Microbiology

Monochloramine for controlling *Legionella* in biofilms: how much we know?

Maria Anna Coniglio[1], Stefano Melada[2], Mohamed H Yassin[3]

[1]Department "G.F. Ingrassia" – Hygiene and Public Health, University of Catania, Catania, Italy. [2]Sanipur srl, via S. Quasimodo, 25 I-25020, Flero (BS), Italy. [3]Department of Infection Control and Infectious Diseases, University of Pittsburgh Medical Center, Pittsburgh, PA, USA

Prevention of *Legionella* in water system is a challenge especially when biofilm is present. Hospitals, in particular, deal with vulnerable population requiring additional protection against *Legionella*. Monochloramine (MC) has been used for small-scale hospital systems in Europe and the US only recently. This review focuses on *Legionella* in biofilm touching major practical challenges with water disinfection using MC. To date there are no published reviews on this topic so a critical and comprehensive update on the progress in the field was necessary. Scientific databases were reviewed for articles published between 1980 and 2013, containing the terms 'Legionella'-'Legionellosis'-'Legionnaires' Disease' and 'Biofilm' and 'Disinfection'-'Legionella' and 'Monochloramine'. In total, 36 articles were considered and divided in 5 groups to evaluate: *(i)* MC biofilm penetration, residual concentrations, production of disinfection by-products; *(ii)* influence of pipe material on biofilm formation and disinfectant penetration; *(iii)* effect of nitrification on MC decomposition rate; *(iv)* MC treatment and VBNC state of *L. pneumophila* in biofilms; *(v)* influence of protozoa on MC disinfection of biofilm. Among the antimicrobial agents, MC appears more effective for decreasing *Legionella* within the biofilms *in vitro* and in plumbing systems. *Journal of Nature and Science, 1(2):e44, 2015.*

Legionella | monochloramine | biofilm control | disinfection

On surfaces exposed to water a majority of microorganisms tend to form a 'biofilm', which is composed of extracellular polymeric substances (EPS) excreted by the microorganisms themselves. In a long distribution system, with wide variations in flow rates, biofilm would eventually be produced due to the sedimentation of organic particles. Because biofilms may contain enough bacteria to give an infective dose, they represent a potential health risk especially in hospitals, where patients constitute a vulnerable population. Furthermore, biofilms on distribution pipes contribute to bacterial regrowth and disinfectant decay in water.

Legionellae are ubiquitous and they could live as free-living planktonic forms or as intracellular parasites of protozoans (Hsu *et al.* 2011). The organisms are also able to survive chlorination and thus enter water supply systems and proliferate in humid thermal habitats, including air conditioners, water systems, cooling towers, water fountains and hospital equipments. Legionellae are frequently found in biofilms on the surfaces of these systems, where they are more resistant to eradication compared to their free-living planktonic counterparts (Lin *et al.* 2011).

One of the key issues for controlling the growth of legionellae in biofilms is to recommend an effective disinfection method. Two main factors interfere with the activity of antimicrobial agents: *(i)* they may fail to penetrate through the EPS or bind to it before they reach legionellae (Simões *et al.* 2010); *(ii)* legionellae may become resistant to conventional chemical antimicrobials (Cooper and Hanlon 2010).

At present, chlorination is the most commonly used treatment for *Legionella* control in water systems. Lapses in chlorination or discontinuous chlorination with chlorine or chlorine dioxide can lead to an increased resistance of biofilm bacteria to chlorine (Casini *et al.* 2008). Copper-silver ionization is currently used for *Legionella* control in water distribution systems and there is evidence that accumulation of copper and silver inside the biofilms is responsible for the prolonged bactericidal effect (Liu *et al.* 1998). Nevertheless, in Europe copper based products for drinking water disinfection have not been allowed since 1st February 2013 due to the Biocidal Product Directive 98/8/EC (EEA Commission Decision 1998). UK, Spain, the Netherlands and Poland, that have been using copper/silver ionization for *Legionella* control for a long period of time, obtained a derogation till 31st December 2017 providing that as of 1st January 2015 users should be actively informed about the immediate need to implement alternative methods.

