The suitability of a glovebox and of a covered still air box design for semi-sterile applications in environmental monitoring

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Abstract

Laminar flow cabinets (LFCs) ensure a safe working space within which product manipulation can be carried out safely excluding contaminations of the product with the environmental microorganisms. However, for environmental monitoring applications mobile laboratories are required and these prefer the lighter gloveboxes (GB; restricted arm movement) or still air boxes (SAB; free arm movement) over the heavier, more expensive LFCs, which need to be regularly maintained. Nevertheless, the efficiency of simple GBs/SABs (no HEPA filter), in providing semi-sterile working conditions has yet to be clearly defined. Consequently, our aim was to assess the suitability of GBs/SABs for semi-sterile applications by using passive and active bioaerosol sample collection procedures within the interior spaces of these boxes. Prior to sample collection the boxes were pre-treated with different spraying preparations (70% ethanol, two percent detergent or sterile water). For a greater restriction of bioaerosol entry, SABs were constructed with covered arm ports and these were classified as partially covered (SAB_{PC}) and completely covered SABs (SAB_{CC}). Results showed that ethanol sprayed GB and SAB_{CC} exhibited microbial aerosol colony counts of zero after one hour of passive sample collection, and active sample collection revealed counts ranging between 1.9 (for GB) - 2.3 $Log_{10}CFU/m^3$ (for SAB_{CC}). However, ethanol sprayed SAB and SAB_{PC} were ineffective having colony counts of 6.9 and 6.5 $Log_{10}CFU/m^3$, respectively. Other spraying regimes resulted in even higher colony counts (up to 7.3 $Log_{10}CFU/m^3$). Therefore, the ethanol sprayed GB and SAB_{CC} could effectively be used for semi-sterile applications, with the SAB_{CC} allowing for an unrestricted arm movement within it.

1. Introduction

Maintaining a safe working space for product/sample manipulation, by preventing the contamination of the product with the environmental microorganisms is a never-ending battle. Laminar flow cabinets (LFC) use high-efficiency particulate air (HEPA) filters to remove particulates from the air (Mcgarrity and Coriell, 1974) and are the most common providers of a sterile preparation space (Kruse et al., 1991). However, unregulated air flow within LFCs can be problematic and may cause product cross-contamination with fungal spores which are easily dispersible in an air flow (Mcgarrity and Coriell, 1974). Consequently, the inlet speed of a LFC has to be checked daily so that the acceptable limits are not breached (acceptable: 0.7-0.9 m/s) (St George's University of London, 2018). The LFC working surface consists of several removable panels. These must be lifted up and the base surface must be cleaned at the end of each session to avoid biofilm occurrence on spilt media (St George's University of London, 2018). Another disadvantage of LFCs is that they are expensive (Peiris et al., 2012) and, because of their size, bulkiness and weight, are strictly stationary devices.

For environmental monitoring, mobile units of cultural heritage conservation institutions frequently employ GBs or SABs (simple boxes without HEPA filters) for semi-sterile analysis of valuable but often mouldy mural paintings. The less expensive light weight boxes are ideal for these units, which need to provide protection and conservation of immovable paintings, which are scattered across the entire country. Additionally, their research budget is often very limited (Bingley and Verran, 2013). Biodeterioration by moulds, involves the production of pigments, degradation of one or more compounds with extracellular enzymes and with secreted

organic acids (Kosel and Ropret, 2021). Moreover, archivists and conservators should be aware of the potential toxicogenic and allergenic properties of moulds from the *Alternaria, Aspergillus*, *Fusarium* and *Penicillium* spp. (Bush and Portnoy, 2001; O'Gorman and Fuller, 2008). They are known to cause immunotoxic diseases such as the sick building syndrome (Burch and Levetin, 2002) and at elevated spore counts are linked to respiratory diseases such as asthma and sinusitis (Peltola et al., 2001; Stryjakowska-Sekulska et al., 2005; Szulc et al., 2020). Therefore, it is important that manipulations during the inspection of paintings in the GB/SAB could be carried out safely and that the fungal spores released could be contained (Moroni and Pitzurra, 2008). To reduce the high risk of cross contamination between different cultural heritage samples, the GB/SAB has to be repeatedly sanitised by disinfectant spraying (Bingley and Verran, 2013). This allows also for a more reliable molecular identification of fungal strains isolated from specific samples or from certain sections of a painting.

Basically, a simple SAB is an enclosed transparent chamber with two frontal openings for manual access (arm ports) designed to provide free arm movement within a clean still air environment (Stuart et al., 2006). A simple GB design is similar to a SAB, however this box is completely sealed off and its arm ports are extended towards the interior with long rubber gloves restricting any possible entry of the surrounding microbial aerosols. Its minor flaw can occur when hands are inserted into the gloves creating a "piston effect", which lifts contaminants within the box (Stuart et al., 2006).

Even though, the efficiency of these simple GBs/SABs in providing semi-sterile working conditions has yet to be clearly defined, their frequent use for private (edible mushroom cultivation) (Stamets and Chilton, 1985; Tunney, 2006) and professional applications (mushroom

packaging) (Anderson and Walker, 2011) is reflected in the Fortune Business Insights report (2020), which claims that the global GB market is expected to rise with an impressive compound annual growth rate (its revenue peaking in 2026). However, it needs to be stated that the use of these simple boxes, which do not contain HEPA filters, is not permissible for compounding sterile preparations. For these applications a HEPA filtered ISO Class five environment is the outmost minimum requirement for a pharmacy (Kienle et al., 2014).

