Influence of Materials, Water Qualities and Disinfection Methods on the Drinking Water Biofilm Community


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Abstract: The composition of biofilm populations in drinking water will mainly depend on the nutrient availability and the temperature. The nutrients may originate either from the raw water or from the installation materials. To determine the effect of drinking water pipe materials on the composition of biofilm populations, biofilms grown on different materials (EPDM materials, PEXb, PEXc and copper) were analyzed by cloning (16S rDNA) and the community fingerprinting method denaturing gradient gel electrophoresis (DGGE). Additionally the long-term effect of mechanical cleaning and disinfection with chlorine dioxide (0.2 mg/l, 24h) on the biofilm populations was tested. The biofilm populations had high diversities on growth supporting materials like EPDM and comparatively low diversity on PEX. The biofilms were dominated by Proteobacteria and the composition of the subdivisions was influenced by the applied material. Flavobacteria and members of the family Sphingomonadaceae, which are known to degrade several organic compounds, were found on EPDM materials and might be suitable as indicators for hygienical relevant materials. Comparison of biofilms previously treated with disinfectants with untreated ones by DGGE revealed a considerable population shift depending on the pipe material.

Keywords: Biofilm; community fingerprinting; disinfection; diversity; drinking water; pipe material

INTRODUCTION

Microorganisms in drinking water colonize surfaces and form biofilms, which are optimal habitats under oligotrophic conditions and offer protection against disinfection. In distribution systems, at least 95% of the total bacterial biomass is found on surfaces (Flemming, 1998). The growth of bacteria in biofilms depends on availability of nutrients (organic and inorganic), which could be allocate by the water or the materials used in the installation (Szewzyk et al., 2000). Drinking water biofilms have a great impact on public health, when they harbour hygienical relevant microorganisms, which might be released into the bulk water phase (Kilb et al., 2003, Bressler et al., 2009).

Depending on the applied installation materials and the ambient conditions, like composition of the drinking water and temperature, different biofilm communities will develop. Approx. 99.9% of the bacterial cells both in biofilms and in the water phase are not culturable on standard media (Szewzyk et al., 2000). And, about 65% of the bacteria in drinking water are in a viable but nonculturable (VBNC) state (Kalmbach et al., 1997). Therefore, the examination of the diversity and community structure of drinking water should be done with culture independent, molecular approaches (e.g. FISH, cloning and fingerprinting). Various studies of drinking water and adjoining biofilms were conducted using these methods for distribution systems (Kalmbach et al., 1997; Schwartz et al., 2003; Emtiazzi et al., 2004; Eichler et al., 2006; Poitelon et al., 2010), shower equipment (Kelley et al., 2004; Feazel et al., 2009) and disinfection methods (Schwartz et al., 2003; Emtiazzi et al., 2004; Williams et al., 2005; Eichler et al., 2006; Mathieu et al., 2009). Only few studies considered the effect of installation materials (Schwartz et al., 2003; Yu et al., 2010).

In this study the biofilm populations on different materials (elastomers, plastomers and copper), used in drinking water systems, were examined by cloning and fingerprinting. In addition it was intended to obtain first data of the long-term influence of mechanical cleaning and chlorine dioxide disinfection on biofilm populations on different materials.
METHODS
Generation of biofilm samples and sampling

Materials. Five different materials used in drinking water systems were exposed at different sites in Germany: Copper, PEXb (silane-cross-linked polyethylene), PEXc (electron-ray cross-linked polyethylene), EPDM (ethylene-propylene-diene-monomer) with (Ew) and EPDM without (Eo) KTW-C and recommendation of the worksheet W270 of the DVGW (German Technical and Scientific Association for Gas and Water).

