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Application of Biofluorescent Particle Counters for Real-Time Bioburden Control in Aseptic Cleanroom Manufacturing

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Abstract: This experimental study examines the use of a real time viable particle counter in pharmaceutical cleanroom operations under ISO class 8/Annex 1 Class C 'in operation' conditions. The used measuring system, a Biofluorescent Particle Counter (BFPC), continuously detected particles >1.0 µm as well as microbe carrying (viable) particles (MCPs). In addition, classic air samplers for the evaluation of Colony Forming Units (CFU) were installed to enable a comparison of counter-provided values with classic, agar-based methods of microbial air sampling required by regulatory guidelines. A test room comparable to typical cleanrooms in the pharmaceutical industry was operated under three different air change rates (ACR). At these ACRs, the operators wore three different garments. With test repetition, 18 experiments were conducted simulating different pharmaceutical process conditions. The results show correlations of measured particles to MCPs and MCPs to CFUs. Furthermore, albeit an industry-wide used and regulatory accepted minimum ARC value of 20 h^{-1}, the study results indicate that an ACR of 10 h^{-1} is capable of keeping cleanrooms within regulatory limits for particles and CFUs, and thus provides the opportunity to save energy for cleanroom operations. The implementation of real time viable particle counters can be used to achieve a quality advantage for ISO class 8 cleanrooms for a continuous, documented control of the cleanroom status, and thus enable a reduction of ACRs with the aim of energy saving. Further long-term studies should validate this.

Keywords: Biofluorescent Particle Counter (BFPC); real time viable particle counter; air change rate; ISO 8; Class C; energy saving; pharmaceutical production; cleanroom 'in operation'; annex 1; aseptic guide

Citation: Behrens, D.; Schaefer, J.; Keck, C.M.; Runkel, F.E. Application of Biofluorescent Particle Counters for Real-Time Bioburden Control in Aseptic Cleanroom Manufacturing. *Appl. Sci.* **2022**, *12*, 8108. https://doi.org/10.3390/app12168108

Academic Editor: Ki-Hyun Kim

Received: 24 June 2022
Accepted: 10 August 2022
Published: 12 August 2022

1. Introduction

Since 2009 continuous microbial monitoring systems for cleanrooms have been available [1,2]. Although these real-time viable particle counters, also described as Biofluorescent Particle Counters (BFPC), provide significant advantages in the detection of bioburden sources, their use for continuous control of microbe carrying particles (MCPs) in the pharmaceutical industry is low [3]. This has its cause in non-existing regulatory MCP limits for this type of measurement technology.

The control of the cleanroom status in aseptic pharmaceutical manufacturing is regulated by good manufacturing practice (GMP) guidelines [4,5]. Specific limit values are given for airborne particles as well as colony forming units (CFU) per cubic meter or cubic foot of air. For example, the limit value for CFUs in operational cleanrooms of ISO class 8 must not exceed 100 CFU/m^3 of air.

To supply clean conditioned air for cleanrooms in aseptic manufacturing by means of heating, ventilation and air conditioning (HVAC) systems, large amounts of energy are required [6–9]. As this not only generates costs, but also has a negative impact on the CO_2 -footprint of the company, manufacturers are in a dilemma: On one hand they need to supply the required amount of air into the production area with a certain safety buffer to

avoid deviations from cleanroom specifications, on the other hand they are faced with the need to save energy as much as possible. A reduction of the air change rate (ACR) would be a possible approach, as various studies have shown [7,10,11].

While particles of various sizes can be detected in real time with commonly used laser particle counters, the regulatory accepted detection of CFUs, using microbial air samplers (MAS) with agar plates or strips, requires subsequent incubation for several days before results are available. This time lag puts manufacturers at risk. If the results are unexpectedly high, the produced batches may be contaminated, and as the production is finished by this time there is no chance to avoid that. As a consequence, manufacturers are still reluctant to reduce air change rates [1], as they would have to rely on particle count only for cleanroom control until CFU results are available. This is despite a good correlation between airborne particles and microorganisms [12]. BFPCs could help to close that gap and offer continuous control of airborne microorganisms.

From the authors' experience, the largest areas in sterile pharmaceutical production facilities are the preparation and supporting areas for product manufacturing prior to sterile filtration and filling. These preparation areas require a Class C environment according to the EU GMP-guide Annex 1, and ISO 8 (Class 100,000) according to the FDA aseptic guide [4,5]. In the same guide, the FDA states: 'Air change rate is another important cleanroom design parameter. For Class 100,000 (ISO 8) supporting rooms, airflow sufficient to achieve at least 20 air changes per hour is typically acceptable' [5]. Though this is not a strict regulatory expectation, many pharmaceutical companies apply the value of 20 h^{-1} as the minimum required ACR for their aseptic preparation and supporting areas. Recent studies have shown that the regulatory 'in operation' limits for particles and CFUs can be achieved with a significantly lower ACR of 10 h^{-1} [10,13,14]. Other studies recommend demand controlled filtration (DCF) with ACRs of 4 h^{-1} during 'at rest' times of the cleanroom to save energy [15–17]. However, with a reduced ACR, the risk of an undetected exceedance of the bioburden increases. In order to control this risk, the continuous monitoring of not only particles but also the number of MCPs in the cleanroom air by use of real-time viable particle counters, could be highly beneficial. This would enable an immediate detection of an increased bioburden, and the ability to react accordingly. By this, it would enable ACR reduction and DCF without enhancing the risk of undetected exceedance of bioburden limits.

BFPC systems work using a laser-induced fluorescent principle [18]. Other than classic laser particle counters, which detect and classify particles by scattered light analysis, a BFPC also measures the wavelength of the scattered light. Substances found in all microorganisms (bacteria and fungi) are dipicolinic acid, nicotinamid adenine dinucteotide (NADH), and riboflavin [19–21]. They create a fluorescent signal that can be detected by the device. The different wavelengths distinguish viable particles from others. However, 'dead' microbes are detected, as they also contain these substances [22].

One drawback of the measuring principal is the potential for false detection. Some substances, which do not belong to the group of viable particles, still cause a fluorescent signal in the BFPC, and are thus detected as viables. One of these substances is 2-propanol, which is widely used in aseptic manufacturing for disinfection purposes. The effect of a false MCP detection by the use of 2-propanol has been observed in previous studies [19,23].

A direct comparison of BFPC-measured values with CFUs counted on agar plates or strips is hardly possible, as described by several scientists [19,24–26]. The reason for this is the dependency on possible growth on agar plates with CFU methods. However, not all microbes will grow on agar, and standard methods for incubation do not support the growth for all microbes. For this reason, the measured values of BFPC systems are typically much higher than the values measured by conventional microbial tests. This is, in fact a big advantage of the BFPC systems, as an immediate overall MCP result can be obtained and knowledge of the general bioburden status of a cleanroom can be provided. Though the specific species of microbes is not known at this point, this is still valuable not only during pharmaceutical operations, but also for an evaluation of the operational readiness

of a cleanroom in preparation for commissioning and qualification [25]. It would minimize the time required for finding bioburden leaks in the cleanroom installation [2,19,27]. A detriment is that industry-wide recommended action limits for MCPs measured by BFPC systems are not available yet [22].

Consequently, the derived research question for this experimental study is: can BFPC systems be used to enable minimized ACRs with the aim of saving energy without compromising on cleanroom air quality, and what can be an appropriate action limit for MCPs? This question led to a study design with three different ACRs combined with three different cleanroom conditions with regard to operator garments and behavior.

2. Materials and Methods

2.1. Study Setup and Test Design

Nine different test setups were chosen for a cleanroom 'in-operation' in which the type of garments and the number of air changes per hour were varied. The three different garments are shown in Figure 1.

I8G: ISO 8 garment: Single use Tyvek®-cleanroom-overall, overshoes, hairnet, face mask, gloves
I9G: ISO 9 garment: Single use fabric cleanroom-overall, overshoes, hairnet, face mask, gloves
NCG: Non-Cleanroom-Garment: Shorts, T-Shirt, face mask, and gloves

Figure 1. Different garments used during the experimental study.

