INTERNATIONAL BioPharm
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Evaluation of an Instantaneous Microbial Detection System in Controlled and Cleanroom Environments

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ABSTRACT

The ability of an instantaneous microbial detection system (IMD-A) to monitor microbial populations in environmental air was evaluated. The IMD-A results were compared with results from conventional environmental air monitoring methods. The comparisons were carried out in controlled microbial barrier test chambers and in cleanroom environments. Additionally, microbial populations in environmental air in an unclassified environment were evaluated using the IMD-A and the all-gas impingement (AGI) method coupled with ScanRDI. In 1-m^3 and 150-m^3 controlled-barrier test chamber studies the mean recoveries with the IMD-A were equal to or greater than the mean recoveries obtained with the Anderson air sampler at various concentrations. The mean microbial recoveries obtained using the AGI were higher, but in the same order of magnitude, as those recovered by IMD-A. In classified environments, microbial recoveries from the SAS air sampler were substantially lower than microbial counts detected by the IMD-A. There were reasonable correlations of microbial recoveries between the IMD-A and the SAS air sampler results in cleanroom environments. Mean microbial recoveries from environmental air in an unclassified environment were similar in the IMD-A and AGI methods coupled with ScanRDI analysis. These results suggest that the IMD-A has the potential to reliably and instantaneously evaluate microbial populations in environmental air to provide a valuable technique for biopharmaceutical manufacturing.

ENVIRONMENTAL MONITORING METHODS

Examining the microbial content of air is a key component of environmental monitoring in pharmaceutical cleanroom environments. Overall environmental air monitoring also includes evaluating the particulate content of the air. Typically, particulate content at 5.0 and 0.5 µm levels is measured using total particulate monitoring systems such as the Climet, PMS, Royco, Lighthouse, APC units, or similar systems.

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Table 1. Statistical evaluation of 1-m^3 microbial barrier test chamber data from the IMD-A and an Anderson air sampler—*Bacillus atropheus* (spores)

Concentration	Instrument	Mean ± SD
1	IMD-A	5 ± 2 (n = 3)
	Anderson air sampler	3 ± 1 (n = 3)
2	IMD-A	5 ± 2 (n = 3)
	Anderson air sampler	6 ± 5 (n = 3)
3	IMD-A	28 ± 8 (n = 11)
	Anderson air sampler	11 ± 3 (n = 11)
4	IMD-A	33 ± 9 (n = 3)
	anderson air sampler	228 ± 39 (n = 3)
5	IMD-A	900 ± 94 (n = 8)
	Anderson air sampler	257 ± 127 (n = 8)

Evaluating microbial content in environmental air involves both active and passive air monitoring. Active microbial content in environmental air is typically evaluated using SAS, MAS, RCS, Mattson Garvin, Anderson air, liquid impinger, or SMA air sampling systems. Active air monitoring often involves the use of a device in which microorganisms from a known volume of air are captured on media plates, or alternatively, air is aspirated into a liquid and the microorganisms in the liquid are captured on a membrane filter and transferred to media plates to evaluate growth. Viable passive air is evaluated by the plate-count method using settling plates. Results from the microbial monitoring of the environmental air are typically not obtained until 3–5 days after sampling.

That need to wait for several days to accommodate microbial growth before acquiring monitoring data has been a major limitation of conventional environmental monitoring methods. Over the past decade, several rapid culture- and nonculture-based methods have been developed to provide much faster turnaround for microbial data.[1]

