

Pearson, A. D., M. Greenwood, T. D. Healing, D. Rollins, M. Shahamat, J. Donaldson, and R. R. Colwell. 1993. Colonization of broiler chickens by waterborne Campylobacter jejuni. Appl. Environ. Microbiol. 59:987-996.

Pedahzur, R., H. Shuval, and S. Ulitzur. 1997. Silver and hydrogen peroxide as potential drinking water disinfectants: their bactericidal effects and possible modes of action. Water Sci. and Technol. 35:87-93.

Pedersen, K. 1990. Biofilm development on stainless steel and PVC surfaces in drinking water. Water Res. 24:239-243.

Percival, S., and J. Walker. 1999. Potable water and biofilms: a review of the public health implications. Biofouling 14:99-115.

Pesti, G. M., S. V. Amato, and L. R. Minear. 1985. Water consumption of broiler chickens under commercial conditions. Poult. Sci. 64:803-808.

Philipsen, I. 2006. Acidifying drinking water supports performance. World Poult 22:20-21.

Poppe, C., R. Irwin, C. Forsberg, R. Clarke, and J. Oggel. 1991. The prevalence of Salmonella enteritidis and other Salmonella spp. among Canadian registered commercial layer flocks. Epidemiol. Infect. 106:259-270.

Poppe, C., D. Barnum, and W. Mitchell. 1986. Effect of chlorination of drinking water on experimental Salmonella infection in poultry. Avian Dis.:362-369.

Prakash, B., BM Veeregowda, and G. Krishnappa. 2003. "Biofilms: A Survival Strategy of Bacteria." Current Sci.85 (9): 1299-1307.

Quichimbo, C., J. Quintana, D. Rodríguez-Saldaña, C. López-Coello, S. Gómez, and F. Castellanos. 2013. Effect of nipple type drinker height on productive parameters of broilers. Int. J. Poult. Sci.12:144.

Radu, J., C. Van Dijk, R. K. Wheelhouse, C. A. Hummant, and P. Gadbois. 1987. Feed and water consumption and performance of male and female broilers fed salinomycin and maduramicin followed by a withdrawal ration. Poult. Sci. 66:1878-1881.

Ragab-Depre, N. J. 1982. Water disinfection with the hydrogen peroxide-ascorbic acid-copper (II) system. Appl. Environ. Microbiol. 44:555-560.

Reeser, Ryan J., Robert T. Medler, Stephen J. Billington, B. Helen Jost, and Lynn A. Joens. "Characterization of Campylobacter jejuni biofilms under defined growth conditions." Appl. and Environ. Microbial. 73, no. 6 (2007): 1908-1913.

Robbins, J. B., C. W. Fisher, A. G. Moltz, and S. E. Martin. 2005. Elimination of Listeria monocytogenes biofilms by ozone, chlorine, and hydrogen peroxide. J. of Food Protect. 68:494-498.

Sanders, S. Q., J. F. Frank, and J. W. Arnold. 2008. Temperature and nutrient effects on Campylobacter jejuni attachment on multispecies biofilms on stainless steel. J. of Food Protect. 71:271-278.

Skyberg, J., K. Siek, C. Doetkott, and L. Nolan. 2007. Biofilm formation by avian Escherichia coli in relation to media, source and phylogeny. J. Appl. Microbiol. 102:548-554.

Sparks, N. 2009. The role of the water supply system in the infection and control of Campylobacter in chicken. Worlds Poult. Sci. J. 65:459-474.

Stacy, Alyssa K., Natalie M. Mitchell, Jacob T. Maddux, A. Miguel, Laura Durán, Jorge A. Girón, Roy Curtiss 3rd, and Melha Mellata. "Evaluation of the prevalence and production of Escherichia coli common pilus among avian pathogenic E. coli and its role in virulence." *PloS one* 9, no. 1 (2014): e86565.

Stern, NJ, MC Robach, NA Cox, and MT Musgrove. 2002. "Effect of Drinking Water Chlorination on *Campylobacter* Spp. Colonization of Broilers." Avian Diseases 46 (2): 401-404.

Stringfellow, K., P. Anderson, D. Caldwell, J. Lee, J. Byrd, J. McReynolds, J. Carey, D. Nisbet, and M. Farnell. 2009. Evaluation of disinfectants commonly used by the commercial poultry industry under simulated field conditions. Poult. Sci. 88:1151-1155. doi:10.3382/ps.2008-00455 [doi].

Thiruvenkadan, A., R. Prabakaran, and S. Panneerselvam. 2011. Broiler breeding strategies over the decades: an overview. Worlds Poult. Sci. J. 67:309-336.

Thurman, R., and C. P. Gerba. 1988. Molecular mechanisms of viral inactivation by water disinfectants. Adv. Appl. Microbiol. 33:75-105.

Tofant, A., M.Vučemilo, Ž. Pavičić, and D. Milić. 2006. "The Hydrogen Peroxide, as a Potentially Useful Slurry Disinfectant." Livestock Sci. 102 (3): 243-247.

Trachoo, N., J. Frank, and N. Stern. 2002. Survival of Campylobacter jejuni in biofilms isolated from chicken houses. J. of Food Protect. 65:1110-1116.

Van der Wende, E., W. Characklis, and D. Smith. 1989. Biofilms and bacterial drinking water quality. Water Res. 23:1313-1322.

Varkoohi, S., M. Moradi Shahr Babak, A. Pakdel, A. Nejati Javaremi, M. Zaghari, and A. Kause. 2010. Response to selection for feed conversion ratio in Japanese quail. Poult. Sci. 89:1590-1598. doi:10.3382/ps.2010-00744 [doi].

Vodela, J. K., J. A. Renden, S. D. Lenz, W. H. McElhenney, and B. W. Kemppainen. 1997. Drinking water contaminants (arsenic, cadmium, lead, benzene, and trichloroethylene). 1. Interaction of contaminants with nutritional status on general performance and immune function in broiler chickens. Poult. Sci. 76:1474-1492.

Waage, AS, T. Vardund, V. Lund, and G. Kapperud. 1999. "Detection of Low Numbers of Salmonella in Environmental Water, Sewage and Food Samples by a Nested Polymerase Chain Reaction Assay." J. of Appl. Microbiol 87 (3): 418-428.

Wang, S., C. Niu, Z. Shi, Y. Xia, M. Yaqoob, J. Dai, and C. Lu. 2011. Effects of ibeA deletion on virulence and biofilm formation of avian pathogenic Escherichia coli. Infect. Immun. 79:279-287. doi:10.1128/IAI.00821-10 [doi].

Watkins, SE. 2009. Broiler Water Consumption. *The poultry site.*

Watkins, S. 2008. Water: Identifying and correcting challenges. Avian Advice 10:10-15.

Watkins, S. 2007. Flocks are healthier and perform better economically when a daily clean water source is provided. *Avitech*.

Watkins, S. 2006. Clean Water Lines for Flock Health. Avian Advice 8:3-5.

Watkins, S and M. Scantling. 2011. " Live Production" Water Line Cleaning: Overrated Or Underappreciated? The Evidence is Growing that Water Sanitation can be a "Crucial Tool in Addressing Poor Performance. Cleaning Water Lines is Essential." Watt Poultry USA 12 (1): 36.

Willems, O., S. Miller, and B. Wood. 2013. Aspects of selection for feed efficiency in meat producing poultry. Worlds Poult. Sci. J. 69:77-88.

Williams, C., G. Tabler, and S. Watkins. 2013. Comparison of broiler flock daily water consumption and water-to-feed ratios for flocks grown in 1991, 2000–2001, and 2010–2011. J.of Appl. Poult. Res. 22:934-941.

Wirtanen, G., S. Salo, I. Helander, and T. Mattila-Sandholm. 2001. Microbiological methods for testing disinfectant efficiency on Pseudomonas biofilm. Colloids and Surfaces B: Biointerfaces 20:37-50.

Xavier, J. B., and K. R. Foster. 2007. Cooperation and conflict in microbial biofilms. Proc. Natl. Acad. Sci. U. S. A. 104:876-881. doi:0607651104 [pii].

Xin, H., R. S. Gates, M. C. Puma, and D. U. Ahn. 2002. Drinking water temperature effects on laying hens subjected to warm cyclic environments. Poult. Sci. 81:608-617.

Yang, H., B. Swem, and Y. Li. 2003. The effect of pH on inactivation of pathogenic bacteria on fresh-cut lettuce by dipping treatment with electrolyzed water. J. Food Sci. 68:1013-1017.

Ziggity Systems, Inc., 2005. Use acids in poultry production with caution. http://ziggity.com/wp-content/uploads/2014/08/cont_article_pdf_160.pdf

Zimmer, M., H. Barnhart, U. Idris, and M. D. Lee. 2003. Detection of Campylobacter jejuni strains in the water lines of a commercial broiler house and their relationship to the strains that colonized the chickens. Avian Dis. 47:101-107.

II. EFFECTS OF CHLORINE AND HYDROGEN PEROXIDE SANITATION ON BIOFILM FORMAITON MODEL OF POULTRY BROODING HOUSE WATERLINES

Abstract

Background: Microbial biofilm can easily be formed in drinker lines in poultry houses over time due to gradual accumulation of various minerals, dirt, rust and algae. Birds remain vulnerable to microbial challenges from biofilms. Further, biofilms clog water pipes and filters, and thus, restrict water flow which can lead to poor flock performance. An *in vitro* experiment was performed to determine if biofilm would develop when polyvinylchloride (PVC) test coupons (material used for poultry waterlines) were exposed to low microbial content warm water and also to determine if biofilm development would be influenced by adding a sanitizer.

Methods: Biofilm was grown using sterile test coupons (PVC sections-2.54 cm long and internal diameter of 1. 90 cm). Two coupons were immersed in 600 ml water in a beaker. Nine beakers were utilized similarly with a total of 18 coupons. Three beakers (T1) were treated with chlorine (Cl) based product (8. 25 % sodium hypochlorite) and the other three (T2) with hydrogen peroxide (HP) product (30 % concentrate). Both products were dosed at the recommended bird drinking rate during the start of experiment. Three untreated beakers served as controls (T3). All beakers and coupons were placed into a water bath shaker under warm and moving water conditions mimicking poultry brooding conditions. Coupons and test water were sampled for treatments for aerobic plate count (APC). The trial was repeated.

Results: Trial 1 used test water with zero cfu bacteria/ml initial APC whereas trial 2 test water initial APC was 3 $\log_{10\ cfu}$/ml. Test water samples and coupons had no bacterial growth for all treatments on sampling occasions for trial 1. In trial 2, T3 (Control) and T2 (HP treated) had

APC growth in both test water (2.5-3.0 $\log_{10}$ cfu/ml) and on coupons (2- 2.5 $\log_{10}$ cfu/cm^2) on sampling days with no difference ($P > 0.05$). Whereas, T1 (Cl treated) completely eliminated bacteria (0 cfu/ml) in test water and inhibited biofilm growth on test coupons (≤ 0.2 $\log_{10}$ cfu/cm^2) during sampling days ($P < 0.05$).

Conclusion: This experiment showed that biofilm can develop in minimally contaminated water even in the presence of sanitizers yet chlorine was more effective than hydrogen peroxide in limiting this development.

Key words: low microbial content water, biofilm, sanitizers

Introduction

Biofilms are complex communities of different species of enclosed microbial cells cooperating with one another for survival and are firmly attached to hydrated surfaces [1, 2]. Microorganisms that form biofilms are different from their free-living counterparts in terms of growth rate, composition and show increased level of resistance to biocides which may be attributed to their up regulation and down regulation of different genes [3, 4].

Biofilm development in poultry water systems plays a crucial role in harboring pathogens [5] which can be a challenge for poultry. Many studies have shown that microbes are capable of forming biofilms in poultry water systems [6-8]. Microbial biofilm are formed in drinker lines in poultry houses over time due to gradual accumulation of various minerals, dirt, rust and algae. Birds, particularly chicks, remain vulnerable to microbial challenges from biofilms [9]. Further, biofilms clog water pipes and filters, and thus, restrict water flow which can lead to poor flock performance [10, 11].

Poultry waterlines, a major portion of poultry water systems, are constructed using polyvinylchloride (PVC) material. PVC surfaces are subject to biofilm formation [12]. Biofilm can form in water systems even when the water supply is clean, potable, and treated [13, 14]. In this experiment, the objective was to understand if biofilm would still develop and at what rate in waterlines of farms supplied with clean and potable water that has low microbial content and under treated conditions, especially when barn house is warm.

Materials and Methods

Two trials, trial 1 and trial 2 were conducted. Trial 2 was a repetition of trial 1. Any differences in methods executed in trial 2 than in trial 1, are stated.

Characterization of test water used for growing biofilms

Two US gallons (1 gallon = 3.78 L) of well water were collected from a commercial poultry farm in a clean 5-gallon bucket and left for 48 hours until no chlorine residual was detected. Once zero ppm free chlorine residual was achieved, the water was tested for microbiological, minerals and other parameters to characterize the type of water used to grow biofilm (Table 1 and Table 2) for both the trials. Test water was distributed into 9 sterile glass beakers [15] each containing 600 ml water.

Test coupons used

Sterile PVC pipe sections of dimension 2.54 cm long and internal diameter of 1.90 cm (this is the dimension of commercial poultry waterlines) were used as biofilm test coupons. Prior to use, the coupons were cleaned by washing with commercial detergent (that had sodium lauryl sulfate as cleaning agent) using municipal water to clear the organic and inorganic debris present in the test coupons and the test coupons were air dried, then dipped in 70% isopropyl alcohol for 15 minutes, dried and then steam autoclaved (121º C (249.8 F), 15 psi for 15 minutes) in order to sterilize them. After the coupons were autoclaved and cooled to room temperature, 2 coupons were immersed in each of the 9 beakers.

Sanitizer application

Three beakers each were randomly assigned to treat with chlorine based product (CBP) (8.25 % sodium hypochlorite) and hydrogen peroxide based product (HPBP) (30 % concentrate) at the dosing rate applied when birds are present in the barns. First the stock solutions were created for each of the sanitizers before they were treated with test water.

Creation of stock solution and test solution:

i) Chlorine stock: 65 ml of fresh CBP stock solution was created by mixing 1 ml the product with 64 ml of deionized water. Next, 4.68 ml of stock solution was added to 600 ml of test water in a beaker for three beakers with coupons to create a 1:128 ratio test solution (Treatment (T) 1).

ii) Hydrogen peroxide stock: 65 ml of fresh HPBP stock solution was created by mixing the 1 ml of the product with 64 ml of deionized water. Next, 4.68 ml of stock solution was added to 600 ml of test water in a beaker for three beakers with coupons to create a 1:128 ratio test solution (T2).

The remaining three untreated beakers with coupons served as control (T3). All beakers were sealed with aluminum foil to retard the rapid loss of residual concentration. Sanitizers were dosed twice in trial 1 (day 1 and day 4) and thrice in trial 2 (day 1, day 4 and day 7). Sanitizer residuals were measured during sampling occasions of test water and coupons after dosing the products using test strips [16].