All three garments were combined and tested with three different air change rates:

1. ACR 20 h^{-1}
2. ACR 15 h^{-1}
3. ACR 10 h^{-1}

Each combination was run twice. Thus, 3 garments × 3 ACRs × 2 runs/test = 18 runs were executed. The test sequence and setup are listed in Table 1.

Table 1. Test Sequence and Setup.

Test Sequence and Setup		
	Preparation Day	
	Cleaning & Disinfection	
Test No.	**ACR**	**Garment**
	1st Day	
Test 1	20 h^{-1}	I8G
Test 2	20 h^{-1}	I8G
Test 3	15 h^{-1}	I8G

Table 1. *Cont.*

Test Sequence and Setup		
Preparation Day		
Cleaning & Disinfection		
Test No.	**ACR**	**Garment**
Test 4	15 h^{-1}	I8G
Test 5	10 h^{-1}	I8G
Test 6	10 h^{-1}	I8G
Test 7	20 h^{-1}	I9G
2nd Day		
Test 8	20 h^{-1}	I9G
Test 9	15 h^{-1}	I9G
Test 10	15 h^{-1}	I9G
Test 11	10 h^{-1}	I9G
Test 12	10 h^{-1}	I9G
Test 13	20 h^{-1}	NCG
Test 14	20 h^{-1}	NCG
3rd Day		
Test 15	15 h^{-1}	NCG
Test 16	15 h^{-1}	NCG
Test 17	10 h^{-1}	NCG
Test 18	10 h^{-1}	NCG

For the cleanroom garments I8G and I9G a 30-min test design was executed with all ACRs. In this time frame, the activities for each run were carried out as shown in Table 2:

Table 2. Test Phases for I8G/I9G.

0–5 min	6–10 min	11–15 min	16–25 min	26–30 min
Start Phase	Particle Loading	Decay Phase	'In Operation' Phase	'Clean up' Phase
HVAC off No operators	HVAC off Two operators moving slowly	HVAC on Two operators moving slowly	HVAC on Two operators moving slowly	HVAC on No operators

With this test design a controlled build-up and decay of particles and MCPs was intended. In the start phase, the HVAC was off. The BFPC and Microbial Air Samplers (MAS) were started, and the operators left the room for 5 min. As no particles and MCPs could be emitted by personnel, it was expected that in this phase the BFPC count would be constant or would marginally decrease by particle sedimentation. After five minutes the operators came back into the room, and moved in a slow, cleanroom-adequate manner. Since the HVAC was still off, particles and MCPs released by the operators would increase the detection level of the measuring instruments (particle loading phase). After 10 min, the HVAC was switched on, and in a 5-min decay phase a reduction of particles and MCPs was expected to be seen at a different velocity, depending on the ACR. Between 16 min and 25 min test time the 'in operation' phase progressed. A steady state of particle and MCP-load was anticipated in a balance of operator emissions, and contamination continuously removed by the HVAC-system. The test ended with a 5 min 'clean up' phase in which no personnel were present. A decay of particles and viables was expected by HVAC operation. The term "clean-up' period' is defined in EU Annex 1 [4] as the time after completion of operations to allow the room to reach the 'at rest' status with lower regulatory limits for particles/m^3. Total experiment time for I8G and I9G was 30 min.

With NCG, a 5-min 'spiking phase' was added between 26 and 30 min in which the operators behaved with uncharacteristic movements for cleanroom personnel. An intentional high generation of particles and MCPs was achieved by fast moving and

waving by the operators. After this phase the personnel left the room and the 5-min 'clean up' phase concluded until the test ended after 35 min.

The tests were conducted on three subsequent days with one preparation day before.

2.2. Cleanroom and Equipment

For this experimental study, a training room at Testo Industrial Services in Kirchzarten, Germany, was used. The room is designed with a Class C/ISO 8 finish with pharma grade floors, walls, doors, and ceilings. A variable HVAC system with two H13 filters, mounted at the room inlet prior to swirl diffusers, allows ACRs from <10 to 70 h^{-1}. This enabled the test execution with the chosen ACRs. The test room was cleaned and disinfected prior to test execution. See Figure 2 for room layout details.

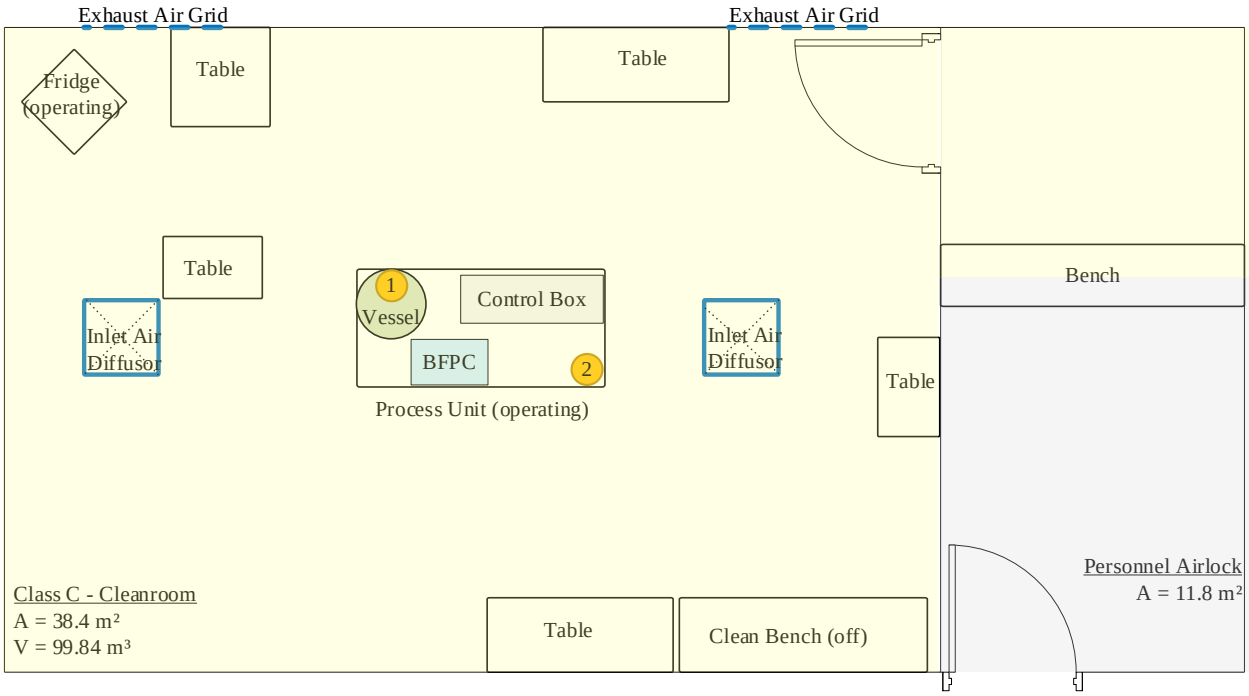

Figure 2. Room layout. For a comparison of the BioTrak® Biofluorescent Particle Counter (BFPC), (TSI Incorporated, Shoreview, MN, USA) with the classic Microbial Air Sampler (MAS), two different measuring systems were used: Merck Millipore RCS® (Merck Millipore, Darmstadt, Germany (1)) and ImpactAir® (Iso-Con Slit-to-Agar, Bridgend, UK (2)). All instruments were placed inside a process unit in the middle of the room.

The BioTrak® BFPC was set up with an airflow rate of 28.3 l/min and an aggregation period of 1 min. This measuring system combined two different detection units: One ISO-compliant particle counter and the MCP counting unit below. A schematic drawing of the system is given in [28]. The laser excites particles with a wavelength of 405 nm. To differentiate viable particles from other fluorescent substances the system takes three parameters for analysis: the normal scattered light at the same wavelength, a re-emission at a higher wavelength between 430–500 nm, and another between 500–650 nm. By this, the system can distinguish biological from non-biological particles and also viable, potentially bacterial, MCPs from other biologic particles, such as pollen [29].

