SAFETY DATA AND QUALITY ASSURANCE ACTIVITIES

BATP® L1700S derives from an accurate selection of 100% naturally occurring, non-pathogenic microorganisms that have been specifically selected for their abilities to degrade target substances, none engineered or genetically modified, none mutagenic or pathogenic for humans, plants or animals.

BATP® L1700S has been developed and realized in according to **Directive 2000/54/ EC**:
- Classification in Risk Group 1: unlikely to cause human disease
- Risk Assessment specific activities with biological agents of risk group1: general Hygiene measures
- Protection level concept 1: general Hygiene procedures

BATP® L1700S is safe for humans.

All the ingredients are non-toxic and non-harmful to man, animals and the environment.

No incidence of adverse health effects related to the use of BATP® L1700S or their by-products has ever been reported.

BATP® L1700S has been tested in order to evaluate in vivo acute toxicity, in vitro irritation potential on eyes, skin and vaginal epithelium, in vitro the prosensitizing potential on human dendritic cells. All tests were carried out by the authorized laboratory, internationally recognized as a way to assure product safety and efficacy and customer satisfaction.

BATP® L1700S is not classified and labeled as a dangerous product.

BATP® L1700S complies with the following codes and regulations:
- Reg. CE n.648/2004 31/03/2004 (about the biodegradability and labeling of detergents)
- European Directive 2001/42/EC
- European Directive 2001/18/EC
- Reg. CE n. 1907/2006 del Parlamento Europeo (REACH)
- Reg. CE n. 834/2007 del 28/06/2007
- Reg. CE n. 1272/2008 del 16/12/2008 (CLP)
- European Directive 2000/54/ EC

BBA Biotech delivers BATP® L1700S in sealed drums identified by progressive numeration and lot numbers.

BBA BIOTECH instituted and implemented a Quality Control System, routine checks and measurements to ensure product integrity, correctness, and completeness, to identify and address errors and omissions in the process and to document and archive all record and activities. The lot of the production is accompanied by an analysis report with the total count data, the number of existing bacterial strains and the pathogen-free results again:

Salmonella/Shigella	*Escherichia Coli*	*Staphylococcus Aureus*
Pseudomonas Aeruginosa	*Mould and Yeast*	*Bile-tolerant Gram neg. bacteria*

BBA Biotech guarantees the quality of the BATP® L1700S with the seal intact, eventual claims, complaints or related notifications must arrive not later than 7 days from the delivery of the product.

SAFETY ASSESSMENT for BATP®L1700S

BATP®L1700S has been evaluated in many models for potential acute effects. Two of the models are standard animal models; rabbit skin for skin irritation potential (OECD 404), and rabbit eyes for eye irritation potential (OECD 405). Two other models, in vitro cell cultures of vaginal epithelium and human dendritic cells, provide in vitro data on the epithelial tissue irritation potential and the skin sensitization potential of a topical product, respectively.

Eye Irritation Not an Irritant to Eyes

BATP®L1700S (0.1 mL) was administered to the right eye of male rabbits. The eyes were evaluated at 60 minutes, 24, 48, and 72 hours for corneal, iritic, and conjunctival (chemosis and congestion) effects. Sixty minutes after administration, two animals showed congestion without chemosis; no other abnormality was observed. All eyes were normal at 24, 48, and 72 hours. BATP®L1700S is not an eye irritant.

Skin Irritation Not an Irritant to Skin

BATP®L1700S (0.5 mL) was administered to the intact skin of rabbits for four hours. The skin was evaluated at 24, 48, and 72 hours for erythema and edema. Neither erythema nor edema occurred at any time in the treated rabbits. BATP®L1700S is not a skin irritant.

Skin Irritation-Vaginal Epithelium Not an Irritant to Vaginal Epithelium

BATP®L1700S was evaluated in a cell survival assay using human vaginal epithelial cells in culture. BATP®L1700S was well tolerated by the vaginal epithelial cells which means it is not likely to irritate human vaginal epithelial tissue and unlikely to irritate other epithelial tissue.

Skin Sensitization-Human Dendritic Cells Not a Skin Sensitization

This in vitro model uses immunocompetent skin cells to determine if a test chemical can induce marker expression in the cells. Stimulation of marker expression is associated with skin sensitization potential. BATP®L1700S did not affect marker expression in this in vitro system. Thus, it is unlikely that BATP®L1700S has any potential to induce immune cellular expression and subsequently, skin sensitization.

Toxicology Expert Assessment

In the toxicology Expert Assessment performed by Intorre Professor of Pharmacology and Toxicology Department – University of Pisa, BATP®L1700S resulted non-toxic, non- harmful, non-irritating for the skin, eyes and human vaginal epithelium and mucosa and does not show any stimulating potential on the immune cellular response.

Challenge Test

In Challenge Test performed by SGS Institute - Life Science Services, BATP®L1700S resulted very active in contrasting the pathogen microbial growth, as Pseudomonas Aeruginosa, Clostridium Sporogenes,Enterococcus Fecalis, Escherichia Coli, Salmonella and Staphilococcus Aureus.

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Sponsor

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VERIFICATION OF BATP®L1700S EFFICACY ON LEGIONELLA PNEUMOPHILA

REPORT

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1 OBJECTIVES

The purpose of this study is to investigate the possibility of using the product BATP ®L1700S as an alternative to the others disinfectants usually used to reduce the microbial load of Legionella pneumophila in artificial water environments.

The study will focus on the verification of the effectiveness of the product against Legionella pneumophila as this organism is responsible for dangerous, even fatal, respiratory diseases.

This study was commissioned by the customer BBA Biotech, producer of the product BATP ®L1700S and author of the patent.

2 REFERENCES

- Study protocol P/2223.3 "Verification of BATP ®L1700S efficacy on Legionella pneumophila".
- ISO 11731-2 "Water quality-Detection and enumeration of Legionella".
- UNI EN ISO 6222 "Water quality - Enumeration of culturable micro-organisms -- Colony count by inoculation in a nutrient agar culture medium.
- EMEA/CVMP/540/03-Revised - Appendix 1

3 SITE

The study was performed by SGS Sertec srl Life Science Laboratory, located in:

Via Cimarosa 95-105 Livorno (57124).

Tel. 0586/852591

Fax 0586/866210

4 STUDY EXECUTION

4.1 MATERIALS AND EQUIPMENT

The verification of the activity of BATP ®L1700S against Legionella required the following materials and equipments.

The preparation of water samples required:

Material	Supplier	Lot	Expire date
Elix water	SGS LSS Laboratory	Sample of 11/12/2014 and the 16/02/2015	-
Livorno aqueduct water	SGS LSS Laboratory	Sample taken the 11/12/2014 and the 19/02/2015	-
Calcium Chloride CaCl2	Sigma Aldrich	SLBL1812V	31/08/2017
L. pneumophila ATCC 33152	Biogenetics	211/324	12/2015
BATP	BBA Biotech Srl	32314	-
Autoclaved flasks	SGS LSS Laboratory	-	-
Autoclaved cellulose caps	SGS LSS Laboratory	-	-
Polypropylene tube	-	-	-
Polyethylene tube	-	-	-
Galvanized steel	-	-	-

The microbial analysis of water samples required:

Material	Supplier	Lot	Expire date
Water Plate Count Agar wPCA	Oxoid	12122014 12022015	12/01/2015 12/03/2015
CYE	Oxoid	12122014 12022015	12/01/2015 12/03/2015
Legionella BCYE Growth supplement	Oxoid	1574235	30/11/2016
Legionella GVPC Selective supplement	Oxoid	1535895	31/08/2016
Legionella BCYE w/o L-cysteine	Oxoid	1513075	30/06/2016
Quantiswab	Biomerieux	883100	03/2016
Gram stain kit	Oxoid	270925	01/03/2015
Sterile plates	VWR	-	-