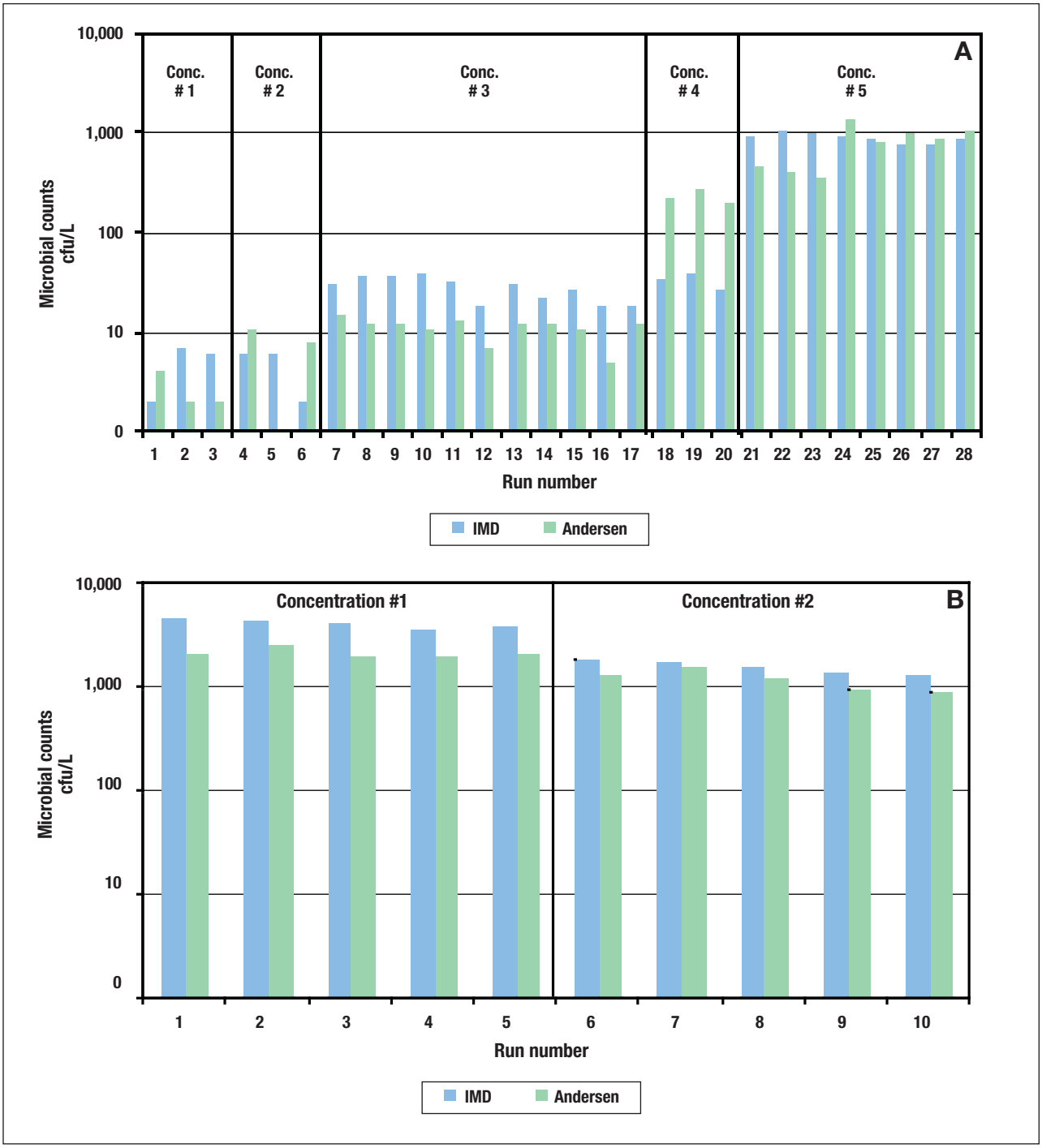
One promising nonculture-based rapid method receiving increased attention in recent years involves the ScanRDI system (Chemunex, France).[2] In this semiautomated system, the total number of viable organisms is determined by filtering samples through a membrane and labeling cells using a nonfluorescent substrate that diffuses across the cell membrane. This labeling differentiates between viable and dead cells based on the presence or absence of esterase activity and intact cell membranes. Only viable cells with membrane activity are able to cleave the dye and retain the fluorescent label. These viable microbial cells are quantified by scanning and counting using laser cytometry. Although the ScanRDI system offers the advantage of rapid evaluation of microbial populations, it is fairly specialized and does not allow for real-time detection of microbial populations. An ideal system for microbial air monitoring in pharmaceutical cleanroom environments would require little or no sample preparation or manipulation and would provide environmental microbial air monitoring data in real time.