Among the antimicrobial agents of relatively most recent application in the disinfection of water, monochloramine (MC) seems to be more effective for decreasing *Legionella* within the biofilms *in vitro* (Lee *et al.* 2010) and in hospital plumbing systems in the US (Kandiah *et al.* 2012) as well as in Italy (Marchesi *et al.* 2012; Casini *et al.* 2014). The aim of this review is to assess the available literature that supports the effectiveness of MC for controlling *Legionella* growth in biofilms.

METHODS

Search for literature was carried out using terms: 'Legionella' OR 'Legionellosis' OR 'Legionnaires' Disease' AND 'Biofilm' AND 'Disinfection NEAR Legionella' AND 'Monochloramine'. The search, including published papers between 1980 and 2013, was conducted in relevant chemical and biomedical databases: ACS Publications, Elsevier, JSTOR, Nature Publishing Group, PubMed, SDOS and Wiley Online Library. Considering the relatively new application of MC, non published research, letters and conference communications were also included. Inclusion criteria were: studies about the effectiveness of MC under *in vitro* conditions, and studies with more than three months of follow up in real conditions. The literature review has been divided in five main parts to evaluate the following aspects of the action of MC: *(i)* biofilm penetration, residual concentrations and production of disinfection by-products; *(ii)* influence of pipe material on biofilm formation and disinfectant penetration; *(iii)* effect of nitrification on decomposition rate; *(iv)* MC treatment and viable but nonculturable (VBNC) *L. pneumophila* in biofilms; *(v)* effect of protozoa on MC disinfection of biofilm.

After this initial search, 125 articles were identified for further review. Each article was evaluated with respect to the use of MC, if the technology was adequately tested for validity and/or accuracy against biofilm, and if the management of the water distribution system was associated with the control of *Legionella.* After this initial process, 32 articles were considered. Of those, 1 was a review article (Simões *et al.* 2010) and the others were surveys.

Table 1 shows a summary of the main studies on the effectiveness of MC against the biofilm.

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Corresponding Author: Dr. Maria Anna Coniglio
Department "G.F. Ingrassia" – Hygiene and Public Health
University of Catania, via Santa Sofia 87, 95123 Catania, Italy.
Tel.: +39 095 3782087; Fax: +39 095 3782175.
E-mail address: ma.coniglio@unict.it

Table 1. Description of noteworthy studies on effectiveness obtained through the application of MC

Authors	Study design	Results
Wolfe *et al.*, 1990	Experimental	Maintaining a chloramine residual of at least 1-2 mg/L could be sufficient to limit nitrifier growth in drinking water
Chen *et al*, 2000	Experimental	Reduction of biofilm viable cells counts better than free chlorine at neutral pH
Pintar *et al.*, 2003	Experimental	Effectiveness in controlling ammonia-oxidizing bacteria activity in the biofilm
Thomas *et al*., 2004	Experimental	Important increase in dead biomass proportion at a concentration of 0.5 mg l^{-1}
van der Kooij *et al.*, 2005	Experimental	A model system of copper can temporarily reduce *Legionella* colonization but, after 2 years, biofilm colonization on copper, stainless steel and cross-linked polyethylene (PEX) pipes is very similar
Park *et al*, 2008	Experimental	High-level MC residual in a low-nutrient water system linked with a reduction in biofilm density on pipe surfaces and to depressed potential functional/metabolic ability of the biofilm community
Türetgen, 2008	Experimental	Decrease of cell cultivability significantly begins at 1 ppm. At 1.5-2 ppm environmental *L. pneumophila* enters VBNC state
van Schalkwyk *et al.*, 2010	Experimental	Even at concentrations as low as 1 ppm MC is able to penetrate complex biofilm matrixes like that in cooling towers
Dupuy *et al*, 2011	Experimental	Similar effectiveness towards free or co-cultured *L. pneumophila* while chlorine and chlorine dioxide were less efficient on co-cultured *L. pneumophila*
Ramseier *et al*., 2011	Experimental	Minimal reaction with organic matter but specific reaction with bacterial membrane at high oxidant exposures
Chien *et al*, 2012	Experimental	More effectiveness in controlling biofouling (accumulation of micro-organisms, plants or algae) in cooling systems employing secondary-treated municipal wastewater compared to free chlorine
Kandiah *et al*, 2012	Observational	Better biofilm penetration than copper-silver ionization
Pressman *et al*, 2012	Experimental	Limiting the free ammonia concentration during MC application slows the onset of nitrification episodes by maintaining the biofilm biomass at a state of lower activity. MC is able to penetrate biofilms 170 times faster than free chlorine
Dupuy *et al*, 2014	Experimental	Less effectiveness than chlorine dioxide against *Acanthamoeba* cysts