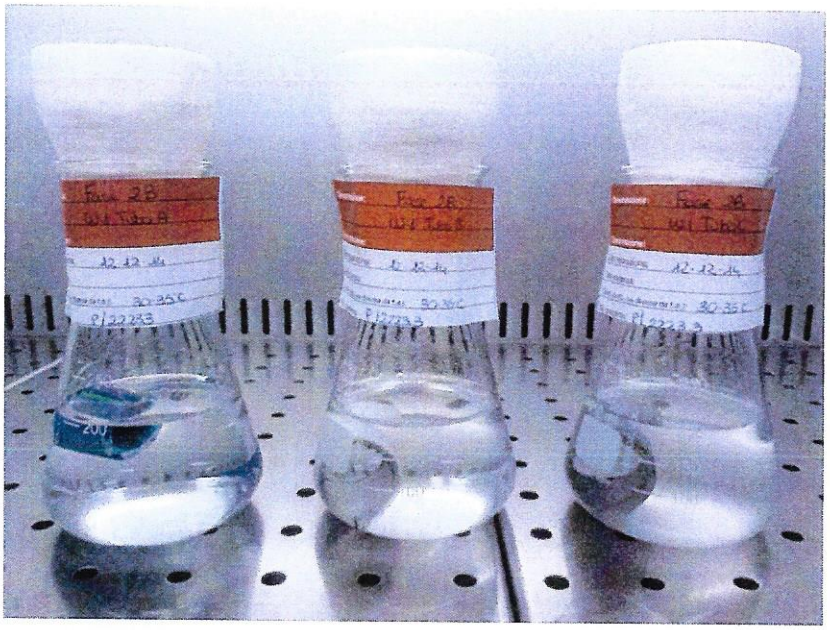
The equipment necessary for the study comprised:

- Biological Flow (tag CQB 22).
- Autoclave (tag CQB 02).
- Incubator (tag CQB 18 at 36 ± 1°C, CQB 16 at 22,5 ± 2,5°C, CBQ 17 at 32,5 ± 2,5°C).
- Refrigerator (tag CQB 25).
- Microscope (tag CQB 48).

4.2 SAMPLES DESCRIPTION

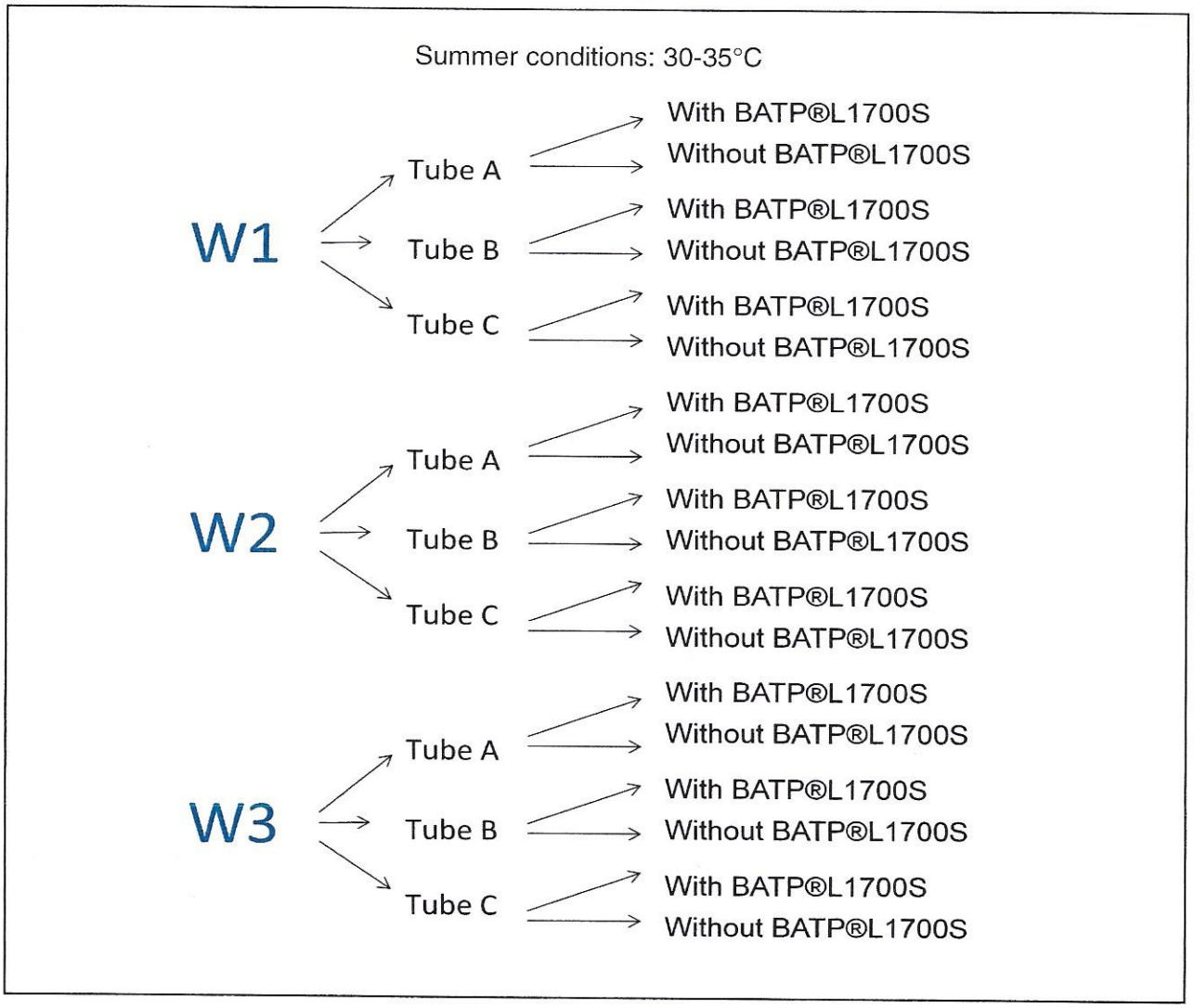
The effect of BATP ®L1700S against Legionella pneumophila was studied on 36 different samples; each sample was prepared in order to test different conditions that simulated the common characteristics of water and type of pipes presets in the aqueduct, where the BATP ®L1700S can act.

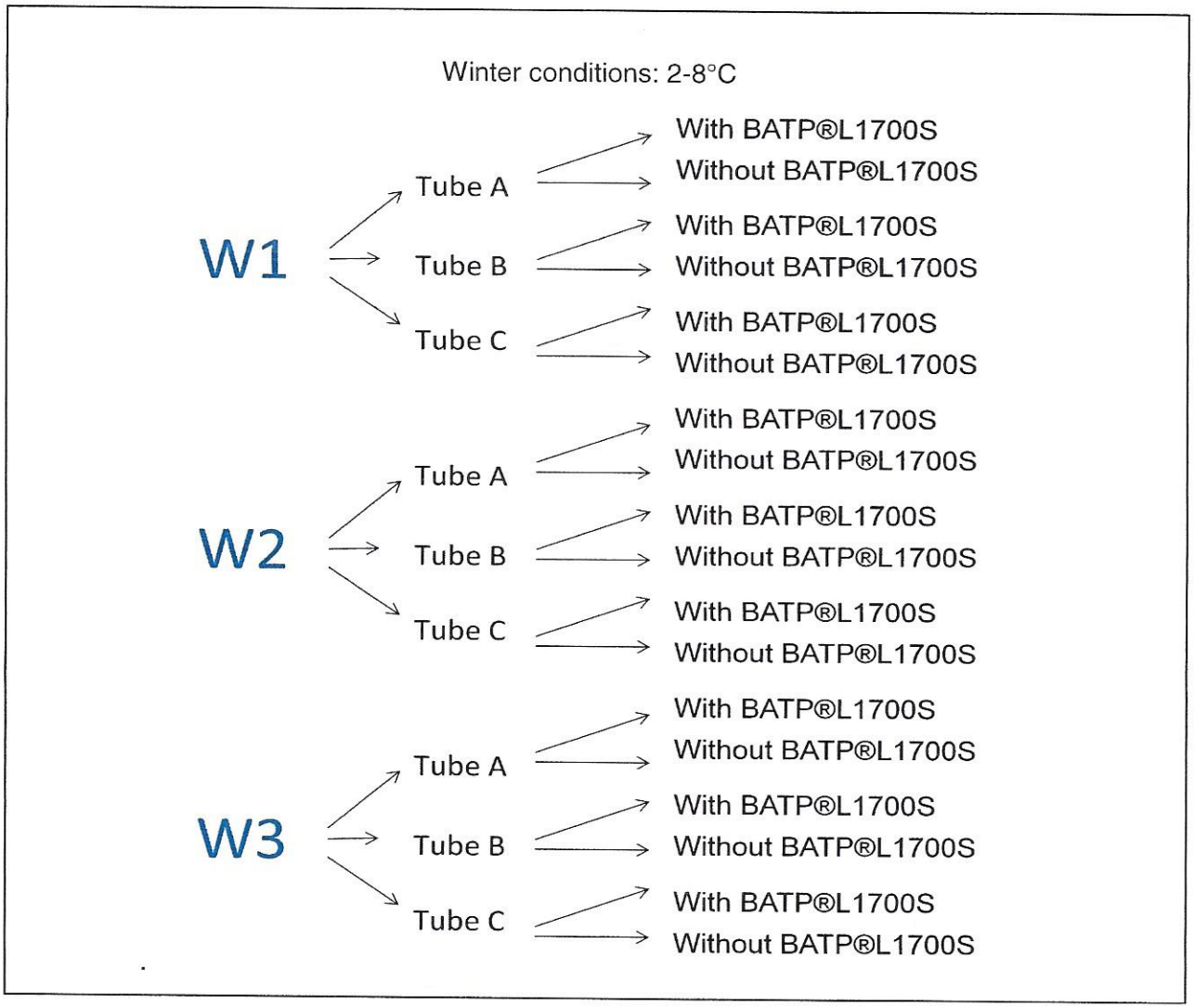
- Three different tube categories were chosen:
  - Tube A: polypropylene.
  - Tube B: polyethylene.
  - Tube C: galvanized steel.
- three different type of water where prepared:
  - Water W1 from Livorno aqueduct without sterilization.
  - Water W2 (Soft water) prepared by adding 29,33 mg/L of $CaCl_2$ to sterile water according to annex 1 of EMEA/CVMP/540/03-Revised.
  - Water W3 (Hard water) prepared by adding 502,4 mg/L of $CaCl_2$ to sterile water according to annex 1 of EMEA/CVMP/540/03-Revised.
- Each sample was composed by a sterile flasks containing 100 ml of each type water and one piece of tube as represented in picture 1.



