Final report on the tests carried out for the evaluation of the microbicidal effect of BIOVITAE® devices inside the model cabin of a cruise ship

In July 2020, with the signing of the Joint Development Agreement between the Company and Nextsense S.r.l, a joint project was launched (hereinafter referred to as the "Biovitae® Project") with the aim of evaluating the effectiveness of Biovitae® devices. for the continuous sanitization of environments in cruise ships.

The Company has identified a prominent laboratory part of intergovernmental organisation as scientific partner for the execution of the tests and the evaluation of the results.

The aim of this document is to provide a commented analysis of the analytical-experimental process conducted by the interested parties and of the results obtained.

1. Development of the project and summary of the results

The Biovitae® Project was divided into three different stages:

Phase 1. **Documentation analysis related and technical-scientific literature** on the microbicidal efficacy of the wavelengths of the visible spectrum and on the results of tests already carried out using Biovitae® devices, including the evaluation of their safety for continuous use in the presence of living beings.

Phase 2. **Performing in vitro tests** at the appointed laboratory to verify the effectiveness of Biovitae® technology, on the human coronavirus SARS-CoV-2 and on a specific strain of E. coli.

Phase 3. **Testing in the environment** through the installation of lighting devices made according to specific indications by the Company in a model booth installed in the laboratories of the Faculty of Engineering of the University of Trieste.

a. The results of Phase 1 and 2

The results of phase 1, which was aimed at verifying the efficacy of Biovitae and its safety even after continuous use in the presence of living beings, were considered very positive and can be summarized in the following conclusions:

"… BIOVITAE® light source complies with IEC 62471 (Photobiological safety of lamps and lamp systems) and IEC TR 62778 (Application of IEC 62471 for the assessment of blue light hazard to light sources and luminaires) Risk Group 0 - RG0 …… Therefore, considering the above classification and the use of blue light in therapy and experiments in vivo, the BIOVITAE® light source could be considered safe in the environment of a cruise ship cabin, provided the positioning prevents passengers' prolonged staring into the light source. "

"... In conclusion, BIOVITAE® has a potential to inactivate SARS-CoV-2 and pathogenic RNA viruses in general ...."

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Following the positive evaluation of Phase 1, through the documents produced by Nextsense and the available scientific literature, we moved on to Phase 2 aimed at confirming in the laboratory the efficacy of Biovitae on SARS-CoV-2 on E. coli, bacterium of specific interest for the Company

In-vitro tests were performed on:

- **Human coronavirus SARS-CoV-2** (FVG_laboratoryS5): The tests carried out in two phases between 2nd and 5th of February and between 16th and 19th of February 2021;
- **E. coli** (DH5α): tests carried out in a single phase between 1 and 15 March 2021.

Tests on SARS-cov2, described below, confirmed the efficacy of BIOVITAE on the specific viral strain.

The experimental setup for all the tests carried out on the virus was the following:

- *LED was switched on with the dimmer at 500 mA / 160V / 80W (ON-ON-ON-ON) see Figure 1. Temperature under the lamp was 26 ° C at the beginning of the experiment and of 28 ° C by the end of experiment (room temperature was 23.4 ° C).*
- *The virus was exposed in duplicate for the following exposure times: 5-15-30-45 minutes.*
- *A second Petri dish was positioned under the lamp at the same experimental conditions,*
- *but covered with Aluminum foil (control).*
- *After exposure the virus was harvested, diluted, and inoculated on Vero E6 cell monolayers*
- *for plaque assay.*
- *After 72 hours of incubation the cells were fixed and stained following standard procedure.*
- *Plaques were counted to measure infectious virus.*

The results of the first test round (February 2-5) are shown in the following table from which it can be seen that **after only 5 minutes the inhibition was 64.71%** to reach **98.13% after 15 minutes** and **99.98% after 30 minutes of exposure**

500 mA (ON-ON-ON-ON)	Light		PFU/ml		Dark		PFU/ml		% inhibition			
5	45000	50000	47500	3535,5	160000	95000	127500	45961,94	64,71	60,78	62,75	2,77
15	2150	1700	1925	318,2	125000	105000	115000	14142,14	98,13	98,52	98,33	0,28
30	5	30	17,5	17,7	125000	90000	107500	24748,74	99,995	99,97	99,98	0,02
45	0	0	0	0,0	90000	60000	75000	21213,20	100,00	100,00	100,00	0,00

