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Formation of natural biofilms during chlorine dioxide and u.v. disinfection in a public drinking water distribution system

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ABSTRACT

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Aims: The influence of two disinfection techniques on natural biofilm development during drinking water treatment and subsequent distribution is compared with regard to the supply of a high-quality drinking water.

Methods and Results: The growth of biofilms was studied using the biofilm device technique in a real public technical drinking water asset. Different pipe materials which are commonly used in drinking water facilities (hardened polyethylene, polyvinyl chloride, steel and copper) were used as substrates for biofilm formation. Apart from young biofilms, several months old biofilms were compared in terms of material dependence, biomass and physiological state. Vital staining of biofilms with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and the DNA-specific 4′,6-diamidino-2-phenylindole (DAPI) staining resulted in a significant difference in physiological behaviour of biofilm populations depending on the disinfection technique. Compared with chlorine dioxide disinfection (0·12–0·16 mg l^{-1}), the respiratory activities of the micro-organisms were increased on all materials during u.v. disinfection (u.v.$_{254}$; 400 J m^{-2}). The biofilm biocoenosis was analysed by *in situ* hybridization with labelled oligonucleotides specific for some subclasses of *Proteobacteria*. Using PCR and additional hybridization techniques, the biofilms were also tested for the presence of *Legionella* spp., atypical mycobacteria and enterococci. The results of the molecular-biological experiments in combination with cultivation tests showed that enterococci were able to pass the u.v. disinfection barrier and persist in biofilms of the distribution system, but not after chlorine dioxide disinfection.

Conclusions: The results indicated that bacteria are able to regenerate and proliferate more effectively after u.v. irradiation at the waterworks, and chlorine dioxide disinfection appears to be more applicative to maintain a biological stable drinking water.

Significance and Impact of the Study: As far as the application of u.v. disinfection is used for conditioning of critical water sources for drinking water, the efficiency of u.v. irradiation in natural systems should reach a high standard to avoid adverse impacts on human health.

Keywords: biofilms, disinfection techniques, enterococci, respiratory activity.

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INTRODUCTION

Biofilms are the product of adhesion and growth of micro-organisms on surfaces. On the one hand, biofilms act as biological filters by mineralizing biologically degradable material from the water and forming locally immobilized biomass. On the other hand, biofilms may unpredictably emerge in the distribution system and may cause diverse problems in terms of bacterial contamination with hygienically relevant bacteria or spontaneous increases of bacterial cell counts in bulk water. Thus, they represent an important factor for the stability of drinking waters. The drinking water distribution net offers a very large surface area for the adhesion of bacteria. At least 95% of the total bacterial biomass in drinking water are found on the walls of the distribution system (Flemming 1998). In oligotrophic compartments, such as drinking water, metabolic activities of the adherent microbial populations are increased in comparison with planktonic cells (Miettinen *et al.* 1996; Kalmbach *et al.* 1997). Nevertheless, a significant biofilm growth also takes place in drinking water systems and offers some advantages for the micro-organisms. Bacteria in biofilms are often described to be more resistant against disinfection agents like chlorine than planktonic micro-organisms. Several mechanisms explain this biofilm resistance to biocides. For large molecules, the exopolymer matrix of biofilms restrict their diffusion and bind antimicrobials (Hoyle *et al.* 1990; Lewis 2001). The negatively charged exopolysaccharides are also efficient in protecting cells from positively charged biocides by restricting their permeation through binding. Additionally, enzymes destroying incoming antibiotics together with the effective synergy between the outer membrane and multi-drug resistance pumps complete bacterial resistance mechanisms in biofilms (Maira-Litrán *et al.* 2000a,b). Alternatively, a small portion of cells (persisters) could survive the common causes of cell death by the induction of quiescence in certain biofilm pockets. Such quiescent cells are noted for their resistance to biocides. With their dormancy broken, post-treatment is possible by the replenished supply of nutrients caused by the death of the majority of the cells (Lewis 2001).

Many water supplies distribute drinking water with a disinfectant residue having the main purpose of limiting re-growth. Chlorination is the most widespread technique, but chlorine dioxide is also commonly used in the USA and European countries (van der Kooij *et al.* 1999). Chemical disinfection is based on unspecific oxidative processes that inactivate micro-organisms. This technique is described to be effective against freely suspended Gram-negative bacteria and viruses. However, a disinfectant residue does not protect the distribution system from recontamination with polluted water or micro-organisms attached to particles. Chemical disinfectants may also entail the formation of unwanted by-products and the use of chlorine dioxide results in the presence of chlorate and chlorite, which may be tasted and smelled. Therefore, application of u.v. disinfection is a promising alternative for drinking water conditioning and widely accepted for the disinfection of potable water, process water and wastewater. The u.v. irradiation damages living cells by direct or indirect changes of the DNA, such as photodimerization between adjacent pyrimidine bases, photohydratization of cytosine, and some inter- and intra-strand-specific cross links (Harris *et al.* 1987; Giese and Darby 2000). The induction of specific DNA damage correlates with distinct wavelengths which are absorbed by the DNA. Gene material is ideally changed by u.v. irradiation in such a way that growth cannot take place any longer. The wavelength region of 250–280 nm (predominantly 254 nm) can be applied in practice for the disinfection, especially of planktonic bacteria (Giese and Darby 2000). If the u.v.-induced DNA damages are not strong enough to kill bacteria, certain repair mechanisms could be activated to restore the injuries. These repair systems are widely spread in the bacterial world and include light-dependent activities, but also dark repair mechanisms (Sommer *et al.* 2000).