Picture 1. Sample example.

Each sample was added with 1 ml of Legionella pneumophila ATCC 211324 from a standard solution concentrated 2,4 x 10^6 CFU/ml to obtain a theoretical concentration of about 1000 CFU/flask.
Half of the samples were added with 1 ml of BATP ®L1700S diluted from a concentrated solution of 5,8 x 10^9 CFU/ml, to have a theoretical concentration of 1000 CFU/flask (Phase 2B-3B). The other half was used as a positive control for Legionella pneumophila without the addition of the product (Phase 2A-3A).
Each sample was incubated at 30-35°C or 2-8°C to simulate summer and winter conditions.
The 36 samples were divided as shown in the pictures below.





3 samples containing water W1 and tubes A, B, C were incubated at room temperature (20-25°C) without the addition of Legionella pneumophila and BATP ®L1700S (Phase 1- microbial growth in a static environment). In order to evaluate the microbial trend in Livorno aqueduct water.

4.3 TEST FREQUENCY

The study to verify the efficacy of BATP ®L1700S against Legionella pneumophila was articulated in two part. Each part of the study was carried out separately one after the other.

- Part 1 (Static condition): started on 12-12-2014 and ended on 7/01/2015
- Part 2 (Dynamic condition): started on 19-02-2015 and ended on 17/03/2015.

The samples resumed in table 1 were prepared twice and analyzed according to the following calendar:

Part	Time of analysis	Date of analysis
Part 1 Static condition	Preparation of the 36 samples	11/12/2014
	T0	12/12/2014
	T3	15/12/2014
	T10	22/12/2014
	T15	27/12/2014
Part 2 Dynamic condition	Preparation of the 36 samples	16/02/2015- 19/02/2015
	T0	19/02/2015
	T3	22/02/2015
	T10	02/03/2015
	T15	07/03/2015

Table1. Calendar of the study

4.4 SAMPLE ANALYSIS

Both part of the study were characterized by the analysis of each sample through the evaluation of the total microbial population according to UNI EN ISO 6222, and the detection of Legionella pneumophila according to ISO 11731-2 (adapted to our study case) for specific medium choice at every time check. The detection of Legionella pneumophila was also accompanied by the utilization of selective media, the gram coloration and the microscope observation in order to confirm presumptive colonies.
The materials used for the study and the raw data obtained from each analysis are collected in the modules provided by the protocol.

Down below it is described step by step the analysis for each sample at every time check.

1) Sample selection: each sample is taken from the incubator CQB 17 or refrigerator CQB 25 at the specific time check and analyzed under biological flow.
2) Swab execution: the tube inside the flask is taken by the operator with sterile pliers and the tube surface is swabbed with an appropriate disposable swab. The swab is then passed on the surface of a plate containing the specific medium for Legionella pneumophila detection (Legionella CYE + BCYE growth supplement). This operation allow to collect any microorganisms that can colonize the surface of the tube.
3) Sample homogenization: the flask is then rotated several time by the operator in order to homogenize the water sample
4) Microbial count for:
    a. Legionella pneumophyla: to detect the presence of Legionella pneumophila in solution 1 ml of sample is taken and spreaded on the specific medium (Legionella CYE + BCYE growth supplement). This operation is repeated three times using also consecutive dilution(e.g. 1:10, 1:100, 1:1000).
    b. Total microbial population: 1 ml of sample is taken and spreaded by inclusion onto a non selective medium (wPCA) according to UNI EN ISO 6222. This operation is repeated three times using also consecutive dilution (e.g.1:10, 1:100, 1:1000).
5) Incubation: the plates produced from all analysis are incubated at the appropriate temperature for the appropriate time according to the reference method; 36 ± 1°C for the detection of Legionella, 36 ± 1°C and 22,2 ±°C for total microbial count.
6) Plate reading: the plates are read after incubation and the results are collected in the appropriate modules provided by the protocol. For CFU reading it is appropriate to count plates with not more than 300 CFU and then multiply for the correct dilution factor.
7) Legionella identification: suspect colonies of Legionella pneumophila are submitted to confirmation tests with specific selective medium:
    a. CYE + BCYE without L-cysteine (it doesn't allow Legionella growth).
    b. CYE + GVPC selective agar (it allow Legionella growth).
    c. Gram test and microscopy observation.

    The same suspect colony is spread onto the two different selective medium and on a slide. The presence of Legionella pneumophila is confirmed if the colony is able to reproduce on GVPC

selective agar and instead of BCYE without L-cysteine and it appears a gram negative rod with the microscopy observation.

8) Report: the results of microbial count at every time check expressed in CFU/ml are reported on the correct modules.
9) Legionella pneumophila reduction calculation: from the results of CFU/ml of Legionella pneumophila obtained from samples with and without the effect of BATP ®L1700S it is possible to calculate the percentage of reduction of the microorganism by this equation.

$$\% = 100 - \frac{CFU\ Legionella\ pneumophila\ with\ the\ effect\ of\ \text{BATP ®L1700S}}{CFU\ Legionella\ pneumophila\ without\ the\ effect\ of\ \text{BATP ®L1700S}}\ x\ 100$$

4.5 RESULTS

4.5.1 PART 1: MICROBIAL GROWTH IN STATIC CONDITION

4.5.1.1 Evaluation of microbial population in Livorno aqueduct (Phase 1A)

The results obtained from the analysis of the microbial population in the three samples of Livorno aqueduct water (phase 1) are reported in the tables below.

Analysis	TMC at 22°C (CFU/ml)				TMC at 36°C (CFU/ml)			
Time analysis	T0	T3	T10	T15	T0	T3	T10	T15
W1A	0	2	36	179	22	39	65	90
W1B	0	7	105	126	3	8	109	187
W1C	0	5	57	94	4	18	84	126

Table2. TMC values expressed in CFU/ml of phase 1 samples.

Analysis	TMC on swabs (CFU/ml)			
Time analysis	T0	T3	T10	T15
W1A	0	0	21	41
W1B	0	0	36	65
W1C	0	0	10	21

Table3. TMC swab results expressed in CFU/ml of phase 1 samples.

