Evaluation of ozone sanitisation did with sanity system tecnology of areas classified in the pharmaceutical field

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ABSTRACT

Ozone is a powerful oxidizing agent produced by air passing through an electric field. Its applications are related to its potential as a biocidal agent and it is widely used for the purification of environments and waters. The aim of this paper is to investigate the efficacy of ozone as an alternative treatment for the sanitisation of non-sterile pharmaceutical production environments. The sanitisation process involves an in-situ production phase with a SanyPlus generator, an 85-minute exposure at room temperature, and finally a degradation and ventilation phase to remove the ozone from the environment at the end of the cycle. The effectiveness of the treatment was evaluated by measuring the CFU/plate before and after exposure to ozone of the microorganisms E*. coli, P. aeruginosa*, *S. aureus* to evaluate bactericidal activity, *C. albicans* and A. *brasiliensis* for fungicidal activity and *B. subtilis* for sporicidal activity *and S. marcescens* and *B. cepacia* to evaluate the effect of ozone on environmental isolates. Samples subjected to the sanitisation cycle showed a 98.4% reduction in the overall vitality. A greater effectiveness of the method emerged on vegetative forms compared to spore-forming ones and phenomena of gas dispersion were highlighted that led to a lower efficiency of the treatment. Overall, a reduction efficacy was demonstrated for a cell titre of 10^5 CFU/plate of 3.2 log for non-spore-forming microorganisms and of 2.6 log for spore-forming ones. Given the overall reduction of at least 1.6 for the lowest inoculum value (10^2 CFU/plate), and considering that on non-sterile class pharmaceutical surfaces there are hardly more than 100 CFU/plate, the effectiveness of ozone as a potential supplement in the sanitisation of pharmaceutical environments has been proven.

Introduction

Microbial contamination of pharmaceutical environments represents one of the fundamental variables to be monitored to ensure purity, quality and safety throughout the pharmaceutical manufacturing process. There are various types of tools and technologies to carry out this activity and thus be able to maintain the production area in the GMP class to which it belongs.

Annex 1 of the GMPs, both the one in force and the one currently being drafted, envisage a maximum number of colony-forming units per surface, depending on the class to which the production facilities belong, ranging from a value <1 for class A, to a maximum of 50 for class D.

In choosing a sanitisation method, it is necessary to consider many factors, primarily the germicidal activity, but also the operating methods, any concentrations and contact times, the safety aspects as well as the compatibility with the materials to be treated (USP current edition).

Among the technologies that can be used, we find disinfection with ozone, a gas with a very high oxidizing power with the particularity of dissolving without leaving traces or chemical residues. Its strong oxidizing power and natural tendency to convert itself back into molecular oxygen (O_2) make it highly usable in many applications for the microbial abatement of air and water (M.Martinelli 2017) (Agnieszka Joanna Brodowska 2017) (N.F.Gray 2014). In its conversion process, ozone produces oxygen and free radicals, which cause the oxidation of various cellular components with consequent damage to the cells. Ozone's target is represented by various cellular components, such as proteins, unsaturated lipids, respiratory membrane enzymes, peptidoglycans, cytoplasmic enzymes and nucleic acids, as well as proteins and peptidoglycans present in viral capsids and spore coatings (Giuseppe Giuliani, 2018).

Its particular instability does not allow it to be stored and consequently it must be produced in the place of use and in the absence of personnel.

The use of ozone gas (O_3) for the sanitisation of pharmaceutical environments has rapid application time. The gas does not corrode production equipment, does not interact with raw materials or finished products and, according to recent studies, does not cause the onset of resistance phenomena (Giuseppe Giuliani, 2018).

Purpose

The purpose of this paper is to verify the efficacy of the bactericidal/fungicidal activity of the ozone produced by the Sany-Plus generator manufactured by the company SANITY SYSTEM ITALIA S.r.l. in reducing a known microbial load in an action time of 85 minutes (the longest cycle preset in the instrument).