Table 2. Description of noteworthy studies comparing the effects of MC vs. other oxidative disinfectants

Authors	Study design	Results
Kandiah *et al.*, 2012	Observational	Appropriate flushing procedures and cleaning of the faucets with a bleach-based solution were unsuccessful in eradication of *Legionella* species despite adequate copper and silver levels. After monochloramine introduction into the hot water system faucets, all sensor faucets converted negative after only three weeks of monochloramine installation.
Marchesi *et al.*, 2012	Observational	*Legionella pneumophila* contamination was followed in comparison with 2 other water networks in the same building using chlorine dioxide. MC significantly reduced the number of contaminated sites compared with baseline (from 97.0% to 13.3%, respectively), chlorine dioxide device I (from 100% to 56.7%, respectively), and device II (from 100% to 60.8%, respectively). MC could represent a good alternative to chlorine dioxide in controlling legionellae contamination in public and private buildings.
Pressman *et al*., 2012	Experimental	The initial MC mass delivery inside a nitrifying biofilm was 170 times greater compared to free chlorine for equivalent chlorine concentrations.
Chen *et al.*, 2000	Experimental	Compared to free chlorine, MC had a longer residual effect in biofilm cells also at neutral pH. While the amount of biofilm removed by chlorine and MC was not statistically significantly different ($p = 0.45$), MC killed bacteria in the biofilm better than did free chlorine at neutral pH ($p = 0.001$).
Ercken *et al.*, 2003	Experimental	The biocidal activity of MC against *Naegleria lovaniensis* was 8x weaker than that of hypochlorite but 2x stronger than that of peracetic acid.
Dupuy *et al.*, 2011	Experimental	Comparison of the efficacy of chlorine, MC and chlorine dioxide against trophozoites of three different *Acanthamoeba* strains showed that MC was more efficient than chlorine and chlorine dioxide at the same level towards free or co-cultured *L. pneumophila.*

Biofilm penetration, residual concentrations and production of disinfection by-products

Despite its relatively low oxidative activity, MC is more effective than the other oxidative disinfectants (e.g. chlorine and chlorine dioxide). The ability of MC to better penetrate biofilms follows a dose-dependent effect. **Table 2** compares MC to other disinfectants in controlling *Legionella.*It has been recently demonstrated that MC is able to penetrate biofilms 170 times faster than free chlorine (Pressman *et al.* 2012) and that even at concentrations as low as 1 ppm it is able to penetrate complex biofilm matrixes like that in cooling towers (van Schalkwyk *et al.* 2010).

Compared to the other oxidative disinfectants, MC has a longer residual effect in biofilm also at neutral pH (Chen and Stewart 2000). Maintenance of MC residual above 3 mg/L is needed to effectively control biofilm in cooling systems employing secondary-treated municipal wastewater as the only source of makeup water (Chien *et al.* 2012). Moreover, MC minimally reacts with organic matter but react specifically with bacterial membrane at high oxidant exposures (Ramseier *et al.* 2011).