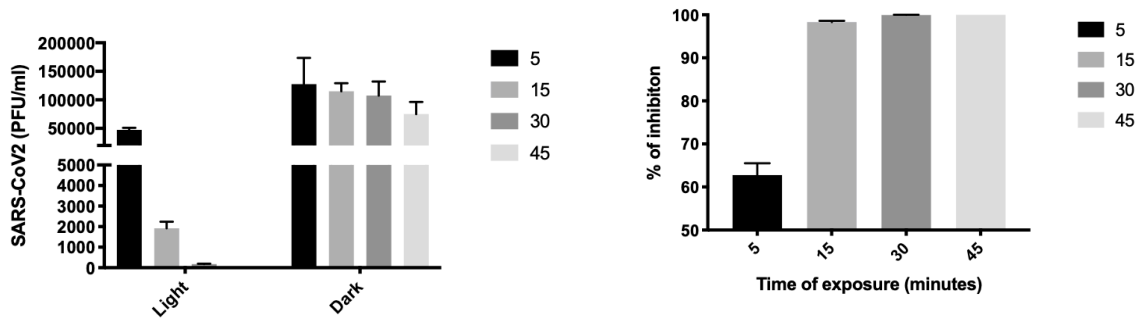

***Fig. 1** - Results of in vitro tests on SARS-Cov2 at 500mA (source: laboratory technical report)*

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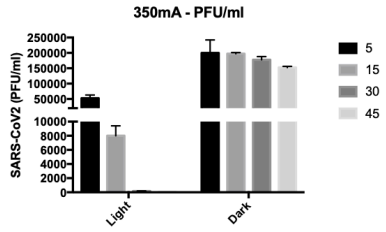
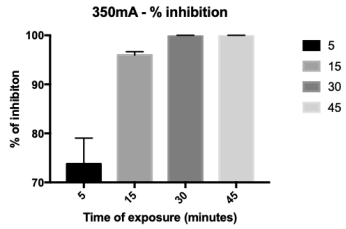
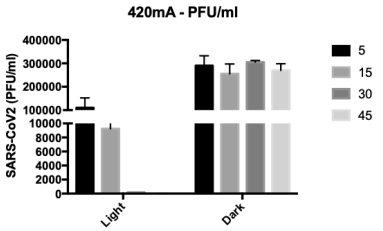
Using the same setup, two different lighting conditions (less powerful, at 350 and 420mA) were used in the second round of testing to assess the impact of power supply on microbicidal efficacy. The results are reported in the following tables.

350 mA (OFF-OFF-OFF-OFF)	Light		PFU/ml		Dark		PFU/ml		% inhibition			
5	60000	45000	52500	10606,6	170000	230000	200000	42426,41	70,00	77,50	73,75	5,30
15	9000	7000	8000	1414,2	195000	200000	197500	3535,53	95,44	96,46	95,95	0,72
30	10	25	17,5	10,6	170000	185000	177500	10606,60	99,994	99,99	99,990	0,01
45	5	0	2,5	3,5	155000	150000	152500	3535,53	99,997	100,000	99,998	0,002





420 mA (OFF-ON-ON-ON)	Light		PFU/ml		Dark		PFU/ml		% inhibition			
5	80000	140000	110000	42426,4	320000	260000	290000	42426,41	72,41	51,72	62,07	14,63
15	11500	7000	9250	3182,0	225000	285000	255000	42426,41	95,49	97,25	96,37	1,25
30	20	15	17,5	3,5	300000	310000	305000	7071,07	99,993	100,00	99,99	0,001
45	25	5	15	14,1	290000	250000	270000	28284,27	99,99	100,00	99,994	0,01



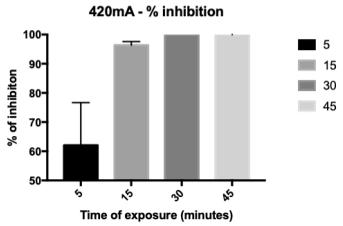


***Fig. 2** - In vitro test results on SARS-Cov2 at 350 and 420mA (source: laboratory technical report)*

As can be seen from the two tables, in all the experimental conditions the tests have demonstrated incontrovertibly the effectiveness of BIOVITAE on SARS-Cov2, confirming the results of the tests previously carried out by other important laboratories (in particular the three military laboratories of Italy, Germany and Sweden) and the **CEA - Commissariat pour l'énergie atomique**).
The conclusions of the ICGEB report are as follows:

"... As shown in the Figure 3 (which refers to the previous tables) below at all intensities tested, we observed a good inhibition starting at 15 'of exposure (data at 500 mA are shown above)"

Tests on Escherichia coli were carried out in the period 1-15 March 2021. Escherichia coli DH5, a non-pathogenic strain commonly used in molecular biology and considered to be at the lowest level of biosafety (BSL1), was used. This is to then allow in vivo tests to be carried out in the ship's cabin with the same viral strain.

