Opportunistic pathogens and faecal indicators in drinking water associated biofilms in Cluj, Romania

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ABSTRACT

Biofouling occurs without exception in all water systems, with undesirable effects such as biocorrosion and deterioration of water quality. Drinking water associated biofilms represent a potential risk to human health by harbouring pathogenic or toxin-releasing microorganisms. This is the first study investigating the attached microbiota, with potential threat to human health, in a public water system in Romania. The presence and the seasonal variation of viable faecal indicators and opportunistic pathogens were investigated within naturally developed biofilms in a drinking water treatment plant. Bacterial frequencies were correlated with microbial loads in biofilms as well as with physical and chemical characteristics of biofilms and raw water. The biofilms assessed in the current study proved to be extremely active microbial consortia. High bacterial numbers were recovered by cultivation, including *Pseudomonas aeruginosa, Escherichia coli, Aeromonas hydrophila,* intestinal enterococci and *Clostridium perfringens.* There were no *Legionella* spp. detected in any biofilm sample. Emergence of opportunistic pathogens in biofilms was not significantly affected by the surface material, but by the treatment process. Implementation of a water safety plan encompassing measures to prevent microbial contamination and to control biofouling would be appropriate.

Key words | biofilm, drinking water treatment, faecal bacteria, human health, opportunistic pathogens

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INTRODUCTION

The availability of safe drinking water represents a global challenge, and authorities sustain substantial efforts in two priority areas: water quantity and water quality assurance. The deterioration of tap water quality primarily involves microbiological hazards, since the majority of evident water-related health problems are the result of microbial contamination (WHO 2008; Riley *et al.* 2011). Two modes of pathogen emergence in drinking water have been described: breakthrough and external contamination, followed by regrowth (Characklis 1988; van der Kooij 2003; Payment & Robertson 2004). In a drinking water system, about 95% of the bacterial numbers are estimated to be located on the surfaces, while only 5% are found in the water phase and detected by routine quality control measures (Flemming *et al.* 2002). Biofouling occurs without

exception throughout drinking water processing and distribution, despite the oligotrophic environment, residual disinfectants and hydraulic conditions. The complex structure of drinking water associated biofilm consists mainly in inoffensive heterotrophs originating from source water and sediment (LeChevallier *et al.* 1987; Szewzyk *et al.* 2000; Payment & Robertson 2004). It offers microniches for corrosion-inducing microbes, as well as for a diversity of waterborne pathogens, opportunistic pathogens and toxin-releasing microorganisms, which are able to contaminate bulk water during dispersion. Consequently, microbial consortia colonising surfaces in a drinking water treatment plant (DWTP) may function as a reservoir for further contamination (Szewzyk *et al.* 2000; Juhna *et al.* 2007; September *et al.* 2007; Wingender & Flemming 2011).

The present study investigated the occurrence of faecal indicators (*Escherichia coli*, intestinal enterococci, *Clostridium perfringens*) and opportunistic pathogens (*Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Legionella pneumophila*) within naturally developed biofilms in a DWTP, in order to evaluate and overcome the potential hazards for human health.

Water pollution with human excreta represents the primary concern about hygiene-relevant contamination and is the main source of waterborne microbial risks. The presence of faecal indicators denotes the potential occurrence of pathogenic bacteria, viruses and cysts of parasitic protozoa (Ashbolt *et al.* 2001).

E. coli, the main representative of thermotolerant coliforms, is the faecal index of choice (Edberg *et al.* 2000; Allen 2011) and is a natural and essential part of the microbiota of the gut of warm-blooded animals. Most strains are harmless, but *E. coli* can also be an excellent opportunistic pathogen. *E. coli* strains causing intestinal diseases have been grouped into at least six different main categories based on epidemiological evidence, phenotypic traits, clinical features of the disease and specific virulence factors: diarrheagenic *E. coli*, enterotoxigenic *E. coli*, enteroinvasive *E. coli*, enteroaggregative *E. coli*, diffusely adherent *E. coli* and Shiga toxin producing *E. coli* (Garrity *et al.* 2005).

Recent advances still emphasise the poor correlation between the concentrations of waterborne human viruses or protozoa and those of coliform organisms, including *E. coli*.

Until more adequate indicators are found, intestinal enterococci (IE) can be used, particularly in conjunction with *E. coli* (Tallon *et al.* 2005; McGuinness 2011). IE are a subgroup of faecal streptococci, represented by the species *Enterococcus faecalis*, *E. faecium*, *E. durans* and *E. hirae* (Ashbolt *et al.* 2001; WHO 2008). Although enterococci are commensal inhabitants of humans, they are frequently isolated from a variety of nosocomial and other infections (Garrity *et al.* 2005).

C. perfringens is the most widespread pathogenic bacterium and an indicator of past pollution. Only type A strains are found as part of the free-living microbiota, in both the soil and intestinal tract. Types B, C, D and E seem to be obligate parasites of animals and occasionally of humans. *C. perfringens* produces a variety of virulence factors; of these, alpha, beta, epsilon and iota toxins are referred to as the major lethal exotoxins (Garrity *et al.* 2005).

