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EFFECT OF DIFFERENT DISINFECTANTS AGAINST BIOFILM BACTERIA

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SUMMARY. Drinking water biofilms represent a potential reservoir for water contamination. The biofilm mode of life provides multiple advantages for its inhabitants, including specific mechanisms of resistance against antimicrobials. The aim of the present study is to assess the effect of several disinfectants on biofilm consortia. The experiment was set in order to address the most stringent issues in drinking water systems: biofilms resilience, microbial diversity and bacterial resistance. Four chlorine-based agents commonly used in drinking water treatment (sodium dichloroisocyanurate, sodium hypochlorite, chloramine-T and chlorine dioxide) and one mixed cleaning agent (containing sulphamic acid, hydrochloric acid, hydrogen peroxide and acetic acid) were tested for their antibacterial properties. The assessment of disinfectants' efficacy on a wide variety of bacteria brings novel outcomes. The average log reduction values (LRV) indicated the mixed cleaning agent as the most efficient product in bacterial inactivation (LRV = 3.673), followed by sodium dichloroisocyanurate (LRV = 1.122), sodium hypochlorite (LRV = 0.979), chloramine-T (LRV = 0.885) and chlorine dioxide (LRV = 0.657).

Keywords: biofilm bacteria, chlorine-based disinfectants, drinking water, mixed cleaning agents.

Introduction

Drinking water safety is the priority of both the professionals in drinking water industry as well as of the public health authorities. Although waterborne diseases occur rarely in developed countries, outbreaks with public health risks were reported in the near past due to malfunctioning of drinking water treatment plants and distribution networks, which failed to maintain an adequate level of disinfectant to

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prevent the growth of pathogens and/or harboured the pathogens (Simões and Simões, 2013). The assessment of treatment efficiency in drinking water treatment plants is accomplished by routine monitoring of inlet and outlet bulk fluids. Microbiological monitoring is based on the values of planktonic bacteria: heterotrophic plate counts (HPC) and faecal indicators (coliform bacteria, Escherichia coli, intestinal enterococci and Clostridium perfringens). HPC offers general and unspecific information on the water microbiota. The presence of faecal indicators warns of microbial contamination with pathogenic bacteria, viruses or protozoa. However, multispecies biofilms proved to be the main mode of bacterial organisation in aquatic environments (Costerton *et al*., 1987). A dynamic exchange of individuals occurs between the attached and planktonic state. The biofilm mode of life provides multiple advantages for its inhabitants, including specific mechanisms of resistance against antimicrobials: slow penetration of disinfectant, stress response defence, metabolic gradients and the presence of persister cells (Chambless *et al*., 2006). Drinking water-associated biofilms may harbour pathogenic species, thus representing a potential reservoir for water contamination (Szewzyk *et al.*, 2000; Wingender and Flemming, 2011; Farkas *et al*., 2012).

In order to overcome the microbiological hazards in drinking water, a number of successive treatment procedures are generally used. Current practices involve multiple barriers to remove raw water pollution and to control bacterial regrowth in distribution systems (Butiuc-Keul, 2014). Chemical disinfection is considered the essential and most direct treatment to inactivate or destroy pathogenic and other microbes in drinking water (Sobsey, 2002). In Europe, it often consists in pretreatment oxidation, primary disinfection and secondary inactivation. While the first two procedures target the optimal removal of raw water contaminants, the last one aims to restrict microbial growth in drinking water distribution systems by maintaining disinfectant residuals at certain levels. Technical barriers (coagulation-flocculation, precipitation, adsorption and filtration) are also designed to modify chemical and physical properties. Such treatments result in assimilable organic carbon reducing, rather than pathogen elimination (Stanfield *et al*., 2003). However, nutrient limitation in bulk water restricts microbial multiplication.