The effect of the ozonizing treatment was evaluated on plates seeded with microorganisms of known titre (range 10^2-10^5 CFU/plate) of the following microorganisms: E*. coli, P. aeruginosa*, *S. aureus* to assess bactericidal activity, *C. albicans* and A. *brasiliensis* for fungicidal activity and *B. subtilis* for sporicidal activity. The study also included the environmental isolates *Serratia marcescens* and *Burkholderia cepacia.*

The aim was to evaluate the logarithmic reduction of a large number of microorganisms exposed to the ozone treatment produced by the Sany-Plus instrument.

Materials and methods

The tests were performed using the following microorganisms:

- *Aspergillus brasiliensis* ATCC 16404
- *Candida albicans* ATCC 10231
- *Bacillus subtilis* ATCC 6633
- *Escherichia coli* ATCC 8739
- *Pseudomonas aeruginosa* ATCC 9027
- *Staphylococcus aureus* ATCC 6538
- *Serratia marcescens wild*
- *Burkholderia cepacia wild*

Media and thinners: Tryptic Soya Agar (TSA), Sabouraud Agar (SA), Physiological solution.

Equipment: Sany-Plus ozone generator (Sanity System Italia s.r.l.).

Preparation of the strains

For the execution of the tests, suspensions of the selected strains were prepared with a McFarland turbidity calculated to achieve a theoretical concentration of 10^8 CFU/ml (Carolina Chiellini 2016). Starting with the suspension with a titre of 10^6 CFU/ml and for the subsequent dilutions, a surface seeding of 100 µl was carried out in TSA plates for bacteria and SA plates for fungi.

To evaluate the actual titre of the suspensions, 100 µl of the 10^3 CFU/ml suspension were seeded in plate (theoretical titre in plate 10^2 CFU).

Incubation conditions: TSA plates at 33 °C for 5 days and SA at 22.5 °C for 5 days.

Test Execution

The SanyPlus ozone generator provides three different cycles to be carried out based on the square footage of the room to be treated. As a precaution, the maximum duration program (85 minutes) was chosen, aimed at saturating rooms up to 400 m^3. The tests were carried out in four different rooms with the following volumes:

	m^3
Room 1	140
Room 2	160
Room 3	220
Room 4	380

Preliminary tests carried out using one instrument per room showed a higher cell survival than initially assumed (the data are not reported in this article). To overcome this problem and to ensure more efficient saturation, the treatment was repeated using two ozonisers in rooms 1, 2 and 3, while in room 4 the program was carried out using three instruments simultaneously.

The tests were carried out in the absence of personnel, with machinery off and the air conditioning system stopped. The doors and SAS of the sanitised premises were sealed to prevent ozone dispersion during the tests.

The plates containing the inocula of the selected microorganisms were placed inside the premises in specific delimited areas. The positioning of the plates was evaluated with the aim of covering the maximum available surface.

At the end of the treatment the plates were incubated under the classic conditions envisaged by the Pharmacopoeia (TSA at 33 °C for 5 days, SA at 22.5 °C for 5 days).

Survival Calculation

At the end of the planned incubation, the count of viable microorganisms on the plates was carried out.

For each strain it was necessary to evaluate the actual titre of inoculated cells against the theoretical titre based on the values obtained in the control plate. The number of colonies was multiplied by the dilution factor, obtaining the actual number of cells of each inoculum.

The logarithmic reduction of viability was then calculated for each microorganism tested (*R*) by applying the following formula:

$$R=log(N_0/N_a)$$

Where N_0 is the actual number of inoculated microorganisms and N_a is the number of microorganisms per plate that survived the test at the end of the contact time.