Table 2. Statistical evaluation of 1-m^3 microbial barrier test chamber data from the IMD-A and an Anderson air sampler—*Staphylococcus aureus* (vegetative cells)

Concentration	Instrument	Mean ± SD (n = 5)
1	IMD-A	4276 ± 451
	Anderson air sampler	2196 ± 192
2	IMD-A	1586 ± 240
	anderson air sampler	1206 ± 276

BioVigilant has developed a nonculture-based instantaneous microbial detection (IMD-A) system based on optical fluorescence sensor technology. The IMD-A system analyzes the quantity and size of biological particles in environmental air and simultaneously determines whether each particle is inert or biologic, all in real time.[3] The IMD-A consists of three components: 1) an optical assembly to measure individual particle size, 2) a concurrent optical detector to detect a UV-laser-induced fluorescence signal from metabolites in microbial cells and spores, and 3) an algorithm for differentiating airborne microbes from inert dust particles. The optical assembly uses the Mie scattering detection scheme to accurately measure airborne particles with sizes ranging from 0.5–20 µm. Exploiting the Mie scattering detection scheme enables the use of ultraviolet (UV) light illumination to concurrently examine each particle for the presence of nicotinamide adenine dinucleotide (NADH) and riboflavin, which are necessary metabolic intermediates of living organisms, including bacteria and fungi. These metabolites are excited by the UV photon energy and subsequently by autofluoresce, which is detected by the sensor. Although the IMD-A cannot identify the genus or species of

Figure 1. Evaluation of microbial counts in environmental air in a 1-m^3 microbial barrier test chamber using the IMD-A and an Anderson air sampler: A) *Bacillus atropheus* (spores), B) *Staphylococcus aureus* (vegetative cells)



microbes, it can instantaneously determine the size of each airborne particle and whether it is biologic or inert.

The overall objective of this study was to evaluate the IMD-A system in controlled and pharmaceutical cleanroom environments by comparing it with other microbial air monitoring methods. Specifically, we set out to do the following:

1. Evaluate BioVigilant's instantaneous

Table 3. Statistical evaluation of 150-m^3 microbial barrier test chamber data from the IMD-A, an Anderson air sampler, and AGI—*Bacillus atropheus* (spores)

Concentration	Aggregate size (µm)	Instrument	Mean ± SD
30 cfu/L	1.0	IMD-A	7 ± 4 (n = 5)
		Anderson air sampler	23 ± 28 (n = 5)
		AGI	23 ± 11 (n = 5)
30 cfu/L	2.5	IMD-A	32 ± 7 (n = 3)
		Anderson air sampler	19 ± 16 (n = 3)
		AGI	140 ± 33 (n = 3)
300 cfu/L	1.0	IMD-A	58 ± 16 (n = 3)
		Anderson air sampler	110 ± 15 (n = 3)
		AGI	119 ± 27 (n = 3)
300 cfu/L	2.5	IMD-A	130 ± 9 (n = 3)
		Anderson air sampler	71 ± 55 (n = 3)
		AGI	237 ± 12 (n = 3)

microbial detection (IMD-A) system in a microbial barrier test chamber (1 m^3 and 150 m^3) in a microbial challenge side-by-side with conventional (Anderson air and air impingement) air-sampling methods

2. Evaluate the IMD-A for use in cleanroom Class E (ISO 9), Class D (ISO 8, Class 100,000 at operational and static), Class C (ISO 7/8, Class 10,000 at static and 100,000 at operational), and A (Class 100, ISO 5) environments at the Bayer Berkeley site by running the IMD-A side-by-side with conventional (SAS) air sampling methods
3. Compare the microbial recoveries in environmental air using the IMD-A with those obtained using ScanRDI.

Note that classified areas mentioned above were monitored using areas that were in control, but not in use for manufacturing.

