BIO³GEN OZONE GENERATOR EFFICIENCY CHECK TEST REGARDING FRUIT TREATMENT

1. ADMINISTRATIVE DATA

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Document prepared by	**Dr.a Maria Bonachini**	**Responsible for quality managenment**	
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CLIENT:

DF PRODUZIONI S.a.S. **Property of Capponi Diego & C**

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TEST LABORATORY:

STUDIO AMBIENTE S.r.l.

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NOTE: Studio Ambiente S.r.l. uses a Quality Management System certified by Cermet (Reg. N. 7173-A – see Annex 1) for the carrying out of technical consulting services in biomedical, pharmaceutical and industrial branches, the carrying out of lab services for microbiological and physical analysis and validation protocol development for products, processes and productive areas.

2. AIM AND APPLICATION FIELD

Ozone is a colorless gas with a high oxidant power that is able to break cellular membranes that are the basis for microbial cells' vital integrity. Ozone's anti-microbial qualities, especially its properties for air, water, food and surface disinfection, have been studied for years and certified by a vast literature available on the subject. However, Ozone is an unstable gas that cannot be stored and must be produced at the time of use. The client *DF PRODUZIONI* produces and sells a device (BIO³GEN) for immediate Ozone production and it is suitable for air disinfection and air purification.

The customer wants to verify the true effectiveness of the Ozone produced by the BIO³GEN device in fruit cleaning by carrying out an analytical test.

This report's aim is to show the methods used and the results obtained in the analytic test that verifies the disinfecting efficiency of the named BIO³GEN product.

3. DEVICE DESCRIPTION

The device used for carrying out the test is delivered directly to the customer together with the instructions for its use, as follows.

Name: **OZONE GENERATOR**

Model: **BIO^{3}GEN**

Lot number: **2011/12**

4. TEST PROCEDURE

4.1. General

In order to reach the aim arranged with the client, the microbial contamination level on the external peel of the apples was tested both before and after treating them by sinking them in water treated with Ozone thanks to the test device for a contact time previously defined and agreed with the client. This way it was possible to verify exhaustion that was obtained.

4.3 Carrying out

5 apples of similar dimension were washed by hand under flowing water for 15 seconds. Consequently they were each positioned in a sterile beaker containing 400 ml of sterile eluent solution (physiologic solution + tween 80): 4 samples underwent the treatment (samples 1 - 4) and 1 was kept for positive check (untreated apple).

An Ozone generator was inserted in each beaker with the sample destined for treatment and it was switched on for 30 minutes.

After this treatment the beakers were agitated in order to assure that microbial cells would detach from the external surface of the sample. Then, the eluent solutions were taken from each beaker containing the samples (treated and untreated) and after an accurate mix they were filtered in order to determine their microbial level. The filters were transferred on Trypton Soy Agar surfaces in order to search for aerobic and anaerobic bacteria by incubating them at (30 ± 1)°C for 5 days.

5. RESULTS AND CONCLUSIONS

The tests started on 15 June 2012 and ended on 18 June 2012. The results are summarized in the following tables and are expressed as u.f.c./apple and percentage and logarithmic reduction.

Sample	Calculation (ufc/amos.)	Logarithm
Untreated Apple	**1,60 x 10^3**	**3,20**
Apple Sample 1	**170**	**2,23**
Apple Sample 2	**149**	**2,17**
Apple Sample 3	**179**	**2,25**
Apple Sample 4	**115**	**2,06**
Average sample value 1 - 4		**2,18**
Logarithmic reduction		**1,02**
Percentage reduction		**90,04%**

The data in the table shows a **great reduction in the amount of bacteria** that contaminated the external surface of the samples once they are treated with **30 minutes** of ozone process.

6. ANNEXES

1. Quality management certificate by Studio Ambiente Reg. N. 7173-A

Document ending, emission and verification date	**01/08/2012**

Document prepared by	**Dr.a Maria Bonachini** **Resp. for Quality Management**
Document verified by	**Dr.a Emanuela Rossignoli** **Technical Director**

BIO³GEN OZONE GENERATOR EFFICIENCY CHECK TEST REGARDING WATER

1. ADMINISTRATIVE DATA

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Document prepared by	**Dr.a Maria Bonachini**	**Responsible for Quality Managenment**
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2. AIM AND APPLICATION FIELD

Ozone is a colorless gas with a high oxidant power that is able to break cellular membranes that are the basis for microbial cells' vital integrity. Ozone's anti-microbial qualities, especially its properties for air, water, food and surface disinfection, have been studied for years and certified by a vast literature available on the subject. However, Ozone is an unstable gas that cannot be stored and must be produced at the time of use. The customer DF produces and sells a device (BIO³GEN) for immediate Ozone production and it is suitable for air disinfection and air purification.

