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Biofilm formation and multiplication of *Legionella* in a model warm water system with pipes of copper, stainless steel and cross-linked polyethylene

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Abstract

Legionella pneumophila was grown in a model warm water system with pipes of copper (Cu), stainless steel (SS) and cross-linked polyethylene (PEX) during recirculation of tap water at 25–35 °C. Subsequently, domestic use of warm (37 °C) water was simulated using tap water with a low AOC concentration (< 10 μg C/L). Two times each week the temperature of the water in the electric heaters (not in the pipes) was elevated to 70 °C for 30 min. ATP concentrations in the water sampled from the pipes over a 2-year period were significantly different for the pipe materials, with median values of 2.1 ng/l (Cu), 2.5 ng/l (SS) and 4.5 ng/l (PEX), respectively. Median values of the biofilm concentration were similar on Cu and SS (about 630 pg ATP/cm^2) and 1870 pg ATP/cm^2 on PEX. *Legionella* multiplied in these biofilms and median values of *Legionella* concentrations in water were 1500 CFU/l (Cu) and about 4300 CFU/l for SS and PEX. *Legionella* to ATP ratios in water had median values of about 0.8 CFU/pg. Hot water flushing (70 °C) of the pipes on day 552, followed by 2 weeks of recirculation at 37 °C, caused strongly increased concentrations of ATP (up to 300 ng/l) and *Legionella* (> 10^7 CFU/l), with about 100 CFU/pg ATP. Concentrations declined to original levels within 1 week of domestic water use, etc. *Legionella* concentrations in water and biofilms were at the same levels for all materials after 2 years. Hence, copper temporarily limited the growth of *Legionella* under the applied conditions and a rapid biomass development strongly increased the *Legionella* to ATP ratio.
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Keywords: Legionella; Warm water; Biofilm formation; ATP; Copper; Stainless steel; PEX

1. Introduction

Multiplication of *Legionella* in hot tap water systems poses a potential health threat when water use leads to aerosol formation (Cordes et al., 1981; Tobin et al., 1980). Conditions ('risk factors') favouring the multiplication of *Legionella* in water systems include: a water temperature between 25 and 45 °C, a long residence time (stagnation) and the presence of biofilms and sediments (Fischer-Hoch et al., 1982; Ciesielsky et al., 1984).

Abbreviations: AOC, assimilable organic carbon; ATP, adenosine triphosphate; BRR, biomass release rate; CFU, colony forming units; LRR, legionella release rate; MWW, model warm water system; SS, stainless steel; PEX, cross-linked polyethylene

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Certain protozoa grazing on bacteria in biofilms and sediments can serve as hosts for *Legionella* (Wadowsky et al., 1988; Abu Kwaik et al., 1998; Kuiper et al., 2004). Investigations in practical situations, in model systems and in laboratory tests have shown that increased biomass concentrations lead to higher numbers of *Legionella* (Rogers et al., 1994; Schoenen et al., 1988; Schofield and Locci, 1984; van der Kooij et al., 2002). However, quantitative information about concentrations of biomass and *Legionella* in biofilms and in the planktonic phase in natural or man-made environments is still scarce. This lack of information is partly due to difficulties related to the quantitative determination of the concentrations of attached and suspended biomass. A variety of methods are available for biomass quantification, e.g. heterotrophic plate counts, total direct cell counts, SEM, or enzymatic methods, but these methods either include only a fraction of the bacterial population, do not differentiate between viable or dead cells, and/or are laborious, requiring extended calibration. In this study adenosine triphosphate (ATP) analysis is used for determining the concentration of active biomass in water and on water exposed surfaces in a model warm water system. This parameter is widely used in microbial physiology and ecology, and data bases exist about ATP concentrations in the environment and under experimental conditions (Holm-Hansen and Booth, 1966; Karl, 1980; van der Kooij, 2003a). The objectives of this study were to (i) determine the impact of the materials stainless steel, copper and cross-linked polyethylene on biofilm formation in a model warm water (MWW) system and (ii) assess the growth of *Legionella* under these conditions in relation to concentrations of both attached and suspended biomass.

2. Materials and methods

2.1. Model warm water (MWW) system

The MWW system included three separate identical electric water heaters (A, B and C), each with a volume of 30 l, an enamel-coated internal surface and a copper heating coil. Each heater was connected to the tap water system with a PVCu pipe (internal diameter 17 mm). The locally available tap water is prepared from anaerobic ground water by using aeration and rapid sand filtration. Typical quality characteristics of the treated water are given in Table 1. Two duplicate pipes of either stainless steel (SS, quality grade AISI 316, seamless, i.d. 16 mm), copper (Cu, phosphorous deoxidised Cu-DHP, half hard, i.d. 13 mm) or polyethylene (PEX, cross-linked using hydrogen peroxide, i.d. 12 mm), each with a length of 5.9 m, were connected to one of these heaters with PVC-C (1.6 m, i.d. 20 mm). These PVC-C pipes also served as outlets via tap 3 (Fig. 1). Connecting pieces and coupling joints were made of SS. Four pipe segments, each with a length of 15 cm, were installed at the outlet side of each test pipe for biofilm analysis. An SS gear pump was installed in each system enabling warm water recirculation via a PVC-C pipe (1.2 m, id 20 mm). Valves were made of PE, the flow meter was polyacrylate, and a Nylon flow control was used to prevent back flow. Domestic warm water use, following a standardised scheme (NEN 5128, 1998) with no use of water for showering, was simulated with magnetic valves steered by a computer-programme. Every 24 h a total volume of 81 l of water passed through each pipe of the selected materials. Temperature in the pipes was registered automatically using calibrated thermocouple devices attached to the pipes. Water samples of 500 ml were collected periodically (usually once a week) from each pipe (at sampling locations tap 1 and tap 2, Fig. 1) and infrequently from the heaters (at tap 3). Pipe segments, which were sampled at larger intervals, were replaced and the day of sampling was recorded. Substances accumulated at the inside surface were collected from the pipe segments with sterile cotton swabs. The entire inner surface of the collected segment was thoroughly swept with two to three swabs, which subsequently were placed in 10 ml of autoclaved tap water. Application of a series of four low-energy sonications of 2 min each in separate 10 ml volumes, using a waterbath, yielded 40 ml of a suspension for chemical and bacteriological analysis.

On day 1 (April) each heater and pipe combination of the MWW system was inoculated with about 10 ml

Table 1
Quality characteristics of the locally available tap water (routine-monitoring data of a 1-year period)

Parameter	Units	Average value
pH		7.9
Conductivity	mS/m	38.8
CO_2	mg/l	6.5
HCO_3	mg/l	257
CO_3^{2-}	mg/l	<2
Chloride	mg/l	9.4
Sulphate	mg/l	<1
Sodium	mg/l	12.8
Potassium	mg/l	0.94
Calcium	mg/l	71.5
Magnesium	mg/l	5.8
Total hardness	mmol/l	2.0
Nitrate–N	mg/l	0.14
Ammonia–N	mg/l	<0.03
Dissolved organic carbon (DOC)	mg/l	2.0
Iron	mg/l	0.03
Manganese	mg/l	0.02

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