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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.

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

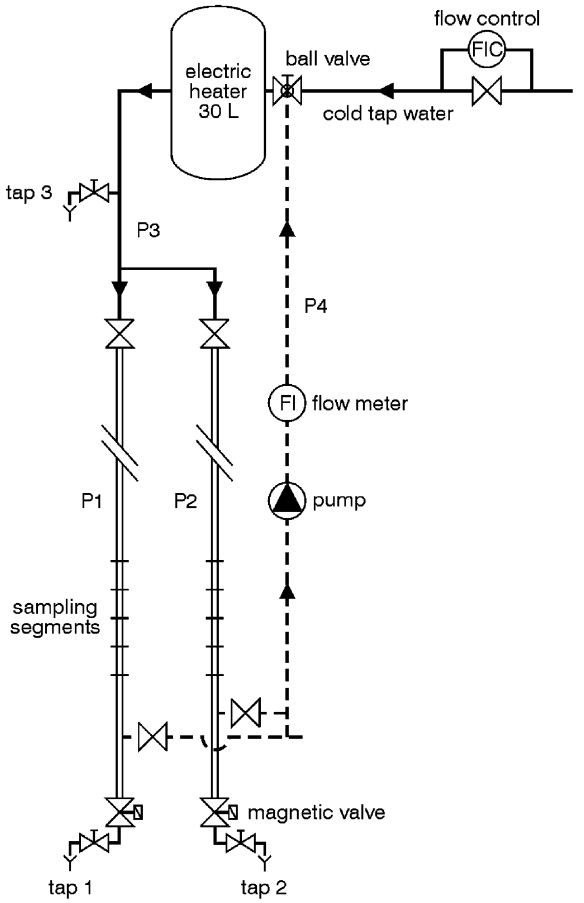

Fig. 1. Scheme (not to scale) of the experimental warm water (MWW) system. P1 and P2, pipes of the test material. P3, PVC-C pipe for the supply of warm water; P4, PVC-C pipe for recirculation; Tap 1 and Tap 2, sampling points for collecting water from the pipes; Tap 3, heater outlet and sampling point for collecting water from the heater.

of a mixed microbial community, including *Legionella pneumophila* grown in water in the presence of pieces of PEX (van der Kooij et al., 2002). The warm water was recirculated in the loops to enable these micro-organisms to multiply in the system. Inoculation was repeated on days 18 and 28 with water from an industrial warm water system. Low concentrations of *Legionella* (50–150 CFU/l) were observed in water from the MWW system for the first time on day 32. Water was replaced on days 37, 64 and 71 and domestic water use was started on day 80. From day 210 on water temperature in the heaters was raised to 70 °C for 30 min two times each week to prevent multiplication of *Legionella*. This hot water was discharged through the outlet pipe via tap 3 and only warm (37 °C) water was supplied to the pipes of the test materials.

2.2. Analytical methods

Adenosine triphosphate (ATP) is an energy-rich compound, which is present in all living organisms. ATP analysis is based on extraction of the compound from biomass, using a nucleotide-releasing agent, followed by the light generating Luciferine–Luciferase reaction. The generated light signal is measured as Relative Light Units, (RLU) after a 2-s delay time and a 10-s integration time with a luminometer (Celsis). The concentration of ATP is calculated from the RLU values using a conversion factor determined in calibration measurements. In all analysis total ATP was determined. Suspensions of accumulated substances obtained from pipe segments were 10-fold diluted prior to ATP analysis to eliminate the toxic effects of copper.

Heterotrophic plate counts (HPC). HPC values were determined with the streak-plate method using R2A medium (Reasoner and Geldreich, 1985). Volumes of 0.05 ml were spread on triplicate plates and colonies were counted after 10 days of incubation at 25 °C.

Legionella concentration. Tap water volumes of 500 ml were membrane filtered (0.4 μm pores), bacteria were resuspended and colony counts of *Legionella* water were determined by spreading volumes of 0.1 ml on plates of BCYE agar with antibiotics which were incubated at 37 °C for 7 days (Edelstein, 1981; NEN 6265, 1991). Suspensions of biomass removed from materials were directly used. Typical colonies were counted and subsequently confirmed using BCYE agar without cysteïne. Serogroup identification was done with commercially available test kits (Oxoid).