Preliminary conclusions:

The microbial population of Livorno aqueduct is very low at the beginning of the test, it grows in time until two log factors. After 10 days of static conditions the microbial populations start also to colonize the tubes surface.

4.5.1.2 Evaluation of BATP ®L1700S effect against Legionella pneumophila in solution

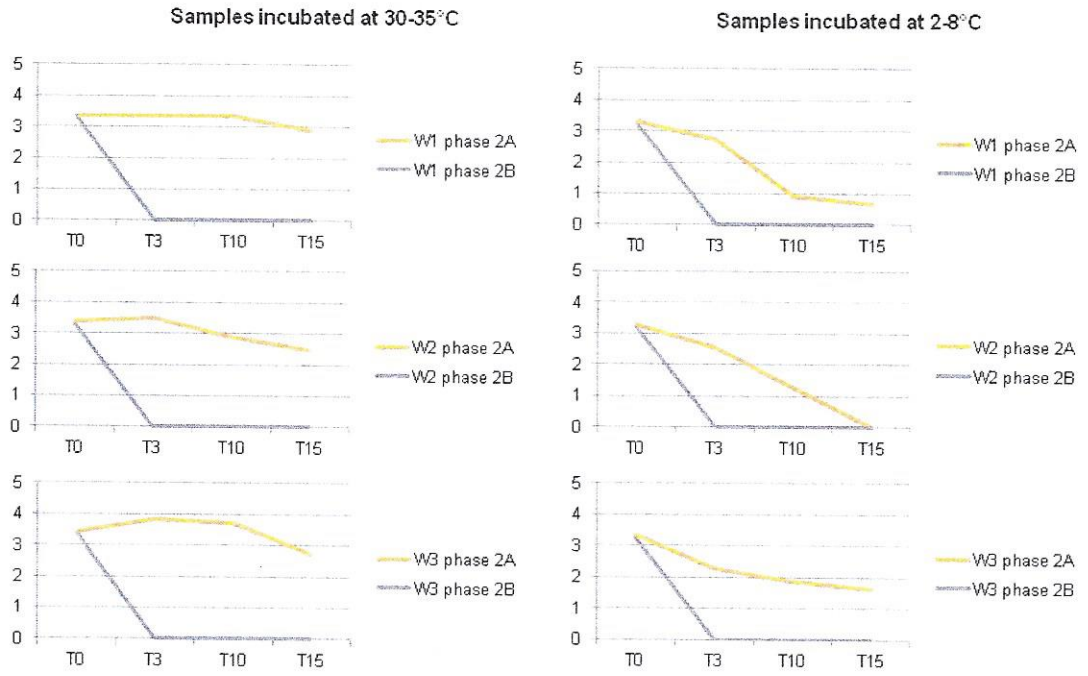
The results obtained from the analysis of Legionella pneumophila in each flasks are reported in the tables below;. the results express the number of Legionella pneumophila CFU/ml calculated for each flask at every time check in presence or absence of BATP ®L1700S (Phase 2A and Phase 2B). Every CFU data was calculated and verified according to paragraph 4.4.

Analysis	CFU/ml of L.pneumophila on phase 2A				CFU/ml of L.pneumophila on phase 2B			
Time analysis	T0	T3	T10	T15	T0	T3	T10	T15
W1A	2512	1580	583	292	2130	0	0	0
W1B	2010	2460	2040	1100	1790	0	0	0
W1C	2230	2440	3900	800	2010	0	0	0
W2A	2550	6500	279	23	1870	0	0	0
W2B	2460	1830	1220	750	2200	0	0	0
W2C	2380	900	750	140	1950	0	0	0
W3A	2420	7200	5760	18	2340	0	0	0
W3B	2950	10520	7680	1400	2680	0	0	0
W3C	2180	2000	1560	130	1840	0	0	0

Table 4. Legionella pneumophila average number of CFU/ml in samples incubated at 30-35°C.

Analysis	CFU/ml of L.pneumophila on phase 2A				CFU/ml of L.pneumophila on phase 2B			
Time analysis	T0	T3	T10	T15	T0	T3	T10	T15
W1A	2150	527	0	0	1700	0	0	0
W1B	1850	870	22	14	1570	0	0	0
W1C	2050	165	4	0	1720	0	0	0
W2A	1600	374	0	0	1360	0	0	0
W2B	1800	635	37	0	1350	0	0	0
W2C	2250	71	21	0	2030	0	0	0
W3A	2450	324	42	16	2060	0	0	0
W3B	1950	165	150	112	1660	0	0	0
W3C	2000	97	27	0	1610	0	0	0

Table 5. Legionella pneumophila average number of CFU/ml in samples incubated at 2-8°C.

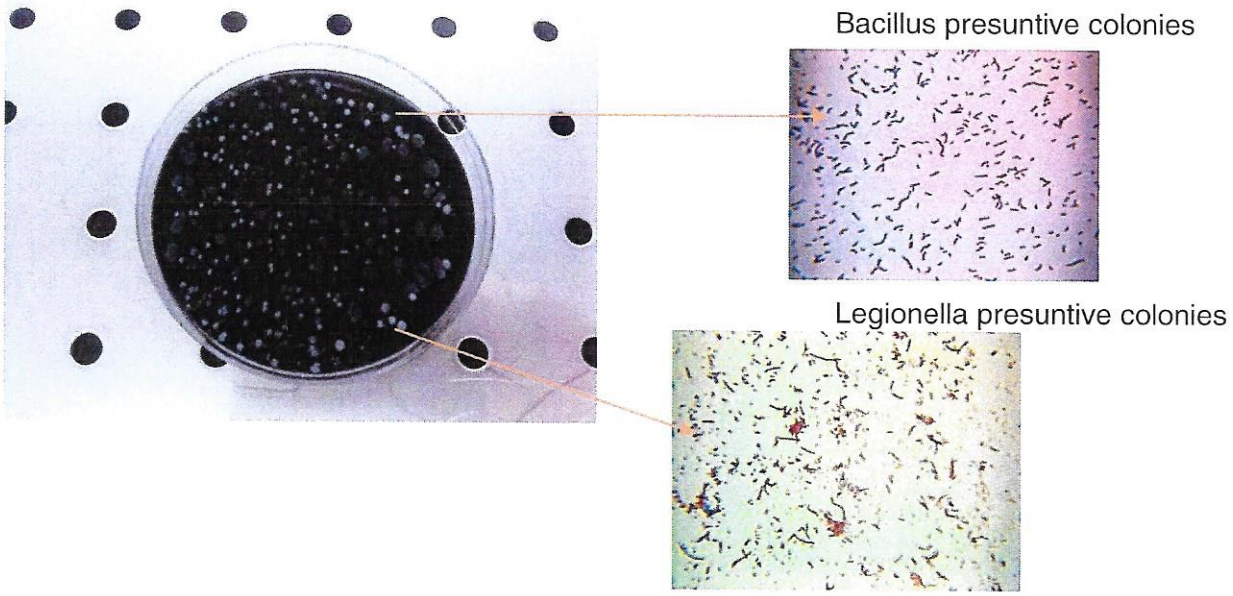


Picture 2. Legionella pneumophila CFU/ml trend from T0 to T15 of samples of Phase 2A and phase 2B for different type of water.

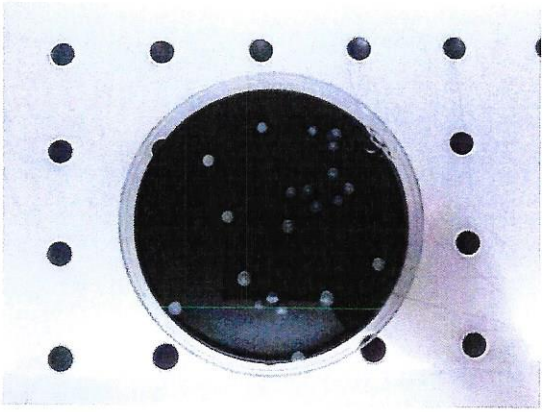
The above representation show graphically the CFU/ml calculated for L. pneumophila on phase 2A and on phase 2B at every time check, from the three different type of water and temperatures of incubation. The CFU/ml values were all transformed into logarithm.