Bacteria inhabiting drinking water biofilms are generally harmless, but some species, referred to as opportunistic pathogens, may cause disease under certain circumstances. Factors making host individuals vulnerable to infections include immunosuppression, malnourishment, diabetes, catheterisation, burns, cancer, AIDS and respiratory conditions; sensitive subpopulations include young children, elderly people or pregnant women (Rusin *et al.* 1997; Ainsworth 2004; Reynolds *et al.* 2008).

The presence of *A. hydrophila*, *P. aeruginosa* and *L. pneumophila* was monitored within DWTP biofilms in the current assessment. These species represent widely occurring bacteria of concern in the continuously increasing category of hospitalised and ambulatory immunocompromised people (Glasmacher *et al.* 2003).

P. aeruginosa is ubiquitous in natural waters and soil, with the ability to survive in oligotrophic environments such as drinking water and even distilled water (Favero *et al.* 1971; van der Kooij *et al.* 1982). It is responsible for infections of the skin, eye, ear, soft tissue, wound, burn, lung and urinary tract. Due to a quorum sensing controlled multifactorial virulence, it causes severe diseases such as pneumonia, septicaemia or chronic infections in cystic fibrosis patients (Van Delden & Iglewski 1998; Head & Yu 2004).

Mesophilic aeromonads are common in worldwide aquatic environments. Producing an impressing array of virulence factors, they have the capacity to infect animals and humans after exposure. New evidence of intestinal and extraintestinal disease produced by *A. hydrophila* suggest it is an emerging human pathogen, but only strains belonging to specific hybridisation groups produce gastroenteritis in humans (Galindo *et al.* 2006; Chopra 2008).

Legionellae are also ubiquitous in natural and artificial water environments. Despite their fastidious nutrional requirements for growth in vitro, water-associated biofilms represent an ecological microniche for *Legionella* species. They have been reported to multiply, as protozoonotic bacteria, in 14 species of amoebae, two species of ciliated protozoa and one slime mould (Murga *et al.* 2001; Fields *et al.* 2002). More than 40 species are included in the genus and all are potentially pathogenic for humans and may cause disease such as legionellosis, Pontiac fever and

Pittsburgh pneumonia, with respect to the frequency and quantity of virulent strains in the environment (Garrity *et al.* 2005). *L. pneumophila* is the most medically relevant member, and its investigation in drinking water associated biofilms is of paramount importance. Epidemic events are usually traced to a contaminated water source (Bartram *et al.* 2007; Moritz *et al.* 2010) and to biofilm detachment (Declerck 2010).

This is the first study investigating the attached microbiota, with potential threat to human health, within a public water system in Romania. The microbial consortia in the DWTP of Cluj were previously assessed, to provide an overview of bacterial composition (Farkas *et al.* 2010a). In the current survey, naturally occurring biofilms in different steps of drinking water processing were monitored over one year. Viable oligotrophic microorganisms, faecal indicators and the opportunistic pathogens mentioned above were quantified in biofilm samples from concrete, steel and sand surfaces. Simultaneously, a series of raw water and biofilm characteristics were determined. Besides the quantification of viable microorganisms, their spatial and temporal variability was investigated. This approach is expected to bring new insights into the growth of microbial contaminants in drinking water associated biofilms. Raw water quality, substrate materials and treatment procedures are known to influence bacterial communities in drinking water systems. The aim of the present study is to identify more precisely the key factors that may further serve as components with predictive value for a water safety plan.

METHODS

Sampling

Naturally occurring biofilms in a DWTP of Cluj, Romania (46°44′39″ N latitude and 23°22′39″ E longitude) were analysed every other month, from May 2010 to March 2011. Sampling points have been set in the settling step and in the granular sand filtration process. All steps in sample collection, processing and analysis were performed using sterile instruments, or in controlled laboratory conditions, according to specific regulations. Biofilm deposits were collected by scraping eight areas with a total surface approximately 1 m^2, from concrete and from steel walls of a clarifier. Sand grains were sampled from three regions of a sand filter operating more than 30 h. To capture the representative microbial composition, weighed samples of biofilms (100 g) and sand grains (1,000 g) were homogenised. From these, 10 and 100 g deposits, respectively, were suspended in sterile tryptone water and were vortexed with sterile glass beads. The homogenised biofilm suspensions and serial dilutions up to 10^{-9} were prepared for inoculation.

Raw water entering the DWTP and bulk fluid from the filter were collected once with biofilm samples.

Bacterial counts

All microbiological determinations in the present study were based on classical techniques, targeting viable and cultivable microorganisms. Inoculation and growth on general or specific culture media was followed by biochemical identification, according to international and local standards. The oligotrophic microbial load was determined as heterotrophic plate count (HPC), by inoculation into the R$_2$A agar medium, with 7 day incubation at 22 °C. The presence of *E. coli* was estimated by the most probable number technique: presumptive determination in MacConkey broth and confirmation in brilliant-green bile lactose broth and in tryptophan broth. The following tests were performed by membrane filtration method. If presumptive growth on *Enterococcus* selective agar according to Slanetz and Bartley had registered any typical colonies, IE were enumerated on bile aesculin azide agar. Presumptive colonies of *C. perfringens* were detected on mCP agar, after exposure to ammonium hydroxide. *P. aeruginosa* was cultured on cetrimide agar, counting the fluorescent, oxidase-positive colonies. *A. hydrophila* incidence in biofilm samples was measured after the inoculation on Ryan's agar. Further confirmatory testing was applied to the colonies that were positive for oxidase, able to ferment trehalose, indole producing and resistant to O129. For *Legionella* isolation, the 'gold standard' method based on cultivation in GVPC agar was used. Typical colonies were subcultured on BCYE agar with L-cysteine and on nutrient agar, as a discriminatory test between *Legionella* and non-*Legionella* species. Merck Duopath *Legionella* test was used for a series of isolates, in order to identify *L. pneumophila* and other *Legionella* species by lateral flow immunoassay. Duopath test has

