Faecal indicator bacteria in river biofilms

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ABSTRACT

Biofilms in surface waters primarily consist of allochthonous microorganisms. Under conditions of pollution faecally derived bacteria may interact with these biofilms. Total coliform bacteria, *Escherichia coli* and intestinal enterococci are used to monitor source water quality, indicating faecal pollution and the possible presence of enteric pathogens. In the present study the occurrence of faecal indicators was investigated in biofilms (epilithic biofilms, sediments) of German rivers. All of the biofilms contained significant concentrations of these bacteria, which were several orders of magnitude lower compared with the total cell number and the number of culturable heterotrophic plate count bacteria indicating that faecal indicator bacteria represented a minor fraction of the whole biofilm communities. The biofilms displayed approximately two orders of magnitude higher concentrations of total coliforms, *E. coli* and enterococci compared with the overlying water. Identification of coliform and enterococcal isolates from the biofilms revealed the presence of species which are known to be opportunistic pathogens. Overall, the results of the present study show that faecal indicator bacteria can survive in the presence of high cell densities of the authochthonous microflora in epilithic biofilms and sediments, suggesting that these biofilms may act as a reservoir for bacterial pathogens in polluted rivers.

Key words | biofilms, coliforms, enterococci, *Escherichia coli*, rivers, sediments

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INTRODUCTION

Contamination of surface waters with faecally derived bacteria can occur through point sources like sewage effluents and non-point sources such as agricultural and urban run-off or waterfowl. *Escherichia coli* and intestinal enterococci represent allochthonous bacteria, which are commonly used as microbiological water quality parameters indicating faecal contamination and thus, the possible presence of enteric pathogens. Coliform bacteria other than *E. coli* can be of faecal, but also of environmental origin (Leclerc *et al.* 2001). Routine monitoring of microbiological quality of bathing waters or source waters used for drinking-water abstraction is usually limited to testing of the water phase. However, field studies and microcosm experiments in the laboratory indicate that, in addition to free cells in the bulk water, bacteria of faecal origin also occur in sediments of marine and freshwater environments (Craig *et al.* 2002*a*; Byappanahalli *et al.* 2003). Pathogenic bacteria can be released from sediments during storm and precipitation events (Craig *et al.* 2002*a*, 2004) or become resuspended during recreational activities, resulting in a rapid increase in the load of pathogens in the water phase. These organisms pose a risk of infection to humans on exposure during water-associated activities (Donovan *et al.* 2008). Because of their hygienic relevance, *E. coli* or alternatively faecal coliforms, and intestinal enterococci have been considered as target organisms in studies of the occurrence and fate of these bacteria in sediments of recreational coastal waters and freshwaters (Valiela *et al.* 1991; Craig *et al.* 2002*a*,*b*, 2004; Byappanahalli *et al.* 2003). Quantification and identification of coliform bacteria other than *E. coli* have usually not been included in these investigations. In addition, the ratio of faecal indicator

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bacteria in relation to the general autochthonous biofilm microflora has generally not been considered.

The focus of the present study was the investigation of biofilms (epilithic biofilms and sediments) in some German streams, which have not been studied before as a potential habitat of hygienically relevant bacteria. The general biofilm microflora was quantified by the determination of total cell counts and the fraction of culturable heterotrophic plate count (HPC) bacteria. To assess the occurrence and proportion of faecal indicator bacteria in these biofilms, the concentrations of total coliforms, *E. coli* and enterococci were determined. In addition, identification of coliforms and enterococci was performed in order to evaluate the presence of clinically important bacterial species among the faecal indicator organisms in the biofilms.

METHODS

Water and biofilm sampling

Ten samplings of water and biofilms were performed at different streams (River Ruhr, Moersbach, Anrathskanal; Table 1) during dry weather conditions in the urbanized and industrialized Ruhr Area (Germany). Epilithic biofilms were scraped from submerged stones; sediments were taken from the upper layers (2–3 cm) of the streambed. Water samples were collected at a distance of approximately 1 m from the river bank. The biofilm and water samples were stored in sterile glass containers under refrigeration and analyzed within 6 h of collection.