The Merck Millipore RCS® High Flow Touch collects air with a flow rate of approx. 100 L/min, and uses centrifugal force for an impaction of MCPs on agar test-strips [30]. The high sampling volume is an advantage of this system. Depending on the used garment the test duration and, consequently, the sampling volume, the set-up was as follows:

I8G/I9G: 7 min/strip—total 683 L (four strips, 28 min + 2 min changeover time)

NCG: 7 min/strip—total 683 L (four strips, 28 min + 2 min changeover time) plus 5 min with last strip (488 L)

The ImpactAir® Iso-Con Slit-to-Agar MAS uses 90 mm tryptic soy agar (TSA) petri dishes. Inside the sampling device, the agar-filled plate slowly rotates below a very narrow inlet slit [31]. The air flow was setup with 15 L/min. The run time and the resulting sampling volume was 7 min/plate, equaling 105 L for I8G/I9G experiments, and 8 min/plate (120 L) for NCG.

For the MAS systems, Merck Millipore 90 mm/30 mL TSA plates and test strips for the RCS® air sampler were used [32,33]. The collected plates and strips were incubated at 32 °C. A daily CFU count was done, starting at day two and finishing after day four, as no further CFUs were observed.

In industrial processes, rooms of this size are typically run by two operators. Consequently, all experiments were performed by two persons to generate realistic cleanroom process operations. As three persons were available, one was exchanged after each test to allow breaks. Hand disinfection during the experiments was done with 2-propanol at the table in the back of the process unit.

A small process unit, consisting of a filled water tank, pump, connecting pipes, and instrumentation, was placed in the middle of the room to simulate a production process (Figure 3). The circulation pump was operated continuously during the three days of the test execution, which also created realistic conditions with regard to equipment heat generation and motor fan-induced air turbulence.

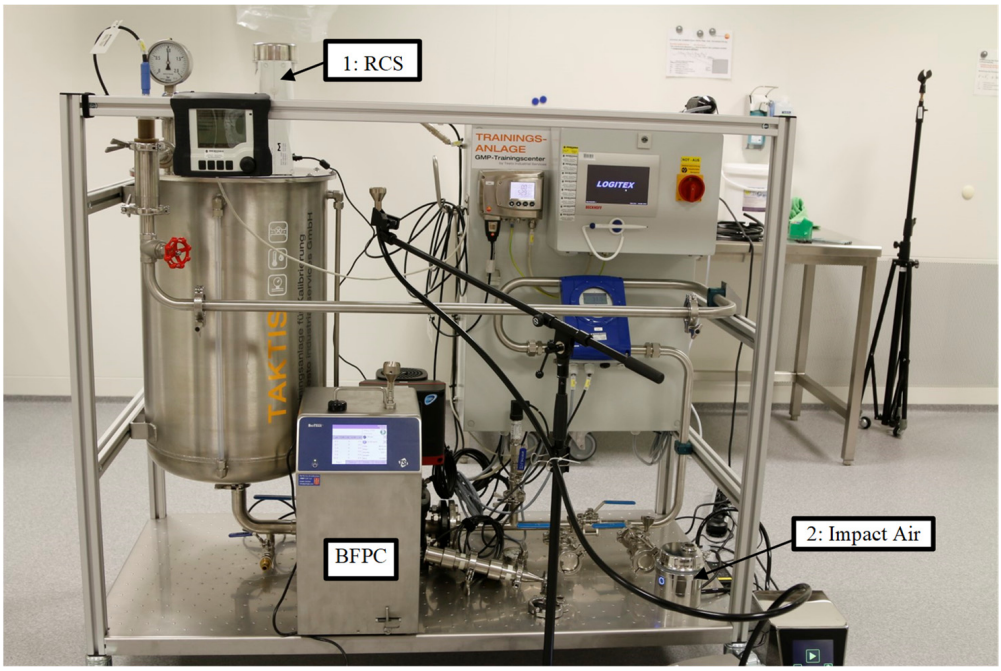

Figure 3. Process Equipment and Measuring Systems.

The air supply rate, to measure and control the anticipated ACR, was determined by a Testo 420 volume flow hood. All test equipment was calibrated before the start of the tests.

All tests were recorded by a full HD-Camera (Canon EOS 5D Mark IV, Tokyo, Japan) for later analysis of the counter results in relation to the operator's behavior.

Descriptive statistics were determined with Microsoft Excel. Comparison of the means was done with JASP software (v.0.14.1), Amsterdam, The Netherlands. Normal distribution and variance homogeneity were determined with Shapiro-Wilk and Levene's tests, respectively. The non-parametric data sets were subjected to a Kruskal–Wallis analysis of variance with subsequent Dunn' post hoc tests [34]. Statistical relationships between the different parameters were investigated by a determination of the respective correlation

coefficients (Spearman's rank correlation coefficient ρ for non-parametric data). Probability values (*p*-values) < 0.05 were considered as statistically significant.

3. Results

The results of this experimental study are shown in the diagrams 4–6, which delineate viables/m^3 on the left *y*-axis, and particles >1.0 µm/m^3 on the right *y*-axis. As MCPs, due to the natural size of microbes, cannot be smaller than 1.0 µm, only particles larger than that are shown in the diagrams to enable a comparison between non-viable and viable particles. The mean values of both test runs were calculated and used for the result analysis. Please see Supplementary Material for details of differences between the first and the second test run.

3.1. Test Phases

In Figure 4 the results for viables/m^3 and particles >1.0 µm/m^3 during the test phases at ACR 20 are illustrated. The expected effects are noticeable: At the beginning of the start phase the operators switched on the measuring systems and left the room. In this timeframe with no personnel present, the values for viables and particles decreased by sedimentation as no airflow turbulence could keep them airborne. With the operator's entry at the end of the start phase, the contamination started to increase. As soon as the HVAC was switched on at beginning of the decay phase after 10 min, viables and particles were effectively removed. In the 'in operation' phase between 16 and 25 min, a balance between the operator-generated and the HVAC-removed contaminants was reached. For garment I8G (Figure 4a), a further reduction of viables and particles became visible only after the staff left the room. Furthermore, a good correlation between BFPC-measured viables and particles was obvious (Spearmans ρ: 0.929; $p < 0.001$).