Preliminary conclusions:

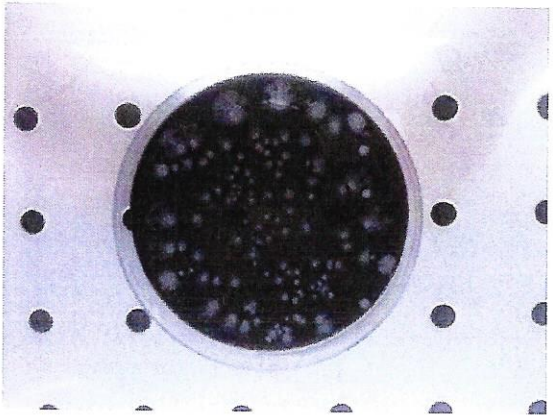
The results showed in both tables and graphs clearly report a different behavior of Legionella pneumophila in presence (phase 2B) and absence (phase 2A) of BATP ®L1700S for both incubation temperatures. The decrease of Legionella pneumophila CFU in presence of BATP ®L1700S starts from Time 3. In fact while at T0 we were able to detect both type of microorganism in the samples of Phase 2B were the two microorganisms had been added with a similar concentration (Picture 3); during the following time checks we were not able to detect Legionella pneumophila any more from samples solution (Picture 4).



Picture 3. Picture representing T0 of the sample containing water W2 with polypropylene tube in phase B spread onto CYE + BCYE growth supplement for Legionella detection; 2 type of microorganism were selected and identified with Gram test. Bacillus colonies with irregular borders, colored in pale gray and Gram positive. Legionella pneumophila white colonies, with a textured, cut-glass appearance, Gram negative.



Picture 4. Picture representing T0 of the sample containing water W2 with polypropylene tube in phase 2A spread onto CYE + BCYE growth supplement for Legionella detection. It is evident the only presence of Legionella pneumophila.



Picture 5. Picture representing T3 of the sample containing water W2 with polypropylene tube in phase 2B spread onto CYE + BCYE growth supplement for Legionella detection. It is evident the only presence of Bacillus spp.

From the results showed in paragraph 4.5 it is also evident the effect of BATP ®L1700S on tube surfaces, as the swab analysis gave the same abatement results.

4.5.1.3 Evaluation of BATP ®L1700S effect against Legionella pneumophila biofilm

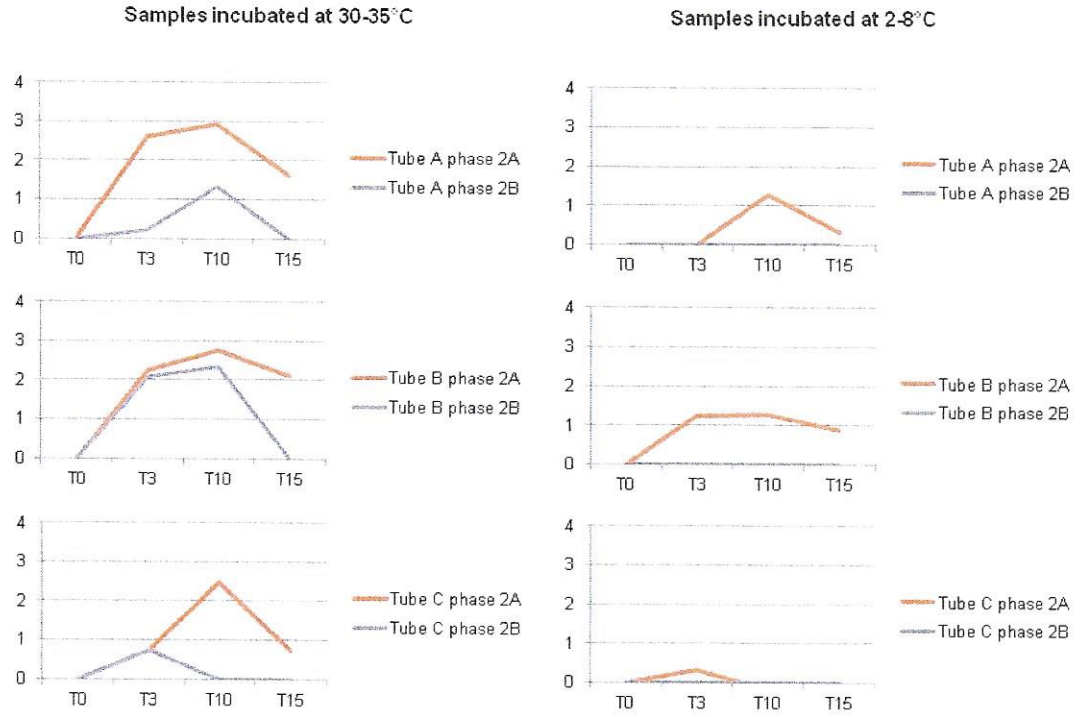
The results obtained from the swabs made on the tube of each flasks are reported in the tables below; the results express the number of Legionella pneumophila CFU observed for each tube at every time check in presence or absence of BATP ®L1700S (Phase 2A and Phase 2B). Every CFU data was calculated and verified according to paragraph 4.4. The presence of a microbial population on the tube indicate the establishment of a biofilm.

Analysis	CFU of L.pneumophila on phase 2A				CFU of L.pneumophila on phase 2B			
Time analysis	T0	T3	T10	T15	T0	T3	T10	T15
W1A	0	0	30	3	0	5	63	0
W1B	0	10	532	272	0	87	4	0
W1C	0	9	896	16	0	14	0	0
W2A	0	0	1280	12	0	0	0	0
W2B	0	0	152	12	0	250	524	0
W2C	0	0	0	0	0	0	0	0
W3A	0	1192	1208	115	0	0	0	0
W3B	0	532	1036	112	0	13	127	0
W3C	0	7	0	0	0	3	0	0

Table 6. Legionella pneumophila CFU swabbed from tubes in samples incubated at 30-35°C.

Analysis	CFU/ml of L.pneumophila on phase 2A				CFU/ml of L.pneumophila on phase 2B			
Time analysis	T0	T3	T10	T15	T0	T3	T10	T15
W1A	0	0	1	0	0	0	0	0
W1B	0	0	54	23	0	0	0	0
W1C	0	0	2	0	0	0	0	0
W2A	0	0	0	0	0	0	0	0
W2B	0	53	0	0	0	0	0	0
W2C	0	0	0	0	0	0	0	0
W3A	0	0	53	6	0	0	0	0
W3B	0	0	2	0	0	0	0	0
W3C	0	6	0	0	0	0	0	0

Table 7. Legionella pneumophila CFU swabbed from tubes in samples incubated at 2-8°C.



Picture 6. Legionella pneumophila CFU trend from T0 to T15 for samples of Phase 2A and phase 2B for different type of tubes.

The above representation show graphically the CFU calculated for L. pneumophila on phase 2A and on phase 2B at every time check, after swabbing on the surfaces of the three different type of tubes and temperatures of incubation. The CFU values obtained were all transformend into logharitm. The different graphs evidence the decrease of Legionella pneumophila CFU onto the tubes surface caused by the presence of BATP ®L1700S.

Preliminary conclusions:

The decrease of Legionella pneumophila CFU by the presence of BATP ®L1700S is showed also from the swab results. However in solution the effect of BATP ®L1700S was immediate at T3, the tubes surface analysis showed in some cases (expecially for tubes incubated at 30-35°C) the persistence of Legionella biofilm also at T10. Though at the end of the experiment Legionella pneumophila colonies in all samples were undetectable for both incubation temperatures.