Biofilms for cloning and sequencing. The different materials were exposed as coupons (2.5 cm x 7 cm) in a biofilm reactor, which meets the requirements of the worksheet DVGW W270 for the measurement of biofilm biomass on materials in drinking water. It consisted of an upflow stainless steel basin of 200 l volume and was continuously supplied (20 l/h on average) with drinking water. The drinking water used was purified water from the river Ruhr (“Mülheimer Verfahren”): After artificial groundwater recharge with surface water, the water is purified in a multistage chemical-biological process with ozone and biological active activated carbon filters. One biofilm reactor was connected to the drinking water installation of the IWW Centre for Water (DWI). The other biofilm reactor was installed directly after the purification (biological active activated carbon filters) before UV disinfection at the waterworks (WW) in Mülheim/Ruhr (Germany), which supply DWI with drinking water. The TOC after purification was on average 0.7 mg/l (median 2009). After 6 weeks of exposure, the coupons were sampled and stored at -80°C until further processing. Biofilms from 2 (EPDM) to 6 (PEX) coupons were scraped off on both sides and collected in PBS for DNA extraction.

Biofilms for DGGE. Biofilms were grown on tubes or pipes of the mentioned materials (length 10 m) in a pilot house installation, which was operated according to DIN 50931-1 at two different temperatures (12 and 37°C). The drinking water (0.8 mg/l DOC) was produced from reduced groundwater in the waterworks Friedrichsgabe (Stadtwerke Norderstedt, Germany). After 8 weeks the biofilms were contaminated with Pseudomonas aeruginosa AdS, Legionella pneumophila AdS and Enterobacter nimipressuralis. 7 months after contamination, the tubes and pipes were mechanically cleaned by air/water impulses (30 min, 5 bar) and chemically disinfected with chlorine dioxide (0.2 mg/l, 24 h). The samples were taken before and 3 weeks after disinfection. Biofilms were scraped off the inner surface of tube sections by shaking with SAZ-pearls (zirconium silicate ER 120 S ZrO₂ 68%, 0.3 – 0.4 mm diameter, Mühlmeier GmbH, Germany) in sterile water. 10 – 50 ml of these samples were stored at -80°C. Before DNA extraction, the samples were thawed and concentrated by centrifugation.

DNA extraction
The total DNA of the biofilm community was isolated using the FastDNA® SPIN Kit for Soil and the FastPrep® Instrument (MP Biomedicals, Santa Ana, CA, USA). The extraction was carried out according to the manufacturer’s instruction with one modification. The first centrifugation step for the separation of cell components was extended to 10 minutes.

Amplification of 16S rDNA fragments for cloning and DGGE
For cloning and sequencing the 16S rRNA gene was amplified with the universal primers 63f (5'-CAGGCTTAACACATGCAAGTC-3') and 1387r (5'-GGCGGWTGTACACAGGCG-3') designed by Marchesi et al. (1998). PCR mixtures (50 µl final volume) contained PCR buffer (50 mM KCl, 10 mM Tris/HCl (pH 9.1), 0.01% Triton-X 100, stabilizers), 200 µM of each deoxynucleotide, 2.5 mM MgCl₂, 120 nM of each primer and 0.5 U OptiTaq DNA Polymerase (Roboklon GmbH, Berlin, Germany). The PCR was performed using a Personal Cycler (Biometra, Göttingen, Germany) with an initial denaturation step for 1 min at 98°C, followed by 30-34 cycles consisting of 45 s of denaturation at 96°C, 1 min annealing at 55°C and 3 min extension at 72°C. The reaction was terminated by a final extension of 10 min at 72°C. The amount of DNA used as template was optimized until sharp bands with optimal yield were produced. The PCR products were cleaned up with the peqGOLD Cycle-Pure Kit (PEQLAB Biotechnologie GMBH, Erlangen)
prior cloning. The amplification products were checked for the correct size by gel electrophoresis in 1% agarose gel.

For DGGE (denaturing gradient gel electrophoresis) the variable V3 region of the 16S rRNA gene was amplified with the universal primers designed by Muyzer et al. (1993). The PCR is prepared as described in Roeder et al. (in press).