proven its capacity to recognise 42 *Legionella* type strains, other than *L. pneumophila* (Helbig *et al.* 2006). In order to increase determination certainty, additional identifications using bioMérieux API20E, API20NE, API20STREP and API20A kits together with apiweb software evaluation were performed.

Physical and chemical parameters

In biofilms, pH, iron and manganese contents were determined. Biofilm pH was recorded after samples homogenisation with narrow-range pH indicator papers (pH 6.0–8.1, Whatman). Iron and manganese contents in biofilm were measured on appropriate dilutions using the atomic absorption spectrophotometer contrAA700 (Analytic Jena), after acid digestion.

Physical and chemical parameters were monitored in raw water entering the DWTP: temperature, turbidity, pH, alkalinity, organic substances, dissolved oxygen, total organic carbon, ammonium, nitrite, nitrate, calcium, magnesium and iron ions; and in bulk water: temperature and pH. Water temperature, pH and turbidity were determined using calibrated laboratory equipments. Alkalinity, organic substances, dissolved oxygen, calcium and magnesium contents were measured by titration. Ammonium, nitrite and nitrate concentrations were measured using Lambda 40Bio (Perkin Elmer) spectrophotometer. Iron and manganese were determined by atomic absorption spectroscopy. Total organic carbon was determined in raw water with a Multi N/C 3100 analyser (Analytic Jena). All microbiological, physical and chemical determinations were performed in a certified laboratory, according to international standard methods.

Culture media and reagents were purchased from bioMérieux, Chimopar, Merck Chemicals, Oxoid, Penta, Sartorius Biotech, Scharlau and Sigma-Aldrich. Negative and positive controls were achieved using MicroBioLogics strains: *A. hydrophila* ATCC 7966, *C. perfringens* ATCC 13124, *E. faecalis* ATCC 19433, *E. coli* ATCC 8739, *L. pneumophila* ATCC 33152 and *P. aeruginosa* ATCC 27853.

Statistics

Descriptive analyses, including average, median, mean values, standard deviation and variability were calculated.

Graphic displays were used to highlight contrasting growth patterns from different organisms. Inferential statistics by co-dependency was analysed in parameter pair series and statistical hypotheses were tested (Rosner 2005; Ţigan *et al.* 2001). First, it was verified whether occurrences of bacterial species are significantly correlated with each other or with water and biofilm physicochemical parameters. Then, the differences in pathogens emergence within the three types of biofilms were explored. Because low sample numbers contributed to uneven variation, statistical differences in non-parametric data were assessed by Wilcoxon signed-rank test. A *p*-value <0.05 was considered statistically significant. Microsoft Office Excel, SPSS and Epi-Info 2000 statistical software were used.

RESULTS AND DISCUSSION

Bacterial development in biofilms

The concrete and steel-associated biofilms investigated revealed similar loads for oligotrophic microorganisms, between 6.59×10^6 and 8.25×10^8 colony forming units (CFU)/g. In filter sand, colony counts were between 1.1×10^3 and 5.6×10^6 CFU/g, with maximum occurrence during spring–summer (Figure 1(a)). Biofilm samples from the clarifier containing more active biomass than the sand grains collected from the filter corresponded to a difference of two or three orders of magnitude in colony counts. Previous findings estimated 6–18% of the cells living in six months-old biofilms to be able to form colony units in R_2A medium (Manuel *et al.* 2007). Such considerations lead to the conclusion that the total number of cells in the examined biofilms may reach more than 10^{10} microorganisms per gram. Similar values were also indicated by other authors investigating bacterial numbers in different types of deposits formed within water systems (Characklis 1988; Barbeau *et al.* 2005; Rubulis *et al.* 2008).