Incubation of coupons in water bath shaker

All the treatment beakers including control were transferred into a shaking water bath [17] and beakers were incubated 7 days for trial 1 and 10 days for trial 2, to induce biofilm

growth. The shaker was set at 40 rpm throughout the trial period (that agitated beakers in a linear forward and backward direction) that provided a gentle movement of water inside beakers, and across coupons to simulate water in poultry waterlines. It was held at 32.2 °C on day 1 of experiment then lowered one °C each day until day (d) 7. Trial 2 was run until d10, where after d7, only half a °C was reduced per day.

Test water and coupons sampling

Test solutions were sampled for all treatments on d3 and d7 for Trial 1, whereas for Trial 2, it was sampled on d3, d7 and d10. 5 ml of test solution was pipetted from each replicate of all three treatments for bacterial enumeration.

Bacterial enumeration of test solution

This was performed following standard plating technique using petrifilm [18]. One milliliter of test solution was directly plated on the petrifilm and another milliliter was subjected to serial dilutions. Serial dilutions, up to 5th dilution level for APC was performed by diluting one ml of test solution in 9 ml of sterile Buffer Phosphate Diluent (BPD) and then spinning the solution in the vortex mixture for 10 seconds. At each dilution level, the plating was performed in duplicate to get the average microbial count. Enumeration (in colony forming units (cfu)) was carried out after 48 hours of incubation at 30°C for APC.

Similarly, one test coupon per replicate for all treatments was aseptically removed for bacterial biofilm development on d 4 and d 7 for trial 1 and d 7 and d 10 for trial 2. The coupons were rinsed to remove the unattached heterotrophic/planktonic cells by aseptically transferring

the coupon into a sterile whirlpack bag containing 30ml of sterile Butterfield Phosphate Diluent (BPDs) and then gently shaking and massaging the coupon-BPD mixture back and forth for 15 seconds. Next the coupon was removed from the BPD solution and the interior of the coupon swabbed to remove the biofilm.

Technique followed for swabbing test coupons

This method of biofilm recovery is similar to swabbing methods discussed in other studies [19, 20]. The coupons were swabbed using a sterile cellulose sponge dipped in 25 ml of sterile BPDs. The entire inner surface of the coupon was swabbed in a clockwise manner for two 360 degree rotations and was performed by same individual for all test coupons swabbed. The sponge was held with sterile forceps during this procedure. After swabbing, the sponge was returned to the BPD solution and the swab/solution was placed in the vortex for 15 seconds using a vortex mixer [21]. After the completion of the vortex mixing, the solution was used for bacterial enumeration following the technique used for test solution plating.

Data analysis

All bacterial counts were converted to $\log_{10}$ prior to analysis to normalize data distribution. Results were analyzed using the GLM procedure of SAS [22]. Means which were significant at the $P < 0.05$ levels were separated using the Least Square Means test.

Results

1. ***Residual results:***

Residuals results recorded for trial 1 and trial 2 are given in table 3 and table 4 respectively. For both the trials and during all sampling occasions, chlorine based product (T1) produced the residual concentration in test solution of 2-3 ppm after the product was introduced, whereas post 72 hours the residual recorded was less than 0.5 ppm for trial 1 and less than 0.25 for trial 2. Similarly, hydrogen peroxide based product (T2) produced the initial residual concentration of more than 50 ppm after the product was introduced for both the trials, whereas post 72 hours, the residual concentration decreased to less than 25 ppm.

The residual concentration of 25 -50 ppm of hydrogen peroxide and 2-5 ppm of free chlorine in drinking water is typical target concentration aimed to effectively decontaminate microbial population in water for poultry drinking purpose [23, 11].

2. Microbial Results

Trial 1. Bacteria were not detected in test water solutions or biofilm cells were not recovered on test coupons sampled during both sampling occasions on day 4 and day 7.

Trial 2: Figure 1 and Figure 2 give the average APC recorded for test water and test coupons during sampling days. For test water samples, APC fluctuated between 2. 5 and 3. 5 $\log_{10}$ cfu/ml during sampling days - d 3, d 7 and d10 with T2 and T3, and were not different in counts between them ($P > 0.05$). Whereas with T1, APC was absent for all sampling days. For test coupons samples, APC fluctuated between 2.17 and 2.4 $\log_{10}$ cfu/cm^2 during sampling days- d 7 and d 10 with T2 and T3, and were not different in counts between them ($P > 0.05$). Whereas with T1, the count was less than a $\log_{10}$ cfu/cm^2, significantly lower than T2 and T3 ($P < 0.05$).

Discussion

In this study, the chlorine and peroxide residuals measured over time were similar to residuals recorded in other studies; however, the water used in those tests had sub optimal microbial quality [24, 25]. The chlorine residuals demands could increase with the increase in temperature [26] regardless of the content of organic matter presence. Under low temperature of water, inhibitory effect on biofilm formation could be anticipated [27]. Therefore, if this experiment were not challenged in warm temperature or were performed at room temperature, further improved efficacy of the chlorine could be possibly anticipated.

Even though this study did not take into account the individual species present in water and their biofilm forming ability, many studies have reported different bacterial species that are potentially found in drinking water are capable of forming biofilms on PVC surfaces. At 32° C *Listeria monocytogenes* has been shown to form biofilm on PVC microtiter plates in less than two days [28] and under various growth conditions [29]. Representative bacteria isolated from human drinking water such as *Acinetobacter calcoaceticus* and *Staphylococcus* spp. were reported to have strong and moderate adhering capability on PVC surfaces [30]. A study reported higher biofilm forming capability of virulent *Legionella pneumophila* (cultured from potable tap water) on chlorinated PVC surfaces at 40° C than at 20° C [31].

This study found an increased efficacy of chlorine as compared to hydrogen peroxide to control microbes or inhibit biofilm formation as observed in other studies [24-25, 32]. Biofilm formation rate on test coupons with hydrogen peroxide treated water was similar to the level of

biofilm formation on test coupons with untreated water, possibly due to microbial regrowth in water [32]. This experiment showed that under conditions simulating a warm poultry brooding environment biofilm can develop in minimally contaminated water even in the presence of sanitizers.

References

1. Davey, ME, O'toole GA: **Microbial Biofilms: From Ecology to Molecular Genetics**. Microbiol and Mol Biol Rev 2008, **64** (4): 847-867.

2. Xavier, JB, Foster KR. **Cooperation and conflict in microbial biofilms.** Proc Natl Aca Sci 2007, **104** (3): 876-881.

3. Donlan, RM, Donlan RM: **Biofilms: Microbial Life on Surfaces.** Emerg Infect Dis 2002, **8** (9): 881.

4. Prakash, B, Veeregowda BM, Krishnappa G. 2003. **Biofilms: A Survival Strategy of Bacteria.** Curr Sci 2003, *85* (9): 1299-1307.

5. Wingender J, Flemming H: **Biofilms in drinking water and their role as reservoir for pathogens.** Int J Hyg Environ Health 2011, **214**(6):417-423.

6. Buswell CM, Herlihy YM, Lawrence LM, McGuiggan JT, Marsh PD, Keevil CW, Leach SA: **Extended survival and persistence of Campylobacter spp. in water and aquatic biofilms and their detection by immunofluorescent-antibody and -rRNA staining.** Appl Environ Microbiol 1998, **64** (2):733-741.

7. Marin C, Hernandiz A, Lainez M: **Biofilm development capacity of Salmonella strains isolated in poultry risk factors and their resistance against disinfectants.** Poult Sci 2009, 88(2):424-431.

8. Trachoo N, Frank J, Stern N: **Survival of Campylobacter jejuni in biofilms isolated from chicken houses.** Journal of Food Protection® 2002, **65**(7):1110-1116.

9. Zimmer M, Barnhart H, Idris U, Lee MD: **Detection of Campylobacter jejuni strains in the water lines of a commercial broiler house and their relationship to the strains that colonized the chickens.** Avian Dis 2003, **47**(1):101-107.

10. Fairchild BD, Ritz CW: **Poultry drinking water primer**. 2009.

11. Watkins, S: **Clean water lines for flock health. Avian Advice**. 2006, **8**(2), 3-5.

12. Pedersen K: **Biofilm development on stainless steel and PVC surfaces in drinking water.** Water Res 1990, **24**(2):239-243.

13. Van der Wende E, Characklis W, Smith D: **Biofilms and bacterial drinking water quality.** Water Res 1989, **23**(10):1313-1322.

14. Percival S, Walker J: **Potable water and biofilms: a review of the public health implications.** Biofouling 1999, **14**(2):99-115.

15.Pyrex Brand 800 Griffin Low-Form Beaker. Cole Parmer Distributor. IL, USA.

16. WaterWorks™ Water-Quality Test Strips. Ben Meadows. WI, USA

17. Heto Maxi Shake. P.O. Box 460 Laurel, MD 20725 www.atrbiotech.com

18. 3M^{TM} PetrifilmTM. 3M Science. Applied to Life.TM 3M Center. St. Paul, MN 55144, USA.

19. Asséré A, Oulahal N, Carpentier B: **Comparative evaluation of methods for counting surviving biofilm cells adhering to a polyvinyl chloride surface exposed to chlorine or drying**. J Appl Microbiol. **2008** ;104(6):1692-702.

20. Gibson H, Taylor JH, Hall KE, Holah JT**: Effectiveness of cleaning techniques used in the food industry in terms of the removal of bacterial biofilms**. J Appl Microbiol. 1999, **87**(1):41-8.

21. IKA MS 3 Basic vortex mixers. Rose Scientific. Canada.

22. SAS® 9.3. 2012. SAS Institute. Inc. Cary, NC.

23. Watkins, S: **Comparison of hydrogen peroxide products for water sanitation.** The poultry site. 2009.

24. Maharjan P, Clark T, Frank M, Martins BB, Foy MK, Watkins S: **Evaluating different hydrogen peroxide products for residuals and efficacy over time.** Int J. Poult. Sci. 2015, **14** (8): 436-440.

25. Maharjan P, Cox T, Clark T, Watkins S. **An in vitro evaluation of AOP versus chemical based poultry water sanitizers for residual and efficacy over time.** Int J. Poult. Sci. Accepted, 2015.26. Ndiongue S, Huck P, Slawson R: **Effects of temperature and biodegradable organic matter on control of biofilms by free chlorine in a model drinking water distribution system.** Water Res 2005, **39**(6):953-964.

27. Hallam N, West J, Forster C, Simms J: **The potential for biofilm growth in water distribution systems.** Water Res 2001, **35**(17):4063-4071.

28. Djordjevic D, Wiedmann M, McLandsborough LA: **Microtiter plate assay for assessment of Listeria monocytogenes biofilm formation.** Appl Environ Microbiol 2002, **68**(6):2950-2958.

29. Moltz AG: **Formation of biofilms by Listeria monocytogenes under various growth conditions.** Journal of Food Protection® 2005, **68**(1):92-97.

30. Simões LC, Simões M, Oliveira R, Vieira MJ: **Potential of the adhesion of bacteria isolated from drinking water to materials.** J Basic Microbiol 2007, **47**(2):174-183.

31. Rogers J, Dowsett AB, Dennis PJ, Lee JV, Keevil CW: **Influence of temperature and plumbing material selection on biofilm formation and growth of Legionella pneumophila in a model potable water system containing complex microbial flora.** Appl Environ Microbiol 1994, **60**(5):1585-1592.

32. Miettinen IT, Vartiainen T, Nissinen T, Tuhkanen T, Martikainen PJ: **Microbial growth in drinking waters treated with ozone, ozone/hydrogen peroxide or chlorine.** 1998, 303-315.

Table 1. Minerals characterization (in ppm) of test water*

	B	**Mg**	**Si**	**Ca**	**P**	**S**	**Na**	Cl	pH
Test Water 1	0.17	2.12	2.69	25.5	0.1	6	7.06	7.8	6.92
Test Water 2	-	2.13	3.1	27	-	-	6.12	7.7	8.2

* Cr, Mn, Fe, Co, Ni, Cu Zn, As, Se, Mo, Cd, Sb, Be, Ba, Al and Pb were measured to be either <0.03 ppm or N. D. in both the test waters

Table 2. Microbiological and other parameters of test water

	APC(cfu/ml)	**Toal coliforms**	**Conductivity (µS/cm)**	***TOC (ppm)**
Test Water 1	0	0	-	-
Test Water 2	1000	0	191	1.28

*TOC is total organic carbon.

Figure 1.

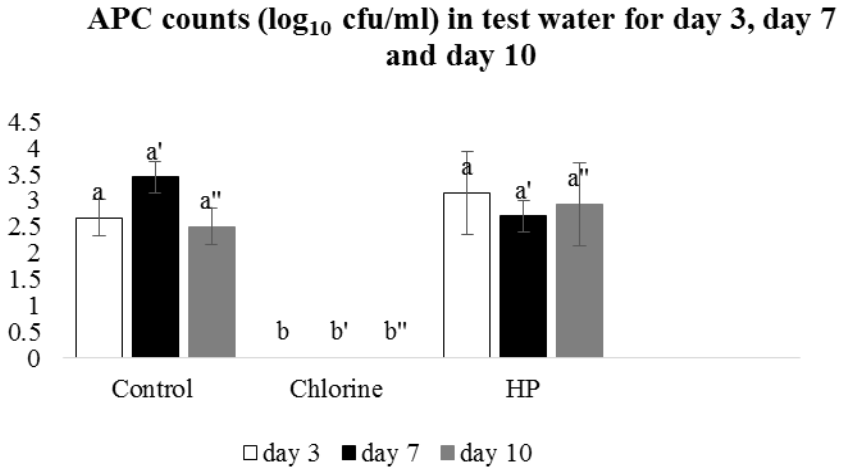


Figure 1. Trial 2. Test water was sampled on day 3, day 7 and day 10. Samples were plated for APC. Treatments were compared for sampled days. Different letters on the top of bars for sampled days are significantly different. Control = no sanitizer; Chlorine = Chlorine based product; HP=Hydrogen peroxide based product

Figure 2.