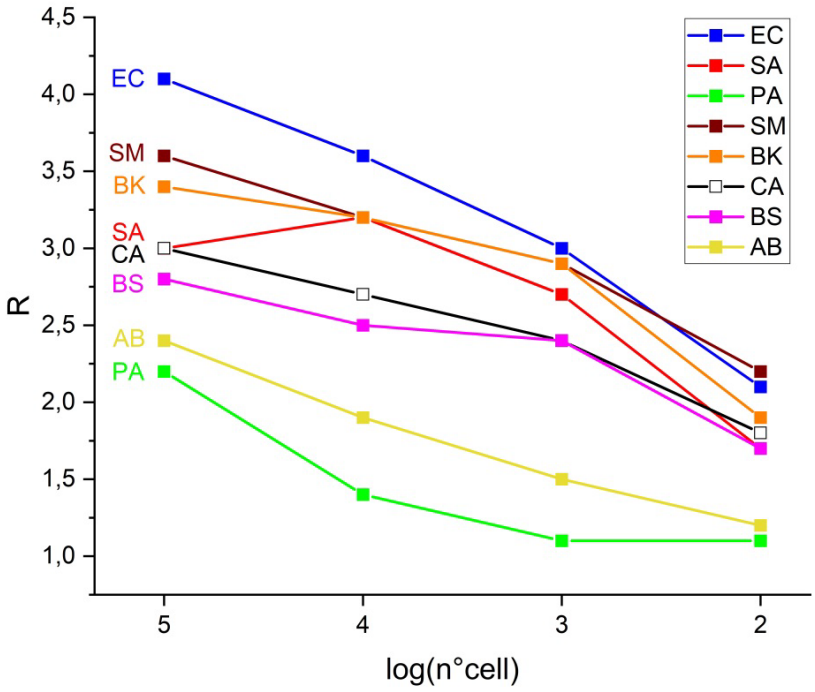
Results

The tests were carried out in the four rooms simultaneously. For each strain, the initial title (N_0) was obtained by averaging the values obtained for the four rooms. Table 1 shows the data obtained:

	N_0	N_a 1	N_a 2	N_a 3	N_a 4	R1	R2	R3	R4	AVERAGE % REDUCTION
Escherichia coli ATCC 8739	136000	6	9	18	17	**4.4**	**4.2**	**3.9**	**3.9**	99.99
	13600	1	2	3	15	**4.1**	**3.8**	**3.7**	**3.0**	99.96
	1360	1	1	2	2	**3.1**	**3.1**	**2.8**	**2.8**	99.89
	136	0	0	0	1	**2.1**	**2.1**	**2.1**	**2.1**	99.26
Staphylococcus aureus ATCC 6538	78000	8	134	135	266	**4.0**	**2.8**	**2.8**	**2.5**	99.83
	7800	1	6	8	20	**3.9**	**3.1**	**3.0**	**2.6**	99.89
	780	1	1	3	2	**2.9**	**2.9**	**2.4**	**2.6**	99.78
	78	0	0	2	2	**1.9**	**1.9**	**1.6**	**1.6**	98.08
Pseudomonas aeruginosa ATCC 9027	107000	357	526	954	1340	**2.5**	**2.3**	**2.0**	**1.9**	99.26
	10700	150	430	493	700	**1.9**	**1.4**	**1.3**	**1.2**	95.86
	1070	54	92	109	15	**1.3**	**1.1**	**1.0**	**0.9**	90.89
	107	8	7	11	14	**1.1**	**1.2**	**1.0**	**0.9**	90.65
Serratia marcescens wild	179000	26	39	68	81	**3.8**	**3.7**	**3.4**	**3.3**	99.97
	17900	5	7	13	25	**3.6**	**3.4**	**3.1**	**2.8**	99.93
	1790	1	0	2	9	**3.3**	**3.3**	**3.0**	**2.3**	99.82
	179	0	0	2	1	**2.3**	**2.3**	**2.0**	**2.3**	99.30
Burkholderia cepacia wild	83000	15	27	52	102	**3.7**	**3.6**	**3.3**	**3.0**	99.95
	8300	4	3	12	17	**3.3**	**2.5**	**2.9**	**2.8**	99.91
	830	0	1	2	2	**2.9**	**3.0**	**2.7**	**2.7**	99.85
	83	0	0	2	1	**1.9**	**2.0**	**1.7**	**2.0**	99.76
Candida albicans ATCC 10231	109000	36	76	136	502	**3.5**	**3.2**	**2.9**	**2.3**	99.83
	10900	4	18	33	94	**3.4**	**2.8**	**2.5**	**2.1**	99.66
	1090	1	2	9	15	**3.0**	**2.7**	**2.1**	**1.9**	99.38
	109	1	0	2	3	**2.0**	**2.0**	**1.7**	**1.6**	98.39
Bacillus subtilis ATCC 6633	128000	114	148	316	385	**3.1**	**2.9**	**2.6**	**2.5**	99.81
	12800	27	32	52	63	**2.7**	**2.6**	**2.4**	**2.3**	99.66
	1280	4	4	5	8	**2.5**	**2.5**	**2.4**	**2.2**	99.59
	128	2	2	3	5	**1.8**	**1.8**	**1.6**	**1.4**	97.66
Aspergillus brasiliensis ATCC 16404	140000	174	784	592	973	**2.9**	**2.3**	**2.4**	**2.2**	99.55
	14000	48	238	165	596	**2.5**	**1.8**	**1.9**	**1.4**	98.13
	1400	7	87	46	150	**2.3**	**1.2**	**1.5**	**1.0**	94.82
	140	4	12	7	21	**1.5**	**1.1**	**1.3**	**0.8**	92.14