Our aim was to assess the suitability of GB and SAB for semi-sterile applications using passive and active bioaerosol sample collection procedures within the boxes. Prior to sample collection, GB and several SABs, constructed from simple household materials, were pre-treated with different spraying preparations (70 % (v/v) ethanol, two percent detergent solution and sterile water). Additionally, we made several arm port modifications to the SAB design, to decrease the size of arm ports, for a greater restriction of bioaerosol entry, and to simultaneously retain its practical free arm movement feature.

2. Methods

Construction of the GB and SAB

The completely enclosed and sealed GB was constructed using a pair of resilient plastic gloves (Guide 4011) and a transparent plastic storage container (Combi Box 67L 59.3 x 38 x 47 cm, Tontarelli S.p.A. Italy) which was turned upside down so that its cover lid was at the bottom of the box (Figures 1A and 1C). Two circular holes for the arm ports were each cut out with a rotary cutter powered by a drilling machine. Each arm port consisted of a PCV pipe, which was 11 cm in diameter and was 5.5 cm long. The pipe was inserted into the cut out hole with three centimetres of pipe sticking into the internal space of the box. Two-component adhesive was applied at the pipe-box interface on both sides of the wall and was let to dry overnight. To reinforce the attachment, each pipe was screwed onto the wall with two angled brackets, and on the outer side additional resistant tape was applied. Gloves were then fitted over each pipe (inside the box) with the thumbs sticking inwards and downwards. Finally, the gloves were secured and tightened in place using plastic zip ties (Figure 1).

The SAB was constructed in the same manner as the GB, with the omission of gloves and glove ports (having two unrestricted and uncovered circular holes for the insertion of hands). A modified version of the SAB, named the SAB_{PC} (partially covered), had circular holes partially covered using an elastic laboratory shoe cover. For this purpose, the cover was cut so that the elastic edge of the cover was in the inner centre of a circle. Each time the operator inserted his/her hands, the inner elastic centre tightened his/her forearms restricting any possible air movement. The third version of the SAB, named the SAB_{CC} (completely covered), was constructed with an additional hanging cover (HC) taped onto the wall from the outside of the SAB_{PC} (only the upper edges of the HC were fixed), covering as much of the arm hole as

possible (Figures 1B and 1D). The HC consisted of a transparent plastic bag and a laboratory glass rod which was glued in place from within the bag, serving as a weight.

For the GB and SAB, the main entry/exit point was via the left/right side by loosening the bottom lid cover and by elevating the box from that side. During work, the operator inserted his/her bare hands within the Guide 4011 gloves of the GB arm ports or used standard laboratory gloves when inserting his/her hands into the SAB/SAB_{PC}/SAB_{CC} arm ports.

