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**MICROBIAL COMMUNITIES DEVELOPING
BIOFILMS WITHIN DRINKING WATER
TREATMENT PLANT AND DISTRIBUTION SYSTEM
OF CLUJ COUNTY**

SUMMARY OF DOCTORAL THESIS

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Keywords:

- biofilm
- drinking water
- microbial activity
- opportunistic pathogens
- disinfection
- resistance genes

Introduction

The access to safe drinking-water is essential to health, a basic human right and a component of effective policy for health protection. In December 2003 the United Nations General Assembly declared the period from 2005 to 2015 as the International Decade for Action, “Water for Life” (WHO, 2008).

The quality of life depends on the key elements such as the quantity and the quality of water available for human consumption. A rational use of resources considering the support capacity of the ecosystems in which we live indicates a sustainable society. Strategies such as the management and protection of water sources, effective treatment methods, the proper distribution and handling of water are intended to be implemented worldwide. Trends to increase productivity are evident in water sectors, as well as in food industry, in order to provide indispensable consumer products for a continuously growing population.

Drinking water is not only a liquid for hydration. It interacts with all industrial, agricultural, economical, social and cultural components characterizing the human society. The paramount importance of human access to safe drinking water is an issue of health.

Drinking water is an important vehicle for spreading microorganisms that interact with human body. Routine testing conducted in drinking water quality monitoring concerns just the bulk water and it may not offer the correct perspective upon the diversity of species nor the complexity of processes taking place in such a dynamic ecosystem. Biofilms, instead, could be an appropriate source for the investigation of microbiota. They harbour a high concentration and a variety of microorganisms, which may survive and proliferate, protected within the biofilm matrix.

Biofouling in water systems generates technical problems, such as biocorrosion, as well as water quality deterioration. The most alarming consequences as a result of biofouling in drinking water distribution systems consist in the presence, multiplication and dispersion into water of bacterial pathogens, opportunistic pathogens, parasitic protozoa, viruses and toxin releasing fungi and algae. Therefore, additional information on the specific composition and the gene pool of the attached microbial consortia could be instrumental in the prevention of microbiological hazards.

The aims of the study

The aims of the study consist in the investigation of biofilms occurring on the surfaces during raw water abstraction, treatment and drinking water distribution in Cluj County. The main objectives are to bring new insights on the bacterial composition and also on the dynamics of microbial activity. This is the first study investigating the biofilms into a public drinking water system in Romania.

The hypothesis of structural and functional complexity of biofilms through water treatment and distribution has led to a two-pronged approach, in the assessment of the potential effect of biofilms in water quality deterioration as well as their impact on the infrastructure.

The spatial and temporal variability of microbial activity, pursuing for the presence of certain bacteria was examined. The total number of germs that offer unspecific information on microbial loads and species indicating a potential risk to human health (faecal indicators and opportunistic pathogens) were targeted. Also, the investigation of enzymatic and physiological activities consisting in the detection of metabolic products with undesirable effects was performed.

The influence of the treatment process upon microbial consortia was assessed. Biofilms from different steps and from different types of surfaces were collected, simultaneously with raw water samples. The influence of temperature and nutrient concentration on biofouling process was tested *in vitro*. The effects of different disinfectants against bacterial consortia embedded into the biofilm matrix were compared.

In the context of a global issue caused by multidrug resistant bacteria, the scientific community has a keen interest in the emergence and dissemination of genes encoding resistance to antibiotics and disinfectants. In this respect, the molecular approach aimed at verifying for the presence of genetic elements responsible for the spreading of bacterial resistance to antimicrobial agents.

1. Biofilm: a paradigm shift in microbiology

Microorganisms and particularly bacteria display two types of behaviour: the planktonic state, characterized by independent float or swimming into the liquid environment, and the attached state, with cells adherent to each other and to the surface, into a solid layer called biofilm (Costerton et al., 1987; Flemming, 2009). Biofilm represents a structured community of sessile microorganisms, adhering to a living or inert surface (Zarnea și Popescu, 2011). These emergent structures are characterized by surface attachment, self-produced exopolymeric matrix, structural, functional and metabolic heterogeneity, capable of intercellular communication by *quorum sensing* and generally plurispecific composition (Costerton, 1994; Donlan, 2002; Dreeszen, 2003).

Biofilms are ubiquitous in natural and anthropogenic environments. Paleontological inventory showed that biofilms are robust fossils which can remain unchanged due to their peculiar chemical composition for up to 3.5 billion years (Westall et al., 2000). They are strong candidates for the most reliable and indisputable category of morphological biomarkers and represent a tool in the search for evidence of life in the oldest terrestrial sites and, by extension, in extraterrestrial materials (Toporski et al., 2003).

Recent findings demonstrate that microorganisms are essential in almost all processes taking place on our planet (Maloy and Schaechter, 2006). The biofilm concept represents a paradigm shift in microbiology: 99% of the microorganisms on Terra live in complex communities associated to surfaces (Costerton et al., 1987). The approach of the attached microbial consortia brings new research perspectives and insights. Cell differentiation, specialization and social behaviour of individuals have launched the hypothesis that biofilms are primitive multicellular systems (Webb, 2007). This new interdisciplinary field involves a complex research work of microbiologists, ecologists, evolutionists, geneticists, chemists, physicists, medical doctors and engineers from all around the world, which continuously bring fundamental contributions in biofilms understanding, preventing, controlling and exploitation (Costerton and Wilson, 2004; West et al., 2007).

2. Biofilms impact on drinking water quality

Biofouling occurs with no exception throughout drinking water processing and distribution, despite the oligotrophic environment, disinfectants' residuals and hydraulic conditions (Moritz, 2011). The main sources of deposits in drinking water treatment and distribution systems are particulate matter transported by water, microbial activity and physicochemical reactions both at the water/walls interface and within the water bulk (Echverría et al., 2009).

In drinking water systems, the high majority of bacteria, estimated at 95%, are located attached at the surfaces, while only 5% are found in water phase and detected by sampling as commonly used for quality control (Flemming et al., 2002). Biofilms play an essential barrier role in biological water treatment through the entrapment of particulate material (including microbial pathogens) as well as through nutrient removal (Fonseca et al., 2001, Långmark et al., 2004; LeChevallier and Au, 2004; Tellen et al., 2010). Bacterial communities associated in biofilms display enhanced biodegradation and bioaccumulation characteristics, compared with planktonic cells (Araya et al., 2003).

On the other hand, occurrence of biofilms in storage and distribution systems may induce water quality deterioration (O'Connor and O'Connor, 2001; Skrabber et al., 2005; Lee et al., 2006; Wingender and Flemming, 2011) and biocorrosion (Videla and Characklis, 1992; Beech and Flemming, 2000; Coetser and Cloete, 2005). Other consequences include water treatment yield loss, efficiency reducing in cooling or heating exchange and transport, as well as in membrane processes.

Quantitative and prescriptive evaluation is the next target of scientific community. Prediction of microorganisms' behaviour in the distribution system water and biofilms requires greater understanding of the effects in microbial attachment, detachment, survival, multiplication and viability of three groups of abiotic and biotic factors: substratum properties, biofilm composition, including microbial intra- and interspecific interactions and bulk water characteristics.

3. The investigation of biofilms developed within the drinking water treatment plant and distribution system of Cluj County

Sustainable development. Drinking water quality in Romania

The National Strategy for Sustainable Development of Romania, prepared in 2008, sets goals, aimed at improvement of human's living standards and relationships between people, in harmony with the natural environment (Popovici et al., 2008). To achieve those goals, sustainable development addresses three elements: resource depletion, ecological and environmental issues and the quality of life.

In 2008, the Sustainable Society Index, calculated for 37 European countries, based on 22 indicators, ranks Romania 23rd place with an overall score of 5.7. Water-relating indicators make a significant contribution (Table 1). Still, statistics indicate that 43% of the population lacks the access to safe drinking water, using private sources (dug wells and boreholes). In our country, water scarcity is not due to limited resources, but to their contamination, mainly with anthropogenic origin (Popovici et al., 2008; Mureşan et al., 2010; Roşu et al., 2010).

Indicator	Romania		Maximum score in Europe		Minimum score in Europe	
	Score	Rank	Score	State	Score	State
3. Sufficient to drink ^a	5.7	37	10	Denmark	5.7	Romania
8. Surface water quality ^b	2.9	37	9.1	Norway	2.9	Romania
16. Use of renewable water resources	8.9	18	10.0	Iceland	0.0	Malta

Table 1. Indexes referring to water and Romanian ranking in 2008 (after Popovici et al., 2008).

a - 43% of the population has no access to an improved water source; b – data from 2003.

In order to ensure a high quality drinking water, the strategies applied include: selection of the best sources, catchments' protection, development of efficient treatment processes and management of water distribution.

Drinking water in Cluj-Napoca

Drinking water in Cluj is supplied by Someș SA Water Company, which provides abstraction, treatment, transport and distribution of drinking water, on the one hand, and collection, transportation, treatment and disposal of sewage on the other. With a tradition of over a century since the establishment of Water and Sewage Works, the company is first class licensed operator in the region, serving two counties: Cluj and Sălaj. It provides drinking water to nearly 700,000 people in eight cities and towns and in 112 villages.

Drinking water in Cluj city comes mostly from high quality surface sources, represented by a series of lakes located in the upper basin of Someșul Mic (Fig. 1), part of Someș-Tisa river basin.



Fig. 1. The main sources of drinking water in Cluj: A – Gilău Lake; B – Someșul Cald Lake; C – Tarnița Lake; D – Someșul Rece River; E – Agârbiciu River.

The waterfall system of dams on the rivers Someșul Cald, Someșul Rece and Someșul Mic allow an accumulation of a huge water volume and perform multiple roles: drinking and industrial water providing, energetic, flood mitigation, for irrigations, recreation, sport fishing and trout farming. In 2008, Tarnița and Fântânele-Beliș lakes were included among the Romania's inland waterways, by HG 665/2008, a controversial issue, since they are part of a strategic basin and represent the main source of drinking water feeding Cluj and surrounding areas. Drinking water treatment plant located in Gilău works since 1973, having three alternative sources of raw water: Gilău Lake, Someșul Cald Lake since 2000 and Tarnița Lake since 2009. Most physico-chemical and microbiological parameters allow raw water's framing in A1 quality category. Potable water meets the standards set by local and European authorities (laboratory and public data).

The treatment process itself was constantly improved, in line with current standards. Steps to potable water (Fig. 2) are:

- microstraining to remove coarse impurities;
- primary disinfection or prechlorination;
- reagents addition, when appropriate (aluminium sulphate for coagulation, calcium carbonate for pH correction and polyelectrolyte as flocculating agent);
- clarification by sedimentation;
- filtration through rapid sand filters in dual current;
- final disinfection by chlorination;
- storage in reservoirs and distribution according to consumption.



Fig. 2. Drinking water treatment process in Cluj: M – microstraining; P – prechlorination; R – reagents addition; D – clarification ; F – rapid sand filtration; C –final disinfection by chlorination; S – storage in reservoirs; L – laboratory.

Premises. Sampling points setting

Microbial activity is one of the most important factors in water purification processes (Hendel et al., 2001). Water quality is so dependent on the specific composition and physiology of microbial communities present during the treatment, storage and transport processes.