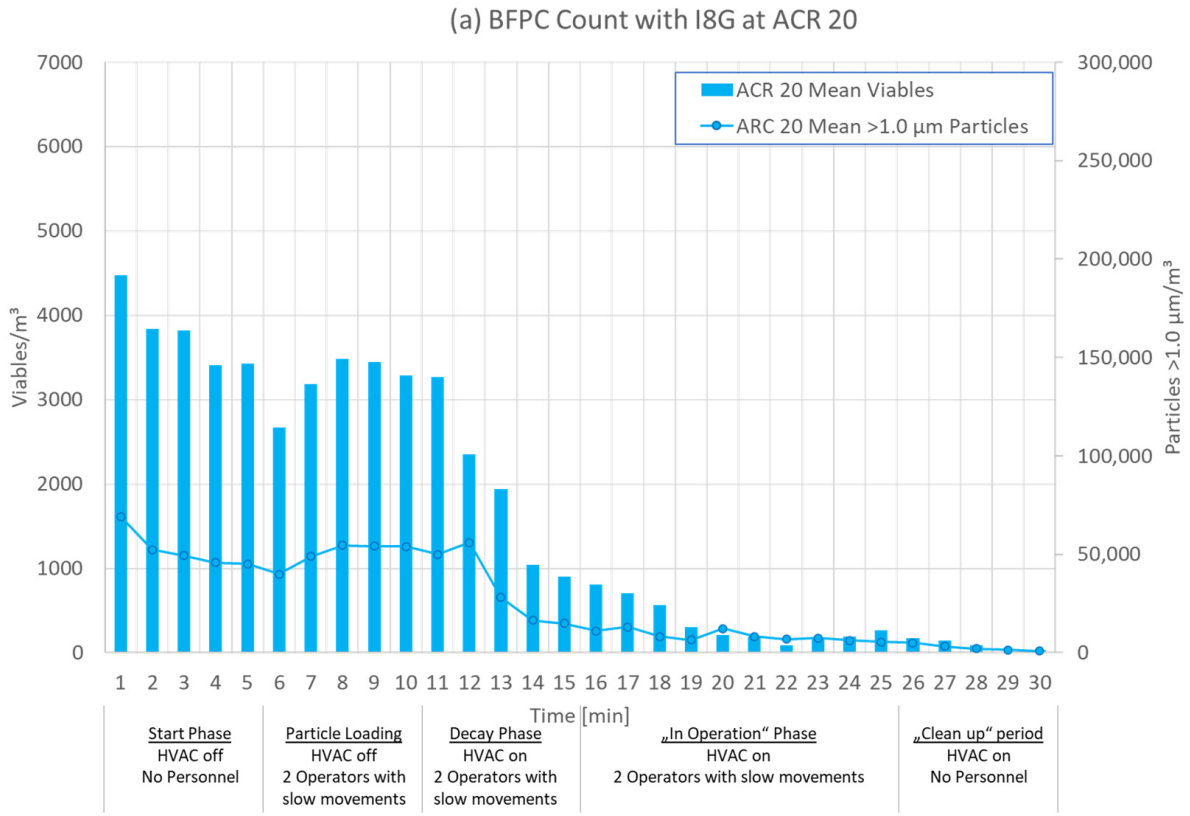

Figure 4. *Cont.*

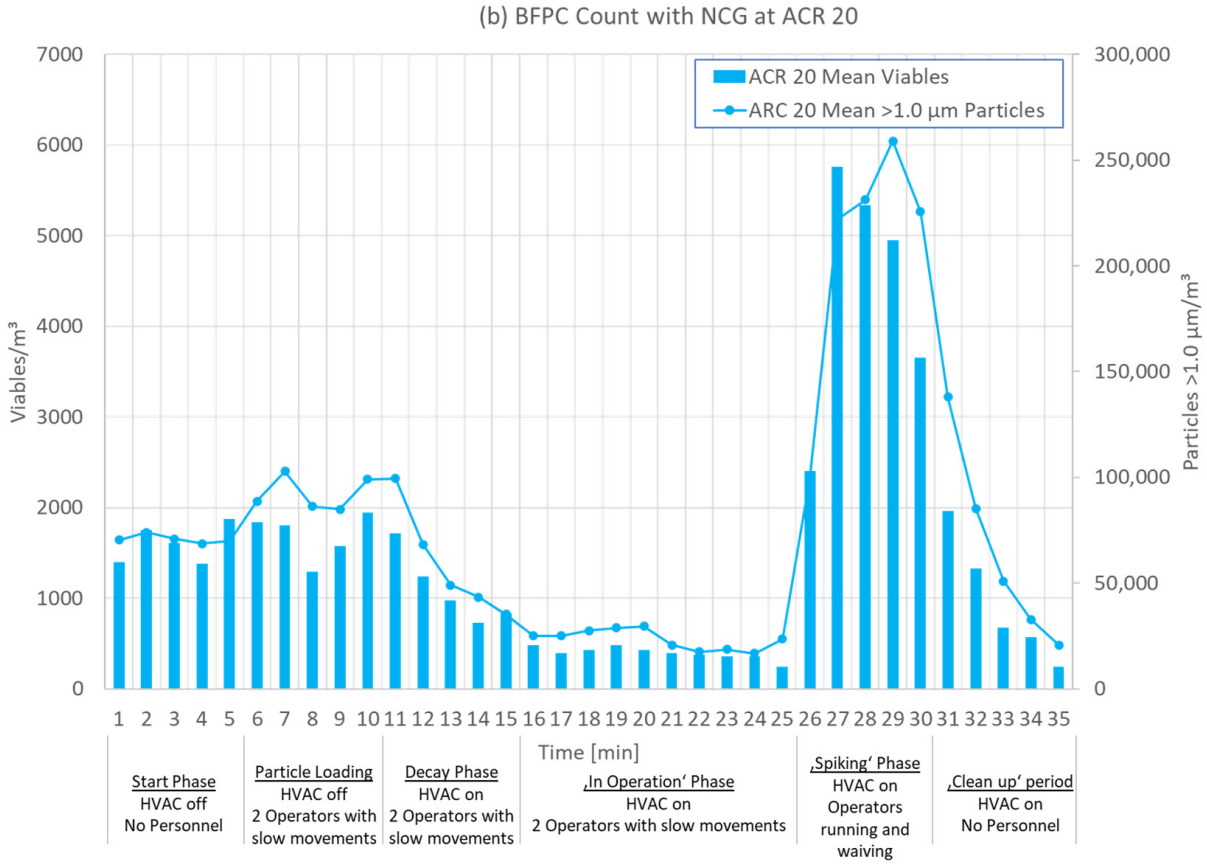

Figure 4. Particles and Viables/m^3 in the different test phases at ACR 20: (**a**) with I8G/(**b**) with NCG (Note the different scale on x-axes in (**b**) to reflect the spiking phase).

For the tests with NCG, the graph looked similar up to 25 min (Figure 4b). Then, with the beginning of the running and waving of the operators, the expected boost in particle and MCP-generation occurred. Again, a correlation between viables and particles was apparent (Spearman's ρ: 0.955; $p < 0.001$). Cf. Supplementary Material for more detailed diagrams.

3.2. Results for Different ACRs

It is not surprising that a higher ACR leads to a lower contamination for a cleanroom 'in operation'. This is represented in Figure 5a. The overall level of viables and particles is higher with ACR 10 than with ACR 15 and 20, wearing I8G. The graph also shows that peaks, which occurred during test execution, are higher for ACR 10 than for ACR 15.

The results for I9G (Figure 5b) are surprising due to high peak values for the supposed better ACR 20 compared to ACR 15 and ACR 10 results. Cf. Section 3.4 for this observation.

In the NCG-graph (Figure 5c), the expectable higher load of particles and viables as a result of the unsuitable cleanroom garment can be observed. Furthermore, the better HVAC-performance at higher ACRs is noticeable. During the 'in operation' phase, the number of viables is in a steady state with ACR 15 and 10 in the same range, while ACR 20 is noticeably lower. In the following spiking phase, the peak number of particles only reaches approx. 50% of the ACR 10 and ACR 15 values.

3.3. Viables Versus CFUs

As expected, the numbers of MCPs detected by the BFPC were much higher than the culturable microbes counted on the agar plates and strips. Figure 6 compares both values for I8G with ACR 20 and ACR 10 (Cf. Supplementary Material for more detailed diagrams). To illustrate this comparison a factor of 80 had to be chosen between the CFU/m^3 and the viable/m^3 axis. Still, it was noticeable that all values decreased during the test runs, and

that the clean-up capability of ACR 20 was higher than that of ACR 10, as the measured values in the second half of the experiments were lower.