Detachment and dispersal of microorganisms

10 g of wet epilithic biofilm or sediment were added to 90 mL of filter-sterilized (0.2 μm filter pore size) water of the sampling site in a 250-mL beaker. Dispersal of epilithic biofilms was performed by treatment in a Stomacher (model 400, Seward) for 20 min at 260 rpm. Sediment suspensions were treated by sonication in an ultrasonic bath (Sonorex RK103H) for 20 min. To determine dry weight of biofilms a known wet weight of material was dried in an oven at 105°C to a constant weight.

Enumeration of microorganisms

Water samples and dispersed biofilms were decimally diluted in filter-sterilized (0.2 μm filter pore size) deionized water. Total cell counts were determined by staining of the bacteria for 20 min with 4′,6-diamidino-2-phenylindole (1.7 μg/ml) and subsequent enumeration of cells by epi-fluorescence microscopy at 1,000-fold magnification. The heterotrophic plate count (HPC) was performed by the spread plate method using R2A medium (Reasoner & Geldreich 1985); colonies were enumerated after incubation at 20°C for 7 days and colony-forming units (cfu) per 100 mL water or 100 g wet weight of biofilms were calculated. Coliform bacteria and *E. coli* were quantified, using the Colilert-18/Quanti-Tray/2000 system (IDEXX). 100-mL volumes of water and biofilm or sediment suspensions were transferred into distribution trays. After incubation at 36°C for 20 h, wells that were yellow (positive for

Table 1 | Sampling data for water and biofilms collected from different German streams

Samples	Date of sampling	Sampling location	Type of biofilm	Water temperature (°C)	pH value
E1	13/04/2004	River Ruhr	Epilithic	9.0	7.7
E2	26/04/2004	River Ruhr	Epilithic	13.2	8.3
E3	27/05/2004	River Ruhr	Epilithic	16.2	7.9
E4	12/04/2005	River Ruhr	Epilithic	9.7	8.5
E5	06/05/2005	River Ruhr	Epilithic	17.7	7.7
S1	12/05/2004	River Ruhr	Sediment	10.7	7.4
S2	07/05/2004	River Ruhr	Sediment	16.8	7.8
S3	26/04/2005	River Ruhr	Sediment	12.4	7.9
S4	10/05/2005	Anrathskanal	Sediment	9.6	8.1
S5	23/05/2005	Moersbach	Sediment	12.1	6.9

coliforms), and yellow and fluorescent upon exposure to long-wavelength UV light (positive for *E. coli*) were counted. Enterococci were quantified using the Enterolert system (IDEXX). After incubation at 41°C for 24 h, wells that fluoresced under long-wave UV light were counted. Results of coliforms, *E. coli* and enterococci were expressed as most probable number (MPN) per 100 mL water or 100 g wet weight of the biofilms. The microbiological analyses were performed in duplicate (*E. coli*, coliforms, enterococci) or triplicate (HPC) and results were given as arithmetic mean values.

Identification of bacteria

Pure cultures of coliform bacteria were prepared from wells of distribution trays of the Colilert-18/Quanti-Tray/2000 system. Aliquots of 50 μL from wells with a positive reaction for coliform bacteria and *E. coli* were subcultured on lactose TTC agar at 36°C for 24 h. Randomly chosen colonies showing a yellow colour development in the agar medium were assayed for their oxidase reaction after growth on tryptic soy agar (TSA) at 36°C for 24 h and for their indole reaction after cultivation in tryptophan broth at 44°C for 24 h according to ISO 9308-1 (2000). Identification of coliform species was performed, using the api 20 E system (bioMérieux) and GN Microplates (Biolog) according to the manufacturers' instructions. Pure cultures of enterococci were prepared from the wells of the Enterolert systems by subculturing 50 μL aliquots from fluorescent wells on kanamycine esculine azide agar at 36°C for 24 h. Presumptive colonies were randomly selected and tested for the presence of catalase and their Gram reaction after cultivation on TSA (36°C, 24 h). Identification of enterococcal species was carried out, using the commercial system rapid ID 32 strep (bioMérieux).