Chemical analysis. Concentrations in water of dissolved organic carbon (DOC), copper, iron and manganese were determined following standardised (ISO) procedures. Water temperature was determined with a calibrated digital thermometer and pH values were determined in accordance with standardised procedures.

Assimilable organic carbon (AOC). The AOC concentration in water was determined with growth measurements of two bacterial pure cultures in pasteurised water samples (600 ml) as described elsewhere (van der Kooij, 1992). The AOC concentration is expressed as acetate-carbon equivalents using the yields of the test strains for acetate.

3. Results

3.1. Start up phase of the MWW system

Water temperature in the recirculation stage (79 days) was between 25 and 35 °C (median 30 °C). Values of pH were 8.1 ± 0.3 (Cu), 7.8 ± 0.2 (SS), and 7.9 ± 0.3 (PEX). The Cu content in the water reached levels of 2–4 mg/l in

the system with the Cu pipes and a level of 0.07–0.2 mg/l in the systems with SS and PEX pipes. Concentrations of ATP in the water sampled from the systems in the recirculation phase reached levels of about 500 ng/l within a few days and dropped to a level of about 20–50 ng/l after 2 weeks. Subsequently, a gradual increase was observed to values of about 300–400 ng ATP/l with SS and PEX after 50 days and about 40 ng/l in the Cu pipes. Median values for ATP concentrations in water in the recirculation period were 30 ng/l (Cu), 50 ng/l (SS) and 60 ng/l (PEX), respectively. HPC values of 8.5×10^3 CFU/ml (Cu), 2.4×10^4 CFU/ml (SS) and 5×10^4 CFU/ml (PEX) reflected the observations with ATP. *Legionella* concentrations reached maximum levels of 2×10^5 CFU/l with Cu and SS, and 2×10^6 CFU/l with PEX. Quality parameters of water sampled from duplicate pipes showed a high degree of similarity.

The ATP concentration of the water dropped to levels <10 ng/l within 1 day after starting simulation of domestic water use on day 80 and also a 10-fold decrease of the *Legionella* concentrations was observed. On day 120 *Legionella* concentrations in the water had attained levels of about 10^4 CFU/l with all materials and further increased to a level of about 4×10^4 CFU/l with Cu and to 10^5 CFU/l with SS and PEX. Hence, domestic water use did not remove *Legionella* from the MWW systems. Water samples collected directly from the heaters showed that *Legionella* multiplied in the heaters. Raising the water temperature in the heaters to 70 °C for 60 min two times each week reduced the *Legionella* concentrations in water sampled at the heater outlets (tap 3, Fig. 1) to below the detection level (33 CFU/l).

3.2. Water quality during continued simulation of domestic water use

From day 210 to day 857, domestic water use was simulated in the MWW system in combination with raising the temperature in the heaters to 70 °C two times each week during 30 min. The temperature of the water sampled from the pipes was 33 ± 4 °C. Occasional technical (e.g. electricity) failures resulted in short periods (up to a few days) of stagnation (no water flow) in the system, viz. on days 283–287, day 357 and day 498. On day 342 (mid-March) the pipes Cu-1, SS-1 and PEX-1 were flushed with hot water (70 °C) for 30 min. On day 552, all pipes were flushed with water at 70 °C for several hours. Immediately thereafter, water was recirculated in the system at 37 °C during 14 days.

The concentration of active biomass (ATP) in the water from the pipes usually remained below 5 ng/l, but incidentally values exceeded 10 ng/l. Elevated ATP concentrations were observed after stagnation of water flow due to technical failures of the system (Fig. 2A). The median values of the ATP concentrations were

