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Effect of Watering Trough Chlorination on Persistence of *Mycobacterium avium* subsp *paratuberculosis*

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Abstract

Continued increases in Johne's disease around the world suggest more information is needed to understand the mechanisms by which the causative agent, *Mycobacterium avium* subsp *paratuberculosis* (MAP), is spread among livestock on the farm site. Livestock watering troughs are frequented by all animals on a farm; they provide a moist, nutrient-rich environment for bacterial survival, and the trough basin provides a surface for bacterial adhesion (i.e., biofilm formation). The goal of this study was to determine whether addition of chlorine to trough water could prevent or reduce biofilms containing MAP on trough materials. MAP was inoculated into trough water containing normal trough water microbial flora. The concentration of MAP in biofilms on suspended 1.5 in x 0.6 in (3.8 cm x 1.5 cm) concrete, plastic, galvanized or stainless steel trough materials was evaluated. Chlorine (2 ppm) was added to the trough water on day 7, and then weekly for 70 days. The concentration of MAP in biofilms on the trough materials was measured using quantitative, real-time PCR to target the MAP-specific IS*900* sequence in DNA extracts. Chlorination was most effective against MAP on galvanized steel and stainless steel trough materials (99% reduction (t_{99}) in biofilm-associated MAP in 15 and 16 days, respectively). This value was two to four times higher for MAP in biofilms on plastic and concrete materials (t_{99} of 33 and 66 days, respectively). Differences in effectiveness of disinfection may result from higher pH (pH 8.23) in troughs with concrete materials and lower chlorine availability in troughs with plastic materials. These results suggest that the effectiveness of chlorine disinfection depends on trough material construction, pH, and chlorine availability. Optimization of disinfection protocols and elimination of biofilms on trough surfaces should reduce persistence of MAP in trough waters.

Key words: *Mycobacterium avium* subsp *paratuberculosis*, paratuberculosis, chlorination, chlorine, trough, water, biofilm, Johne's disease

Résumé

L'accroissement soutenue de la prévalence de paratuberculose partout dans le monde suggère qu'il est nécessaire d'avoir plus d'information afin de mieux comprendre les mécanismes par lesquels l'agent causal de la maladie, *Mycobacterium avium* subsp *paratuberculosis* (MAP), se transmet entre les animaux dans une ferme. Les abreuvoirs sont fréquentés par tous les animaux d'une ferme et constituent un environnement humide et riche en nutriments favorisant la croissance bactérienne. La surface de l'abreuvoir est propice à l'adhérence bactérienne (i.e. formation de biofilm). Le but de cette étude était de déterminer si l'addition de chlore à l'eau des abreuvoirs peut prévenir ou réduire la formation de biofilm contenant des MAP sur les parois de l'abreuvoir. Les bactéries MAP ont été inoculées dans l'eau d'abreuvoirs contenant une flore microbienne d'eau normale. L'évaluation de la concentration de MAP dans les biofilms était faite sur des plaques suspendues mesurant 1.5 par 0.6 pouces (3.8 cm x 1.5 cm) et constituées de béton, de plastique ou d'acier galvanisé ou inoxydable. Le chlore (2 ppm) était ajouté à l'eau de l'abreuvoir au jour 7 et ensuite à chaque semaine pendant 70 jours. La concentration de MAP dans les biofilms sur les différents matériaux était déterminée avec la réaction d'amplification en chaîne par polymérase quantitative en temps réel ciblant des séquences IS900 spécifiques à MAP dans des extraits d'ADN. L'ajout de chlore était le plus efficace contre MAP sur les matériaux en acier galvanisé ou inoxydable (réduction de 99% (t_{99}) des MAP associées au biofilm aux jours 15 et 16, respectivement). Cette valeur était de deux à quatre fois plus élevée pour le MAP dans les biofilms associés aux plaques en plastique et en béton (t_{99} de 33 et 66 jours, respectivement). L'efficacité différentielle de la désinfection peut être causée par un pH plus élevé (pH 8.23) dans les abreuvoirs en béton et par une plus faible disponibilité du chlore dans les abreuvoirs en plastique. Ces résultats démontrent que l'efficacité de la désinfection au chlore dépend du matériel de fabrication des abreuvoirs, du pH et de la

disponibilité du chlore. L'optimisation des protocoles de désinfection et l'élimination de biofilms sur la surface des abreuvoirs devraient réduire la persistance de MAP dans l'eau des abreuvoirs.