The growth of *E. coli* in biofilms collected from concrete and steel surfaces was also comparable during the year, with minimum 45 and maximum 7×10^5 CFU/g. In filter sand, minimal load of 1 and maximum 4.8×10^4 CFU/g occurred. Highest values in summer–autumn, as shown in Figure 1(b), are usual.
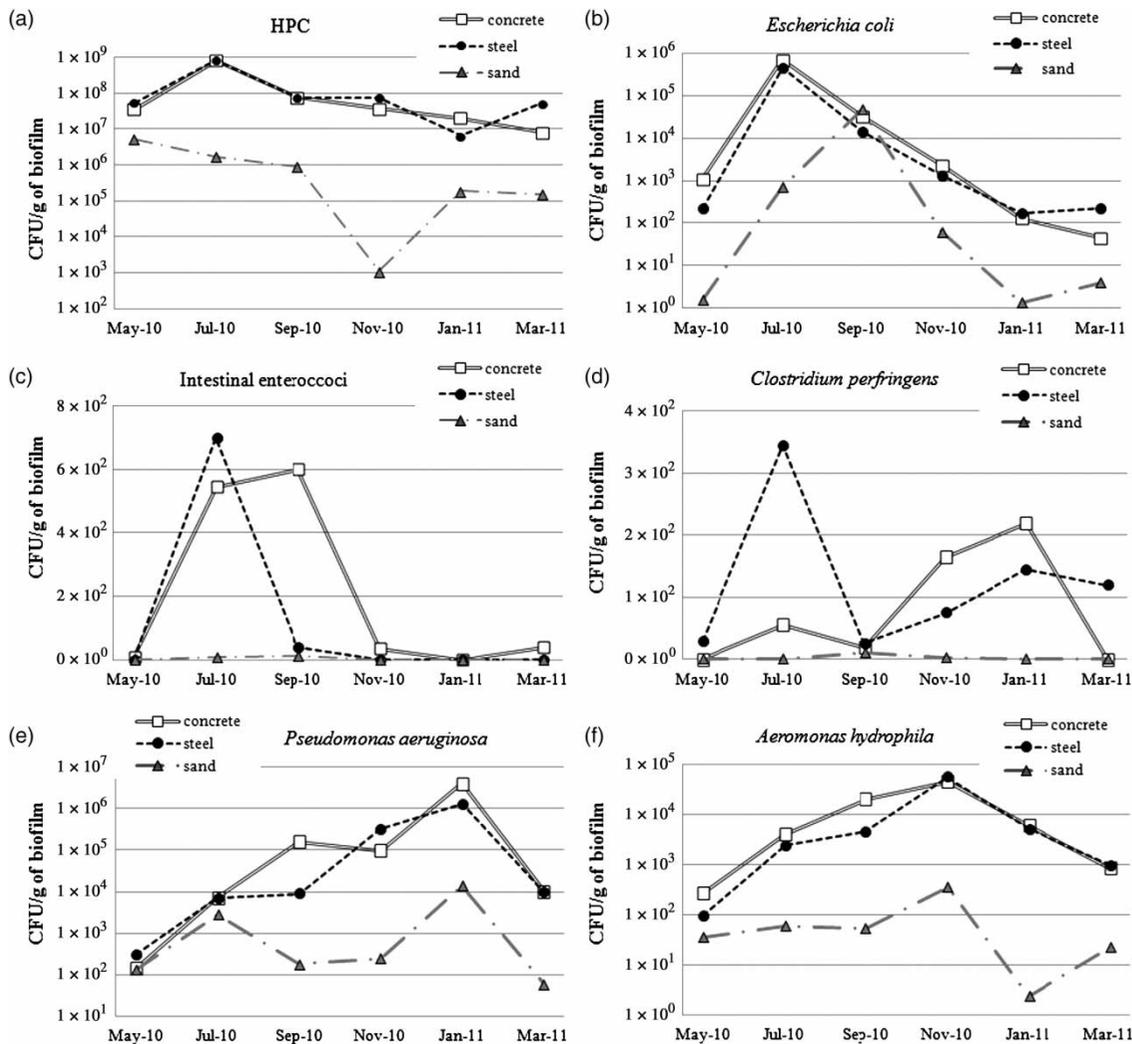
Figure 1 | Development of microbial in DWTP biofilms.

In a study by LeChevallier *et al.* (1987), iron tubercles were found to support coliform growth in pipelines. Hence, the presence of iron oxides, nutrient syntropy, surface roughness and protection from disinfection are considered key factors for coliform colonisation at corroded surfaces. Nevertheless, *E. coli* showed slightly elevated concentrations in concrete-attached communities analyed in the present monitoring.

There is no strong evidence of *E. coli* multiplication in temperate environments, although cases of drinking water contamination with coliforms and even *E. coli* emanated from pipeline biofilms have been reported (LeChevallier *et al.* 1987; Wingender & Flemming 2004). Recent studies

attest to its growth ability under anaerobic conditions in biofilms (Latimer *et al.* 2010). An extensive investigation of drinking water biofilms in pipes from four European countries has found *E. coli* in all networks, except one. It contributed from 0.001 to 0.1% of the total bacterial numbers (Juhna *et al.* 2007).

Intestinal enterococci displayed maximum values of 7×10^2 CFU/g in steel-associated biofilm in July, and 6×10^2 CFU/g in biofilm collected from the concrete substratum, in September 2010. Biofilms on steel surface proved to be unfriendly in hosting enterococci during the cold season. Four out of six samples were negative (from November 2010 to March 2011), while biofilm enterococci

were detected in all except one concrete-associated sample (January 2011). In all filter sand biofilms, IE colony counts revealed concentrations from 0.6 to 13 CFU/g (Figure 1(c)). Enterococci have been assigned to indicate the presence of viruses, due to their persistence and no evidence of multiplication in the environment, including in water-associated biofilms (Bitton 2005).