Planktonic systems were used extensively for dose-response measurements, but do not yield any information about biofilms (Sommer *et al.* 2000). Because of the importance of biofilms, knowledge of their response to disinfection exposure is critical for predictions of process efficiency. Thus, the incubation of platelets at representative sites allows to periodically examine the adhering biofilms. In the present work, the development of such biofilms in drinking water distribution nets is used to investigate the kinetics and physiological state under the influence of different disinfection techniques.

MATERIALS AND METHODS

Physical system

The installation of biofilm devices was fundamental for this study (Manz *et al.* 1993). The main item of these devices is a hollow stainless steel cylindrical element (260 mm in length and 150 mm in diameter), into which 15 stainless steel bolts holding platelets of test materials can be screwed. Each test material platelet ($15 \times 30 \times 2{\cdot}5$ mm) is attached to the end of a bolt with a small screw and serves as adhesion area for biofilms. No organic adhesives influencing the bacterial growth are used to fix the coupons. Perforated plates just behind the inlet and in front of the outlet of the device provide for a uniform distribution of water flow.

To study the natural formation of biofilms, two biofilm devices were installed at different sampling points downstream of an embankment filtration waterworks. The two

devices were installed in house branch connections of the distribution system (D1 and D2) and were completely full of drinking water at work. The platelets were positioned in an asymetrical manner around the device. Stop tabs up- and downstream of the devices allowed the removal of the platelets. Both sampling points were supplied with drinking water from the waterworks mentioned above and located at a distance of 1–2 km from the plant.

The raw water was conditioned by sand filtration, granular activated-carbon filtration, and finally disinfected by chlorine dioxide in the first investigation period and by u.v. irradiation in a second phase. Chlorine dioxide was added at a concentration of 0·12–0·16 mg l^{-1} according to the German Drinking Water Regulations (Anon. 2001). The conditioned water contained 0·05–0·11 mg l^{-1} of chlorine dioxide residues. The dissolved organic carbon content of the disinfected drinking water during both test phases was about 1·4 mg l^{-1} (data of the municipal drinking water supplier).

The influence of chlorine dioxide disinfection on biofilm formation was investigated from 1995 to 1997. In November 1997, the waterworks completely switched to u.v. disinfection. The biofilm analyses were performed up to the end of 1999. For biofilm sampling, all kinds of platelets were incubated for short-term periods of 4 weeks (12–14 times for each sampling point) and additionally for long-term periods of 6 months (six times for each sampling point). Incubation time of the platelets was shortened to 1 week only for population analysis via *in situ* hybridization. Previous experiments with drinking water biofilms had demonstrated that longer incubation times resulted in weak hybridization signals (Schwartz *et al.* 1998a).

The following materials which meet all standard requirements for drinking water pipelines and are widely used in drinking water facilities were tested: (i) hardened polyethylene (PE-HD, MW > 200·000), (ii) hardened polyvinyl chloride (PVC), (iii) copper and (iv) stainless steel.

All materials were incubated in parallel for biofilm formation and three platelets of each material at least were tested each time a sample was taken.

Vital and DNA staining

Vital staining is based on the intracellular enzymatic reduction of the tetrazolium salt 5-cyano-2,3-ditolyl tetrazolium chloride (CTC; Polyscience, Eppelheim, Germany) to red fluorescent, water-insoluble formazan crystals (Schwartz *et al.* 1998a; Lisle *et al.* 1999). For maximum enumeration of respiring bacteria, the CTC solution was freshly prepared at a concentration of 5 mM with pyruvic acid being added (2 mM). It was necessary to start the incubation of the platelets immediately after removal from the devices (D1 and D2). After a 3-h incubation period in darkness and at room temperature, the stained cells could be studied under the microscope. Because of the autofluorescence of the plastic materials tested, the biofilms were scraped from the platelets using a sterile cell scraper (Nunc, Wiesbaden, Germany), suspended, and homogenized in the native water by intensive vortex treatment (3 min). To control the complete removal of bacteria, microscopic analysis of DAPI (4′,6-diamidino-2-phenylindole; Merck, Darmstadt, Germany) stained material platelets was performed. The sample of the final cell suspension was filtered through a polycarbonate 0·2-μm-pore-size membrane filter (25 mm in diameter; Nucleopore, Tübingen, Germany). The filter was covered with 0·5 ml DAPI solution (1 μg ml^{-1}) in the filter funnel apparatus (Sartorius, Gröttingen, Germany). The DAPI solution was removed by applying vacuum to the filter funnel apparatus. Then, the filter was rinsed with sterile water, removed, air-dried, embedded with Citifluor (Citifluor Ltd, London, UK) on a slide and analysed by epifluorescence microscopy. To quantify the total and vital cell counts, the same microscopic fields were analysed with different filter combinations. Bacteria on the filters were examined by epifluorescence microscopy with a magnification of 1000 (Axioplan; Zeiss, Oberkochen, Germany) using light filters for DAPI (BP 365, FT 395, LP 397) or CTC and Cy3 (BP 546, FT 580, LP 590). For statistical evaluation, the number of cells observed in 10 microscopic ocular grid fields per sample were counted. The number of cells per cm^2 testing material was calculated from the average number of cells per ocular grid (0·0156 mm^2), the filter area, and the filtered sample volume.