Introduction

Mycobacterium avium subsp *paratuberculosis* (MAP), the causative agent of Johne's disease, is an obligate pathogen thought to be capable of growth only inside of a suitable host. However, studies have shown that the organism survives well in the environment; MAP has been detected for 200 to 600 days in water and soils.[4,25,34] It is possible that the organism persists in a dormant state in the environment until ingested by a suitable host.[35] MAP has been found in many areas on the farm, particularly those that are moist, nutrient-rich, and commonly frequented by livestock.[1,14,25,26,34] Although research has not been done to evaluate MAP transmission rates from the environment to livestock, data suggest there is a strong correlation between the number of positive environmental samples and MAP infection status on the farm.[1,19,22,26]

Livestock watering troughs are one of the most commonly frequented locations on farm sites, and a potential source of MAP sequestration. In a recent study, Cook *et al*[5] showed that MAP readily forms mixed-community biofilms on trough materials and persists within them for up to one year. Survival of other pathogens in livestock drinking troughs has also been well documented.[17,18,21,27,29] Several studies have shown that detection of pathogens in livestock drinking troughs on a farm is correlated with fecal shedding of the same pathogen in the herd.[27,29] Control of pathogens such as MAP in livestock drinking water sources may serve as a critical control point for slowing spread of the disease.[18,21,29]

Mycobacteria are often found in terrestrial and aquatic environments[10] and have been shown to readily colonize surfaces.[6,11,30,32] Mycobacteria are common contaminants in biofilms which form on drinking water distribution system pipes, and are often resistant to disinfectants used to eliminate them.[6,9,20,30] The unique, extremely hydrophobic cell wall structure of mycobacteria increases the tendency for these organisms to adhere to surfaces and increases their resistance to chemical disinfectants.[2,3,31] Trough water biofilms provide the organism with protection from washout, predation, and other toxic substances.[7] Given the tendency for MAP to form biofilms on trough materials and the known resistance of mycobacteria to chemical disinfection, it is important to evaluate the susceptibility of MAP to disinfection within trough water systems. Therefore, the goal of this study was to gain a better understanding of the importance of troughs as a reservoir for this organism by evaluating the effect of disinfection (chlorination) on reducing the occurrence of MAP on trough materials.

Materials and Methods

Bacterial culture preparation

A culture of MAP isolated from the ileum of a clinically infected, Johne's-positive dairy cow was inoculated into flasks containing Middlebrooks 7H9 broth[a] (one liter total volume) with 2 ppt glycerol, 10% Middlebrooks OADC,[b] and 2 ppm Mycobactin J.[c] Cells were grown for 21 days at 98.6°F (37°C) with constant mixing. The culture was pelleted and washed three times and the final pellet was re-suspended in 100 mL phosphate buffered saline. Twelve mL of this inoculum (6.7±0.93 x 10^8 cells mL^{-1}) was used to inoculate each trough on day 0 of the experiment.

Trough setup and chlorine addition

Glass tanks[d] were filled with 8.5 gallons (gal) (32 L) of trough water (diluted in half with tap water) which was collected from a dairy farm located in western Kentucky. Eight glass tanks were used to hold the trough water and the different watering trough materials in a common, inert vessel (Figure 1). To avoid chemical interactions, trough materials were not mixed in the tanks. The four most commonly used livestock watering trough materials were used for the tests: plastic,[e] concrete,[f] stainless steel,[g] and galvanized steel.[h] Tanks contained