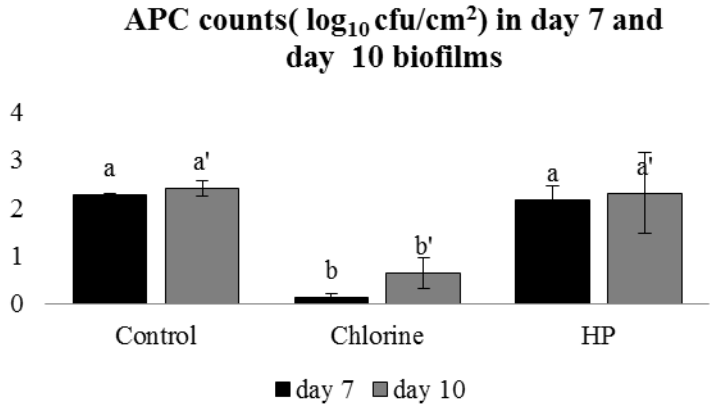


Figure 2. Trial 2. Test coupons were sampled on day 3, day 7 and day 10. Biofilms were swabbed and were plated for APC. Treatments were compared for sampled days. Different letters on the top of bars for sampled days are significantly different. Control = no sanitizer, Chlorine = Chlorine based product; HP=Hydrogen peroxide based product

III. EFFICACY OF CHLORINE AND HYDROGEN PEROXIDE TREATMENT FOR MICROBIAL REDUCTION AND BIOFILM PREVENTION ON POLYVINLYLCHLORIDE SURFACES EXPOSED TO SUBOPTIMAL MICROBIAL WATER

ABSTRACT

Birds remain vulnerable to microbial challenges through contaminated water and biofilm in drinker lines. Further, biofilms clog water pipes and filters, and thus, restrict water flow which can lead to poor flock performance. An *in vitro* experiment was performed to determine chlorine and hydrogen peroxide treatment in suboptimal microbial water (test water > 3 $\log_{10}$ cfu/ml) for its efficacy to reduce microbial population and prevention of biofilm formation on polyvinylchloride (PVC) surfaces exposed to the test water. Biofilm was grown using sterile test coupons (PVC sections-internal surface area of 15. 16 cm^2) immersed in test water in beakers incubated in a water bath shaker maintained at for 3- day period (d1 through d3, in $^{\circ}$C – 32. 2, 31.6, 31.1). The idea was to mimic water and waterline conditions in a typical brooded poultry house. Beakers were treated either with chlorine based product (CBP, 8. 25 % sodium hypochlorite) or hydrogen peroxide based product (HPBP, 30 % concentrate). Both products were dosed at the recommended bird drinking rate at the start of experiment. Untreated beakers served as controls (T3). Test water and coupons were sampled for all treatments for aerobic plate count (APC) and mold counts over time. Results showed that there was an increase ($P < 0.05$) in APC over time (from > 3 $\log_{10}$ cfu/ml to > 4 $\log_{10}$ cfu/ml) in untreated test water samples. Both the sanitizers tested showed a significant reduction in microbial counts in test water by 1-hour post treatment ($P < 0.05$), whereas CBP was more effective than HPBP (> 2 $\log_{10}$ reduction vs > 1 $\log_{10}$ reduction). Similarly, biofilm growth in treated samples at 48 hours was significantly lower than in control. Results showed that treating water significantly reduced microbial counts in suboptimal water to acceptable poultry drinking water standards. While biofilm can still

develop in contaminated water, even in the presence of sanitizers, chlorine was more effective than hydrogen peroxide in limiting biofilm growth.

Key words: Poultry waterlines, bacteria, biofilm, chlorine, hydrogen peroxide

INTRODUCTION

Providing clean and safe drinking water to poultry is a basic requirement for optimizing production. One prime factor that determines the quality of drinking water is its microbial content. For poultry drinking purposes an acceptable level of bacteria in water is 1000 cfu/ml, beyond this range is considered as sub optimal microbial water for poultry operation (Watkins, 2007; Watkins, 2008). Microbial contamination above the acceptable levels in drinking water can affect health and performance (King, 1996). Both top and bottom producing farms suffer equally from contamination with pathogens such as *Escherichia coli* and *Pseudomonas* (Barton, 1996). This shows that water is vulnerable to microbial contamination regardless of good management practices. Health and production related issues in birds, including breeders, have been reported in various farms due to poor microbial water quality (Grizzle *et al.,* 1997 a, b; Pearson *et al.,* 1993; Gregory *et al.,* 1997; Sparks, 2009). Fecal contaminated well water is a source of coliforms, such as *E. coli* that causes colibacillosis in poultry flocks (Jafari *et al.,* 2006). Water and water systems including water tanks and drinker lines act as potential sources for *Salmonella* and *Campylobacter* (including viable but non-culturable forms) in chickens (Sparks, 2009; Waage *et al.,* 1999; Johnson *et al.,* 2003; Marin *et al.,* 2009) and water treatment is a viable control strategy at the farm level (Doyle and Erickson, 2006; Vandeplas *et al.,* 2010).

In commercial production barns, newly hatched chicks and poults are provided water that is warmed to prevent chilling in birds. It has been documented that chicks less than a week old drink 5-10 gallons per thousand birds in a 24- hour period (Williams *et al.,* 2013). This minimal volume of water usage means water often remains in waterlines for several hours. This results in

loss of efficacious sanitizer residuals which could leave birds vulnerable to microbial challenges from biofilms.

In this study, chlorine and hydrogen peroxide based poultry drinking water sanitizers were evaluated for their efficacy in microbial killing and biofilm prevention on PVC surfaces exposed to sub optimal quality warm microbial water that mimicked the brooding environment.

MATERIALS AND METHODS

Characterization of test water used for growing biofilm

Two US gallons (1 gallon = 3.78 L) of well water were collected from a commercial poultry farm in a clean 5-gallon bucket and left for 48 hours until no chlorine residual was detected. Once zero ppm free chlorine residual was achieved, the water was tested for mineral parameters to characterize the type of water used to grow biofilm (Table 1). Test water was distributed into 9 sterile glass beakers (Pyrex Brand, Cole Palmer, Vernon Hills, IL 60061) each containing 600 ml water.

Test coupons used

Sterile PVC pipe sections of dimension 2.54 cm long and internal diameter of 1.90 cm (this is the dimension of commercial poultry waterlines) were used as biofilm test coupons. Prior to use, the coupons were cleaned by washing with commercial detergent (that had sodium lauryl sulfate as cleaning agent) using municipal water to clear the organic and inorganic debris present in the test coupons and the test coupons were air dried, then dipped in 70% isopropyl alcohol for

15 minutes, dried and then steam autoclaved (121º C (249.8 F), 15 psi for 15 minutes) in order to sterilize them. After the coupons were autoclaved and cooled to room temperature, 2 coupons were immersed in each of the 9 beakers.

Sanitizer application

Three beakers each were randomly assigned to treat with chlorine based product (CBP) (8.25 % sodium hypochlorite) and hydrogen peroxide based product (HPBP) (30 % concentrate) at the dosing rate applied when birds are present in the barns. Sanitizers were applied once the coupons were immersed in beakers. First the stock solutions were made for each of the sanitizers before they were treated with test water.

Creation of stock solution and test solution:

Chlorine stock: 33 ml of fresh CBP stock solution was created by mixing 1 ml the product with 32 ml of deionized water. Next, 4.68 ml of stock solution was added to 600 ml of test water in a beaker for three beakers with coupons to create a 1:128 ratio test solution (Treatment (T) 1).

Hydrogen peroxide stock: 33 ml of fresh HPBP stock solution was created by mixing the 1 ml of the product with 32 ml of deionized water. Next, 4.68 ml of stock solution was added to 600 ml of test water in a beaker for three beakers with coupons to create a 1:128 ratio test solution (T2).

The remaining three untreated beakers with coupons served as control (T3). All beakers were sealed with aluminum foil to retard the rapid loss of residual concentration.

Sanitizer residuals were measured during each sampling occasion of test water and coupons after dosing the products using test strips (WaterWorks™, Ben Meadows Company, Janesville, WI 53547).

Incubation of coupons in water bath shaker

All the treatment beakers including control were transferred into a shaking water bath (Heto Maxi Shake, MD 20725, USA) and beakers were incubated for 72 hours to determine the effect of sanitizers on microbial reduction, and to understand the nature of biofilm growth on PVC surface under treated/untreated conditions. The shaker was set at 40 rpm throughout the trial period that provided a gentle linear movement of water inside beakers, and across coupons to simulate water in poultry waterlines. It was held at 90 °F (32. 2 °C) on day (d) 1 of the experiment and then lowered one °F each day until d 3 (in °C, from d 2-d 3 - 31.6, 31.1).

Test water and coupons sampling

Test solutions were sampled for all treatments before sanitizer application (0 hour), and at 1 hour, 6 hours, 24 hours and 48 hours post application. Five ml of test solution was pipetted from each replicate of all three treatments for bacterial and mold enumeration. Test coupons, one coupon per replicate, were sampled for all treatments at 48 hours and 72 hours post sanitizer application.

Bacterial and mold enumeration of test solution

Petrifilm™ (3M Company, St Paul, MN 55144) were used for plating using manufacturers recommendations. One milliliter of test solution was directly plated on the

petrifilm and another milliliter was subjected to serial dilutions. Serial dilutions, up to the third dilution level for APC and 2nd dilution level for mold were performed by diluting one ml of test solution in 9 ml of sterile Buffer Phosphate Diluent (BPD) and then spinning the solution in the vortex mixture for 10 seconds. At each dilution level, the plating was performed in duplicate to get the average microbial count. Enumeration (in colony forming units (cfu)) was carried out after 48 hours of incubation at 30°C for APC, and 72 hours of incubation at room temperature for mold.

Test coupons sampling

The coupons were rinsed to remove the unattached heterotrophic/planktonic cells by aseptically transferring the coupon into a sterile whirlpack bag containing 30ml of sterile BPD and then gently shaking and massaging the coupon-BPD mixture back and forth for 15 seconds. Next the coupon was removed from the BPD solution and the interior of the coupon swabbed to remove the biofilm.

Technique followed for swabbing test coupons

This method of biofilm recovery is similar to swabbing methods discussed in other studies (Assere *et al.,* 2008; Gibson *et al.,* 1999). The coupons were swabbed using a sterile cellulose sponge dipped in 25 ml of sterile BPDs. The entire inner surface of the coupon was swabbed in a clockwise manner for two 360 degree rotations and was performed by the same individual for all test coupons swabbed. The sponge was held with sterile forceps during this procedure. After swabbing, the sponge was returned to the BPD solution and the swab/solution was placed in the vortex for 15 seconds using a vortex mixer. After the completion of the vortex

mixing, the solution was used for bacterial enumeration following the technique used for test solution plating.

Data analysis

All bacterial counts were converted to $\log_{10}$ prior to analysis to normalize data distribution. Results were analyzed using the GLM procedure of SAS. Means which were significant at the $P < 0.05$ levels were separated using the Least Square Means test (SAS, 2012).

RESULTS

Residual results

Residual results recorded are shown in Table 2. Chlorine based product (T1) produced the residual concentration in test solution of 2-3 ppm after the product was introduced, whereas the residual dropped to less than 1 ppm by 24 hours and by the end of 72 hours, the residual concentration measured was approximately 0.1 ppm. Similarly, the HPBP (T2) produced the initial residual concentration of more than 50 ppm after the product was introduced. It was maintained at an effective residual concentration range of 25- 50 ppm for the first 24 hours, and dropped to slightly > 10 ppm by 72 hours of treatment.

Microbial Results

Test water

Initial bacterial or mold levels detected in all treatments were between 3 and 3.5 $\log_{10}$ cfu/ml (Figure 1), and 1. 5 and 2 $\log_{10}$ cfu/ml (Figure 2) respectively, and were not different between

treatments ($P > 0.05$). Post one hour of sanitizer application, there was a significant drop ($P < 0.05$) in bacterial and mold counts (> 2 $\log_{10}$ reduction in CBP vs. > 1 $\log_{10}$ reduction in HPBP) in treated water as compared to control. At other sampling occasions, HPBP did not produce a further drop and remained above 2 $\log_{10}$ cfu/ml throughout the trial period, whereas with CPB, the bacterial population was absent at 6 and 24 hours but increased to > 2 $\log_{10}$ cfu/ml at 48 hours (Figure 1).

Mold counts in the control decreased significantly at 24 and 48 hours. A significant drop ($P < 0.05$) in mold counts was observed with the HPBP starting at 6 hours, and further decreasing at 24 and 48 hours, whereas CBP completely eliminated mold population post 1 hour treatment lasting throughout 48 hours (Figure 2).

Test coupons

Figures 3 and 4 respectively show the bacterial and mold populations retrieved from test coupon samples. Control had > 4 $\log_{10}$ cfu/cm2 bacteria recovered at 48 and 72 hours sampling occasions, significantly higher ($P < 0.05$) than bacteria recovered from treated test coupons (< 2 $\log_{10}$ cfu/cm^2). The CBP treatment eliminated bacteria at 72 hours (Figure 3). Similarly, mold was present at low levels in the control (> 1 $\log_{10}$ cfu/$cm^{2)}$ at both sampling occasions, whereas with both HPBP and CBP treated coupons, mold was not detected (Figure 4).

DISCUSSION

In this study, the rate of microbial reduction in suboptimal water using chlorine or peroxide based products or the free chlorine and peroxide residuals recorded over time in treated water

were similar to those we previously reported (Maharjan *et al.*, 2015a, b). The residual concentration of 25 -50 ppm of hydrogen peroxide and 2-5 ppm of free chlorine in drinking water is typical target concentration to effectively decontaminate microbial population in water for poultry drinking purpose (Watkins, 2009). This residual range was observed for both the products tested in this study. The disinfectant residual demand can increase with increase in temperature (Ndiongue *et al.,* 2005) regardless of the content of organic matter present. Under low temperature of water, an inhibitory effect on biofilm formation could be anticipated (Hallam *et al.*, 2001). Therefore, if this experiment were not challenged conducted using warm water temperature a further improved efficacy of the chlorine and lower levels of bacterial biofilm could be anticipated.

Even though this study did not take into account the individual species present in water and their biofilm forming ability, many studies have reported different bacterial species that are potentially found in drinking water are capable of forming biofilms on PVC surfaces. At 32° C *Listeria monocytogenes* has been shown to form biofilm on PVC microtiter plates in less than two days (Djordjevic *et al.*, 2002) and under various growth conditions (Moltz *et al.*, 2005). Representative bacteria isolated from human drinking water such as *Acinetobacter calcoaceticus* and *Staphylococcus* spp. were reported to have strong and moderate adhering capability on PVC surfaces (Simões *et al.,* 2007). A study reported higher biofilm forming capability of virulent *Legionella pneumophila* (cultured from potable tap water) on chlorinated PVC surfaces at 40º C than at 20º C (Rogers *et al.,* 1994).

Various brands of water sanitizers or water line cleaners are available in the market under a few classes of disinfectants advocating their efficacy under worst case conditions. These products should be monitored for their true efficacy, applicability and cost effectiveness along with safety aspects. This study found an increased efficacy of chlorine as compared to hydrogen peroxide to control microbes or inhibit biofilm formation as observed in other studies (Miettinen *et al.,* 1998; Maharjan *et al.,* 2015 a, b). This experiment showed that under conditions simulating a warm poultry brooding environment, sanitizers significantly reduced bacterial and mold counts in sub optimal microbial water, however, biofilm can still develop in the presence of sanitizers.

REFERENCES

Asséré A, Oulahal N, Carpentier B: Comparative evaluation of methods for counting surviving biofilm cells adhering to a polyvinyl chloride surface exposed to chlorine or drying. J Appl Microbiol. 2008; 104(6):1692-702.

Barton,T.L., 1996. Relevance of water quality to broiler and turkey performance. Poult. Sci., 75: 854-856.

Djordjevic D, Wiedmann M, McLandsborough LA: Microtiter plate assay for assessment of Listeria monocytogenes biofilm formation. Appl Environ Microbiol 2002, 68(6):2950-2958.

Doyle, MP and MC Erickson. 2006. "Reducing the Carriage of Foodborne Pathogens in Livestock and Poultry." *Poultry Science* 85 (6): 960-973.

Gibson H, Taylor JH, Hall KE, Holah JT**:** Effectiveness of cleaning techniques used in the food industry in terms of the removal of bacterial biofilms. J Appl Microbiol. 1999, 87(1):41-8.

Grizzle, J.M., T.A. Armbrust, M.A. Bryan and A.M. Saxton, 1997a. Water quality II: The effect of water nitrate and bacteria on broiler growth performance. J. Appl. Poult. Res., 6: 48-55.