RESULTS AND DISCUSSION

Distribution of total cells and HPC bacteria in water and river biofilms

Ten samplings of water and biofilms (epilithic biofilms, sediments) were performed at three different German streams. The water temperatures varied between 9.0°C and 17.7°C, and the pH values between pH 6.9 and 8.5 (Table 1). The dry weight of the five epilithic biofilm and five sediment samples was 15.1% ± 9.1% and 73.8% ± 12.8% (arithmetic mean values), respectively, indicating that the main component of epilithic biofilms was water in contrast to the sediments with a significantly lowered water content.

The general microbial populations in water and biofilms were quantified by the determination of total cell counts and the fraction of culturable (HPC) bacteria. Total cell counts in the water and biofilm samples ranged from 9.9×10^7 to 2.4×10^9 cells per 100 mL (geometric mean, 3.3×10^8 cells/100 mL) and 2.2×10^{11} to 4.9×10^{12} cells per 100 g wet weight (geometric mean, 1.1×10^{12} cells/100 g), respectively (Figure 1).

The concentration of the culturable (HPC) bacteria ranged from 8.9×10^5 to 1.7×10^7 cfu per 100 mL (geometric mean, 5.9×10^6 cfu/100 mL) and 9.9×10^8 to 1.8×10^{11} cfu per 100 g wet weight (geometric mean, 3.6×10^9 cfu/100 g) in the water and biofilm samples, respectively (Figure 1). The fractions of culturable bacteria (HPC percentages of total cell counts) were 0.2 to 17.2% for water and 0.04 to 8.2% for biofilms with a trend towards slightly lower culturability of organisms in biofilms compared with the water phase.

These results indicate that the epilithic biofilms and sediments characteristically displayed high cell densities of the autochthonous bacteria compared to the overlying water. The distribution of total cells and HPC bacteria in river sediments and free surface water has usually not been considered in the literature before. One exception is a study of Griebler *et al.* (2001) who also determined total cell counts in sediments from various freshwater environments (lakes and rivers) and found high cell numbers in the sediments compared with the surrounding pore water. Possible explanations for the relatively low culturability of water and biofilm bacteria determined in the present study may be unfavourable culture conditions, the occurrence of bacteria in a viable but non-culturable state or the presence of dead cells which are still intact and contain DNA to be recognized by the microscopic cell count method.

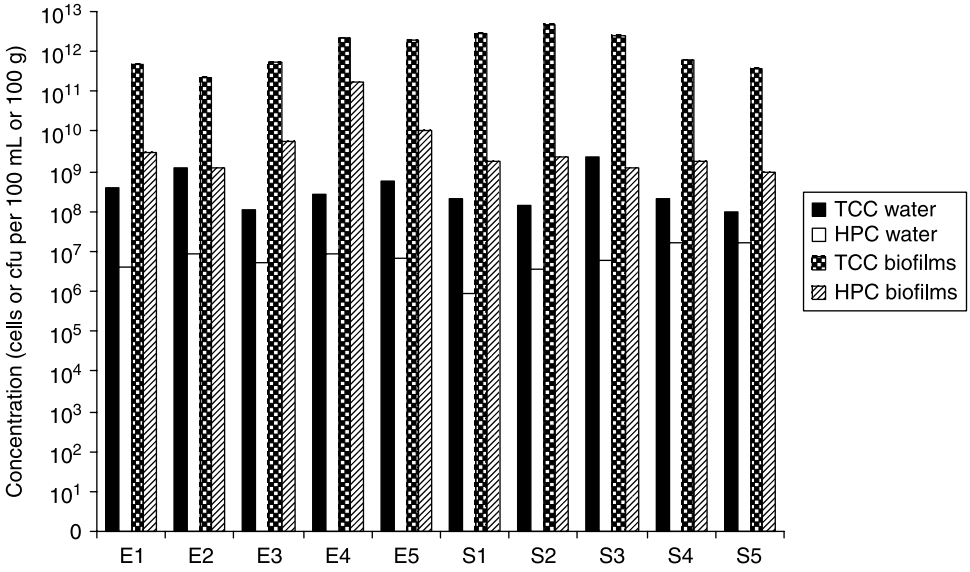

Figure 1 | Distribution of total cells and HPC bacteria in river water and biofilms. E1 to E5 refer to samplings including epilithic biofilms, S1 to S5 to samplings including sediments (Table 1). TCC, total cell counts.