Cloning and sequencing
The purified PCR products of the 16S rRNA gene were cloned using the TOPO TA Cloning® Kit for Sequencing (Invitrogen, USA) with chemically competent One Shot® TOP10 cells according to the manufacturer’s specifications. Depending on the diversity determined by DGGE for each clone library, ca 11 to 60 clones were picked from LB plates containing 100 µg/ml ampicillin and grown in liquid LB medium with ampicillin (100 µg/ml). Plasmid DNA were purified using the Plasmid Mini Prep Kit EasyPrep® Pro (Biozym, Germany) for sequencing. Sequence data (Location DWI: 139 clone sequences (without PEXb), location WW: 173 clone sequences) were obtained by capillary electrophoresis (SMB Services in Molecular Biology GmbH, Germany and Macrogen Inc., Korea) under the BigDye Terminator cycling conditions (Applied Biosystems Inc., USA) with the primer 610r (5’-ACCGCGGCTG CTGGCAC-3’) (SMB GmbH) or 63f (Macrogen Inc). Each useable sequence read was approximately 440 bp (SMB GmbH) or 730 bp (Macrogen Inc), respectively.

All sequences were compared with the BLAST function (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and with EMBL-EBI Nucleotide database (http://www.ebi.ac.uk/Tools/blast2/nucleotide.html) for the detection of the closest cultivated relatives and for grouping. Each clone sequence and its closest cultivated relative were imported into the ARB software package (http://www.arb-home.de/). Sequences were aligned automatically with FastAligner and manually checked using the ARB software package. The topology of the tree was based on neighbor-joining with Jukes-Cantor correction. Bootstrapping under parsimony criteria was performed with 1000 replicates. The short clone sequences were integrated using the “Quick add Parsimony” function. The trees were rooted by using Deinococcus radiodurans as an outgroup.

Analysis of 16S rDNA fragments by DGGE
The DGGE analysis was performed as described in Roeder et al. (in press) with a 8% (w/v) polyacrylamid gel with a linear chemical denaturing gradient ranging from 40-75%.

The DGGE fingerprints were analysed with the software BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium). The band based Dice coefficient was used to calculate the similarity matrix with a position tolerance of 1%. For clustering the UPGMA (Unweighted Pair Group Method with Arithmetic mean) method was applied.

RESULTS AND DISCUSSION
Clone library analysis
Clone libraries were constructed for 6 weeks old biofilms grown on EPDM with and without recommendation, PEXb and PEXc. Depending on the expected microbial diversity on these materials, determined by DGGE, 11 to 60 clones were analysed.

The percentages of 16S rRNA gene sequences belonging to the major bacterial taxonomic groups in the clone libraries of EPDM without recommendation (left) and PEXc (right) for location DWI (top) and WW (bottom) are presented in Figure 1.

All drinking water biofilms were dominated by Proteobacteria (78.3% and higher). The compositions of the Proteobacteria subdivisions on the different materials varied. On PEXc Alphaproteobacteria dominated the sequenced clones of both locations (60-81.8%). On EPDM without recommendation (Eo) the Alphaproteobacteria constituted only 33% of cells in biofilms of the drinking water installation (DWI). In addition on this material the percentage of clones affiliated to the Gammaproteobacteria was nearly doubled (35%) in comparison to PEXc (18.2%). At location WW (treated water) 77.5% of the clone sequences obtained from Eo could be related to the
Betaproteobacteria. The proportion of Gammaprotobacteria on PEXc and Eo at WW was nearly equal (7.5-10%). On Eo the Flavobacteria appeared with a percentage of 15 to 20%.