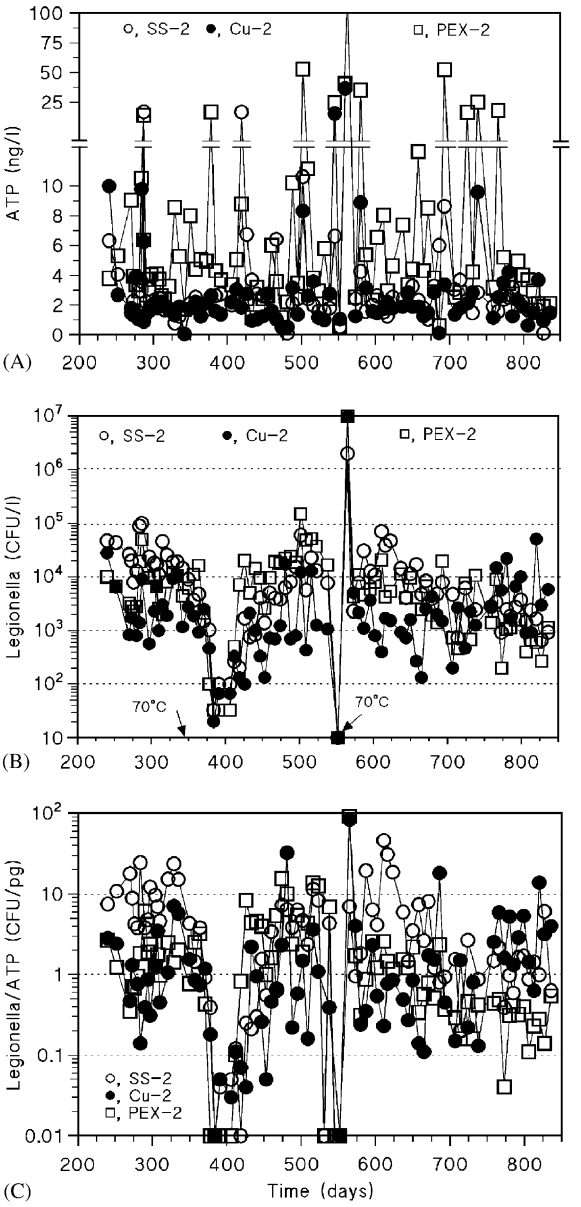

Fig. 2. Concentrations of ATP (A), *Legionella* (B), the *Legionella* to ATP ratio (C) in water collected from the pipes of stainless steel, Cu and PEX, respectively in the period with simulation of domestic water use. On day #342 the odd numbered pipes were flushed with hot (70 °C) water for 30 min. All pipes were flushed with hot (70 °C) water on day 552. *Legionella* concentrations exceeding 10^7 CFU/l were observed in some pipes on day 565 (B).

2.1 ng/l (Cu pipes), 2.5 ng/l (SS) and 4.5 ng/l (PEX) with high similarities for values of duplicate pipes (Table 2). Statistical analysis (of variance) revealed that the ATP concentrations in water from the pipes were significantly different from each other ($p<0.05$) for the tested materials.

Table 2
Statistics of the concentrations of biomass and Legionella in the water collected from the MWW system

Material-pipe nr	Biomass (ng ATP/l)[a]			*Legionella* (CFU/l)[b]			*Legionella*/ATP (CFU/pg)		
	P10	Med	P90	P10	Med	P90	P10	P50	P90
Cu–1	1.1	2.1	5.6	33	1665	5330	0.02	0.7	3.1
Cu–2	1.1	1.8	5.9	133	1400	10800	0.07	0.8	5.2
SS–1	1.3	2.5	6.3	<33	2000	22000	0.01	0.6	11
SS–2	1.3	2.4	6.4	665	5700	26300	0.05	2.7	15
PEX—1	2.2	4.5	20.0	70	2870	17000	0.01	0.8	3.1
PEX–2	2.2	4.1	17.0	400	5930	21000	0.04	1.0	5.4

[a] $n = 83$.
[b] $n = 80$ (Cu-1), 79 (Cu-2); 77 (PEX-1); 80 (PEX-2); 80 (SS).

HPC values in the water from the pipes were determined with a low frequency ($n = 8$) and the median values for the three different materials were 1.0×10^3 CFU/ml (Cu), 1.6×10^3 CFU/ml (SS) and 4.9×10^3 CFU/ml (PEX), respectively. These observations confirmed the ranking as observed with the ATP values.

The continuing presence of *Legionella* in the water demonstrated the ability of this organism to multiply in the pipes under the conditions of domestic use (each day 70–120 volume replacements). The *Legionella* concentration ranged from values below the detection limit (33 CFU/l) to values over 10^4 CFU/l with all materials (Fig. 2B). In the course of the investigation, the *Legionella* colonies changed from spherical to flat, with more than 99% flat colonies at the end of the experiment. The spherical and flat colonies were identified as *L. pneumophila* serogroups 1 and 6, respectively. No *Legionella* (<33 CFU/l) were observed directly after flushing the pipes Cu-1, SS-1 and PEX-1 with water at 70 °C on day 342. Low and increasing numbers were observed after 2 months (PEX), 3 months (Cu) and 4 months (SS), respectively and the levels before heating were attained on day 447 (PEX), 488 (Cu) and day 502 (SS), respectively. On day 378 (end of April), concentrations of *Legionella* in the water from the even numbered pipes declined to low numbers for unknown reasons but increased again within 6 weeks (Fig. 2B).