Only minor amounts of presumptive *C. perfringens* were found in drinking water associated biofilms. Two peak events registered in samples of clarifier origin: 3.45×10^2 CFU/g on the steel surface, in July 2010, and 2.20×10^2 CFU/g on concrete, in January 2011. *C. perfringens* was not detected in two samples from the latter, in May 2010 and March 2011. All sand filter samples were positive for *C. perfringens*, in low concentrations, from 0.12 to 11.1 CFU/g (Figure 1(d)). *C. perfringens* may indicate sources liable to intermittent contamination, and has been proposed as an index for enteric viruses and protozoa in drinking water supplies, as well as for the effectiveness of filtration processes (Ashbolt *et al.* 2001; WHO 2008).

P. aeruginosa reached elevated concentrations in all biofilm samples (Figure 1(e)). From 1.45×10^2 to 4×10^6 CFU/g counts occurred in biofilm on concrete walls. Sand filter biofilm was also rich in pseudomonads: from 60 to 1.4×10^4 CFU/g occurred. The highest loads were recorded in winter for all samples. Established biofilms represent a reservoir for *P. aeruginosa*, due to its great colonisation ability. Therefore, attached consortia act as a source of contamination in distribution systems (Bressler *et al.* 2009; Moritz *et al.* 2010).

A. hydrophila proved to be one of the commonest inhabitants of DWTP biofilms of all investigated bacteria (Figure 1(f)). As for the other species, homologous communities from concrete and steel surfaces in the clarifier developed together, with 96 to 5.6×10^4 CFU/g cultured. Increased levels of aeromonads were recorded from sand filter biofilms, from 2.35 to 3.5×10^2 CFU/g. Seasonal variation occurs with the highest loads registered in autumn.

There is increasing concern about *A. hydrophila* incidence during water treatment and distribution with regard to public health impact and its role in indicating chlorine resistance, disinfection efficacy, regrowth potential and biofouling, even at 4 °C (Garrity *et al.* 2005). WHO recommends control of aeromonads in piped distribution systems as a

risk-management strategy (Sartory 2002; WHO 2008), while US Environmental Protection Agency placed *A. hydrophila* on the Contaminant Candidate List (USEPA 2002). The persistence of *Aeromonas* spp. within biofilms (Bomo *et al.* 2004) as well as the ability of strains isolated from drinking water to produce enterotoxins (Razzolini *et al.* 2008), justifies the need monitor it in water quality assessment. Proper biochemical identification, DNA fingerprinting and virulence signature could be instrumental in differentiating pathogenic from non-pathogenic strains (Chopra 2008).

A seasonal variation can be observed in microbial growth, as expected. HPC, *E. coli* and intestinal enterococci harboured by DWTP biofilms showed seasonality with peak events in summer, as commonly characterise bacterial occurrences in raw water (Farkas *et al.* 2009; 2010b; 2011). *A. hydrophila* showed the highest colony counts in autumn, while *P. aeruginosa* populations increased during the cold season. A time lag is observed when comparing clarifier- with filter-developed biofilms (Figure 1). Such events may be explained by detachment and transportation of microorganisms within the DWTP, followed by colonisation or secondary contamination downstream. The bacteria may attach both as primary and secondary colonisers, initiating the adhesion or integrating in pre-existing biofilms (Wingender & Flemming 2011). The presence of pseudomonads and aeromonads in drinking water networks may be related to their remarkable biofouling ability rather than disinfectant resistance or multiplication in bulk water (van der Kooij 2003; Bressler *et al.* 2009).

Drinking water associated biofilms investigated in the present study harbour extremely active microbial consortia, with high concentrations of cultivable bacteria, except for *Legionella* (Table 1). No species of *Legionella* was detected in any sample, despite different techniques applied: direct inoculation by spreading, filtration of suspensions through membrane filters, acid or heat treatment. Even if they failed L-cysteine discrimination, a few colonies grown on BCYE agar were tested by lateral flow immunoassay. The results were always negative for *L. pneumophila* as well as for other species of *Legionella*. The results seem surprising, since *Legionella* spp. are described as common in freshwater biofilms (Garrity *et al.* 2005). *L. pneumophila* is a secondary coloniser of major concern, with important health

Table 1 | Microbiological parameters investigated in DWTP biofilms with maximum, minimum, mean counts (cfu/g) and standard deviation

Parameter	Sampling site	Maximum	Minimum	Mean	Standard deviation
HPC	C	825×10^6	8.3×10^6	167.5×10^6	322.9×10^6
	S	825×10^6	6.5×10^6	182.3×10^6	315.9×10^6
	F	5.4×10^6	1100	1395729	2.1×10^6
E. coli	C	7.0×10^5	45	1.2×10^5	2.8×10^5
	S	4.6×10^5	170	0.8×10^5	1.9×10^5
	F	0.5×10^5	1	8127	0.2×10^5
Intestinal enterococci	C	600	<1	205	285.47
	S	700	<1	123	282.96
	F	13	<1	4	5.54
C. perfringens	C	220	<1	77	93.39
	S	345	26	124	118.44
	F	11	<1	3	4.09
A. hydrophila	C	0.5×10^5	264	0.1×10^5	0.2×10^5
	S	56499	96	11619	0.2×10^5
	F	351	2	88	130.77
P. aeruginosa	C	4.0×10^6	145	0.7×10^6	1.6×10^6
	S	1.3×10^6	318	0.3×10^6	0.5×10^6
	F	14100	60	2932	5578.13
L. pneumophila	C	n.d.	n.d.	–	–
	S	n.d.	n.d.	–	–
	F	n.d.	n.d.	–	–