Cultivation of bacteria

Bacteria were removed from the platelets surface with a sterile scraper and pooled in a total volume of 10 ml water. The heterotrophic plate count (HPC) of bacteria was evaluated by plating serial dilutions of the obtained bacterial suspension on R2A agar (Difco) (Reasoner and Geldreich 1985). Plate counts were enumerated after 5 days of incubation at 20°C. In parallel, enterococci in biofilms were cultivated on Slanetz and Bartley agar (Oxoid) and subsequently identified using a commercial test kit (BioMérieux, Nürtingen, Germany).

In situ hybridization with fluorescent-labelled oligonucleotide probes

Following the removal of the test platelets, the biofilms on the surfaces were fixed immediately with 50% (v/v) ethanol for 30 min. The biofilms were scraped from the surface after fixation, and the solution was filtered through a polycarbonate membrane. All oligonucleotide probes labelled at the 5′-end with Cy3 (Cyanine dye; Biological Detection Systems, Pittsburgh, PA, USA) were purchased from MWG-

Biotech (Ebersberg, Germany). The sequences were based on the data published by Amann *et al.* (1995) and Manz *et al.* (1995). The following oligonucleotides were complementary to 16S or 23S rRNA sequences of the different subclasses of the *Proteobacteria*: (i) β-42a (5′-GCCTTCCC-ACTTCGTTT-3′) and γ-42a (5′-GCCTTCCCACATC-GTTT-3′) are complementary to regions of the 23S rRNA and (ii) α-1b (5′-CGTTCGYTCTGAGCCAG-3′) is complementary to a region of the 16S rRNA. The hybridizing reaction was performed with a 50 ng oligonucleotide probe in a moisture chamber at 37°C for 2 h according to Schwartz *et al.* (1998a). For β and γ subclasses, the formamide content of the hybridization buffer was 35% and for α subclass 20% formamide was used. The hybridization buffer contained NaCl (0·9 M), Tris/HCl (pH 7·2, 10 mM) and SDS (0·1%). The hybridized filters were then washed with the hybridization buffer without probe for 25 min at 37°C and 25 min at 42°C. In addition, the bacteria on the filter were stained with DAPI (1 μg ml^{-1}) for 10 min, dried and embedded in Citifluor for microscopic analysis.

PCR and Southern blot hybridization

The biofilms were scraped from the platelets and suspended in autochthonous water. Aliquots of these samples were centrifuged at 15 000 × *g* for 15 min. The pellet was re-suspended in 10 μl sterile water for PCR (Seradest Labwater, Giershahn, Germany). Aliquots of the scraped biofilms were used for PCR using primer combinations specific for 23S or 16S rRNA-related sequences of *Eubacteria*.

A primer combination for an unspecific amplification of 23S rRNA-related DNA fragments of *Eubacteria* was used for PCR detection of enterococci. The primer and probe sequences were based on published data (Amann *et al.* 1995; Manz *et al.* 1995; Frahm *et al.* 1998). For PCR the HotStar Polymerase (Qiagen, Hilden, Germany) was used. Aliquots of the PCR solution were put on a 1% agarose gel and separated by electrophoresis. The DNA molecules were stained with ethidium bromide to verify PCR and transferred to nylon membranes (Qiagen). The DNA was cross-linked with the membranes by u.v. irradiation for 3 min. The filters were hybridized with 15 pmol of an oligonucleotide probe specific for *Legionella* spp. (5′-CTG-GTGTTCCTTCCGATC-3′), faecal streptococci (*E. faecalis:* 5′-TAGGTGTTGTTAGCATTTCG-3′), *E. faecium* (5′-CACACAATCGTAACATCCTA-3′) and *E. gallinarum* (5′-CACAACTGTGTAACATCC-3′) or a nonspecific probe for *Eubacteria* as a control. All probes were labelled at the 3′-end of the sequence with digoxigenin by a terminal transferase reaction (Roche Biochemical Diagnostics, Mannheim, Germany). Oligonucleotide probes hybridized with specific target DNA molecules were visualized by a digoxigenin-specific antibody conjugated with alkaline phosphatase. The bound probe was detected by a chemiluminescence reaction according to the manufacturer's instruction (Roche Biochemical Diagnostics) using CSPD (di-sodium-3-[4-methoxyspiro1, 2-dioxethane-3,2′-(5′-chloro)-tricyclo-[3·3·1·13·7]decan-4-yl] phenyl phosphate) as substrate and the LumiImager workstation.

Atypical mycobacteria were detected according to Schwartz *et al.* (1998b) and based on the amplification of specific 16S rRNA-related DNA sequences. In detail, a genus-specific PCR was performed using the *Eubacteria*-specific Eub338R primer (5′-ACTCCTACGGGAGG-CAGC-3′) together with the mycobacteria-specific primer Myc1 (5′-AAGGAAGGAAACCCACAC-3′). PCR was followed by a Southern blot hybridization with the labelled Myc3 probe (5′-TTCACGAACAACGCGACAA-3′) specific for the internal target region of the amplified PCR amplicon. Another PCR system was applied for the specific detection of facultatively pathogenic, atypical mycobacteria like *Mycobacterium avium, M. intracellulare, M. kansasii* or *M. szulgai*. This PCR system is based on the primer combination of Eub338R and Myc2 (5′-CCACCTACCGT-CAATC-3′). The unspecific probe for *Eubacteria* was hybridized with all samples blotted on the membrane and used as a positive control with regard to the hybridization and detection reactions.