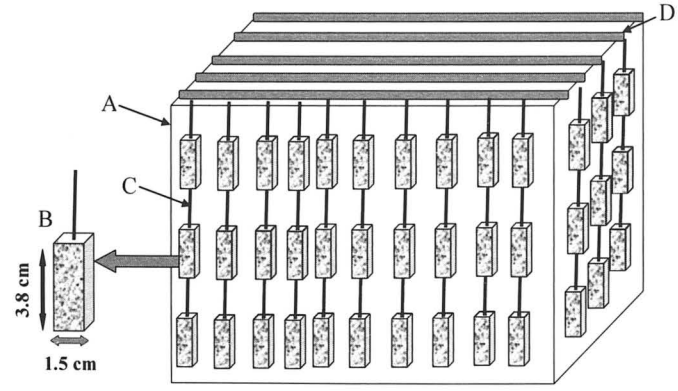

Figure 1. Schematic showing the tank design with trough materials. Each 10 gallon (gal) tank (A) was filled with 8.5 gal (32 L) of trough water and contained around 100 trough material plates (B) of either concrete, plastic, galvanized or stainless steel. Trough materials measured 1.5 in X 0.6 in (3.8 cm X 1.5 cm) and were strung together in groups of three using monofilament line (C). Trough materials were suspended in trough water from wooden dowels (D) which were suspended across the tanks. Three trough material plates were randomly removed at each sampling point.

around 100 plates of different trough materials, which were suspended vertically and submerged in the trough water (Figure 1). To supply surface area for biofilm formation and provide uniform, independent samples, the trough materials (plastic, galvanized and stainless steel) were cut into 1.5 in X 0.6 in (3.8 cm X 1.5 cm) plates (Figure 1). Concrete coupons were produced by pouring concrete into wooden molds of the same dimensions. The width of trough materials varied by construction material (plastic, 0.65 cm; galvanized steel, 0.061 cm; stainless steel, 0.064 cm; and concrete, 1.1 cm). Tanks containing the trough material plates were maintained in the dark in large incubators[i] at 77°F (25°C). Moisture was not controlled, but pans filled with deionized water were kept in the chambers near the fans to humidify the chambers and reduce trough water evaporation.

For each trough material type, around 100 plates were placed into one tank that contained only trough water, while a second tank contained around 100 plates that received trough water which was treated with chlorine for disinfection. Sodium hypochlorite bleach[j] (5.25%) was first added on day 7, then weekly for five weeks and then every two to three weeks for 149 days. Chlorine (2 ppm) was added to provide a residual-free chlorine level of approximately 0.2 ppm after 24 hours. Free and total chlorine levels were measured[k] before and after chlorination (which occurred following sampling of trough materials), and on occasion after 24 hours. Fresh trough water was added as needed to maintain tank volume at or around 8.5 gal. Trough water pH was determined using a combination electrode,[l] and was measured in the tanks prior to most samplings. Every time trough water was taken from the farm and added to the trough water tanks, 20 mL was extracted and analyzed for MAP as described below. MAP was never detected in trough water collected from the farm, and trough water addition had no effect on total or MAP cell concentrations in the biofilms or in the tank trough water (data not shown).

Sample collection and quantification of total and MAP cells

Triplicate trough material plates were collected on day 3, then weekly for six weeks and again on weeks 10, 14, and 21. Trough material samples were placed in centrifuge tubes containing 10 mL of filter-sterilized deionized water with approximately 10-20 glass beads and vortexed on high speed for two minutes to dislodge cells. Supernatant was filtered and DNA on the filters was extracted according to manufacturer's specifications.[m] Quantitative, real-time PCR (qPCR) was used to determine the concentration of total bacteria (16S rRNA gene) and MAP (IS*900* sequence). Template DNA (4 to 20 ng) was run by qPCR in triplicate as previously described.[4] Cell concentrations were calculated by dividing the copy number per cm^2 of trough material by the number of IS*900* copies per cell (14 to 20; 14 was used in this study). Q-PCR analysis of total cells (targeting 16S rRNA genes) was carried out as previously described.[12] Cell concentrations were calculated by dividing the copy number per cm^2 of trough material by 4.0, the average copy number of 16S rRNA genes per cell.[16]

Statistical analysis

Differences between treatments were assessed by fitting the log-linear portion of the cell concentration data with a first-order decay model:

$$C_t = C_0 e^{-kt}$$

where C_t is the measured cell concentration at time t, C_0 is the cell concentration at time zero, and k is the decay rate. Parameter estimates and uncertainties for both k and C_0 were obtained by fitting a linearized version of Eq.[1] to log-transformed cell concentration data. From the fitted values of k, the time to reach 99% reduction in cell concentration (t_{99}) was calculated. All data fitting was performed using the software program JMP 8.0.[n]