C = concrete, S = steel, F = filter sand, n.d. = not detected.

significance in drinking water sources, processing and distribution. Its persistence in biofilms, mostly in a viable but non-cultivable state, has been demonstrated (Moritz *et al.* 2010). This suggests the need of a further culture-independent approach. Cotuk *et al.* (2005) indicated how important is the ecological balance for the growth of *L. pneumophila* in water systems. Their results showed that the growth of *L. pneumophila* serogroup 1 could be controlled by heterotrophic bacteria such as *Pseudomonas* and *Aeromonas* that live normally in the same water body.

Pseudomonads, coliforms and aeromonads dominated the DWTP biofilms, with elevated concentrations in all samples, during all seasons. Intestinal enterococci and sulphite reducing clostridia registered lower proportions in microbial communities characterising these attached microniches. Drinking water-associated biofilms harbour microorganisms adapted to oligotrophic conditions and help them to persist over long time periods. Survival and multiplication of pathogens in these environments represent a subject of general interest (Wingender & Flemming 2011).

The presence of faecal indicators is a sign of the potentially pathogenic risk in drinking water associated biofilms and on further dispersion of microorganisms. Biofilm analysis targeting bacteria with health significance should be included in a comprehensive water safety plan to secure water quality (LeChevallier 2003). Although the investigation of local strains associated with drinking water, e.g. *A. hydrophila*, would need additional adequate DNA fingerprinting and virulence biomarkers identification in order to reveal their threat to humans, their elevated levels in biofilms are a warning sign of contamination.

Physical and chemical characteristics of biofilm and raw water

Conditions influencing microbial growth at the interface include water temperature, residence time, biological stability, disinfectant characteristics, surface materials and corrosion products (LeChevallier *et al.* 1987; LeChevallier 2003; van der Kooij 2003; Cerrato *et al.* 2006). The present data confirm that biofilms developed on steel surface have lower pH values but higher iron and manganese contents than concrete-associated deposits (Table 2). This may be caused by the accumulation of metal oxides in corrosion

Table 2 │ Physical and chemical parameters investigated in DWTP biofilms, with their maximum, minimum, average values and standard deviation

Parameter	Sampling site	Maximum	Minimum	Mean	Standard deviation
pH	C	7.60	6.80	7.133	0.26583202
	S	7.20	6.20	6.740	0.40099875
	F	7.55	6.50	6.854	0.49857797
Iron (mg/g)	C	166.737	58.663	102.475	40.856
	S	910.455	123.053	316.762	316.762
Manganese (mg/g)	C	96.684	29.865	50.977	26.370
	S	195.113	11.956	62.321	75.012

C = concrete, S = steel, F = filter sand.

products, followed by tuberculation and chloride penetration in tubercles (Herro 1989). Disinfectants may have an accelerated decay in drinking water treatment, an increase in their demand determined by manganese or iron oxides deposited on the pipe walls being indicated also by other findings (Cerrato *et al.* 2006). In biofilms, pH, iron and manganese concentrations are essential features for microbial proliferation that is necessary for biocorrosion. The proximal environment may influence biofilm composition, considering the specific requirements of bacteria, e.g. *Legionella* and *Clostridium* demand for iron salts. Iron concentration, as an environmental signal, induces synthesis of pigments and expression of virulence determinants: exotoxins and quorum sensing mediators in *P. aeruginosa* or exotoxins and proteases in *E. coli* (Mekalanos 1992; Garrity *et al.* 2005).

Increased deposition of iron and manganese in biofilms was observed during winter (Figure 2), in contradiction to conclusions of Ginige *et al.* (2011), whose results indicated a higher biological activity correlated with elevated iron
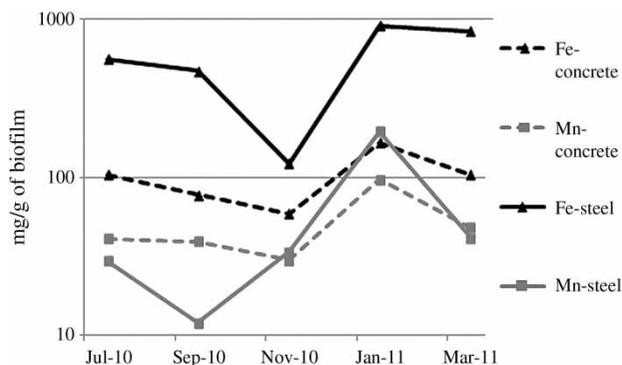
Figure 2 │ Iron and manganese concentrations in DWTP biofilms.

and manganese concentrations during late summer and autumn.

Physical and chemical characteristics of raw water entering the DWTP were also recorded (Table 3). Results revealed that biofilms could harbour faecal indicators and opportunistic pathogens even when inlet water has excellent chemical composition.