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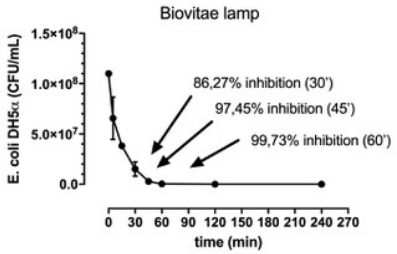
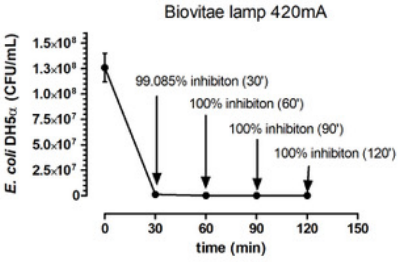
Three different power intensities have always been used (350-420-500ma) and, also in this case, as for Sars-Cov2, tests have confirmed the effectiveness of Biovitae on Escherichia coli, as can be seen from the figures of reported below.

500 mA				
Time (min)	CFU/mL		PI (percentage of inhibition)	
0	1.12e+008	1.08e+008	0	0
5	8.06e+007	5.08e+007	26.73	53.82
15	3.82e+007			65.27
30	1.00e+007	2.02e+007	90.91	81.64
45		2.80e+006		97.45
60	3.00 e+005	3.00 e+005	99.73	99.73
120	0.00	0.00	100.00	100.00
240	0.00	0.00	100.00	100.00



420 mA				
Time (min)	CFU/mL		PI (percentage of inhibition	
0	1.16e+008	1.36e+008	0.00	0.00
30	7.00e+005	1.6e+006	99.44	98.73
60	0.00	0.00	100.00	100.00
90	0.00	0.00	100.00	100.00
120	0.00	0.00	100.00	100.00



350 mA				
time	CFU/mL		PI	
0.	1.45e+008	1.40e+008	0.00	0.00
30.	1.40e+006	2.20e+006	99.02	98.46
60.	0.000000	0.000000	100.00	100.00
90.	0.000000	0.000000	100.00	100.00
120.	0.000000	0.000000	100.00	100.00

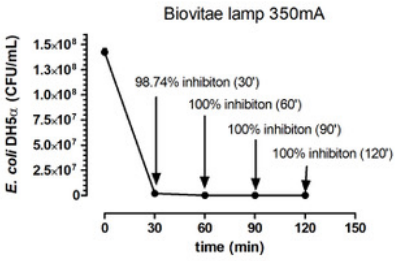


Fig. 3 *- Results of in vitro tests on E.coli at 350, 420 and 500 mA (source: ICGEB technical report)*

Both phases therefore concluded with a positive outcome, confirming the solidity of the technical-scientific evidence supporting the efficacy of the Biovitae® technology on viruses and bacteria, further confirmed by the in vitro tests performed in the ICGEB laboratories, whose team, headed by Prof. Marcello, drew up the following conclusions:

"Firstly, we analyzed the effect of the BIOVITAE® LED on SARS-CoV-2 observing a very good virucidal effect at all illumination conditions. These data confirm those provided by the Company and conducted independently in several laboratories. Next, we tested the ability of the configuration to inhibit E. coli. Again, we observed a very good performance of the LED as already shown (see previous Phase 1 report). Clearly, the two systems may differ a lot with respect to the mechanism of inactivation. The effect appears virucidal for SARS-CoV-2, while, in the case of bacteria, it is instead likely a combined bacteriostatic germicidal effect…. "

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2. Description of the efficacy tests in the environment (Phase 3)

The purpose of Phase 3 was to test the BIOVITAE® devices in the mockup of a cabin of a cruise ship reproduced at the Department of Architecture and Engineering of the University of Trieste (figure below).

***Fig. 4** – Mock-up cabin (source: ICGEB technical report)*

Tests were conducted using lighting devices with power 40 times lower than those used in Phase 2 laboratory tests using ceiling lights supplied by the Company and with LED strips, respectively modified (to integrate them with Biovitae® technology) and specially designed by the Nextsense technical group. This is because the objective of the test was to demonstrate the continuous containment of the infectious risk in real conditions over a time period of 24 in conditions of normal use of the cabin during a cruise.