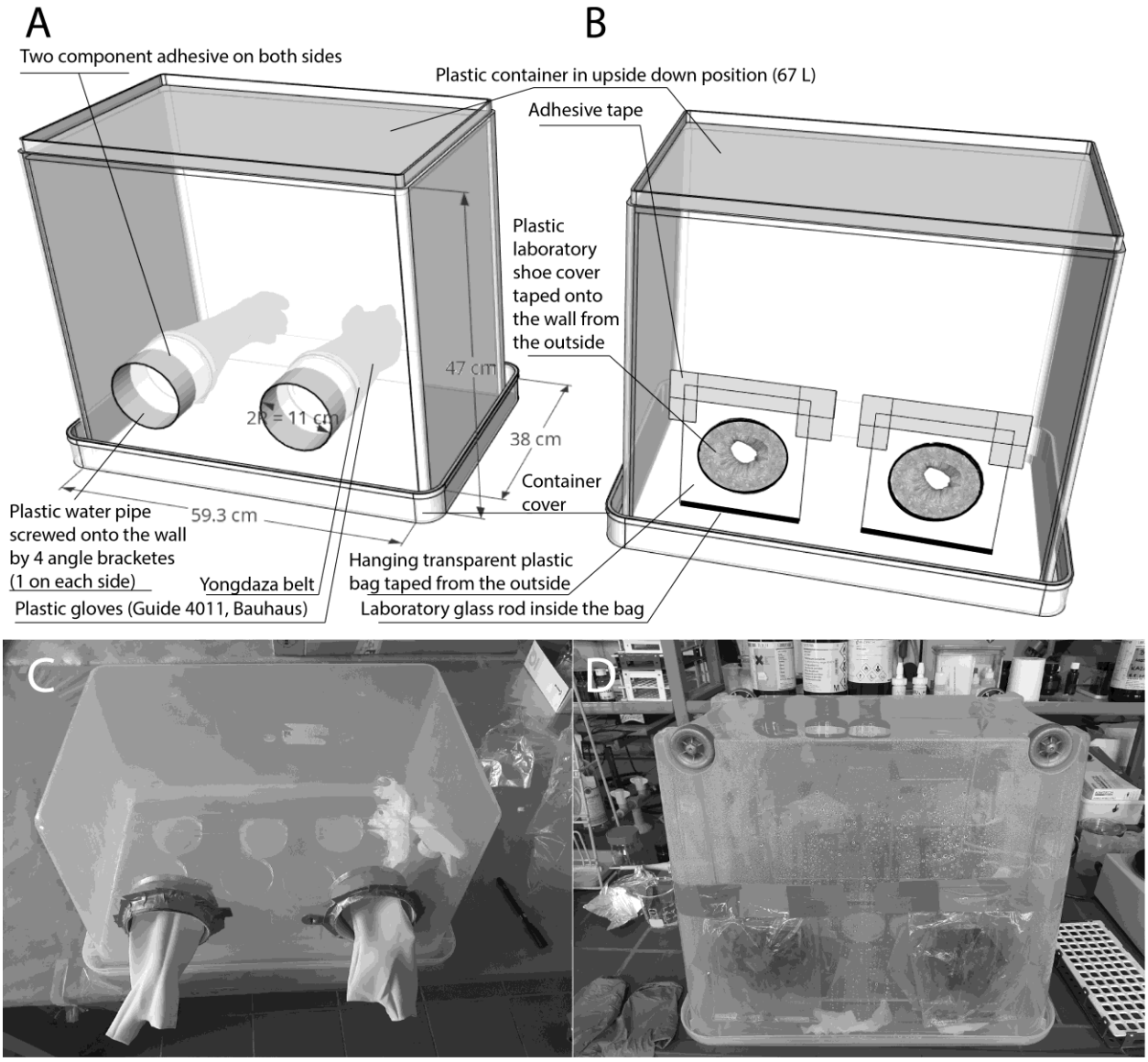

Figure 1: Schematic representation of GB (A), SAB_{CC} (B) and of their collective parts. Their experimental set-ups, during the passive sample collection procedure, are presented in frames C (GB) and D (SAB_{CC}).

Passive sample collecting

Before each experiment, the lid cover of a clean GB/SAB was removed and the internal space of the box was fully exposed to the outside air for 15 min. After this exposure period, the box was closed and the entire enclosed internal space as well as the gloves were sanitized by spraying (70

% (v/v) ethanol in distilled water; sterile autoclaved distilled water; or a two percent solution of a household detergent in autoclaved distilled water) and the roof and bottom parts of the box were briefly wiped using two paper towel sheets. Enclosed sterile Petri plates were inserted into the GB/SAB by elevating the box by five centimetres above the bottom lid cover (only on one side – left or right). For sample collection, microbial fallout was tested on Tryptic Soy Agar (TSA: 15 g/L of agar (Difco™, Laboratories, Detroit, Mich.), 15 g/L of casein peptone (Difco™), 5 g/L of sodium chloride (Difco™) with a pH value of 7.3) and on Sabouraud chloramphenicol dextrose agar plates (SabC, Becton-Dickinson, Heidelberg, Germany) (de Hoog et al., 2000). Petri plates with a diameter of nine centimetres and a surface area of 0.0055 m^2 (A) were left open to internal air for either 15 min, 30 min or 60 min (t). After a 10-day long incubation at 30 ^oC (for fungi on SabC medium) or a two day long incubation at 37 ^oC (for bacteria on TSA medium) colonies were counted (N) and the Total Viable Count (TVC) in CFU/m^2/h (in Log_{10}CFU/m^2 for representation) was calculated by taking into account the corresponding exposure time and the surface area:

$$TVC_P \left[\frac{\frac{\text{CFU}}{\text{m}^2}}{\text{h}}\right] = \frac{N}{A}/t \quad (1)$$

where N [CFU] is the number of colonies; A [m^2] is the culturable surface area of a Petri plate; and t [h] is the time of growth medium exposure. TVC_P was measured by three independent samplings (each sampling consisted of three Petri plates; and therefore nine plates were used in total).

The laboratory air was sampled according to the 1/1/1 scheme (plates exposed for one hour, one meter above the floor, about one meter away from walls or any major obstacles) which defines the Index of Microbial Air Contamination (IMA_P); (Pasquarella et al., 2000).

Active sample collecting

Twenty millilitres of deionized water (V_1) was poured into a 30 mL all-glass AGI impinger, the openings of the impinger were covered with aluminium foil and its sterilization was carried out by autoclaving (121 °C, 1.1 bar, 15 min). Box preparations were done in the same manner as for the passive method. A sterilised impinger and a vacuum pump (EMC Italy; AC 230 V, 50 Hz, 80 W) were inserted into the box and the enclosed internal space was sprayed. Before sampling, the sterile impinger was placed in the middle insides of the GB/SAB and the aluminium foil was removed. The impinger's exit was then connected to the silicone tube (diameter of five mm) of the pump and seven separate air draws of 75 L each (five minutes of operation) were made at a flow rate of 15 L/min for a total sampled air volume of 500 L (V_A of 0.5 m^3), with intervals of five minutes between draws. For analysis, samples were initially serially diluted (dilution factor (D) of 1, 10 or 100) in aseptic conditions (next to a laboratory butane burner) and one millilitre of sample (V_2) (or its dilution) was pipetted onto sterile SabC and TSA Petri plates. After a 10 day long incubation at 30 ºC (for moulds) or a two day long incubation at 37 ºC (for bacteria) colonies on Petri plates were counted (N). The Total Viable Count (TVC) in CFU/m^3 (in Log_{10}CFU/m^3 for representation) was calculated according to the following equation (Burton et al., 2005):

$$TVC_A \left[\frac{\mathrm{CFU}}{\mathrm{m}^3}\right] = \frac{N \times D \times (V_1/V_2)}{V_A} \quad (2)$$

where N [CFU] is the number of colonies; D is the dilution factor; V_1 [mL] is the amount of extraction fluid used; V_2 [mL] is the eluate amount used for analysis, and V_A [m^3] is the volume

of air sampled (flow rate of the sampler multiplied by the sampling time). TVC_A was measured by five independent samplings with three technical repeats.

Lastly, the laboratory air was also sampled using the active sample collection method which adhered to the 1/1/1 scheme of the IMA index (IMA_A).