Naturally formed biofilms were collected from the following sampling points (Fig. 3):

I. Water abstraction step: the feeding pipe from Someșul Cald Lake; material: HOBAS (glass fibre reinforced); age: 10 years;

II. Treatment plant: - from concrete and steel walls of a clarifier in the settling step;
- from a rapid sand filter operating more than 30 hours;

III. Distribution and storage: - from a steel pipe in Maramureşului Street, operating from 1972-1973.

- from reservoirs Zorilor I, Zorilor II and Baciului;
material: concrete; capacity: 2.500 m³. Of these points, sediment samples from the bottom of tanks were collected, as there was not registered any biofilm accumulation on the vertical walls.

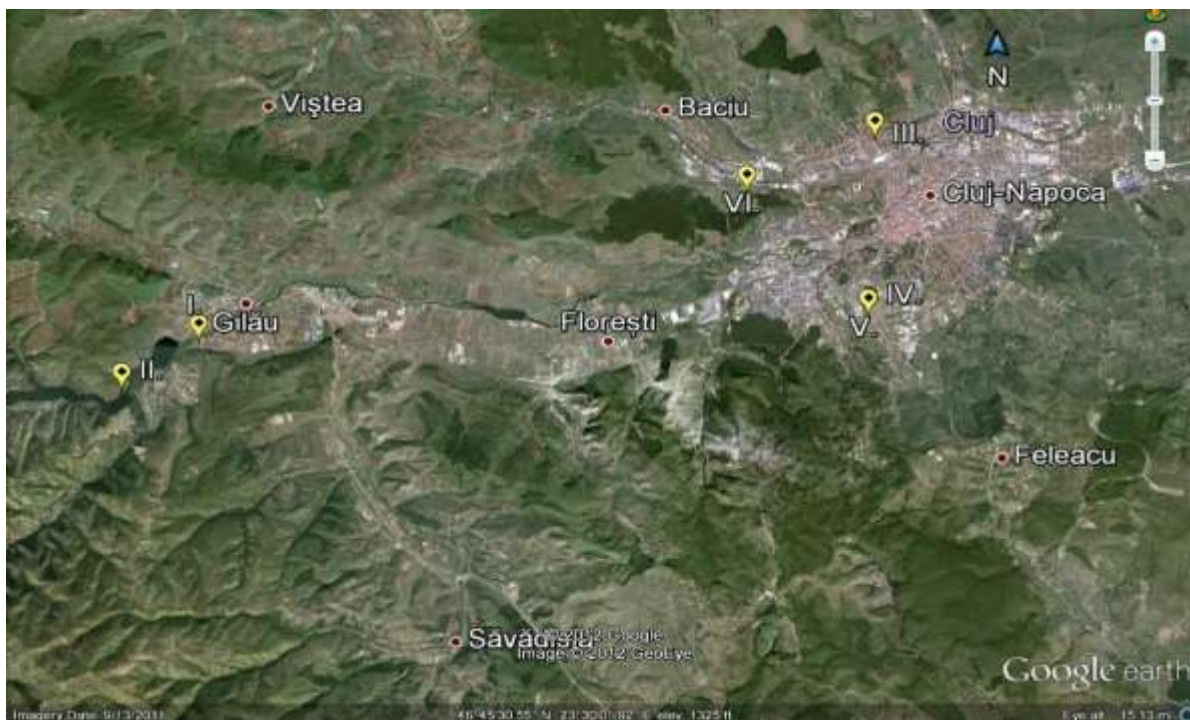


Fig. 3. Sampling points for biofilm samples: I. Drinking water treatment plant, Gilău; II. Feeding pipe from Someşul Cald Lake; III. Distribution system, Maramureşului Street; IV. Zorilor I Reservoir; V. Zorilor II Reservoir; VI. Baciului Reservoir.

4. Materials and methods

Sampling and sample preparation

Biofilm specimens were harvested by scraping the surfaces in contact with water, from preset sampling points, complying with the protocol. All procedures in biofilm collecting and processing in microbiological analysis were performed under sterile conditions. In order to capture a representative sample, biofilms were collected by the following protocol:

- Setting of sampling sites in the settler, sand filter, tank or pipeline;
- Harvesting of excess amounts of biofilm or sediment;

- Transport in the laboratory;
- Homogenization and subsampling.

At once with the investigation of biofilms from the water treatment plant, monitoring of raw water physico-chemical, chemical and microbiological parameters was conducted.

All microbiological, physico-chemical and chemical analyses were performed in a certified laboratory within the water treatment plant of Someș SA Water Company, Cluj-Napoca. Enzymatic and molecular analyses were performed in the laboratories of the Faculty of Biology and Geology, Babeș-Bolyai University, Cluj-Napoca.

Microbiological methods

Microbiological analyses were performed by conventional cultivation techniques, pursuing the development of viable and cultivable microorganisms. Biofilm samples or appropriate dilutions were inoculated in general or selective-differential growth media. Successive series of dilutions, up to 10^{-9} were prepared in sterile peptone water.

Three methods were used for cultivation:

- **Inoculation of agar medium** by incorporating the inoculums (heterotrophic plate counts, HPC) or by their distribution on the surface of solid agar (manganese oxidizing bacteria).

- **Membrane filter method:** biofilm suspensions were filtered through sterile microbiological membranes, with 0.45 mm porosity, followed by placing them on specific culture media (intestinal enterococci, *Clostridium perfringens*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Legionella pneumophila*).

- **Most probable number (MPN) method:** inoculation of at least three successive dilutions in series of 5 tubes (coliform bacteria and *Escherichia coli*, ammonifying, denitrifying, sulfur-reducing, sulfur-oxidizing, sulfate reducing and iron reducing bacteria).

After the inoculation and incubation according to required conditions for each species or target group, typical colonies were counted on solid media and specific reactions were followed in broths (Krumbein and Altman, 1973; Alexander, 1982; Cușa, 1996; Drăgan-Bularda, 2000; Atlas, 2004). Additional biochemical tests and phenotypic identification using API kits were performed.

Analysis characterizing microbial activities associated with drinking water biofilms included enzymatic determinations, assessing the activity of dehydrogenase, phosphatase and catalase (Drăgan-Bularda, 2000).

Assessing the influence of temperature and nutrient concentration on local communities of bacteria in biofilms, a colorimetric assay was performed to determine the density of biofilm (biomass estimation by crystal violet staining).

In order to evaluate the effect of disinfectants on biofilm bacteria, their recovery on growth media was tested. Coupons with biofilm as control and biofilms subjected to the action of disinfectant solutions were used (Table 2). The antimicrobial effect of each disinfectant was estimated by calculating the log reduction in bacterial growth (log reduction value = LRV) for each type of bacteria targeted (Hamilton, 2010).

Active components	Commercial name	Producer
Sodium dichloroisocyanurate	Clorom	G&M, Romania
Sodium hypochlorite	Sodium hypochlorite solution	Penta, Czech Republic
<i>N</i> -chloro- <i>p</i> -toluene sulphonamide	Chloramine-T	Sintofarm, Romania
Chlorine dioxide	TwinOxide	TwinOxide, Netherlands
Sulphamic acid Hydrochloric acid Hydrogen peroxide, Acetic acid, Peracetic acid	Floran: Topix 40% vol Filtrasan 40% vol Oxis 20% vol	Mosslein, Germany

Table 2. Disinfectants experimented for antibacterial effect against biofilm bacteria.

Investigation of bacterial resistance by molecular methods

The presence of genetic elements encoding antimicrobial resistance in the microbiota of drinking water associated biofilms was investigated by molecular methods.

There were selected 96 isolates: 58 (60%) colonies harvested from culture media targeting environmental bacteria and 38 (40%) colonies collected from culture media targeting faecal indicators and opportunistic pathogens.

The following genetic determinants were targeted: class 1 integrons, integron-integrase gene (*intI1*) and specific gene cassettes (*qacG*, *qacH*, *qacE*), genes that encode resistance to quaternary ammonium compounds (*qacE*, *qacEΔ1*), the gene encoding resistance to sulphonamide (*sulI*) and the transposition module (*tni*) of class 1 integrons in transposons. Due to a high phenotypic variability expected to occur in the autochthonous bacterial populations, 23 combinations of the 33 selected primers were tested (Table 3).

The protocol was designed to accomplish the following steps:

- isolation of pure colonies on different agar media;
- biochemical testing;
- selection of isolates;
- verification for the presence of the target gene by polymerase chain reaction (PCR), using specific primers;

- purification of amplicons;
- amplicons' sequencing;
- confirmation of results by analysing the similarity with the sequences from NCBI GenBank database.

Symbol	Primers	Target	Amplicon size (bp)	References
P1	MRG284 MRG285	Class 1 integron	variable 400-2000	Gillings et al., 2009
P2	MRG284 MRG286	Class 1 integron	variable 400-2000	
P3	HS915 HS916	<i>intI1</i>	≈350	Márquez și colab, 2008
P4	HS458 HS459	5'CS-3'CS <i>intI1</i>	1300	Holmes et al., 2003
P5	HS714 HS715	<i>intI1</i> +IRi	≈500	Stokes et al., 2006;
P6	HS463a HS464	<i>intI1</i>	473	
P7	HS722 HS715	<i>oriV</i> + <i>intI</i>	1320	
P8	HS714 HS726	<i>intI1</i>	1320	Chuanchuen et al., 2007
P9	intF intR	<i>intI1</i>	497	
P10	HS464 HS721	<i>intI1</i> + IRi	1520	Stokes et al., 2006
P11	HS458 HS723	<i>intI1</i>	2100	
P12	HS714 HS463a	<i>intI1</i>	-	
P13	qacEF qacEΔ1R	<i>qac</i> cassettes	363	Chuanchuen et al., 2007
P14	qacEF qacER	<i>qac</i> cassettes	363	
P15	qacEF sulR	<i>qac</i> , <i>sulI</i> cassettes	1112	
P16	MRG287 MRG288	<i>qacG</i> family	281	Gillings et al., 2009a
P17	MRG289 MRG290	<i>qacH</i> family	235	
P18	MRG291 MRG292	<i>qacE</i> family	193	
P19	MRG287 MRG293	<i>qacG</i> cassettes	354	
P20	MRG289 MRG294	<i>qacH</i> cassettes	301	
P21	MRG291 MRG295	<i>qacE</i> cassettes	277	
P22	HS549 HS550	<i>sulI</i>	1100	Stokes et al., 2006;
P23	HS724 HS725	<i>miAB</i>	520	

Table 3. Combinations of primers and fragments targeted in the investigation of genes encoding bacterial resistance.

In order to determine the bacterial species positive for genetic elements responsible for antimicrobial resistance, molecular identification was performed based on 16S ribosomal RNA gene sequencing.

The steps performed in molecular identification:

- amplification of 16S rRNA gene fragments by PCR, using universal bacterial primers (16S-8F and 16S-1493R);
- purification of amplicons;
- amplicons' sequencing;
- identifying sequence similarity with that of known sequences from NCBI GenBank database.

Sequencing was performed by Sanger method using Applied Biosystems 3730XL automated sequencer at Macrogen Inc., Netherlands.