Effects of substrate material and raw water properties on opportunistic pathogen emergence in biofilms

Comparing the characteristics in the investigated biofilms, Wilcoxon signed-rank test results emphasise the absence of significant differences ($p > 0.05$) between the means of oligotrophic bacteria, faecal indicators and opportunistic pathogens developed on concrete compared to steel surfaces. However, significant differences ($p < 0.05$) appear when parameters in biofilms with sand filter origin were compared with those of samples from the clarifier. Wilcoxon test results (Table 4) verify the hypothesis that the treatment process affects the evolution of bacterial populations in biofilms, but the interface material has no significant influence on the composition of microbial communities. Bacteria of concern are able to colonise all the concrete, steel and sand surfaces.

Data interpretation revealed multiple correlations, with different levels of significance, between the parameter pairs considered. The moderate and strong Pearson common correlations ($R > 0.5$) are summarised in Table 5. A high similarity may be observed when comparing concrete and steel-related biofilms. From a total of 144 parameter pairs considered, 58 statistically significant correlations were recorded in clarifier biofilms (concrete and

Table 3 | Maximum, minimum, average values and standard deviation for physical and chemical parameters investigated in DWTP water

Parameter	Sampling site	Maximum	Minimum	Mean	Standard deviation
Temperature (°C)	Raw water	14.00	3.30	8.93	4.436965
	Clarifier	15.80	4.00	10.07	4.656035
	Filter	15.70	4.20	10.22	4.629650
Turbidity (NTU)	Raw water	2.18	0.91	1.47	0.450377
pH	Raw water	7.37	7.00	7.25	0.131364
	Clarifier	7.50	6.20	7.11	0.529622
	Filter	7.60	7.20	7.46	0.151051
Alkalinity (mEq/L)	Raw water	0.75	0.58	0.67	0.073416
Organic subst. (mg/L)	Raw water	2.91	1.73	2.39	0.384326
Dissolved O_2 (mg/L)	Raw water	12.51	8.64	10.56	1.589948
TOC (mg/L)	Raw water	2.91	2.37	2.60	0.214639
Ammonium (mg/L)	Raw water	0.05	0.02	0.03	0.009831
Nitrites (mg/L)	Raw water	0.02	0.00	0.01	0.007527
Nitrates (mg/L)	Raw water	3.82	2.33	2.74	0.572587
Calcium (mg/L)	Raw water	12.89	10.82	11.57	0.700447
Magnesium (mg/L)	Raw water	4.33	1.85	2.81	0.938267
Iron (mg/L)	Raw water	0.15	0.02	0.06	0.050892

Table 4 | *p*-values of Wilcoxon signed ranks test for microbial parameters in DWTP biofilms

Parameter	*p*-value
HPC concrete – HPC sand	0.028 based on positive ranks
HPC steel – HPC sand	0.028 based on positive ranks
IE concrete – IE sand	0.046 based on positive ranks
C. perfringens steel – *C. perfringens* sand	0.028 based on positive ranks
A. hydrophila concrete – *A. hydrophila* sand	0.028 based on positive ranks
A. hydrophila steel – *A. hydrophila* sand	0.028 based on positive ranks
P. aeruginosa concrete – *P. aeruginosa* sand	0.028 based on positive ranks
P. aeruginosa steel – *P. aeruginosa* sand	0.028 based on positive ranks

steel), a total of 33 in steel and filter sand samples and 33 in concrete and filter sand biofilms. The annual (seasonal) development of bacterial populations was similar, to a great extent, in biofilms developed in the clarifier, on concrete and steel walls (40.3% common correlations). Bacterial populations were not significantly influenced by substrate materials, as Wilcoxon test confirms. The same percentage of significant common correlations (27.0%), but not referring to the same parameter pairs, resulted when comparing biofilms from the clarification step with samples from the sand filter. Drinking water treatment process seems to significantly influence the opportunistic pathogens occurring not only in bulk water, but in associated biofilms, as well.

HPC and faecal indicators developed positive significant common correlations with each other and with temperature, organic substances and TOC. Negative correlations resulted with dissolved oxygen, except for anaerobic *C. perfringens*. Increments in bacterial numbers with water temperature and nutrients availabilities were predictable. Pseudomonads evolved in agreement with manganese concentration, in both biofilms from concrete and steel surfaces. Increases in water alkalinity and calcium content appear to inhibit *P. aeruginosa* multiplication. More alkaline inlet water inhibited clostridia, and magnesium restricted the populations of enterococci. Aeromonads positively correlated with iron ions that were found at low levels in raw water, but negatively with high iron content in biofilms. This may suggest that insoluble ferric oxides in corrosion products act as growth-limiting compounds for *Aeromonas* in microbial consortia.