Chemical disinfection procedures are designed based on the type (surface or groundwater) and the quality of the source (LeChevallier and Au, 2004), using gaseous chlorine, monochloramine, chlorine dioxide, ozone, as well as UV irradiation. Chlorination is traditionally applied in drinking water treatment, especially as primary disinfection. Gaseous chlorine is either used for shock chlorination, or dosed as a residual disinfectant. A disadvantage of chemical treatment is the release of trihalometanes (THM) and of other halogenated disinfection by-products (DBP). However, the risks to human health from DBP are extremely small in comparison with the risks associated with inadequate disinfection. By-product formation may be controlled by treatment process optimization (WHO,

2008). The poor efficacy of residual chlorine disinfectant in drinking water to inactivate waterborne pathogens in distribution systems has been observed previously (Payment, 1999). Alternative agents available to water systems exceeding drinking water standards in DBP precursors removal include chlorine dioxide and monochloramines. While chlorine dioxide is increasingly used as either primary or secondary disinfectant, monochloramine is recommended and used as secondary disinfectant only, due to its longer persistence and biofilm penetration (WHO, 2000; LeChevallier and Au, 2004).

The present study investigates the bactericide and bacteriostatic effect of five solutions against biofilm consortia. The experiment was set in order to adress the most stringent issues in drinking water systems: biofilms resilience, microbial diversity and bacterial resistance. Antimicrobial substances commonly used in water industry as well as commercially available products recommended for water disinfection were chosen to be tested. The aim of the paper is to compare the bacterial inactivation performance of conventional chlorine-based disinfectants with the antimicrobial efficiency of a mixed cleaning agent. The hypothesis to be tested is that an innovative solution to be used for drinking water systems cleaning and disinfection should have bactericidal effects and also be capable to disintegrate the biofilm matrix.

Materials and methods

Sampling

In order to obtain mature and structured biofilms, polypropylene coupons with a surface area of 60 cm^2 were immersed for 90 days (Boe Hansen *et al.*, 2002; Martiny *et al.*, 2003) in the settling step of a drinking water facility in Cluj (Fig. 1).

The raw water is abstracted from Tarniţa Lake, falling within A1 quality category (Farkas *et al*., 2011). During the biofilm growth, the average values for few physico-chemical parameters were recorded, as following: temperature 10.8 °C, turbidity 1.52 NTU, pH 7.35, organic substances 2.32 mg/L, dissolved oxygen 10.16 mg/L, total organic compounds 2.64 mg/L, ammonium 0.04 mg/L, nitrites 0.015 mg/L and nitrates 2.78 mg/L.

Disinfection procedure

The polypropylene coupons containing the 90-day-old biofilms were collected and transported to laboratory. All laboratory procedures were performed in sterile conditions. Five biofilm coupons were exposed to disinfectant solutions, while a control coupon was kept in sterile raw water, for two hours. Three chlorine compounds-generating reagents (sodium dichloroisocyanurate, sodium hypochlorite and chloramine-T), one chlorine dioxide-generating product and one mixed cleaning agent (containing hydrochloric acid, sulphamic acid and hydrogen peroxide) were tested for their antibacterial properties (Table 1). Disinfectant solutions were

prepared in sterile raw water, based on laboratory or commercially available products, as shown in Table 1. Solid compounds (Clorom, Chloramine-T tablets) were dissolved in sterile raw water. Liquid reagents (sodium hypochlorite) were diluted in sterile raw water. Aliquots of stock solutions were diluted in sterile raw water up to a concentration of 1.1mg/L free chlorine/chloramine (SR EN ISO 7393-1/2002) and of 1.1mg/L chlorine dioxide. Chlorine dioxide was generated by mixing the precursors delivered in kits, as per manufacturers' instructions. The mixed cleaning agent Floran was prepared using three solutions delivered by the producer: Topix, Filtrasan and Oxis.

Chlorine and chloramine consumptions were measured after disinfection. Disinfectant residuals were inactivated by the addition of 0.5% sodium thiosulphate solution (SR EN ISO 19458/2007).

Table 1.

Disinfectants tested for their antibacterial effect on drinking water biofilms

Active components	**Commercial name**	**Producer**
Sodium dichloroisocyanurate	Clorom	G&M, Romania
Sodium hypochlorite	Sodium hypochlorite solution	Penta, Czech Republic
N-chloro-*p*-toluene sulphonamide	Chloramine-T	Sintofarm, Romania
Chlorine dioxide	TwinOxide	TwinOxide, Netherlands
Topix 40% vol: sulphamic acid, phosphatidic acid; Filtrasan 40% vol: hydrochloric acid, sodium triphosphate, lactic acid, ascorbic acid; Oxis 20% vol: hydrogen peroxide, acetic acid, peracetic acid.	Floran	Mosslein, Germany

Biofilm analyses

Biomass was harvested from each coupon and 7 g biofilm were homogenized. Two subsamples of 1 g weight each were further used for preparing the stock suspensions. Two series of dilutions up to 10^{-9} (control biofilm) and 10^{-6} (disinfected biofilm) were prepared from each subsample (SR EN ISO 6887-1/ 2002).

