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EVALUATION OF THE BIOCIDAL ACTIVITIES OF TRISTEL CHLORINE DIOXIDE SOLUTION AGAINST BIOFILM

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CONTENT

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I : DESCRIPTION OF THE STUDY :

✓ **Title:**	Evaluation of the biocidal activities of Tristel chlorine dioxide solution against biofilm.
✓ **Internal reference :**	254.TRI.04
✓ **Sponsor :**	TRISTEL Lynx Business Park, Fordham Road Snailwell, Cambridgeshire CB8 7NY United Kingdom *Contact:* Paul SWINNEY
✓ **Test period :**	From 16/08/04 to 26/10/04
✓ **Study manager :**	Lionel PINEAU
✓ **Operation manager :**	Gwenola LENOEN
✓ **Test laboratory :**	Laboratoire BIOTECH-GERMANDE Parc Scientifique de Luminy 163 Av. de Luminy – Case 927 13288 Marseille cedex 9

II : PURPOSE OF THE STUDY :

The aim of the study is to evaluate:

- The efficacy of Tristel chlorine dioxide solution to produce "microbiologically controlled rinse water".
- The ability of Tristel chlorine dioxide solution to control biofilm formation.
- The ability of Tristel chlorine dioxide solution to remove biofilm.

III : PRINCIPLE :

Four test lines made up of 2 Tygon® tube pieces [one contaminated with *Pseudomonas aeruginosa* CIP A22 biofilm grown on the inner surface (see figure 1 point [1]) (obtained as described in pr EN ISO 15883-1:2003 [1], see V.a) and the second one sterile (see figure 1 point [2])] are supplied 10 times per day with 10 liters of tap water to reproduce a daily use (flow : 1.0 to 1.5 liter/minute).

- The first test line is continuously treated with addition of Tristel chlorine dioxide solution in order to have a 5 ppm ClO_2 concentration in the water line : "Preventive treatment" (see figure 1.A),
- The second one is disinfected 2 times per day with a 50ppm Tristel ClO_2 solution for 5 minutes : "Curative treatment" (see figure 1.B).
- The third one is fitted with a 0.2µm filter sterilized each test day "Filtered water" (see figure 1.C).
- The last one not treated is used as a control (see figure 1.D).

The efficacy of each treatment system is compared by analyzing:

- the evolution of the number of viable bacteria and of the amount of residual proteins and polysaccharides content per surface unit of the initially contaminated Tygon® tube (biofilm removal) and initially sterile tube (biofilm control).
- the microbiological quality of the water produced (see figure 1 point [3]) (filtration of 100ml of water sample on 0,45µm membrane and incubation on PCA)

For each test line, 2 water samples (see figure 1 point [3]) and 2 pieces of each Tygon® tube portion (see figure 1 points [1] and [2]) are taken and analyzed each day (one at the beginning and the other one at the end of the day) during 4 weeks (20 working days).

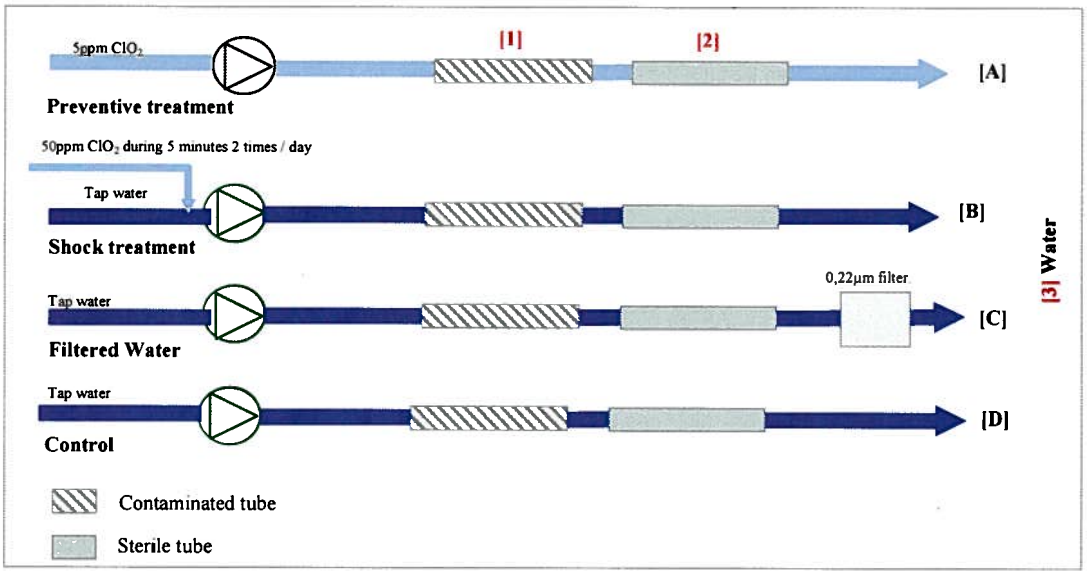

Figure 1: Test lines description.

IV : MATERIAL :

a) Reagents for chlorophenol red titration:

i) Chlorophenol red (CPR) :

As described by Francis W. Yau [2], 3.33 x 10^{-4} M chlorophenol red (CPR) was prepared by dissolving 0.1436 g of Chlorophenol red (Aldrich 19,952-4 batch n° 00604KW-034) in 100 ml of 0.01N NaOH (SigmaS-0899batch n°119H0169) and diluting to 1 litre with distilled water. The solution was allowed to stand overnight, and then filtered through 0.45µm filter before use as a titrant.

ii) pH 7.2 phosphate buffer :

Buffer solution at pH 7.2 was prepared by dissolving 1.76 g KH_2PO_4 (Sigma P-0662batch n° 29H0005) and 3.64g of $Na_2HPO_4.2H_2O$ (Sigma S-9763 batch n° 042K0186) in 60 ml of distilled water and diluting to 100 ml.

b) Tristel chlorine dioxide generator:

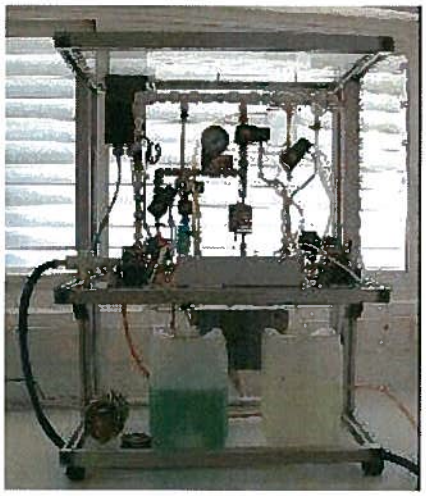

Figure 2 : Tristel chlorine dioxide generator:

Automatic system for the production of chlorine dioxide by mixing an activator and a base in a specific coil; Different coils can be used according to chlorine dioxide concentration range required.

c) Chemicals for chlorine dioxide generator :

Batch n°:----------Not communicated
Internal reference:----------Activator (255.TRI.04.542) base (255.TRI.04.556)
Manufacturer: ----------Tristel Company Limited
Date of acceptance:----------08/03/2004
Storage conditions:----------Room temperature and darkness
Active substance and concentration:----------Not specified

d) Chemicals for protein and polysaccharide measurements :

i) Proteins dosage :

Kit UPTIMA ---------- UP75860A

ii) Polysaccharides dosage :

Phosphate buffer---------- SIGMA, P-0662, N° lot: 29H0005.
Phenol---------- SIGMA, P-4161, N° lot: 39H0910.
H_2SO_4---------- FLUKA, 84727, N° lot: 443960/1
Glucose---------- SIGMA, G-5767, N° lot: 51K0062

e) Bacterial strains :

Pseudomonas aeruginosa CIP A22

f) Culture medium :

i) Maintaining and counting medium :

Tryptcase soy agar:---------- Biomérieux, 51044

Internal reference(s): 914.1.1, 917.1.4.

ii) Biofilm broth :

Composition of the nutrient broth used to supply the loop and promote biofilm formation:

Casamino acids (Difco 228820, batch n°: 1128005)---------- 0.1g/l
Yeast extract (Sigma Y1625 batch n°: 81K0354)---------- 0.1 g/l
$MgSO_4$, $2H_2O$ (Fluka 61138, batch n°: 419094/114101)---------- 0.2 g/l
$FeSO_4$, $7H_20$ (Sigma F2387, batch n°: 49H3647)---------- 0.0005 g/l
Na_2HPO_4 anhydrous (Sigma S9763, batch n°: 42K0186)---------- 1.25 g/l
KH_2PO_4 (Sigma P0662, batch n°: 29H0005) ---------- 0.5 g/l
Lactose (Prolabo 24946294, batch n°: D97G)---------- 0.025 g/l

iii) Neutralizing agent :

The composition of the neutralizing agent used to inactivate the biocidal activity of the tested product is based on the indications provided by the collection of standards published by AFNOR. The validity of the neutralizing agent is controlled for each bacterial strain used and each product tested.

Composition:
Lecithin (SIGMA, P-5394, batch n°: 128H8003)---------- 2%(p/v),
Sodium thiosulphate (SIGMA, S-8503, batch n°: 41K0256)---------- 0.5%(p/v)
Tween 80 (SIGMA, P-1754, batch n°: 024K7043)---------- 10% (v/v)
L-Histidine (SIGMA, H-8000, batch n°: 112K0929)---------- 4% (p/v)
Tryptcase soy broth (Biomérieux, 51019, batch n°: 774802201)---------- q.s.p. 100 ml.

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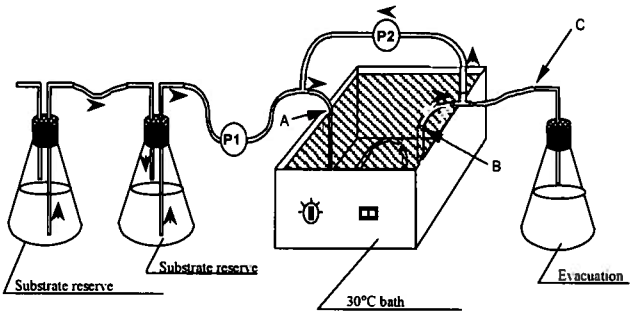
V : METHODS :

a) Biofilm formation :

The method used to obtain Tygon® tubes portion contaminated with *Pseudomonas aeruginosa* CIP A22 biofilm on the inner surface is described hereafter:

i) Experimental device (Figure N° 1):



P1 : Pump for the adhesion broth supply (2,5 à 3 ml/min).
P2 : Agitation pump (100 ml/ min).
A-B : Sampling part for the contaminated Tygon® tube portions. (90 cm).

Figure 3: experimental device used to obtain biofilm on Tygon® tube (6.4 mm internal diameter).

ii) Pumps :

Two pumps are respectively used:
- To supply the loop with the adhesion broth (pump N°1). The flow is adjusted between 2.5 and 3 ml/min.
- To agitate the loop content (pump N°2) with a speed rotation of about 40 rpm, that is to say 100 ml/min (laminar flow).

iii) Inoculation of the loop :

After completion of the circuit with the adhesion broth, the loop is inoculated by injecting (at point C level) 5 to 10 ml of a bacterial suspension containing approximately 10^8 bacteria per ml (pump N°1 turned off). The system is maintained under agitation (pump n°2 turned on) for 30 minutes before turning on the supply pump.