Exposure to *Aspergillus niger*

In order to simulate the effect of an intense GB/SAB exposure to airborne microorganisms, the internal space of a box was sprayed with a spore suspension of *Aspergillus niger* prior to its sanitization with 70 % (v/v) ethanol. *Aspergillus niger* ZIM F42, acquired from the ZIM Collection of Industrial Microorganisms, Slovenia, was cultured at 28 °C on SabC plates. Spores from fresh culture plates (48 h old) were harvested, suspended and diluted in sterile distilled water. Spore concentration was determined with the light microscope Zeiss LSM 800 using a haemocytometer and was adjusted to a final concentration of 1.0×10^7 cells mL^{-1}. One hundred millilitres of spore suspension was then pipetted into a sterilised mini air brush spray Gun (Cosscci) and the interior of the enclosed GB/SAB was sprayed.

Simulating normal working conditions

Because the experiments described above were conducted under ideal experimental conditions (boxes were closed entire time with minimal hand movement) we have additionally tested the ethanol sanitised (sprayed) GB and SAB_{CC} in conditions which more closely simulate real (normal) work. Therefore, after each active or passive sample collecting, the operator's arms were inserted into the arm ports and the operator exchanged the position of an empty but sterile plastic Petri plate

six times from the outmost left side to the outmost right side. After this, his hands were removed from the arm ports. This simulation procedure was repeated after 15, 30 and 60 min of passive sampling, and after five, 15 and 33 min of active sampling.

Potential inhibition by residual ethanol

Residual ethanol within a box, after its sanitation and after its interior wiping with a paper towel, may potentially still interfere with the final colony counts of microbial fallout (may reduce the viability of collected cells on the agar surface). Therefore, if a box is used for passaging of monocultures, the residual ethanol could inhibit or reduce their desired growth. Therefore, our next experiment was aimed to test how residual ethanol within a box can affect the viability of ethanol sensitive bacteria *Escherichia coli* (Dombek and Ingram, 1984; Sawada and Nakamura, 1987). This control experiment was only performed on the two most air restrictive boxes, namely the GB and the SAB_{CC} (completely covered (SAB_{CC}) or sealed (GB) boxes to restrict the surrounding air from entering into the interior space of a box). *E. coli* CB390, kindly provided to us by Guzmán et al. (2008), was cultured at 37 °C on TSA plates. Colonies from fresh culture plates (two days old) were harvested and suspended in sterile saline solution (0.9 % NaCl). Bacterial cell concentration was determined under the light microscope Zeiss LSM 800 using a haemocytometer and was adjusted to a final concentration of around 1×10^4 cells mL^{-1}. In sterile conditions, 40 µL of bacterial suspension was pipetted onto 16 sterile TSA plates. The first fourth of the inoculated plates (four plates) was immediately set for incubation at 37 °C for two days, however the second fourth (four plates) was firstly sprayed with ethanol (70 % v/v) in a GB/SAB_{CC} (one pressing 20 cm from the open plate) and was only then placed within the incubator (two days at 37 °C). The remaining inoculated plates were firstly processed within a GB/ SAB_{CC} (four plates) according the

procedures described under passive sample collecting protocol (using 70 % (v/v) ethanol as a sanitation reagent) and were then incubated at 37 ^{o}C for two days. Colony counts of CB390 for all these different treatments were determined.

Statistical analysis

Mean values and their standard deviation values were calculated, and the differences between mean values were assessed using the Tukey's studentized range test (one-way ANOVA) (SAS Institute, 1988) within the statistics software package OriginPro version 8.0 (OriginLab, Northampton, MA). ANOVA results are presented within Tables 1 and 2 using the Compact Letter Display (CLD) technique. This technique goes through each comparison, and assigns a letter to each group of mean values. Groups, which are given the same letter, are not significantly different at an alpha value of 0.05 (at $p < 0.05$). A group may also be marked with two neighbouring letters if its mean values fall in between (mean value within a group, which is marked by two neighbouring letters, does not statistically differ from mean values in both of the neighbouring groups).

3. **Results**

Testing of boxes (GB, SAB, SAB_{PC} and SAB_{CC}) to provide a sufficiently sterile working environment using passive and active sample collection procedures is presented in Figure 2.

Passive sampling after ethanol spraying treatments of the GB and SAB_{CC} revealed no aerosol colony counts regardless of the sampling time (Figure 2A). However, for SAB and SAB_{PC}, after 60 min of exposure, the viable aerosol count reached 3.4 $Log_{10}CFU/m^2$ and 2.9 $Log_{10}CFU/m^2$, respectively. Furthermore, 33 min of active sampling (500 L of air sampled) after ethanol spraying treatments of the GB and SAB_{CC} revealed only 1.9 $Log_{10}CFU/m^3$ and 2.3 $Log_{10}CFU/m^3$, respectively. On the other hand, 33 min of active sampling within ethanol sprayed SAB and SAB_{PC}, determined considerably higher viable aerosol counts (7.0 $Log_{10}CFU/m^3$ for SAB and 6.5 $Log_{10}CFU/m^3$ for SAB_{PC}).

Regardless of the sampling method employed for any of the box types, the initial spraying with *A. niger* and the subsequent ethanol spraying treatment (Figure 2B) resulted in very similar results to those observed when only ethanol treatment was performed (Figure 2A).