Results and Discussion

The effect of chlorination on both the total and MAP populations present in mixed-community biofilms on trough construction materials was assessed. The ability of MAP to form mixed-community biofilms with the normal trough water microbial population was evaluated by inoculating MAP ($6.7\pm0.93 \times 10^8$ cells of MAP mL^{-1}) into trough water on day 0, and monitoring biofilm formation on plates made of four commonly used trough construction materials (concrete, plastic, galvanized, and stainless steel). Biofilm plates or "coupons" are commonly used for assessing biofilm formation on surfaces. Coupons have been used to measure biofilm formation on stainless steel plates made from materials used in the food processing industry,[24] seawater transport pipelines for the oil industry,[28] and drinking water materials[15] among many others, including glass, plastics, rubber, and Teflon materials. In this case, trough material plates permitted sampling of triplicate biofilms growing on trough construction materials of the same dimensions.

Chlorine is an inexpensive, effective biocide commonly used for disinfection of water and water distribution systems. In this study, the effect of chlorine on the total microbial population and the MAP population present in biofilms on the surface of water trough materials was determined. Chlorination was initiated on day 7, continued on a weekly basis for 70 days, and then every two to three weeks thereafter. There was no chlorine detected in the trough water before chlorine addition started on day 7, and chlorine was not detected in the tanks that were not treated with chlorine. Chlorine dos-

age testing was used to determine the concentration of chlorine needed to give an initial total chlorine concentration of 2 ppm, with the goal of maintaining a chlorine 24-hour residual of 0.2 to 0.6 ppm. This value is similar to that used in other studies of chlorine disinfection of drinking and trough water biofilms.[18,23,30,33] The concentration of free and total chlorine averaged between 0.9 and 2.3 ppm following addition to trough water (Figure 2). The ratio of free chlorine to total chlorine was lower in trough water containing plastic coupons (54.1±24.9%) as compared to concrete, galvanized, and stainless steel coupons (61.0±26.7%, 71.3±33.3%, and 71.1±25.6%, respectively). In this study, approximately 20-25% of free and 30-40% of total chlorine remained in the water after 24 hours, but values were variable.

Chlorination had little effect on the background (i.e., non-MAP) microbial population in biofilms on trough materials. When compared to cell concentrations in biofilms on trough materials with no chlorine exposure, there were few differences (P=0.05) between chlorinated and non-chlorinated materials (Figure 3). In a study of the effect of chlorine disinfection on biofilms of *Pseudomonas aeruginosa* and *Klebsiella pneumonia*, DeBeer *et al*[8] suggested that chlorine efficacy depended on the cell density and reducing capacity of the biofilm, which in turn affected chlorine penetration. In this study, high microbial cell concentrations in biofilms on all trough materials (cell concentrations of 0.4 to 7 x 10^6 cells per cm^2 of trough material within three days) may have limited the effectiveness of chlorination. Chlorination may be more effective if trough surfaces are cleaned regularly, thereby reducing biofilm and sediment formation, both of which may serve as reservoirs for bacteria.

MAP rapidly became incorporated into mixed microbial community biofilms on trough construction materials. Within three days, concentrations of the organism were 1.6 ± 0.8 x 10^5 cells per cm^2 of trough material (Figure 4). The concentration of MAP was 1%, 22%, 31%, and 28% of the total microbial popula-