Table 1 - Results for rooms 1,2 3 and 4 subjected to an 85-minute ozone cycle.
***N_0** average of actual number of inoculated microorganisms; **N_a** plate organisms surviving at the end of the contact time for rooms 1, 2, 3 and 4.* **R** *logarithmic reduction calculated on each strain and for each room.*

The graph below shows the average values of the logarithmic reductions of each strain. Each point represents the average of the four values obtained according to the dilutions applied:



Picture 1 - Results related to the average of the four tests carried out.
EC=E. coli, PA=P.aeruginosa, SA=*S.aureus, CA=C.albicans,* AB=A.*brasiliensis,* BS=*B.subtilis, SM=S.marcescens, BK=B.cepacia*

The lowest inoculum, determined by an initial titre of 10^2, constitutes to all intents and purposes the most representative point of the possible microbial contaminations found in the pharmaceutical production premises. As can be seen from the data, for most of the strains in this range, values on average of 0 and 1 CFU/plate were found following an ozone cycle. Six microorganisms (*E. coli, S. aureus, C. albicans, B. subtilis, S. marcescens, B. cepacia)* are indeed at the limit of the sensitivity of the treatment, with an average cell mortality of 98.6%, while two microorganisms reported lower mortality rates (90.7% for *P. aeruginosa* and 92.1% for A. *brasiliensis*). It is important to consider that such low initial titre values lead to an underestimation of the sanitisation efficiency. In order to have a more representative evaluation of the effectiveness of the method, the data obtained from higher initial cell titres were therefore analysed, even though they were far from the values normally found in the pharmaceutical field. Observing the data relating to higher concentrations, it can be noted that the efficiency of the method tends to increase with the increase in the number of cells. Indeed, in all microorganisms the highest value of logarithmic reduction is obtained for the inoculum of the greatest order of magnitude (10^5 CFU/plate). The absence of a plateau on the higher values could imply a possible increase in efficacy at even greater cell contraction values. However, at the experimental level, higher cell contamination values were not taken into consideration, since the aim of the method is to demonstrate a reduction of the bacterial load with plausible values for pharmaceutical production environments. The maximum value of the order of magnitude of 10^5 represents a titre far higher than the values normally found in production environments, therefore it is a more than acceptable threshold value.

Considering the overall trend of the various microorganisms, the graph shows the differences in the efficiency of the method according to the microorganism and proportionally for each dilution applied. In the highest initial contamination value (10^5 CFU/plate) the bactericidal activity is optimal for both ATCC microorganisms (E. coli *and S. aureus) and* for environmental isolates *(S. marcescen* and *B. cepacia),* with an average logarithmic reduction of 3.51. The fungicidal activity on *C. albicans* is slightly lower in terms of logarithmic reduction (R=2.97), values very similar to that obtained for *B. subtilis* (R=2.78). Significantly lower values were obtained for *P. aeruginosa* (R=2.18) and A. *brasiliensis* (R=2.42).