Also, in this case the positive performances of Biovitae have been confirmed, so much so that at the end of 24 hours, the average abatement compared to the initial population of 1000 CFU / ml was greater than 70%, ranging from 53% of the poorly irradiated areas up to 91% of the best irradiated areas.

It is considered useful to describe in more detail the methodology adopted to underline the value of the results.

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Materials and methods

The model cabin in which the Phase 3 tests were carried out was installed in the laboratories of the Department of Engineering and Architecture of the University of Trieste.

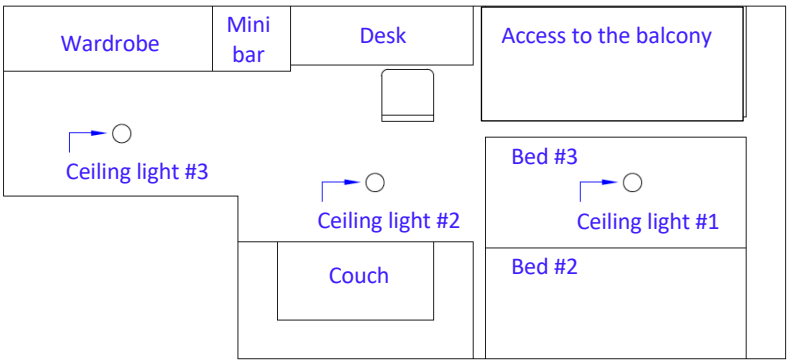
Normally, sampling tests in the environment are performed through the analysis of environmental contamination in two distinct phases, ie before and after the installation of the devices.
In this case, being a model booth, a "hybrid" sampling method was chosen for the evaluation of the results, ie using pre-contaminated open plates.

Due to the particular constitution of the testing environment, it was not possible to monitor some very important variables for the growth of microorganisms such as, for example, environmental temperature and humidity, nor to implement procedures that could prevent recontamination of the plates by the microorganisms present in the environment (for this purpose, for example, in the laboratory tests, Prof. Marcello had made use of an open flame positioned near the open plates and exposed to light to prevent other microorganisms from contaminating them and invalidating the test; but , naturally in the model cabin it was not possible to use open flames due to the risk of fire represented by the presence of furnishing materials).

Inside the cabin, 112 Petri dishes were placed, inoculated with E. coli and prepared with a culture medium suitable for the growth of microorganisms and distributed with the following arrangements:

- p1) Bed #2,
- p2) Bed #3,
- p3) Sofa,
- p4) Floor (ceiling lamp #2),
- p5) Desk,
- p6) Mini-bar,
- p7) Suspended (ceiling lamp #3, reference point).

The device called ceiling light 3 positioned in the anteroom of the room was used as a point of comparison between the tests carried out in the laboratory and those in vivo, positioning the plates at 25 cm from the light source with the aid of a support, visible in the photo, which served as a base for the petri dishes.



The plates were then irradiated with ceiling lights supplied by the Company and with LED strips, respectively modified and specially designed by the Nextsense technical group to integrate them with Biovitae® technology. It is specified that the changes were made following the lighting specifications indicated by the Company and that the devices were installed in the normal installation positions without any modification and / or change.

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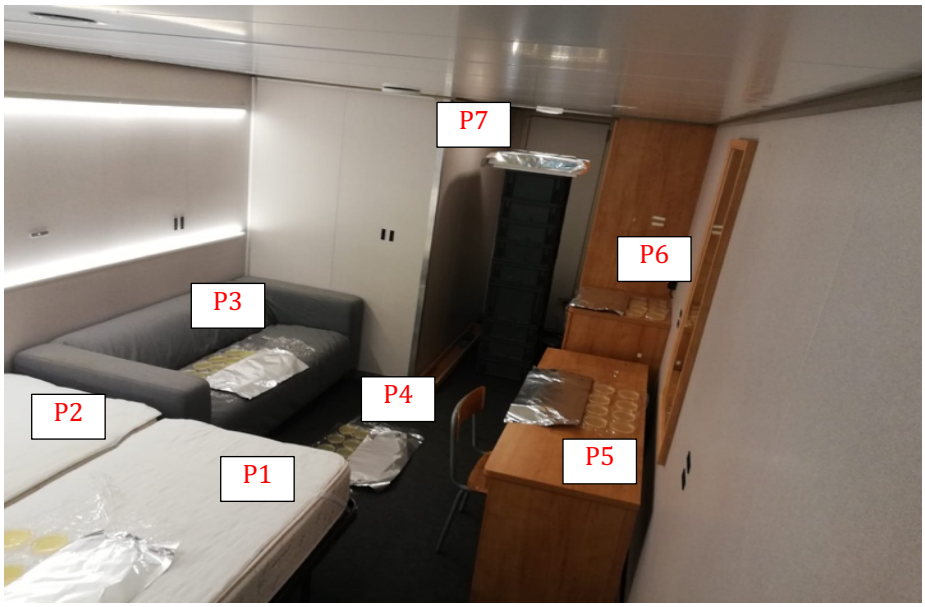
The following table shows the irradiance values obtained by direct illumination of the surfaces at the various control points and the percentage of radiated power with respect to the reference (the control plates, which were not to be irradiated with Biovitae® devices, were covered with aluminum foil).