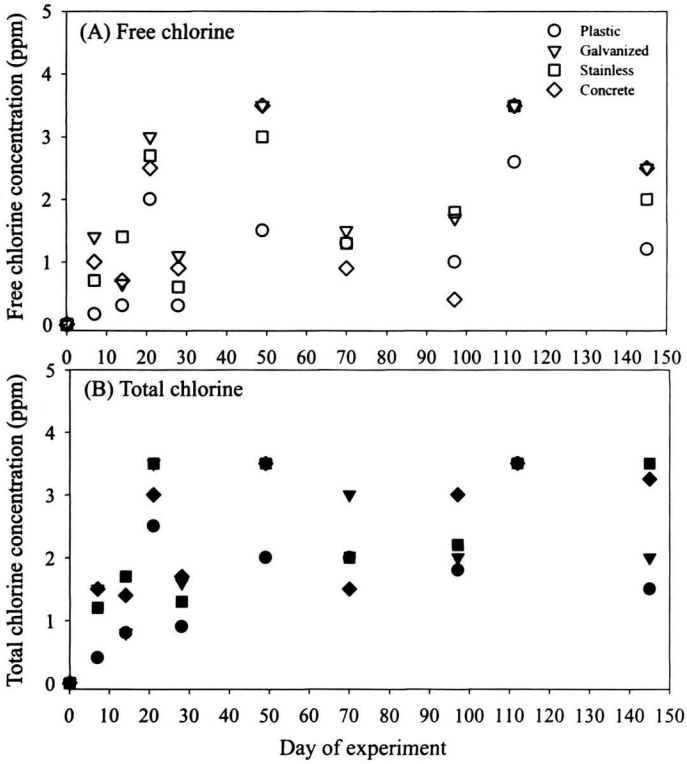

Figure 2. (A) Free (open symbols) and (B) total (closed symbols) chlorine concentrations in chlorine-treated trough water containing plastic, concrete, galvanized, and stainless steel trough material plates.

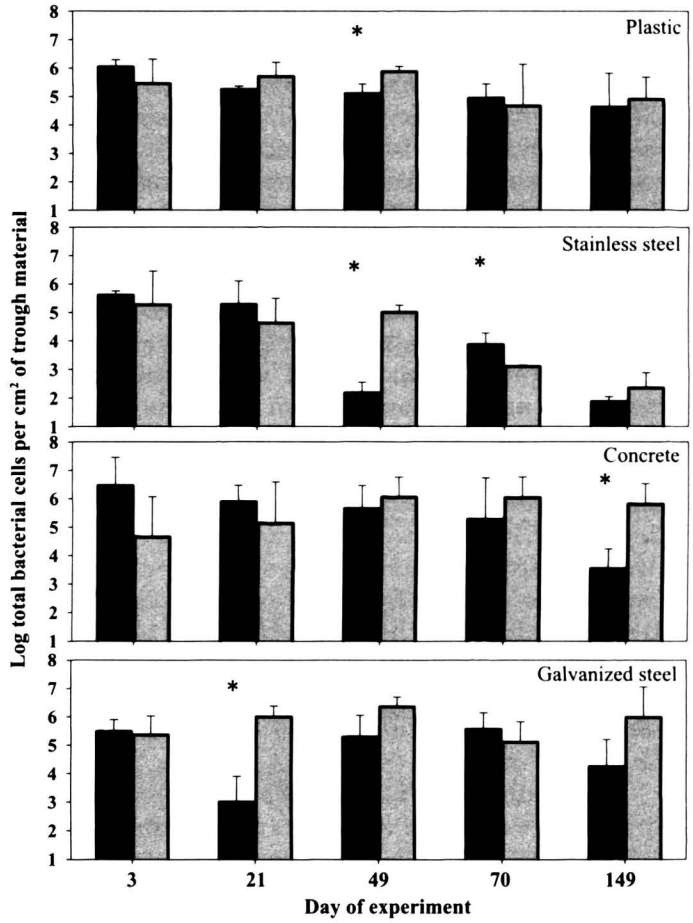

Figure 3. Q-PCR analysis of total cell concentrations in biofilms present on chlorine (dark bars) or non-chlorine (light bars) treated plastic, stainless steel, concrete or galvanized steel trough materials taken on days 3, 21, 49, 70, and 149. Values are the mean ± standard deviation of triplicate samples, each run in duplicate. Asterisks indicate statistically significant differences (P = 0.05) between sample types.