RESULTS

Total and respiratory active cell counts during chemical and u.v. disinfection

The total cell count measured by DAPI staining as well as the CTC reduction activities were used to illustrate the material dependency of cell growth and cell activities after different exposure times (4 weeks and 6 months). The results of short-term incubation were based on five to seven independent experiments for each sampling point (D1/D2) during the two different disinfection periods. Long-term incubation experiments were repeated three times during both chlorine dioxide disinfection and u.v. disinfection.

The enumeration and statistical calculation of the bacterial densities in biofilms from different materials suggested a slight material-dependent growth on plastic after the short incubation period during chlorine dioxide disinfection (Table 1). The average metabolic activity was determined to be 24% of the total cell count using PE-HD, PVC and steel coupons. On copper, the smallest bacterial adhesion and vitality was measured.

After 6 months of incubation of the different platelets in the drinking water distribution system, the total cell count increased only slightly on all materials (Table 1). Even on copper, a biofilm was formed, which reached a cell density of $3{\cdot}3 \times 10^6$ bacteria per cm^2. This density was equivalent to

Table 1 Comparison of short- and longtime exposure of coupons concerning DAPI and vital staining, and HPC of biofilms after chlorine dioxide disinfection

	House branch connections within the distribution system							
	Incubation (4 weeks)				Incubation (6 months)			
Detection method	PE-HD	PVC	Steel	Copper	PE-HD	PVC	Steel	Copper
Total bacterial cell count of the biofilms (DAPI)*	$1{\cdot}3 \times 10^6$ ($\pm 2{\cdot}7 \times 10^5$)	$1{\cdot}1 \times 10^6$ ($\pm 1{\cdot}7 \times 10^5$)	$0{\cdot}7 \times 10^6$ ($\pm 1{\cdot}5 \times 10^5$)	$0{\cdot}16 \times 10^6$ ($\pm 2{\cdot}8 \times 10^4$)	$4{\cdot}6 \times 10^6$ ($\pm 1{\cdot}1 \times 10^6$)	$1{\cdot}4 \times 10^6$ ($\pm 2{\cdot}5 \times 10^5$)	$7{\cdot}5 \times 10^6$ ($\pm 2{\cdot}5 \times 10^5$)	$3{\cdot}3 \times 10^6$ ($\pm 7{\cdot}2 \times 10^5$)
Respiratory activity of the biofilms (CTC)†,‡	25 (±6·2) ($3{\cdot}25 \times 10^5$)	28 (±7·9) ($3{\cdot}0 \times 10^5$)	20 (±5·2) ($1{\cdot}4 \times 10^5$)	4 (±1·0) ($6{\cdot}4 \times 10^3$)	15 (±3·4) ($6{\cdot}9 \times 10^5$)	20 (±4·2) ($2{\cdot}8 \times 10^5$)	21 (±6·2) ($1{\cdot}6 \times 10^6$)	12 (±2·5) ($4{\cdot}0 \times 10^5$)
CFU cm^{-2} on R2A agar§	$2{\cdot}0 \times 10^5$	$1{\cdot}5 \times 10^5$	$1{\cdot}8 \times 10^5$	$0{\cdot}02 \times 10^5$	$2{\cdot}3 \times 10^5$	$2{\cdot}3 \times 10^5$	$0{\cdot}5 \times 10^5$	$0{\cdot}014 \times 10^5$

*Mean values from D1 and D2 (given in cells per cm^2) ± S.E.

†All values are percentages of the total cell counts determined by DAPI ± S.E.

‡Mean values of respiratory active cells are given in brackets.

§Mean values of heterotrophic plate count (HPC); the standard errors were measured with ±20–30%.

Table 2 Comparison of short- and longtime exposure of coupons concerning DAPI and vital staining, and HPC of biofilms after u.v. disinfection