In biofilms on PEXc in the drinking water installation (DWI) the Alphaproteobacteria could be mainly assigned to the unclassified Candidatus Reyranella massiliensis with an identity of 98-99% (Figure 2). On both EPDM materials representatives with 99-100% identity to members of the family Sphingomonadaceae (Sphingomonas sp., Sphingobium sp.) could be detected as the main Alphaproteobacteria. Also members of the family Caulobacteraceae could be determined on both EPDM materials (Caulobacter spp., 99% identity) and PEXc (Brevundimonas sp., 100% identity). The Gammaproteobacteria sequences for PEXc were found to be affiliated to Legionella rubrilucens (96%) and for Eo mainly to Pseudoxanthomonas mexicana (99%). Within the Betaproteobacteria class relatives of the members Aquabacterium, Leptothrix, Ideonella, Acidovorax and Thauera were assigned on the EPDM materials. Flavobacterium sp. were besides Pseudoxanthomonas sp. the dominating species in biofilm grown on Eo. The biofilm of EPDM with recommendation (Ew) was dominated by relatives of Aquabacterium spp. (98%) and Ideonella sp. (97-98%).

Biofilms grown in the treated water at the waterworks (WW) had different dominating representatives of the bacterial classes on the different materials (Figure 3). In the biofilm on PEXc Alphaproteobacteria of the family Caulobacteraceae could be assigned, but mainly a distantly related species to Phenyllobacterium composti (88%) was detected. The PEXb biofilm was dominated by relatives of Aquabacterium parvum (99-100%). The Alphaproteobacteria on PEXb could mainly be affiliated to Hyphomicrobium spp. (96-99%). Dominating species of biofilms of

**Figure 1:** Taxonomic abundance of 16 S rDNA sequences from each library: a EPDM without recommendation and b PEXc of location DWI (drinking water installation); c EPDM without recommendation and d PEXc of location WW (after purification at the waterworks)
Ew and Eo were assigned to Aquabacterium spp. (98%), Acidovorax spp. (99-100%) and Flavobacterium sp. (99%).

Figure 2: Phylogenetic tree of the bacterial clones and closest cultivated relatives found at location DWI (drinking water installation) on the different materials: EPDM without recommendation (Eo), EPDM with recommendation (Ew), PEXc (Pc), PEXb (Pb). Bootstrap values greater than or equal to 50% are indicated at the nodes. Numbers behind the strains are the accession numbers of GenBank [GB] or the European Bioinformatic Institute [EBI]. The clones are named in following way: Location (DWI or WW) material (Eo, Ew, Pc or Pb) – clone numbers. Scale bar indicates 10% sequence divergence. The tree was rooted by using Deinococcus radiodurans as an outgroup.

The 16S rDNA clone libraries indicated differences in the biofilm community structure for the different materials. PEXc releases only traces of organic compounds, which support thin biofilm growth. On these surfaces species will settle which have the potential to survive oligotrophic conditions. In contrast, on rubber materials like EPDM, copiotrophic species will develop. On Eo...
more Flavobacteria and Betaproteobacteria occurred than on PEXc (Figure 1). This indicates that the bacteria, which appeared in the EPDM biofilms, use the released organic compounds as substrate. Species of the genus *Flavobacterium* and members of the family Sphingomonadaceae were mainly observed on the EPDM materials. Sphingomonads isolated from various soil, water, and clinical environments, are known to degrade several organic compounds (White et al., 1996; Leys et al., 2004). Sphingomonads were also found on shower curtain and showerhead biofilms (Kelley et al., 2004; Feazel et al., 2009). Also Flavobacteria have a pronounced degradative potential (Bissett et al., 2008). Biofilm populations from the two locations were different when developing on the same material. The biofilm community on PEXc in purified artificial recharged groundwater after the biological active activated carbon filter from WW was more diverse than in the drinking water in the installation DWI. Both PEXc biofilms were dominated by Alphaproteobacteria species. Relatives of *Acidovorax* sp. were, besides *Aquabacterium spp.*, dominant in EPDM biofilms only at location WW. The genus *Acidovorax* was isolated from water, wastewater sludge, soil and also from clinical environments (Willems et al., 1990). *Aquabacterium spp.* were dominant in biofilms on Ew at both locations and on Eo and PEXb at WW. The genus *Aquabacterium* was already described as a dominant group in Berlin drinking water biofilms by Kalmbach et al. (1997, 1999) and was isolated from glass and PE slides. A similar difference in the community structure of biofilms concerning the Proteobacteria was observed for biofilms grown in groundwater, in water from the main pipes and in drinking water installations (Kalmbach et al., 1997).