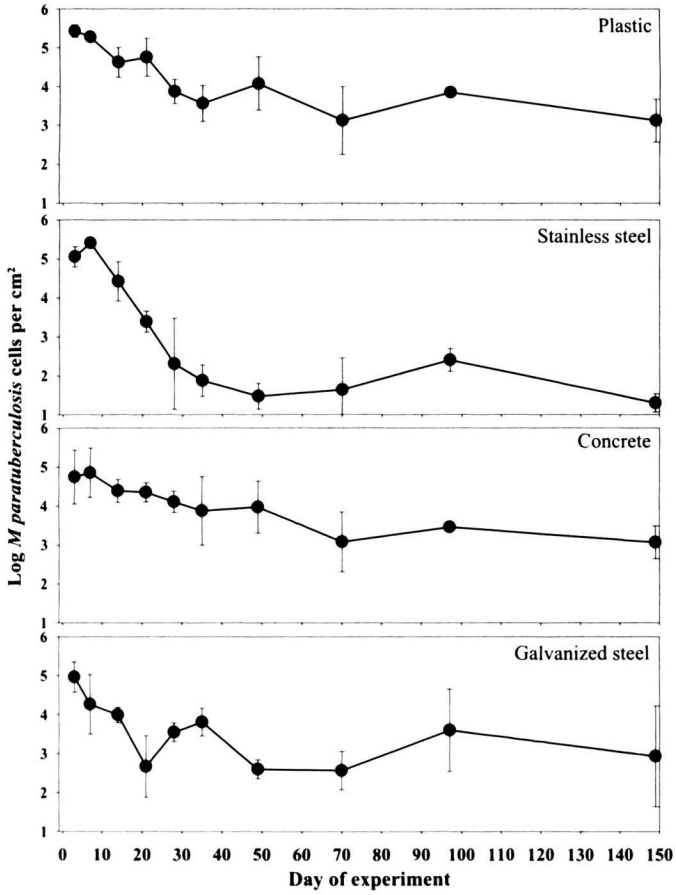

Figure 4. Q-PCR analysis of MAP cell concentrations on chlorine-treated plastic, stainless steel, concrete or galvanized steel trough materials. Values are the mean ± standard deviation of triplicate samples, each run in duplicate.

tion present on concrete, plastic, stainless, and galvanized steel trough materials, respectively. Rapid and sustained biofilm formation by mycobacteria has been well-documented.[5,6,11,30,31] It is thought that the unique hydrophobic nature of the cell wall of the organism aids it in adherence to surfaces. Results from this study suggest that when present in trough water, MAP readily attaches to the surface of trough materials. Rapid biofilm formation on solid surfaces is common and was to be expected in a livestock trough system given the nature of the troughs; solid surfaces for attachment, agitation during drinking, and unpredictable input of organic matter from cud or fecal material on the animal's mouth. Biofilm formation would be expected to provide bacteria with protection from washout, predation, and toxic substances, as well as enhance nutrient availability.[7] Further studies are needed to determine the concentration of MAP in trough water that would provide an infective dose for susceptible animals.

In the presence of chlorine, the loss of MAP was most rapid on stainless steel and galvanized steel trough materials, followed by plastic and concrete (Table 1). Time required for a 99% (t_{99}) reduction in MAP cells from biofilms on galvanized and stainless steel trough materials was 15 and 16 days, respectively (Table 1). This value was two to four times longer for cells in biofilms on plastic and concrete materials. Decay rates for MAP in biofilms on the stainless and galvanized steel trough materials were nearly identical, but were significantly faster than the decay rates obtained for the concrete or plastic trough materials. In addition to faster decay rates, stainless and galvanized steel trough materials with chlorine added on a weekly basis had significantly reduced concentrations of MAP as compared to materials that were not treated with chlorine (Figure 5). In a study of the microbial quality of livestock drinking water, LeJeune *et al*[18] found that metal troughs had lower concentrations of *E. coli* and fecal coliforms than concrete or plastic troughs. They also found that chlorination significantly reduced concentrations of coliforms, but cautioned that differences may not be biologically significant. Similarly, we found that chlorine reduced MAP concentrations, but did not eliminate the organism from any of the materials (Figure 4).

Differences in MAP concentrations in biofilms on chlorinated and non-chlorinated galvanized steel in this study suggest that chlorine can be an effective disinfectant, even for this chemically-resistant organism. In a previous study, we found that without chlorination, MAP was able to persist without die-off in biofilms on the surface of galvanized steel trough materials.[5] The reductions in MAP concentration (3 orders of magnitude over 70 days) in this study suggest that chlorination

Table 1. Decay rate (k) and time required for a 99% reduction (t_{99}) in concentration of MAP on trough materials.