Passive sampling (60 min) after detergent spraying of the GB and SAB_{CC} revealed aerosol colony counts of 2.7 $Log_{10}CFU/m^2$ and 3.1 $Log_{10}CFU/m^2$, respectively (Figure 2C) (these were zero $Log_{10}CFU/m^2$ when ethanol spraying was employed). In comparison to the ethanol treatments, for the SAB (3.9 $Log_{10}CFU/m^2$) and SAB_{PC} (3.6 $Log_{10}CFU/m^2$), an approximately 0.5 $Log_{10}CFU/m^2$ increase (60 min) in viable aerosol count was observed when detergent was employed. With the active sampling method, we also observed a significant increase in viable

aerosol counts when detergent instead of ethanol was used. Specifically, after detergent treatments and after 33 min of active sampling within the GB and SAB_{CC}, 5.5 $Log_{10}CFU/m^3$ and 6.9 $Log_{10}CFU/m^3$ of viable aerosol counts were recorded, respectively (an increase of 3.6-4.6 $Log_{10}CFU/m^3$ in compassion to the ethanol spraying). On the other hand, for the detergent treatments, active sampling within the SAB and the SAB_{PC} revealed viable aerosol counts of 7.2 $Log_{10}CFU/m^3$ and of 7.0 $Log_{10}CFU/m^3$, respectively (an increase in 0.2-0.5 $Log_{10}CFU/m^3$ in comparison to the ethanol spraying).

Passive sample collection (60 min) after sterile distilled water spraying treatment of the SAB revealed aerosol colony counts (3.8 $Log_{10}CFU/m^2$) similar to those obtained after detergent spraying (Figure 2D). Nevertheless, aerosol counts for the GB (2.1 $Log_{10}CFU/m^2$), SAB_{CC} (2.6 $Log_{10}CFU/m^2$) and the SAB_{PC} (3.1 $Log_{10}CFU/m^2$) after distilled water spraying were lower (approximately 0.5 $Log_{10}CFU/m^2$ lower for each box type). Active sampling within the GB (5.5 $Log_{10}CFU/m^3$), SAB (7.3 $Log_{10}CFU/m^3$) and the SAB_{PC} (6.9 $Log_{10}CFU/m^3$) revealed that after sterile distilled water treatments aerosol counts were similar to those obtained after detergent spraying treatments (active sampling). Nevertheless, aerosol counts for the SAB_{CC} after sterile distilled water spraying, were for 0.8 $Log_{10}CFU/m^3$ lower to those threated with the diluted detergent.

When spraying was completely omitted, the aerosol counts for all box types was around 3.9 $Log_{10}CFU/m^2$ and around 6.9 $Log_{10}CFU/m^3$ for the passive (60 min) and active samplings (33 min), respectively.

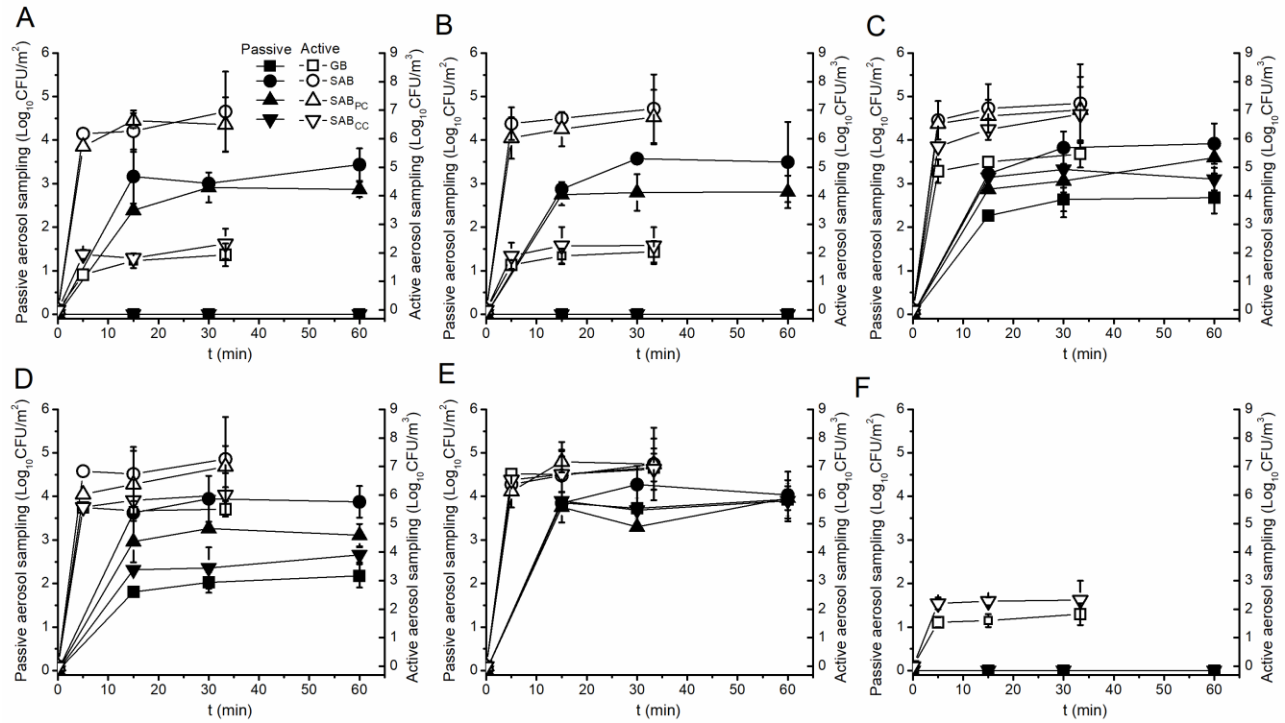

Figure 2: Assessing semi-sterility within the GB, SAB, SAB_{PC} and the SAB_{CC} employing both passive (TVC_P) and active sample collection methods (TVC_A). Prior to sampling, the interior was sprayed either by 70 % ethanol (A); by a suspension of *A. niger* and subsequently by ethanol (B); by 2 % detergent solution (C), by sterile distilled water (D) or was left untreated (no spraying E). In ethanol sanitised GB and SAB_{CC}, object movement every 10 min (empty Petri plate) simulated normal working conditions (F). In graphs, means values are presented and error bars indicate their corresponding standard deviation values.