	House branch connections within the distribution system							
	Incubation (4 weeks)				Incubation (6 months)			
Detection method	PE-HD	PVC	Steel	Copper	PE-HD	PVC	Steel	Copper
Total bacterial cell count of the biofilms (DAPI)*	$7{\cdot}4 \times 10^6$ ($\pm 3{\cdot}9 \times 10^5$)	$7{\cdot}0 \times 10^6$ ($\pm 2{\cdot}5 \times 10^5$)	$7{\cdot}5 \times 10^6$ ($\pm 2{\cdot}5 \times 10^5$)	$3{\cdot}1 \times 10^6$ ($\pm 3{\cdot}8 \times 10^4$)	$7{\cdot}5 \times 10^6$ ($\pm 1{\cdot}8 \times 10^6$)	$6{\cdot}7 \times 10^6$ ($\pm 1{\cdot}5 \times 10^6$)	$2{\cdot}0 \times 10^6$ ($\pm 3{\cdot}2 \times 10^5$)	$0{\cdot}7 \times 10^6$ ($\pm 1{\cdot}7 \times 10^5$)
Respiratory activity of the biofilms (CTC)†,‡	72 (±16) ($5{\cdot}3 \times 10^6$)	80 (±25) ($5{\cdot}6 \times 10^6$)	76 (±25) ($5{\cdot}7 \times 10^6$)	10 (±1·0) ($3{\cdot}1 \times 10^5$)	47 (±13) ($3{\cdot}5 \times 10^5$)	42 (±14) ($2{\cdot}8 \times 10^6$)	30 (±6·7) ($6{\cdot}0 \times 10^5$)	25 (±5·0) ($1{\cdot}8 \times 10^5$)
CFU cm^{-2} on R2A agar§	$1{\cdot}8 \times 10^5$	$8{\cdot}4 \times 10^5$	$7{\cdot}6 \times 10^5$	$0{\cdot}23 \times 10^5$	$0{\cdot}7 \times 10^5$	$2{\cdot}2 \times 10^5$	$2{\cdot}5 \times 10^5$	$0{\cdot}37 \times 10^5$

*Mean values from D1 and D2 (given in cells per cm^2) ± S.E.

†All values are percentages of the total cell counts determined by DAPI ± S.E.

‡Mean values of respiratory active cells are given in brackets.

§Mean values of heterotrophic plate counts (HPC); the standard errors were measured with ±20–30%.

that of the other materials. Concerning the respiratory activities of the biofilms, the average values ranged from 12% of total cell counts in biofilms on copper to 21% of total cell counts of biofilms on steel.

Biofilm formation was analysed after the change of the disinfection technique at the waterworks in order to study the influence of u.v. disinfection on the biofilm microorganisms within a real drinking water distribution system.

No initial material dependency could be observed after 4 weeks of incubation. The average of the total cell count on PE-HD, PVC and steel was 9–15% higher than the counts of biofilms investigated after chlorine dioxide disinfection (Table 2). However, statistical calculation revealed that the average values reflecting the standard errors were similar to the results of biofilm analysis during chemical disinfection (Table 1). Concerning the metabolic activities in these biofilms, the differences were even more significant. After the short incubation period (4 weeks), metabolically active microbes on PE-HD, PVC and steel reached 70–80% of all bacteria. Even on copper, this proportion amounted to 19%. After 6 months of incubation, these high counts for metabolic activities in biofilms decreased, but still were twice as high as the values obtained during chlorine dioxide disinfection (Table 2).

The increased metabolic activities in biofilms after 4 weeks and 6 months of incubation during u.v. disinfection made us decide to perform additional experiments after 3 months of incubation. The results measured for the total cell number of the plastic materials and steel were found to be equivalent to the short-term incubation experiments. However, with copper, bacterial adhesion was measured to increase by about 50% to $6{\cdot}3 \times 10^6$ bacteria per cm^2. The metabolic activities during u.v. disinfection reached percentages of 75–90% for plastics, 60% for steel and 36% for copper. The data of 3 months were averages of both sampling points D1 and D2, and represented the results of two independent samplings during u.v. disinfection at waterworks.

Table 3 *In situ* hybridization for population analysis of biofilms with specific probes for *Proteobacteria* after UV and chloride dioxid disinfection

	House branch connections within the distribution system					
	Chlorine dioxide disinfection			Ultraviolet disinfection		
Detection method	PE-HD	PVC	Steel	PE-HD	PVC	Steel
Total bacterial cell count of the biofilms (DAPI)*	$6{\cdot}9 \times 10^5$ ($\pm 1{\cdot}4 \times 10^5$)	$7{\cdot}2 \times 10^5$ ($\pm 1{\cdot}8 \times 10^5$)	$3{\cdot}2 \times 10^5$ ($\pm 7{\cdot}2 \times 10^4$)	$4{\cdot}5 \times 10^6$ ($\pm 1{\cdot}0 \times 10^6$)	$5{\cdot}7 \times 10^6$ ($\pm 8{\cdot}5 \times 10^5$)	$3{\cdot}0 \times 10^6$ ($\pm 8{\cdot}0 \times 10^5$)
In situ hybridization†						
α-1b	<10	15	18	<10	20	15
β-42a	30	30	42	38	36	38
γ-42a	25	27	20	31	23	20

*Mean values (given in cells per cm^2) ± S.E.

†All values are percentages of total cell counts determined by DAPI staining.

CFU and population analysis of pipe material biofilms

Bacteria from the platelets were spread on R2A plates to estimate the culturable proportions of bacteria in biofilms. Results of the HPC were smaller than the total cell counts found by the DAPI-staining technique by about one order of magnitude (Tables 1 and 2). The HPC remained almost constant on all materials during chlorine dioxide disinfection and u.v. disinfection. Differences which might have been induced by the adjacent disinfection technique were mostly within the statistical variability range. Although the metabolic activities of bacteria in biofilms increased during u.v. disinfection, this result was not clearly demonstrated by the cultivation experiments.