Coupon type	k (day-1)[1]	t_{99} (days)[2]	P[3]
Concrete	0.07 (0.017)[c]	66[a]	<0.001
Galvanized steel	0.30 (0.048)[a]	15[c]	<0.001
Plastic	0.14 (0.013)[b]	33[b]	<0.001
Stainless steel	0.29 (0.026)[a]	16[c]	<0.001

[1]Obtained by fitting a log-transformed data using linear regression (standard errors in parentheses)
[2]The time required for a 99% reduction in biofilm-associated cells (t_{99}) was calculated based on the fitted value of k.
[3]P=probablility that the value of the fitted slope is significantly different from zero.
*Values with the same letter have 95% confidence intervals which overlap.

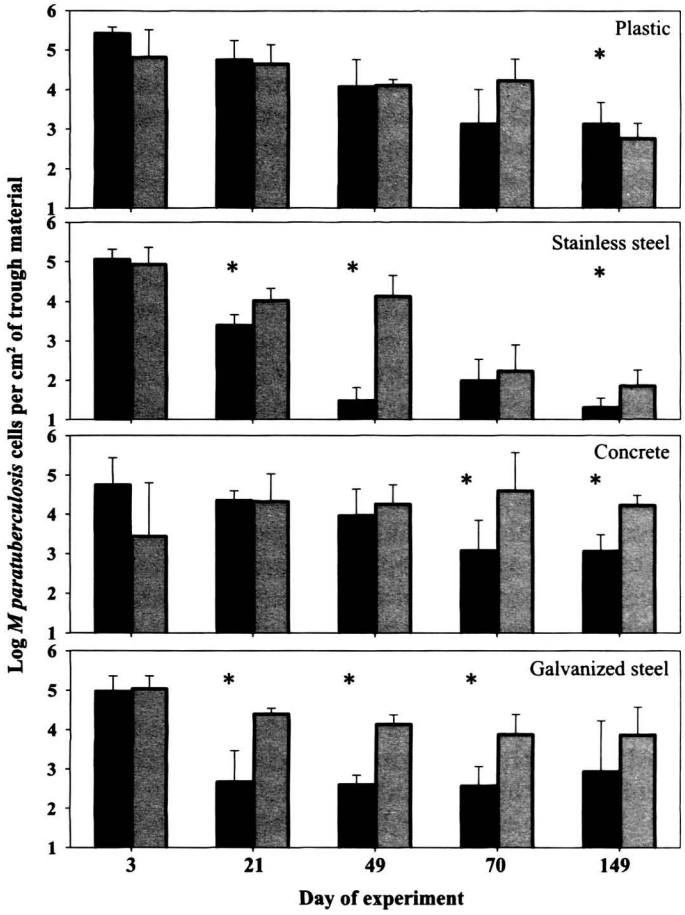

Figure 5. Comparison of MAP cell concentrations in biofilms present on chlorine (dark bars) or non-chlorine (light bars) treated plastic, stainless steel, concrete or galvanized steel trough materials. Values are the mean ± standard deviation of triplicate samples, each run in duplicate. Asterisks indicate statistically significant differences ($P = 0.05$) between sample types.

has a significant impact on the pathogen. However, when chlorine was added every two to three weeks, the effect of chlorination was reduced (day 149, Figure 5). Preliminary work suggests that after four days the total chlorine concentration was still around 1 ppm, however, free chlorine was below detection (data not shown). These results emphasize the importance of maintaining a 24-hour free chlorine residual of around 0.2 to 0.6 ppm.

MAP made up between 30% and 100% of the bacterial population in biofilms on stainless steel trough materials. Therefore, despite the lower total concentration of bacteria (Figure 3) and MAP (Figure 4) in biofilms on stainless steel materials, the pathogen was a more significant part of the population. In a study of *M. avium* biofilms on drinking water pipes, Norton *et al*[23] found that disinfection eliminated competitive bacteria on copper pipe surfaces and resulted in a population of nearly 100% *M. avium*. Therefore, chlorination may eliminate bacteria that compete with pathogens, thereby permitting those organisms to become dominant on some surfaces. However, despite being dominated by MAP, it is important to point out that concentrations of MAP in biofilms on stainless steel materials was consistently lower (up to 3 orders of magnitude lower) than on any of the other trough materials (Figure 4). Furthermore, chlorination significantly reduced MAP concentrations to levels nearing detection limits (Figure 4). These results suggest that the use of stainless steel troughs, properly maintained and disinfected, should minimize biofilm formation and reduce survival of biofilm-associated pathogens such as MAP.