Peak concentrations of *Legionella* ranging from 1.3×10^6 to 2.7×10^7 CFU/l were observed in water sampled from the pipes after heating at 70 °C on day 552 followed by recirculation for 2 weeks (Fig. 2B). In these samples also elevated ATP concentrations (73–160 ng/l for Cu, 170 ng/l for SS, and 300 ng/l for PEX) were observed. Within 1 week after continuation of domestic use concentrations of *Legionella* and ATP declined to levels observed before heating.

Legionella concentrations in water from the SS and PEX pipes periodically were more than 10 times higher than those from the Cu pipes. After about 760 days, *Legionella* concentrations were at the same level for all materials. Statistics including median values of the *Legionella* concentrations in water from the pipes are given in Table 2. Analysis of variance revealed that over a 2-year period only *Legionella* concentrations in water from the Cu pipes were significantly different ($p < 0.05$) from those in the water from the other pipes (SS and PEX).

The *Legionella* to ATP ratio in water usually varied from 0.1 to 10 CFU/pg ATP. The median value of the *Legionella* to ATP ratio was slightly below 1 CFU/pg ATP in all but one pipe (Table 2). Maximum values of about 100 CFU/pg were observed during recirculation following heating on day 552 (Fig. 2C).

AOC concentrations of cold tap water were 2–6 μg C/l, but water collected from the heater outlets showed elevated AOC concentrations (up to 16 μg C/l). This increase was largely due to an increase of compounds available to test strain *P. fluorescens* P17 (Fig. 3). AOC concentrations in water sampled from the SS pipes were similar to those in the cold tap water, indicating AOC uptake during pipe passage. Storage of tap water at 37 °C for 16 h in a thoroughly cleaned Erlenmeyer flask (TWS) did not affect the AOC concentration.

3.3. Biofilm formation

Concentrations of biomass on the inner surface of pipe segments sampled at the end or shortly after the recycling period were about 500–600 pg ATP/cm^2 for the different materials. This level was maintained on the pipes of Cu and SS, but higher levels developed on PEX following the introduction of domestic water use (Fig. 4A). Biofilm concentrations on pieces cut from the pipes on days 542 (only Cu) and 857 confirmed the effects of the pipe materials on biofilm development. For

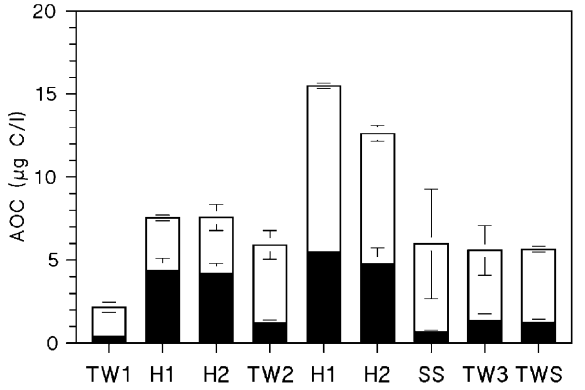

Fig. 3. AOC concentrations (with standard deviations) in cold tap water (TW), in water from two heaters (H), in water after passage of SS pipes (SS), and tap water after 16 h storage at 37 °C (TWS). Black bar represent AOC-fraction available to strain P17; open bar, strain NOX. Samples were collected on day 597 (TW1, H1, H2), day 650 (TW2, H1, H2, SS) and day 665 (TW3, TWS), respectively.