After an incubation of 72 hours pumps 1 and 2 are turned off, the external surface of the tube is disinfected with 95% alcohol and Tygon® tube portions are removed from the loop (between points A and B) in order to be used for the test.

b) Main test :

Four test lines made up of 2 pieces of Tygon® tubing [one contaminated with *Pseudomonas aeruginosa* CIP A22 biofilm grown on the inner surface and the second one sterile are used to evaluate the biocidal activities of Tristel chlorine dioxide solution against biofilm (see figure 1).

- The first test line is treated 10 times per day with 10 liters of 5 ppm chlorine dioxide solution to evaluate the effect of a "preventive treatment" on biofilm removal and control,
- The second one is supplied 10 times per day with 10 liters of filtered tap water but also disinfected 2 times per day with a 50ppm Tristel ClO_2 solution for 5 minutes (after 1 and 7 rinsing stages with 10 liters of filtered tap water) to evaluate the effect of a "curative treatment" on biofilm removal and control,
- The third test line is also supplied 10 times per day with 10 liters of filtered tap water but in this case the test line is fitted (at the distal end) with a 0,2µm filter (sterilized each test day). This test line will be used as a "reference system" for the production of bacteria free water.
- The fourth test line used as a control is supplied 10 times per day with 10 liters of filtered tap water.

The efficacy of each treatment system is compared by analyzing:

- The evolution of the number of viable bacteria (see V.b.i) and of the amount of residual proteins and polysaccharides content (see V.b.ii) per surface unit of the initially contaminated Tygon® tube (biofilm removal) and initially sterile tube (biofilm control).
- The microbiological quality of the water produced (filtration of 100ml of water sample on 0,45µm membrane and incubation on PCA)

For each test line, 2 water samples and 2 x 2 portions of each Tygon® tube portion are taken and analyzed each day during 4 weeks (20 working days).
The first water sample is collected each morning before any chemical treatment, water circulation and before the installation of the filter for test line C and it corresponds to water which has stagnated in the test line all the night. Those samples are taken to evaluate the contamination of test line water due to the growth of bacteria remaining on the test surface after treatment or circulation of water.
The second water sample is collected at the end of the working day to evaluate the microbiological quality of the water produced after treatment or water circulation (see figure 1 test lines A, B and D) or after the installation of the 0.2µm filter (see figure 1 test line C).
Among the 4 portions of tubes taken, two are used for the counting of viable fixed bacteria and two for the determination of protein and polysaccharide contents.

i) Determination of the number of viable bacteria still fixed to the Tygon® tube:

Twice a day, 2 cm of the tested Tygon® tube portion taken is dipped in 10 ml of neutralizing agent for 10 minutes in order to stop any residual biocidal activity, cut lengthwise and scraped thoroughly. The purpose of this operation is to detach all residual fixed micro-organisms of the biofilm.
The Tygon® tube portions and the neutralizing solution are then shaken for 1 minute with vortex (vortex 2, shake 5, Bioblock, France) and a tenfold serial dilution of this solution is then performed. For each dilution, 2 x 1 ml are included in counting medium and agar plates are incubated aerobically for 48 hours at 37°C. Results are expressed as the log number of viable and still adherent microorganisms per cm^2 of biofilm on the inner surface of the Tygon® tube.

Note: to facilitate the graphic representation and the interpretation of the results, when no viable bacteria are detected on the tested tube, results are mentioned by the value "0 log/cm2 ". However, regarding the threshold of detection of the counting method used these values should be considered as "<0.7 log/cm^2 ".

ii) Determination of the residual amounts of proteins and polysaccharides on the internal surface of the tested Tygon® tube:

One of the two Tygon® tube portion taken twice a day for each test line is dipped in 3 ml of sterile distilled, cut lengthwise and scraped thoroughly. The residual amount of proteins and polysaccharides par cm² of Tygon® tube ($[Pol._{assay}]_t$($[Prot_{assay}]_t$) are determined from these 3 ml using respectively the MicroBC and Dubois test methods.

c) Chlorine dioxide controls :

Concurrently to main test, controls are performed periodically to verify chlorine dioxide concentrations in solutions produced by Tristel generator (chlorophenol red titration).

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VI : RESULTS :

a) **Control:**

Time (hours)	Contaminated tube			Sterile Tube			Solution
	Bacteria Nb. UFC/cm²	[prot.] µg/cm²	[polys.] µg/cm²	Bacteria Nb. UFC/cm²	[prot.] µg/cm²	[polys.] µg/cm²	Nb. UFC/100ml
0	$8.0\ 10^{8}$	50.0	19.8	0	2.9	1.3	0
8	$1.5\ 10^{2}$	23.1	8.5	$7.5\ 10^{1}$	1.7	0.4	$5.5\ 10^{3}$
24	$3.4\ 10^{4}$	28.2	8.0	$5.0\ 10^{0}$	0.5	0.9	$7.3\ 10^{5}$
32	$6.0\ 10^{1}$	19.9	4.9	$3.5\ 10^{1}$	1.9	1.4	$7.0\ 10^{2}$
48	$6.2\ 10^{5}$	25.1	5.2	$6.8\ 10^{4}$	1.2	3.3	$5.2\ 10^{6}$
56	$6.7\ 10^{5}$	23.8	6.1	$2.0\ 10^{4}$	3.1	3.0	$5.9\ 10^{3}$
72	$7.0\ 10^{7}$	17.3	4.6	$6.5\ 10^{4}$	1.1	1.4	$2.7\ 10^{8}$
80	$4.2\ 10^{6}$	17.6	8.0	$1.9\ 10^{4}$	1.8	1.9	$3.3\ 10^{4}$
144	$2.7\ 10^{8}$	11.0	7.0	$5.8\ 10^{7}$	0.2	2.6	$1.9\ 10^{9}$
152	$9.5\ 10^{7}$	12.4	5.9	$5.4\ 10^{4}$	2.4	1.3	$1.8\ 10^{4}$
168	$6.3\ 10^{7}$	1.3	7.2	$2.7\ 10^{6}$	2.4	2.2	$1.1\ 10^{8}$
176	$3.3\ 10^{7}$	10.4	3.9	$8.6\ 10^{4}$	1.3	11.7	$3.6\ 10^{4}$
192	$4.3\ 10^{6}$	12.5	6.0	$1.2\ 10^{5}$	1.6	1.8	$4.3\ 10^{6}$
200	$1.8\ 10^{4}$	11.9	5.4	$1.6\ 10^{2}$	2.0	1.8	$1.6\ 10^{1}$
216	$1.3\ 10^{5}$	8.4	6.6	$8.5\ 10^{2}$	2.5	2.2	$1.1\ 10^{5}$
224	$3.7\ 10^{4}$	7.1	9.5	$5.0\ 10^{1}$	2.8	3.7	$2.0\ 10^{2}$
240	$3.2\ 10^{4}$	5.3	2.6	$3.5\ 10^{1}$	2.0	1.3	$1.3\ 10^{3}$
248	$1.7\ 10^{4}$	4.4	5.0	$1.5\ 10^{1}$	2.1	2.2	$3.0\ 10^{2}$
312	$2.5\ 10^{4}$	2.5	4.5	$1.7\ 10^{3}$	3.3	7.6	$1.8\ 10^{4}$
320	$1.3\ 10^{3}$	1.0	3.1	$1.5\ 10^{1}$	3.7	3.5	$5.8\ 10^{1}$
336	2.310^{2}	1.3	3.0	$1.5\ 10^{1}$	4.7	5.6	$6.0\ 10^{2}$
344	$5.0\ 10^{0}$	1.8	2.5	$<5.0\ 10^{0}$	4.1	7.1	$4.0\ 10^{0}$
360	$5.6\ 10^{2}$	1.9	2.5	$<5.0\ 10^{0}$	4.3	7.1	$2.0\ 10^{2}$
368	$<5.0\ 10^{0}$	1.8	2.2	$<5.0\ 10^{0}$	4.2	4.9	$2.0\ 10^{0}$
384	$3.1\ 10^{2}$	2.3	3.0	$<5.0\ 10^{0}$	6.1	5.8	$3.0\ 10^{2}$
392	$1.6\ 10^{2}$	4.5	2.5	$<5.0\ 10^{0}$	6.3	4.6	$1.5\ 10^{1}$
408	$1.1\ 10^{2}$	1.9	3.2	$1.5\ 10^{2}$	5.9	4.1	$1.0\ 10^{2}$
416	$<5.0\ 10^{0}$	3.9	2.6	$<5.0\ 10^{0}$	4.3	3.2	$3.0\ 10^{0}$
480	$<5.0\ 10^{0}$	1.8	2.3	$<5.0\ 10^{0}$	9.5	4.6	$7.0\ 10^{2}$
488	$1.0\ 10^{1}$	1.9	2.3	$<5.0\ 10^{0}$	5.7	3.8	$9.0\ 10^{0}$
504	$<5.0\ 10^{0}$	1.6	1.8	$<5.0\ 10^{0}$	7.5	6.6	$3.0\ 10^{2}$
512	$<5.0\ 10^{0}$	2.3	0.1	$<5.0\ 10^{0}$	6.0	7.0	$1.0\ 10^{0}$
528	$<5.0\ 10^{0}$	5.2	2.9	$<5.0\ 10^{0}$	6.8	8.6	$1.0\ 10^{2}$
536	$<5.0\ 10^{0}$	9.0	7.8	$<5.0\ 10^{0}$	8.0	7.6	$7.0\ 10^{0}$
552	$<5.0\ 10^{0}$	1.2	0.7	$<5.0\ 10^{0}$	5.0	7.9	$1.0\ 10^{2}$
560	$<5.0\ 10^{0}$	2.9	0.7	$<5.0\ 10^{0}$	7.1	4.8	$1.7\ 10^{1}$
576	$1.5\ 10^{1}$	1.5	1.0	$1.5\ 10^{1}$	10.8	8.5	$2.9\ 10^{1}$
584	$<5.0\ 10^{0}$	11.3	2.0	$<5.0\ 10^{0}$	8.7	6.7	$9.0\ 10^{0}$
648	$<5.0\ 10^{0}$	3.2	2.0	$5.0\ 10^{0}$	12.2	9.8	$9.0\ 10^{0}$
656	$<5.0\ 10^{0}$	5.0	2.0	$<5.0\ 10^{0}$	9.1	7.5	$7.0\ 10^{0}$
672	$<5.0\ 10^{0}$	3.9	1.5	$<5.0\ 10^{0}$	8.6	7.8	$1.0\ 10^{2}$
680	$<5.0\ 10^{0}$	0.7	1.6	$<5.0\ 10^{0}$	8.7	9.1	$6.0\ 10^{0}$

Table I: Control: Number of viable bacteria (fixed to the test surface and in suspension in the water produced) and residual amounts of proteins and polysaccharides per surface unit of the contaminated Tygon® tubes (sterile and contaminated) when the test line is supplied 10 times a day with 10 litters of 0.2µm filtered tap water.