The application of chloramines, as well as chlorine, may cause increased formation of highly carcinogenic nitrosamines and other disinfection by-products (DBPs) (Chang *et al.* 2011). **Table 3** summarizes studies describing the effect of MC on DBPs. A recent study investigating the effects of corrosion products of copper in plumbing systems on *N*-nitrosodimethylamine (NDMA) formation from DMA found that the transformation of MC to dichloramine and complexation of copper with DMA were involved in elevating the formation of NDMA by copper at pH 7.0 (Zhang and Andrews 2013). Anyway, MC generally results in lower concentrations of DBPs compared to the other oxidative disinfectants. It has been recently demonstrated that DBPs levels in filtered river waters, as well as in coagulated surface waters collected from water treatment plants are generally higher after chlorination than after chloramination (Farré *et al.* 2013).

Table 3. Description of noteworthy studies on the production of DBPs through the application of MC*

Authors	Study design	Results
Chang et al., 2011	Experimental	NDMA was the dominant species of nitrosamines and the levels of other nitrosamines were too low to show specific formation characteristics following treatments. The presence of bromide shifted the DBPs species into brominated DBPs, and treatment with MC generated a higher proportion of brominated DBPs than those obtained with sodium hypochlorite.
Zhang *et al*., 2013	Experimental	The investigation on the effects of corrosion products of copper on NDMA formation from DMA showed that the transformation of MC to dichloramine and complexation of copper with DMA were involved in elevating the formation of NDMA by copper at pH 7.0.
Lu *et al.*, 2009	Experimental	Chloramination of the dissolved natural organic matter (DOM) fractions yields much less THMs and HAAs than chlorination with the increase of disinfectant dosage, contact time and dissolved organic carbon content.
Farré *et al*., 2013	Experimental	Chlorination formed higher concentrations of DBPs or more potent DBPs in waters collected from three different drinking water treatment plants, along with reverse osmosis permeate from a desalination plant.

* DBP:Disinfection byproducts, NDMA: *N*-nitrosodimethylamine, DMA:Dimethylamine, THM: Trihalomethanes , HAA: halogenated acetic acid

Influence of pipe material on biofilm formation and monochloramine effectiveness

Another factor on formation and persistence within the biofilms of *Legionella* is the influence of pipe material. Copper piping has been in use in water systems to minimize the risk of Legionellosis because copper may prevent colonization of the pipe and could inhibit the biofilm growth. Anyway, a model system of copper can temporarily reduce *Legionella* colonization but, after 2 years, biofilm colonization on copper, stainless steel and cross-linked polyethylene (PEX) pipes was very similar (van der Kooij *et al.* 2005).

The decomposition rate of MC may be enhanced by copper due to the formation of a Cu(II)-humic acid complex (Fu *et al.* 2009). A laboratory experiment has confirmed that MC could decay rapidly only in the presence of new copper pipes, providing a possible explanation for the rapid disinfectant loss in the new buildings (Nguyen *et al.* 2012). Nevertheless accumulation of Cu(II) ions could occur also after years of use of copper/silver ionization. **Figure 1** shows a longitudinal section of a water pipe after more than 10 years of use of copper/silver ionization. Note the severe corrosion within the pipe wall. Copper corrosion products can thus affect the MC disinfection by affecting its decomposition rate. An adequate chloramination system should be put in place to reduce MC decomposition rate and to avoid accumulation of DBPs.

Figure 1. Longitudinal section of a water pipe after more than 10 years of use of copper/silver ionization. (Picture from Boffardi BP, Hannigan JM. A limited evaluation of pitting corrosion of copper piping in a hospital domestic hot water system using copper-silver ionization for *Legionella* control. AWT, Mohegan, 2013.)

Effect of nitrification on decomposition rate of monochloramine into biofilm

Chloramination provides a source of ammonia promoting the growth of nitrifying bacteria within the biofilms. Nitrifying bacteria can grow in the presence of MC due to their ammonia and nitrite oxidizing characteristics. Biofilm grown on pipe surfaces can harbor nitrifiers, belonging primarily to *Nitrosomonas*, *Nitrobacter* and *Nitrosospira* (Lipponen *et al.* 2004). Nitrification in drinking water distribution systems may result in water quality degradation and subsequent noncompliance with existing regulations. Accelerated chloramine decay is related to high levels of nitrification and when nitrification is absent chloramine is expected to better penetrate iron biofilms (Lee *et al.* 2011). A possible explanation for these evidences is that the presence of high free ammonia concentration due to MC decomposition allows the microorganisms deeper within the biofilm to remain active during MC application. Moreover, once initiated, nitrification is very hard to stop because CT value (product of disinfectant concentration and contact time) is too low in biofilm where the right residual MC cannot be maintained. In fact, limiting the free ammonia concentration during MC application could slow the onset of nitrification episodes by maintaining the biofilm biomass at a state of lower activity (Pressman *et al.* 2012).