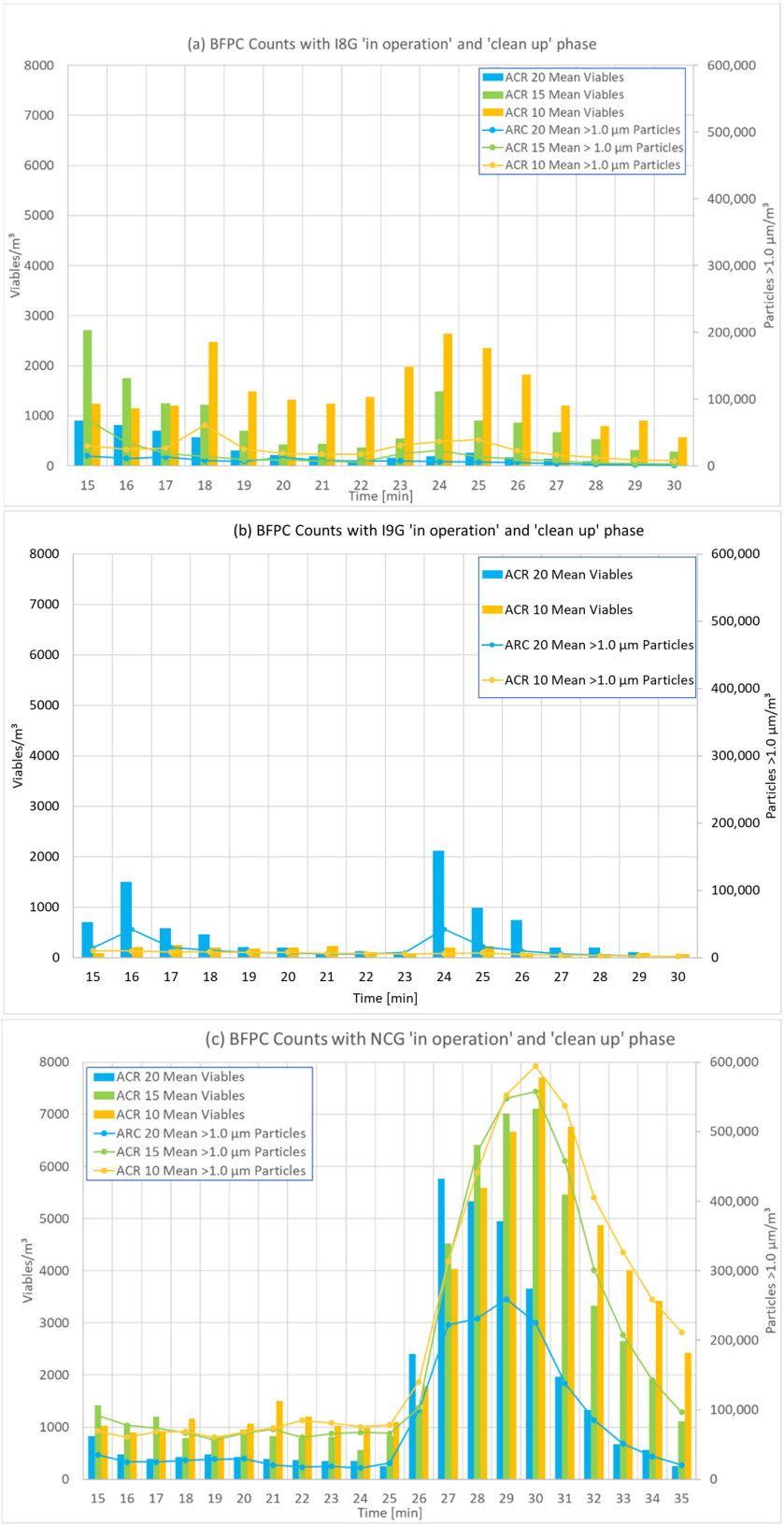

Figure 5. Particle and Viable/m^3 results at different ACRs: (**a**) with I8G/(**b**) with I9G/5c with NCG (Please note the different scale on *x*-axes in Figure (**c**) to reflect the 'spiking phase').

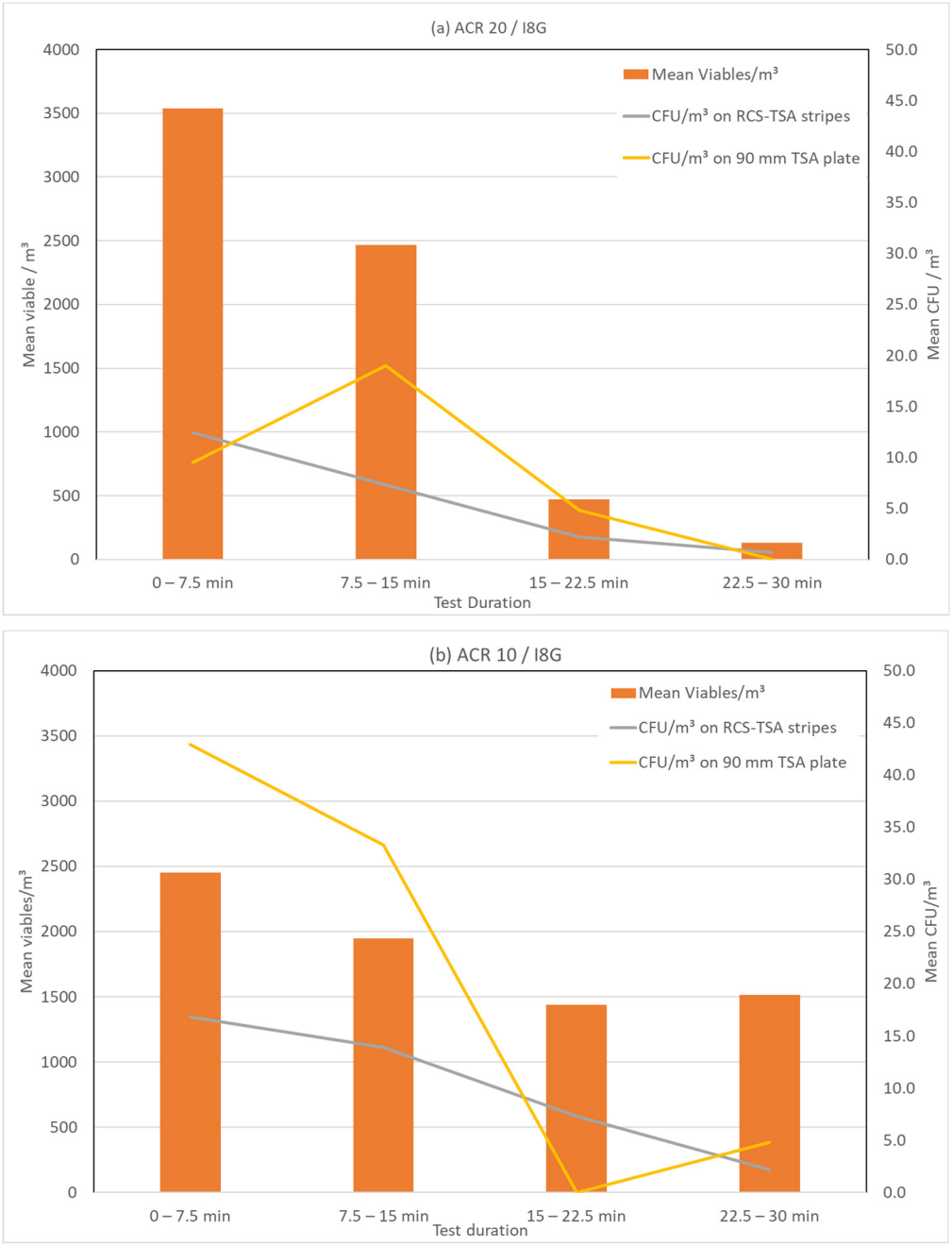

Figure 6. BFPC measured viables vs. CFUs with I8G.

3.4. Influence of 2-Propanol

For hand disinfection, two different bottles with 2-propanol were available on a table behind the process unit: normal dispensing bottles and spray bottles. The operators chose any of these until the test No. 7 (ACR 20/I9G—first run). During this test, it was recognized that the use of the spray bottle, even though applied with a distance to the BFPC, and close to the exhaust air grid, influenced the particle count. Consequently, the use of spray bottles was stopped from test No. 8 onwards.

The effect of induced peaks by use of a 2-propanol spray bottle for hand disinfection is visible in Figure 7. The peaks occurred only in the first of the two test runs with I9G at ACR 20. The comparison of particles and viables at these peak times, at first sight led to the assumption of operator activity, which released a high load of MCPs, and which was subsequently detected by the particle counter as well as by the BFPC. However, this would

also have become visible as increased CFUs, although a related increase in CFUs could not be found either on RCS-strips or on agar-plates. By reviewing the recorded videos, the cause of the peaks could be traced back to the use of the 2-propanol spray bottles, which was also observed in other studies [19,23,24]. Once observed, the use of disinfection spray was stopped after test 7.