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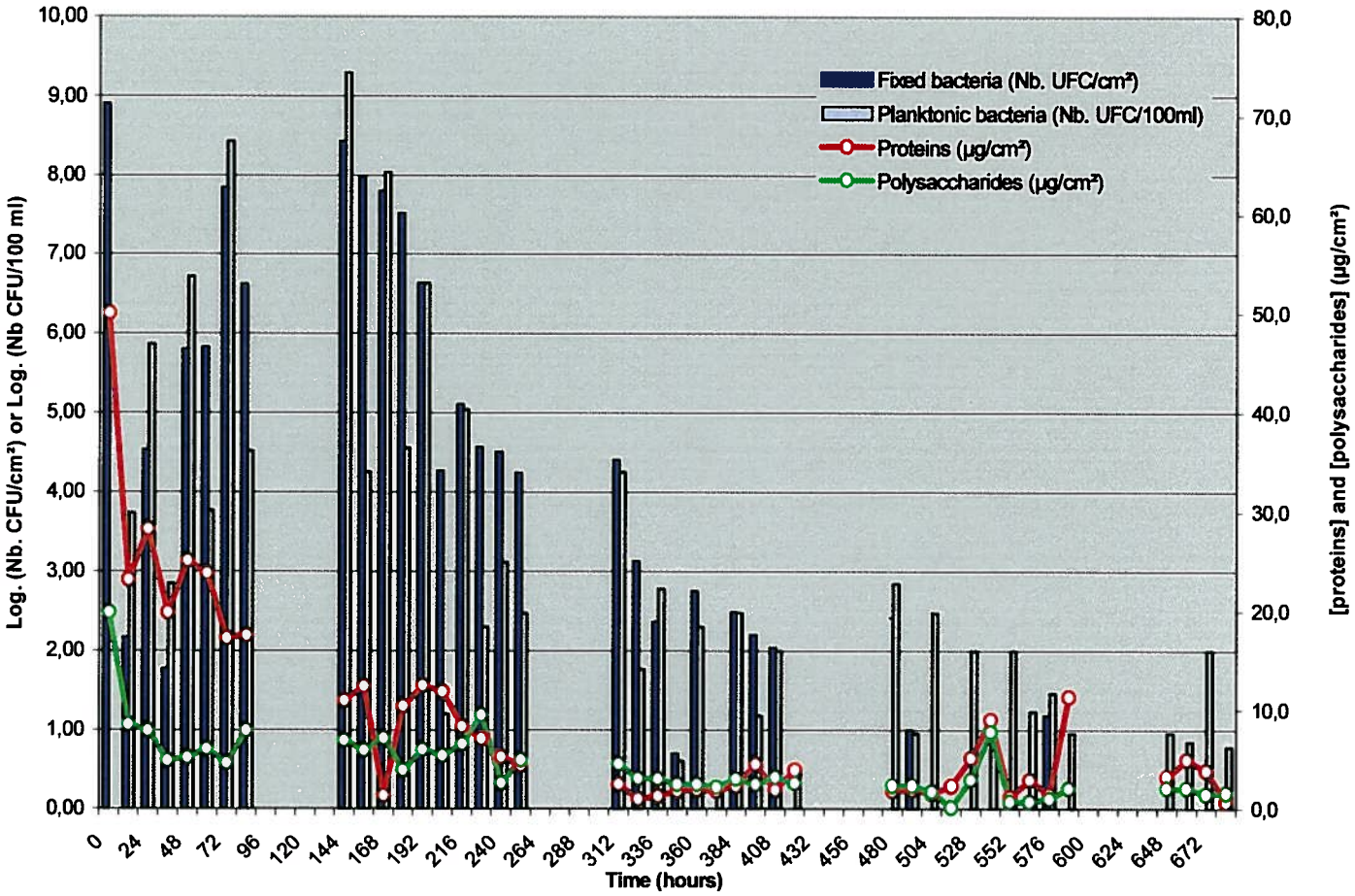

Figure 4: Control : Evolution of the number of viable bacteria (fixed to the test surface and in suspension in the water produced) and residual amounts of proteins and polysaccharides per surface unit of the contaminated Tygon® tubes according to contact time, when the test line is supplied 10 times a day with 10 litters of 0.2µm filtered tap water.

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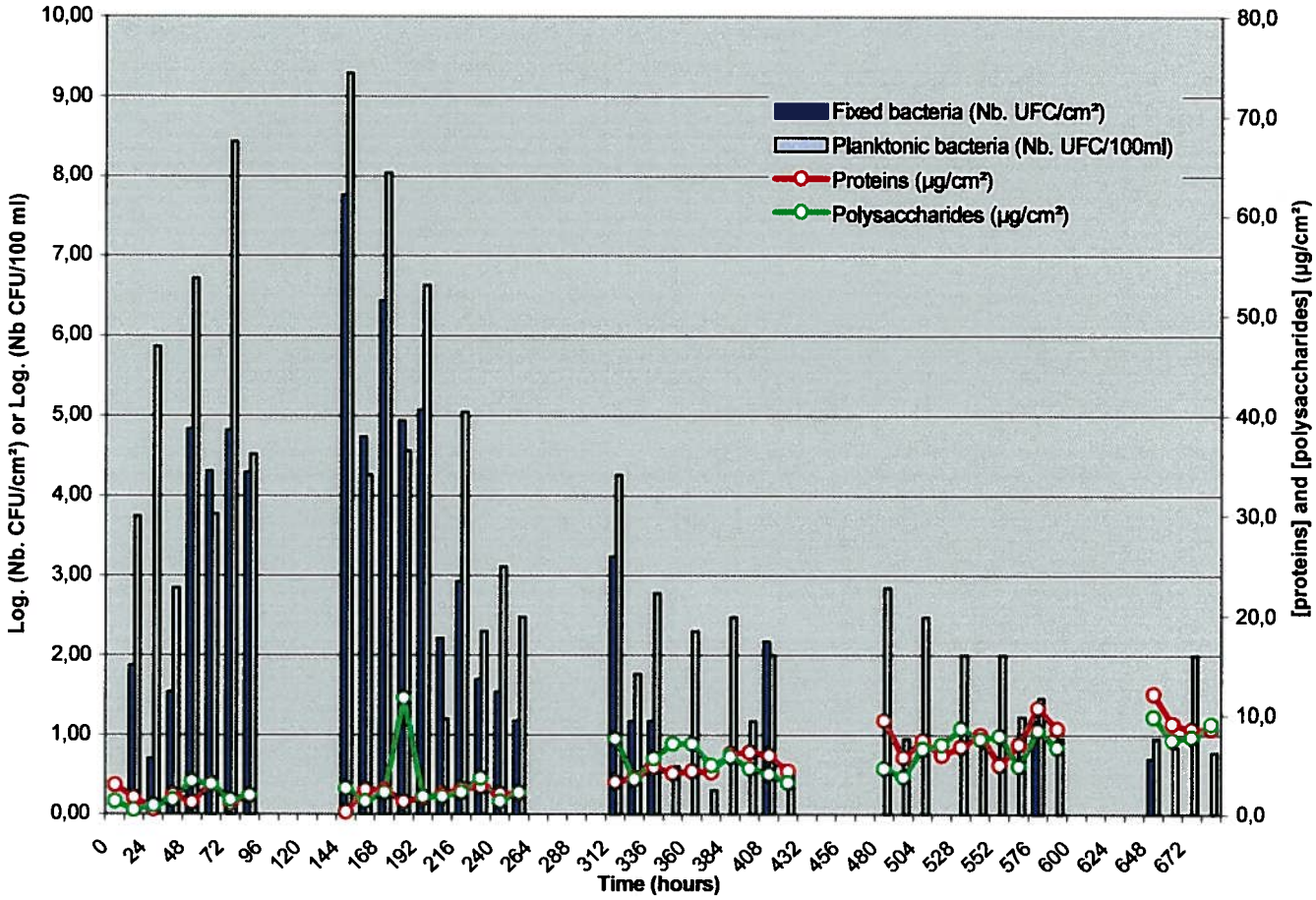

Figure 5: *Control*: Evolution of the number of viable bacteria (fixed to the test surface and in suspension in the water produced) and residual amounts of proteins and polysaccharides per surface unit of the sterile Tygon® tubes according to contact time, when the test line is supplied 10 times a day with 10 litters of 0.2µm filtered tap water.

The analysis of experimental data presented in Table I and figure 4 indicate that overall, the circulation of filtered tap water through the Tygon® tube contaminated with a *Pseudomonas aeruginosa* biofilm, induces a reduction of the number of viable bacteria fixed to the test surface. This effect is irregular during the first three days during which a first important reduction followed by an increase of the number of viable fixed bacteria is observed. After 80 hours of treatment, the number of viable bacteria fixed to the tested surface is about 4.2 10^6 CFU/cm² whereas the initial contamination level was about 8.0 10^8 CFU/cm². The reduction of the number of fixed bacteria is much more regular during the second and third test week and after 408 hours the number of viable bacteria on the surface of the test tube is about 1.1 10^2 UFC/cm². For longer contact time (more than 480 hours), no viable bacteria are recovered from the initially contaminated test surface except for 488 and 576 hours.

The analysis of the evolution of the amount of proteins and polysaccharides per surface unit of Tygon® tube confirms also that the circulation of 10 x 10 litters of water each day through the test line induces a weakening of the biofilm. Residual amounts of proteins and polysaccharides decrease respectively from 50.0 µg/cm² and 19.8 µg/cm² to 3.9 µg/cm² and 2.6 µg/cm² after 416 hours. For the fourth and fifth test weeks (between 480 and 680 hours), the residual amount of proteins and polysaccharides per surface unit remains low (about 3.6 µg/cm² for proteins and 2.6 µg/cm² for polysaccharides) even if sporadic increases are observed (536, 584 hours).

The evolution of the number of viable bacteria in the water collected in or circulating through the test line is comparable to what it has been observed for fixed bacteria or proteins and polysaccharides, i.e. an irregular evolution during the first three days followed by an important reduction during the second and third test week. Contamination level observed vary from 1.9 10^9 UFC/100 ml to 1.0 10^2 UFC/100 ml for water samples collected in the morning and from 3.6 10^4 UFC/100 ml to 20 UFC/100 ml for the water sample collected at the end of the working day.
Contamination levels of water samples collected each morning are always higher than those of water samples collected at the end of the working day. This difference is due to the fact that the first sample corresponds to water which has stagnated in the test line all the night and includes bacteria not or slightly adhered to surfaces. On the opposite, for the second water sample collected at the end of the working day, the circulation of filtered tap water through the test line has already led to an elimination of all bacteria not or slightly fixed.
For the fourth and fifth test weeks (between 480 and 680 hours), the mean residual amount of viable bacteria is about 175 UFC/100 ml for the water which has stagnated in the test line all the night and 8 UFC/100 ml for the water produced at the end of the working day.

The analysis of the results obtained for the sterile Tygon® tube portion inserted in the test line (see table I and figure 5) show that the number of fixed bacteria increases quickly from 0 to 5.8 10^7 UFC/cm² after 144 hours and then decrease during the second and the third test week. Like for the contaminated tube, no viable bacteria are recovered from the test surface for contact time longer than 480 hours, except after 576 and 648 hours. On the opposite, residual amounts of proteins and polysaccharides increase regularly during all the test period to reach a value of about 10µg/cm² for both biofilm constituents. If the presence of bacteria on the test surface during the 300 first hours is more linked to a reversible adhesion of bacteria coming from the contaminated Tygon® tube and present in the water circulating through the test line, the increase of proteins and polysaccharide amounts seems to indicate that an active adhesion process is in progress.

To conclude, the circulation of filtered tap water through the Tygon® tube contaminated with a *Pseudomonas aeruginosa* biofilm induces a weakening of the biofilm with a partial removal of biofim constituents (fixed bacteria, proteins and polysaccharides) and leads to an improvement of the microbiological quality of the water produced. Nevertheless this removal effect due to the wash-off effect of the water circulating through the tube is only partial and some bacteria remain on the test surface and in the water circulating through the test line The tests performed on the initially sterile Tygon® tube portion reveals also that an active colonisation of initially non contaminated surface by the bacteria coming from the contaminated Tygon® tuibe portion is possible.