Different culture media, specific for the fourteen types of bacteria targeted were inoculated with three to five successive dilutions. Viable and cultivable heterotrophic bacteria, faecal indicators, opportunistic pathogens, bacteria involved in nitrogen and sulphur cycling together with iron and manganese bacteria were estimated per gram of biofilm.

HPCs were enumerated in R2A Agar, after 7 days incubation at 22°C (SR EN ISO 6222/2004). The presence of opportunistic pathogens (*Aeromonas hydrophila* and *Pseudomonas aeruginosa*) and of faecal indicators (*Escherichia coli*, intestinal enterococci, *Clostridium perfringens*) was assessed by membrane filtration, as described by available standard methods.

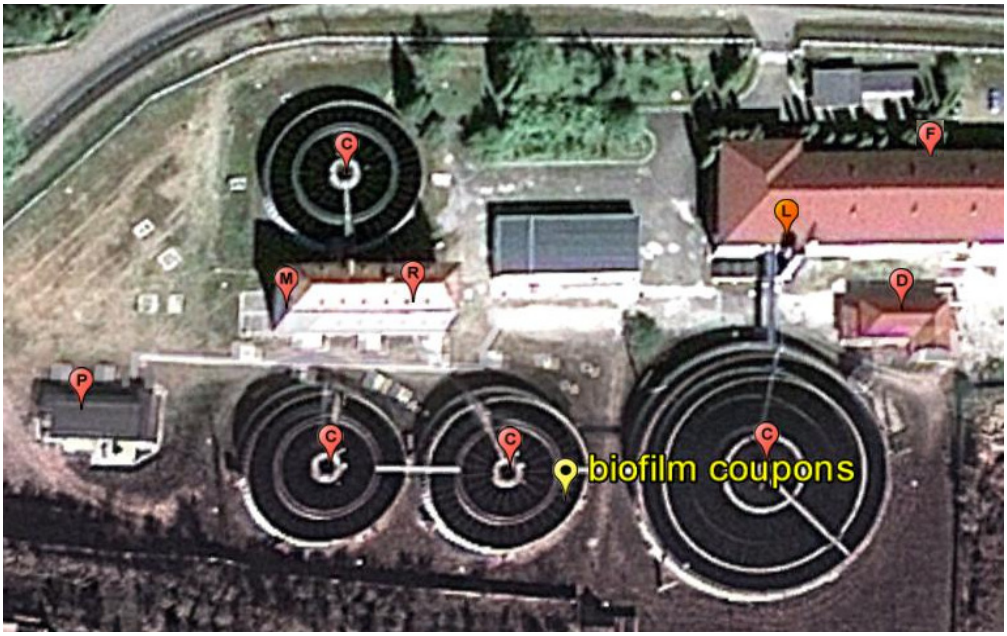

Figure 1. The drinking water treatment plant of Cluj with the position of the immersed polypropylene coupons. M – microstraining; P – prechlorination; R – reagents addition for coagulation and flocculation; C – clarification; F – rapid sand filtration; D – final disinfection by chlorination; L – laboratory.

The incidence of *A. hydrophila* in biofilm samples was measured by inoculation in 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 vibriostatic agent O129 (2,4-diamino-6,7-diisopropyl pteridine) (HPA W9, US EPA 1605). *P. aeruginosa* was cultured on cetrimide agar, counting the fluorescent, oxidase-positive colonies (HPA W6, SR EN ISO 16266/2008). Typical yellow colonies with yellow discoloration developed on Chapman Agar were considered *E. coli* if oxidase-negative and indole positive (SR EN ISO 9308-2/1990). Red to brown colonies on Slanetz and Bartley Agar were enumerated as enterococci if able to produce bile aesculin hydrolysis (SR EN ISO 7899-2/2002). Presumptive colonies of *C. perfringens* were detected on mCP agar, if turned pink to purple after exposure to ammonium hydroxide (Council Directive 98/83/EEC).