The customer wants to verify the true effectiveness of the Ozone produced by the BIO³GEN device in drinking water disinfection/purification by carrying out an analytical test.

This report's aim is to show the methods used and the results obtained in the analytic test that verifies the water disinfecting efficiency of the named BIO³GEN product.

3. DEVICE DESCRIPTION

The device used for carrying out the test is delivered directly to the customer together with the instructions for its use, as follows (see ANNEX 2).

Name: **OZONE GENERATOR**

Model: **BIO^{3}GEN**

Lot number: **2011/12**

4. TEST PROCEDURE

4.1. General

In order to reach the aim arranged with the client, an extreme microbial contamination situation (worse than what happens normally for drinking water) of a certain quantity of water by using standard known microbial strains.

Consequently the ozone generating device was switched on at specified intervals that were compatible to the producer's instructions for use and specimen from the treated sample were withdrawn.

The elimination of the contaminated microbial amount in the sample was verified at different intervals and it was compared to the microbial amount previously contaminating the sample.

4.2 Microbial strains

In order to carry out the test, the following microbial strains have been used, stored and managed complying to operative procedures internal to Studio Ambiente.

Strain	ATCC	Development conditions
Staphylococcus aureus	ATCC 6538	Thermostat (37 ± 1)°C, aerobiosis, 48 - 72 hours
Escherichia coli	ATCC 10536	Thermostat (37 ± 1)°C, aerobiosis, 48 - 72 hours

4.3 Carrying out

The strains were sub-cultivated in such a way to obtain developing cultivations. Standard quantities of microbial cells were withdrawn from each cultivation and were used to prepare a suspension in sterile physiologic solution containing about 1 x 10^7 ufc/ml. In order to know the exact number of micro-organisms in each suspension, 1 ml of it was filtered using a double membrane. The membranes were transferred on surfaces containing suitable cultivable soil and kept in the thermostat at specified conditions in order to let them grow. Once developed, the number of colonies that had grown on each membrane was counted and registered and the number of units forming the colony per millimeter (ufc/ml) of test suspension (N) was determined.

Using 0,1 ml of each microbial suspension to infect a bottle containing 1000 ml of sterile drinking water, the contamination concentration resulted almost equals to 10^3. This was considered a "worst case" process for drinking water. The microbial water contamination was determined by double filtration of 1 ml of water and after that the colonies were counted; the number of obtained ufc/ml was considered the reference value at time 0 (NT_0).

After the contamination the water purification process was carried out by using ozone and by following the producer's instructions regarding the BIO^{3}GEN device. See following detailed instructions:

- BIO^{3}GEN must be connected to an electrical socket;
- The tube that is part of the kit must be pressure connected to the appliance;
- The round pebble must be pressure inserted in the end of the tube;
- The tube must be introduced inside the bottle together with the pebble;
- BIO^{3}GEN must be switched on and kept running for the following contact times: 2, 5, 10, 20 minutes.

When each contact time came to an end, 2 quotas of water, 1 ml each, were withdrawn from the bottle and the ufc/ml concentration of surviving micro-organisms was determined (NT_2, NT_5, NT_{10}, NT_{20}).

5. RESULTS E CONCLUSIONS

The tests started on 17 April 2012 and ended on 27 April 2012. The results are expressed in u.f.c./ml and percentage reduction compared to the initial reference (T0) and are summarized in the following tables.

Strains	Intcial reference (N)	T0 Ref. (NT0)	2 min Ref. (NT2)	5 min Ref. (NT5)	10 min Ref. (NT10)	20 min Ref. (NT20)
S. aureus ATCC 6538	**2,51 x 10^8**	**2,44 x 10^4**	**0**	**0**	**0**	**0**
E. coli ATCC 10536	**8,0** x 10^7	**9,6** x 10^3	**0**	**0**	**0**	**0**

	Percentage reduction			
Strain	**2 minutes**	**5 minutes**	**10 minutes**	**20 minutes**
S. aureus ATCC 6538	**> 99,9%**	**> 99,9%**	**> 99,9%**	**> 99,9%**
E. coli ATCC 10536	**> 99,9%**	**> 99,9%**	**> 99,9%**	**> 99,9%**

Reading the data in the tables, there is an evident **reduction in the microbial amount** that contaminates the water , both positive (S. aureus) and negative (E. Coli) bacteria just after having undergone **2 minutes** of theozone process.