Grizzle, J.M., T.A. Armbrust, M.A. Bryan and A.M. Saxton,1997b. Water Quality III: The Effect of Water Nitrate and Bacteria on Broiler Breeder Performance. J. Appl. Poult. Res., 6: 56-63

Gregory, Eric, H. Barnhart, DW Dreesen, NJ Stern, and JL Corn. 1997. "Epidemiological Study of *Campylobacter Spp.* in Broilers: Source, Time of Colonization, and Prevalence." *AvianDiseases*: 890-898.

Hallam N, West J, Forster C, Simms J: The potential for biofilm growth in water distribution systems. Water Res 2001, 35(17):4063-4071.

Heto Maxi Shake. P.O. Box 460 Laurel, MD 20725 www.atrbiotech.com

Jafari, RA, A. Fazlara, and M. Govahi. 2006. "An Investigation into *Salmonella* and Fecal Coliform Contamination of Drinking Water in Broiler Farms in Iran." *International Journalof Poultry Science* 5 (5): 491-493.

Johnson, J. YM, JE Thomas, TA Graham, I. Townshend, J. Byrne, LB Selinger, and V. PJ Gannon. 2003. "Prevalence of *Escherichia coli* O157: H7 and *Salmonella* Spp. in Surface Waters of Southern Alberta and its Relation to Manure Sources." *Canadian Journal ofMicrobiology* 49 (5): 326-335.

King, A. J. 1996. Water quality and poultry production. Poult. Sci. 75:852-853.

Maharjan P, Clark T, Frank M, Martins BB, Foy MK, Watkins S: Evaluating different hydrogen peroxide products for residuals and efficacy over time. Int J. Poult. Sci. 2015a, 14 (8): 436-440.

Maharjan P, Cox T, Clark T, Watkins S. An in vitro evaluation of AOP versus chemical based poultry water sanitizers for residual and efficacy over time. Int J. Poult. Sci. Accepted, 2015b.

Marin, C., A. Hernandiz, and M. Lainez. 2009. "Biofilm Development Capacity of *Salmonella* Strains Isolated in Poultry Risk Factors and their Resistance Against Disinfectants." *Poultry Science* 88 (2): 424-431.

Miettinen IT, Vartiainen T, Nissinen T, Tuhkanen T, Martikainen PJ: Microbial growth in drinking waters treated with ozone, ozone/hydrogen peroxide or chlorine. 1998, 303-315.

Moltz AG: Formation of biofilms by Listeria monocytogenes under various growth conditions. Journal of Food Protection® 2005, 68(1):92-97.

3M™ PetrifilmTM. 3M Science. Applied to Life.TM 3M Center. St. Paul, MN 55144, USA.

Ndiongue S, Huck P, Slawson R: Effects of temperature and biodegradable organic matter on control of biofilms by free chlorine in a model drinking water distribution system. Water Res 2005, 39(6):953-964.

Pearson, A. D., M. Greenwood, T. D. Healing, D. Rollins, M. Shahamat, J. Donaldson, and R. R. Colwell. 1993. Colonization of broiler chickens by waterborne Campylobacter jejuni. Appl. Environ. Microbiol. 59:987-996.

Pyrex Brand 800 Griffin Low-Form Beaker. Cole Parmer Distributor. IL, USA.

Rogers J, Dowsett AB, Dennis PJ, Lee JV, Keevil CW: Influence of temperature and plumbing material selection on biofilm formation and growth of Legionella pneumophila in a model potable water system containing complex microbial flora. Appl Environ Microbiol 1994, 60(5):1585-1592.

SAS® 9.3. 2012. SAS Institute. Inc. Cary, NC.

Simões LC, Simões M, Oliveira R, Vieira MJ: Potential of the adhesion of bacteria isolated from drinking water to materials. J Basic Microbiol 2007, 47(2):174-183.

Sparks, NHC. 2009. "The Role of the Water Supply System in the Infection and Control of *Campylobacter* in Chicken." *World's Poultry Science Journal* 65 (03): 459-474.

Waage, AS, T. Vardund, V. Lund, and G. Kapperud. 1999. "Detection of Low Numbers of *Salmonella* in Environmental Water, Sewage and Food Samples by a Nested Polymerase Chain Reaction Assay." *Journal of Applied Microbiology* 87 (3): 418-428.

WaterWorks™ Water-Quality Test Strips. Ben Meadows. WI, USA

Watkins, S: Comparison of hydrogen peroxide products for water sanitation. The poultry site. 2009. http://www.thepoultrysite.com/articles/1411/comparison-of-hydrogen-peroxide-products-for-water-sanitation/

Watkins,S., 2008. Water: Identifying and correcting challenges. The poultry site. http://www.thepoultrysite.com/articles/1216/water-identifying-and-correcting-challenges/

Watkins,S., 2007. Water line sanitation. Ross Tech. Notes. http://www.aviagen.com/assets/Tech_Center/Broiler_Breeder_Tech_Articles/English/AviaTech_WaterLineSanitation_Aug07.pd

Williams, C. L., G. T. Tabler, and S. E. Watkins. "Comparison of broiler flock daily water consumption and water-to-feed ratios for flocks grown in 1991, 2000–2001, and 2010–2011." *The Journal of Applied Poultry Research* 22, no. 4 (2013): 934-941.

Vandeplas, S., RD Dauphin, Y. Beckers, P. Thonart, and A. Thewis. 2010. "*Salmonella* in Chicken: Current and Developing Strategies to Reduce Contamination at Farm Level." *Journal of Food Protection*73 (4): 774-785.

Table 1. Minerals characterization (in ppm) of test water*

	Fe	**Mg**	**Mn**	**Ca**	**P**	**S**	**Na**	Cl	pH
Test Water	0.05	3.29	0.02	65.43	<5	4.72	<5	0.01	7.57

* Cr, Co, Ni, Zn, Mo, Cd, Al and Pb were measured to be either <0.03 ppm or N. D. in both the test wate

Table 2. Residual concentration measured in test solutions treated with HPBP and CBP over time during sampling occasions[1]

Post sanitizer application (hours)	**[2]HPBP(ppm)**	**[3]Free Chlorine (ppm)**
0	>50	~2.5
1	≥ 50	1-2.5
6	<50	1
24	~ 30	<1
48	< 30	0.1
72	10 to 30	0.1

[1] Residuals measured in ppm, n= 3
[2]HPBP: Hydrogen peroxide based product; 3CBP: Chlorine based produc**t;**

Figure 1.

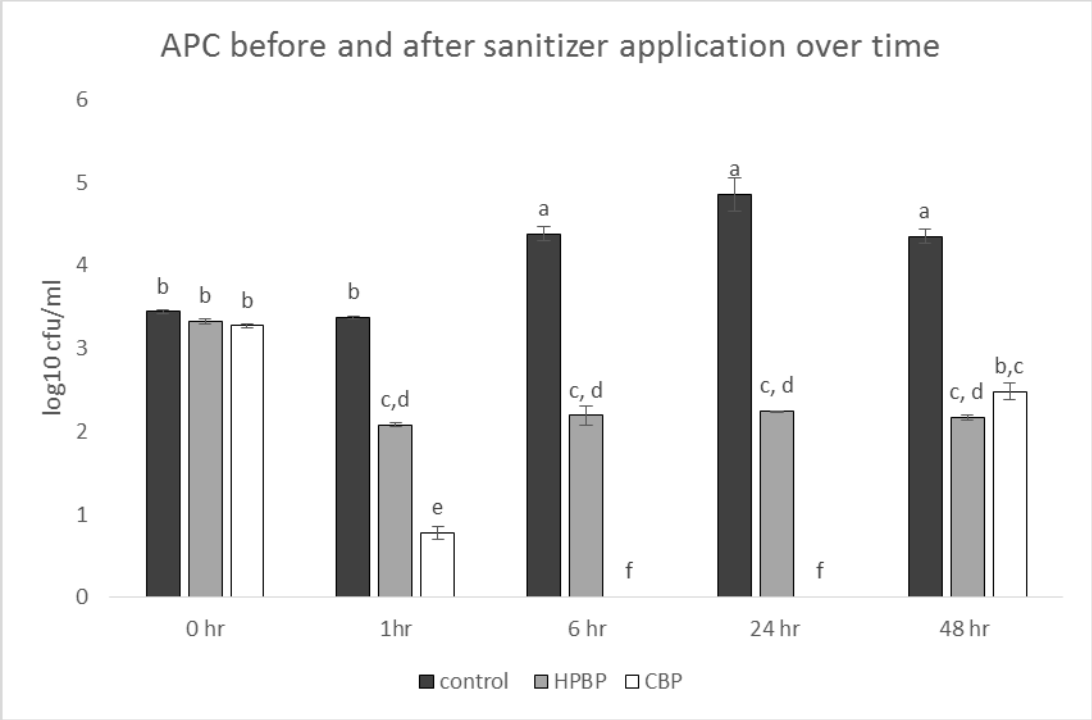


Figure 1. Test water was sampled at 0 hour, 1 hour, 6 hours, 24 hours and 48 hours post sanitizers treatment and were plated for APC. Treatments were compared for sampled occasions. Different letters on the top of bars for sampled days are significantly different. Control = no sanitizer; HPBP= Hydrogen peroxide based product; CBP = Chlorine based product.

Figure 2.

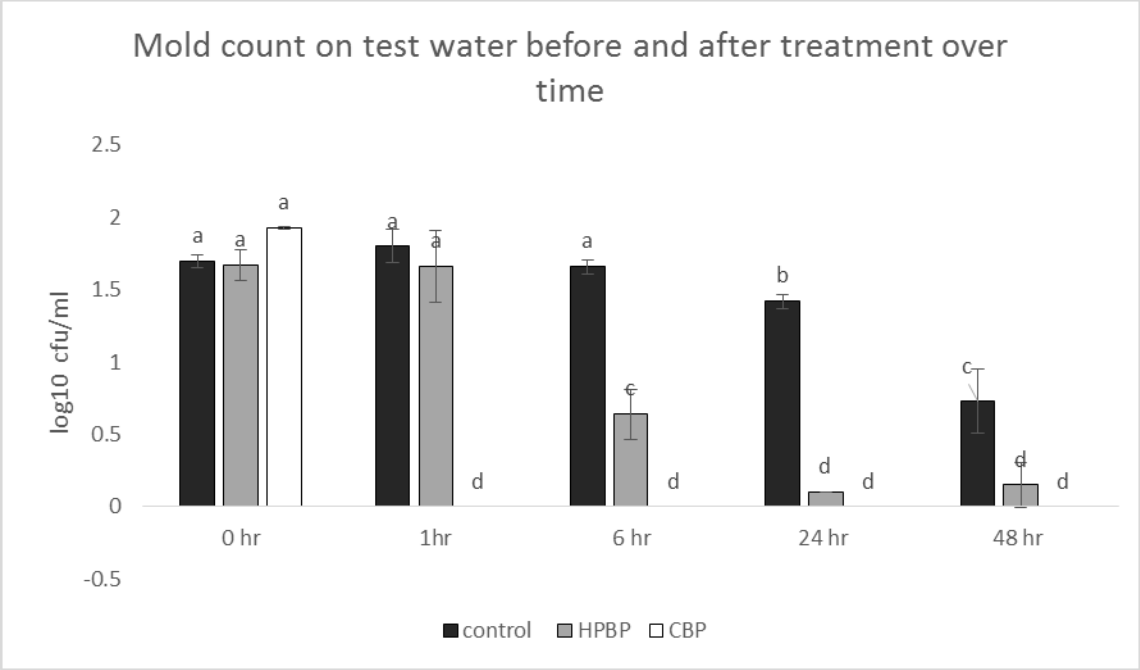


Figure 2. Test water was sampled at 0 hour, 1 hour, 6 hours, 24 hours and 48 hours post sanitizers treatment and were plated for mold. Treatments were compared for sampled occasions. Different letters on the top of bars for sampled days are significantly different. Control = no sanitizer; HPBP= Hydrogen peroxide based product; CBP = Chlorine based product.

Figure 3.

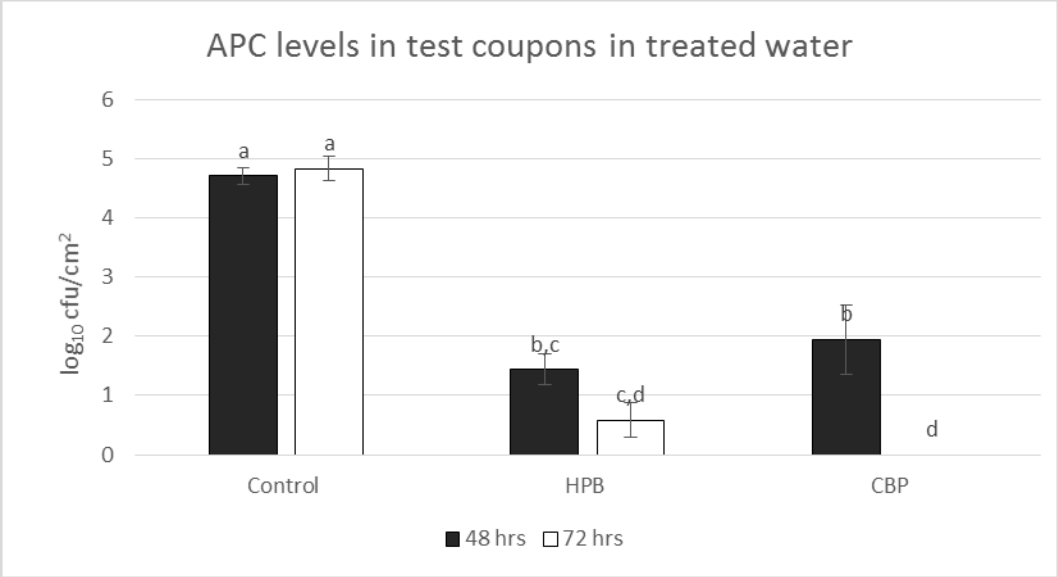


Figure 3. Test coupon one per replicate for all treatments were sampled at 48 hours and 72 hours post sanitizer application. Biofilm present in test coupons were swabbed and were plated for APC. Treatments were compared for sampled occasions. Different letters on the top of bars for sampled days are significantly different. Control = no sanitizer; HPB= Hydrogen peroxide based product; CBP = Chlorine based product.

Figure 4.