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b) filtered water:

Time (hours)	Contaminated tube			Sterile Tube			Solution
	Bacteria Nb. UFC/cm²	[prot.] µg/cm²	[polys.] µg/cm²	Bacteria Nb. UFC/cm²	[prot.] µg/cm²	[polys.] µg/cm²	Nb. UFC/100ml
0	$8.2\ 10^{8}$	69.2	20.3	0	4.6	1.0	0 (a)
8	$5.2\ 10^{4}$	29.3	8.0	$3.1\ 10^{4}$	26.7	0.4	$2.0\ 10^{0}$
24	$1.4\ 10^{5}$	31.4	9.1	$1.8\ 10^{4}$	2.0	0.0	$2.2\ 10^{7}$ (a)
32	$5.2\ 10^{3}$	28.8	7.4	$1.8\ 10^{5}$	2.3	2.6	$1.4\ 10^{1}$
48	$4.2\ 10^{6}$	10.6	4.3	$1.4\ 10^{6}$	2.2	1.3	$6.3\ 10^{7}$ (a)
56	$2.3\ 10^{6}$	16.8	5.6	$2.8\ 10^{4}$	2.3	1.2	$<1.0\ 10^{0}$
72	$7.2\ 10^{7}$	12.2	4.6	$5.0\ 10^{6}$	0.8	0.3	$1.2\ 10^{8}$ (a)
80	$3.2\ 10^{5}$	10.0	3.6	$1.1\ 10^{4}$	1.3	2.3	$4.0\ 10^{0}$
144	$6.5\ 10^{6}$	9.6	5.1	$3.0\ 10^{7}$	1.4	2.6	$6.0\ 10^{8}$ (a)
152	$9.5\ 10^{7}$	8.3	5.2	$2.3\ 10^{5}$	1.6	1.3	$4.0\ 10^{2}$
168	$1.7\ 10^{8}$	12.8	6.9	$4.2\ 10^{6}$	2.9	0.4	$3.5\ 10^{8}$ (a)
176	$4.1\ 10^{6}$	14.8	6.3	$5.6\ 10^{4}$	1.7	1.5	$5.1\ 10^{1}$
192	$1.5\ 10^{7}$	13.2	8.0	$3.2\ 10^{5}$	1.3	0.0	$1.7\ 10^{7}$ (a)
200	$3.3\ 10^{4}$	8.2	3.5	$1.7\ 10^{3}$	2.5	0.3	$4.0\ 10^{0}$
216	$2.1\ 10^{5}$	8.6	5.9	$2.4\ 10^{3}$	3.1	0.9	$1.7\ 10^{5}$ (a)
224	$2.9\ 10^{4}$	6.2	6.4	$1.2\ 10^{2}$	5.8	0.7	$<1.0\ 10^{0}$
240	$4.1\ 10^{5}$	9.6	5.3	$1.9\ 10^{2}$	2.2	0.7	$1.8\ 10^{4}$ (a)
248	$6.2\ 10^{4}$	6.6	2.6	$7.0\ 10^{1}$	3.1	2.6	$1.0\ 10^{0}$
312	$1.2\ 10^{5}$	3.2	5.3	$1.4\ 10^{4}$	3.7	5.3	$4.8\ 10^{5}$ (a)
320	$1.2\ 10^{4}$	3.9	5.6	$2.0\ 10^{3}$	4.0	4.8	$<1.0\ 10^{0}$
336	$1.7\ 10^{3}$	4.3	4.1	$7.4\ 10^{2}$	3.9	3.5	$1.8\ 10^{3}$ (a)
344	$<5.0\ 10^{0}$	5.2	4.3	$<5.0\ 10^{0}$	3.9	2.5	$<1.0\ 10^{0}$
360	$3.5\ 10^{2}$	4.9	3.3	$6.7\ 10^{1}$	3.3	3.5	$3.7\ 10^{3}$ (a)
368	$1.2\ 10^{2}$	4.5	2.3	$2.0\ 10^{1}$	3.9	4.2	$<1.0\ 10^{0}$
384	$1.4\ 10^{2}$	3.7	2.3	$<5.0\ 10^{0}$	4.9	5.1	$1.4\ 10^{3}$ (a)
392	$2.0\ 10^{1}$	2.3	2.6	$<5.0\ 10^{0}$	3.4	2.6	$<1.0\ 10^{0}$
408	$2.0\ 10^{1}$	2.3	3.8	$1.0\ 10^{1}$	5.3	4.1	$8.0\ 10^{2}$ (a)
416	$5.0\ 10^{0}$	2.5	2.3	$<5.0\ 10^{0}$	5.7	5.1	$1.0\ 10^{0}$
480	$6.5\ 10^{1}$	0.8	2.8	$1.7\ 10^{2}$	5.3	5.5	$2.0\ 10^{2}$ (a)
488	$<5.0\ 10^{0}$	2.4	1.7	$<5.0\ 10^{0}$	5.6	4.9	$1.0\ 10^{0}$
504	$<5.0\ 10^{0}$	0.9	1.9	$1.0\ 10^{1}$	2.2	6.3	$2.0\ 10^{2}$ (a)
512	$<5.0\ 10^{0}$	1.8	1.3	$<5.0\ 10^{0}$	6.1	4.4	$1.0\ 10^{0}$
528	$3.0\ 10^{1}$	0.7	1.2	$<5.0\ 10^{0}$	6.0	10.5	$2.0\ 10^{2}$ (a)
536	$1.0\ 10^{1}$	6.0	1.5	$<5.0\ 10^{0}$	7.1	5.9	$<1.0\ 10^{0}$
552	$1.0\ 10^{1}$	2.3	1.6	$<5.0\ 10^{0}$	8.7	7.5	$1.0\ 10^{2}$ (a)
560	$<5.0\ 10^{0}$	3.9	1.0	$<5.0\ 10^{0}$	11.7	6.0	$<1.0\ 10^{0}$
576	$<5.0\ 10^{0}$	1.4	0.9	$1.0\ 10^{1}$	12.2	8.2	$1.0\ 10^{2}$ (a)
584	$<5.0\ 10^{0}$	2.9	1.0	$<5.0\ 10^{0}$	3.0	2.8	$<1.0\ 10^{0}$
648	$1.1\ 10^{1}$	2.2	3.1	$1.1\ 10^{1}$	9.0	7.5	$1.1\ 10^{3}$ (a)
656	$<5.0\ 10^{0}$	1.6	2.0	$<5.0\ 10^{0}$	7.8	7.0	$<1.0\ 10^{0}$
672	$1.5\ 10^{1}$	2.0	1.8	$5.0\ 10^{0}$	7.6	7.9	$6.0\ 10^{2}$ (a)
680	$<5.0\ 10^{0}$	2.5	2.8	$<5.0\ 10^{0}$	11.8	7.5	$<1.0\ 10^{0}$

Table II: Filtered water : Number of viable bacteria (fixed to the test surface and in suspension in the water produced) and residual amounts of proteins and polysaccharides per surface unit of the contaminated Tygon® tubes (sterile and contaminated) when the test line is fitted with a 0.2µm filter and supplied 10 times a day with 10 litters of 0.2µm filtered tap water. (a) non filtrated water sample collected at the beginning of the working day (stagnant water).

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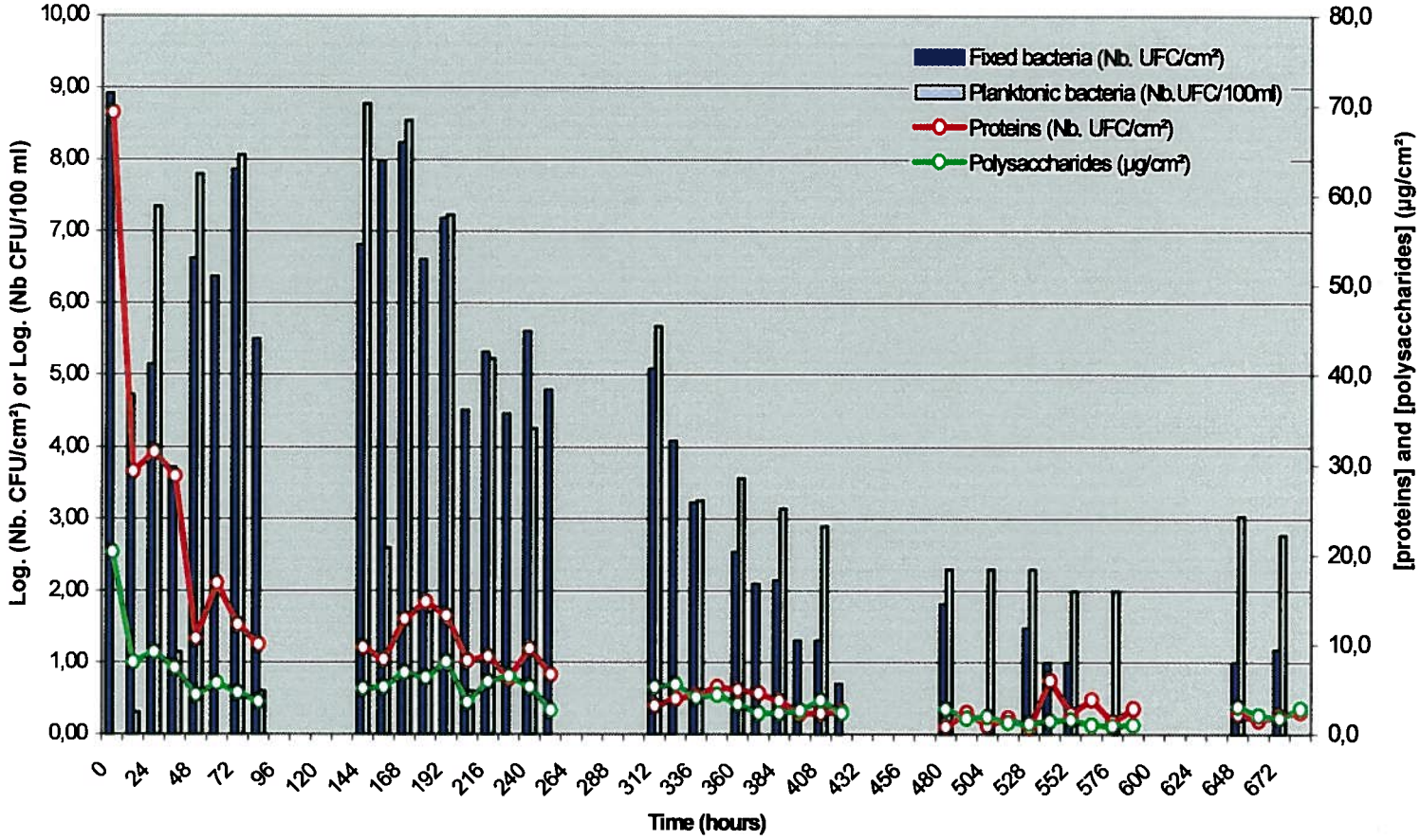

Figure 6: Filtered water : Evolution of the number of viable bacteria (fixed to the test surface and in suspension in the water produced) and residual amounts of proteins and polysaccharides per surface unit of the contaminated Tygon® tubes according to contact time, when the test line is fitted with a 0.2µm filter and supplied 10 times a day with 10 litters of 0.2µm filtered tap water.