4.5.1.4 Evaluation of the total microbial count

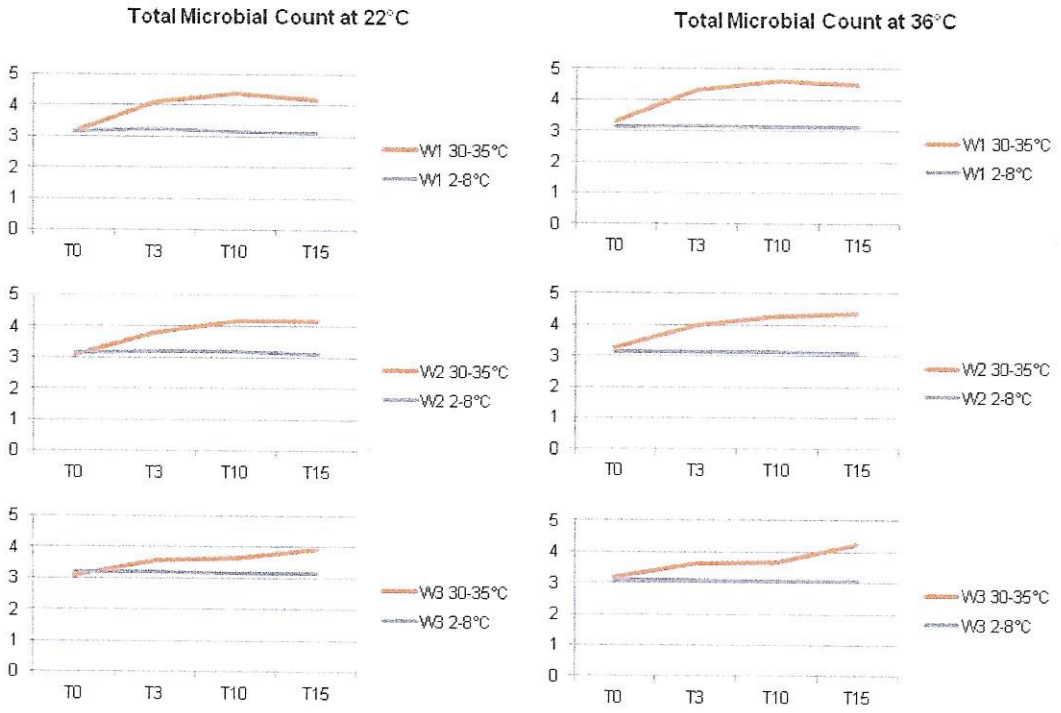
The results obtained from the total microbial count, TMC, are reported on the tables below. This parameter was measured only for phase 2B in order to evaluate the microbial trend in the samples where the BATP ®L1700S was added.

Analysis	TMC at 22°C CFU/ml				TMC at 36°C CFU/ml			
Time analysis	T0	T3	T10	T15	T0	T3	T10	T15
W1A	1690	10500	4200	11000	1970	20000	9800	34000
W1B	1210	12200	56000	18000	1670	18000	83000	31000
W1C	1450	14000	13000	14000	1990	25000	23000	22000
W2A	1020	9000	8900	2200	1560	14000	11000	4300
W2B	1320	940	29000	32000	1840	1300	38500	44000
W2C	1240	8800	5200	10200	1830	13000	9000	17000
W3A	1280	950	1360	1900	1600	1000	890	2200
W3B	1100	1290	880	9700	1360	720	1030	28000
W3C	1050	8500	10500	15000	1650	10300	12100	21000

Table 8. Total microbial count expressed in CFU/ml of samples incubated at 30-35°C.

Analysis	TMC at 22°C CFU/ml				TMC at 36°C CFU/ml			
Time analysis	T0	T3	T10	T15	T0	T3	T10	T15
W1A	1680	1590	1630	1250	1360	1460	1250	1540
W1B	1100	1540	1200	1180	1250	1280	1320	1010
W1C	1560	1960	1420	1370	1480	1650	1390	1320
W2A	1420	1630	1690	1250	1670	1690	1460	1380
W2B	1580	1290	1630	1420	1330	1230	1440	1160
W2C	1260	1590	1430	1270	1220	980	1120	960
W3A	1380	1290	1490	1260	1250	1020	1300	1190
W3B	1650	1820	1630	1520	1380	1530	1100	1310
W3C	1460	1600	1250	1320	1160	1210	1040	940

Table 9. Total microbial count of samples incubated at 2-8°C.



Picture 7. Total microbial count (TMC) trend from T0 to T15 for samples of phase 2B for different type of water and temperature of incubation.

Preliminary conclusions:

These results show a proliferation of microorganisms in samples incubated at 30-35°C, suggesting that higher temperature help the microbial growth. The microbial population in samples incubated at 2-8°C remains constant.

4.5.2 PART 2: MICROBIAL GROWTH IN DYNAMIC CONDITION

4.5.2.1 Evaluation of BATP effect against Legionella pneumophila in solution

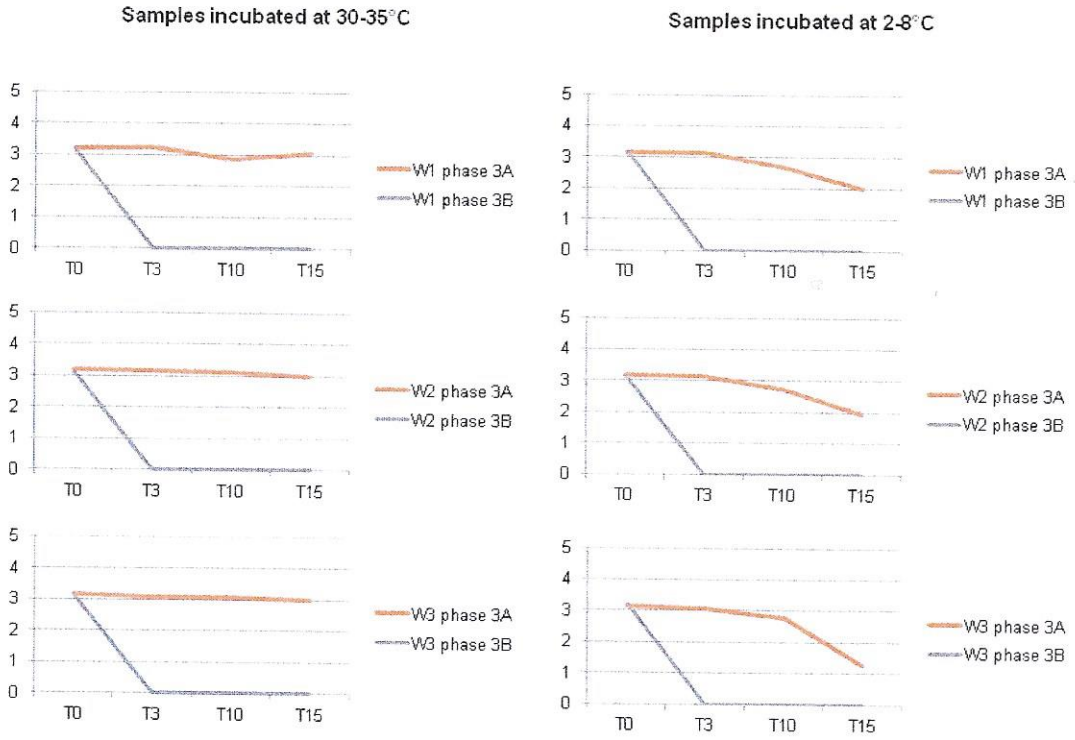
The results obtained from the analysis of Legionella pneumophila in each flasks during the dynamic phase are reported in the tables below; the results express the number of Legionella pneumophila CFU/ml calculated for each flask at every time check in presence or absence of BATP ®L1700S (Phase 3A and Phase 3B). Every CFU data was calculated and verified according to paragraph 4.4.

Analysis	CFU/ml of L.pneumophila on phase 3A				CFU/ml of L.pneumophila on phase 3B			
Time analysis	T0	T3	T10	T15	T0	T3	T10	T15
W1A	1480	1650	580	1230	1350	0	0	0
W1B	1590	1820	720	1350	1130	0	0	0
W1C	1610	1480	890	1130	1660	0	0	0
W2A	1750	1250	1760	1200	1280	0	0	0
W2B	1396	1470	960	650	1340	0	0	0
W2C	1488	1380	1100	1230	1350	0	0	0
W3A	1650	890	1240	1130	1550	0	0	0
W3B	1400	1830	1080	700	1220	0	0	0
W3C	1390	965	1220	1030	1120	0	0	0

Table 10. Legionella pneumophila CFU in samples incubated at 30-35°C.