Bacterial communities of biofilms from the early stage (1–4 weeks old) of development were analysed by *in situ* hybridization with probes specific for *Proteobacteria* subclasses (Table 3). As the biofilm layers were very thin, the bacteria from at least two platelets of each material were combined to increase the microscope detection limit and enhance statistical evaluation. Several months old biofilms could not be analysed with *in situ* hybridization because of the low hybridization signal during epifluorescence microscopy. The average total cell counts of all tested materials (PE-HD, PVC and steel) was found to be $5{\cdot}7 \times 10^5$ ($\pm 2{\cdot}2 \times 10^5$) bacteria per cm^2 on the polycarbonate membrane used for *in situ* experiments during chlorine dioxide disinfection. The total cell counts on the membranes reached an average of $4{\cdot}4 \times 10^6$ ($\pm 1{\cdot}3 \times 10^6$) bacteria per cm^2 during u.v. treatment. Copper was not used for *in situ* hybridization.

The percentages of the three subclasses of *Proteobacteria* are also shown in Table 3. The β and γ species in particular were found to be the predominant subclasses of *Proteobacteria* colonizing the tested materials in the house branch connections. Bacteria of the α subclass were detected in a much lower percentage. The standard errors of the *in situ* hybridization results, which were calculated from at least 10 ocular field grids of the microscope, were found to reach 35% at the maximum of the shown subclass-specific percentages (Table 3). In general, the hybridizing results did not reveal any differences of the percentage ratio of the α, β and γ subclasses of the *Proteobacteria*, which was caused by the disinfection technique used.

Hygienically relevant bacteria

Molecular-biological methods were employed for the detection of enterococci, *Legionella* spp. and atypical mycobacteria. Table 4 illustrates the occurrence of *Legionella* spp., faecal streptococci, atypical mycobacteria and *Eubacteria* as a positive control.

Biofilms were screened for the existence of enterococci as organisms indicating insufficient drinking water conditioning. Both molecular-biological methods based on the 23S rRNA sequence detection (Frahm *et al.* 1998) as well as cultivation methods were used. Enterococci were never found at the sampling points D1 and D2 after chemical disinfection. However, they were detected at D2 after disinfection with u.v. irradiation (Table 4). A total of eight samplings for detection of enterococci were performed during u.v. disinfection. Five samples distributed over time were found to be positive concerning the molecular biological detection of enterococci, whereas these microorganisms were only detected in biofilms from plastic material. The molecular-biological results were confirmed by cultivation experiments using selective media (Slanetz and Bartley medium; Oxoid) for enterococci in three independent samples. The isolates were identified as *Enterococcus faecium* using the rapid ID 32 STREP identification system (BioMérieux). Neither enterococci nor enterococci-specific sequences could be isolated or detected in bulk water at this sampling point. The cultivation experiments for enterococci were performed in accordance with the German Drinking Water Regulation (Anon. 2001).

Table 4 Molecular biological detection of hygienically relevant bacteria in biofilms

	D1				D2			
Assay for	PE-HD	PVC	Steel	Copper	PE-HD	PVC	Steel	Copper
Chlorine dioxide disinfection								
Legionella spp.	+	++	+	–	+	+	+	+
Enterococci	–	–	–	–	–	–	–	–
Atypical mycobacteria	+	++	+	–	+	++	+	+
Eubacteria	++	++	++	++	++	++	++	++
Ultraviolet disinfection								
Legionella spp.	+	+	+	–	+	++	+	+
Enterococci	–	–	–	–	++	+	–	–
Atypical mycobacteria	+	–	+	–	+	++	–	+
Eubacteria	++	++	++	++	++	++	++	++

Enterococci, *Legionella* spp., atypical mycobacteria and *Eubacteria* were detected by PCR and Southern blot hybridization. *Eubacteria* detection was used as positive control. Strong signals are indicated by (++), weak signals are indicated by (+) and no signal is described by (–).

Water samples (100 ml) were filtered on a membrane and subsequently incubated on an enterococci-selective agar medium.

In agreement with previous results (Schwartz *et al.* 1998a), *Legionella* spp. was found in nearly all biofilms, independently of the kind of disinfection. Because of the specificity of the hybridization probe used (Manz *et al.* 1995), it was not possible to specify the *Legionella* species present in the biofilms. Cultivation experiments for *Legionella* on a BCYE-α synthetic medium (Merck), which is especially used for the cultivation of *Legionella pneumophila*, did not reveal any *Legionella*-specific colony growth.

Atypical mycobacteria sequences were detected in biofilms after both chemical and u.v. disinfection. In all cases, only saprophytic, nonpathogenic atypical mycobacteria could be detected by molecular-biological and cultivation experiments.

No molecular-biological signals specific for both *Legionella* and atypical mycobacteria could be detected in bulk water samples.

DISCUSSION

The biological stability of water in distribution systems is a major concern of drinking water conditioning. Biodegradable dissolved organic carbons that are not retained by conditioning procedures are one factor that contributes to biofilm formation. The efficiency of disinfection is also important to reduce the total bacterial cell counts in bulk water and to avoid contamination of drinking water with pathogenic or facultative pathogenic micro-organisms.