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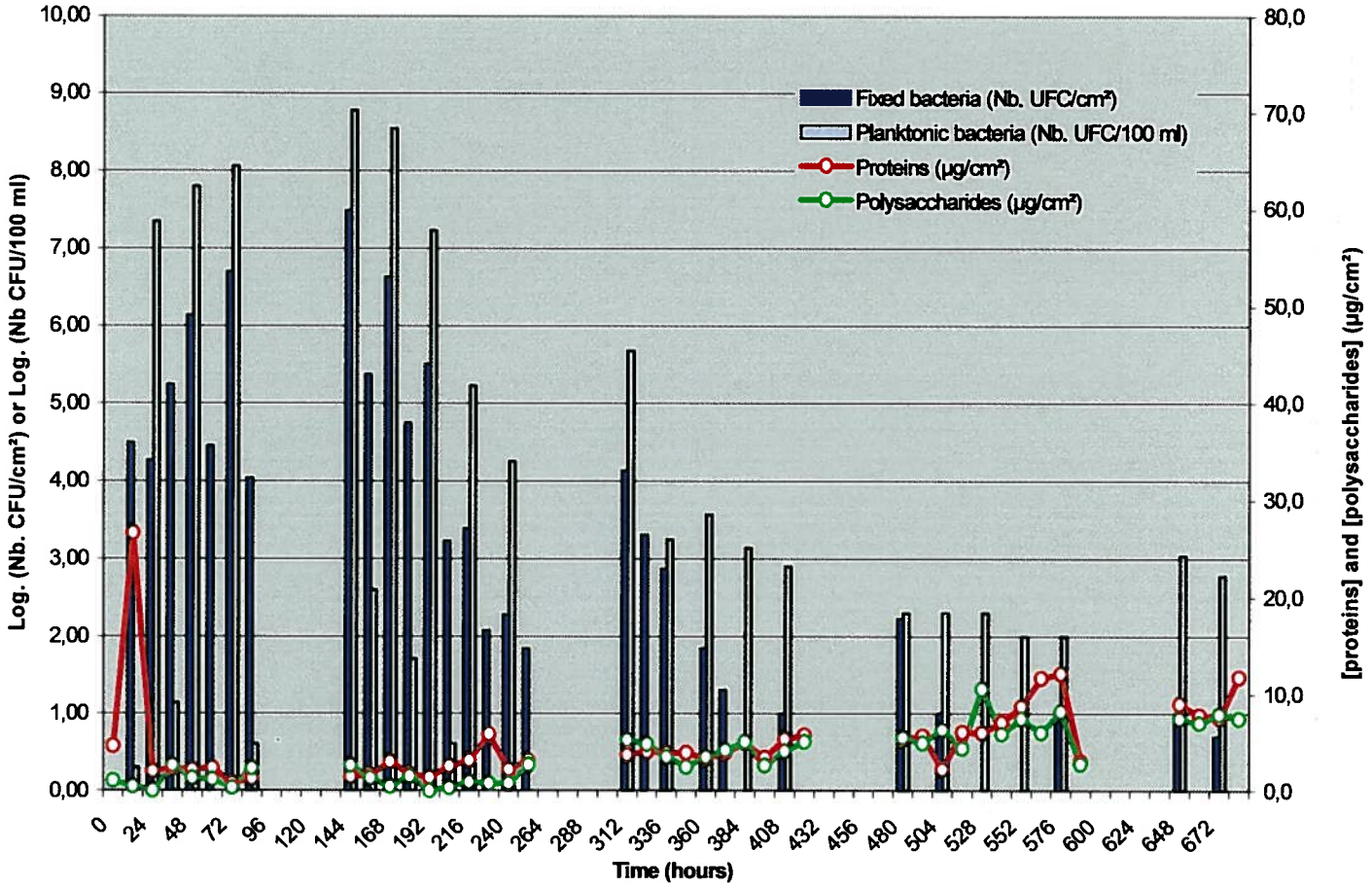

Figure 7: Filtered water : Evolution of the number of viable bacteria (fixed to the test surface and in suspension in the water produced) and residual amounts of proteins and polysaccharides per surface unit of the sterile Tygon® tubes according to contact time, when the test line is fitted with a 0.2µm filter and supplied 10 times a day with 10 litters of 0.2µm filtered tap water.

The analysis of experimental data presented in Table 2 and figure 6 indicate that results obtained with the test line fitted with a 0.2µm filter at the distal end are comparable to those obtained with the control test line (without filter – see Table 1, Figures 4 and 5).

Like for the control test line (see table I figure 4), the circulation ten times a day of 10 litters of filtered tap water through the Tygon® tube contaminated with a *Pseudomonas aeruginosa* biofilm induces within the first seven days a reduction of the number of viable fixed bacteria per surface unit followed by an increase of this parameter, to reach a maximum contamination level of 1.7 10^8 UFC/cm² after 168 hours. During the second and the third week of test, the number of fixed bacteria decreases regularly and after 416 hours, the number of viable bacteria on the surface of the test tube is about 5 UFC/cm². For longer contact time (> 416 hours) no viable bacteria are recovered from 8 out of the 14 Tygon® tube portions analysed against 12 out of 14 for the control test line (see table 1). I we consider only Tygon® tube portions taken at the end of the working day this ratio is 6 out of 7 for both test lines.

The reduction of the amount of proteins and polysaccharides per surface unit of the contaminated Tygon® tube resulting from the circulation of 10 x 10 litters of water each day through the test line is also confirmed for the test line fitted with a 0.2µm filter. Residual amounts of proteins and polysaccharides decrease respectively from 69.2 µg/cm² and 20.3 µg/cm² to 2.5 µg/cm² and 2.3 µg/cm² after 416 hours. For test weeks four and five (i.e. between 480 and 680 hours), the residual amount of proteins and polysaccharides per surface unit remains low (about 2.2 µg/cm² for proteins and 1.7 µg/cm² for polysaccharides) even if sporadic increases are observed (536, 560 hours).

The analysis of the evolution of the number of viable bacteria in the water collected need to be considered separately according to the time of the sampling:

- ✓ Water samples collected each morning correspond to water which has stagnated in the test line all the night. These samples are taken before the installation of the 0.2µm filter and their analysis permit to evaluate the contamination of the water due to the growth of bacteria remaining on the test tube. Results obtained for such water samples are comparable to those obtained for the control test line (increase from 0 to 6.0 10^8 UFC/100ml during the first 144 hours followed by a regular reduction during the second and third test week, to reach a minimal value of about 1.0 10^2 UFC/100 ml. This contamination level is then maintained until the end of the test period (i.e. between 480 and 672 hours).

- ✓ The second water sample is collected at the end of the working day to evaluate the microbiological quality of the water produced after the installation of the 0.2µm filter. Contrary to what it has been observed for the control test line, 10 out of 21 samples analyzed do not present any contamination and 71.5% of all samples collected present a contamination level lower or equal to 1 CFU/100 ml whereas for the control test line all samples presented a contamination level included between 1 and 3.6 10^4 CFU/100 ml.

Results obtained for the sterile Tygon® tube portion inserted in the test line fitted with a 0.2 µm filter is also comparable to what is has been observed for the control test line. The number of fixed bacteria increases quickly from 0 to 3.0 10^7 UFC/cm² after 144 hours and then decrease during the second and the third test week. During the fourth and fifth test week the number of viable bacteria fixed to the test tube remains very low (less than 11 UFC/cm²) except for the Tygon® tube portion taken at the beginning of week five (480 hours) for which the contamination level determined is 1.7 10^2 CFU/cm². However this results is not surprising seeing that this Tygon® tube portion has been taken after an incubation of two days without submitted it to any treatment favouring the growing of all residual bacteria. It also important to mention than between 480 hours and 680 hours, 9 Tygon® tube portions out of 14 analyzed do not present any contamination whereas for the control test line (see table 1) 12 were not contaminated.

Even if the number of fixed bacteria remains low, the increase of residual amounts of proteins and polysaccharides on the initially non contaminated test tube from 0 to 10µg/cm² (for both constituents) seems to indicate that an active adhesion process is in progress.

To conclude, like for the control test line, the circulation of filtered tap water through the Tygon® tube contaminated with a *Pseudomonas aeruginosa* biofilm induces a weakening of the biofilm with a partial removal of biofim constituents (fixed bacteria, proteins and polysaccharides). Nevertheless this removal effect due to the wash-off effect of the water circulating through the tube is only partial and remaining bacteria can colonized nearby surfaces and/or contaminate the water circulating though the test tube. The addition of a 0.2 µm filter at the outlet of the test line permits to control the microbial quality of the water produced even if all water sample collected after the filter are not free from microbial contamination.