The lower efficacy values found in spore-forming microorganisms are probably related to a greater resistance of the spores (M.Martinelli 2017). The handbook of disinfectants and antiseptics (Ascenzi s.d.) describes the intrinsic mechanisms of resistance of spores, however some studies have shown that the mechanism for killing spores by ozone is related to damage to their inner membrane, which makes their germination difficult (Joseph P. Wood 2020) (S.B. Young 2004). The conditions for the inactivation of the spores, however, require concentrations of ozone higher than those produced by a normal generator, in addition to the control of relative humidity. (Joseph P. Wood 2020) (Ahmet Aydogan 2012). These conditions are not reproducible with the instrumentation used for this study; therefore the sporicidal activity was found to be significantly lower than that on the vegetative forms. The nature of the substrate on which the spores are placed also affects the inactivation rate (Ahmet Aydogan 2012), while the porosity of the materials was correlated with the efficiency rate of ozone on various microorganisms (M.Y.Mentrez 2009).

Among the vegetative forms tested, *P. aeruginosa* demonstrated a much lower susceptibility to ozone than the others. This characteristic is perhaps attributable to the tendency of the microorganism to form aggregates on the surface of the plate, a mechanism correlated with ozone resistance phenomena. (Stefanie Heß 2015). However, this evidence should be subjected to further investigation.

In order to obtain a more complete evaluation of the data, the results for each individual strain were also analysed. The graphs below show the reduction curves divided by microorganism, with the aim of obtaining a comparison of the results for each room.