Specific growth media were prepared for diverse physiological groups of bacteria, estimated by the most probable number method (Farkas *et al*., 2013). Ammonifying bacteria grown in Peptone Broth supplemented with mineral salts and red phenol were detected by the addition of Nessler reagent. Denitrifying bacteria cultured in Allen Broth were able to produce gaseous nitrogen and nitrogen oxides. Peptone medium, as well as Oppenheimer and Gunkel Broth, Starkey Broth and Postgate Broth were used for the recovery of bacteria involved in sulphur cycling, based on their ability to produce hydrogen sulphide (sulphur reducing bacteria), to precipitate sulphide and iron salts (sulphate reducing bacteria) or to generate sulphur deposits (sulphur oxidizers). Iron reducing bacteria were detected in Ottow's culture medium, recognized by the pink staining of the bivalent iron ions resulted from Fe^{3+} reduction with α-α-dipiridil. Manganese bacteria were grown in modified Manganese Agar and stained with leucoberbelin blue.

Statistics

The experimental procedure was performed in duplicate. Descriptive analyses including average, mean and standard deviation were calculated. Statistical significance testing was performed to assess the differences between data obtained in the two series of biofilm suspensions. Student test was carried out to verify whether the differences in the two series of biofilm suspensions were significant. Because of the limited replications, Wilcoxon rank-sum test was run for each parameter separately. Microsoft Office Excel and Wessa Statistics Software (Holliday, 2012) were used.

The efficiency of each disinfectant was assessed based on the log reduction value (LRV) for each type of bacteria (Hamilton, 2010), based on Eq. (1):

$$LRV = \log10 \text{ (viable bacteria in control / viable bacteria in disinfected biofilm)} \quad (1)$$

Results and discussion

Viable and cultivable HPC, opportunistic pathogens, faecal indicators, bacteria involved in nitrogen cycling, iron and manganese bacteria and bacteria involved in sulphur cycling were measured in control and in disinfected biofilms (Fig. 2). This is the first extensive research on such a wide variety of bacteria. Previously, the antimicrobial effect of different types of disinfectants was assessed mostly on HPC and faecal indicators, especially *E. coli* (LeChevallier *et al*., 1988; Gagnon *et al*., 2005; Volk *et al*., 2010; Kephart and Stoeckel, 2011).

A similar eficacy in reducing bacterial growth was observed in biofilms treated with sodium dichloroisocyanurate and sodium hypochlorite. Differences were observed in their action against opportunistic pathogens and sulphur bacteria.

Sodium dichloroisocyanurate proved to better inactivate aeromonads, pseudomonads and *Clostridium*. It also reduced the viability of sulphur reducers to a greater extent, compared to sodium hypochlorite, while the last registered a higher antibacterial effect against sulphur oxidizers.

When added to water, sodium dichloroisocyanurate rapidly hydrolyses to release free available chlorine and establishes a complex series of equilibria involving six chlorinated and four non-chlorinated isocyanurates (Kuznesov, 2004). The overall hydrolysis reaction can be considered as (Eq. 2):

$$C_3N_3O_3Cl_2Na + 2H_2O \leftrightarrow C_3N_3O_3H_2Na + 2HOCl \quad (2)$$

The antimicrobial effect of chlorine in water is based on its products of dissociation, hypoclorous acid (HOCl) and hypoclorite ions (OCl-), where act as oxidants. They can remove or assist in the removal of some chemicals: pesticides, manganese (II), iron (II), arsenite, hydrogen sulphide, sulphite, bromide, iodide, and nitrite (WHO, 2000; 2003; 2007).

$$Cl_2 + H_2O \rightarrow HOCl + H^+ + Cl^- \quad (3)$$

$$HOCl \leftrightarrow H^+ + OCl^- \quad (4)$$

Sodium hypochlorite disinfection is also based on the action of hypochlorite ions resulted from hypochlorous acid dissociation:

$$NaOCl + H_2O \rightarrow HOCl + Na^+ + OH^- \quad (5)$$

When using sodium dichloroisocyanurate in water disinfection, hypochlorous acid is consumed into an oxidation reaction with organic material, while chloroisocyanurates function as reservoir chlorine, rapidly dissociating to release more HOCl (WHO, 2007). This may explain the higher efficiency of sodium dichloroisocyanurate in biofilm bacteria inactivation, when compared with sodium hypochlorite.