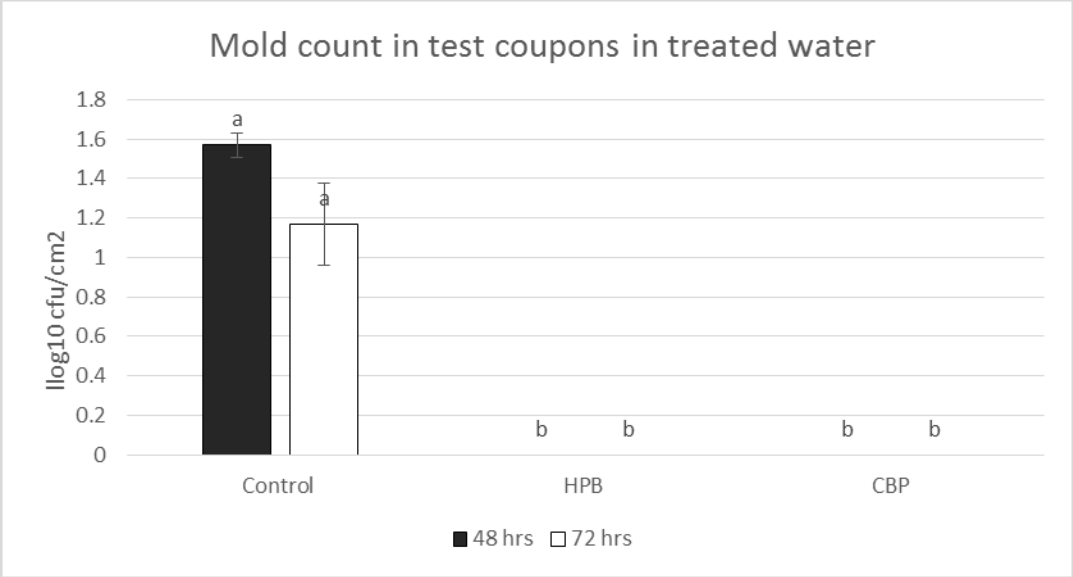


Figure 4. Test coupon one per replicate for all treatments were sampled at 48 hours and 72 hours post sanitizer application. Biofilm present in test coupons were swabbed and were plated for mold. Treatments were compared for sampled occasions. Different letters on the top of bars for sampled days are significantly different. Control = no sanitizer; HPB= Hydrogen peroxide based product; CBP = Chlorine based product.

IV. BIOFILM GROWTH ON POLYVINYLCHLORIDE SURFACE INCUBATED IN SUBOPTIMAL MICROBIAL WARM WATER AND EFFECT OF SANITIZERS ON BIOFILM REMOVAL POST BIOFILM FORMATION

ABSTRACT

An in vitro experiment was conducted to understand the nature of biofilm growth on polyvinylchloride (PVC) surface when exposed to sub optimal quality microbial water (> 4 $\log_{10}$ cfu/ml) obtained from poultry drinking water source mimicking water in waterlines during the first week of poultry brooding condition. PVC sections (internal surface area of 15. 16 cm^2) were utilized in the study to grow biofilms. After 7 days of test period, test coupons with 7 d old biofilm were transferred into autoclaved municipal water and then treated with either chlorine based or hydrogen peroxide based sanitizer at bird drinking water rate, to observe the impact on removal of biofilm formed on test coupons. Two trials (T1 and T2) were conducted. Test coupons used in trial 1 and trial 2 had the bacterial growth of 3.67 (SEM 0.04) and 3.97 (SEM 0.11) $\log_{10}$ cfu/cm^2 on day 7. After sanitizer application, chlorine based sanitizer removed bacteria in biofilm completely (0 cfu/cm^2) within 24 hours post treatment whereas hydrogen peroxide based sanitizer reduced the counts to 1.68 $\log_{10}$ cfu/cm^2 ($P < 0.05$) by 48 hours post sanitizer application. Control remained the same ($P > 0.05$). Results indicated that biofilm formation can occur quickly under suboptimal water condition on PVC surface, and sanitizer application help mitigate already formed biofilm.

Key words: suboptimal microbial water, waterline biofilm, sanitizers

INTRODUCTION

The introduction of enclosed water systems such as nipple drinkers during the early 1990's revolutionized poultry industry by dramatically improving water quality. Unfortunately, the industry became complacent with water system sanitation, primarily because this type of system removed water supplies from being visually inspected and created a sense of "out of sight, out of mind" mentality. Since then more has been learned about biofilm and their role in creating microbial populations which survive and thrive within water lines and drinker systems and create health challenges that are not easily addressed. Further, biofilms clog water pipes and filters, and thus, restrict water flow which can lead to poor flock performance (Fairchild, 2009; Watkins, 2007).

Many studies have shown that microbes are capable of forming biofilms in poultry water systems (Buswell *et al.,* 1998, Marin *et al.*, 2009; Trachoo *et al.*, 2002). Even when the water supplies are clean, biofilm formation can still occur (Momba *et al.,* 1998), Maharjan *et al.,* 2015). Biofilm can harbor pathogens (Wingender and Flemming, 2011) which remains as a challenge to birds for several flocks. Birds, particularly chicks, remain vulnerable to microbial challenges from biofilm (Zimmer *et al.,* 2003). Further, biofilm bacteria are more resistant than unattached bacteria to disinfectants (LeChevallier *et al.,* 1988).

Considering biofilm growth could be impacted by temperature or flow of water (Silhan *et al.,* 2006; Lehtola *et al.,* 2006); this study was conducted *in vitro* to understand the rate of biofilm formation over a 7-d period on polyvinylchloride (PVC), the material generally used to

build poultry waterlines, utilizing PVC sections as test coupons subjected to warm and moving sub optimal microbial water (> 4.5 $\log_{10}$ cfu/ml) to mimic waterlines during poultry brooding situation. Next objective was to evaluate efficacy of disinfectants in removal of already established biofilm if treated at poultry drinking water rate.

METHODS

Two trials, trial 1 and trial 2 were conducted. Trial 2 was repetition of the trial 1. Any differences in methods executed in trial 2 than in trial 1, are stated.

Characterization of test water used for growing biofilms

Test water for the trials were collected (directly from well) from two different poultry farms that use underground water. Water was tested for microbiological, minerals and other parameters to characterize the type of water used to grow biofilm (Table 1 and Table 2) for both the trials. 600 ml of test water was distributed in a sterile glass beaker (Pyrex brand, Cole Parmer Distributor. IL, USA) for 9 beakers.

Test coupons used

Sterile PVC pipe sections of dimension 2.54 cm long and internal diameter of 1.90 cm (this is the dimension of commercial poultry waterlines) were used as biofilm test coupons. Prior to use, the coupons were cleaned by washing with commercial detergent (that had sodium lauryl sulfate as cleaning agent) using municipal water to clear the organic and inorganic debris present in the test coupons and the test coupons were air dried, then dipped in 70 % isopropyl alcohol for

15 minutes, dried and then steam autoclaved (121º C (249.8 F), 15 psi for 15 minutes) in order to sterilize them. After the coupons were autoclaved and cooled to room temperature, 3 coupons per beaker were immersed in all of the 9 beakers that had test water.

Incubation of Coupons in Water Bath Shaker

All the beakers were transferred into a water bath shaker (Heto Maxi Shake, ATR, Laurel MD) and beakers were incubated 7 days to induce biofilm growth. Beakers were covered with aluminum foil to prevent to outwardly contamination. The shaker was set at 40 rpm throughout the trial period (that agitated beakers in a linear distance in a backward and forward direction) to provide a gentle movement of water inside beakers, and across coupons to simulate water in poultry waterlines. It was held at 90 °F (32.2 °C) on day 1 of experiment then lowered one °F each day until day (d) 6 (in °C, from d 2-d 6 - 31.6, 31.1, 30.5, 30, 29.4) in order to mimic water temperatures in waterlines in poultry house under brooding condition.

Transferring Day 7 Coupons to Autoclaved Water

On day 7, beakers were taken out of the shaker. Coupons from each beaker were individually transferred to 400 ml of autoclaved water (set at room temperature) in a beaker after they were rinsed in BPDs to remove heterotrophic/planktonic cells. This transferring of coupons was done for all nine beakers and the remaining period of experiment were carried out at room temperature (25 °C). The water quality parameters of autoclaved municipal water for the trials are presented in table 3.

Rinsing Procedure

Each test coupon that had 7-day old biofilm was aseptically taken out from the beaker and placed into a sterile whirlpack bag that has 30 ml of sterile Butterfield Phosphate Diluent (BPDs) and then gently shaking and massaging the coupon-BPD mixture back and forth for 15 seconds. Then the coupon was transferred to another beaker that had autoclaved water.

Test Coupon Sampling Occasions

One test coupon/beaker was swabbed for all treatments immediately after the coupons were transferred into the autoclaved water to determine bacterial levels in day 7 biofilms. Before they were swabbed, they were again rinsed following the same rinsing procedures while transferring coupons as described earlier. After coupons were sampled, three beakers were treated with sanitizers and coupon samples were taken 24 and 48 hours post treatment.

Technique followed for swabbing

This method of biofilm recovery is similar to swabbing methods discussed in other studies (Assere *et al.,* 2008; Gibson *et al.,* 1999). The coupons were swabbed using a sterile cellulose sponge dipped in 25 ml of sterile BPDs. The entire inner surface of the coupon was swabbed in a clockwise manner for two 360 degree rotations. The sponge was held with sterile forceps during this procedure. After swabbing, the sponge was returned to the BPD solution and the swab/solution was placed in the vortex mixer for 15 seconds. After the completion of the vortex mixing, the solution was used for bacterial enumeration.

Bacterial Enumeration

This was done following standard plating technique using petrifilm (3M™ Petrifilm™, MN, USA) One milliliter of swabbed solution was directly plated on the petrifilm and another milliliter was subjected to serial dilutions. Serial dilutions, up to 5th dilution level for APC was performed by diluting one ml of test solution in 9 ml of sterile Buffer Phosphate Diluent (BPD) and then spinning the solution in the vortex mixture for 10 seconds. At each dilution level, the plating was performed in a duplicate to get the average microbial count. Enumeration (in colony forming units (cfu)) was carried out after 48 hours of incubation at 30°C for APC.

Sanitizers Application

Three beakers each were randomly assigned to treat with chlorine based product (CBP) (8.25 % sodium hypochlorite) and hydrogen peroxide based product (HPBP) (30 % concentrate) at the dosing rate applied when birds are present in the barns. First the stock solutions were created for each of the sanitizers before they were treated with test water.

Creation of stock solution:

i) CBP stock: Stock solution was created by mixing 1ml regular bleach product (8. 25 % sodium hypochlorite) with 32 ml of deionized water. Next, 3.12 ml of stock solution was added to 400 ml of autoclaved water in a beaker for three beakers with coupons to create a 1:128 ratio test solution (Treatment (T) 1).

ii) HPBP stock: Stock solution was created by mixing 1ml hydrogen peroxide based product (30% concentrate) with 32 ml deionized water. Next, 3.12 ml of stock solution was added to 400 ml of test water in a beaker for three beakers with coupons to create a 1:128 ratio test solution (T2).

Remaining three untreated beakers with coupons served as control (T3). All beakers were sealed at its mouth with aluminum foil to retard the rapid loss of residual concentration except during sampling or residual measurement. Sanitizer residuals were measured immediately after the stock solution was introduced and during sampling occasions of test coupons 24 and 48 hours post treatment using test strips (WaterWorks^{TM,} WI). Coupons were sampled following the same procedures as carried out for the day 7 coupon sampling.

Data Analysis

All bacterial counts were converted to $\log_{10}$ prior to analysis to normalize data distribution. Results were analyzed using the GLM procedure of SAS (SAS Institute. Inc 9.3, 2012). Means which were significant at the $P < 0.05$ levels were separated using the Least Square Means test.

RESULTS

Residual Results

Trial 1 and trial 2 residual results recorded for test solutions were given in table 4. Immediately after the sanitizers were introduced, free chlorine residuals recorded were ~ 4. 5 ppm in trial 1 and ~ 3 ppm in trial 2 with CBP, whereas with HPBP, it was ~ 100 ppm in trial 1 and above 50 ppm in trial 2. Post 24 and 48 hours, free chlorine residual dropped 1 ppm each time from its initial values for both the trials. Until post 48 hours, hydrogen peroxide residuals were above 25 ppm.

Microbial Results

The bacterial counts for test coupons sampled for trial 1 and trial 2 are presented in Figure 1. The average bacterial counts recovered from d 7 test coupons were 3. 67 (SEM 0.04) and 3.97 (SEM 0.11) $\log_{10}$ cfu/cm^2 for trial 1 and trial 2 respectively. When these readings are further considered treatment wise, the bacterial counts between treatments on day 7 was in between 3. 6 and 3. 7 $\log_{10}$cfu/cm^2 in trial 1 and between 3.7 to 4.2 $\log_{10}$ cfu/cm^2 in trial 2 and the readings were not different between treatments for both the trials ($P > 0.05$). Post addition of sanitizers, CBP removed the bacterial counts to zero cfu/cm^2 ($P < 0.05$) whereas HPBP had still some levels of bacteria recovered from test coupons (> 1. 68 $\log_{10}$ cfu/cm^2) in both the sampling occasion in both the trials. APC in Control remained same in both the sampling occasion ($P > 0.05$).

DISCUSSION

Biofilms are complex communities of a matrix of different species of enclosed microbial cells cooperating with one another for survival and are firmly attached to hydrated surfaces (Davey and O'toole, 2000; Xavier and Foster, 2007). Microorganisms that form biofilms are different from their free-living counterparts in terms of growth rate, composition and show increased level of resistance to biocides which may be attributed to their up regulation and down regulation of different genes (Donlan and Donlan, 2002; Prakash *et al.,* 2013). Microorganism profoundly show transition from planktonic (free-swimming) cells to the complex, surface attached entity (O' Toole *et al.,* 2000), henceforth, it is worthwhile to evaluate the biofilm formation in poultry waterlines using sub optimal microbial water mimicking poultry grow-out

scenario. In this experiment, water exposed to test coupons were agitated to simulate the flow in waterlines, as flow/non flow conditions affect biofilm formation rates (Manuel *et al.*, 2007; Stoodley *et al.*, 1999). Water quality parameters used in the test were taken into consideration as the nutrients availability and composition of water also govern the biofilm progression (Teodósio *et al.*, 2011; Stoodley *et al.*, 1999)

Even though, this study did not take into account the individual species present in water and their biofilm forming ability, many studies have reported different bacterial species that are potentially found in drinking water are capable of forming biofilms on PVC surfaces. At temperature (32° C), *Listeria monocyogenes* has been shown to form biofilm on PVC microtiter plate in less than two days (Djordjevic *et al.*, 2002) and under various growth conditions (Moltz, 2005). Representative bacteria isolated from human drinking water such as *Acinetobacter calcoaceticus* and *Staphylococcus* spp. were reported to have strong and moderate adhering capability on PVC surfaces (Simões *et al.*, 2007). A study has reported higher biofilm forming capability virulent *Legionella pneumophila* (cultured from a potable tap water) in chlorinated PVC surface at 40 °C than in 20 °C (Rogers *et al.*, 2004).

Pederson, 1990 did a study on biofilm formation on PVC surface exposing it to drinking water moving with a certain velocity and correlated that growth could occur exponentially with a doubling time of 11 days in the first 122 days. This finding reiterates the importance that treating water could be a good option to mitigate continually increasing biofilm mass or its removal from water system. In this study, water was treated with either chlorine or hydrogen peroxide based sanitizers, commonly available poultry drinking water sanitizers, to understand their efficacy in

biofilm removal. Disinfectant residual concentration or types has crucial role in rate of accumulation of biofilm or biofilm communities (Ollos *et al.,* 2003; Williams *et al.,* 2005). In the experiment, the chlorine and peroxide residuals measured over time were similar to residuals recorded in other studies, where sub optimal quality microbial water, as used in this study, were used (Maharjan et al., 2015a, b). The chlorine residuals demand could increase with the increase in temperature (Ndiongue *et al.,* 2005) regardless of the content of organic matter presence. Under low temperature of water, inhibitory effect on biofilm formation could be anticipated (Hallam *et al.,* 2001). Therefore, if this experiment were not challenged in warm temperature, improved efficacy of the chlorine or reduced level of biofilm formation could be anticipated than observed in the study. However, chlorine proved to be a potent disinfectant to control biofilm growth as observed in other studies (Butterfield *et al.,* 2002; Hallam *et al*., 2001) compared to hydrogen peroxide.