Within the framework of the recent study, biofilms from a real public drinking water distribution system were analysed to show the influences of different disinfection techniques on the natural biofilm formation. The development, bacterial metabolic activities and phylogenetic diversity of biofilms on PE-HD, PVC, steel and copper were analysed at different sampling points of house branch connections. It was not the major concern of this study to investigate material dependencies. However, material influences were to be excluded, when differences occurred because of the disinfection applied. Additionally, no significant changes in water temperature or seasonal fluctuations were observed during the time period. Compared with many investigations in bench scale units, it was an advantage to perform biofilm investigations in a real technical drinking water asset that conditioned river water to drinking water after bank filtration. The disinfection technique of the municipal waterworks subjected to testing changed from chlorine dioxide to u.v. disinfection during the period of study. In spite of this, the chemical parameters of the conditioned drinking water remained constant during the whole investigation period. Even the dissolved organic carbon (DOC) did not change and was measured to be 1·4 mg l^{-1} as a matter of routine by the municipal drinking water supplier. Disinfection with chlorine dioxide followed the German Drinking Water Regulation (0·05–0·1 mg l^{-1}). However, biofilm formation was found to be related to the depletion of the residual disinfectant concentration (Momba *et al.* 1998). In this respect, residual chlorine dioxide concentrations were measured in the conditioned drinking water at the outlet of the waterworks as mentioned above, but no residues of the disinfectant were found at the sampling point within the distribution system (private communication of the local drinking water supplier). The results demonstrated that the metabolic activities of the biofilm bacteria were increased after u.v. disinfection compared with chlorine dioxide disinfection, whereas the total bacteria densities of the

biofilms did not vary significantly. Even on copper, a well-developed biofilm formed after several months of incubation. Re-growth of bacteria in the distribution system could be associated with the carbon fines from the granular activated-carbon filters at the waterworks, which may contribute to the colonization of the platelets with bacteria. Previous studies showed that bacteria associated with carbon fines were carried through the disinfection barrier without injuries (Servais *et al.* 1992; Morin *et al.* 1996). Therefore, these particles attach to pipelines of the distribution system and could be an important factor of biofilm re-growth. Apart from these studies, a passage of organisms not associated with carbon fines through the treatment is possible. In fact, these observations explained the re-growth of biofilms, but did not reflect the metabolic differences during the two different disinfection periods.

The total cell count together with vital staining reflected the changes in the biofilms more distinctly than the HPC. One of the applications of the HPC is that of monitoring the reductions that occur in the bacterial load of the water as it passes through the various unit processes of drinking water treatment (Reasoner 1990). Very little information is available on disinfection of the general heterotrophic bacterial population in water; most disinfection studies have focused on specific microbial pathogens or on the coliform group of bacteria, but did not reflect the metabolic activities of natural biocoenosis (Momba *et al.* 2000). Therefore, this study noted a lack of relationship between CTC and plate counts. It may hence be concluded that plate counts are problematic. A number of items have also been considered in the application of the CTC method, such as toxicity (1–20 mM), temperature (Choi *et al.* 1999), effect of inorganic constituents and pH (Lisle *et al.* 1999) as well as growth phase (Sherr *et al.* 1999). In fact, all these parameters did not change during both periods of disinfection and, thus, did not cause any increased metabolic activities in biofilms during u.v. disinfection. As a consequence, it has to be speculated that the disinfection methods used at the waterworks gave rise to the differences observed in biofilms after u.v. treatment.

According to German regulations, an u.v. irradiation of 400 J m^{-2} is considered necessary to obtain a safe and reliable disinfection in water treatment. In this case, drinking water-relevant pathogens (bacteria and viruses) are supposed to be reduced by 4 logs (killed or inactivated) of vegetative bacteria, viruses, and fungi at least, the latter being far more sensitive to u.v. than bacterial spores and parasites. Selected bacteria were used in most of the investigations to optimize the disinfection technique. Natural variation of planktonic and adherent microbes in response to u.v. irradiation was not taken into consideration.

However, vegetative bacteria can repair DNA damages caused by u.v. irradiation, but not damages caused by chemical disinfectants, like chlorine dioxide, because of their high numbers of target molecules. Injured, but not dead micro-organisms can use two different repair mechanisms to restore u.v.-mediated DNA alterations. One is activated by light (Schoenen and Kolch 1992; Tosa and Hirata 1999), while the other also works in the dark (Sommer *et al.* 2000). As far as the dark activation of repair mechanisms is concerned, control methods are not described by regulations, although these processes might play a very important role in pipelines. Bacteria use at least three different dark-activated repair mechanisms, which are regulated by the expression of the *rec*A gene. The RecA protein is found in all bacterial genomes and possesses a highly conserved amino acid sequence. As a consequence, RecA-dependent repair mechanisms can be induced by u.v. light in most aquatic bacteria, if the u.v.-mediated DNA damages are not irreversible. Only a few publications are available on both dark repair and photoreactivation (Sommer *et al.* 2000). They focus on selected pathogens, such as *Escherichia coli* O157 and other pathogenic *E. coli* strains, and state that an irradiation intensity of 400 J m^{-2} is sufficient to inactivate pathogenic *E. coli*. Still, microbial response to disinfectants in natural communities and the consequences in terms of re-growth and biofilm formation are not well understood. Thus, it is critical to extrapolate the natural bacterial behaviour in processes using data from selected micro-organisms investigated in bench scale units.