Chloramine-T and chlorine dioxide had similar effects against biofilm bacteria, with one major exception. Intestinal enterococci displayed an increased resistance to chlorine dioxide, while no enterococcal growth was observed in any other disinfected sample. Chlorine dioxide was the second most efficient solution against *E. coli* bacteria, after Floran mixed cleaning agent.

Chloramine-T used in this experiment is an organic chloramine, different than generic chloramine resulted from the combination of chlorine with ammonia. In water, chloramine-T, as a sodium salt of chlorinated arylsulphonamide, dissociates to yield hypochlorite and the sulphonamide moiety. It is therefore used as a mild disinfectant and a biocide.

$$CH_3\text{-}C_6H_4\text{-}SO_2NClNa + H_2O \leftrightarrow CH_3\text{-}C_6H_4\text{-}SO_2NH_2 + OCl^- + Na^+ \quad (6)$$

Chlorine dioxide is a powerful oxidizing agent that dissolves in water, decomposing with the slow formation of chlorite and chlorate:

$$2ClO_2 + H_2O \rightarrow ClO_2^- + ClO_3^- + 2H^+ \quad (7)$$

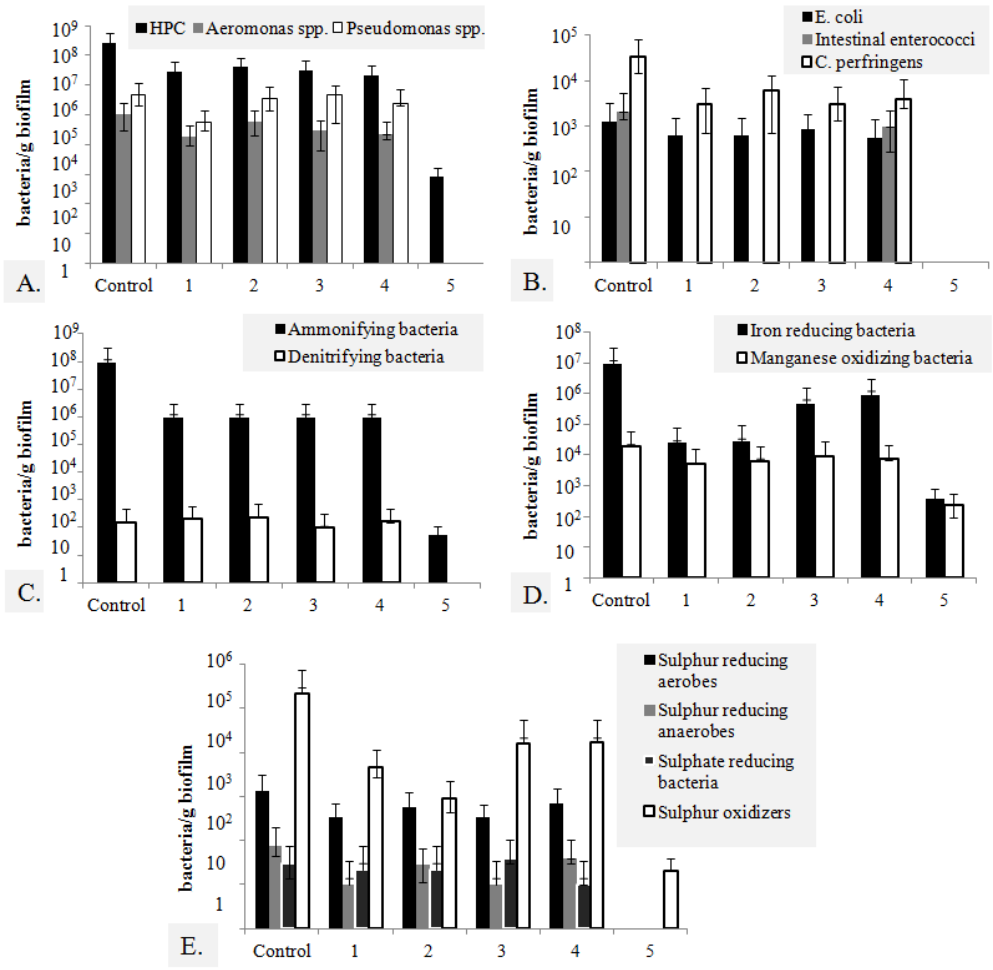

Figure 2. Comparative colony counts in control biofilm and in disinfected biofilms after applying: 1 – sodium dichloroisocyanurate; 2 – sodium hypochlorite; 3 – chloramine-T; 4 – chlorine dioxide; 5 – Floran. A – HPC and opportunistic pathogens; B – faecal indicators; C – bacteria involved in nitrogen cycling; D – iron and manganese bacteria; E – bacteria involved in sulphur cycling