Environment	Bed 3	Bed 2	Sofa	Ceiling light 2 (floor)	Writing desk	Fridge bar	Ceiling lamp 3 (25 cm)
Position	P1	P2	P3	P4	P5	P6	P7
mW cm-2	0.00344	0.02519	0.00454	0.00166	0.00022	0.00015	0.06948
%	4.94%	36.26%	6.54%	2.38%	0.31%	0.21%	100.00%

Tab. 1 *Irradiance values obtained by direct illumination of the surfaces in the various control points and percentage of radiated power with respect to the reference (ceiling light 3).*

In order to adequately evaluate the results obtained it is appropriate to highlight (see photo below shown in laboratory report) that during the test the ceiling lights 2 (P4) and 3 (P7) were kept off while, relative to those on, in some cases the light beams did not completely reach the surfaces to be radiate as they are blocked by other elements, as in the case of the plates in positions P4 (floor) and P6 (mini-bar). It is clear that these situations affected the cumulative test results in a negative way.



Fig, 5 *- Experimental setup (source: laboratory technical report)*

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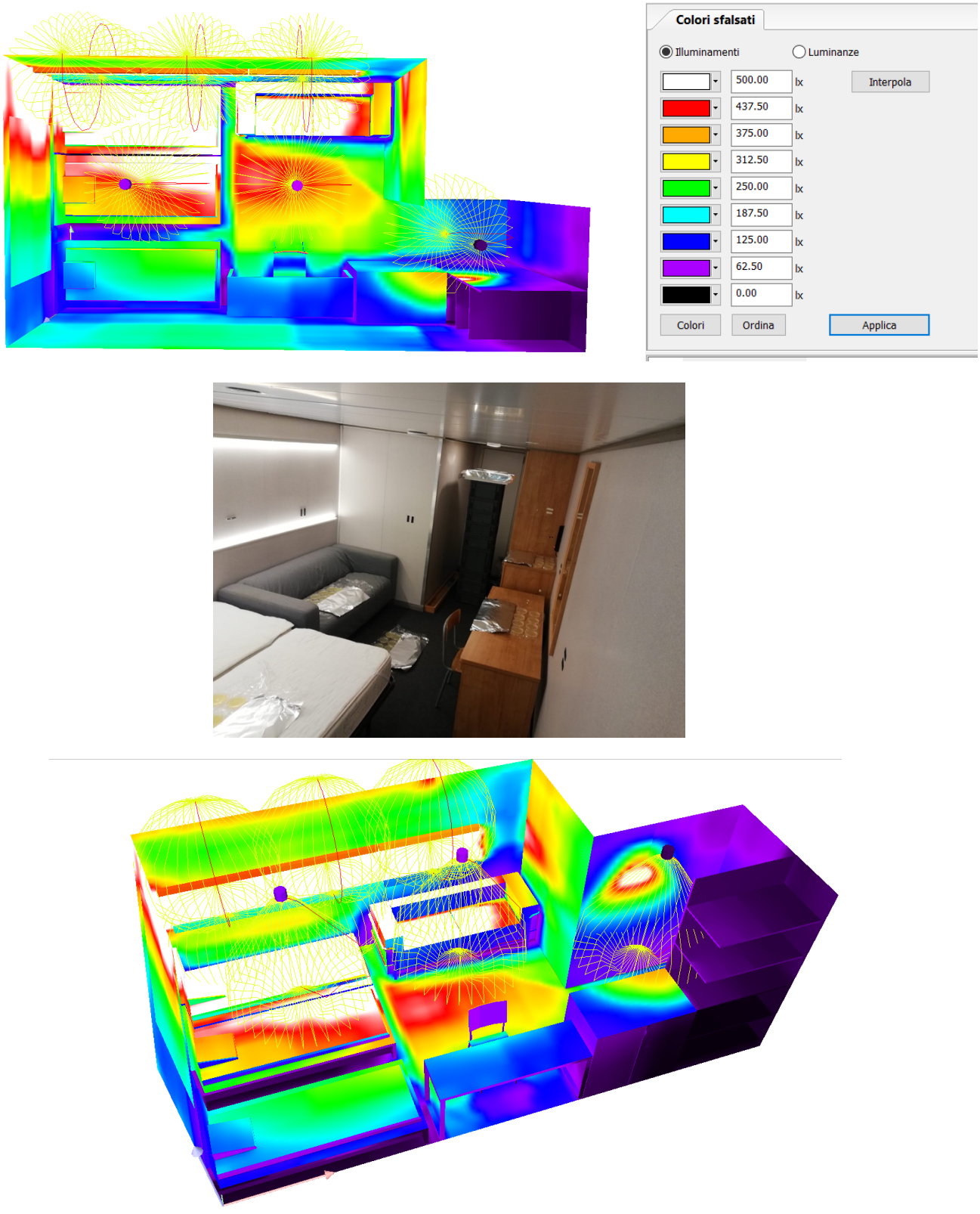
Fig. 6 - *3D representation of the illuminance levels inside the model cabin.*
The staggered colors identify the areas from the most irradiated (in white) to the least irradiated (in black)