Nonetheless, although there is evidence that maintaining a chloramine residual of at least 1-2 mg/L could be sufficient to limit nitrifier growth in drinking water (Wolfe *et al.* 1990), it has been shown that greater MC residuals may be required to inactivate bacteria inside the biofilms (Park and Kim 2008).

The degradation of the hypochlorite used to produce MC can lead to an increased level of free ammonia and this can affect the disinfection effectiveness of the *in situ* produced MC. Increased levels of *Legionella* occurred for a severe degradation of the hypochlorite reagent despite MC levels were in the desired range and, only after draining and cleaning of the reagent tank, free ammonia and *Legionella* were reduced to negligible levels. A major US University-based medical system experienced similar issue as high temperature in the storage room caused degradation of hypochlorite (personal data).

Monochloramine treatment and viable but nonculturable (VBNC) *Legionella pneumophila* in biofilms

After disinfections with chlorine compounds, *L. pneumophila* can completely lose its cultivability but do not lose viability entering the viable but nonculturable (VBNC) state. Decrease of cell cultivability significantly begins at 1 ppm dosage of MC, while at 1.5-2 ppm environmental *L. pneumophila* enters VBNC state (Türetgen 2008).

It has also been shown that up to 20 ppm MC concentration, *Legionellae* enter VBNC state and are still able to synthesize virulence factors. Nonetheless, at this concentration, attempts to resuscitate VBNC cells with amoebas failed (Alleron *et al.* 2013). This suggests that the accumulation of virulence factors by VBNC cells may not be sufficient to maintain their virulence.

Anyway, disinfectants' *in vitro* activity is less effective in field applications and at the moment there is a lack of studies evaluating the effect of long exposures to MC in real water systems.

Effect of protozoa on monochloramine disinfection of biofilm

Within biofilms, free-living amoebae (FLA) may favor the multiplication, dissemination and virulence of *Legionella*. Intracellular parasitism of FLA is probably at the origin of the rapid re-colonization of water distribution systems by *Legionella* generally observed immediately after stopping a disinfection program (Thomas *et al.* 2004). A recent experience in an Italian hospital (personal data) showed a rapid and massive re-colonization of the water distribution system only two weeks after stopping a 2-months disinfection program with MC caused by a temporary failure of the device. The possible presence of FLA throughout the entire water system could explain the above results, thus indicating the necessity of a continuous disinfection with MC.

Up to date, only a few studies have been focused on the effectiveness of MC against pathogenic FLA. Under laboratory conditions the biocidal activity of MC against *Naegleria lovaniensis* was 8x weaker than that of hypochlorite but 2x stronger than that of peracetic acid (Ercken *et al.* 2003). This evidence makes MC a good candidate for inactivation of pathogenic *Neaegleria* species and an ecologically less harmful alternative to hypochlorite.

Moreover, comparison of the efficacy of chlorine, MC and chlorine dioxide against trophozoites of three different *Acanthamoeba* strains showed that MC was more efficient than chlorine and chlorine dioxide at the same level towards free or co-cultured *L. pneumophila* (Dupuy *et al.* 2011). Nonetheless, despite its better effectiveness against amoeba trophozoites, MC appears less effective than chlorine dioxide against *Acanthamoeba* cysts (Dupuy *et al.* 2014).

CONCLUSIONS

The presence of *Legionella* within a biofilm makes eradication from water system very difficult. Among the antimicrobial agents, MC seems to be more effective for decreasing *Legionella* within the biofilms *in vitro* as well as in model plumbing systems. As of to date there are no published reviews on this topic, a critical and comprehensive update on the progress in the field is necessary.

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