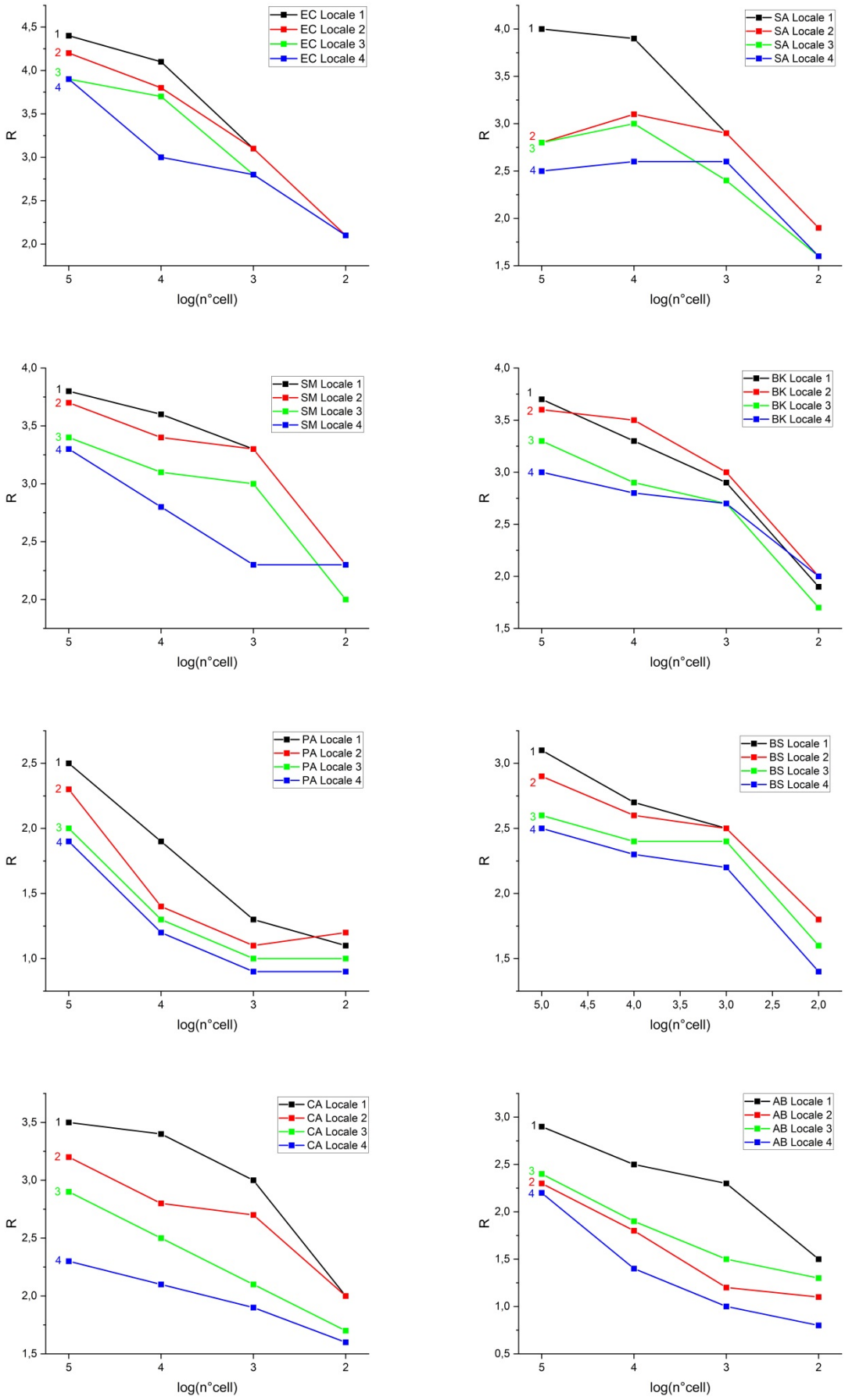


Image 2 - Results obtained for each strain tested. Each graph shows the values obtained in each room, compared. *EC=E.coli, PA=P.aeruginosa*, SA=*S.aureus, CA=C.albicans,* AB=A.*brasiliensis,* BS=*B.subtilis, SM=S.marcescens, BK=B.cepacia*

The results obtained in room 1 show overall higher R values than in the other rooms. This result has been attributed to the fact that rooms 2, 3 and 4 are connected by a single air recirculation system with a consequent greater dispersion of ozone inside the air conditioning system. Before starting a study on the effectiveness of ozone as a germicidal agent, it is important to evaluate the volume of the ventilation system, since ozone tends to diffuse and this volume must therefore be added to that of the room to be treated.

Conclusion

The instrument proved to be a valid aid in the sanitisation process of pharmaceutical production facilities. The easy use of the instrument and the possibility of using the room at the end of the cycle after 15 minutes of ventilation make it a valid choice in the sanitisation of non-sterile pharmaceutical production rooms.

The data obtained showed in general a strong reduction of the charges of the microorganisms exposed to ozone. However, some critical strains emerged. Among the microorganisms tested, fungi and spore-forming microorganisms were less susceptible to ozone exposure. Furthermore, also *P. aeruginosa* has been shown to be less susceptible than other microorganisms.

Taking into account this variability, it is necessary to consider the numerous factors that interfere with the effectiveness of ozone. Primarily, physical parameters such as temperature and relative humidity of the air (Ginny Moore 2000). Before carrying out the sanitisation process, it is important to evaluate these variables, together with the size of the room and the dispersion phenomena connected to the ventilation system and any unsealed doors.

It is also necessary to consider the fact that in the tests carried out the microorganisms were in optimal conditions for their growth (agarised media). The presence of an organic substrate was correlated as a possible interfering phenomenon with respect to the effective abatement caused by the gas (Agnieszka Joanna Brodowska 2017). Since various types of materials are used within pharmaceutical environments, subsequent studies on the efficiency of ozone on a given surface to be decontaminated will be useful for the integration of the results obtained so far.

In conclusion, the results obtained highlighted the effectiveness of ozone as a potential supplement in the sanitisation of pharmaceutical environments. The tests have shown a reduction effectiveness of an initial titre of 10^5 cells by 99.7%, with a logarithmic reduction of 3.2 for non-spore-forming microorganisms and 2.6 for spore-forming ones. The efficacy calculated on inoculum values of 10^2 CFU/plate, where the statistical variation is greater, corresponds to a logarithmic reduction of 1.81 for non-spore-forming microorganisms and 1.42 for spore-forming ones. Given the overall reduction of at least 1.6 for the lowest inoculum value, and with the assumption that on non-sterile class pharmaceutical surfaces there are hardly more than 100 CFU/plate, the instrument is suitable for the envisaged purpose.

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