Summing up, results of this study indicate that bacterial biofilm formation (> 3.5 $\log_{10}$ cfu/cm^2) can occur quickly (≤ 7 d) in poultry waterlines under brooding condition and when water supplies are sub optimal microbial quality. However, chlorine or hydrogen peroxide based sanitizers can help mitigate already formed biofilm.

REFERENCES

Asséré A, N. Oulahal, and B. Carpentier. 2008. Comparative evaluation of methods for counting surviving biofilm cells adhering to a polyvinyl chloride surface exposed to chlorine or drying. J Appl Microbiol. 104(6):1692-702.

Buswell, C. M., Y. M. Herlihy, L. M. Lawrence, J. T. McGuiggan, P. D. Marsh, C. W. Keevil, and S. A. Leach. 1998. Extended survival and persistence of Campylobacter spp. in water and aquatic biofilms and their detection by immunofluorescent-antibody and -rRNA staining. Appl. Environ. Microbiol. 64:733-741.

Butterfield, P. W., A. K. Camper, B. D. Ellis, and W. L. Jones. 2002. Chlorination of model drinking water biofilm: implications for growth and organic carbon removal. Water Res. 36:4391-4405.

Davey, M.E., and G.A. O'toole. 2000. Microbial Biofilms: From Ecology to Molecular Genetics.Microbiol. Mol. Biol. Rev. 64 (4): 847-867.

Donlan R.M., and R.M. Donlan. 2002. Biofilms: Microbial life on surfaces. Emerg. Infect.Dis.8 (9): 881-890.

Djordjevic, D., M. Wiedmann, and L. A. McLandsborough. 2002. Microtiter plate assay for assessment of Listeria monocytogenes biofilm formation. Appl. Environ. Microbiol. 68:2950-2958.

Fairchild, B. D., and C. W. Ritz. 2009. Poultry drinking water primer.

Gibson, H., J.H. Taylor, K.E. Hall, and J. T Holah. 1999. Effectiveness of cleaning techniques used in the food industry in terms of the removal of bacterial biofilms. J Appl Microbiol. 87(1):41-8.

Hallam, N., J. West, C. Forster, and J. Simms. 2001. The potential for biofilm growth in water distribution systems. Water Res. 35:4063-4071.

Heto Maxi Shake, ATR research equipment. 460 Laurel, MD 20725

LeChevallier, M. W., C. D. Cawthon, and R. G. Lee. 1988. Inactivation of biofilm bacteria. Appl. Environ. Microbiol. 54:2492-2499.

Lehtola, M. J., M. Laxander, I. T. Miettinen, A. Hirvonen, T. Vartiainen, and P. J. Martikainen. 2006. The effects of changing water flow velocity on the formation of biofilms and water quality in pilot distribution system consisting of copper or polyethylene pipes. Water Res. 40:2151-2160.

Maharjan, P., T. Clark, M. Frank, B. Martins, M. Foy, and S. Watkins. 2015a. Evaluating Different Hydrogen Peroxide Products for Residuals and Efficacy over Time. International Journal of Poultry Science 14:436-440.

Maharjan, P., S. Cox, T. Clark, and S. Watkins. 2015b. An in vitro Evaluation of AOP Versus Chemical Based Poultry Water Sanitizers for Residual and Efficacy over Time. International Journal of Poultry Science 14:506-510.

Manuel, C., O. Nunes, and L. Melo. 2007. Dynamics of drinking water biofilm in flow/non-flow conditions. Water Res. 41:551-562.

Marin, C., A. Hernandiz, and M. Lainez. 2009. Biofilm development capacity of Salmonella strains isolated in poultry risk factors and their resistance against disinfectants. Poult. Sci. 88:424-431.

Moltz, A. G. 2005. Formation of biofilms by Listeria monocytogenes under various growth conditions. Journal of Food Protection® 68:92-97.

Momba, M. N., T. E. Cloete, S. N. Venter, and R. Kfir. 1998. Evaluation of the impact of disinfection processes on the formation of biofilms in potable surface water distribution systems. Water science and technology 38:283-289.

3M™ PetrifilmTM. 3M Science. Applied to Life.TM 3M Center. St. Paul, MN 55144, USA.

Ndiongue, S., P. Huck, and R. Slawson. 2005. Effects of temperature and biodegradable organic matter on control of biofilms by free chlorine in a model drinking water distribution system. Water Res. 39:953-964.

Ollos, P. J., P. M. Huck, and R. M. Slawson. 2003. Factors affecting biofilm accumulation in model distribution systems. Journal (American Water Works Association):87-97.

O'Toole, G., H. B. Kaplan, and R. Kolter. 2000. Biofilm formation as microbial development. Annual Reviews in Microbiology 54:49-79.

Pedersen, K. 1990. Biofilm development on stainless steel and PVC surfaces in drinking water. Water Res. 24:239-243.

Prakash, B., B. Veeregowda, and G. Krishnappa. 2003. Biofilms: a survival strategy of bacteria. Curr. Sci. 85:1299-1307.

Pyrex Brand 800 Griffin Low-Form Beaker. Cole Parmer Distributor. IL, USA.

Rogers, J., A. B. Dowsett, P. J. Dennis, J. V. Lee, and C. W. Keevil. 1994. Influence of temperature and plumbing material selection on biofilm formation and growth of Legionella pneumophila in a model potable water system containing complex microbial flora. Appl. Environ. Microbiol. 60:1585-1592.

SAS® 9.3. 2012. SAS Institute. Inc. Cary, NC

Silhan, J., C. Corfitzen, and H. Albrechtsen. 2006. Effect of temperature and pipe material on biofilm formation and survival of Escherichia coli in used drinking water pipes: a laboratory-based study. Water Science & Technology 54:49-56.

Simões, L. C., M. Simões, R. Oliveira, and M. J. Vieira. 2007. Potential of the adhesion of bacteria isolated from drinking water to materials. J. Basic Microbiol. 47:174-183.

Stoodley, P., I. Dodds, J. Boyle, and H. Lappin-Scott. 1999. Influence of hydrodynamics and nutrients on biofilm structure. J. Appl. Microbiol. 85:19S-28S.

Teodósio, J., M. Simões, L. Melo, and F. Mergulhão. 2011. Flow cell hydrodynamics and their effects on E. coli biofilm formation under different nutrient conditions and turbulent flow. Biofouling 27:1-11.

Trachoo, N., J. Frank, and N. Stern. 2002. Survival of Campylobacter jejuni in biofilms isolated from chicken houses. Journal of Food Protection® 65:1110-1116.

WaterWorks™ Water-Quality Test Strips. Ben Meadows. WI, USA

Watkins, S. 2007. Water line sanitation. Ross tech notes.

Williams, M. M., J. W. Santo Domingo, and M. C. Meckes. 2005. Population diversity in model potable water biofilms receiving chlorine or chloramine residual. Biofouling 21:279-288.

Wingender, J., and H. Flemming. 2011. Biofilms in drinking water and their role as reservoir for pathogens. Int. J. Hyg. Environ. Health 214:417-423.

Xavier, J. B., and K. R. Foster. 2007. Cooperation and conflict in microbial biofilms. Proc. Natl. Acad. Sci. U. S. A. 104:876-881. doi:0607651104 [pii].

Zimmer, M., H. Barnhart, U. Idris, and M. D. Lee. 2003. Detection of *Campylobacter jejuni* strains in the water lines of a commercial broiler house and their relationship to the strains that colonized the chickens. Avian Dis. 47:101-107.

Table 1. Minerals characterization (in ppm) of test water*

	Ba	**B**	**Mg**	**Se**	**Ca**	**Zn**	**P**	**Mn**	**S**	**Na**	Cl	pH
Test Water 1	0.04.	0.04	2.64	0.03	28.7	N.D.	1.38	N.D.	1.10	3.75	7.82	7.25
Test Water 2	0.02	0.74	3.97	N.D	23.3	0.13	N.D	0.03	1.20	75.3	35.9	7.98

* Cr, Fe, Co, Ni, Cu, As, Mo, Cd, Sb, Be, Ba, Al and Pb were measured to be N. D. in both the test waters

Table 2. Microbiological and other parameters of test water

	APC	**N (Nitrate + Nitrite)**	**TOC (ppm)**
Test Water 1	5.08	4.15	4.48
Test Water 2	5.25	3.02	2.32

*TOC is total organic carbon; N = Nitrogen

Table 3. Minerals characterization (in ppm) of autoclaved water*

	Ba	**B**	**Mg**	**Ni**	**Ca**	**Zn**	**P**	**Mo**	**S**	**Na**	Cl	pH
Trial 1	0.02	0.33	2.07	N.D	28.4	0.01	2.41	N.D.	6.99	8.53	8.30	7.24
Trial 2	0.02	0.11	2.17	0.01	30.4	N.D.	N.A	0.01	N.A	7.89	N.A	7.35

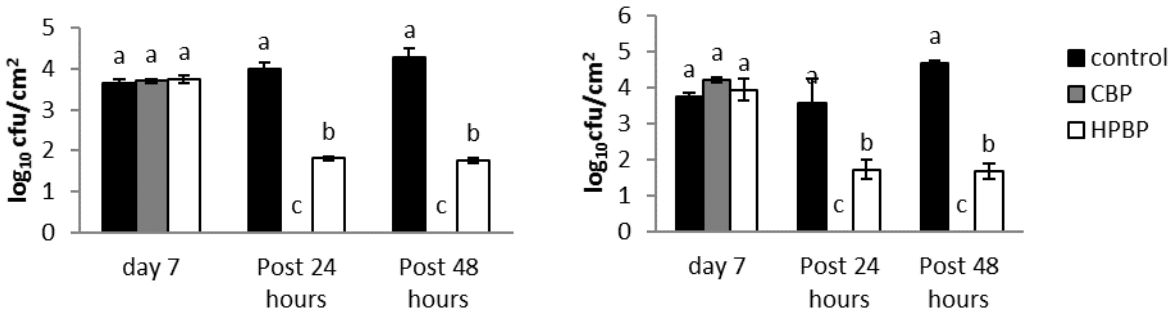
* Cr, Fe, Co, Se, Cu, As, Mo, Mn, Cd, Sb, Be, Ba, Al and Pb were measured to be N. D. in both the test waters; N.A = not available

Table 4. Trial 1: Average residuals recorded in test solutions[1]

	[4]Post 0 hour		Post 24 hours		Post 48 hours	
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
CBP[2]	4 - 5	2-3	3-4	2	2 - 3	>1
HPB[3]	50-100	>50	>50	25-50	25-50	>25

[1] Residuals measured in replicates (n= 3) measured the same for both the treatments.
[2]CBP: Chlorine based product; [3]HPB: Hydrogen peroxide based product; [4]Post 0 hour is the residual measurement immediately after sanitizer application in test water

Figure 1.



Trial 1 Trial 2

Figure 1. Trial 1 and Trial 2: Bacterial biofilm levels observed on PVC test coupons when exposed to suboptimal microbial water (> 4 $\log_{10}$ cfu/ml) for 7 days. Post 7 days, test coupons that had 7 d old biofilms were transferred to autoclaved municipal water and treated with sanitizers. Biofilm levels were measured post 24 and 48 hours sanitizer treatment. CBP: Chlorine Based Product; HPBP: Hydrogen Peroxide Based Product. [a,b,c] **Different letters on top of the bars are significantly different ($P < 0.05$).**

V. EFFECT OF CHLORINE TREATMENT ON INHIBITION OF AVIAN PATHOGENIC *ESCHERICHIA COLI* INCORPORATION INTO 7-DAY OLD BIOFILM ON POLYVINYLCHLORIDE SURFACE

ABSTRACT

Poultry waterlines are constructed using polyvinylchloride (PVC) material on which bacterial biofilms can easily form. Biofilm can harbor pathogens including *Escherichia coli.* Two trials were conducted in an attempt to understand the rate of biofilm growth on PVC surfaces using sub-optimal microbial test water (> 4.5 $\log_{10}$ cfu/ml) and the ability of sanitizers to remove biofilm. A second objective was to determine whether *E. coli* sero group O2 can incorporate into an established biofilm on PVC surface within 48 hours if present in water supplies. PVC test coupons (15.16 cm^2) were used in the study and were immersed in test water in beakers (3 coupons/beaker; 6 beakers) to grow biofilm for a 7-day period. The temperature of the test water was set dynamic over time, 90°F (32.2 °C) on d1 and then a one °F drop (in °C, from d 2-d 6 - 31.6, 31.1, 30.5, 30, and 29.4) each day over a 7-day period. The water that bathed the coupons was gently agitated. The goal was to mimic the flowing water in waterlines during the first week of brooding. On day 7, coupons (n=6) were swabbed and cultured for bacterial growth. Then, coupons were transferred into pathogen free water utilizing 6 beakers (300ml /beaker). A 0.1 ml aliquot of of tryptose phosphate broth containing 7 $X10^7$cfu/ml *E. coli* O2 was seeded into each of the beakers and then treated with chlorine (3 beakers) producing ~ 3 ppm free chlorine. Three beakers that did not receive chlorine served as control. Beakers were set at 83°F (28.3 °C) for 24 hours post treatment and 82 °F (27.7 °C) for the next 24 hours and were similarly agitated. Another experiment with similar set up was also tested to understand the *E. coli O2* attachment rate on PVC surface, but used the sterile PVC test coupons without already formed biofilm in it. Coupons were sampled for both average plate count (APC) and *E. coli* enumeration post 24 and 48 hours of treatment. Day 7 APC recovered was 4. 35 $\log_{10}$ cfu/cm^2 in Trial 1 and

3. 66 $\log_{10}$ cfu/cm^2 in Trial 2. Upon chlorine application, bacteria were not recovered *(P* < 0.05); whereas, the control had similar (*P* > 0.05) levels of bacteria in Trial 1 or greater levels (*P* < 0.05) in Trial 2. *E. coli* was not recovered in chlorine treated test coupon samples, whereas it was detected in untreated controls (> 3 $\log_{10}$ cfu/cm^2 in Trial 1 and > 2 $\log_{10}$ cfu/cm^2). This study suggests that biofilm can quickly (≤ 7d) develop on PVC surfaces in contaminated water and *E. coli* O2 can incorporate into established biofilm within 24 hours if water is not treated, while the attachment time of the pathogen was prolonged in absence of already formed biofilm.

Key words: poultry waterlines, biofilm, chlorine, *E. coli*

INTRODUCTION

The introduction of enclosed water systems such as nipple drinkers during the early 1990's revolutionized the poultry industry by dramatically improving water quality. Unfortunately, the industry became complacent with water system sanitation, primarily because this type of system removed water supplies from being visually inspected and created a sense of "out of sight, out of mind" mentality. Since then more has been learned about biofilm and their role in creating microbial populations which survive and thrive within water lines and drinker systems and create health challenges that are not easily addressed. Further, biofilms clog water pipes and filters, and thus, restrict water flow which can lead to poor flock performance (Fairchild and Ritz, 2009; Watkins, 2006).