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c) **Curative treatment:**

Time (hours)	Contaminated tube			Sterile Tube			Solution
	Bacteria Nb. UFC/cm²	[prot.] µg/cm²	[polys.] µg/cm²	Bacteria Nb. UFC/cm²	[prot.] µg/cm²	[polys.] µg/cm²	Nb. UFC/100ml
0	$5.7\ 10^{9}$	59.0	12.6	0	5.0	0.6	0
8	$7.8\ 10^{3}$	3.7	7.8	$7.5\ 10^{2}$	4.0	1.1	NR
24	$2.5\ 10^{1}$	8.7	3.1	$4.0\ 10^{1}$	1.0	2.3	$>1.5\ 10^{2}$
32	$3.0\ 10^{1}$	11.4	1.7	$3.0\ 10^{1}$	2.0	0.0	NR
48	$5.0\ 10^{0}$	14.1	2.6	$5.0\ 10^{0}$	1.9	0.8	$2.8\ 10^{1}$
56	$5.0\ 10^{0}$	5.0	0.8	$<5.0\ 10^{0}$	2.3	0.8	NR
120	$<5.0\ 10^{0}$	17.7	4.4	$<5.0\ 10^{0}$	3.4	1.2	$<1.0\ 10^{0}$
128	$<5.0\ 10^{0}$	16.3	2.8	$<5.0\ 10^{0}$	2.9	0.0	NR
144	$<5.0\ 10^{0}$	16.3	2.3	$<5.0\ 10^{0}$	1.8	0.0	$<1.0\ 10^{0}$
152	$<5.0\ 10^{0}$	15.3	1.2	$<5.0\ 10^{0}$	2.0	0.0	NR
168	$<5.0\ 10^{0}$	19.2	8.6	$<5.0\ 10^{0}$	3.1	0.9	$<1.0\ 10^{0}$
176	$<5.0\ 10^{0}$	16.9	4.1	$<5.0\ 10^{0}$	3.6	1.2	NR
192	$<5.0\ 10^{0}$	12.9	5.6	$<5.0\ 10^{0}$	3.4	0.9	$<1.0\ 10^{0}$
200	$<5.0\ 10^{0}$	8.6	2.2	$<5.0\ 10^{0}$	4.0	1.9	$<1.0\ 10^{0}$
216	$<5.0\ 10^{0}$	8.5	5.9	$<5.0\ 10^{0}$	1.8	1.9	$<1.0\ 10^{0}$
224	$<5.0\ 10^{0}$	8.0	4.3	$<5.0\ 10^{0}$	4.1	1.5	$<1.0\ 10^{0}$
288	$<5.0\ 10^{0}$	13.7	6.5	$<5.0\ 10^{0}$	5.6	2.2	$<1.0\ 10^{0}$
296	$<5.0\ 10^{0}$	5.3	1.5	$<5.0\ 10^{0}$	2.8	1.5	$<1.0\ 10^{0}$
312	$<5.0\ 10^{0}$	5.4	4.6	$<5.0\ 10^{0}$	3.0	1.5	$<1.0\ 10^{0}$
320	$<5.0\ 10^{0}$	4.1	1.5	$<5.0\ 10^{0}$	3.5	2.1	$<1.0\ 10^{0}$
336	$<5.0\ 10^{0}$	4.2	2.6	$<5.0\ 10^{0}$	1.3	0.0	$<1.0\ 10^{0}$
344	$<5.0\ 10^{0}$	4.0	2.4	$<5.0\ 10^{0}$	1.3	0.9	$<1.0\ 10^{0}$
360	$<5.0\ 10^{0}$	4.1	1.1	$<5.0\ 10^{0}$	1.7	0.0	$<1.0\ 10^{0}$
368	$<5.0\ 10^{0}$	3.3	0.5	$<5.0\ 10^{0}$	4.0	1.5	$<1.0\ 10^{0}$
384	$<5.0\ 10^{0}$	12.0	2.0	$<5.0\ 10^{0}$	3.8	0.5	$<1.0\ 10^{0}$
392	$<5.0\ 10^{0}$	1.7	0.2	$<5.0\ 10^{0}$	1.4	0.0	$<1.0\ 10^{0}$
456	$<5.0\ 10^{0}$	2.0	2.0	$<5.0\ 10^{0}$	0.9	1.5	$<1.0\ 10^{0}$
464	$<5.0\ 10^{0}$	1.1	2.3	$<5.0\ 10^{0}$	1.0	1.4	$<1.0\ 10^{0}$
480	$<5.0\ 10^{0}$	1.9	2.1	$<5.0\ 10^{0}$	0.6	1.4	$<1.0\ 10^{0}$
488	$<5.0\ 10^{0}$	2.2	0.0	$<5.0\ 10^{0}$	1.1	0.0	$<1.0\ 10^{0}$
504	$<5.0\ 10^{0}$	2.8	3.0	$<5.0\ 10^{0}$	2.3	1.8	$<1.0\ 10^{0}$
512	$<5.0\ 10^{0}$	2.5	1.5	$<5.0\ 10^{0}$	1.2	2.1	$<1.0\ 10^{0}$
528	$<5.0\ 10^{0}$	2.1	1.7	$<5.0\ 10^{0}$	0.8	0.8	$<1.0\ 10^{0}$
536	$<5.0\ 10^{0}$	2.0	1.4	$<5.0\ 10^{0}$	2.5	1.7	$<1.0\ 10^{0}$
552	$<5.0\ 10^{0}$	2.1	1.5	$<5.0\ 10^{0}$	2.8	1.1	$<1.0\ 10^{0}$
560	$<5.0\ 10^{0}$	1.9	2.7	$<5.0\ 10^{0}$	1.4	1.5	$<1.0\ 10^{0}$
624	$<5.0\ 10^{0}$	1.8	2.3	$<5.0\ 10^{0}$	0.7	0.6	$<1.0\ 10^{0}$
632	$<5.0\ 10^{0}$	2.0	2.0	$<5.0\ 10^{0}$	0.7	1.7	$<1.0\ 10^{0}$
648	$<5.0\ 10^{0}$	1.9	2.1	$<5.0\ 10^{0}$	1.6	0.9	$<1.0\ 10^{0}$
656	$<5.0\ 10^{0}$	0.5	1.1	$<5.0\ 10^{0}$	0.6	0.2	$<1.0\ 10^{0}$

Table III : *Curative treatment*: Number of viable bacteria (fixed to the test surface and in suspension in the water produced) and residual amounts of proteins and polysaccharides per surface unit of the contaminated Tygon® tubes according to contact time, when the test line is treated 2 times per day for 5 minutes with a 50ppm ClO_2 solution.

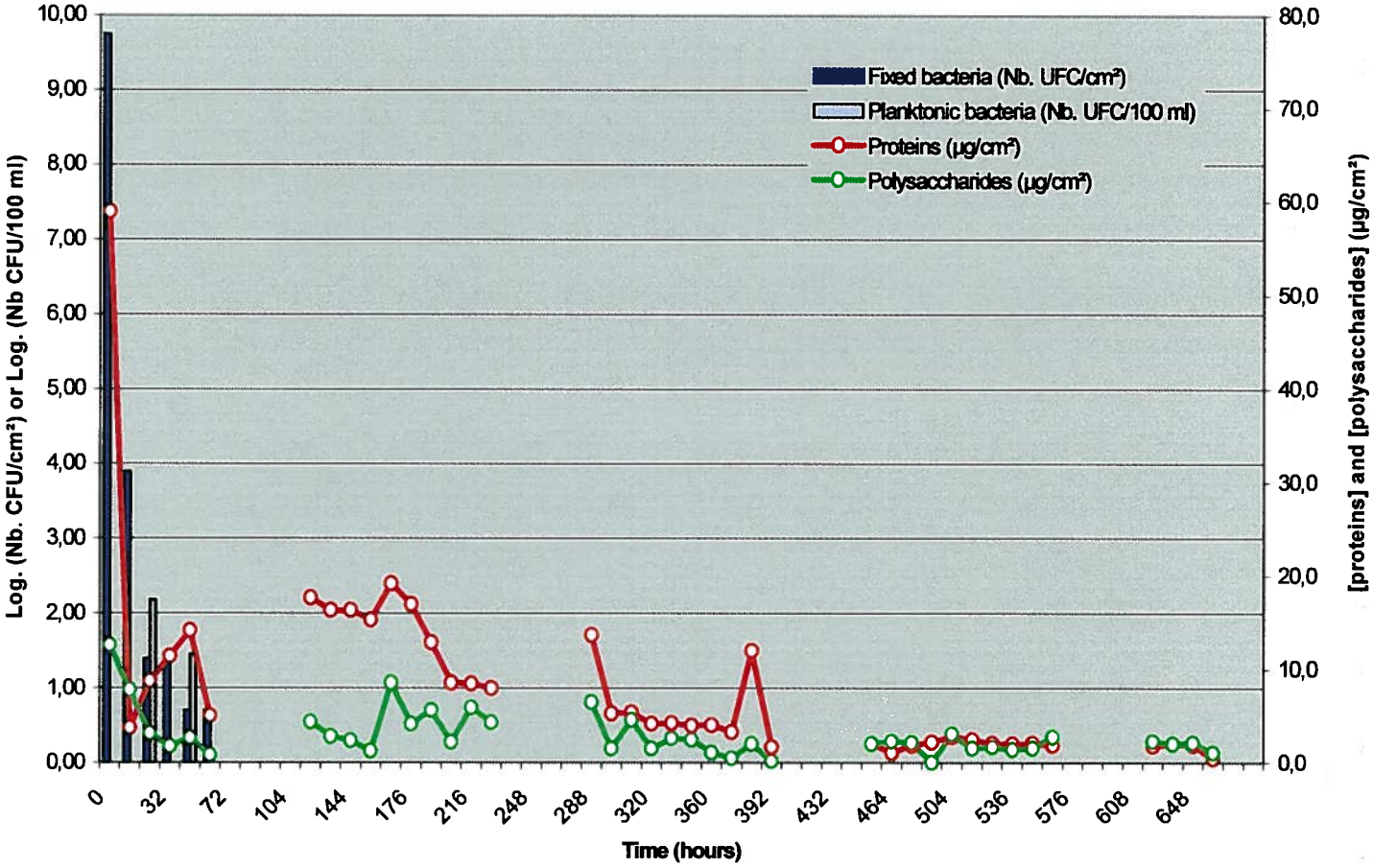

Figure 8: Curative treatment : Evolution of the number of viable bacteria (fixed to the test surface and in suspension in the water produced) and residual amounts of proteins and polysaccharides per surface unit of the contaminated Tygon® tubes according to contact time, when the test line is treated 2 times per day for 5 minutes with a 50ppm ClO_2 solution.

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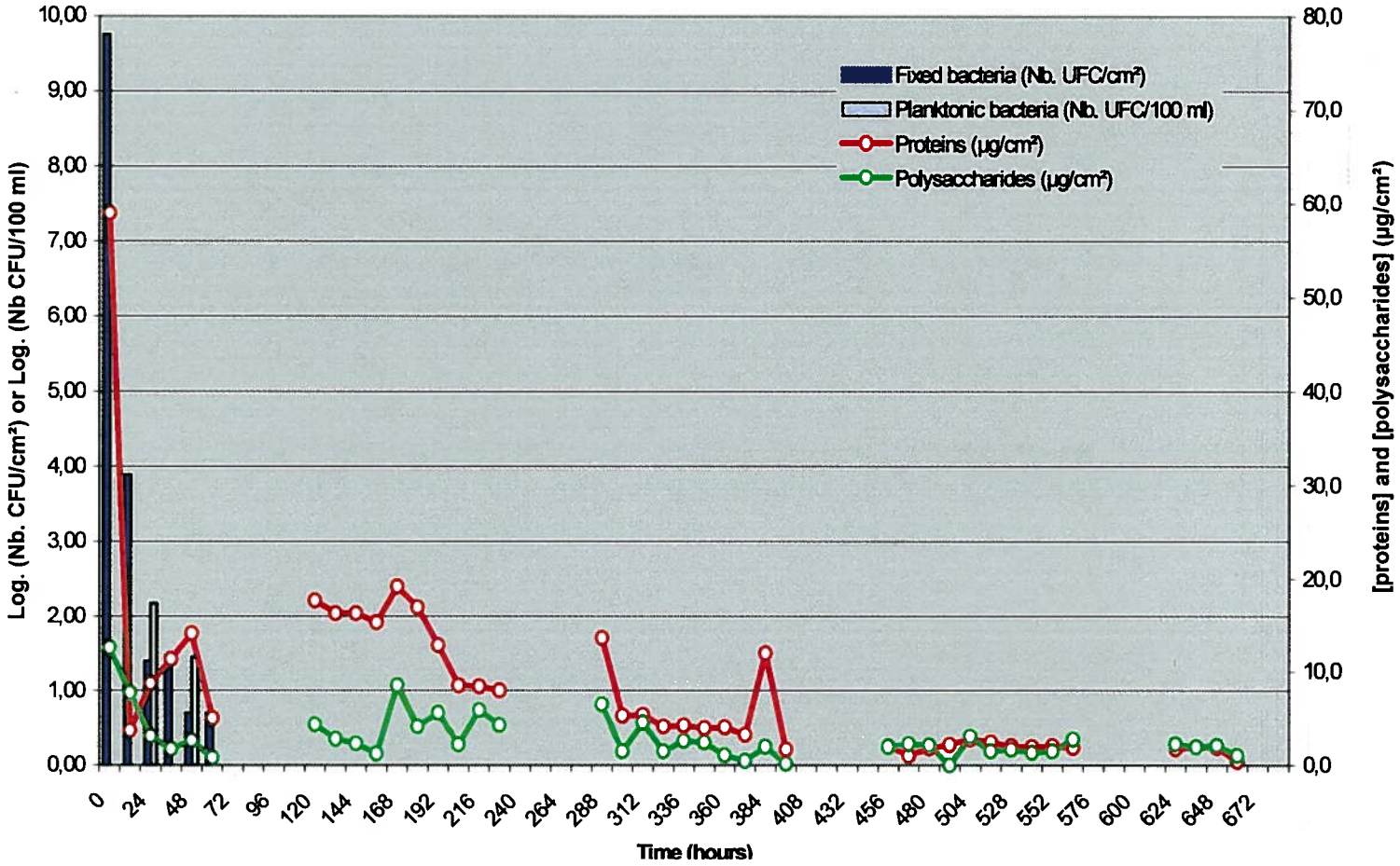

Figure 9: *Curative treatment* : Evolution of the number of viable bacteria (fixed to the test surface and in suspension in the water produced) and residual amounts of proteins and polysaccharides per surface unit of the sterile Tygon® tubes according to contact time, when the test line is treated 2 times per day for 5 minutes with a 50ppm ClO_2 solution.