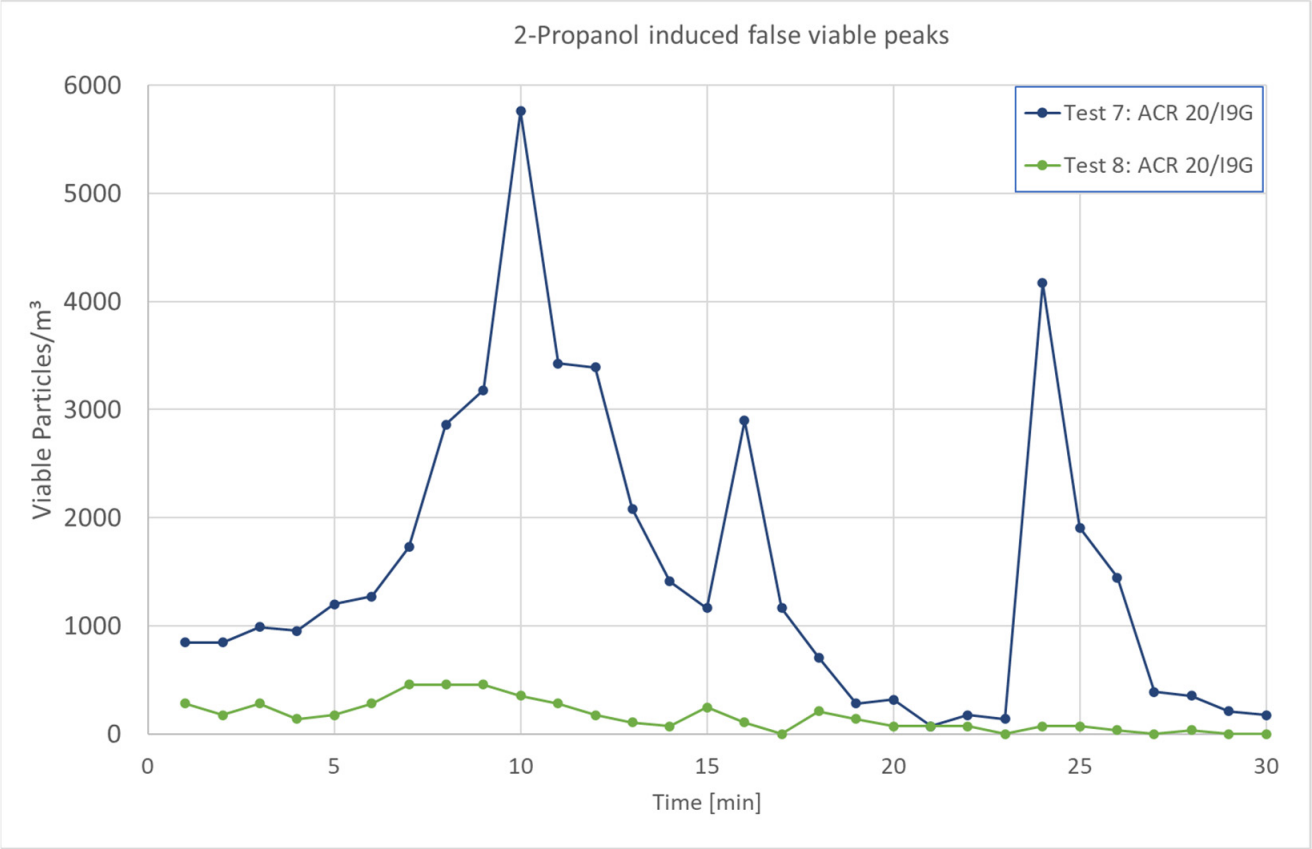

Figure 7. Effect of spray bottle use for hand disinfection.

The results of all particle, MCP and CFU counts 'in operation' between 16 and 25 min are presented in Table 3. The difference between cleanroom garments in contrast to NCG is clearly visible. However, the regulatory limit of 100 CFU/m^3 [4,5] was only exceeded twice at the Impact Air-MAS with 90 mm plates at ACR 15 (Test No. 15) and ACR 10 (Test No. 18). For the cleanroom garments I8G and I9G, the highest CFU values 'in operation' reached only 10% of the allowed limit. The missing IA values at ACR 15/I8G (Tests 3 + 4) were caused by sampling failures.

In Figure 8, the ratio of CFUs/m3 to viables/m3 wearing pharmaceutical garments at the highest (ACR 20) and lowest (ACR 10) airflow are shown. The values of both MAS systems correlate (Spearman's ρ: 0.55/p = 0.027). For CFUs vs. viables the median value was at the same level for RCS and IA (0.5%). The distribution for IA with 90 mm agar plates was higher due to a leveraging effect caused by the low sampling volume and short runtime per plate (cf. Section 4 for discussion of this effect).

The distribution of particles/viables 'in operation' values for the three different garments is presented in Figure 9. It is clearly detectable that the mean and median values differed between the type of garments. A correlation between the different garments is not given. Furthermore, the distribution of values was higher with I9G and NCG.

Table 3. 'In Operation' results.

No.	ACR	Garment	Particles > 1.0 µm/m³	MCPs/m³	Mean MCPs/m³	Ratio	IA CFU/m³	Mean IA CFU/m³	RCS CFU/m³	Mean RCS CFU/m³	Spray Bottles Used
Test 1	20	I8G	5819	224	185.5	26.0	9.5	5	4.4	2	No
Test 2	20	I8G	9817	147		66.8	0		0		No
Test 3	15	I8G	11596	518	518	22.4	-	-	0	2	No
Test 4	15	I8G	13592	not valid due to disinfection spray bottle use		15.6	-		4.4		Yes
Test 5	10	I8G	23969			13.7	0	0	5.9	3	Yes
Test 6	10	I8G	29240			15.6	0		0		Yes
Test 7	20	I9G	22750			20.1	0	5	1.5	1	Yes
Test 8	20	I9G	5218	59	59	88.4	9.5		0		No
Test 9	15	I9G	5277	100	82.5	52.8	0	0	1.5	2	No
Test 10	15	I9G	3345	65		51.5	0		1.5		No
Test 11	10	I9G	8846	183	174	48.3	9.5	10	8.8	6	No
Test 12	10	I9G	4164	165		25.2	9.5		2.9		No
Test 13	20	NCG	5430	159	356.5	34.2	33.3	58	48.3	57	No
Test 14	20	NCG	37767	554		68.2	83.3		65.9		No
Test 15	15	NCG	81331	931	798	87.4	**158.3**	113	82	75	No
Test 16	15	NCG	51449	665		77.4	66.7		67.3		No
Test 17	10	NCG	68993	895	1145.5	77.1	83.3	129	48.3	64	No
Test 18	10	NCG	84717	1396		60.7	**175**		80.5		No