The decay rate and time required for a 99% reduction in MAP on concrete and plastic trough materials was significantly longer than for stainless and galvanized steel. The lack of significant differences in the concentration of MAP on chlorine and non-chlorine treated concrete trough materials may be due to differences in pH in the trough water containing the different trough materials: plastic, 7.82±0.32; galvanized, 8.04±0.36; stainless 8.08±0.23; and concrete 8.23±0.41. Concrete mixed with water produces hydroxide ions that elevate pH. Chlorine disinfection is most effective at pH values between 6.5 and 7.5. In this range, hypochlorous acid, a strong sanitizing agent, dominates. At higher pH values hypochlorous acid is converted to the hypochlorite ion which is less effective as a disinfectant. Previous studies have shown that inactivation of *M. avium*, a close relative of MAP, by chlorine decreases as pH increases.[20] In the concrete troughs, with a pH of 8.2, the chlorine may have been converted to the non-sanitizing ionic form. It is also possible that the porosity of concrete materials made it difficult for the chlorine to penetrate all available surfaces.

Lack of differences in MAP concentrations between chlorine and non-chlorine treated plastic was more surprising. Although the pH of the trough water (7.82) was in the range expected to be optimal for disinfection, we found that the total and free-chlorine available in trough water with plastic materials was consistently lower than for the other materials (Figure 2). The high density polyethylene (HDPE) plastic trough construction material has been shown in other studies to retain lower disinfection residual than either polyvinyl chloride or glass materials.[13] The authors speculate this may be due to the interaction of hypochlorous acid with either antioxidants from the manufacturing process or with the HDPE polymer. These results suggest that chlorination of concrete and plastic troughs is less effective than for stainless steel and galvanized troughs, however, optimization of chlorine addition schemes may improve disinfection.

Results from this study suggest that MAP may persist on farm sites by attaching to surfaces of water trough materials where the organism is protected from

washout, predation, and disinfectants. Norton *et al*[23] found that biofilm formation by *M. avium* was dependent upon a complex interaction between the surface material, nutrient levels, and disinfectants; data from this study support that conclusion. Although chlorination had no influence on the background microbial population, it did affect survival of MAP on galvanized and stainless steel trough materials. Trough construction material affected both MAP biofilm formation and the effectiveness of chlorination.

Conclusions

This study shows that MAP readily forms biofilms on four of the most commonly used livestock watering trough materials: stainless steel, galvanized steel, concrete, and plastic. Although this is a laboratory study, these results clearly demonstrate the importance of watering troughs as potential reservoirs of MAP. Chlorination significantly reduced (two to three orders of magnitude) concentrations of MAP on galvanized and stainless steel trough materials, but the organism never completely disappeared from the trough surfaces. It was shown that the effect of chlorination depends on trough construction material, pH, and chlorine availability. After 70 days of chlorine addition on a weekly basis, MAP was less than 10% of the population in biofilms on all trough materials except stainless steel, and concentrations were significantly lower in chlorine-treated than in non-chlorinated tanks. Based on these data, it is suggested that cleaning of troughs be performed at least every 50 to 70 days to minimize sediment and biofilm formation and improve chlorine effectiveness. Given the low cost and convenience of chlorination as a disinfection strategy, further studies are required to optimize disinfection methods and develop regimes to improve the effectiveness against this important pathogen.

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Endnotes

[a]Remel, Lenexa, KS
[b]Becton Dickinson, Sparks, MD
[c]Allied Monitor, Fayette, MO
[d]All Glass Manufacturing, Franklin, WI
[e]Rubbermaid, Fairlawn, OH
[f]Quikrete concrete, Atlanta, GA
[g]McMaster-Carr, .025" thick, 1/2" wide, 10' coil, Aurora, OH
[h]McMaster-Carr, low carbon sheet .024" thick, 48"x 48"
[i]Perceval Scientific, Inc., Perry, IA
[j]Chlorox, Oakland, CA
[k]Hach, Loveland, CO
[l]Fisher Scientific, Hampton, NH
[m]Qbiogene, Irvine, CA
[n]SAS Version 9.1; SAS Institute, Inc., Cary, NC

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