PEX a biofilm formation rate (BFR) of 2.8 ± 0.4 ($r^2 = 0.86$) pg ATP/(cm^2 d) was calculated. The concentrations of attached biomass were much higher than the concentrations in the water from the pipes. Indicative values for these ratios (as calculated from the median values presented in Tables 2 and 3) were 260 (SS), 320 (Cu) and 430 (PEX) cm^{-1}, respectively. On day 700, segments were cut from the PVC-C pipes connecting the heater with the pipes of the selected materials. Biofilm concentrations on these materials were low (125 ± 65 pg ATP/cm^2), demonstrating the effect of periodic flushing of these pipes with hot (70 °C) water. On day 829, samples were collected from the inner surface of the electric heater, to which the SS pipes were connected. Biofilm concentrations ranged from 1900 pg ATP/cm^2 at the top and middle to 2800 pg ATP/cm^2 at the lower part of the wall. Obviously, periodic heating to 70 °C did not prevent biofilm formation in the heaters.

HPC values as determined in a number of biofilm samples were 4.4 ± 0.2 log 10 (Cu), 4.2 ± 0.4 log 10 (SS) and 4.7 ± 0.6 log 10 (PEX) CFU/cm^2 and only with PEX values exceeding 10^5 CFU/cm^2 were observed. These values generally confirmed the differences in biofilm concentrations on the materials as observed with ATP, but no correlation was observed between CFU values and ATP concentrations. *Legionella* concentrations in the biofilms depended on the material type. The lowest values were observed with Cu and the highest values with PEX (Fig. 4B). *Legionella* concentrations increased on the Cu segments with increasing exposure time (Fig. 4B). On day 857, *Legionella* concentrations in pieces cut from the pipes were at the same range for the different materials. The median values for the *Legionella*

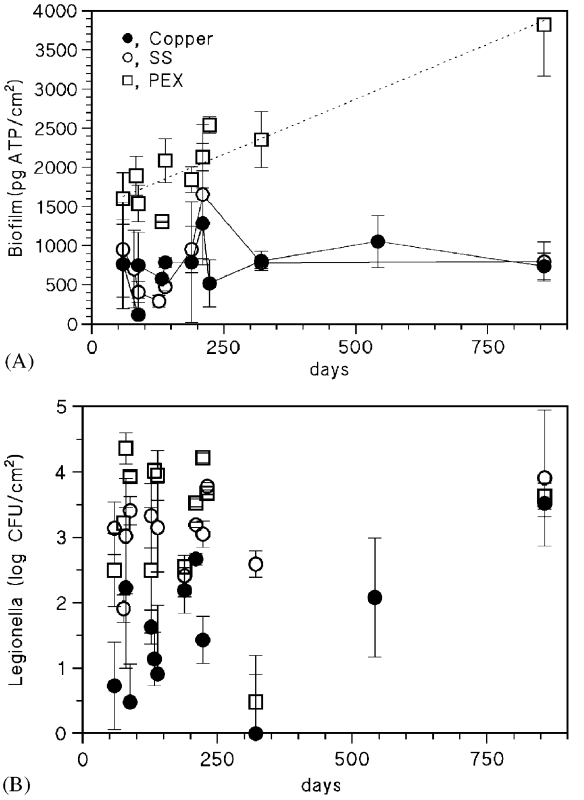

Fig. 4. Concentrations (averages with standard deviations) of biomass (A) and *Legionella* (B) on pipe segments in relation to exposure time in the MWW system during simulation of domestic water use. On days 532 and 857, samples were cut from the pipes.

to ATP ratio in the biofilm resembled those observed in water from SS and PEX, but a much lower value was obtained with Cu (Fig. 3).

Legionella was not observed (<1 CFU/cm^2) on the PVC-C pipe segments collected on day 700. Also no *Legionella* was detected on the inner wall of the heater (day 829).

3.4. Copper, iron and manganese

The concentration of Cu in the cold tap water in most cases was <10 µg/l. The Cu concentration in the warm water from the SS and PEX pipes usually ranged from 30 to 40 µg/l, indicating that some copper was released by the heating coil. Cu concentrations in the warm water from the Cu pipes ranged from 200 to 700 µg/l (average 370 µg/l). Cu compounds on the surface of copper pipe segments initially accumulated linearly with time but the concentrations on pipes pieces tested on days 542 and 857 suggest a decreasing accumulation rate (Fig. 5) Cu concentrations remained below 10 mg/m^2 in the SS and

Table 3
Statistics of the concentrations of biomass and Legionella in the biofilm on the materials in the MWW system

Pipe material	Biomass[a] (pg ATP/cm^2)			*Legionella*[a] (CFU/cm^2)			*Legionella*/ATP (CFU/pg)		
	P10	Med	P90	P10	Med	P90	P10	P50	P90
Cu	310	720	1520	1	40	2500	0.001	0.09	2.9
SS	340	635	1405	150	1020	8220	0.14	1.4	21
PEX	725	1810	2615	65	3700	15225	0.05	2.1	7.9

[a] $n = 47$ (Cu); 41 (SS); 41 (PEX).