Within the ethanol sanitised GB and SAB_{CC}, object movement every 10 min (empty Petri plate) simulated normal working conditions (Figure 2F). Our results, after passive and active sampling within the GB and SAB_{CC}, show that aerosol colony counts do not significantly deviate if minimal movement (ethanol sanitised boxes in Figure 2A) or simulated normal working movement (Figure 2F) is employed.

Tables 1 and 2 summarise the results presented in Figure 2. Within the tables, the TVC values are also compared with the laboratory's IMA values, which represent the air quality outside the boxes. Microbial aerosol reduction efficiencies (*E*) were calculated by subtracting the logarithmic values of TVC from the logarithmic values of IMA. The IMA values measured using the passive sedimentation method and the active air suction method were estimated at 4.3 $Log_{10}CFU/m^2/h$ (Table 1, IMA_P) and at 7.2 $Log_{10}CFU/m^3$ (Table 2, IMA_A), respectively.

When comparing different spraying treatments, ethanol spraying and spraying by a suspension of *A. niger* and subsequently by 70 % ethanol resulted in the highest overall *E* values. Specifically, for both spraying treatments, passive sampling revealed a complete reduction (*E* value of 4.3 $Log_{10}CFU/m^2/h$) within the GB and the SAB_{CC} (Table 1). Moreover, *E* values were in range of 4.8 - 5.1 $Log_{10}CFU/m^3$ when active sampling was employed (Table 2).
In comparison, the *E* values of other spraying treatments (diluted detergent and sterile distilled water) were much lower and when spraying was completely omitted the *E* values were only around 0.3 $Log_{10}CFU/m^2/h$ (for passive sampling) and 0.2 $Log_{10}CFU/m^3$ (for active samplings).

Interestingly, *E* values for sterile distilled water treatments were significantly higher to those of detergent spraying treatments (*E* value difference of 0.5 for GB, of 0.1 for SAB, of 0.5 for SAB_{PC} and of 0.4 for SAB_{CC} (all in $Log_{10}CFU/m^2/h$)). However, this was only evident when passive sampling was employed and when active sampling was conducted within the SAB_{CC} (*E* value difference of 0.9 $Log_{10}CFU/m^3$).

When the entire experimental frame is considered, GB and SAB_{CC}, in comparison to SAB and SAB_{PC}, clearly provide a higher degree of semi-sterility. Their higher protective potential was evident even for less effective spraying treatments such as when using sterile distilled water or diluted detergent sprayings.

Table 1: Microbial aerosol counts after 60 min of passive sampling (TVC_P) within the GB, SAB, SAB_{PC} and the SAB_{CC}. Microbial aerosol reduction efficiency (*E*) was calculated by subtracting the TVC_P value from the value of IMA_P.

Passive sample collection *(IMA_P of 1.8 x 10^4 $CFU/m^2/h$ = 4.3 $Log_{10}CFU/m^2/h$)*	***GB***	***SAB***	***SAB_{PC}***	***SAB_{CC}***
Microbial aerosol count *(TVC_P in $Log_{10}CFU/m^2/h$)*				
Ethanol spraying	$0^{A, \alpha}$	$3.4 \pm 0.4^{B, \alpha}$	$2.9 \pm 0.2^{B, \alpha}$	$0^{A, \alpha}$
1. A. niger spraying; 2. ethanol spraying	$0^{A, \alpha}$	$3.5 \pm 0.9^{B, \alpha}$	$2.8 \pm 0.3^{B, \alpha}$	$0^{A, \alpha}$
Diluted detergent spraying	$2.7 \pm 0.4^{A, \beta}$	$3.9 \pm 0.5^{B, \alpha}$	$3.6 \pm 0.4^{B, \alpha\beta}$	$3.1 \pm 0.3^{AB, \beta}$
Sterile dH_20 spraying	$2.2 \pm 0.3^{A, \beta}$	$3.8 \pm 0.4^{B, \alpha}$	$3.1 \pm 0.2^{AB, \alpha}$	$2.6 \pm 0.2^{A, \beta}$
No spraying	$3.9 \pm 0.4^{A, \gamma}$	$4.0 \pm 0.5^{A, \alpha}$	$3.9 \pm 0.3^{A, \beta}$	$3.9 \pm 0.5^{A, \gamma}$
Normal working conditions after ethanol spraying	$0^{A, \alpha}$	n.c.	n.c.	$0^{A, \alpha}$
Microbial aerosol reduction efficiency (E_P) *(in $Log_{10}CFU/m^2/h$)*				
Ethanol spraying	4.3	0.8	1.4	4.3
1. A. niger spraying; 2. ethanol spraying	4.3	0.8	1.5	4.3
Diluted detergent spraying	1.6	0.3	0.7	1.2
Sterile dH_20 spraying	2.1	0.4	1.2	1.6
No spraying	0.3	0.2	0.3	0.4
Normal working conditions after ethanol spraying	4.3	n.c.	n.c.	4.3

Table Legend: presented values are mean values and values behind "±" symbols are their corresponding standard deviations; "n.c." represents that the experiment was "not conducted"; Index of Microbial Air Contamination obtained using the passive sampling method (IMA_P). Mean values marked with Upper Case Letters of English alphabet represent ANOVA mean comparisons (alpha value of 0.05) between different kinds of GB or SABs, which were treated with the same spraying preparation/operation. Mean values marked with Lower Case Letters of Greek alphabet represent ANOVA mean comparisons (alpha value of 0.05) between different spraying preparations/operations within the same box (GB/SAB).