Analysis	CFU/ml of L.pneumophila on phase 3A				CFU/ml of L.pneumophila on phase 3B			
Time analysis	T0	T3	T10	T15	T0	T3	T10	T15
W1A	1250	1360	540	0	1450	0	0	0
W1B	1320	1280	520	310	1580	0	0	0
W1C	1450	1350	101	0	1610	0	0	0
W2A	1300	1350	420	147	1130	0	0	0
W2B	1570	1420	590	18	1790	0	0	0
W2C	1630	1520	650	93	1310	0	0	0
W3A	1390	1240	820	2	1590	0	0	0
W3B	1420	1100	235	0	1610	0	0	0
W3C	1340	1260	720	52	1720	0	0	0

Table 11. Legionella pneumophila CFU in samples incubated at 2-8°C.



Picture 8. Legionella pneumophila CFU/ml trend from T0 to T15 of samples of Phase 3A and phase 3B for different type of water.

Preliminary conclusions:

The results obtained during the static phase where confirmed also in the dynamic one by the disappearance of Legionella pneumophila after 3 days of experiment, for both temperature of incubation.

4.5.2.2 Evaluation of BATP effect against Legionella pneumophila biofilm

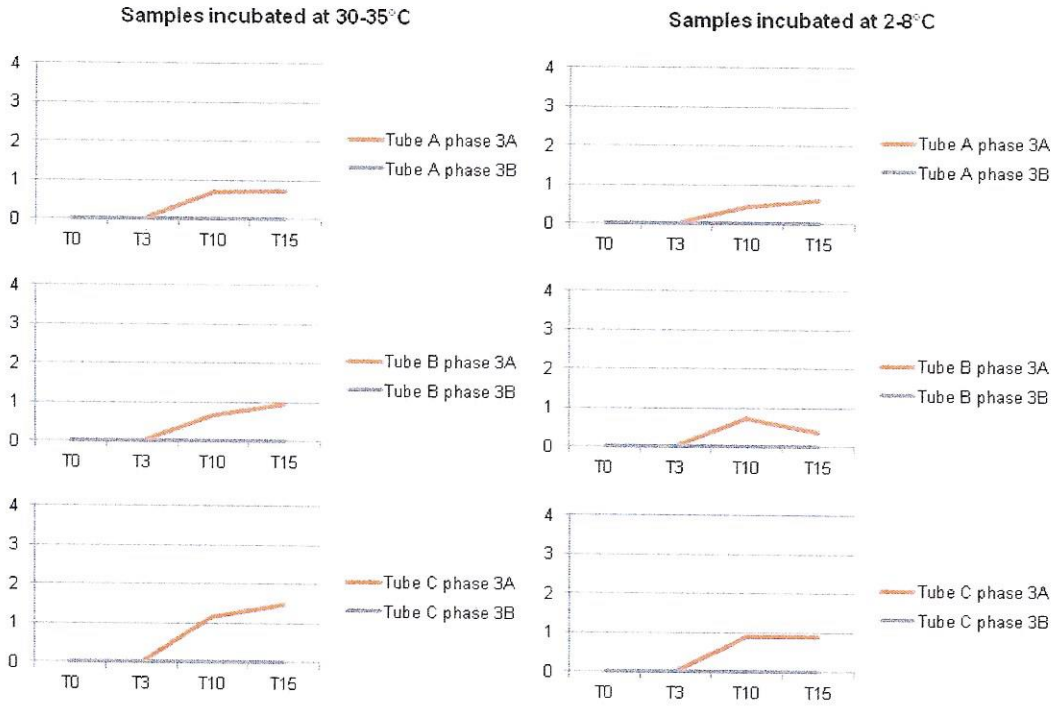
The results obtained from the swabs made on the tube of each flasks are reported in the tables and picture below;. the results express the number of Legionella pneumophila CFU calculated for each tube at every time check in presence or absence of BATP ®L1700S (Phase 3A and Phase 3B). Every CFU data was calculated and verified according to paragraph 4.4. The presence of a microbial population on the tube indicate the establishment of a biofilm.

Analysis	CFU of L.pneumophila on phase 3A				CFU of L.pneumophila on phase 3B			
Time analysis	T0	T3	T10	T15	T0	T3	T10	T15
W1A	0	0	12	16	0	0	0	0
W1B	0	0	5	9	0	0	0	0
W1C	0	0	2	12	0	0	0	0
W2A	0	0	0	0	0	0	0	0
W2B	0	0	7	19	0	0	0	0
W2C	0	0	22	52	0	0	0	0
W3A	0	0	3	0	0	0	0	0
W3B	0	0	2	0	0	0	0	0
W3C	0	0	18	27	0	0	0	0

Table 12. Legionella pneumophila CFU on tubes in samples incubated at 30-35°C.

Analysis	CFU of L.pneumophila on phase 3A				CFU of L.pneumophila on phase 3B			
Time analysis	T0	T3	T10	T15	T0	T3	T10	T15
W1A	0	0	3	0	0	0	0	0
W1B	0	0	13	0	0	0	0	0
W1C	0	0	8	9	0	0	0	0
W2A	0	0	1	0	0	0	0	0
W2B	0	0	3	4	0	0	0	0
W2C	0	0	10	8	0	0	0	0
W3A	0	0	4	12	0	0	0	0
W3B	0	0	0	3	0	0	0	0
W3C	0	0	6	7	0	0	0	0

Table 13. Legionella pneumophila CFU on tubes in samples incubated at 2-8°C.



Picture 9. Legionella pneumophila CFU trend from T0 to T15 of samples of Phase 3A and phase 3B for different type of tubes.

Preliminary conclusions:

The results obtained from the swabs analysis show the effectiveness of BATP ®L1700S against Legionella pneumophila biofilm and confirm the previous results.

4.5.2.3 Evaluation of the total microbial count (dynamic phase)

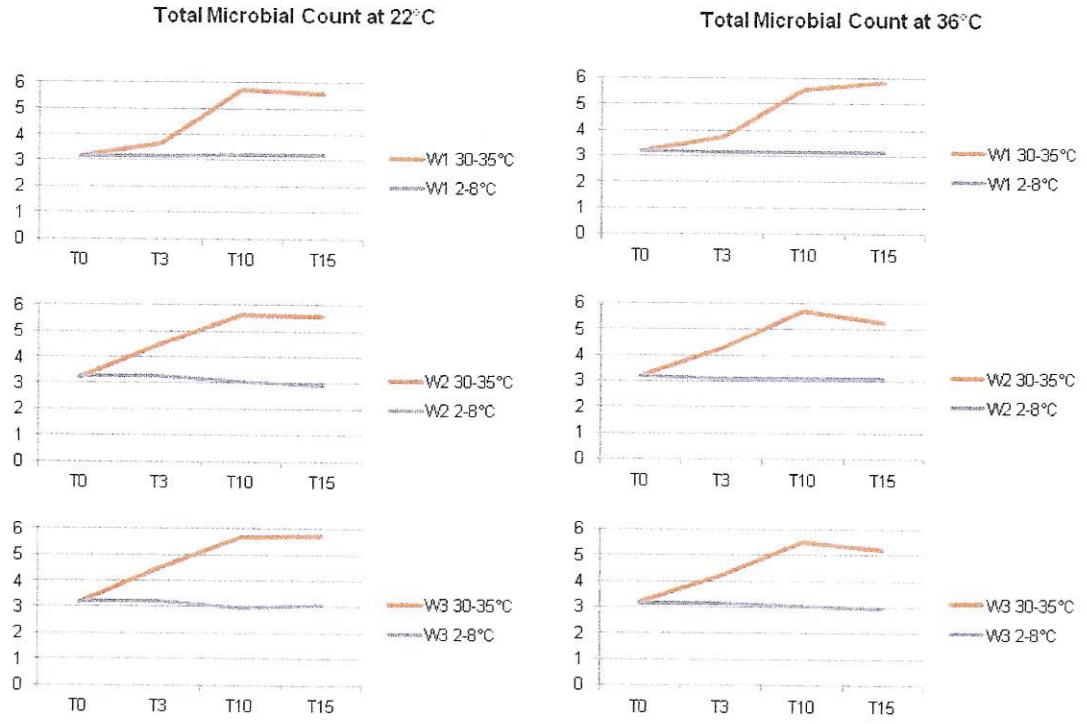
The results obtained from the total microbial count, TMC, are reported on the tables below. This parameter was measured only for phase 3B in order to evaluate the microbial trend in the samples where the BATP ®L1700S was added. These results show a proliferation of microorganisms in samples incubated at 30-35°C, suggesting that higher temperature help the microbial growth. The microbial population in samples incubated at 2-8°C remains constant.