In drinking water, 50-70% of chlorine dioxide is converted to chlorite and 30% to chlorate and chloride (Werdehoff and Singer, 1987). It can be involved in a variety of redox reactions, such as the oxidation of iodide, sulphide, iron (II) and manganese (II) ions (WHO, 2000).

Chloramine-T solution was observed to be the only disinfectant with antimicrobial effect against denitrifying bacteria. It had no inhibitory action against sulphate reducing bacteria (SRB). Rather the contrary, an increment in sulphate reduction occurred within the biofilms exposed for two hours in 1.1 mg / L chloramine-T solution. Such results may explain the enhanced corrosion rates in pipe surfaces when applying chloramine-T disinfection, SRB being recognised as one important physiological group of bacteria involved in biocorrosion (Beech and Flemming, 2000; Coetser and Cloete, 2005). Sungur *et al*. (2010) also found chloramine (1.5 ppm, for 3 hours) to be efficient in HPC reduction in water and biofilms, as well as in planktonic SRB inactivation, but significantly ineffective against SRB in biofilms.

A p-value < 0.05 was considered to indicate that data obtained from two series of biofilm suspensions were significantly different. Student's test showed that no significant differences registered between the means of bacterial populations in the two subsamples of control, as well as in disinfected biofilms. Wilcoxon test revealed the absence of any significant difference for 13 of the 14 parameters investigated. Significant differences in the two series of biofilm suspensions (p-value < 0.05) occurred in the case of manganese bacteria (Table 2).

Residual chlorine concentrations after two hours disinfection are presented in Table 3. Sodium hypochlorite was the most rapidly consumed (residual 0.15 mg / L), while chloramite-T dissociated slowly (residual 0.8 mg / L). Chlorine dioxide proved to be able to persist longer, when compared with sodium dichloroisocyanurate and sodium hypochlorite.

No cells of aeromonads, pseudomonads, faecal indicators, denitrifiers, sulphur and sulphate reducing bacteria were recovered from biofilms treated with the mixed cleaning agent Floran. Recommended by the manufacturer for biofilms control in drinking water systems, Floran proved a high efficiency in bacterial inactivation. The performance of Mosslein cleaning solutions was tested previously in the drinking water treatment plant of Voila. Effects such as biofilm removal and sand filters conditioning were observed (Niculaie *et al.*, 2010).

Based on the average log reduction values of all bacterial types, the most effective disinfection in present investigation was achieved by the use of the mixed cleaning agent Floran (LRV = 3.673), followed by sodium dichloroisocyanurate (LRV = 1.122), sodium hypochlorite (LRV = 0.979), chloramine-T (LRV = 0.885) and chlorine dioxide (LRV = 0.657). As it can be seen in Table 4, of the five disinfectants tested, Floran was the most powerful agent in inactivating biofilm bacteria. It was able to reduce microbial viability in a percentage range from 96.66% (LRV = 1.477) to 99.99994% (LRV = 6.227).

Other studies on the comparative efficiency of disinfectants, targeting an inactivation of 99% (2 log) for planktonic HPC and *E. coli*, indicated the following ranking: hypochlorous acid followed by chlorine dioxide, hypochlorite ion and monochloramine (LeChevallier *et al.*, 1988; LeChevallier and Au, 2004).

Table 2.

Bacterial counts and standard deviations in control and disinfected biofilms. 1 – sodium dichloroisocyanurate; 2 – sodium hypochlorite; 3 – chloramine-T; 4 – chlorine dioxide; 5 – Floran. I, II – replicates. In rows – p-values for Wilcoxon test, assessing significant differences for each parameter, in replicates. In columns – p-values for Student's test, assessing significant differences for the 14 parameters in the two series of biofilm suspensions.