6. ANNEXES

1. Quality management certificate by Studio Ambiente Reg. N. 7173-A
2. BIO3GEN device instructions for use

Document ending emission and verification date	**02/05/2012**

Document prepared by	**Dr.a Maria Bonachini Resp. for Quality Management**
Document verified by	**Dr.a Emanuela Rossignoli Technical Director**

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Dear
DF PRODUZIONI SAS de Capponi Diego & C.
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TEST FOR THE ELIMINATION OF LEGIONELLA PNEUMOPHYLISPREVIOUSLY INOCULATED IN PRIMARY WATER SAMPLES THANKS TO THE DISINFECTING TREATMENT CARRIED OUT BY THE BIO^{3}GEN OZONE GENERATOR DEVICE.

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Ariccia, 28/06/2012

INDEX

1 INDEX 2
2 ANALYSIS AIM 3
3 NECESSARY EQUIPMENT FOR THE TEST 4
4 CARRYING OUT OF THE TEST 4
6 CONCLUSIONS 7

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2 ANALYSIS AIM

This aim of the survey carried out on 01.06.2013 is to eliminate the legionella pneumofhylis contained in primary water (previously infected with a pure strain) thanks to the BIO^{3}GEN device.

This appliance produces ozone 400 mg/h concentrated and 3-3,6 L7minute flow.

The production can be managed by using the display on the machine body and that regulates gas emission in the environment at intervals of 2, 5, 10, 20 and 30 minutes.

In the lab the appliance's silicon tube whose end part is attached to a 20 mm pebble is inserted in sterile food sash containing 1 liter drinking water infected by a *legionella pneumophylis* ATCC 33152 strain at a known concentration level. The process has been carried out three times at different intervals:

- At time 0 (immediately after contamination);
- At time T1 after 5 minutes of the appliance being switched on;
- At time T2 after 10 minutes of the appliance being switched on;
- At time T3 after 20 minutes of the appliance being switched on.

The company supplying the strain for the test is Biogenetics S.r.l.

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3 NECESSARY EQUIPMENT FOR THE TEST

Bacterial suspension preparation:

The *Legionella pneumophyli*s strain is reconstructed in a sterile diluent solution in order to have a 7,0 x 10^2 U.F.C./ml concentration.

(U.F.C. = units forming a colony).

The analysis method used for the water samples is described in the document "Permanent Conference for the relationship between State, Regions and the Independent Provinces of Trento and Bolzano, Doc. 04/04/2000 GU n° 103 05/05/2000". The Aedes lab is accredited by Ente Accredia for this method.

4 CARRY OUT OF THE TEST

On its end part the BIO^{3}GEN appliance has a silicon tube with a 20 mm pebble attached to it and it emitted ozone for 5 minutes in 1 liter of drinking water infected by 700 U.F.C. of *Legionella pneumophylis*. Later, the sample was filtered on a sterile 0,2 micron membrane and then it was processed following the method described in Document 04/04/2000 GU n° 103 05/05/2000.

Consequently, another liter of primary water was contaminated with 700 additional U.F.C. of bacterial strain. It underwent 10 minutes of ozone process. This procedure repeated itself till the primary water sample underwent a 20 minute ozone immersion.

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The elimination percentages for each analysis have been calculated in reference to the following formula:

Reduction%= (A-B /A) x 100

i.e.

A= microbial population at T=0, expressed in U.F.C./ml

B= microbial population at analysis time T=Tx (Tx= 5 minutes; Tx= 10 minutes; Tx= 20 minutes), expressed in U.C.F./L.

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WORK SURFACE RESULTS

TABLE 1

Analysis time (minutes)	Legionella pneumophylis U.F.C./L
T= 0 minutes	7,0 x 10
T= 5 minutes	<10
T= 10 minutes	<10
T= 20 minutes	<10

It is important to note that the wording "< 10 U.F.C./L" means that no colony has been detected on the analyzed sample. This result is due to the mathematical formula that is an essential part of the analysis method.

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TABLE 2

Analysis time (minutes)	Bacterial population reduction (%) compared to the first Legionella pneumophylis inoculation at T= 0
T= 5 minutes	100 %
T= 10 minutes	100 %
T= 20 minutes	100 %

6 CONCLUSIONS

Relating to the obtained results the BIO^{3}GEN appliance proves itself as "EFFICIENT" in *Legionella pneumophylis* elimination in previously contaminated drinking water samples, as evident in tables 1 and 2.

Il Responsabile Laboratorio
Il Chimico (Dott.ssa Marina Icovi)

Responsible for the Laboratory
Research Chemist (Dr. Marina Icovi)
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