Another explanation of the increased metabolic activities in biofilms after u.v. treatment is based on the availability of nutrients for bacterial re-growth. Here, various types of disinfection could influence the content and accessibility of dissolved organic nutrients. It is not well understood, to which extent chemical disinfectants may alter dissolved organic fractions like DOC or cell components (proteins, polysaccharides, etc.) released into the water after cell lysis. The u.v. irradiation at a wavelength commonly used for drinking water disinfection ($u.v._{254}$) is known to convert organically combined phosphorous to orthophosphate and to degrade natural organic matter. It could be shown that $u.v._{254}$ dose (15–50 mW s cm^{-2}) used in waterworks reduced the concentration of assimilable organic carbon and the sum of the molecular size fraction. The release of microbially available phosphorous needed higher doses (204 mW s cm^{-2}) of $u.v._{254}$ radiation (Lehtola *et al.* 2003). In consequence, the increased metabolic biofilm activities during u.v. disinfection did not result from higher amounts of fraction of bioavailable dissolved organic carbons or nutrients in conditioned drinking water. Hygienically relevant bacteria, such as enterococci, could be detected by molecular-biological methods, which did not consider the state of their viability in biofilms after u.v. disinfection. However, viable adhesive enterococci were also found

sporadically at the sampling points downstream of the waterworks during u.v. disinfection only. The occurrence of enterococci in drinking water biofilms suggested that a recurrent passage of hygienically relevant bacteria through the u.v. barrier might be possible. Furthermore, an increased bioavailability of nutrients during u.v. disinfection as mentioned above could support the re-growth of bacteria which survived disinfection or were resident in biofilms within the distribution net in low cell numbers.

Legionella spp. as well as atypical saprophytic mycobacteria were detected in most of the investigated biofilms of the drinking water independently of the disinfection technique used. Both are described to find their natural habitats in surface waters, which indicates that they might have been transferred to the distribution system via embankment filtration and conditioning steps (Lye *et al.* 1997; Schwartz *et al.* 1998b). *Legionella* spp. are described to be adapted to warm water systems, but Lye *et al.* (1997) also detected *Legionella* in significant amounts in groundwater samples and potable water supplies. Here, *Legionella* could reach a density percentage of about 10%, which was confirmed by former investigations (Schwartz *et al.* 1998a). Concerning its nutrient supply, *Legionella* spp. depends on a symbiotic life together with other bacteria and protozoa (Rogers *et al.* 1993). Therefore, life in community with other micro-organisms in biofilms seems to promote their growth. The symbiotic lifestyle of these bacteria could be responsible for the inefficiency of disinfection and their subsequent occurrence in drinking water biofilms. Their intracellular occurrence in protozoa, for example, could provide a selective protection against different disinfection measures. Moreover, other killed micro-organisms in biofilms could serve as a nutrient source.

For atypical mycobacteria, water plays an important role as the natural habitat and source of transmission of these bacteria (Neumann *et al.* 1997; Schwartz *et al.* 1998b). Apart from harmless saprophytes, some facultatively pathogenic atypical mycobacteria, such as *M. avium, M. kansasii* or *M. szulgai*, have been isolated from water samples. Infections caused by atypical mycobacteria increased significantly during the earlier decades. Human immunodeficiency virus patients, other immuno-deficient people, and children during their first decade of life were mostly affected by the micro-organisms (Falkinham 1996). Many atypical mycobacteria were found to be resistant against chemical disinfectants and able to pass this barrier of the drinking water treatment process without any injury (Pelletier *et al.* 1988; Russell 1995; Schwartz *et al.* 1998b). Within the framework of this study, only harmless mycobacteria were identified in drinking water biofilms, but it cannot be excluded that also facultatively pathogenic mycobacteria could be transferred from surface water to drinking water. The re-growth of these micro-organisms within the distribution system could be promoted by an increase in the bioavailable fractions of the water components.

Thus, it has to be considered more generally that in addition to the metabolic activities in biofilms also the potential risk of contamination increases when disinfecting conditioned drinking water by u.v. irradiation in the waterworks investigated. Resistance to u.v.-imposed loss of viability can also be partially attributed to agglomeration during the irradiation process (Blatchley *et al.* 2001). Thus, the bacterial population may comprise two sub-populations: one with a low resistance (discrete or paired cells) and a second with a high resistance (bacterial aggregates). Population analysis with subclass specific oligonucleotides for *Proteobacteria* demonstrated that no shift within the bacterial biocoenosis occurred during the investigation periods. Similar to other studies, the β subclass bacteria were found most frequently in the drinking water biofilms (Kalmbach *et al.* 1997; Schwartz *et al.* 1998a).

Regarding the present data, induction of bacterial repair mechanisms together with changed nutrient conditions after u.v. irradiation might contribute to an increased metabolism in biofilms in the distribution system. Further investigations with natural drinking water biofilms will show to what an extent cellular repair mechanisms are induced by u.v. disinfection so as to explain more specifically the presented different implications of chlorine dioxide and u.v. disinfection on biofilm formation.

Therefore, investigations should be performed to study the extent to which natural phenomena, e.g. the turbidity of local waters and occurrence of particles, influence the u.v. intensities. Further investigations concerning the induction of DNA repair mechanisms by u.v. irradiation should be carried out to confirm the efficiency of u.v. disinfection in real drinking water conditioning facilities. As far as the application of u.v. disinfection in developing countries, where contaminated surface water is frequently used for drinking water conditioning, is concerned, the efficiency of u.v. irradiation in natural systems should reach a high standard to avoid adverse impacts on human health.

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