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Table 2: Microbial aerosol counts after 33 minutes of active sampling (TVC_A) within the GB, SAB, SAB_{PC} and the SAB_{CC}. Microbial aerosol reduction efficiency (*E*) was calculated by subtracting the TVC_A value from the value of IMA_A.

Active sample collection *(IMA_A of 1.47 x 10^7 CFU/m^3= 7.2 Log_{10}CFU/m^3)*				
	GB	***SAB***	***SAB_{PC}***	***SAB_{CC}***
Microbial aerosol count				
(TVC_A in Log_{10}CFU/m^3)				
Ethanol spraying	$1.9 \pm 0.4^{A, \alpha}$	$6.9 \pm 1.4^{B, \alpha}$	$6.5 \pm 0.9^{B, \alpha}$	$2.3 \pm 0.5^{A, \alpha}$
1. A. niger spraying; 2. ethanol spraying	$2.0 \pm 0.4^{A, \alpha}$	$7.0 \pm 1.2^{B, \alpha}$	$6.8 \pm 1.0^{B, \alpha}$	$2.3 \pm 0.6^{A, \alpha}$
Diluted detergent spraying	$5.5 \pm 0.5^{A, \beta}$	$7.2 \pm 1.4^{A, \alpha}$	$7.0 \pm 1.1^{A, \alpha}$	$6.8 \pm 1.0^{A, \beta}$
Sterile dH_20 spraying	$5.5 \pm 0.2^{A, \beta}$	$7.3 \pm 1.5^{B, \alpha}$	$6.9 \pm 0.7^{B, \alpha}$	$6.0 \pm 0.8^{AB, \beta}$
No spraying	$6.9 \pm 0.5^{A, \gamma}$	$7.1 \pm 1.3^{A, \alpha}$	$7.0 \pm 0.9^{A, \alpha}$	$6.9 \pm 0.7^{A, \beta}$
Normal working conditions after ethanol spraying	$1.8 \pm 0.4^{A, \alpha}$	n.c.	n.c.	$2.3 \pm 0.7^{A, \alpha}$
Microbial aerosol reduction efficiency (E_A)				
(in Log_{10}CFU/m^3)				
Ethanol spraying	5.2	0.2	0.7	4.8
1. A. niger spraying; 2. ethanol spraying	5.1	0.1	0.4	4.9
Diluted detergent spraying	1.7	-0.1	0.2	0.3
Sterile dH_20 spraying	1.7	-0.1	0.2	1.2
No spraying	0.2	0.1	0.1	0.3
Normal working conditions after ethanol spraying	5.4	n.c.	n.c.	4.9

Table Legend: presented values are mean values and values behind "±" symbols are their corresponding standard deviations; "n.c." represents that the experiment was "not conducted"; Index of Microbial Air Contamination obtained using the active sampling method (IMA_A). Mean values marked with Upper Case Letters of English alphabet represent ANOVA mean comparisons (alpha value of 0.05) between different kinds of GB or SABs, which were treated with the same spraying preparation/operation. Mean

values marked with Lower Case Letters of Greek alphabet represent ANOVA mean comparisons (alpha value of 0.05) between different spraying preparations/operations within the same box (GB/SAB).

Potential inhibition by residual ethanol, within the GB or SAB_{CC}, after their sanitation and wiping, was tested on *E. coli* CB390 cells which were initially plated onto TSA plates. The high sensitivity of CB390 to ethanol was evident, since the colony count of inoculated and ethanol sprayed plates (within a box) was zero Log_{10}CFU/mL (Figure 3). In comparison, inoculated and directly incubated CB390 plates (IDC plates) reached ~2.5 Log_{10}CFU/mL. Interestingly, the colony counts of CB390 plates, which were processed within the GB or SAB_{CC} according to the procedures described under passive sample collecting protocol, remained similar to that of the IDC plates (Figure 3). Therefore, residual ethanol, within the GB or SAB_{CC}, after their wiping, did not affect the cultivability of ethanol sensitive *E. coli.*

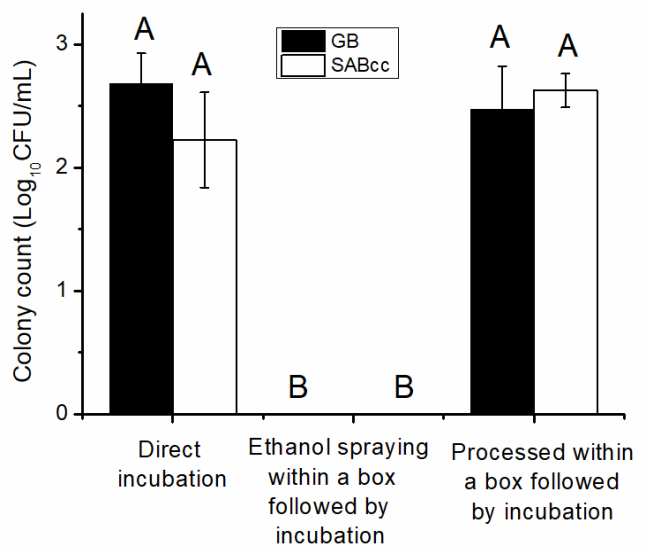

Figure 3: Potential inhibition of plated *E. coli* CB390 cells by residual ethanol, within the GB and SAB_{CC}, after their sanitation and wiping. In the graph, mean values are presented and error bars indicate their corresponding standard deviation values. Mean values marked with Upper Case Letters of English alphabet represent ANOVA mean comparisons (alpha value of 0.05).