Several epidemiological studies have revealed that water source and water systems in broiler houses can act as both high and low risk factors for flock infection of several microbial pathogens (Humphrey *et al.,* 1993; Jacobs-Reitsma *et al.,* 1995; Pearson *et al.,* 1993). Microbes form biofilm in poultry water systems (Buswell *et al.,* 1998, Marin *et al,* 2009; Trachoo *et al.,* 2002). Biofilms can harbor pathogens (Wingender and Flemming, 2011) which remain as a challenge to birds for several flocks. There is evidence that *Escherichia coli* forms biofilms both in broiler and layer water systems (Ahmad *et al*., 2008). The poultry industries suffer from devastating economic losses due to avian pathogenic *E. coli* (APEC) which cause systemic infections in birds (Dho-Moulin and Fairbrother, 1999; Ewers *et al.,* 2003). APEC strains exhibit variable degrees of biofilm forming ability (Skyberg *et al.,*2007); studies have reported

that many strains of APEC exhibit strong and moderate biofilm forming ability (Dou *et al.*, 2016).

This study was conducted to determine if the biofilm derived from sub optimal quality water on PVC coupons would incorporate APEC into its biofilm community and if this phenomenon would be affected by the presence of chlorine sanitizer.

METHODS

Two trials, Trial 1 and Trial 2 were conducted. Trial 2 was a repetition of Trial 1. Any differences in methods executed in Trial 2 than in Trial 1, are stated.

Characterization of test water used for growing biofilm

Test water for the trials were collected (directly from well) from two different poultry farms that use underground water. Water was tested for bacterial count for the test water used (4. 82 $\log_{10}$ cfu/ml in Trial 1 and 4. 53 $\log_{10}$ cfu/ml in Trial 2). Mineral characteristics of the test water used in both trials to grow biofilm are shown in Table 1. Test water (600 ml) was distributed into six sterile glass beakers (Pyrex brand, Cole Parmer Distributor. IL, USA).

Test coupons

Sterile PVC pipe sections of dimension 2.54 cm long and internal diameter of 1.90 cm (this is the dimension of commercial poultry waterlines) were used as biofilm test coupons. Prior to use, the coupons were cleaned by washing with commercial detergent (that had sodium lauryl

sulfate as cleaning agent) using municipal water to clear the organic and inorganic debris present in the test coupons and the test coupons were air dried, then dipped in 70% isopropyl alcohol for 15 minutes, dried and then steam autoclaved (121º C, 15 psi for 15 minutes) in order to sterilize them. After the coupons were autoclaved and cooled to room temperature, 3 coupons per beaker were immersed in all of the six beakers.

Incubation of coupons in water bath shaker

All of the treatment beakers were transferred into a water bath shaker (Heto Maxi Shake, ATR Research Equipment, Laurel, MD 20725) and beakers were incubated 7 days to induce biofilm growth. The mouths of the beakers were covered with aluminum foil to prevent outward contamination. The shaker was set at 40 rpm throughout the trial period (that agitated beakers in a linear distance that produced water inside beakers backward and forward motion) to provide a gentle movement of water inside beakers, and across coupons to simulate water in poultry waterlines. Temperature was held at 90 ºF (32.2 ºC) on day 1 of the experiment then lowered one ºF (in ºC, from d 2-d 6 - 31.6, 31.1, 30.5, 30, and 29.4) each day until day 7 in order to mimic water temperatures in waterlines during the first week of brooding.

Transferring day 7 coupons to autoclaved water and day 7 biofilm sampling

On day 7, beakers were taken out of the shaker. Coupons from each beaker were individually transferred to 300 ml of autoclaved water (set at room temperature) in a beaker after they were rinsed to remove heterotrophic/planktonic cells. This transferring of coupons was done for all six beakers. The water quality parameters of autoclaved municipal water for the trials are presented in Table 2.

One test coupon/beaker was swabbed for all treatments immediately after the coupons were transferred into the autoclaved water to determine aerobic bacterial levels in day 7 biofilm. The coupons were also tested for *E. coli* in the biofilm community (presence/absence test) recovered on day 7 by plating each sample on MacConkey agar to assure the absence of *E. coli* O2 in the biofilm community. Before they were swabbed, they were again rinsed following the same rinsing procedures while transferring coupons.

Rinsing procedures followed

Each test coupon that had 7-day old biofilm was aseptically taken out from the beaker and placed into a sterile whirlpack bag (Whirl-Pak, Nasco-Fort Atkinson, WI, USA) containing 30 ml of sterile Butterfield Phosphate Diluent (BPD) and then gently shaken and massaged back and forth for 15 seconds. Then the coupon was transferred to a different beaker containing autoclaved water.

Technique followed for swabbing

This method of biofilm recovery is similar to swabbing methods discussed in other studies (Assere *et al.,* 2008; Gibson *et al.,* 1999). The coupons were swabbed using a sterile cellulose sponge dipped in 25 ml of sterile BPD. The entire inner surface of the coupon was swabbed in a clockwise manner for two 360 degree rotations. The sponge was held with sterile forceps during this procedure. After swabbing, the sponge was returned to the BPD solution and the swab/solution was placed in the vortex mixer for 15 seconds. After the completion of the vortex mixing, the solution was used for bacterial enumeration.

Seeding of *E. coli* O2, sanitizer application and post seeding test coupon sampling

Immediately after the day 7 samples were pulled, all six beakers were seeded with *E. coli* (0.1 ml with inoculum size 7X 10^7 cfu/ml), and then three beakers were randomly selected and then treated with a chlorine based sanitizer (CBP) with the dose rate as described below in the method. The remaining three untreated beakers served as control. All beakers were set at 83°F (28.3 °C) for the first 24 hours and then at 82 °F (27. 7 °C) for the next 24 hours in the water bath shaker and similarly agitated (at 40 rpm). Coupon samples (1 coupon/replicate) were plated at 24 and 48 hours post treatment for APC and *E. coli* enumeration.

During each sampling occasion and immediately after introducing products, residuals in test solutions were measured using test strips for free chlorine (WaterWorks™, Ben Meadows, WI).

Description of E. coli O2 used, inoculum preparation technique and its seeding

A lactose negative, non-motile strain of *E. coli* serotype O2, which had originally been isolated from chickens with colisepticemia was used (Huff *et al.,* 1998) The inoculum was prepared by adding the first quadrant growth of an overnight culture on blood agar to 100 ml of tryptose phosphate broth (TPB) and incubating for 2.5 h in a 37 ºC shaking water bath. The inoculum bottle was put on ice immediately at end of water bath incubation. A 1 ml aliquot of the inoculum was pipetted to the first tube of a series of tenfold dilutions in TPB broth for a standard plate count. The inoculum was stored at 4ºC.

Creation of positive control experiment

Positive control experiment was set up in order to understand if the *E. coli* O2 attachment on PVC surface would be solely facilitated by presence of already formed biofilm on the surface. PVC test coupons that had no preformed biofilm were used to study *E. coli* O2 attachment over time in presence or absence of sanitizer.

Six beakers (300 ml autoclaved water/beaker; mineral quality parameters of autoclaved water used listed in table 3) had three sterile test coupons immersed in each beaker. All six beakers were seeded with 0.1 ml aliquot of TPB inoculum ($7X10^7$ cfu/ml) and then three of the beakers were randomly treated with the chlorine based sanitizer as described below in the method, and three untreated beakers served as control. All beakers were set at 83°F (28.3 ˚C) for the first day and then at 82 °F (27. 7 ˚C) for the next 4 days in a water bath shaker and similarly agitated as described earlier while growing biofilm in test coupons using sub optimal water. Coupons were sampled at the end of d1, d2 and d5 for the presence of *E. coli* in test coupons for both treatments. Residuals were recorded in test water with treated coupons during sampling occasions.

Sanitizer dose rate used

Chlorine based sanitizer was tested. Stock solutions were created for the product before it was treated with test water* (autoclaved water seeded with *E. coli* O2) by mixing 1 ml regular bleach product (8. 25 % sodium hypochlorite) with 32 ml of deionized water. Next, 2.34 ml of stock solution was added to 300 ml of test water*in only those beakers as described above that had test coupons to create a 1:128 ratio test solution.

Bacterial enumeration

This was done following standard plating technique using petrifilm (3M™ Petrifilm™, St. Paul, MN, 55144). One ml of swabbed solution was directly plated on the petrifilm and another ml was subjected to serial dilutions. Serial dilutions, up to 5th dilution level for APC was performed by diluting one ml of test solution in 9 ml of sterile BPD and then spinning the solution in the vortex mixture for 10 seconds. At each dilution level, the plating was performed in duplicate to get the average microbial count. Enumeration was carried out after 48 hours of incubation at 30°C for APC.

For *E. coli* enumeration, MacConkey agar plates were utilized. Individual colonies were counted. Only 0.1 ml of test water was plated onto MacConkey agar for all treatments following the standard plating technique.

Data analysis

All bacterial counts were converted to $\log_{10}$ prior to analysis to normalize data distribution. Results were analyzed using the GLM procedure of SAS (SAS Institute, 2012). Means which were significant at the $P < 0.05$ levels were separated using the Least Square Means test.

RESULTS

Residual Results

Trial 1 and Trial 2 residual results recorded for test solutions are given in Table 4. Immediately after the CBP was introduced, free chlorine residuals recorded were ~ 2. 5 ppm in

in both trials. Free chlorine residual dropped to slightly more than 1 ppm post 24 hours of treatment and then to 1 ppm post 48-hour treatment in both trials.

For positive control experiment, the residual recorded were ~ 5 ppm on d 1 whereas, ~ 2. 5 on d 2, and between 0.1 and 0.2 ppm on d 5.

Bacterial Results

The aerobic bacterial counts for test coupons sampled for Trial 1 and Trial 2 are presented in Figure 1 and Figure 2. The average bacterial counts recovered from d 7 test coupons were 4. 35 (SEM 0.09) and 3.87 (SEM 0.06) $\log_{10}$ cfu/cm^2 for Trial 1 and Trial 2 respectively. Post addition of sanitizer, CBP decreased bacterial counts to zero cfu/cm^2 (P <0.05) for both of the sampling occasions in both trials. APC in the controls remained either the same (P> 0.05) in Trial 1 or increased (P <0.05) in Trial 2 at each sampling occasion. For *E. coli* enumeration, neither biofilm nor *E. coli* were detected post addition of CBP at both sampling occasions in both of the trials. Whereas, in the controls, biofilm samples contained more than 3 $\log_{10}$ cfu/cm^2 *E. coli* in Trial 1 and more than 2 $\log_{10}$ cfu/cm^2 in Trial 2 by 24 hours (Figures 3 and 4). At 48 h post-seeding in Trial 2 there was significantly less *E. coli* incorporated into biofilm as compared to the level at 24 h (Figure 4).

In the positive control experiment, Trial 1 had no *E. coli* O2 retrieved in test coupon samples from d 1 and d 2 for both treated and untreated test coupons. At d 5 untreated coupons sampled, it had 2.87 (SEM = 0.27)) $\log_{10}$ cfu/cm^2, whereas the pathogen was not retrieved from treated coupons (data not shown). Trial 2 did not observe any *E. coli O2* enumeration with treated and untreated coupons for all three sampled occasions.

DISCUSSION

Biofilms are complex communities of a matrix of different species of enclosed microbial cells cooperating with one another for survival and are firmly attached to hydrated surfaces (Davey and O'toole, 2000; Xavier and Foster, 2007). Microorganisms that form biofilms are different from their free-living counterparts in terms of growth rate and composition and show increased level of resistance to biocides which may be attributed to their up regulation and down regulation of different genes (Donlan and Donlan, 2002; Prakash *et al.,* 2003). Bacteria show a transition from planktonic (free-swimming) cells to the complex, surface attached biofilm entity (O' Toole *et al.,* 2000), henceforth, it is worthwhile to evaluate biofilm formation in poultry waterlines using sub optimal microbial water mimicking the poultry grow-out scenario. In this experiment, water exposed to test coupons was agitated in dynamic temperature conditions to simulate the flow in brooded waterlines in poultry houses, as flow/non flow conditions or water temperatures do impact biofilm formation rates (Stoodley *et al.,* 1999; Sanders *et al.,* 2008). Water quality parameters used in the test were taken into consideration as the nutrient availability and composition of water also govern the biofilm progression (Teodósio *et al.,* 2011; Stoodley *et al.,* 1999).

In this study, water was treated with chlorine based sanitizer, a commonly available poultry drinking water sanitizer, to understand its effect in biofilm removal and its effect on inhibiting incorporation of *E. coli* O2 present in water into an established biofilm. Disinfectant residual concentration or type has crucial role in rate of accumulation of biofilm or biofilm communities (Ollos *et al.,* 2003; Williams *et al.,* 2005). In the experiment, the chlorine residuals

measured over time were similar to residuals recorded in other studies, where sub optimal quality microbial water, as used in this study, were used (Maharjan *et al.,* 2015a, b). The chlorine residuals demands could increase with the increase in temperature (Ndiongue *et al.,* 2005) regardless of the content of organic matter presence.

Various factors determine *E. coli* attachment to established biofilm on PVC such as age and physical properties of the biofilm, and also the physico-chemical properties of water (Janjaroen *et al.,* 2013). The Trial 2 noticed the decreased retrieval of *E. coli* O2 at 48-hour sampling occasion from test coupons as compared to counts retrieved at 24-hour sampling time, which could be due to numerically low APC recovered from the test coupons at 48 hour than at 24 hour sampling occasion. Even though this study didn't consider the genes responsible in APEC strains for biofilm forming capability, *E. coli* common pilus (ECP) and an invasion protein, ibeA genes, have been reported to have role in biofilm formation and thus the virulence of the strains (Stacy *et al.,* 2014; Wang *et al.,* 2011).

In summary, this study showed that biofilm quickly (≤ 7d) developed on PVC surfaces in contaminated water under conditions simulating the poultry brooding environment, and *E. coli* O2 did incorporate into an established biofilm in untreated water in less than 24 hour of contact time. However, results from positive control showed that absence of already formed biofilm on PVC surface could delay *E. coli* O2 attachment into the surface. Treating water with chlorine prevented *E. coli* O2 from being attaching to PVC surface or being incorporated into already formed biofilm.

REFERENCES

Ahmad, S., Ashraf, M., Siddique, M., Mehmood, F., Arshad, M. and Khan, H.A., 2008. Biofilm formation and drinking water quality in relation to Escherichia coli at commercial poultry farms. J of Agri. Soc. Sci. 4 (2): 77.

Asséré A, N. Oulahal, and B. Carpentier. 2008. Comparative evaluation of methods for counting surviving biofilm cells adhering to a polyvinyl chloride surface exposed to chlorine or drying. J Appl Microbiol. 104(6):1692-702.

Buswell, C. M., Y. M. Herlihy, L. M. Lawrence, J. T. McGuiggan, P. D. Marsh, C. W. Keevil, and S. A. Leach. 1998. Extended survival and persistence of Campylobacter spp. in water and aquatic biofilms and their detection by immunofluorescent-antibody and -rRNA staining. Appl. Environ. Microbiol. 64:733-741.

Davey, M. E., and G. A. O'toole. 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev. 64:847-867.