5. Results and discussion

5.1. Microbiological analysis

Viable and cultivable bacteria in biofilms

The evolution of microbial loads assessed by heterotrophic plate counts (HPC) and expressed as colony forming units per gram of biofilm (CFU/g), in the attached consortia, developed within the treatment plant, is shown in Fig. 4. There was a similar development of the microbiota on the three substrates (concrete, steel and sand), with fluctuations in bacterial density and seasonal development. The maximum values were recorded during the summer or autumn and minimum during winter. Biofilms from the settler, regardless of substrate type, supported microbial communities with a similar trend and similar values, while the sand filter biofilms were poor in microorganisms.

To get an overview of the dynamics of microbiota attached to surfaces during the abstraction, processing and distribution of drinking water in Cluj, the average values of microbial loads were compared in biofilms developed before, during and after treatment process (Fig. 5). Applying correct procedures in drinking water treatment and disinfection is estimated to have reduction efficiency over 99% for planktonic microbiota (Le Chevallier and Au, 2004). Less tremendous decrements in bacterial numbers can be observed in the investigated biofilms.

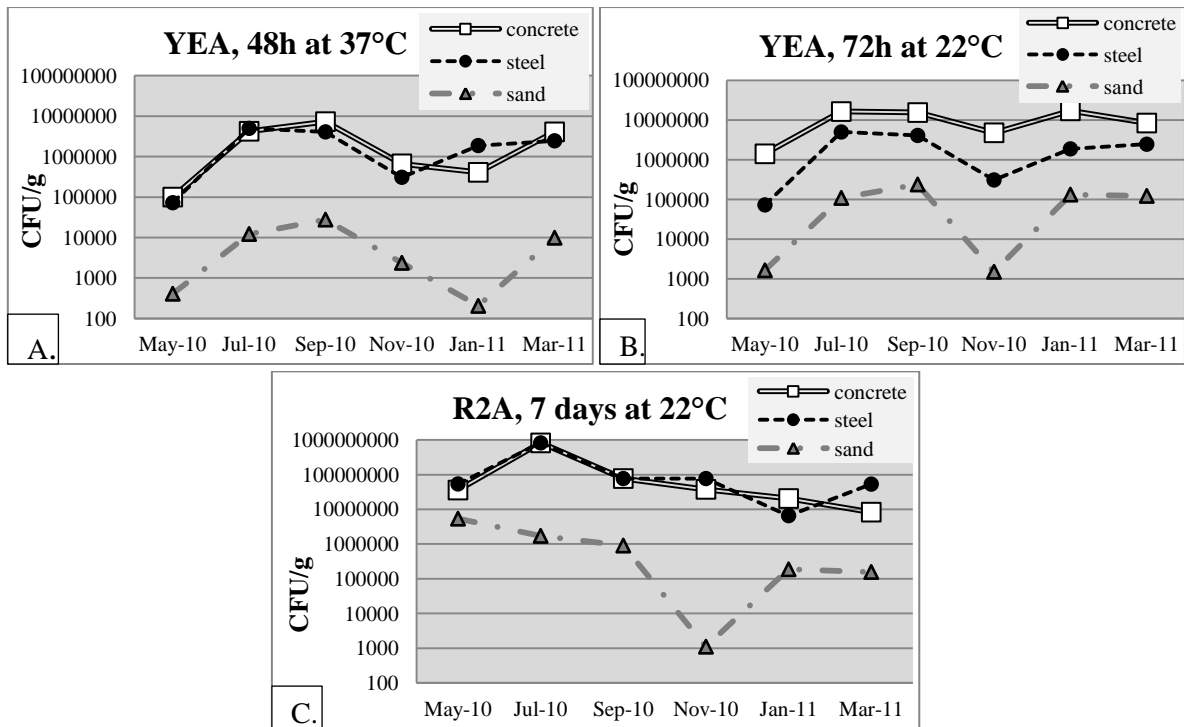


Fig. 4. The evolution of HPC in biofilms developed within the drinking water treatment plant, on three substrates: concrete, steel and sand. A – HPC at 37°C, 48 hours incubation on yeast extract agar (YEA); B – HPC at 22°C, 72 hours incubation on YEA; C – HPC at 22°C, 7 days incubation on R2A agar.

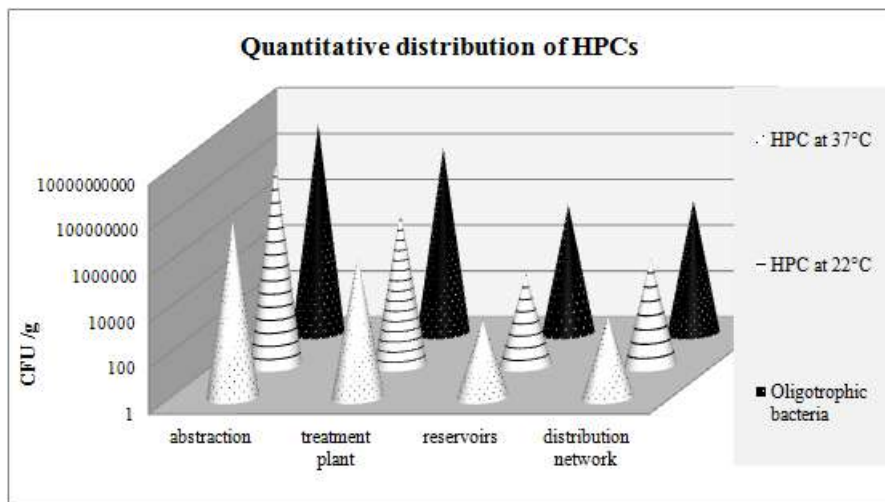


Fig. 5. Comparison of microbial loads in biofilms occurring before, during and after the treatment process – average values 2010-2012.

Significance of microbial plate counts in drinking water associated biofilms

Although only a small percentage of metabolically active microorganisms are able to grow *in vitro*, in common culture media, the estimation of HPC provides general information of the microbial load in biofilms. Further, this allows the assessment of biofilms potential in water contamination, as reservoirs of microorganisms.

The presence of heterotrophic and oligotrophic bacteria in drinking water and associated biofilms does not necessarily represent an infectious risk and a concern in terms of consumer's health (Glasmacher et al., 2003). Non-pathogenic bacterial populations should not be overlooked, because of their primary role in biofilm formation (LeChevallier, 2003).

Faecal indicators and opportunistic pathogens

The results of indicators of faecal contamination and opportunistic pathogenic bacteria monitoring in biofilms associated with drinking water indicates that pseudomonads, coliforms and aeromonads populations prevailed in surface attached consortia, the whole year. Intestinal enterococci and sulphite reducing clostridia, although present, were detected in extremely small scale, compared with the abundant species.

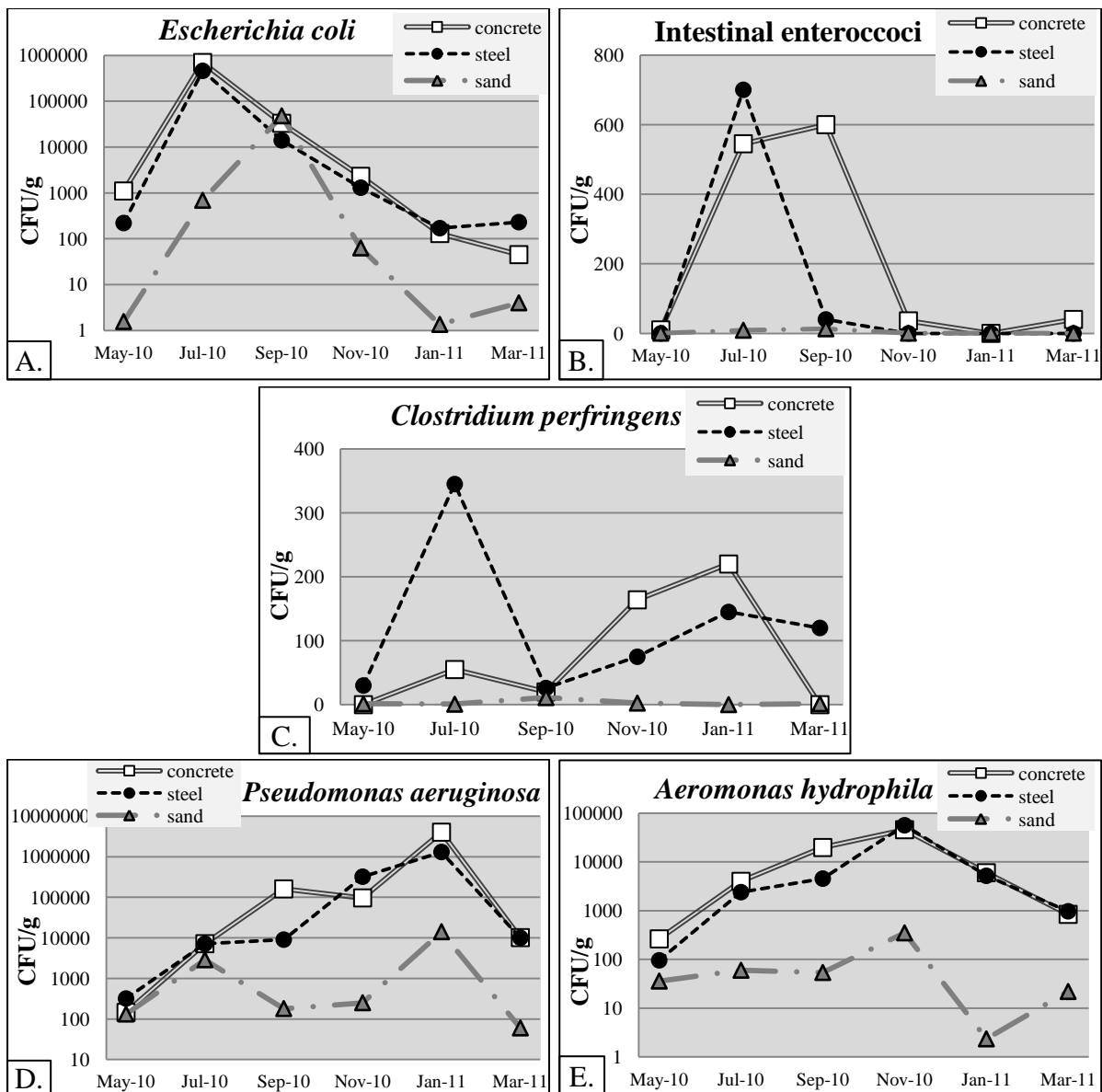


Fig. 6. The evolution of faecal indicators and opportunistic pathogens in biofilms developed within the drinking water treatment plant, on three substrates: concrete, steel and sand.

The observed seasonal variation of *Escherichia coli* and intestinal enterococci in treatment plant's biofilms is characterized by increments in their populations, probably due to a higher degree of contamination of raw water during the warmer season. The fluctuation in *C. perfringens* evolution is characterized by intermittent episodes of contamination, which led to of the peak events in July 2010 and in January 2011. Aeromonads were abundant in biofilms during the autumn, while pseudomonads development reached unprecedented peaks in winter (Fig. 6).