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Analysis of the results

Despite the criticalities highlighted above, the count of microbial colonies in the exposed plates, at the end of the experiment, compared to the initial population of 1000 CFU / ml, recorded an average reduction of more than 70%, ranging from 51% (P6: mini bar) and 91% (P7: ceiling lamp 3), as shown in the following graph:

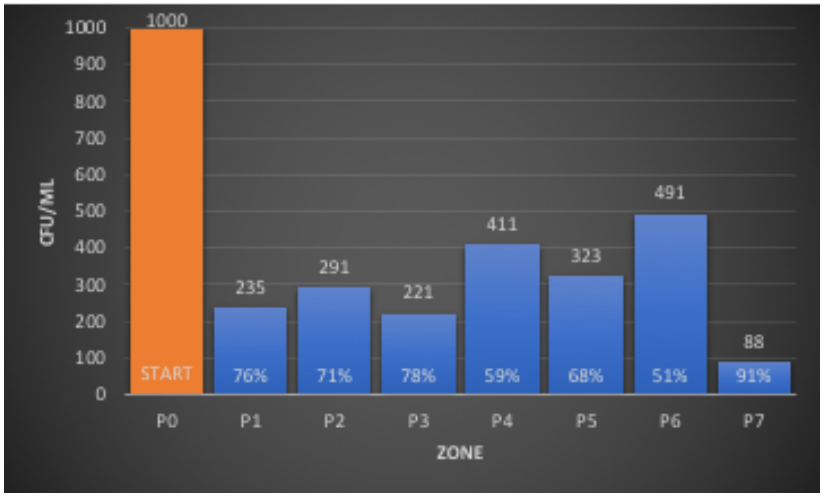

***Fig. 7** - Abatements after 24h exposure (source: laboratory technical report).*

Wanting to evaluate the results also on the basis of the comparison between positive samples (exposed plates) and negative samples (unexposed plates), it can be seen from the graph below that only in two cases there was a growth in the exposed samples with an average variation of 15 % (P4: floor and P6: mini bar).

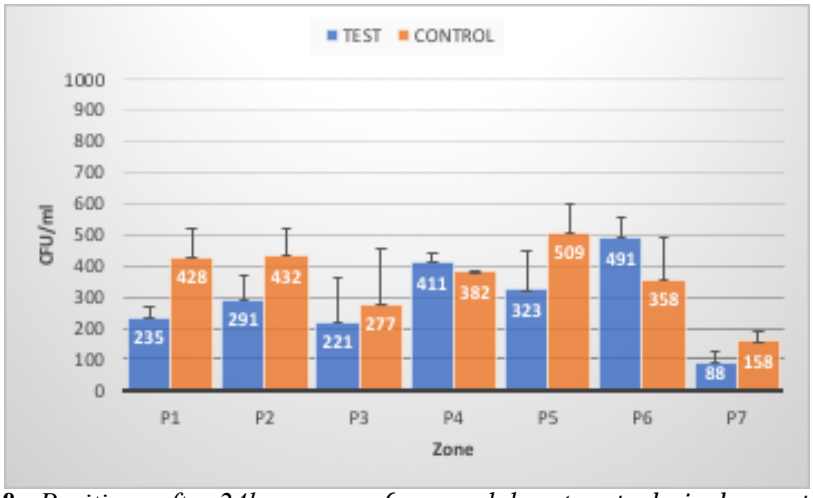

***Fig. 8** - Positions after 24h exposure (source: laboratory technical report).*

But in both cases, there were some critical issues which are reported below:

1) **the presence of elements that obstruct the passage of light beams**, limiting the irradiance values on the surfaces (sofa and beds for position P4, wardrobe for position P6);

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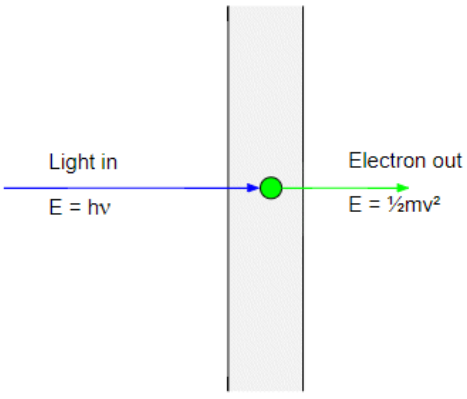
2) that the only device that could have acted on the samples in position P4, i.e. the ceiling light 2, is off; while the luminous flux of the ceiling light 3 in position P6 is hindered by the presence of the supports at 25 cm; and, consequently, the samples were clearly deprived of the microbicidal action that would have been induced by the light beams.

Furthermore, there are further elements to consider:

1. Biovitae® systems are designed to be used in real conditions of environmental contamination, while the tests conducted in the model cabin - for obvious methodological needs - were carried out "on colonies of bacteria inoculated in culture media that favored their replication". In real conditions, the bacteria are found on “normal” surfaces on which replication is very slow and, therefore, this element supports even more the results obtained considering the conditions of induced contamination;
2. the death rate during the exponential growth of E. coli is 0.7 CFU per 1000 CFU in one hour [9]. Therefore, if we consider replication times of 30 minutes, we obtain that in 24 hours, in the dark, the initial colony of 1,000 CFU would have had to grow to 277,000,000,000,000,000 CFU (including the mortality rate). This shows that Biovitae® is able to contain the proliferation of the microbial load even in real environments;
3. since the colonies in the control samples followed a mortality curve close to that of the colonies in the test samples, we highlight that the adoption of aluminum foil as a means of protecting the plates did not protect the controls but, on the contrary, produced mortality in colonies due to the generation of a photoelectric effect, i.e. the transfer of energy from the photons present in the light beams to the electrons present in the metal surface hit by the light beams, causing the emission of photoelectrons. Since in Einstein's interpretation the interactions take place between single electrons and single photons instantaneously, the photoelectric effect occurs even at very low light intensities, the interaction between a single electron and a single photon being necessary and sufficient.



4. The lower the thickness of the culture medium within the exposed plates compared to the culture medium in the unexposed plates is a further confirmation of the effective action of Biovitae on bacteria.
   Since bacteria replicate both in the dark and - more rapidly - in light, when colonies are inoculated on a culture medium their growth is a fact that should have been recorded in both exposed and covered specimens.

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However:

a. in the case of the exposed colonies, the death of the bacteria was caused by an uncontrollable acceleration of the metabolism triggered by the light of Biovitae®. In fact, in the first phase of irradiation, Biovitae causes a violent increase in metabolism which generates an uncontrollable production of toxic molecules (radicals) in the bacteria which leads to their death (Biovitae® effect); at the same time, Biovitae® leads to an exponential increase in the replicative activity of the bacteria. In both cases, the consequence is a more rapid consumption of the nutrients available to them.

b. in the case of non-exposed colonies, the lack of consumption of culture medium is the evidence that the death of the bacteria was caused by the beams of photoelectrons released by the photoelectric effect, a phenomenon caused by the interaction between light and aluminum sheets. This immediately prevented their replicative capacity, consequently reducing the consumption of the culture medium.

3. CONCLUSIONS

Only by constantly keeping the environmental microbial load under control is it possible to safeguard the continuous interaction between living beings and non-infectious doses of microorganisms [6], thus preserving the correct functioning of the immune system of the frequenters of that environment.

For this reason, the Biovitae® philosophy is based on the principle that an effective sanitization system must preserve this interaction without indiscriminately eliminating all the microbial load, as it is now established that the sterilization of environments, especially those of daily life, causes a weakening of the immune system.

Not even chemical disinfectants effectively protect against infectious risks as the sanitization routines with chemical agents are performed in the absence of people - when the infectious risk is practically non-existent - and, due to their limited effectiveness over time, they are unable to guarantee any protection when environments are frequented.