Total coliforms and *E. coli* in river biofilms

Coliform bacteria and *E. coli* were detected in all water and biofilm samples. Total coliform concentrations were between 2.9×10^2 and 9.0×10^3 MPN per 100 mL (geometric mean, 1.4×10^3 MPN/100 mL) in the water samples and between 2.9×10^4 and 2.4×10^7 MPN per 100 g wet weight (geometric mean, 3.9×10^5 MPN/100 mL) in the biofilms (Figure 2). The concentrations of *E. coli* ranged from 1.3×10^2 to 3.4×10^4 MPN/100 mL of water (geometric mean, 2.5×10^2 MPN/100 mL) and from 1.0×10^3 to 1.5×10^6 MPN/100 g wet weight (geometric mean, 2.0×10^4 MPN/100 mL) of the biofilms (Figure 3).

The elevated concentrations of total coliforms and *E. coli* in all biofilms compared with those in water (Figures 2 and 3) indicate that these bacteria persist in these environments independent of the type of biofilm (epilithic or sediment). At least one order of magnitude higher concentrations of *E. coli* or faecal coliforms in sediments than in the overlying water have also been described in a number of studies of freshwater streams and coastal waters in various other geographic regions (Valiela *et al.* 1991; Obiri-Danso & Jones 1999; Craig *et al.* 2002*a*,*b*, 2004; Byappanahalli *et al.* 2003). Byappanahalli *et al.* (2003) reported the ubiquitous and persistent occurrence of *E. coli* in stream sediments, which could not be explained solely by direct faecal input. Long-term survival and/or multiplication was assumed to be likely. This observation was confirmed by microcosm studies which showed that *E. coli*/faecal coliforms persisted in marine and freshwater sediments for several days to weeks (Burton *et al.* 1987; Marino & Gannon 1991; Davies *et al.* 1995; Craig *et al.* 2002*b*, 2004). Survival of these bacteria was supposed to be limited by competition/antagonism with the native microflora and protozoan predation (Marino & Gannon 1991). Survival of *E. coli* in sediments was shown to be enhanced compared with that in overlying water (Craig *et al.* 2004). Thus, several mechanisms may underlie the consistent presence of *E. coli* in biofilms of anthropogenically

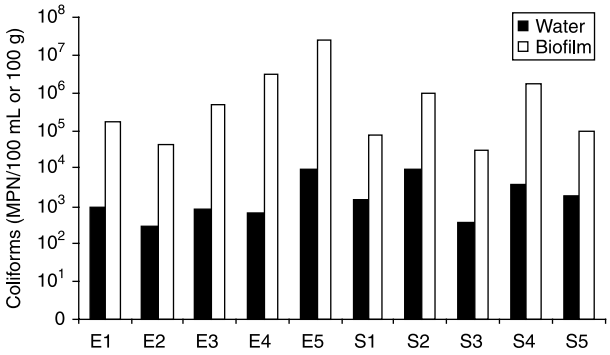

Figure 2 | Distribution of total coliform bacteria in river water and biofilms. E1 to E5 refer to samplings including epilithic biofilms, S1 to S5 to samplings including sediments (Table 1).

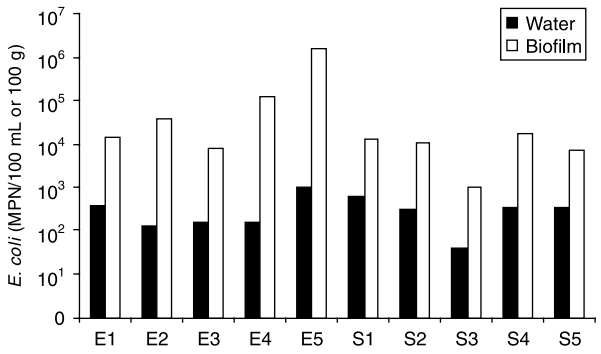

Figure 3 | Distribution of *E. coli* in river water and biofilms. E1 to E5 refer to samplings including epilithic biofilms, S1 to S5 to samplings including sediments (Table 1).

influenced surface waters such as the constant input from external sources, transport and incorporation into biofilms as well as the adoption of survival strategies to persist in the competitive environment of aquatic biofilms.