There were no species belonging to *Legionella* genera detected in any sample, despite different techniques applied. The results seem to surprise, since species belonging to *Legionella* genera are described as common in freshwater biofilms, although nutritionally fastidious (Garrity et al. 2005). *L. pneumophila* is a secondary coloniser of major concern, with important health significance in drinking water sources, processing and distribution. Its persistence in biofilms, mostly in a viable but non-cultivable state, has been demonstrated (Moritz, 2011). This suggests the need of a further culture-independent approach.

A dynamic situation in the populations of faecal indicators and opportunistic pathogens harboured was registered in biofilms attached to surfaces during the abstraction, processing and distribution of drinking water in Cluj (Fig. 7). Overall, one can observe a decrease in the number of bacteria per gram of biofilm, in the distribution network, after water treatment. It was less evident in case of the species *A. hydrophila* and *P. aeruginosa*.

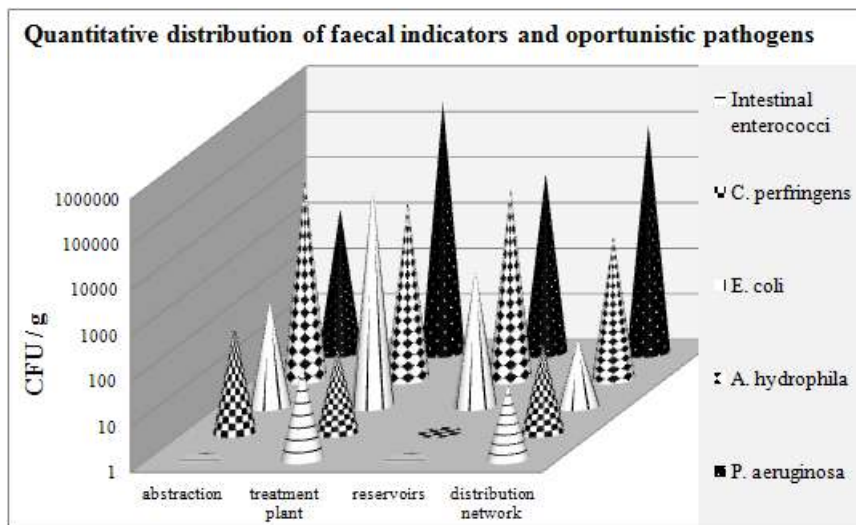


Fig. 7 Comparison of faecal indicators and opportunistic pathogens in biofilms occurring before, during and after the treatment process – average values 2010-2012.

Significance of faecal indicators and opportunistic pathogens in drinking water associated biofilms

Biofilms developed within abstraction, treatment and distribution of drinking water in Cluj County proved to harbour extremely active microbial consortia, with high concentrations of cultivable bacteria, except for *Legionella* species.

Detection of faecal indicators in drinking water associated biofilms has multiple significance: on the one hand are themselves a potential risk in terms of consumers' health, on the other hand they indicate the possible presence of other bacteria, viruses or protozoan pathogens.

Dispersion of opportunistic pathogens hosted in biofilms may lead to contamination of the aqueous phase, carriage with water flow and downstream colonization. Such aspects should be further investigated.

Ecophysiological groups of bacteria in biofilms

Ammonification, iron reduction and manganese oxidation proved to be the predominant processes in all the investigated biofilms. All the other ecophysiological groups of microorganisms, i.e. denitrifying bacteria and bacteria involved in sulphur cycle were found in smaller percentage, representing less than 0.1% of the germs detected in biofilms.

The bacteriological indicator of biofilm quality (BIBQ) indicates reduced microbial activities during the winter and in the direction of water flow (Fig. 8).

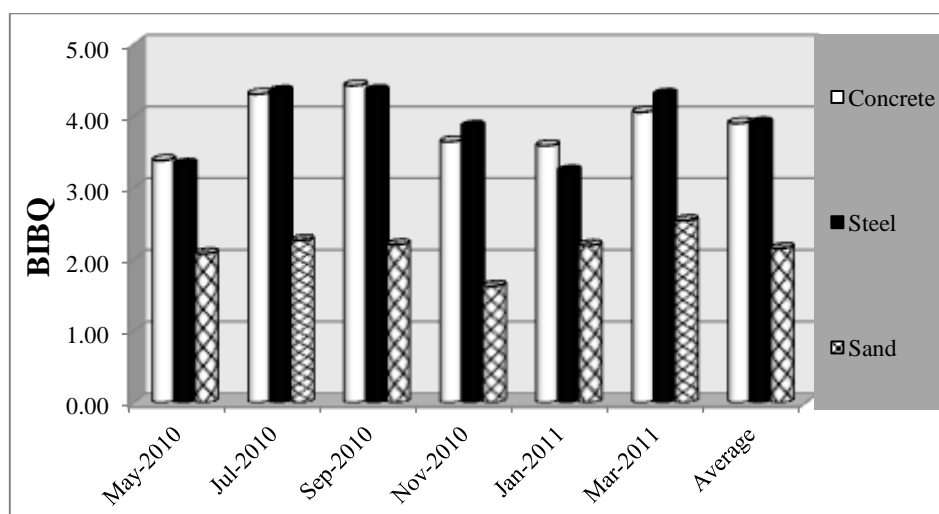


Fig. 8. The evolution of BIBQ in biofilms developed within the drinking water treatment plant, on three substrates: concrete, steel and sand.

Dynamics of bacteria belonging to different ecophysiological groups, attached to surfaces during the abstraction, processing and distribution of drinking water in Cluj, was shown in Fig. 9. A decrease in the number of bacteria may be observed after water treatment, in distribution network's biofilms, less obvious for ammonifying, sulphur oxidizing and iron reducing bacteria.

Biochemical identification with API kits (bioMérieux) of colonies isolated on agar culture media was carried out for all eight physiological groups of bacteria. Bacteria capable of ammonification, sulfur and iron reduction were found to belong to the genera *Aeromonas*,

Pseudomonas and *Burkholderia*. Isolates involved in manganese oxidation proved to belong to the species *Bacillus cereus*, *B. mycoides*, *B. subtilis* and *Paenibacillus polymyxa*.

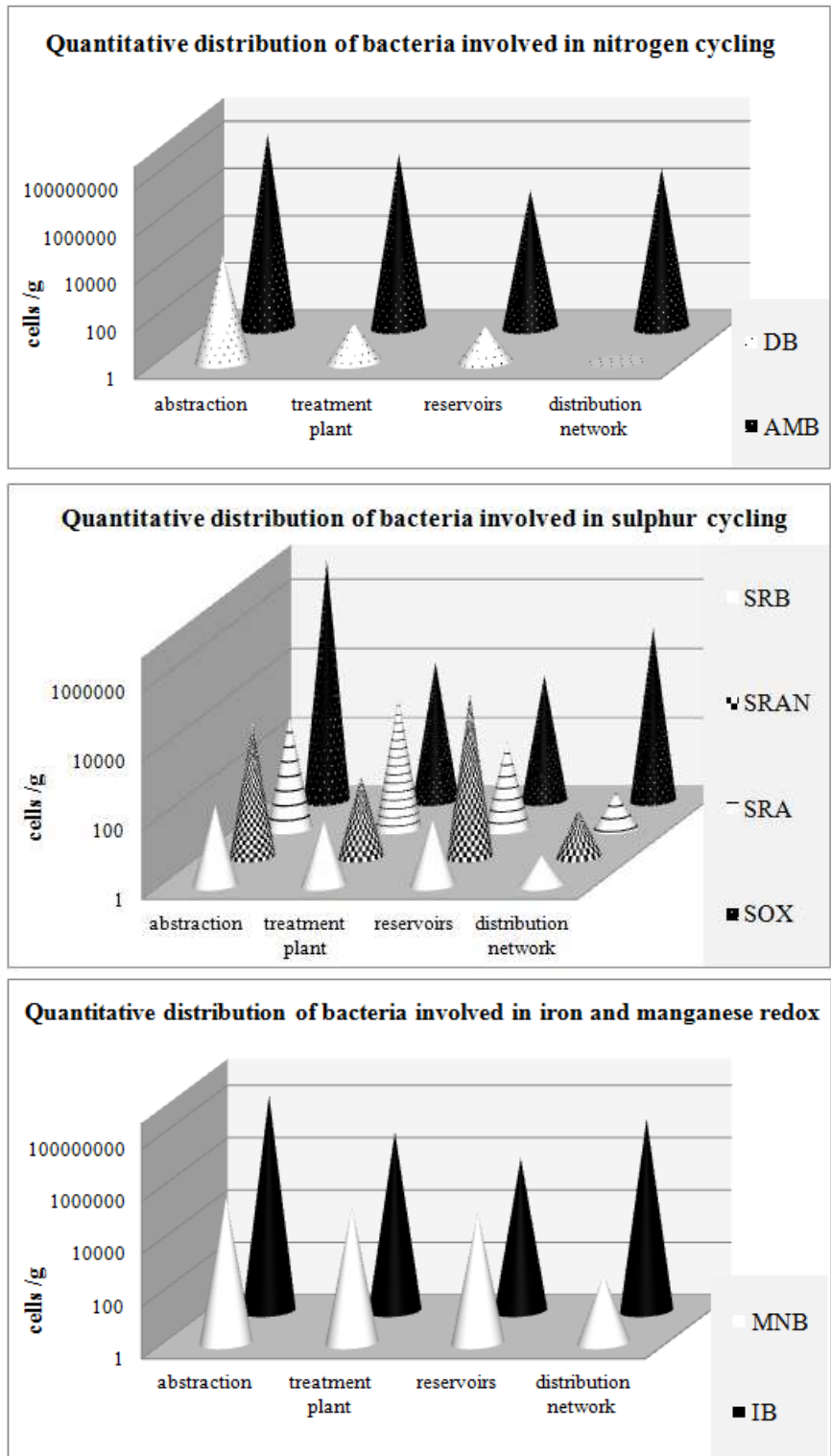


Fig. 9. Comparison in the distribution of ecophysiological groups of bacteria in biofilms occurring before, during and after the treatment process – average values 2010-2012: ammonifying (AMB), denitrifying (DB), sulphate reducing bacteria (SRB), sulphur reducing anaerobes (SRAN), sulphur reducing aerobes (SRA), sulphur oxidizing (SOX), iron reducing (IB) and manganese oxidizing bacteria (MNB).

Significance of the distribution of physiological groups of bacteria in drinking water associated biofilms

Microbial diversity and the intensity of activities in biofilms associated with drinking water result in the release of metabolites such as organic and inorganic acids, ammonia, hydrogen sulphide, as well as in the accumulation of extracellular enzymes. Such compounds may interact with surface materials, inducing biocorrosion, or may be released into the water bulk, affecting its aesthetic properties.

Routine monitoring shows that drinking water quality of Cluj is not negatively affected by the physiological groups of bacteria present in biofilm communities, in terms of ammonium, nitrite, and nitrate concentrations (laboratory and public data). Seldom consumer complaints about the organoleptic properties are typically associated with old buildings facilities and long residence time of water in the pipelines.