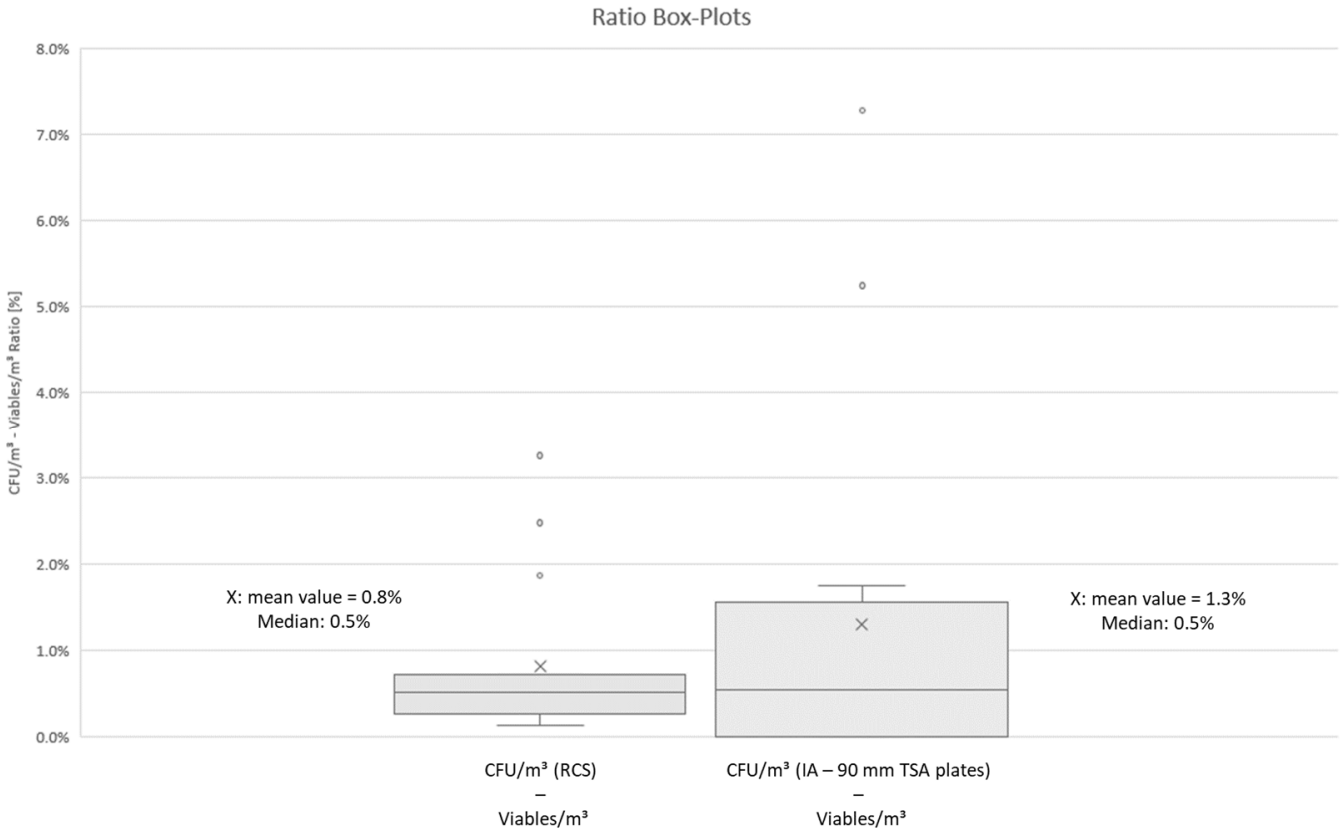

Figure 8. I8G + I9G/ACR 20 + ACR 10 Ratios of CFUs vs. viables.

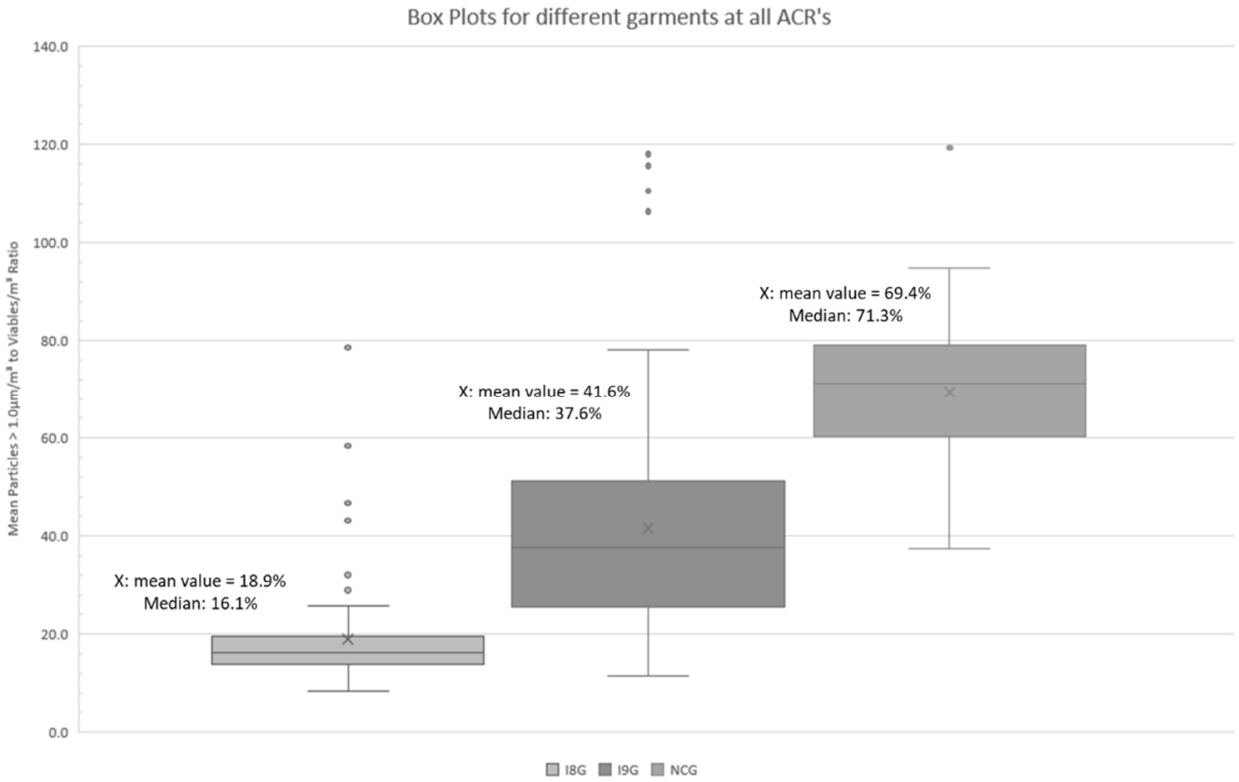

Figure 9. Particle/Viable ratio for different garments at all ACRs.

4. Discussion

Regulatory guidelines for aseptic pharmaceutical production [4,5] do not provide limits for particles >1.0 µm. The ISO 14644-1 [35] gives a limit at 832,000 particles/m^3 for this particle size in ISO class 8. This value was not reached in any of the conducted experiments, not even in spiking phases with NCG (highest value: 594,523 particles >1.0 µm/m^3 equivalent to 71% of the limit value).

In Table 4, the statistical evaluation of all data regarding BFPC-counted particles and viables vs. CFU/m^3 are presented. While a weak correlation is shown for viables vs. CFUs on TSA plates, all other comparisons correlate well. Similar correlations of particles to CFUs have also been described by Parat et al. [12] and Sandle et al. for particles to viables [20].

Table 4. Correlation of particles/viables vs. CFUs.

Overall Correlation in Experimental Study			Spearman's ρ	*p*
Particles > 1.0 µm/m^3	-	Viables/m^3	0.864	<0.001
Particles > 1.0 µm/m^3	-	CFU/m^3 on RCS-TSA strips	0.778	<0.001
Particles > 1.0 µm/m^3	-	CFU/m^3 on 90 mm TSA plate	0.705	<0.001
Viables/m^3	-	CFU/m^3 on RCS-TSA strips	0.623	<0.001
Viables/m^3	-	CFU/m^3 on 90 mm TSA plate	0.472	<0.001
CFU/m^3 on RCS-TSA strips	-	CFU/m^3 on 90 mm TSA plate	0.758	<0.001

A good correlation between the two different MAS systems was expected, as both are based on microbial growth on agar. The different correlations between BFPC-measured viables vs. CFUs on RCS® strips or 90 mm plates are interesting. It is most likely that this deviation has its cause in the highly different sampling volumes per minute of the used devices. The absolute CFU counts on plates and strips must be converted to CFU/m^3 by division through the sampled air volume in m^3. For the 90 mm TSA-plates, a total volume of 0.105 m^3 was sampled per plate, which transforms each single colony on the plate into 9.5 CFU/m^3. This is a big lever with respect to the generally low numbers of CFUs found

on the plates, especially during the 'in operation' phase with a maximum of one CFU found on individual plates. Generally, the collected data from 18 test runs gives a good indication of airborne CFUs in an operational cleanroom at different ACRs. Still, single test run results and single plate counts for a test phase underlie a random factor whether a microbe finds its way into the probe head or not. With the RCS® system, the sampling volume was significantly higher. For one TSA strip, 0.683 m^3 of air was sampled. Hence, one CFU on the strip means 1.5 CFU/m^3. With respect to the generally low numbers of CFUs found on the agar plates or strips, this leads to steadier sampling with a lower chance of random effects, as also visible in Figure 6 for RCS® vs. IA CFU results in which the RCS® achieves steadier results.