Results presented in table III and figure 8 show that the treatment of a Tygon® tube contaminated with a *Pseudomonas aeruginosa* CIP A22 biofilm, 2 times per day with a 50ppm Tristel ClO_2 solution for 5 minutes (after 1 and 8 rinsing stages with 10 liters of filtered tap water) induces an important reduction of biofilm constituents.

The number of viable bacteria fixed per surface unit of Tygon® tube decreases quickly from 5.7 10^9 CFU/cm² to less than 5 CFU/cm² in less than 120 hours. For longer contact times, no viable bacteria are recovered from the surface of the initially contaminated test tube.

The reduction of residual amounts of proteins and polysaccharides is slower and 392 hours are necessary to reduce the residual amount of proteins and polysaccharides from respectively 59.0 µg/cm² to 1.7 µg/cm² and from 12.6 µg/cm² to 0.2 µg/cm². For contact time longer than 392 hours the mean residual amount of protein and polysaccharide remains stable to a value of about 1.9 µg/cm² for proteins and 1.8 µg/cm² for polysaccharides.

The analysis of water samples collected demonstrate that whatever water sample is considered (stagnant or circulating water) no viable bacteria are recovered from the water produced except for samples taken after 24 hours and 48 hours.

Results obtained on the initially sterile Tygon® tube portion (see table III and figure 9) indicate that after disinfection twice a day with a 50 ppm Tristel ClO_2 solution for 5 minutes there is no accumulation of proteins and polysaccharides on the test surface like this has been described for the control test line (see table I) and the test line fitted with a 0.2µm filter (see table II). Results of bacterial counting indicate also that except samples taken during the first three test days, no viable bacteria are recovered from the surface of the initially sterile Tygon® tube portion of in the water produced.

Those results demonstrate that the treatment of a Tygon® tube contaminated with a *Pseudomonas aeruginosa* CIP A22 biofilm, two times per day with a 50ppm Tristel ClO_2 solution for 5 minutes (after 1 and 8 rinsing stages with 10 liters of filtered tap water) induces a total and irreversible elimination of biofilm constituents (fixed bacteria, proteins and polysaccharides), permits to control the biofilm formation on nearby surfaces but also permits to ensure the microbial quality of the water produced.

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d) Preventive treatment:

Time (hours)	Contaminated tube			Sterile Tube			Solution
	Bacteria Nb. UFC/cm²	[prot.] µg/cm²	[polys.] µg/cm²	Bacteria Nb. UFC/cm²	[prot.] µg/cm²	[polys.] µg/cm²	Nb. UFC/100ml
0	$2.7\ 10^{9}$	44.5	13.8	0	4.6	0.8	0
8	$1.1\ 10^{4}$	24.1	4.2	$8.9\ 10^{1}$	2.5	0.0	NR
24	$6.0\ 10^{5}$	10.6	2.6	$1.5\ 10^{5}$	1.0	3.0	$<1.5\ 10^{2}$
32	$1.5\ 10^{1}$	17.5	3.9	$2.0\ 10^{1}$	1.2	0.0	NR
48	$2.0\ 10^{1}$	19.6	11.0	$9.9\ 10^{0}$	3.3	0.0	$1.4\ 10^{3}$
56	$9.9\ 10^{0}$	15.8	5.0	$<5.0\ 10^{0}$	2.5	0.6	NR
120	$1.4\ 10^{2}$	17.7	11.8	$3.0\ 10^{1}$	1.6	0.0	$3.7\ 10^{6}$
128	$5.0\ 10^{0}$	17.1	5.1	$5.0\ 10^{0}$	3.4	0.0	NR
144	$5.0\ 10^{0}$	18.0	10.33	$<5.0\ 10^{0}$	2.9	0.0	$7.0\ 10^{2}$
152	$<5.0\ 10^{0}$	17.2	2.5	$<5.0\ 10^{0}$	2.9	0.0	NR
168	$<5.0\ 10^{0}$	18.3	6.8	$2.5\ 10^{1}$	3.5	0.7	$2.5\ 10^{3}$
176	$<5.0\ 10^{0}$	7.5	0.5	$<5.0\ 10^{0}$	4.5	1.2	NR
192	$<5.0\ 10^{0}$	18.9	7.7	$<5.0\ 10^{0}$	3.5	3.4	$1.3\ 10^{3}$
200	$<5.0\ 10^{0}$	9.2	9.6	$<5.0\ 10^{0}$	6.4	2.1	$<1.0\ 10^{0}$
216	$<5.0\ 10^{0}$	7.9	7.4	$5.0\ 10^{0}$	1.2	7.1	$8.0\ 10^{2}$
224	$<5.0\ 10^{0}$	5.2	7.1	$<5.0\ 10^{0}$	4.9	0.3	$<1.0\ 10^{0}$
288	$8.2\ 10^{3}$	7.5	7.2	$2.7\ 10^{3}$	13.4	2.7	$3.2\ 10^{5}$
296	$<5.0\ 10^{0}$	4.2	3.7	$5.0\ 10^{0}$	0.9	1.2	$4.0\ 10^{0}$
312	$<5.0\ 10^{0}$	6.2	5.0	$<5.0\ 10^{0}$	1.5	0.6	$2.0\ 10^{3}$
320	$<5.0\ 10^{0}$	4.0	1.6	$<5.0\ 10^{0}$	1.6	2.2	$<1.0\ 10^{0}$
336	$<5.0\ 10^{0}$	4.1	1.1	$<5.0\ 10^{0}$	1.4	0.6	$3.3\ 10^{3}$
344	$<5.0\ 10^{0}$	3.2	0.6	$<5.0\ 10^{0}$	1.3	0.2	$1.0\ 10^{0}$
360	$<5.0\ 10^{0}$	2.0	0.2	$<5.0\ 10^{0}$	0.4	1.1	$7.0\ 10^{2}$
368	$<5.0\ 10^{0}$	1.7	0.3	$<5.0\ 10^{0}$	1.2	0.3	$<1.0\ 10^{0}$
384	$<5.0\ 10^{0}$	2.4	0.6	$<5.0\ 10^{0}$	1.2	0.0	$1.2\ 10^{3}$
392	$<5.0\ 10^{0}$	2.2	0.5	$<5.0\ 10^{0}$	2.0	0.0	$2.0\ 10^{0}$
456	$2.7\ 10^{3}$	1.7	2.4	$1.3\ 10^{3}$	1.2	1.2	$2.5\ 10^{3}$
464	$<5.0\ 10^{0}$	0.9	1.4	$<5.0\ 10^{0}$	1.1	1.8	$<1.0\ 10^{0}$
480	$2.0\ 10^{1}$	1.3	1.4	$<5.0\ 10^{0}$	1.1	1.8	$6.0\ 10^{2}$
488	$5.0\ 10^{0}$	1.3	0.0	$<5.0\ 10^{0}$	1.1	0.0	$<1.0\ 10^{0}$
504	$2.5\ 10^{1}$	1.7	2.3	$<5.0\ 10^{0}$	1.8	1.3	$1.4\ 10^{3}$
512	$<5.0\ 10^{0}$	1.8	2.3	$<5.0\ 10^{0}$	2.4	2.0	$<1.0\ 10^{0}$
528	$2.0\ 10^{1}$	1.0	1.5	$5.0\ 10^{0}$	1.3	0.9	$2.6\ 10^{3}$
536	$<5.0\ 10^{0}$	2.5	1.2	$<5.0\ 10^{0}$	3.3	2.3	$2.0\ 10^{0}$
552	$<5.0\ 10^{0}$	3.1	2.1	$<5.0\ 10^{0}$	2.7	2.6	$8.0\ 10^{2}$
560	$<5.0\ 10^{0}$	0.8	2.3	$<5.0\ 10^{0}$	1.1	0.3	$3.0\ 10^{0}$
624	$1.0\ 10^{6}$	1.1	1.5	$6.4\ 10^{4}$	1.1	1.8	$1.3\ 10^{5}$
632	$<5.0\ 10^{0}$	0.8	0.8	$<5.0\ 10^{0}$	1.9	1.5	$7.0\ 10^{0}$
648	$9.9\ 10^{0}$	1.2	1.5	$1.4\ 10^{2}$	2.1	1.7	$5.4\ 10^{3}$
656	$<5.0\ 10^{0}$	0.6	0.9	$<5.0\ 10^{0}$	0.8	0.2	$3.0\ 10^{0}$

Table IV : Preventive treatment: Number of viable bacteria (fixed to the test surface and in suspension in the water produced) and residual amounts of proteins and polysaccharides per surface unit of the contaminated Tygon® tubes according to contact time, when the test line is continuously treated with 5ppm ClO_2 solution.

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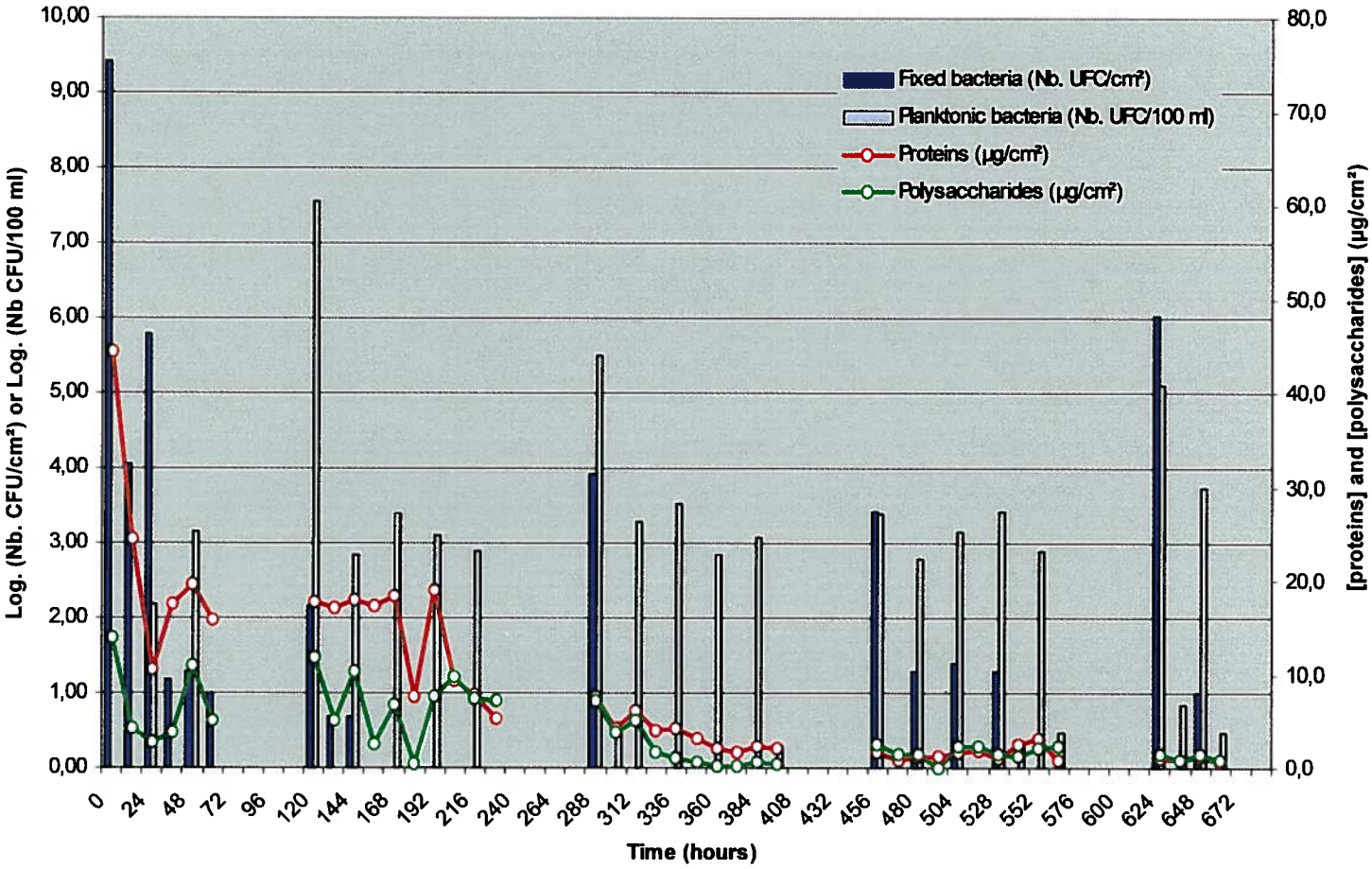