The enhanced mineralization processes occurring within bacterial communities suggest they are playing an important purification effect in drinking water treatment. Biofilms function as biofilters, displaying bioaccumulation and bio-adsorption properties.

In situ observations revealed that corrosion and tuberculation occurred mainly on steel surfaces. On concrete walls, consistent biofilm accumulation developed rapidly, within days during the warm season. The biocorrosion and bio-clogging potential effects of the predominant microorganisms indicated by the present study (ammonifying, iron and manganese reducing/oxidizing bacteria) should be further considered in water processing distribution in Cluj County. However, corrosion rates may not correlate perfectly with the bacteria detected in biofilms or in water, but rather to the intensity of their metabolic activity (Beech, 2003). Microbial communities associated with drinking water in Cluj proved to be very active, generating a significant bacterial recovery by *in vitro* cultivation.

Among the identified species as involved in physiological processes, the massive presence of *Aeromonas*, *Pseudomonas* and *Bacillus* members is of high significance, both from a human health perspective, as well as in terms of their role in biofouling.

Enzymatic activity in drinking water associated biofilms

In accordance with the absolute values of enzymatic activities (dehydrogenase, phosphatase and catalase), the enzymatic index of biofilm quality (EIBQ) recorded maximum values for biofilms developed in the settler, during the winter. Minimum EIBQ values were reached in the sand filter, during the warm season (Fig. 10). Two possible explanations can be suggested: the accumulation of extracellular enzymes in the biofilm matrix and cells entering a state of physiological stress.

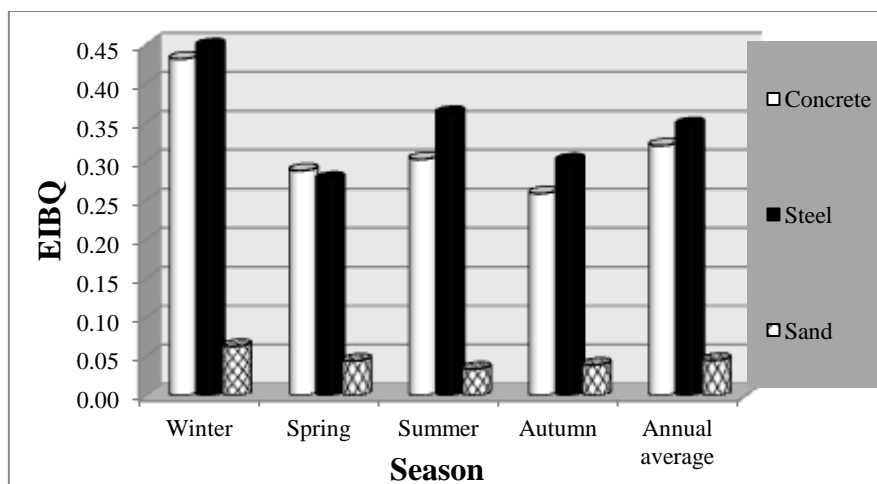


Fig. 10. Evolution of the enzymatic index of biofilm quality within the drinking water treatment plant, on three substrates: concrete, steel and sand, during 2011.

Significance of the enzymatic activity in drinking water associated biofilms

Bacterial enzyme activity in biofilms has both positive and negative effects on the infrastructure and hence on the quality of drinking water. Biofilms can be exploited in water purification, by virtue of the enhanced biodegradation and bioaccumulation properties. On the other hand, enzyme activity is one of the factors generating corrosion in pipes.

Affecting the composition and properties of the exopolymeric matrix, extracellular enzymes are influencing biofilm development (Tielen et al., 2010).

In the present study, intense enzymatic activities were registered in the settler. The clarification process is static, allowing biofilm formation and deposits accumulation. In the sand filter, where water is dynamically processed, a decrease in enzymatic activity was observed. Reductions in enzyme activities during the purification process from surface water to drinking water reflect an improvement in its trophic status, indicating that the purification process principally fulfilled its function (Emtiazi et al., 2004). A decrement in dehydrogenase activity in sand filter's biofilms, both in terms of intensity, but also the share in the total enzyme activity suggests a decrease of microbial activity during the treatment process. There were no sharp differences in catalase activity levels in clarifier's biofilms compared with those in the sand filter. The share of catalase activity in the total enzymatic activity increased in sand filter's biofilms, compared to that in the settler. This indicates an enhanced oxidative stress that bacteria have undergone, catalase being involved in cell protection in the face of environmental disturbance (Bonnineau et al., 2010). In biofilms formed during water purification, oxidative stress is represented by the treatment procedures and by the presence of chemicals, such as disinfectants.

Factors influencing bacterial communities in biofilms

Assessing data dispersion across the biofilm consortia, the coefficient of variation was less than 3%, indicating the homogeneity in all the bacterial populations investigated.

Correlation analysis of biofilm bacteria with water and biofilm's physicochemical properties, as well as statistical significance tests indicate that bacterial populations in the clarification step evolved similarly. Greater differences were registered between microbial populations attached in the clarifier, when compared with those developed in the sand filter.

It can thus be concluded that the type of the immersed material has no significant influence upon the evolution of microbial communities, rather the treatment step, suggesting that drinking water treatment processes restrict microbial growth not only in bulk fluid but in biofilms, too. These insights are particularly important regarding the ability of the three types of materials in contact with water to support growth of bacteria that can deteriorate water quality and infrastructure.

***In vitro* biofilm models**

Effect of different disinfectants against biofilm bacteria

Based on the mean values of log reduction values (LRV), the most effective product in bacterial inactivation was found to be the mixed cleaning agent Floran, followed by sodium dichloroisocyanurate, sodium hypochlorite, chloramine T and chlorine dioxide (Table 4). Floran solution has the ability to reduce bacterial viability in a percentage scale from of 96.66% (LRV = 1.477) in case of SRB, up to 99.99994% (LRV = 6.227) in case of ammonifying bacteria.

When comparing results of similar investigations, realized on planktonic cells, to disinfectants efficacy on the attached bacteria, revealed by present study, biofilm organization proves its protective features. The reduced antimicrobial impacts may be explained by slow penetration of disinfectants into the exopolymeric matrix (de Beer et al., 1994). Another explanation resides in the presumably sublethal antimicrobial dosage, which may result in adaptative stress responses within the bacterial cells. Injured bacteria may react through a series of cellular repair and response mechanisms. The effects in terms of public health risks include the emergence of resistant variants, pathogens exhibiting enhanced virulence and bacteria entering the viable but nonculturable state (Wesche et al., 2009).

The increments registered in the recovery of denitrifying bacteria from biofilms exposed to chlorine-based agents and the increased sulphate reduction registered in biofilms treated with chloramine-T may represent hormetic effects. Hormesis is a biphasic dose-response phenomenon characterized by low-dose stimulation and high-dose inhibition (Calabrese, 2008; Kaplan, 2011).

Type of bacteria	LRV (bacteria/g of biofilm)				
	1	2	3	4	5
HPC	0.969	0.826	0.929	1.108	4.523
<i>E. coli</i>	0.287	0.298	0.155	0.345	3.097
Intestinal enterococci	3.305	3.305	3.305	0.305	3.305
<i>C. perfringens</i>	1.045	0.744	1.046	0.920	4.523
<i>P. aeruginosa</i>	0.955	0.141	0.026	0.288	5.687
<i>A. hydrophila</i>	0.777	0.245	0.562	0.667	5.023
AMB	2.004	2.004	2.004	2.004	6.227
DB	-0.096	-0.176	0.203	-0.032	2.199
SRA	0.600	0.362	0.607	0.277	3.125
SRAN	0.889	0.442	0.889	0.298	1.889
SRB	0.125	0.125	-0.114	0.477	1.477
SOX	1.684	2.373	1.124	1.114	4.036
IB	2.580	2.506	1.301	1.010	4.386
MNB	0.577	0.503	0.346	0.418	1.928
Average LRV	1.122	0.979	0.885	0.657	3.673

Table 4. Log reduction value in biofilm bacteria achieved by the use of: 1 – sodium dichloroisocyanurate; 2 – sodium hypochlorite; 3 – chloramine-T; 4 – chlorine dioxide; 5 – Floran solutions.

Selection of the appropriate treatment procedures in order to achieve drinking water safety is essential. Biofilm regeneration after inefficient treatment could lead to populations of resistant bacteria, which may be recalcitrant to subsequent disinfection (Simões et al., 2004). To achieve efficient disinfection by the use of chlorine-based disinfectants, mechanical removal of biofilms prior to water sanitization is recommended. Also, investigations about the composition of microbial consortia and the physiological activities in the associated biofilms should be considered.

In conclusion, all chlorine-based agents failed to completely inactivate faecal indicators or opportunistic pathogenic bacteria. It is even more necessary to implement a water safety plan, including measures to control the associated biofilms, these reservoirs of drinking water contamination.

Molecular analysis. The presence of class 1 integrons and gene cassettes encoding antimicrobial resistance

Seven out of the 23 primer pairs tested revealed positive PCR results. Nine (9.375%) of the 96 isolates tested were found to possess class 1 integron-integrase or gene cassettes encoding resistance to antimicrobial agents (Table 5).

The presence of class 1 integrons was assessed with two pairs of primers, which target the whole integron, from *attII* site beyond the *attC* site of the last cassette in the array (Gillings et al., 2009). PCR results were negative for all isolates.

Eight isolates (8.33%) were found to possess the *intI1* integrase gene. Investigating for the presence of multidrug efflux *qac* families of genes, *qacEΔ1* was detected in 9 isolates (9.375%). The presence of *sul1* gene was identified in three isolates (3.125%). In the same cells it was found a genetic linkage between *qacEΔ1* and *sul1* genes (Fig. 11).

Isolate no.	Primer pairs / target genes						
	P3	P5	P6	P9	P13	P15	P22
	<i>intI1</i>	<i>intI1</i>	<i>intI1</i>	<i>intI1</i>	<i>qacEΔ1</i>	<i>qac,sul1</i>	<i>sul1</i>
14					x		
84	x	x			x		
88	x				x		
91	x	x			x		
92	x				x		
93	x				x		
94		x		x	x	x	x
95	x	x	x	x	x	x	x
96	x		x	x	x	x	x

Table 5. Bacterial isolates from drinking water treatment plant's biofilms, positive for class 1 integrons and gene cassettes encoding resistance to antibacterial agents.