Type of bacteria	Sample	Control	1	2	3	4	5	Wilcoxon test, p-value
HPC	I	28272727	3214348	4307878	3398919	2420760	856	0.5887
	II	26818181	2704894	3908485	3091989	1879543	798	
	SD	1028519	360238	282413	217032	382698	41	-
A. hydrophila	I	127272	24000	75000	33235	33300	0	0.5211
	II	83692	11240	45120	24600	12100	0	
	SD	30815	9022	21128	6105	14990	0	-
P. aeruginosa	I	633333	75000	454000	496000	400000	0	0.2615
	II	340000	33000	250000	420000	102000	0	
	SD	207417	29698	144249	53740	210717	0	-
E. coli	I	1700	490	460	950	330	0	0.7393
	II	800	800	800	800	800	0	
	SD	636	219	240	106	332	0	-
Intestinal enterococci	I	3000	0	0	0	1200	0	0.7750
	II	1040	0	0	0	800	0	
	SD	1385	0	0	0	282	0	-
C. perfringens	I	43540	2500	5500	3950	5820	0	0.6303
	II	23166	3520	6520	2050	2200	0	
	SD	14406	721	721	1343	2559	0	-
Amonifying bacteria	I	5400000	1800000	1800000	1800000	1800000	55	0.2442
	II	80000000	36000	36000	36000	36000	55	
	SD	23460844	1247336	1247336	1247336	1247336	0	-
Denitrifying bacteria	I	36	54	54	18	60	0	0.0533
	II	280	340	420	180	280	0	
	SD	172	202	258	114	155	0	-
Iron reducing bacteria	I	470000	3600	3600	3600	3600	330	0.0869
	II	18000000	45000	54000	920000	1800000	430	
	SD	12395581	29274	35638	647992	1270246	71	-
Manganese oxidizing bacteria	I	35455	9090	11364	15455	12273	290	0.0411
	II	3000	1100	700	1900	2400	164	
	SD	22949	5649	7540	9584	6981	89	-

Table 2.continued

Sulphur reducing aerobes	I	1700	340	640	330	790	0	0.6279
	II	970	330	520	330	620	0	
	SD	516	7	84	0	120	0	-
Sulphur reducing anaerobes	I	110	20	20	20	60	0	0.2504
	II	45	0	36	0	18	0	
	SD	45	14	11	14	29	0	-
Sulphate reducing bacteria	I	20	45	45	60	20	0	0.0552
	II	40	0	0	18	0	0	
	SD	14	31	31	29	14	0	-
Sulphur oxidizing bacteria	I	430000	6400	1240	32000	32000	20	0.1481
	II	4700	2600	600	680	1400	20	
	SD	300732	2687	452	22146	21637	0	-
Student's test	p-value	0.2959	0.2234	0.2067	0.5429	0.7500	0.6409	-

No evidence of a direct correlation between chlorine consumption and the average LRV was found in the present experiment.

Previous studies assessing the effectiveness of chlorine dioxide as a disinfectant on planktonic bacteria in sewage systems indicated LRV ranging from 4.06 to 6.57 CFU/100ml (Kephart and Stoeckel, 2011). Ozone plus chlorine dioxide disinfection revealed LRV greater than 5 in removal of planktonic *C. perfringens* (Payment and Franco, 1993). In the present study, the action of chlorine-based agents resulted in *C. perfringens* log inactivation from 1.046 to 0.744 (91% to 81.98%), while Floran solution was able to reduce the active *Clostridium* cells with 99.997% (LRV = 4.523).

Even if chlorine dioxide was the most efficient chlorine-based disinfectant against heterotrophic bacteria (LRV = 1.108), as well as against *E. coli* (LRV = 0.345), its overall inhibitory effect against the fourteen types of bacteria was weaker than expected. Previous research studies reported a log-inactivation of 1.6-1.8 for suspended cells and just less than 1 log inactivation of biofilm heterotrophs (at a low concentration of 0.25mg/l, for 12 weeks) (Gagnon *et al.*, 2005). It is possible that longer contact time to be needed in order to achieve a more effective disinfection.

Table 3.

Chlorine residuals after two hours disinfection

Disinfectant	**mg / L**
Sodium dichloroisocyanurate	0.3
Sodium hypochlorite	0.15
Chloramine-T	0.8
Chlorine dioxide	0.6

Table 4.