PEX pipes. Concentrations of iron and manganese on the inner surface of the pipes increased linearly with time, with iron accumulation rates of 0.09 (on Cu), 0.13 (on SS) and 0.17 (on PEX) mg Fe/(m^2 d), respectively. Accumulation of manganese occurred at lower rates.

4. Discussion

4.1. Effect of water and materials on biomass production

Biomass production in the Cu pipes confirms other studies demonstrating biofilm development on Cu surfaces in contact with water (Tuschewitzki, 1990; Rogers et al., 1994). Biofilm formation in the pipes of Cu and SS in the MWW system, which was operated for more than 2 years, was caused by the presence of biodegradable compounds in the water. The low AOC concentration (<10 μg C/l) of the tap water increased upon passage of the heater, but storage of water in an Erlenmeyer at 37 °C did not cause such an increase (cf. Fig. 3). The increase of the P17 fraction indicates that biomass components were introduced. This AOC increase in the heater may be related to the presence of a biofilm (1900–2800 pg ATP/cm^2) on the inner surface of the heater and periodic heating to 70 °C. The hydraulic load of the pipe surface as calculated from the exposed surface areas of the pipes (2400 cm^2 for Cu, 2960 cm^2 for SS and 2220 cm^2 for PEX) and the daily flow through each pipe (81 litre) was 33 (Cu), 27 (SS) and 37 (PEX) cm^3/cm^2 d). Obviously, these water volumes provided sufficient AOC to support biofilm formation.

ATP concentrations of the water from the Cu and SS pipes were relatively low as compared to values observed in drinking water from 240 treatment plants (Van der Kooij, 2003b). The significantly elevated ATP concentrations in the water from the PEX pipes and in the biofilm on this material show that PEX enhanced biomass production for a period of more than 2 years (Fig. 4A). This observation may be explained by the release of organic compounds from PEX, as has been demonstrated with chemical analysis (Skjevrak et al., 2003). The high ATP concentrations of the water in all pipes after 14 days of recirculation following heating to 70 °C, with the highest concentrations in the PEX pipes (300 ng/l) suggest that biodegradable compounds had been liberated from the accumulated biofilms.

Median biofilm concentrations of 0.15 μg C/cm^2 (on Cu and SS) and 0.25 μg C/cm^2 (PEX), respectively, were calculated by using an ATP to biomass C ratio of 250 (Karl, 1980). These values and the values expressed as pg ATP/cm^2 are similar to those reported for water distribution systems (van der Kooij et al., 1999; Niquette et al., 2001). The logarithmic mean of the HPC values in the biofilms on Cu, SS, and PEX, were all below 10^5 CFU/cm^2. These values are relatively low in comparison to those observed in other model systems (10^5–10^6 CFU/cm^2, Volk and LeChevallier, 1999; Rogers et al., 1994). However, effects of population composition on the culturability hamper comparisons on the basis of HPC values. The ATP concentrations as observed in water collected from the pipes enable estimations of the Biomass Release Rate (BRR, pg ATP/cm^2 d). Median BRR values are estimated at about 60–70 pg ATP/(cm^2 d) for Cu and SS and 150–160 pg ATP/(cm^2 d) for PEX. From these BRR values and the median values of the biofilm concentrations (Table 3) estimations of the average net exponential growth rates of the biofilms are obtained (0.08–0.1 d^{-1}; doubling time: 7–9 days). These estimated doubling times are close to values observed in other experimental systems and in practice (Pedersen, 1990; Niquette et al., 2001; Boe-Hansen et al., 2002).