Table 5 | Significant common correlations between microbial, physical and chemical parameters in DWTP biofilms and water

Parameter pairs	Correlation coefficient		
	Concrete	Steel	Filter sand
HPC – *E. coli*	$R_C = 0.999$	$R_S = 0.997$	
HPC – Temperature	$R_C = 0.595$	$R_S = 0.581$	
HPC – Organic substances	$R_C = 0.644$	$R_S = 0.648$	
HPC – Dissolved oxygen	$R_C = -0.645$	$R_S = -0.622$	
HPC – TOC	$R_C = 0.842$	$R_S = 0.828$	
HPC – Nitrites	$R_C = 0.608$	$R_S = 0.540$	
E. coli – IE	$R_C = 0.619$	$R_S = 0.999$	$R_F = 0.807$
E. coli – *C. perfringens*		$R_S = 0.909$	$R_F = 0.979$
E. coli – Temperature	$R_C = 0.569$	$R_S = 0.557$	$R_F = 0.568$
E. coli – Organic substances	$R_C = 0.657$	$R_S = 0.658$	
E. coli – Dissolved oxygen	$R_C = -0.623$	$R_S = -0.612$	$R_F = -0.571$
E. coli – TOC	$R_C = 0.834$	$R_S = 0.827$	
E. coli – Nitrites	$R_C = 0.512$	$R_S = 0.524$	
IE – *C perfringens*		$R_S = 0.902$	$R_F = 0.741$
IE – Temperature	$R_C = 0.865$	$R_S = 0.574$	$R_F = 0.839$
IE – Dissolved oxygen	$R_C = -0.905$	$R_S = -0.630$	$R_F = -0.881$
IE – TOC	$R_C = 0.886$	$R_S = 0.839$	$R_F = 0.791$
IE – Dissolved iron	$R_C = -0.512$		$R_F = -0.515$
IE – Dissolved magnesium	$R_C = -0.5635$		$R_F = -0.656$
C. perfringens – NTU	$R_C = 0.536$	$R_S = 0.609$	
C. perfringens – Alkalinity	$R_C = -0.582$		$R_F = -0.502$
P. aeruginosa – NTU	$R_C = 0.750$	$R_S = 0.709$	$R_F = 0.845$
P. aeruginosa – pH (water)	$R_C = -0.943$	$R_S = -0.914$	
P. aeruginosa – Alkalinity	$R_C = -0.597$	$R_S = -0.603$	$R_F = -0.512$
P. aeruginosa – Dissolved calcium	$R_C = -0.542$	$R_S = -0.534$	$R_F = -0.555$
P. aeruginosa – Manganese (biofilm)	$R_C = 0.963$	$R_S = 0.969$	–
A. hydrophila – Dissolved iron	$R_C = 0.799$	$R_S = 0.924$	$R_F = 0.842$
A. hydrophila – Iron (biofilm)	$R_C = -0.693$	$R_S = -0.814$	–

C = concrete, S = steel, F = sand surface.

Inferential statistics revealed no correlations between oligotrophic or faecal bacteria and *P. aeruginosa*, *A. hydrophila* or *L. pneumophila* within the monitored microbial communities.

Biofilms were monitored within the DWTP in conditions of periodic raw water prechlorination and routine sand filters back flushing, before the final disinfection. The tremendous number of heterotrophic bacteria and opportunistic pathogens may be related to the quality of the water source and sediments.

Significant efforts are made to ensure drinking water quality in Cluj: selection of best available sources, catchment protection, upgrading and control over the treatment process. The water sources of Cluj, although of excellent chemical quality, proved to be faecally contaminated (Curticăpean & Drăgan-Bularda 2007; Farkas *et al.* 2011). The waterfall system of dam reservoirs on Someşul Mic River, as a strategic environment, needs additional disturbance mitigation measures (Farkas *et al.* 2009; 2010a).

The occurence of bacteria with health risk potential in drinking water environments is of great concern. Since their appearance is most likely related to the contamination of the sources, additional measures for protection of catchments would be appropriate. Biofilms within the DWTP represent a reservoir for opportunistic pathogens. Their dispersal in bulk water and eventually escaping disinfection should be further considered. A comprehensive water safety plan (LeChevallier 2003) that includes preventive and corrective actions in order to control biofouling and microbial contamination would be appropriate.

CONCLUSIONS

This is the first study investigating opportunistic pathogens interrelated with the attached microbiota within a public water system in Romania. Not only the presence but also the seasonal variation of viable opportunistic pathogens was monitored in biofilms. The extremely active microbial consortia allowed the recovery of numerous cultivable bacteria, such as *P. aeruginosa, E. coli, A. hydrophila,* intestinal enterococci and *C. perfringens.* There were no *Legionella* spp. detected in any biofilm sample.

The raw water showed an excellent chemical quality, but naturally developed biofilms harboured faecal bacteria and opportunistic pathogens of concern, independent of the surface materials. They emerged throughout the whole period of investigation, registering a seasonal variation and a dependency on the treatment process. No statistically significant correlations between oligotrophic or faecal bacteria and aeromonads or pseudomonads occurred within biofilms. Opportunistic pathogens were significantly correlated with raw water physical and chemical parameters. Dissolved calcium appeared to reduce *P. aeruginosa* populations, while magnesium and iron ions restricted intestinal enterococci. Low levels of iron in water exerted stimulatory effects on *A. hydrophila* populations, but the high concentrations of ferric oxides limited their numbers in biofilms.

The occurrence of opportunistic pathogens in DWTP biofilms represents a concern from a public health perspective. The implementation of a water safety plan encompassing additional preventive and corrective measures is required.

As the international authorities recommend, biofilm surveillance and control are important tools to ensure safe drinking water.

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