Log reduction values in biofilm bacteria disinfected with:
1 – sodium dichloroisocyanurate; 2 – sodium hypochlorite; 3 – chloramine-T;
4 – chlorine dioxide; 5 – Floran solutions

Type of bacteria	***Log reduction value*** *(bacteria/g of biofilm)*				
	1	**2**	**3**	**4**	**5**
HPC	0.969	0.826	0.929	1.108	4.523
A. hydrophila	0.777	0.245	0.562	0.667	5.023
P. aeruginosa	0.955	0.141	0.026	0.288	5.687
E. coli	0.287	0.298	0.155	0.345	3.097
Intestinal enterococci	3.305	3.305	3.305	0.305	3.305
C. perfringens	1.045	0.744	1.046	0.920	4.523
Ammonifying bacteria	2.004	2.004	2.004	2.004	6.227
Denitrifying bacteria	-0.096	-0.176	0.203	-0.032	2.199
Iron reducing bacteria	2.580	2.506	1.301	1.010	4.386
Manganese oxidizing bacteria	0.577	0.503	0.346	0.418	1.928
Sulphur reducing aerobes	0.600	0.362	0.607	0.277	3.125
Sulphur reducing anaerobes	0.889	0.442	0.889	0.298	1.889
Sulphate reducing bacteria	0.125	0.125	-0.114	0.477	1.477
Sulphur oxidizing bacteria	1.684	2.373	1.124	1.114	4.036
Average LRV	**1.122**	**0.979**	**0.885**	**0.657**	**3.673**

When comparing results of similar investigations, realized on planktonic cells, to disinfectants efficacy on attached bacteria, as revealed by the present study, biofilm organization proves its protective features. The reduced antimicrobial impacts may be explained by slow penetration of disinfectants into the biofilm matrix. A direct measurement by the use of microelectrodes showed that chlorine concentrations in biofilms were typically only 20% or less of the concentration in the bulk liquid (de Beer *et al.*, 1994). Another explanation resides in the possibility of 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).

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

Therefore, drinking water treatment strategies should consider both the lower susceptibility of biofilm bacteria to disinfectants and the increased resistance of detached cells (Steed and Falkinham, 2006), which can survive and adhere to other surfaces to initiate biofilm formation downstream. Removal of deposits from the distribution systems also contributes to drinking water quality improvement (Lehtola *et al.,* 2004).

The mixed cleaning agent Floran demonstrated not just a bactericidal/bacteriostatic effect, but it was able to disintegrate the whole biofilm structure. As known, the efficient cleaning and disinfection implies not only killing the cells within the biofilm, but also disintegration of the biofilm matrix, so that the biofilm can be completely removed from the surface. Any leftover organic material provides nutrients that facilitate the rapid formation of a new biofilm (Wirtanen *et al.,* 2002; Luppens *et al.,* 2003).

Selection of the appropriate procedures in order to achieve drinking water safety is essential, since biofilm recovery after inefficient treatment could lead to populations of resistant bacteria, which may be recalcitrant to subsequent disinfection process (Simões *et al.,* 2004). To achieve efficient disinfection by the use of chlorine-based disinfectants, mechanical removal of biofilms prior to water disinfection is recommended. Also, investigations about the composition of microbial consortia and the physiological activities in the associated biofilms should be considered, due to the specificity of metabolic interactions between bacteria with different physiological requirements (Farkas *et al*., 2013).

Conclusions

After two hours exposure to disinfectant solutions based on the commonly used and commercially available chlorine-based products, in the recommended concentrations, significant numbers of bacteria were able to survive in drinking water biofilms.

None of the chlorine-based antimicrobials tested were able to completely inactivate the opportunistic pathogens or faecal bacteria embedded in biofilms. Moreover, increments in rates of sulphate reduction or in denitrification occurred in biofilms after applying chlorine-based disinfectants. Such aspects are important, considering the role of biofilms in biocorrosion.

The use of mixed cleaning agents proved to be the most efficient procedure for the inactivation of bacterial populations in drinking water associated biofilms. The maximum antimicrobial effect was achieved in biofilms treated with Floran (LRV = 3.673). Neither opportunistic pathogens nor faecal bacteria were viable in biofilms after treatment and the biofilm matrix was disintegrated.

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