**DGGE analysis of the biofilm samples**

First results for fingerprinting analysis of biofilms before and 3 weeks after a mechanical cleaning and disinfection with chlorine dioxide were obtained for different materials (Figure 4). The copper pipes in warm water had a reddish brown (cuprite, CuO₂) and in cold water a greenish (malachite, Cu₂CO₃(OH)₂) coating, which were partially detached during biofilm removal.

The fingerprints of the drinking water biofilms were very diverse depending on material, water temperature and the disinfection (Figure 4). At different water temperatures different biofilm communities developed on the same material. A relatively low diversity (number of bands) was found on the PEX materials in warm and cold water before disinfection. In contrast, on the EPDM materials a high bacterial diversity was found. Biofilms on copper had a low diversity in warm and a high diversity in cold water before disinfection.

In Figure 5 the clusters analysis of the similarities (%) of the DGGE band profiles are shown. The similarities were calculated with the band based Dice coefficient, which consider common bands in the pattern, but not the different diversities of the samples. The warm water (WW) biofilms form, except for PEXb, a cluster on the bottom of the dendrogram. The similarity in this cluster (43%) is due to the different diversities on the materials not high. Also most of cold water (CW) biofilms cluster with 38%. Here the biofilms of PEXc are separated from this cluster due the low diversity. The diversity on Ew and Eo is more comparable. They form a cluster of 70.6% in cold water. In warm water the Ew cluster with 72.7%.

The mechanical cleaning and the disinfection had different long-term effects on biofilm populations on different materials. On PEXc the similarity before and 3 weeks after disinfection was low (36.4% CW, 40% WW). Also on Ew and Eo the populations were before and three weeks after treatment very similar (>70%) except Eo in warm water (54.5%) (Figure 4). For copper, the biofilm communities (before and after disinfection) were not very similar at both temperatures (42.9% CW, 45.5% WW). In warm water the low diversity of the copper biofilm increased after disinfection; in cold water the high diversity of the copper biofilm decreased. The copper pipes had different coatings in WW and CW, which was possibly detached during the mechanical treatment. Thus the surface of copper had before and after treatment different properties for the biofilm population.
Figure 3: Phylogenetic tree of the bacterial clones and closest cultivated relatives found at location WW (after waterworks) on the different materials. Details see Figure 2.
The results of the fingerprinting of the biofilms showed a high impact of the material and temperature on the developing biofilm population. Both EPDM materials support the growth of a highly diverse biofilm population, but at the same time with high similarity between the materials. The microorganisms obviously use the leaching organic substances from the material as nutrients. The high diversity in the fingerprints indicates that various microorganisms use them as easily degradable carbon source. No matter if the recommendations of the DVGW W270 are maintained by the EPDM or not, a relative high similarity between the populations was found on these materials. With the worksheet DVGW W270 drinking water materials are tested by measuring the developed biofilm biomass. The high population similarities indicate, that in both EPDM qualities similar organic compounds were released in different proportions and that these could by used by a similar biofilm population. The utilized organic compounds could originate from additives like fatty acids, solubilizers, paraffin oils, and other compounds (Schmeisser et al., 2003). The long-term effect of the mechanical treatment and disinfection with chlorine dioxide on the biofilm population on the EPDM materials was low, because of the released organic compounds. The organic compounds had a stronger influence on the biofilm community than the disinfection. Bacteria that survived the disinfection inside the biofilm or inside amoebae and new bacteria from the water will reconstitute the biofilms with similar members and diversities.
Figure 5: Cluster analysis of the DGGE band profiles of biofilms before (untreated) and three weeks after treatment with mechanical cleaning and chemical disinfection with chlorine dioxide. The differences between the DGGE patterns are indicated by similarity percentage. The similarity is calculated with the band based Dice coefficient with 1% position tolerance and clustered with UPGMA method.