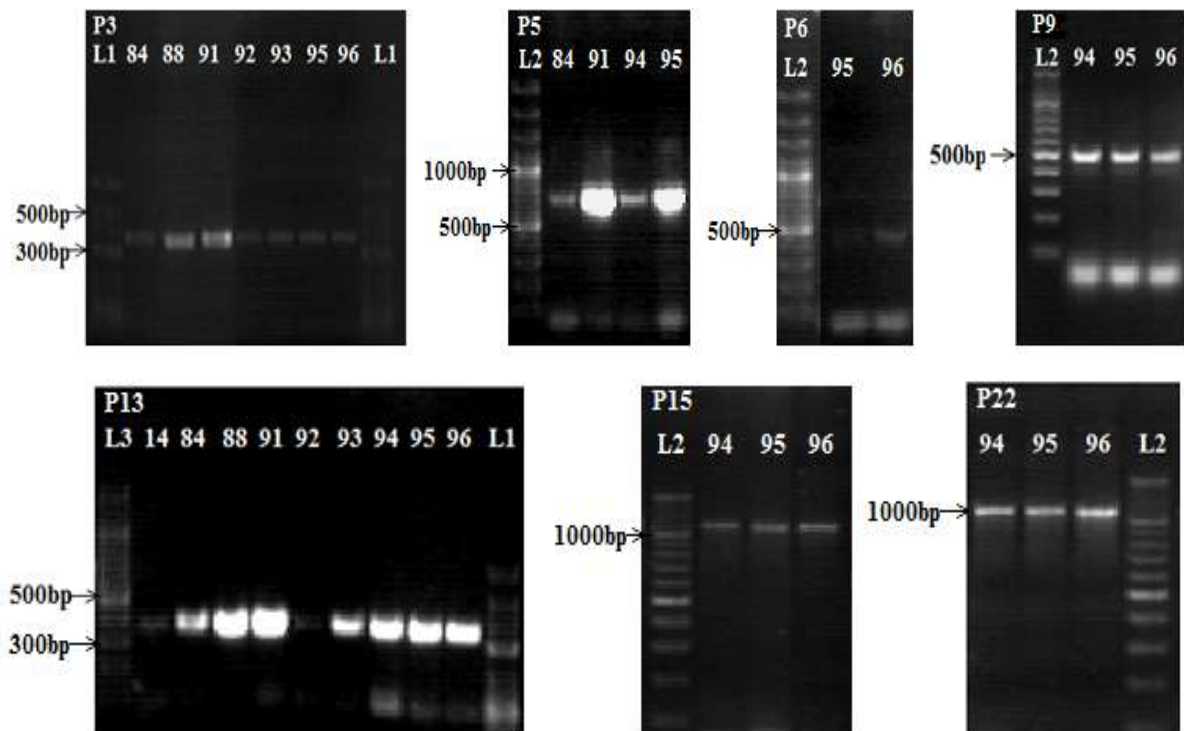


Fig. 11. PCR amplifications targeting class I integron-integrase gene (P3 – P9), *qacEΔ1* gene (P13, P15) and *sul1* gene (P15, P22). L = 1kb ladder (Fermentas SM0311), L1 = 700bp ladder (Fermentas SM1203), L2 = 1500bp ladder (Fermentas SM0623), L3 = 1000bp ladder (Fermentas, SM1133). Amplicons were separated in 1.5% w/v agarose gels in 1 x TAE buffer, stained with ethidium bromide 0.5μg/ml.

The horizontal transfer of integron-related resistance cassettes was suggested to explain the association of integrase and transposase genes (Dawes et al., 2010). No evidence of linkage between class 1 integron genes and Tn402 transposition genes was found in the present study, since none site of origin of replication (*oriV*) and transposition module (*tni*) were detected. This suggests that either other types or hybrid transposons are involved (Labbate et al., 2008), or class 1 integrons are located in bacterial chromosomes (Stokes et al., 2006; Gillings et al., 2008).

The major percent of isolates tested were collected from culture media targeting environmental microorganisms (60%) and only 40% of the colonies were selected as potential bacteria of concern. Nevertheless, the great majority of class 1 integron/cassettes bearing cells proved to be bacteria with presumable faecal origin. Additional biochemical tests and phenotypic profile based on API kits confirmed the hypothesis that mobile genetic elements triggering microbial resistance may be connected to faecal contamination. Subsequent identification of positive isolates, based on 16S rRNA gene (Fig. 12) revealed certain discrepancies at the species level (Table 6).

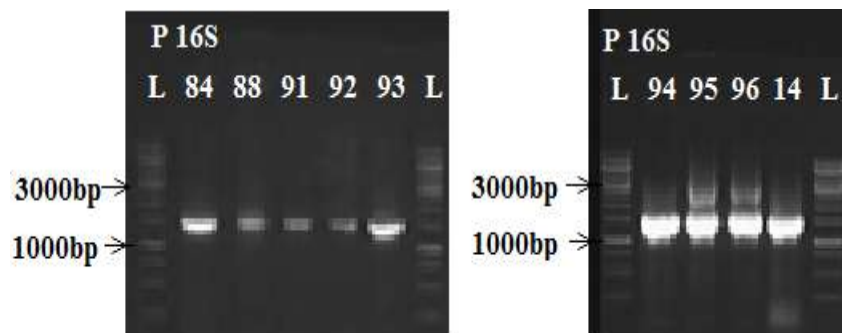


Fig. 12. PCR amplifications targeting 16S rRNA gene. L= 1kb ladder (Fermentas SM0311).

Identification results based on 16S rRNA gene sequencing showed that only one out of nine isolates (11.11%), positive for resistance determinants belongs to an environmental species, identified as *Pseudomonas fragi*. Eight isolates (88.88 %) represent enteric species or may be linked to a human or animal origin (Garrity et al., 2005). They were identified as *Clostridium barati*, *Enterococcus faecalis*, *Enterococcus saccharolyticus*, *Escherichia fergusonii*, *Klebsiella oxytoca*, *K. pneumoniae*, *Staphylococcus vitulinus* and *S. warneri*. Molecular identification validated the hypothesis that most of the bacteria triggering bacterial resistance in drinking water microbiota may be attributed to anthropogenic disturbance.

Microbiological contamination with faecal origin in Cluj drinking water sources was previously demonstrated (Lumperdeanu și Drăgan-Bularda, 2002; Curticăpean și Drăgan-Bularda, 2007; Muntean et al., 2010; Farkas et al., 2010a; b). Human activities represent a selective pressure, increasing the frequency of lateral gene transfer and influencing bacterial evolution (Stokes and Gillings, 2011).

In order to control the spread of existing and novel resistance genes, additional investigations of dissemination mechanisms in biofilms and drinking water would be appropriate. The gene pool potential in this environment should not be overlooked, although

integron-bearing strains may not always express the antimicrobial phenotype (Roe et al., 2003).

Isolate no.	Biochemical testing according to specific reference methods	Phenotypic identification with API kits			16S rRNA gene identification	
14	<i>Pseudomonas aeruginosa</i>	<i>P. putida</i>	API 20NE	64.5%	<i>P. fragi</i>	99%
84	<i>Clostridium perfringens</i>	<i>C. perfringens</i>	API 20A	97.7%	<i>C. barati</i>	95%
88	Coliform organism	Unacceptable profile	API 20STREP	-	<i>Staphylococcus vitulinus</i>	99%
91	<i>Escherichia coli</i>	<i>E. coli</i>	API 20E	91.8%	<i>Escherichia fergusonii</i>	99%
92	<i>Escherichia coli</i>	<i>Raoultella ornithinolytica</i>	API 20E	99.9%	<i>Klebsiella oxytoca</i>	99%
93	Coliform organism	<i>Klebsiella oxytoca</i>	API 20E	98.6%	<i>Klebsiella pneumoniae</i>	99%
94	Intestinal enterococcus	<i>Enterococcus faecalis</i>	API 20STREP	55.5%	<i>Enterococcus faecalis</i>	99%
95	Intestinal enterococcus	Unacceptable profile	API 20STREP	-	<i>Staphylococcus warneri</i>	99%
96	Intestinal enterococcus	<i>Enterococcus faecium</i>	API 20STREP	99%	<i>Enterococcus saccharolyticus</i>	95%

Table 6. The identification of bacterial isolates from drinking water treatment plant's biofilms, positive for class 1 integrons and gene cassettes encoding resistance to antibacterial agents.

Assessing the significance of human carriage of antimicrobial resistant *E. coli* with contaminated drinking water, Coleman (2008) concluded that the intake of resistant bacteria was 40% higher for individuals using contaminated water than for subjects consuming uncontaminated water or drinking water containing sensitive *E. coli* strains.

Besides the genetic mechanisms, factors promoting survival of bacteria in chlorinated water supplies include aggregation, attachment, starvation but also ineffective disinfection (LeChevallier et al., 1988). Biofilm insusceptibility to biocides is sometimes considered to be a tolerance due to a physiological adaptation, rather than to the presence of genetic determinants (Bridier et al., 2011). This assessment proves that genes encoding antibacterial resistance are present in biofilm consortia occurring throughout drinking water processing.

However, it is unlikely that the treatment process may have contributed to the selection of resistant variants within the investigated facility. A primary disinfection with gaseous chlorine is applied with intermittence, quarterly. The responsible use of biocides, following a professional procedure is not discouraged in situations where there is a real benefit (Gilbert and McBain, 2003), as in case of drinking water purification.

Conclusions

- The present study has analysed the microbial consortia inhabiting biofilms attached to surfaces during raw water abstraction, treatment and drinking water distribution in Cluj County, in 2010-2012. This is the first study investigating bacterial populations from biofilms occurring into a public drinking water system in Romania.
- The investigated biofilms proved to be extremely active microbial consortia, with high concentrations of cultivable bacteria: HPC, faecal indicators, opportunistic pathogens and bacteria belonging to different ecophysiological groups.
- The observed structural and temporal variability in microbial activity indicates a dynamic situation in biofilms colonizing the infrastructure, from raw water abstraction to drinking water distribution. There is a reduction in bacterial numbers per gram of biofilm in water systems downstream, suggesting that treatment and disinfection procedures act not only on planktonic microbiota but also on the attached consortia.
- Faecal indicators were detected in all the biofilm samples investigated, with few exceptions: intestinal enterococci were present neither in the abstraction pipeline's biofilms nor in reservoirs' sediment. *Clostridium perfringens* was not detected in reservoirs. Large populations of *Escherichia coli* were detected in all biofilms analyzed, especially those developed in the treatment plant.
- *Aeromonas hydrophila* were numerous in biofilms developed in the abstraction pipeline, while *Pseudomonas aeruginosa* dominated the biofilms developed in drinking water treatment plant.
- Neither *Legionella pneumophila*, nor other species belonging to *Legionella* genera were detected in any biofilm sample, in spite of different techniques applied.
- Dynamics of physiological processes indicates the predominance of ammonification, iron reduction and manganese oxidation during drinking water abstraction, processing and distribution in Cluj. All other ecophysiological groups of microorganisms, i.e. denitrifying bacteria and bacteria involved in sulphur cycle were found in smaller percentage, representing less than 0.1% of the germs detected in biofilms.
- Biochemical identification with API kits (bioMérieux) reveal that ammonifying bacteria, sulfur and iron reducers belong to the genera *Aeromonas*, *Pseudomonas* and *Burkholderia*. Bacteria involved in manganese oxidation proved to belong to the species *Bacillus cereus*, *B. mycoides*, *B. subtilis* and *Paenibacillus polymyxa*.