Figure 10: Preventive treatment : Evolution of the number of viable bacteria (fixed to the test surface and in suspension in the water produced) and residual amounts of proteins and polysaccharides per surface unit of the contaminated Tygon® tubes according to contact time, when the test line is continuously treated with 5ppm ClO_2 solution.

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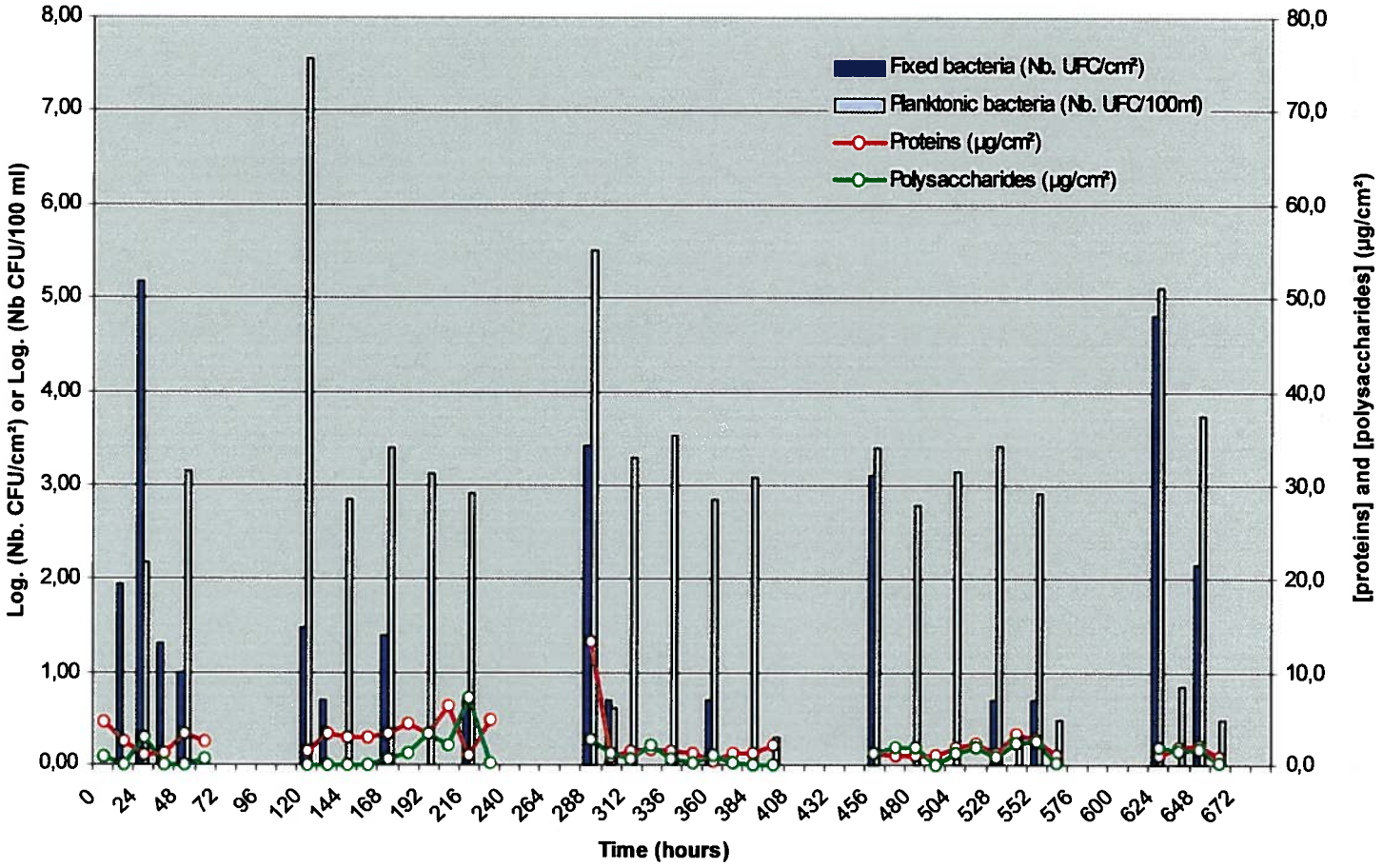

Figure 11 : Preventive treatment : Evolution of the number of viable bacteria (fixed to the test surface and in suspension in the water produced) and residual amounts of proteins and polysaccharides per surface unit of the sterile Tygon® tubes according to contact time, when the test line is continuously treated with 5ppm ClO_2 solution.

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The analysis of experimental data presented in Table IV and figure 8 indicate that the circulation 10 times a day of 10 litters of 5 ppm chlorine dioxide solution through a Tygon® tube contaminated with a *Pseudomonas aeruginosa* biofilm, induces an important and quick reduction of the number of viable bacteria fixed to the test surface. The number of viable bacteria fixed to the tested surface decreases from 2.7 10^{9} CFU/cm² to less than 1.0 10^{2} CFU/cm² after only 56 hours (i.e. after 30 x 10 litters of 5 ppm ClO_2 solution). For longer contact time, the contamination level of the test surface remains low (less than 25 CFU/cm²) except for the Tygon® tube samples collected after each week end for which higher contamination levels are observed (from 1.4 10^{2} CFU/cm² to 1.0 10^{6} CFU/cm²).

Repetitive treatment with a 5 ppm ClO_2 solution leads also to a reduction of the amount of proteins and polysaccharides per surface unit of Tygon® tube. Residual amounts of proteins and polysaccharides decrease respectively from 44.5 µg/cm² and 13.8 µg/cm² to 2.2 µg/cm² and 0.5 µg/cm² after 392 hours and remain stable until the end of the test period to a mean value of about 1.4 µg/cm² for proteins and 1.5 µg/cm² for polysaccharides.

The analysis of the microbiological quality of the water produced indicates that the contamination level of the first water sample collected each morning (corresponding to water which has stagnated in the test line all the night) remains unchanged during all the test period (from 1.5 10^{2} CFU/100 ml to 5.4 10^{3} CFU/100 ml) except for water samples collected after each week end for which higher contamination levels are observed (from 2.5 10^{3} CFU/100 ml to 3.7 10^{6} CFU/100 ml). On the other hand, all water samples collected at the end of the working day present a contamination level lower than 7.0 CFU/100 ml and 50% of those water samples are free from microbial contamination.

Results obtained for the sterile Tygon® tube portion inserted in the test line (see table IV and figure 11) are comparable to those described for the contaminated test tube with :

An important reduction of the number of fixed bacteria with a contamination level which remains very low (62.5% of the Tygon® tube portions analyzed present a contamination level lower to the detection limit of the counting medium i.e. 5 CFU/cm²) except for Tygon tube samples taken after each week-end for which higher contamination levels are observed (from 3.0 10^{1} CFU/cm² to 6.4 10^{4} CFU/cm²).
Contrary to what it has been observed for the control test line (see table I and figures 4 and 5) and the test line fitted with a 0.2µm filter (see table II and figures 6 and 7) there is no increase of the residual amounts of proteins and polysaccharides on the initially sterile test surface. Proteins and polysaccharides contents remain respectively to a mean value of about 2.4 µg/cm² and 1.2 µg/cm².

To conclude, the circulation 10 times a day of 10 litters of 5 ppm chlorine dioxide solution through a Tygon® tube contaminated with a *Pseudomonas aeruginosa* biofilm induces a reduction of the number of fixed bacteria but also a reduction of the residual amount of proteins and polysaccharides per surface unit. Nevertheless this effect is only partial and bacteria remaining on the test line can lead after prolonged unused test periods, to an increase of the contamination level of test surfaces or of the water produced.

Lastly, repetitive treatment with 5 ppm ClO_2 solution permit to control biofilm formation on nearby surfaces and except the water which has stagnated in the test line all the night this treatment leads to an improvement of the microbiological quality of the water produces (compared to the control test line).

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VII : CONCLUSION :

Tests performed to evaluate the biocidal activities of Tristel chlorine dioxide solution against biofilm demonstrate that the circulation of filtered tap water through a Tygon® tube contaminated with a *Pseudomonas aeruginosa* biofilm induces a weakening of the biofilm with a partial removal of biofilm constituents (fixed bacteria, proteins and polysaccharides). Nevertheless this removal effect due to the wash-off effect of the water circulating through the tube is only partial and remaining bacteria can colonized nearby surfaces and/or contaminate the water circulating though the test tube.
The addition of a 0.2 µm filter at the outlet of the test line permits to control the microbial quality of the water produced even if all water sample collected after the filter are not free from microbial contamination.

In the same test conditions, the treatment of the contaminated test line, two times per day with a 50ppm Tristel ClO_2 solution for 5 minutes (curative treatment) induces a total and irreversible elimination of biofilm constituents (fixed bacteria, proteins and polysaccharides), permits to control the biofilm formation on nearby surfaces but also permits to ensure the microbial quality of the water produced.

The circulation 10 times a day of 10 litters of 5 ppm chlorine dioxide solution through the contaminated test line (preventive treatment) induces a reduction of biofilm constituents. Nevertheless this effect is also partial and bacteria remaining on the test line can after prolonged unused test periods induce an increase of the contamination level of test surfaces and/or of the water produced. Nevertheless, compared to control and filtered test lines, repetitive treatment with 5 ppm ClO_2 solution permits to control biofilm formation on nearby surfaces. Lastly, those results seem to indicate that, associated to a specific cycle run each morning to eliminate the potentially contaminated water which has stagnated in the test line all the night (ex: curative treatment or empty cycle) this preventive treatment could be envisaged for the production of "microbiologically controlled rinse water".

VIII : REFERENCES :

1. pr EN ISO 15883 Washer-disinfectors Part 1: General requirements definitions and tests.

2. F. W. Yau. A rapid microdetermination of chlorine dioxide in the presence of active chlorine compounds. Microchemical journal 23, 160-164 (1978).

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