4.2. Multiplication of legionella

Legionella multiplied in the biofilms in the MWW system. The delay of growth in the start up phase may have been caused by the absence of biofilms, possibly in combination with elevated concentrations of Cu originating from the new heating coils and the pipes. The *Legionella* concentrations on the Cu pipe segments exposed for less than 250 days to warm water in the MWW system were much lower than those on the segments of SS and PEX (Fig. 4B), thus confirming earlier studies on the effect of Cu (Rogers et al., 1994). However, biofilm concentrations in Cu pipes were

similar to those in the SS pipes and therefore it is postulated that the community of these Cu tolerant microorganisms is an unfavourable environment for the multiplication of *Legionella*. After about 2 years of operation *Legionella* concentrations in water from the Cu pipes were at the same level as those in the SS and PEX pipes (Fig. 2B). Moreover, relatively high *Legionella* concentrations were observed on samples cut from the Cu pipes on day 857. These observations suggest that the inhibitory effect of Cu on *Legionella* had disappeared as a result of accumulating corrosion products (Cu carbonates and hydroxides) covering the metallic surface (cf. Fig. 5). Hence, the *Legionella* concentrations observed on the Cu segments within 250 days of exposure do not reflect the concentrations in the Cu pipes at longer exposure time, because replacement of cleaned segments amplified the effects of Cu on *Legionella*.

Legionella concentrations in water from hot water systems in buildings generally are below 10^5 CFU/l and concentrations exceeding 10^6 CFU/l have only incidentally been reported (Lück et al., 1993; Zacheus and Martikainen, 1994; Pringler et al., 2002). In this study, *Legionella* concentrations were above 10^5 CFU/l in a few samples from the MWW system (Fig. 2B). Values above 10^6 CFU/l were observed in the PEX pipes (2×10^6 CFU/l) in the initial recirculation phase. During recirculation following heating to 70 °C at day 552 concentrations even exceeded 10^7 CFU/l in the water in the PEX pipes and in one of the Cu pipes. Hence, the highest *Legionella* concentrations were observed under conditions that favoured a rapid increase of the biomass concentration.

The *Legionella* concentrations in water sampled from the pipes represent the concentration in the water from the system, because most (39 of 47) released volumes were 11 or less. Hence, estimations of the Legionella Release Rate (LRR, CFU/cm^2 d) can be obtained from the statistics of the *Legionella* concentrations shown in Table 3. Estimations for median LRR values of the even numbered pipes range from about 150 (Cu) to 500 CFU/(cm^2 d) for SS and PEX. P90 values were about 3–5 times higher. Estimations of the net growth rates of *Legionella* in the biofilm, as derived from these LRR values, range from 0.17 d^{-1} (PEX) to 0.5 d^{-1} (SS) (doubling times: 1.4–4 days). No estimation is made for the growth rate on Cu because *Legionella* concentrations on the reused pipe segments do not represent concentrations after prolonged exposure time.

The *Legionella* concentrations in the water from the pipes showed much larger variations than the ATP concentrations, resulting in strongly fluctuating *Legionella* to ATP ratios (Fig. 2C). The highest *Legionella* to ATP ratios (up to 100 CFU/pg) were observed after periods of rapid biomass production, i.e. after water replacement in the recirculation phase and during recirculation following heating of the pipes to 70 °C. Consequently, the relatively strong growth of *Legionella* in these situations is related to the increase of the biomass concentration combined with an increase of the *Legionella* to ATP ratio. Such conditions also exist directly after incubation of tap water samples in a batch