PEX materials can also leach organic compounds (mixture of phenols, quinones, antioxidants), although at low concentrations (Denberg et al., 2007). The examined PEX materials had a relative low biofilm diversity and low similarities compared to EPDM. This indicates that these materials release different organic compounds in low concentration and that these compounds are not easy degradable. On PEX materials the biofilm populations before and three weeks after disinfection were not very similar. In thin biofilms the cells are not well protected against disinfection. Development of new biofilm communities will be supported and influenced by the dead biomass, which in the beginning can be used as easily accessible carbon source.

Copper induced a very different biofilm population in warm and cold water in terms of diversity. The higher diversity in the cold water biofilm suggests that the bacteria are better protected against copper ions, when the pipes are coated with copper carbonate than with copper oxide. During the mechanical treatment the coating possibly partially detached and the new developed biofilm population was less divers. The increase of diversity in the warm water biofilm after the disinfection could be a result of remained dead cells, which could be used by various bacteria as a carbon source.

Also other studies of different pipe materials suggest that the material affect the microbial diversity in biofilms (Yu et al., 2010). There are several studies, which obtained effects of chlorination on biofilm community in drinking water and biofilms (Williams et al., 2005; Eichler et al., 2006; Mathieu et al., 2009; Poitelon et al., 2010). The disinfection with chlorine dioxide might cause a similar population shift in the proteobacteria subclasses like Mathieu et al. (2009) reported for PVC and chlorine. They observed a reversible shift to beta- and gammaproteobacteria subclasses when high chlorine residuals maintained in the water. Nevertheless the material has also a wide impact on the biofilm population and this shift could be controlled by both: disinfection method and the material. Also the origin of the raw water and disinfection treatments had a significant effect on the drinking water and biofilm community (Emtiazi et al., 2004;
Eichler et al., 2006). The biofilm community analysis with DGGE of different drinking water treatment steps at a waterworks done by Emtiliazi et al. (2004) revealed a high impact of the different process steps like UV irradiation on biofilm communities. Despite UV irradiation they detected hygienically relevant bacteria like *Legionella spp.* and sometimes *Pseudomonas aeruginosa* in the drinking water biofilms. In contrast the investigation of the influence of UV irradiation on drinking water communities in a model distribution system and the analysis of intergenic transcribed spacer (ITS) fingerprints did not show a consistent impact of UV irradiation on the biofilm community (Pozos et al., 2004). These different observations could be due either to differences in the setup between a real and a model distribution system or to the different community fingerprint methods used. Eichler et al. (2006) determined a strong influence of chlorination before distribution on bulk water communities with RNA based fingerprints. Monochloramine and chlorine residuals in drinking water were shown to affect the community composition of biofilms in a model water distribution system (Williams et al., 2005).

**CONCLUSIONS**

Different factors influence the diversity of the biofilm community in drinking water installations. Besides the condition of the drinking water and the water temperature, also the applied materials and disinfection with chlorine dioxide showed long-term effects on the community composition of drinking water biofilms. Elastomeric materials, which provide organic compounds, induced biofilms with a higher diversity and different bacterial composition compared to PEX. The biofilm population was more affected by these components than by the disinfection with chlorine dioxide. Hygienic relevant bacteria may occur and persists on elastomeric installation materials, which promote their growth, and they may be released in the bulk water phase (Kilb et al., 2003; Bressler et al., 2009). Thus growth-supporting materials like elastomers in drinking water installations pose a threat to human health.

Some bacteria like Flavobacteria and members of the family Sphingomonadaceae, known for their degradative potential, were found mainly on EPDM and not on PEX. These bacteria might be suitable as indicators for health risks associated with materials used in installation systems.

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**REFERENCES**