4. **Discussion**

The function of a GB is to provide a completely enclosed environment restricting any possible entry of environmental microorganisms (Stuart et al., 2006). If the insides of the GB are sprayed, the aerosolized droplets perforate this still air and land on the inner surfaces of the box. During the spraying process, the introduced droplets capture any particulates floating in the air flow and remove them by attaching them onto the walls of the GB (Tessum and Raynor, 2017). If the spraying liquid is a disinfectant, a significant portion of the captured microbial aerosol will be deactivated. If surfactants are used, their net charged nature forms electrostatic interactions with cell wall proteins, facilitating the immobilisation and deactivation of microorganisms (Otzen, 2011). The principle mode of action of a SAB is similar to that of a GB. However, most SABs are only partially enclosed and consequently offer a lower degree of protection. Nevertheless, they allow for significantly less strenuous work conditions, omitting restraint of the operator's hands for complete freedom of movement (Stuart et al., 2006).

Napoli et al. (2012) reported a strong correlation between the active and the passive bioaerosol collection methods. Contrary to their findings, our results show a clear divergence for some of the treated boxes. For example, when samples of ethanol-treated GB (completely enclosed and sealed) and SAB_{CC} (completely enclosed but not sealed) were analysed, passive sample collection revealed no aerosol counts, however active sampling was able to detect ≈1 $Log_{10}CFU/m^3$ (within GB) and ≈ 2 $Log_{10}CFU/m^3$ (within SAB_{CC}). This could be due to the vacuum pump likely causing detachment of a small portion of spores initially attached to the inner walls of the boxes. Moreover, for SAB_{CC}, significant portions of outside air

likely entered through the completely covered but not sealed arm ports, increasing the aerosol count to 2 $Log_{10}CFU/m^3$.

Regardless of the sample collection method employed or of the experimental setup, most of the microbial aerosol was collected until the lowest applied sampling time (15 min for passive and five minutes for the active sample collection). Most probable reason is that, after initiating sample collection, the operator's hands were removed from the box resulting in air stilling due to the elimination of the air current source (hand movement) (Davies, 1973).

When comparing different spraying treatments, spraying with 70 % ethanol proved to be far superior, reducing microbial sedimentation counts within GB and SAB_{CC} to zero. This level of ethanol effectiveness was also observed when the GB and the SAB_{CC} were deliberately exposed to a high aerosol spore count of *A. niger*. Despite indications that ethanol is generally ineffective against spores (Thomas, 2004), ethanol wipes continue to be employed for disinfecting surfaces etc. (Thomas, 2012). Ethanol disinfection occurs mainly through protein coagulation (Marreco et al., 2004). Additionally, ethanol has been shown to inhibit spore germination by affecting the enzymes necessary for the spore germination process (Trujillo and Laible, 1970).
Spraying of GB and SABs with a household suspension of detergent proved to be even less effective than sterile distilled water treatments. We assume that the detergent in use was contaminated and its original packaging already harboured viable microorganisms. For instance, Chaturvedi and Kumar (2011) demonstrated that bacteria isolated from ponds contaminated with detergent can degrade surfactants such as sodium dodecyl sulphate.

To sum up, ethanol spraying within the GB or the SAB_{CC} created a reliable and safe sterile working environment free of microbial aerosol, with the SAB_{CC} having the added advantage of allowing greater freedom of arm movement. On the other hand, ethanol spraying within the SAB or the SAB_{PC} did not show satisfactory results, indicating that covering the arm ports to a certain degree is necessary to restrict the inflow of microbial aerosols.

Conclusions

In this study we defined the microbial aerosol reduction efficiency of the GB and of three SAB designs after a specific spraying pre-treatment with either 70 % (v/v) ethanol, diluted detergent or with sterile water. The following conclusions can be drawn:

1. *E* values of GB and SAB_{CC}, sprayed with ethanol, were 4.3 $Log_{10}CFU/m^2/h$ (100 % reduction) for passive sample collection and 4.8 (SAB_{CC}) - 5.2 $Log_{10}CFU/m^3$ (GB) for active sample collection. Even when GB and SAB_{CC} were deliberately exposed to a high aerosol spore count of *A. niger*, the *E* values after ethanol spraying and sampling were still high (zero $Log_{10}CFU/m^2/h$ for passive and 5.1 (GB) - 4.9 $Log_{10}CFU/m^3$ (SAB_{CC}) for active sample collection).
2. However, when SAB or SAB_{PC} were sprayed with ethanol, *E* values were only around one $Log_{10}CFU/m^2/h$ for passive sampling and around 0.5 $Log_{10}CFU/m^3$ for active sampling.
3. Spraying with diluted detergent and sterile distilled water yielded relatively low *E* values, even for GB (*E* value of two $Log_{10}CFU/m^2/h$ for passive and of 1.7 $Log_{10}CFU/m^3$ for active sample collection) and SAB_{CC} (*E* value of 1.5 $Log_{10}CFU/m^2/h$ for passive and from 0.3- 1.2 $Log_{10}CFU/m^3$ for active sample collection).

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