Analysis	TMC at 22°C CFU/ml				TMC at 36°C CFU/ml			
Time analysis	T0	T3	T10	T15	T0	T3	T10	T15
W1A	1610	10880	492000	480000	1620	13600	422000	1048000
W1B	1540	1660	696000	187000	1460	1310	560000	370000
W1C	1490	1340	356000	448000	1470	1260	132000	760000
W2A	1500	41000	592000	508000	1560	35900	428000	520000
W2B	1790	51600	696000	660000	1370	21900	986000	15400
W2C	1520	1650	1400	2300	1520	1360	1300	1500
W3A	1530	26800	654000	568000	1480	32800	382000	320000
W3B	1320	69300	812000	992000	1370	16500	606000	144000
W3C	1570	1620	1700	16900	1620	1260	1110	1470

Table 14. Total microbial count of samples incubated at 30-35°C.

Analysis	TMC at 22°C CFU/ml				TMC at 36°C CFU/ml			
Time analysis	T0	T3	T10	T15	T0	T3	T10	T15
W1A	1560	1380	1980	1240	1490	1280	960	1480
W1B	1289	1420	1360	1820	1630	1320	1620	1460
W1C	1850	1690	1820	1750	1490	1610	1400	1280
W2A	1630	1630	1250	980	1350	1030	1490	1750
W2B	1920	1850	1960	1020	1710	1180	1660	1240
W2C	1730	1810	520	600	1490	1410	420	850
W3A	1480	1320	1100	1230	1250	1830	1200	982
W3B	1460	1650	1360	1480	1720	1290	1350	1270
W3C	1760	1860	420	750	1240	980	630	470

Table 15. Total microbial count of samples incubated at 2-8°C.



Picture 10. Total microbial count (TMC) trend from T0 to T15 for samples of phase 3B for different type of water and temperature of incubation.

Preliminary conclusions:

Also the dynamic phase show a proliferation of microorganisms in samples incubated at 30-35°C, suggesting that higher temperature help the microbial growth. The microbial population in samples incubated at 2-8°C remains constant.

4.6 DISCUSSION

4.6.1 LEGIONELLA PNEUMOPHILA REDUCTION

This test was performed in order to simulate the effect of BATP ®L1700S that can be added to several artificial water environments to reduce the Legionella pneumophila colonization. The reduction of Legionella pneumophila was calculated from the results obtained by the comparison between:

$$\% = 100 - \frac{CFU\ Legionella\ pneumophila\ with\ the\ effect\ of\ \text{BATP ®L1700S}}{CFU\ Legionella\ pneumophila\ without\ the\ effect\ of\ \text{BATP ®L1700S}}\ x\ 100$$

The results of all the test are reported in tables below; they show the ability of BATP ®L1700S to reduce completely the presence of Legionella pneumophila after three days of experiment, for both temperature of analysis and for both static and dynamic conditions. All samples present in fact a 100% abatement of Legionella pneumophila from T3. In samples were the Legionella pneumophila CFUs were undetectable, the abatement percentage was not calculated (NA). The results in the tables below do not consider time zero for the calculation of the abatement.

Phase	2B			2B		
Time analysis	T3 30-35°C	T10 30-35°C	T15 30-35°C	T3 2-8°C	T10 2-8°C	T15 2-8°C
W1A	100	100	100	100	100	100
W1B	100	100	100	100	100	100
W1C	100	100	100	100	100	100
W2A	100	100	100	100	100	100
W2B	100	100	100	100	100	100
W2C	100	100	100	100	100	100
W3A	100	100	100	100	100	100
W3B	100	100	100	100	100	100
W3C	100	100	100	100	100	100

Table 16. Legionella pneumophila percentage reduction calculated for samples of the static phase.

Phase	3B			3B		
Time analysis	T3 30-35°C	T10 30-35°C	T15 30-35°C	T3 2-8°C	T10 2-8°C	T15 2-8°C
W1A	100	100	100	100	100	100
W1B	100	100	100	100	100	100
W1C	100	100	100	100	100	100
W2A	100	100	100	100	100	100
W2B	100	100	100	100	100	100
W2C	100	100	100	100	100	100
W3A	100	100	100	100	100	100
W3B	100	100	100	100	100	100
W3C	100	100	100	100	100	100

Table 17. Legionella pneumophila percentage reduction calculated for samples of the dynamic phase.

Phase	2B			2B		
Time analysis	T3 30-35°C	T10 30-35°C	T15 30-35°C	T3 2-8°C	T10 2-8°C	T15 2-8°C
W1A	0	0	100	NA	100	NA
W1B	89	99	100	NA	100	100
W1C	0	100	100	NA	100	NA
W2A	NA	100	100	NA	NA	NA
W2B	0	0-	100	100	NA	NA
W2C	NA	NA	NA	NA	NA	NA
W3A	100	100	100	NA	100	100
W3B	98	88	100	NA	100	NA
W3C	57	NA	NA	100	NA	NA

Table 18. Legionella pneumophila percentage reduction calculated from the swab results of the static phase.

Phase	3B			3B		
Time analysis	T3 30-35°C	T10 30-35°C	T15 30-35°C	T3 2-8°C	T10 2-8°C	T15 2-8°C
W1A	NA	100	100	NA	100	NA
W1B	NA	100	100	NA	100	NA
W1C	NA	100	100	NA	100	100
W2A	NA	NA	NA	NA	100	NA
W2B	NA	100	100	NA	100	100
W2C	NA	100	100	NA	100	100
W3A	NA	100	NA	NA	100	100
W3B	NA	100	NA	NA	NA	100
W3C	NA	100	100	NA	100	100

Table 19. Legionella pneumophila percentage reduction calculated from the swab results of the dynamic phase.

4.6.2 DIFFERENCES OBSERVED BETWEEN INCUBATION TEMPERATURES

The results obtained from the two different temperature of incubation show that BATP ®L1700S can act against Legionella pneumophila both at 30-35°C and at 2-8°C.

The better temperature for its proliferation is 30-35°C which is also the optimal temperature growth for most bacteria, especially Bacillus genera.

4.6.3 DIFFERENCES OBSERVED BETWEEN STATIC AND DYNAMIC CONDITION

The results obtained from the two different temperature of incubation show that BATP ®L1700S can act against Legionella pneumophila in both static and dynamic condition.

We can observe an interesting difference on the swab analysis:

- During the static phase Legionella pneumophila is able to colonize the tubes also at T10 and create biofilm in presence of BATP ®L1700S, while in solution the effect of the BATP is

immediate at T3. Though at the end of the experiment Legionella pneumophila colonies in all samples were undetectable for both incubation temperatures.

- During the dynamic phase, the continuous agitation of the samples makes Legionella pneumophila cells more attackable from BATP ®L1700S. So that they cannot stick on tubes.

4.6.4 DIFFERENCES OBSERVED BETWEEN DIFFERENT TYPES OF WATER

The abatement of Legionella pneumophila in presence of BATP ®L1700S is 100% after 3 days of experimentation for all types of water. The different hardness of water does not interfere with BATP ®L1700S functionality against Legionella.

4.6.5 DIFFERENCES OBSERVED BETWEEN DIFFERENT TYPES OF TUBES

The test performed show no significant differences also in tubes choice, demonstrating the ability of BATP ®L1700S in interfering with the formation of Legionella pneumophila biofilms.

5 CONCLUSIONS

Tests results obtained by the application of protocol P/2223.3 demonstrate the efficacy of BATP ®L1700S towards Legionella pneumophila.

The test preformed, concerning these experimental conditions, prove that:

- BATP ®L1700S is able to eliminate Legionella pneumophila after three day from treatment in condition of equal concentration both in static and dynamic condition.
- BATP ®L1700S is able to eliminate Legionella pneumophila biofilm at least after 15 days from treatment in static condition.
- BATP ®L1700S is able to eliminate Legionella pneumophila biofilm after 3 days from treatment in dynamic condition.
- BATP ®L1700S activity is independent from temperature; The microorganisms that compose the product can tolerate winter temperatures of 2-8°C.