- The bacteriological indicator of biofilm quality (BIBQ) indicates a decrement in microbial activities during the winter and in the direction of water flow.
- The intense enzymatic processes, assessed by dehydrogenase activity, phosphatase and catalase activities, especially during winter may be attributed to enzymes accumulation in the biofilm matrix.
- Statistical analysis indicates that the immersed material has no significant influence on the composition of bacterial consortia in biofilms. All three types of materials in contact with water (concrete, steel and sand) have the ability to harbour bacterial biofilms that may deteriorate water quality and infrastructure. Treatment process was found to restrict microbial growth not only in bulk fluid but in biofilms, too.
- *In vitro* biofilm models indicate that raw water brings in the clarifier both heterotrophic mesophilic bacteria and oligotrophic bacteria. The treatment process contributes in the selection of oligotrophic psychrophilic bacteria.
- Based on the mean values of log reduction values (LRV), the most effective product in bacterial inactivation was found to be the mixed cleaning agent Floran, followed by sodium dichloroisocyanurate, sodium hypochlorite, chloramine T and chlorine dioxide
- Assessing the effect of different disinfectants against biofilm bacteria, by estimating the logarithmic reduction for fourteen types of bacteria, the most effective product proved to be the mixed cleaning agent Floran. It was able to disintegrate the biofilm matrix and to greater reduce microbial viability. Of chlorine-based disinfectants, the most effective proved to be sodium dichloroisocyanurate, followed by sodium hypochlorite, chloramine T and chlorine dioxide.
- A percentage of 9.375% of the bacterial isolates tested were identified to carry class 1 integrons and genes encoding resistance to quaternary ammonium compounds and/or to sulphonamide.
- Molecular identification based on 16S rRNA gene sequencing showed that most of bacteria positive for resistance determinants represent either enteric bacteria or species that can be attributed to a human or animal origin. They were identified as *Clostridium barati*, *Enterococcus faecalis*, *Enterococcus saccharolyticus*, *Escherichia fergusonii*, *Klebsiella oxytoca*, *K. pneumoniae*, *Staphylococcus vitulinus* and *S. warneri*.

Future perspectives

The correlated presence of faecal indicators (*Escherichia coli*, intestinal enterococci and *Clostridium perfringens*) warns about a potential risk represented by drinking water associated biofilms. Detection of faecal indicators in drinking water associated biofilms has multiple significance: on the one hand are themselves a potential risk in terms of consumers' health, on the other hand indicate the possible presence of other bacteria, viruses or protozoan pathogens.

Molecular identification validated the hypothesis that most of the bacteria triggering bacterial resistance in drinking water microbiota may be attributed to anthropogenic disturbance.

Opportunistic pathogens hosted in biofilms, such as *Escherichia coli*, *Aeromonas hydrophila* and *Pseudomonas aeruginosa* may be dispersed in the water phase. The perpetuation and dissemination of genetic elements responsible of resistance to antimicrobials is also of high concern. Their carriage with the water flow, downstream colonization, drinking water contamination and the risk of consumers' exposure should be further investigated.

Safe drinking water providing in Cluj as well as in other Romanian cities requires the effective implementation of measures such as:

- Catchments' protection;
- Control of biofilms;
- Compliance with cleaning and disinfection protocols;
- Maintenance of the integrity of the distribution systems;
- Water safety plans designing and implementation.

Routine monitoring, according to standard regulations, involves the analysis of few microbiological and physic-chemical parameters. They provide very limited information about drinking water microbiota, so it might be impossible to predict and prevent a potential hazard due to the presence of pathogenic microorganisms.

A periodic biofilm analysis, sampled from critical points, according to a water safety plan may represent a viable alternative that would comply with the recommendations of World Health Organization.

Selected references

- Alexander, M. 1982. Most probable number method for microbial populations. In: Page, A.L., Miller, R.H., Keeney, D.R. (Eds.) Methods of soil analysis, Part 2, Chemical and microbiological properties. 2nd ed. American Society of Agronomy, Madison, pp. 815-820.
- Araya, R., Tani, K., Takagi, T., Yamaguchi, N., Nasu, M. 2003. Bacterial activity and community composition in stream water and biofilm from an urban river determined by fluorescent in situ hybridization and DGGE analysis. FEMS Microbiology Ecology, 43: 111-119.
- Atlas, R.M. 2004. Handbook of Microbiological Media. 3rd ed. CRC Press, New York.
- Beech I.B., Flemming H.C. 2000. Microbiological fundamentals. In: Beech, I., Bergel, A., Mollica, A., Flemming, H.C., Scotto, V., Sand, W. (Eds.) Simple methods for the investigation of the role of biofilms in corrosion. Biocorrosion, 00-02: 3-15.
- Beech, I.B. 2003. Sulfate-reducing bacteria in biofilms on metallic materials and corrosion. Microbiology Today, 30: 115-117.
- Bonnineau, C., Bonet B., Corcoll N., Guasch H. 2010. Catalase in fluvial biofilms: a comparison between different extraction methods and example of application in a metal-polluted river. Ecotoxicology, 20: 293-303.
- Bridier, A., Briandet, R., Thomas, V., Dubois-Brissonet, F. 2011. Resistance of bacterial biofilms to disinfectants: a review. Biofouling, 27(9): 1017-1032.
- Calabrese E.J. 2008. Hormesis: why it is important to toxicology and toxicologists. Environmental Toxicology and Chemistry, 27: 1451-1474.
- Chuanchuen, R., Khemtong, S., Padungtod, P. 2007. Occurrence of *qacE/qacEA1* genes and their correlation with class 1 integrons in *Salmonella enterica* isolates from poultry and swine. Southeast Asian Journal of Tropical Medicine and Public Health, 38(5): 855-862.
- Coetser S.E., Cloete T.E. 2005. Biofouling and biocorrosion in industrial water systems. Critical Reviews in Microbiology, 31: 213-232.
- Coleman, B.L. 2008. The role of drinking water as a source of transmission of antimicrobial resistant *Escherichia coli*. PhD Thesis, University of Toronto, Department of Public Health Sciences, Toronto, Canada.
- Costerton, J.W., Cheng, K.J., Gessey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M., Marrie, T.J. 1987. Bacterial biofilms in nature and disease. Annual Reviews in Microbiology, 41: 435-464.
- Costerton, J.W. 1994. Structure of biofilms. In: Geesey, G.G., Lewandowski, Z., Flemming, H.C. (Eds.) Biofouling and biocorrosion in industrial water systems. CRC Press, pp. 1-15.
- Costerton, J.W., Wilson, M. 2004. Introducing biofilms. Biofilms. Cambridge University Press, UK, pp. 1-4.
- Curticăpean, M.C., Drăgan-Bularda, M. 2007. The microbial distribution from water and sediment of Tarnița dam reservoir. Studia UBB Biologia, 52: 67-78.
- Cușa, V. 1996. Instrucțiuni metodologice pentru analiza microbiologică a sedimentelor acvatice. Institutul de Cercetări și Ingineria Mediului, București, pp. 2-30.
- Dawes, F.E., Kuzevski, A., Bettelheim, K.A., Hornitzsky, M.A., Djordjevic, S.P., Walker, M.J. 2010. Distribution of class 1 integrons with IS26-mediated deletions in their 3'-conserved segments in *Escherichia coli* of human and animal origin. PLoS One, 5(9): e12754.
- de Beer D., Srinivasan R., Stewart P.S. 1994. Direct measurement of chlorine penetration into biofilms during disinfection. Applied and Environmental Microbiology, 60: 4339-4344.

- Donlan, R.M. 2002. Biofilms: microbial life on surfaces. *Emerging Infectious Diseases*, 8(9): 881-890.
- Drăgan-Bularda, M. 2000. *Microbiologie generală. Lucrări practice*, Editura Universității Babeș-Bolyai, Cluj-Napoca, pp.70, 175-192; 218-232.
- Dreeszen, P.H. 2003. *Biofilm*. Edstrom Industries, INC, pp. 2-18.
- Echverría, F., Castaño, J.G., Arroyave, C., Peñuela, G., Ramírez, A., Morató, J. 2009. Characterization of deposits formed in a water distribution system. *Ingeniare. Revista Chilena de Ingeniería*, 17: 275-281.
- Emtiazi, F., Schwartz, T., Marten, S.M., Krolla-Sidenstein, P., Obst, U. 2004. Investigation of natural biofilms formed during the production of drinking water from surface water embankment filtration. *Water Research*, 38: 1197-1206.
- Farkas, A., Bocoș, B., Țigan, Ș., Ciatarăș, D., Drăgan-Bularda, M., Carpa, R. 2010a. Surveillance of two dam reservoirs serving as drinking water sources in Cluj, Romania. *Balkans Regional Young Water Professionals Conference Proceedings*, Belgrad, pp. 91-97.
- Farkas, A., Ciatarăș, D., Bocoș B., Țigan, Ș. 2010b. Monitoring of water source Gilău and its affluent Someșul Rece during 2005-2009. *Applied Medical Informatics*, 26(1-2): 27-34.
- Flemming, H.C. Percival, S.L., Walker, J.T. 2002. Contamination potential of biofilms in water distribution systems. *Water Supply*, 2(1): 271-280.
- Flemming, H.C. 2009. Why microorganisms live in biofilms and the problem of biofouling. In: Costerton, J.W. (Ed.) *Springer series on Biofilms*, Vol. 4. Marine and industrial biofouling. Springer Verlag, Berlin Heidelberg, p 3-11.
- Fonseca, A.C., Summers, R.S., Hernandez, M.T. 2001. Comparative measurements of microbial activity in drinking water biofilters. *Water Research*, 35(16): 3817-3824.
- Garrity, G.M., Brenner, D.J., Krieg, N.R., Staley, J.T. 2005. *Bergey's Manual of Systematic Bacteriology*. Bergey's Manual Trust, USA.
- Gilbert, P., McBain, A.J. Rickard AH. 2003. Formation of microbial biofilm in hygienic situations: a problem of control. *International Journal of Biodeterioration and Biodegradation*, 51: 245-248.
- Gillings, M.R., Krishnan, S., Worden, P.J., Hardwick, S.A. 2008. Recovery of diverse genes for class 1 integron-integrases from environmental DNA samples. *FEMS Microbiology Letters*. 287: 56-62.
- Gillings, M.R., Xuejun, D., Hardwick, S.A., Holley, M.P. 2009. Gene cassettes encoding resistance to quaternary ammonium compounds: a role in the origin of clinical class 1 integrons? *The ISME Journal*, 3: 209-215.
- Glasmacher, A., Engelhart, S., Exner, M. 2003. Infections from HPC organisms in drinking-water amongst the immunocompromised. In: Bartram, J., Cotruvo, J., Exner, M., Fricker, C., Glasmacher, A. (Eds.) *Heterotrophic Plate Counts and Drinking-water Safety*. IWA Publishing, Londra, UK, pp. 137-145.
- Hamilton, M.A. 2010. The log reduction measure of disinfectant efficacy. Center for Biofilm Engineering, (<http://www.biofilm.montana.edu/files/CBE/documents/KSA-SM-07.pdf>).
- Hendel, B., Marxsen, J., Fiebig, D., Preuß, G. 2001. Extracellular enzyme activities during slow sand filtration in a water recharge plant. *Water Research*, 35(10): 2484-2488.
- Holmes, A.J., Gillings, M.R., Nield, B.S., Mabbutt, B.C., Helena Nevalainen, K.M., Stokes, H.W. 2003. The gene cassette metagenome is a basic resource for bacterial genome evolution. *Environmental Microbiology*, 5(5): 383-394.
- Kaplan J.B., Jabbouri, S., Sadovskaya, I. 2011. Extracellular DNA-dependent biofilm formation by