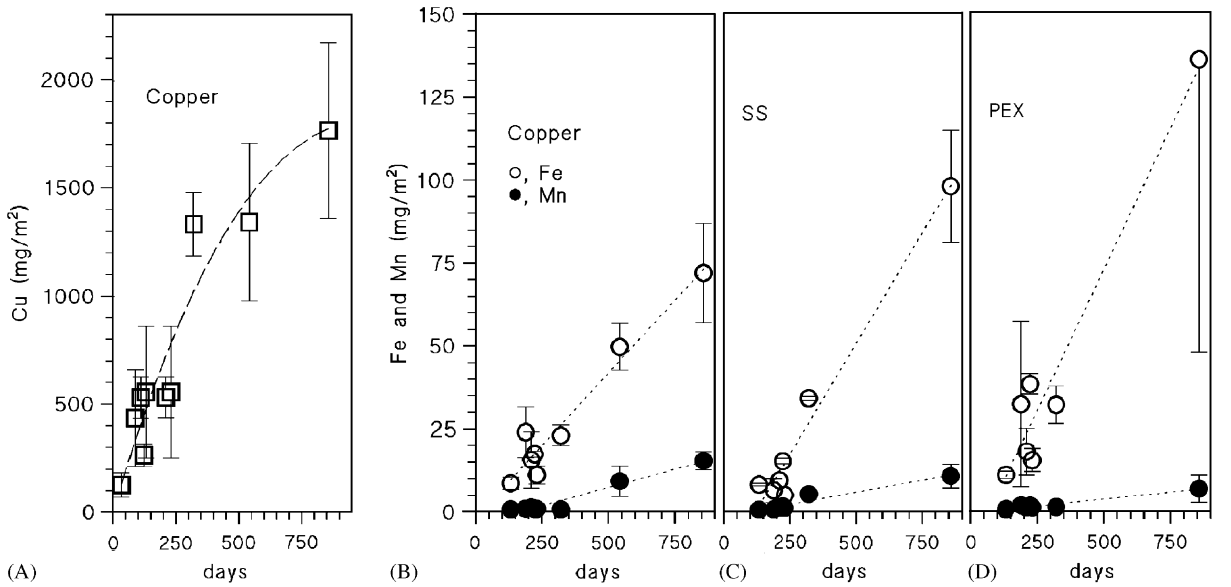

Fig. 5. Concentrations of iron, manganese and copper on the pipe wall as a function of time. (A) Copper in copper pipes, (B) Fe and Mn in copper pipes; (C) Fe and Mn in stainless steel pipes; (D) Fe and Mn in PEX pipes.

test (Yee and Wadowsky, 1982). These observations and the need of protozoa for proliferation of *Legionella* suggest that situations with a rapid production of attached biomass enhance the growth of protozoa serving as hosts for *Legionella* (Wadowsky et al., 1988; Murga et al., 2001; Kuiper et al., 2004). The median ('normal') values of the *Legionella* to ATP ratios in the situation with domestic use were about 1 CFU/pg ATP in water and slightly higher on the materials (Tables 2 and 3). The *Legionella* to ATP ratio in water may have been affected by the reduced recovery of the membrane filtration procedure applied for concentrating *Legionella* from water. This recovery is estimated at about 30% based on a number of samples, which were spread directly on the plates (results not shown).

The observations show that up to a 10,000-fold increase of the *Legionella* concentration may occur in water installations within a short period of time. The high values rapidly declined during normal water use and regular or incidental monitoring sampling may not reveal the elevated values of *Legionella* (or ATP) occurring under specific conditions in water installations. *Legionella* concentrations in the samples from the MWW system also declined when water volumes larger than the pipe volume (about 1 l) were collected from the system. A 10-fold decline was observed within 10 min at a flow of 4 l/min (data not shown). These observations may partly explain the lack of correlation between observed concentrations of *Legionella* in water systems and cases of legionellosis (Kool, 2000).

5. Conclusions

The following conclusions can be drawn from the presented study:

(1) *Legionella* can survive and multiply at biofilm concentrations as low as about 500 pg ATP/cm^2 (<0.12 μg C/m^2) in pipes at a high rate of volume replacement and water temperatures ranging from 30 to 37 °C.
(2) Tap water with a low AOC concentration (<10 μg C/l), after passage of an electric heater, promoted sufficient biofilm formation in the pipes of SS and Cu to support the growth of *Legionella*. Heater passage (37 °C) caused elevated AOC concentrations.
(3) Biofilm formation on Cu surfaces was similar to those on SS surfaces, but significantly lower concentrations of *Legionella* were observed in water from the Cu pipes and on Cu surfaces. After about 2 years of operation also the *Legionella* concentrations were similar to those observed with the SS pipes.
(4) The concentrations of attached and suspended biomass (ATP) in the PEX pipes were 2–3-fold higher than those in the SS pipes, but under the experimental conditions no significant differences were observed between the *Legionella* concentrations in water from these pipes during domestic use. The long-term effect of PEX on biomass production remains unclear.
(5) Conditions promoting a rapid biomass development caused a large increase (about 100-fold) of the *Legionella* to ATP ratio, thus resulting in strongly elevated concentrations of *Legionella* in the water. Median *Legionella* to ATP ratios during normal operation were about 1–3 CFU/pg ATP.
(6) Incidentally elevated *Legionella* concentrations may remain undetected at a low monitoring frequency.
(7) Limiting the growth potential of *Legionella* in installations for warm tap water requires a combination of structural measures, viz. reduction of the biofilm formation potential of water and materials and prevention of a rapid biomass development.

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