Identification of coliform isolates from epilithic biofilms and sediments revealed a variety of species (Table 2), which fell into three ecological categories described for coliforms by Leclerc *et al.* (2001). *E. coli* representing the thermotrophic group of faecal origin was isolated from all biofilms. The *Citrobacter* and *Klebsiella* species identified as well as *Enterobacter aerogenes* and *Enterobacter cloacae* (Table 2) belong to the ubiquitous group of coliforms, which may be of faecal origin, but can also be found in natural environments such as soil, vegetation and surface waters,

Table 2 | Coliform species identified in ten epilithic biofilms and sediments (see Table 1). In brackets, number of biofilms which were positive for the species identified

Coliform species identified	
Escherichia coli (10)	*Enterobacter asburiae* (2)
Klebsiella pneumoniae (6)	*Enterobacter cloacae* (2)
Klebsiella oxytoca (5)	*Kluyvera* sp. (2)
Buttiauxella agrestis (4)	*Serratia marcescens* (2)
Citrobacter braakii (4)	*Citrobacter koseri/farmeri* (1)
Citrobacter freundii (4)	*Enterobacter intermedius* (1)
Enterobacter nimipressuralis (4)	*Kluyvera cryocrescens* (1)
Serratia fonticola (4)	*Proteus mirabilis* (1)
Citrobacter youngae (3)	*Rahnella aquatilis* (1)
Pantoea sp. (3)	*Serratia rubidea* (1)
Serratia liquefaciens (3)	*Yersinia enterocolitica* (1)
Enterobacter aerogenes (2)	*Yersinia intermedia* (1)
Enterobacter amnigenus (2)	*Yersinia mollaretii* (1)

where their occurence is not necessarily related to faecal contamination (Leclerc *et al.* 2001). In addition, members of the environmental group of coliforms were detected such as *Buttiauxella agrestis*, *Enterobacter amnigenus*, *Enterobacter intermedius*, *Kluyvera* sp., *Pantoea* sp., *Rahnella aquatilis*, *Serratia* and *Yersinia* species. Thus, the coliforms identified in the river biofilms of the present study represent different ecological species of both faecal and environmental origin.

In addition to their function as indicator bacteria for enteric pathogens, *E. coli* and other coliform bacteria may also include pathogenic strains. *E. coli* which are usually harmless commensals for humans, also include pathogenic variants such as enterohaemorraghic *E. coli* O157:H7 which were involved in waterborne outbreaks (Leclerc *et al.* 2001) and are potentially able to survive in sediments of lakes and rivers (Czajkowska *et al.* 2005). Thus, although no attempts were made to differentiate pathogenic *E. coli* strains in the present study, their occurrence in river water sediments cannot be excluded. In addition, some of the other coliform species identified (Table 2) are known as opportunistic pathogens (Guentzel 1996). Examples are *K. pneumoniae* (Struve & Krogfelt 2004), *Enterobacter* spp. (Sanders & Sanders 1997), *Citrobacter* spp., *S. marcescens* (Hejazi & Falkiner 1997) and *P. mirabilis*.

Other studies usually focused on the quantitative detection of *E. coli* in marine and freshwater biofilms, while the diversity of coliforms has only rarely been considered and almost no information exists as to the composition of coliform populations in these habitats. One exception is a study of sediment (gravel) biofilms of the River Danube, where *Rahnella aquatilis* and the opportunistic pathogen *Enterobacter agglomerans* were identified (Zalmun *et al.* 1998).