- Staphylococcus epidermidis* RP62A is response to subminimal inhibitory concentrations of antibiotics. *Research in Microbiology*, 162(5): 525-541.
- Krumbein W.E., Altman H.J. 1973. A new method for the detection and enumeration of manganese oxidizing and reducing microorganisms. *Helgolander Wissenschaftliche Meeresuntersuchungen*, 15: 347-356.
- Labbate, M., Chowdhury, P.R., Stokes, H.W. 2008. A class 1 integron present in a human commensal has a hybrid transposition module compared to Tn402: evidence of interaction with mobile DNA from natural environments. *Journal of Bacteriology*, 190 (15): 5318-5327.
- Långmark J., Storey, M.V., Ashbolt, N.J., Stenström, T.A. 2004. Artificial groundwater treatment: biofilm activity and organic carbon removal performance. *Water Research*, 38: 740-748.
- LeChevallier, M.W., Cawthon, C.D., Lee, R.G. 1988b. Factors promoting survival of bacteria in chlorinated water supplies. *Applied and Environmental Microbiology*. 54(3): 2492-2499.
- LeChevallier, M.W. 2003. Conditions favouring coliform and HPC bacterial growth in drinking-water and on water contact surfaces. In: Bartram, J., Cotruvo, J., Exner, M., Fricker, C., Glasmacher, A. (Eds.) *Heterotrophic Plate Counts and Drinking-water Safety*. IWA Publishing, London, UK, pp. 177-197.
- LeChevallier M.W., Au K.K. 2004. *Water treatment and pathogen control*. IWA Publishing, London, (http://www.who.int/water_sanitation_health/dwq/en/watreatpath.pdf).
- Lee, D.G., Kim, S.J., Park, S.J. 2006. Effect of reservoirs on microbiological water qualities in a drinking water distribution system. *Journal of Microbiology and Biotechnology*, 16:1060-1067.
- Lumperdeanu, M.C., Drăgan-Bularda, M. 2002. Bacteriological and enzymological researches on the water and sediment in the Gilău dam reservoir – Cluj County. *Contribuții Botanice*, 37: 239-249.
- Maloy, S., Schaechter, M. 2006. The era of microbiology: a Golden Phoenix. *International Microbiology*, 9: 1-7.
- Márquez, C., Labbate, M., Raymondo, C., Fernández, J., Gestal, A.M., Holley, M., Borthagaray, G., Stokes, H.W. 2008. Urinary tract infections in South American population: dynamic spread of class 1 integrons and multidrug resistance by homologous and site-specific recombination. *Journal of Clinical Microbiology*, 46(10): 3417-3425.
- Moritz, M.M. 2011. Integration of hygienically relevant bacteria in drinking water biofilms on domestic plumbing materials. PhD Thesis. Biofilm Centre. Duisburg-Essen University, Germania, pp. 2; 21-24; 123-126; 134-137.
- Muntean, V., Maier, C.G., Carpa, R., Mureșan, C., Farkas, A. 2010. Microbiological and enzymological study on sediments and water of the river Someșul Mic upstream the Gilău treatment Plant. *Studia UBB, Biologia*, 55(1): 131-138.
- Mureșan, C., Farkas, A., Vele, D., Chakirou, C. 2010. Studiu asupra surselor proprii de apă – fântâni și izvoare – din județul Cluj. *Provocările Noilor Tehnologii în Managementul Apei*. Arad, Asociația Română a Apei. Fascicula conferinței, pp. 7-18.
- O'Connor, T.L., O'Connor, T.J. 2001. Water quality deterioration in distribution systems. *Water Engineering and Management*, 148: 16-19.
- Popovici, C., Veraart, R., van de Kerk, G. 2008. România, către o societate durabilă. *Fundația pentru o Societate Durabilă*, (<http://www.romaniadurabila.net/romania%20catre%20o%20societate%20durabila.pdf>).

- Roe, M.T., Vega, E, Pillai, S.D. 2003. Antimicrobial resistance markers of class 1 and class 2 integron-bearing *Escherichia coli* from irrigation water and sediments. *Emerging Infectious Diseases*, 9(7): 822-826.
- Roșu, C., Costan, C., Arghiuș, V., Costin, D., Baciu, C. 2010. Water quality in Cluj's private drinking water wells. *Studia UBB Ambientum*, 55(1-2): 119-126.
- Simões M., Pereira M.O., Vieira M.J. 2004. Biofilm recovery after treatment with an anionic and cationic surfactant at sublethal concentrations. *Biofilms Conference 2004: Structure and Activity of Biofilms*, Las Vegas, USA, pp. 200-204.
- Skraber, S., Schijven, J., Gantzer, C., de Roda Husman, A.M. 2005. Pathogenic viruses in drinking water biofilms: a public health risk? *Biofilms*, 2: 105-117.
- Stokes, H.W., Nesbø, C.L., Holley, M., Bahl, M.I., Gillings, M.R., Boucher, Y. 2006. Class 1 integrons potentially predating the association with Tn402-like transposition genes are present in a sediment microbial community. *Journal of Bacteriology*, 188(16): 5722-5730.
- Stokes, H.W., Gillings, M.R. 2011. Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into Gram-negative pathogens. *FEMS Microbiology Reviews*, 35(5): 790-819.
- Tellen, V., Nkeng, G., Dentel, S. 2010. Improved filtration technology for pathogen reduction in rural water supplies. *Water*, 2: 285-306.
- Tielen, P., Rosenau, F., Wilhelm, S., Jaeger, K.E., Flemming, H.C., Wingender, J. 2010. Extracellular enzymes affect biofilm formation of mucoid *Pseudomonas aeruginosa*. *Microbiology*, 156: 2239-2252.
- Toporski, J., Steele, A., McKay, D.S. 2003. Bacterial biofilms in astrobiology: the importance of life detection. In: Krumbein, W.E., Paterson, D.M., Zavarzin, G.A. *Fossil and recent biofilms: a natural history of life on earth*. Kluwer Academic Publishers, Dordrecht, Olanda, pp. 429-446.
- Videla H.A., Characklis, W.G. 1992. Biofouling and microbially influenced corrosion. *International Biodegradation and Biodeterioration*, 29: 195-207.
- Webb, J.S. 2007. Differentiation and dispersal in biofilms. In: Kjelleberg, S., Givskov, M. (Eds.) *The biofilm mode of life: mechanisms and adaptations*. Horizon Bioscience, Norfolk, UK, pp. 165-174.
- Wesche A.M., Gurthler J.B., Marks B.P., Ryser E.T. 2009. Stress, sublethal injury, resuscitation, and virulence of bacterial foodborne pathogens. *Journal of Food Protection*, 5: 1121-1138.
- West, S.A., Diggle, S.P., Buckling, A., Gardner, A., Griffin, A.S. 2007. The social lives of microbes. *The Annual Review of Ecology, Evolution and Systematics*, 38: 53-77.
- Westall, F., Steele, A., Toporski, J., Walsh, M., Allen, C., Guidry, S., McKay, D., Gibson, E., Chafetz, H. 2000. A 3.8 b.y. history of bacterial biofilms and their significance inn the search for extraterrestrial life. 31st Annual Lunar and Planetary Science, Texas, USA. Abstract 1707.
- Wingender, J., Flemming, H.C. 2011. Biofilms in drinking water and their role as reservoir for pathogens. *International Journal of Hygiene and Environmental Health*, 213(3): 190-197.
- WHO 2008. *Guidelines for drinking-water quality*, Vol. 1: 3rd ed., Recommendations, Geneva, pp. 1-294.
- Zarnea, G., Popescu, O.V. 2011. *Dicționar de microbiologie generală și biologie moleculară*. Editura Academiei Române, București, pp. 187-188.

List of publications

Personal publications related to the thesis

1. **Farkas, A.**, Bocoș B., Țigan, S., Mureșan, C., Chira R. 2009. Experimental biofilms with drinking water treatment plant origin; evaluation of nutrient concentration and temperature influences upon their development. *Analele Universității din Oradea. Fascicula Biologie*, 16(2): 66-69.
2. **Farkas, A.**, Ciatarăș, D. 2010. Biofilmele din stația de tratare a apei: grupe fiziologice de bacterii implicate în coroziune. *Romaqua*, 74: 10-21.
3. **Farkas, A.**, Ciatarăș, D., Bocoș, B., Țigan, Ș. 2012. Biofilms impact on drinking water quality. In: Vouduris, K., Voutsas, D. (Eds.) *Ecological Water Quality - Water Treatment and Reuse*. In-Tech, Rijeka, Croatia, pp. 141-160.
4. **Farkas, A.**, Drăgan-Bularda, M., Ciatarăș, D., Bocoș B., Țigan Ș. 2012. Opportunistic pathogens and faecal indicators in drinking water associated biofilms in Cluj, Romania. *Journal of Water and Health*. doi:10.2166/wh.2012.148. IF = 1,367.
5. **Farkas, A.**, Carpa, R., Muntean, V., Drăgan-Bularda, M. 2012. Spatial and temporal variations of enzymatic activity in biofilms occurring into a drinking water treatment plant in Cluj, Romania. *Studia Universitatis Babeș-Bolyai, Biologia*, 57(1), 83-98.
6. **Farkas, A.**, Drăgan-Bularda, M. 2012. Biofilmul: o nouă paradigmă în microbiologie, *Revista de Studii și Cercetări – Biologie*. Muzeul Județean Bistrița (*in press*).

Other publications of the author

1. **Farkas, A.**, Chira, R., Bocoș, B., Țigan, Ș. 2009. Dezvoltarea durabilă și apa supusă potabilizării. Studiu de caz: monitorizarea în anul 2008 a unei ape de suprafață utilizată pentru potabilizare în județul Cluj. *Romaqua*, 61: 37-42.
2. **Farkas, A.**, Ciatarăș, D., Bocoș B., Țigan, Ș. 2010. Monitoring of water source Gilău and its affluent Someșul Rece during 2005-2009. *Applied Medical Informatics*, 26(1-2): 27-34.

3. **Farkas, A.**, Bocoș B., Țigan, Ș., Ciatarăș, D., Drăgan-Bularda, M., Carpa, R. 2010. Surveillance of two dam reservoirs serving as drinking water sources in Cluj, Romania. Balkans Regional Young Water Professionals Conference Proceedings, Aprilie 2010, Belgrad, Serbia, pp. 91-97.

4. Muntean, V., Maier, C. G., Carpa, R., Mureșan, C., **Farkas, A.** 2010. Microbiological and enzymological study on sediments and water of the river Someșul Mic upstream the Gilău (Cluj County) treatment plant. Studia Universitatis Babeș-Bolyai, Biologia, 55(1): 131-138.

5. Mureșan C., **Farkas A.**, Vele D., Chakirou, C. 2010. asupra surselor proprii de apă (fântâni și izvoare) din județul Cluj. Romaqua, 73: 38-42.

6. **Farkas, A.**, Bogătean, M., Ciatarăș, D., Bocoș, B., Țigan, Ș. 2011. The new water source of Cluj brings improvements in raw water quality. Danube – Black Sea Regional Young Water Professionals Conference Proceedings, București, pp. 3-9.