For the relevant I8G garment in a pharmaceutical cleanroom environment, the median value for the particle/viable ratio was 16.1. (Figure 9). Applying this value to the ISO 8 particle limit of 832,000/m^3, the corresponding limit value for BFPC measured viables would be 832,000/m^3/16.1 = 51,700/m^3. This value was not reached in any of the 18 experiments. The highest measured value was reached with street clothes in test No. 17 (ACR 10/NCG) with 7880 viable particles/m^3. An exceedance of regulatory limits for CFUs was observed at lower MCP counts. Hence, a derivation based on the particle/viable ratio is obviously not a feasible approach to find a BFPC limit value for cleanrooms.

The maximum number of CFUs found during 'in operation' phases throughout all tests with pharmaceutical garments I8G + I9G was 10 CFU/m^3, being ten times lower than the regulatory limit of 100 CFU/m^3. An exceedance of regulatory CFU/m^3 limits was observed during the tests with NCG. The corresponding MCP/m^3 values measured by the BFPC in these test phases were around 1000/m^3. For that reason, an MCP limit for the BFPC, used in the given cleanroom in this experimental study, can be set at 1000 MCP/m^3. BFPC values measured below this number are not likely to lead to an exceedance of the regulatory limit value of 100 CFU/m^3.

The good responsiveness of the BFPC on peak generation of MCPs as triggered in the test runs with NCG also shows the applicability of the system to detect bioburden leaks during ongoing production or for cleanroom commissioning and qualification. This possible application has been proposed by Bhupathiraju et al. [36] and Weber et al. [19].

Continuous viable particle control may also be used to reduce the number and frequency of microbial air sampling. Scott et al. summarized the results of a meeting with the FDA to get authority feedback on regulatory acceptance of BFPCs for cleanroom control in which the FDA expressed a positive position [3]. However, these systems are regarded as an additional tool for quality assurance and are (today) not seen as a full alternative to classic CFU analysis. This study provides data which support the aim of air sampling reduction.

As reported by other authors [19,23], the effect of a false viable detection by fluorescent substances such as 2-propanol also became visible during these experiments. Though spray bottles were used in the back of the detection systems and close to the exhaust air grid, the generated fluid particles had a clear effect on particle detection as well as MCP detection by the BFPC. Therefore, manufacturers should avoid the use of disinfection spray bottles in rooms where such detection systems are used.

Energy savings by means of reduced ACRs has been described by various authors [6,11,37]. In a previous publication by Behrens et al. [10], which focused on a regulatory limit values for particles >0.5 µm and >5 µm, it was stated that ISO 8 cleanroom operations can be performed with lower ACRs than 20 h^{-1}. The results of this study support that outcome, as the BFPC results correlate well with particles and may also be used to continuously control the bioburden status of a cleanroom with early warning possibilities in case of high microbial values. This is in line with the study results of Mičko et al. [11], who found that 'lower flow rates reduce unwanted turbulence and the magnitude of velocity fluctuations, making the ventilation system more efficient at removing contaminants . . . '. If enhanced particle or heat generation within a cleanroom can be excluded as a reason for higher ACRs, manufacturers should test and qualify their individual cleanrooms with an ACR of 10 h^{-1} BFPC systems cannot, today, replace regulatory required CFU recording

by classic air sampling but can support manufacturers in lowering ACRs in cleanrooms without compromising the control of air quality. This would reduce the energy demand for cleanroom operations.

Study Limitations

The test room was a training room, which was not operated and cleaned as regularly as typical process rooms in industry. Thus, the cleanroom status and its individual microbiome could still have been different, though cleaning and disinfection efforts were made prior to test start.

This study provides results from one series of different test setups in one room only. Thus, the test results cannot be universally transferred to other, real-world manufacturing sites. However, they give a strong motivation to conduct further, long-term experimental studies with direct comparison of MCPs, CFUs and particles in a real operational cleanroom within the pharmaceutical industry. This should provide sufficient data to provide clear recommendations for reducing ACRs and MCP alert limits.

This study focused only on numbers of MCPs detected by the BFPC, and CFUs found on agar samples, without identification of collected microbes (bacteria and fungi). As the regulatory limits refer to absolute numbers of any CFU the specific type of bacteria or fungi is not relevant. However, in real pharmaceutical operations an identification of the contaminating species should be performed in any case of deviation.

5. Conclusions

The results of this study show good applicability of BPFC systems in pharmaceutical cleanroom operations regulated by the FDA aseptic guide and the European Annex 1 [4,5]. This is especially visible in the fast detection of an intentionally created MCP generation during the test runs with NCG. The immediate detection of microbial contamination can be of big advantage in identifying limit exceedance during ongoing operations. It would enable the pharmaceutical industry to run cleanrooms with a reduced ACR of 10 h^{-1} to save energy without compromising the quality requirements for ISO 8/Class C cleanrooms. A second advantage is the accelerated commissioning and qualification of cleanrooms in renovation or new building projects.

As a result of this study, an MCP value of max. 1000 /m^3 was evaluated as a probable limit for MCP/m^3 in ISO Class 8 cleanrooms. Further (long-term) studies are required in real pharmaceutical production rooms to provide more data from other cleanroom setups. This should enable the verification or adjustment of the value of max. 1000 MCP/m^3 found in this study. Once a generally applicable limit has been found, this will likely enhance the use and acceptance of BFPC systems in the pharmaceutical industry.

The study results indicate that cleanrooms can be operated with ACR 10 to save energy. A further long term-study is recommended to validate this outcome. The continuous control of particles and viables using BFPC systems could provide the necessary reliability that the cleanrooms run within limits. Significant cost savings for reduced energy consumption, as well as an improved CO_2-footprint, would be of significant benefit.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app12168108/s1. Figure S1: Viable particle counts in test runs 1–6. Figure S2: Viable particle counts in test runs 9–12. Figure S3: Differences between different garments with ACR 20. Figure S4: Differences between different garments with ACR 10. Figure S5: Particles at ACR 20 with I8G vs. I9G. Figure S6: MCP vs. CFU with ACR 20 and garment I9G. Figure S7: MCP vs. CFU with ACR 10 and garment I9G. Table S1: Statistical data for particles and different garments. Table S2: Statistical data for particles vs. MCPs with I8G.

Author Contributions: Conceptualization, methodology, test execution, data analysis, writing and visualization: D.B.; Data statistics: C.M.K. Review and editing: D.B., J.S., C.M.K., F.E.R.; Supervision: C.M.K., F.E.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Please review to Supplementary Material for further information. Raw data can be obtained from the corresponding author.

Acknowledgments: The authors express special thanks to Testo Industrial Services, Kirchzarten, Germany and PMT, Heimsheim, Germany for the provision of rooms, equipment, and personnel. Open Access funding provided by the Open Access Publication Fund of Philipps-University Marburg with support of the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation).

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ACR	Air Change Rate
BFPC	Biofluorescent Particle Counter
CFU	Colony Forming Units
FDA	Food and Drug Administration
GMP	Good Manufacturing Practice
HVAC	Heating, Ventilation and Air Conditioning
IA	Impact Air MAS, low inside process unit
ISO	International Organization for Standardization
MAS	Microbial Air Sampler
MCP	Microbe-Carrying Particle
RCS	RCS® High Flow Touch MAS, high inside process unit
TSA	Tryptic Soy Agar

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