Enterococci in river biofilms

Enterococci were quantified in eight water and biofilm samples. The levels of enterococci in water samples varied between 6.0×10^0 and 2.4×10^2 cfu/100 mL (geometric mean, 4.1×10^1 MPN/100 mL), while in biofilms the values ranged from 6.5×10^2 to 1.9×10^5 cfu/100 g wet weight (geometric mean, 4.4×10^3 MPN/100 mL) (Figure 4). Only a few studies also reported the presence of enterococci in

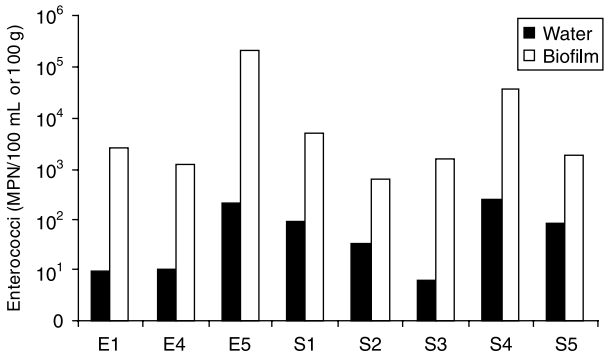

Figure 4 | Distribution of enterococci in river water and biofilms. E1, E4 and E5 refer to samplings including epilithic biofilms, S1 to S5 to samplings including sediments (see Table 1).

river biofilms (Obiri-Danso & Jones 1999; Donovan *et al.* 2008). In a microcosm study, intestinal enterococci were shown to persist for more than 28 d in river sediments with higher survival compared to the water column (Craig *et al.* 2002*b*), indicating the potential of enterococci to survive in river sediments for extended periods.

Identification of enterococci was performed on isolates from three biofilms of the River Ruhr, Anrathskanal and Moersbach (samples E5, S4 and S5, respectively). *Enterococcus faecalis* was detected in all three biofilms, *Enterococcus faecium* in two biofilms, *Enterococcus hirae* and *Enterococcus durans* in only one biofilm. These four species represent the relevant enterococcal species of true faecal origin (Pinto *et al.* 1999). Other enterococcal species identified in all biofilms were *Enterococcus casseliflavus* and *Enterococcus gallinarum*, which are regarded as environmental organisms (Pinto *et al.* 1999). Among the species identified, *E. faecalis* and *E. faecium* are a frequent cause of a wide variety of infections in humans, while the other enterococcal species are clinically less important (Jett *et al.* 1994).

CONCLUSIONS

In the river biofilms investigated in the present study, the faecal indicators *E. coli* and intestinal enterococci were consistently present in epilithic biofilms and sediments in higher concentrations compared to the overlying water. To the authors' knowledge, a similar observation has not been reported for German rivers before. However, a similar occurrence and distribution of faecal indicator bacteria has been shown for river water and sediments in other geographic regions as for example in Australia (Craig *et al.* 2002*a*,*b*), the United States (Byappanahalli *et al.* 2003) and the UK (Obiri-Danso & Jones 1999). Thus, it seems to be a general property of *E. coli* and intestinal enterococci to survive in biofilms of polluted rivers at relatively high concentrations compared to the bulk water.

Among the bacterial groups considered in the present study, *E. coli* and intestinal enterococci can be regarded as allochthonous organisms of faecal origin, while coliforms and other enterococci, also included environmental species which are expected to be more adequately adapted to the aquatic habitats of biofilms. The presence of *E. coli* and intestinal enterococci in biofilms of polluted surface waters suggests that these allochthonous bacteria can survive in the presence of high cell densities of autochthonous biofilm organisms represented by the HPC bacteria.

From a health perspective, the occurrence of faecal indicator bacteria in river biofilms is of hygienic relevance, since it indicates the potential presence of obligately pathogenic enteric bacteria. Furthermore, it has to be considered that faecal indicator organisms also include species that are known to be opportunistic pathogens. Thus, river biofilms act as a reservoir for pathogenic bacteria and can represent a health hazard when these pathogens are resuspended from the biofilms into the water, e.g. due to mobilization of sediments during heavy rains (Craig *et al.* 2004) or during recreational activities, and are transmitted to humans upon exposure to the contaminated water (Donovan *et al.* 2008).

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