Dho-Moulin, M., and J. M. Fairbrother. 1999. Avian pathogenic Escherichia coli (APEC). Vet. Res. 30:299-316.

Donlan R.M., and R.M. Donlan. 2002. Biofilms: Microbial life on surfaces. Emerg. Infect.Dis.8 (9): 881-890.

Dou, Xinhong, Jiansen Gong, Xiangan Han, Ming Xu, Haiyu Shen, Di Zhang, Linlin Zhuang, Jiasheng Liu, and Jianmin Zou. "Characterization of avian pathogenic Escherichia coli isolated in eastern China." Gene 576, no. 1 (2016): 244-248.

Ewers, C., T. Janssen, and L. H. Wieler. 2003. Avian pathogenic Escherichia coli (APEC). Berl. Munch. Tierarztl. Wochenschr. 116:381-395. (In German.)

Fairchild, B. D., and C. W. Ritz. 2009. Poultry drinking water primer. University of Georgia.

Gibson, H., J.H. Taylor, K.E. Hall, and J. T Holah. 1999. Effectiveness of cleaning techniques used in the food industry in terms of the removal of bacterial biofilms. J Appl Microbiol. 87(1):41-8

Huff, W.E., Balog, J.M. & Rath, N.C. (1998). The effects of dexamethasone immunosuppression on turkey osteomyelitis complex in an experimental Escherichia coli respiratory infection. Poult. Sci. 77: 654–661.

Humphrey, T. J., A. Henley, and D. G. Lanning. 1993. The colonization of broiler chickens with Campylobacter jejuni: some epidemiological investigations. Epidemiol. Infect. 110:601-607.

Jacobs-Reitsma, W. F., A. W. van de Giessen, N. M. Bolder, and R. W. Mulder. 1995. Epidemiology of Campylobacter spp. at two Dutch broiler farms. Epidemiol. Infect. 114:413-421.

Janjaroen, Dao, Fangqiong Ling, Guillermo Monroy, Nicolas Derlon, Eberhard Mogenroth, Stephen A. Boppart, Wen-Tso Liu, and Thanh H. Nguyen. "Roles of ionic strength and biofilm roughness on adhesion kinetics of Escherichia coli onto groundwater biofilm grown on PVC surfaces." Water Res. 47, no. 7 (2013): 2531-2542.

Maharjan, P., T. Clark, M. Frank, B. Martins, M. Foy, and S. Watkins. 2015a. Evaluating Different Hydrogen Peroxide Products for Residuals and Efficacy over Time. Int. J. Poult. Sci. 14:436-440.

Maharjan, P., S. Cox, T. Clark, and S. Watkins. 2015b. An *in vitro* Evaluation of AOP Versus Chemical Based Poultry Water Sanitizers for Residual and Efficacy over Time. Int. J. Poult. Sci. 14:506-510.

Marin, C., A. Hernandiz, and M. Lainez. "Biofilm development capacity of Salmonella strains isolated in poultry risk factors and their resistance against disinfectants." Poult. Sci. 88, no. 2 (2009): 424-431.

Ndiongue, S., P. Huck, and R. Slawson. 2005. Effects of temperature and biodegradable organic matter on control of biofilms by free chlorine in a model drinking water distribution system. Water Res. 39:953-964.

Ollos, P. J., P. M. Huck, and R. M. Slawson. 2003. Factors affecting biofilm accumulation in model distribution systems. Journal (Am. Water Works Assoc.):87-97.

O'Toole, G., H. B. Kaplan, and R. Kolter. 2000. Biofilm formation as microbial development. Annual Reviews in Microbiology 54:49-79.

Pearson, A. D., M. Greenwood, T. D. Healing, D. Rollins, M. Shahamat, J. Donaldson, and R. R. Colwell. 1993. Colonization of broiler chickens by waterborne Campylobacter jejuni. Appl. Environ. Microbiol. 59:987-996.

Prakash, B., BM Veeregowda, and G. Krishnappa. 2003. "Biofilms: A Survival Strategy of Bacteria." Current Sci. 85 (9): 1299-1307.

SAS Institute, Inc. SAS® 9.3. 2012. Cary, NC

Sanders, S.Q., Frank, J.F. and Arnold, J.W., 2008. Temperature and nutrient effects on Campylobacter jejuni attachment on multispecies biofilms on stainless steel. J. Food Protect. 71(2), pp.271-278.

Skyberg, J.A., Siek, K.E., Doetkott, C. and Nolan, L.K., 2007. Biofilm formation by avian Escherichia coli in relation to media, source and phylogeny. J. of Appl. Microbiol. 102(2), pp.548-554.

Stacy, Alyssa K., Natalie M. Mitchell, Jacob T. Maddux, A. Miguel, Laura Durán, Jorge A. Girón, Roy Curtiss 3rd, and Melha Mellata. "Evaluation of the prevalence and production of Escherichia coli common pilus among avian pathogenic E. coli and its role in virulence." PloS one 9, no. 1 (2014): e86565.

Stoodley, P., I. Dodds, J. Boyle, and H. Lappin-Scott. 1999. Influence of hydrodynamics and nutrients on biofilm structure. J. Appl. Microbiol. 85:19S-28S.

Teodósio, J. S., Simões, M., Melo, L. F., & Mergulhão, F. J. (2011). Flow cell hydrodynamics and their effects on E. coli biofilm formation under different nutrient conditions and turbulent flow. Biofouling, 27(1), 1-11.

Trachoo, N., J. F. Frank, and N. J. Stern. "Survival of Campylobacter jejuni in biofilms isolated from chicken houses." J. Food Protect. 65, no. 7 (2002): 1110-1116.

Wang, Shaohui, Chunling Niu, Zhenyu Shi, Yongjie Xia, Muhammad Yaqoob, Jianjun Dai, and Chengping Lu. "Effects of ibeA deletion on virulence and biofilm formation of avian pathogenic Escherichia coli." Infect. and Imm. 79, no. 1 (2011): 279-287.

Watkins, S. 2006. Clean Water Lines for Flock Health. Avian Advice 8:3-5.

Whirl-Pak. Nasco-Fort Atkinson, WI- 53538-0901. USA

Williams, M. M., J. W. Santo Domingo, and M. C. Meckes. 2005. Population diversity in model potable water biofilms receiving chlorine or chloramine residual. Biofouling 21:279-288.

Wingender J, Flemming H: Biofilms in drinking water and their role as reservoir for pathogens. Int J Hyg Environ Health. 2011, 214(6):417-423.

Xavier, J. B., and K. R. Foster. 2007. Cooperation and conflict in microbial biofilms. Proc. Natl. Acad. Sci. U. S. A. 104:876-881. doi:0607651104 [pii].

Table 1. Mineral characterization (in ppm) of test water*

	Ba	**Fe**	**Mg**	**Cu**	**Ca**	**Zn**	**P**	**Mn**	**K**	**S**	**Na**	pH
Test Water 1	0.04.	0.03	2.35	0.06	45.64	0.07.	<5	0.02	1.87	-	<5	7.98
Test Water 2	0.03	0.02	2.25	0.03	36.54	0.02	<5	0.01	2.82	1.73	<5	7.96

* Cr, Co, Ni, Cd, Ba, Al and Pb were measured to be either 0.03 or less. N. D. in both the test waters

Table 2. Mineral characterization of autoclaved water

	Al	**Ba**	**Fe**	**Mg**	**Cu**	**Ca**	**Zn**	**P**	**Mn**	**K**	**Na**	pH
Water 1	0.08	0.02.	<0.02	1.17	0.03	15.35	0.01	<5	<0.01	1.79	<5	7.54
Water 2		0.02	<0.01	1.23	0.03	17.91	0.02	<5	<0.01	2.10	5.16	7.64

* Cr, Co, Ni, Cd, Ba, and Pb were measured to be either 0.03 or less. N. D. in both the test waters

Table 3. Mineral characterization of autoclaved water used in positive control experiment

	Al	**Ba**	**Fe**	**Mg**	**Cu**	**Ca**	**Zn**	**P**	**Mn**	**K**	**Na**	pH
Water 1	0.10	0.03.	<0.02	1.17	0.03	24.52	0.01	<5	<0.01	1.96	6.35	7.74
Water 2	0.04	0.03	<0.02	1.73	0.08	23.36	0.02	<5	<0.01	1.85	5.98	8.34

* Cr, Co, Ni, Cd, Ba, and Pb were measured to be either 0.03 or less. N. D. in both the test waters. Water 1 and Water 2 refer test water used in Trial 1 and Trial 2.

Table 4: Free chlorine recorded (ppm) in test solution in Trial 1 and Trial 2.

	0 hour (immediately after application)	**24 hours post treatment**	**48 hours post treatment**
Trial 1	2.5	> 1	1
Trial 1	2.5	> 1	1

*residuals recorded in triplicate (n= 3)

Figure 1.

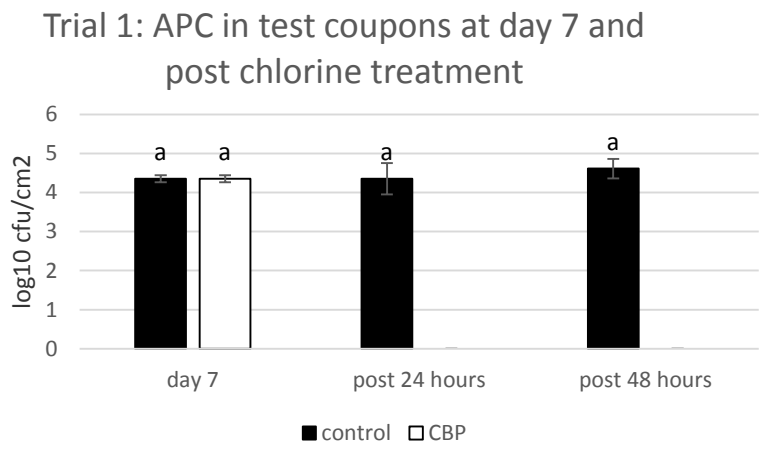


Figure 2.

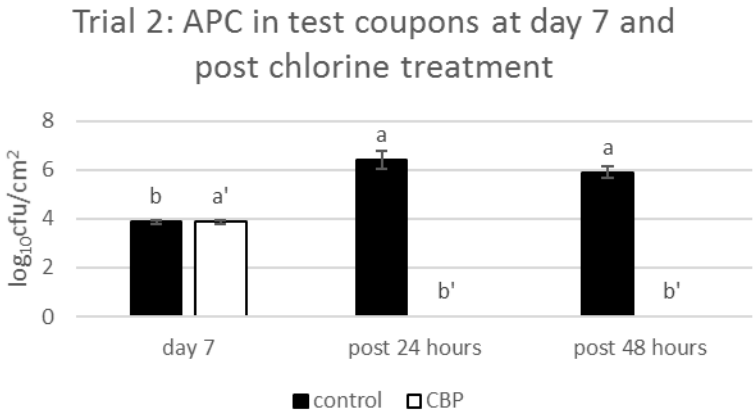


Figure 1 and Figure 2: APC (Aerobic Plate Count) recovered in test coupons on day 7, and post 24 and 48 hours of chlorine application. Different letters on the top of bars for sampled days are significantly different. CBP= Chlorine based product.

Figure 3.

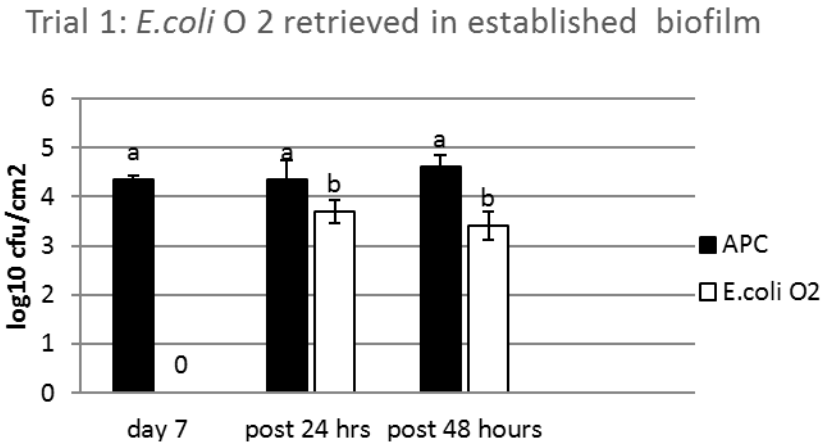


Figure 4.

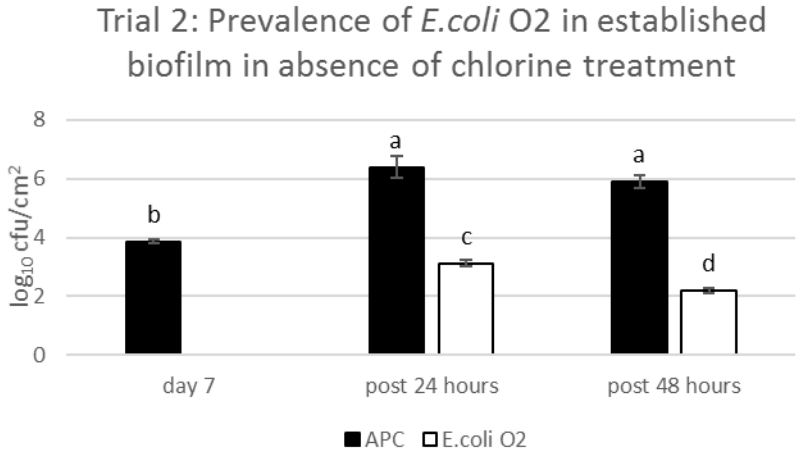


Figure 3 and 4: *E. coli O2* was seeded into pathogen free water that had 7 d old biofilm test coupons to study its biofilm forming capability. For the coupons swabbed post 24 hours and 48 hours for *E. coli* enumeration, the samples were also enumerated for aerobic plate count (APC). Different letters on the top of bars for sampled days are significantly different.

CONCLUSIONS

Water is a vital nutrient for birds that is consistently vulnerable to contamination. It is crucial that water supplies are within the acceptable level of microbial and mineral content to ensure flock health and performance, and to improve food safety. Therefore, drinking water supplied for poultry should be routinely tested for microbiological and physico-chemical parameters in order to apply appropriate water sanitation intervention. Birds are more prone to water borne infection during the early grow out period if water supplies are contaminated or water system is fouled with biofilm growth. Therefore, water supplies require daily and uninterrupted treatment especially during the early grow out period to maintain microbiologically safe water and to keep the water system hygienic. Water sanitation practice must be a consistent program that needs to be employed throughout the entire flock grow out period.

This study demonstrates that regardless of a clean and treated water supply, water systems are susceptible to biofilm growth especially when barn temperature is warm during the early grow out period. Biofilm growth more than 4 $\log_{10}$ cfu/cm^2 can occur quickly (within a few days) if water is not treated and water supply is sub optimal (> 3 $\log_{10}$ cfu/ml) type. When the water supply is contaminated with poultry pathogens (for instance, avian pathogenic *E. coli* (APEC) strain O2), the established biofilm in the water system can facilitate attachment of these pathogens into its community within a day and pose a prolonged health risk to birds. This evaluation also shows that treating water either with chlorine or hydrogen peroxide based

sanitizer can be an effective water sanitation measure to address microbial problems in water or to mitigate biofilm related issues in water systems.