For all these reasons, the **new sanitation paradigm introduced by Biovitae® is that of continuous and passive sanitation (i.e., without human intervention) which is safe for both living beings and the ecosystem**. Its proven effectiveness guarantees a continuous sanitizing action even in the presence of living beings since it is absolutely safe in any condition of use and, by not generating resistance from microorganisms, it does not cause imbalances in the balance of ecosystems.

In conclusion, it being understood that the best way to test a device is a laboratory environment (the only one in which it is possible to control all the variables that can alter the conditions of execution of the tests), we believe that the test as a whole - and individually for each phase - **can be considered concluded with a positive outcome**.

In particular, **the results of Phase 3 of the tests confirmed the positive results already obtained in the laboratory**, unquestionably demonstrating the effectiveness of Biovitae® even in uncontrolled real conditions, despite the non-optimal installation conditions which reduced the irradiation and, therefore, the microbicidal power of the devices, both elements that can be improved in case of specific needs.

The results obtained (**average abatements of 70% with peaks of 91%**) are perfectly in line with both expectations and with respect to what can be considered an adequate level of microbiological hygiene within a confined environment such as a cruise ship's cabin.

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APPENDIX

Side considerations on sanitation systems

Ships, especially passenger ships that host thousands of people, are closely linked to public health: they can be not only a place for the transmission of infectious diseases, but a main source of infections (for example through contaminated water, surfaces and food).

The health risks deriving from cross-border movements represent a significant challenge, both for industrialized and less developed countries, due to the spread of carriers and the development of fast connections on a continental and intercontinental level.

Already in 2006, the SHIPSAN Project, funded by the European Community, emphasized the need to control the spread of infectious diseases on board vessels, with the main objective of implementing an integrated and sustainable strategy at European level for the protection of passenger and crew health in passenger and cargo ships, preventing the cross-border spread of diseases and improving public health.

The role of interaction with environments in the contamination process of living beings, with particular attention to surfaces and furnishing accessories, is a crucial issue, since both surfaces and furnishing accessories act as reservoirs for potentially pathogenic microorganisms [1], increasing the risk of cross-contamination through direct and indirect contact and, for this reason, they are the main object of the sanitation routines, the purpose of which is to contain the risk of contamination with the progressive reduction of the quantity of microorganisms present in them.

The common sanitation routines are based exclusively on the use of chemical disinfectants, which in addition to having a very consistent environmental impact, have several disadvantages that are often little known [2, 7, 8], including:

- ***Effectiveness dependent on external factors**, including a) the type of microorganism, b) the concentration of the disinfectant, b) the contact time of the disinfectant with the surface, c) the pH and temperature of the disinfectant, d) the presence of other organic material on the surfaces , e) the material with which the treated surface is made.*

- ***Limited duration of the biocidal effect over time**. Chemical disinfection covers a maximum time frame of containment of the microbial load which rarely reaches 30 minutes. At the end of this short period, the microbial load begins to grow uncontrollably.*

- ***Uncontrolled effects on the ecosystem.** When we use disinfectants recklessly, we destroy the part of microorganisms that represent the natural environmental microbiota and help establish a so-called "environmental dysbiosis", that is a condition that leaves open spaces for colonization to pathogenic microorganisms. The great evolutionary success obtained by microorganisms suggests that they played the fundamental role in the evolution of life; and thinking of eliminating them completely appears to be a reckless choice.*

Furthermore, the excessive use of chemical sanitizers / disinfectants, even more so if at high concentrations, favors the development of resistance by bacteria to these materials both through the

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acquisition of exogenous mobile genetic elements and through the process of intrinsic genetic adaptation [1]. Furthermore, such excessive use has a significant impact on the environment with the spread of substances harmful to the ecosystem in the air, water and soil.

The increase in bacterial species resistant to both disinfectants and antibiotics, also in light of the lessons learned from the SARS-CoV-2 pandemic, has made it necessary to change the paradigm in sanitation to ensure the safety of confined environments through the use of modern technologies a) safe for living beings, b) ecological, c) without the problems induced by chemical biocides [2].

The new frontier of sanitation technologies is increasingly moving towards the use of physical agents capable of limiting the negative impacts of the materials / devices used up to now.

Among these, light, and in particular the radiations of visible light in the 400-420nm range, has already been the subject of numerous selective efficacy tests with respect to multiple microbial species, amply demonstrating their validity in countless scientific studies conducted by many universities and research centers [3, 4, 5]. And precisely the possibility of using visible light as an element common to all confined spaces realizes the new paradigm of sanitation: continuous sanitation.

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