COMPARISON TESTING OF THE IMD-A

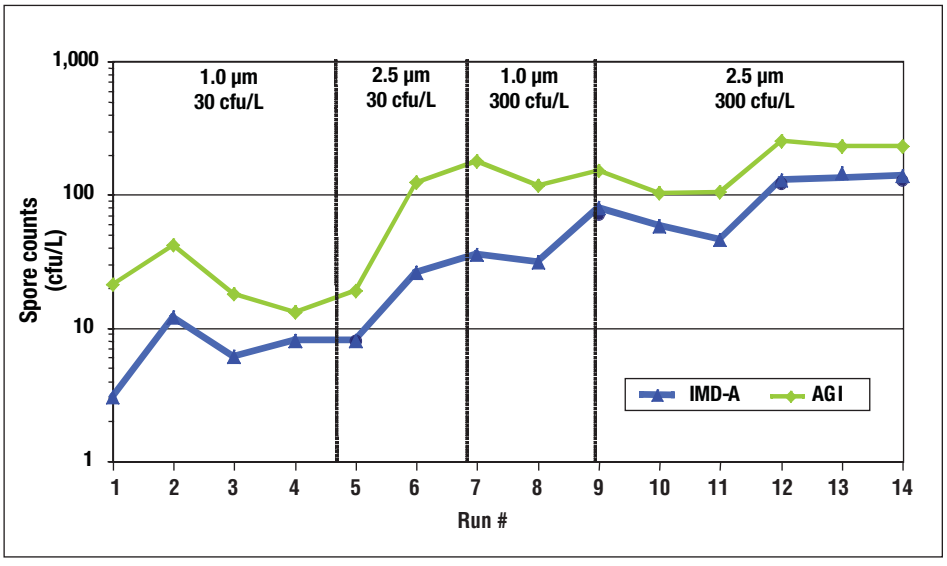
The 1-m^3 microbial barrier test chamber challenge study was performed at Nelson Laboratories (Salt Lake City, UT). In the first study, cultures of *Bacillus atropheus* spores were inoculated onto soybean casein digest agar (SCDA) and incubated for 2–7 days at 30–35 °C. The cultures were harvested in sterile water and the suspension was subjected to heat shock at 80–85 °C for 10 minutes to destroy vegetative cells. The titers of the spore suspensions were determined by serial dilution and plating onto SCDA, then adjusted to yield concentrations of 10^4–10^8 cfu/mL.

In the 1-m^3 barrier test chamber, bacteria were disseminated using a Chicago nebulizer. We performed experiments at five different spore concentrations, with tests repeated at least three times at each concentration. Microbial measurements of the environmental air in the barrier test chamber were done using the IMD-A (BioVigilant Systems, Inc., Tucson, AZ) and the Anderson air sampler (Anderson Instrument Company, Fultonville, NY), a conventional device that relies on an impaction method to enumerate microorganisms in environmental air. SCDA plates from the Anderson air sampler were incubated for four days at 37 °C. In a second study, vegetative cells of *Staphylococcus aureus* were used. Culture preparation was similar to that described above for *B. atropheus* except that no heat shock treatment was performed. Experimental set-up was similar to that described above for *B. atropheus*, except that vegetative cells of *S. aureus* were tested at two different concentrations and the tests were repeated at least five times at each concentration. For both studies, data were normalized to account for the different flow rate between the Anderson air sampler and the IMD-A.

The 150-m^3 microbial barrier test chamber aerosol challenge study was performed at the US Army's Dugway Proving Ground (Dugway, UT). In this study, *B. atropheus* spores were prepared similarly to the method described above for the 1-m^3 microbial barrier test chamber challenge study. *B. atropheus* spores were disseminated in the 150-m^3 barrier test chamber using a proprietary ultrasonic methodology that can specify the size and likely number of bacteria per aggregate to a high degree of accuracy. Nominal concentrations of *B. atropheus* spores were dispersed over four runs: 30 cfu/L at 1.0 µm, 30 cfu/L at 2.5 µm, 300 cfu/L at 1.0 µm, and 300 cfu/L at 2.5 µm. Microbial measurements of the environmental air in the barrier test chamber were per-

formed using the Anderson air sampler and using two IMD-A instruments, each placed at opposite ends of the chamber, and an all glass impinger (AGI), a glass bubble tube used to sample air. In the AGI method, a known volume of air is actively pumped through an impinger containing a liquid medium. SCDA plates from the Anderson air sampler were incubated for four days at 37 °C. To assess the AGI, liquid obtained was filtered onto a membrane to capture cells and the membrane was placed on a media plate and incubated to evaluate microbial growth. The membrane filters were transferred to SCDA media plates and incubated for four days at 37 °C. The results obtained from the Anderson air sampler, AGI, and the two IMD-A units were normalized to account for different flow rates in the three types of instruments.

Figure 2. Evaluation of *Bacillus atropheus* (spores) counts in environmental air in a 150-m^3 microbial barrier test chamber using IMD-A and AGI



In another comparison test, microbial populations in cleanroom Class E (ISO 9), Class D (ISO 8, Class 100,000 at operational and static), Class C (ISO 7/8, Class 10,000 at static and 100,000 at operational), and Class A (Class 100, ISO 5) environments at the Bayer Berkeley Site were evaluated using the IMD-A, running it side-by-side with a conventional SAS air sampling method (Bioscience International, Rockville, MD). SAS air sampling uses sieve impaction: air is aspirated for a specific time period through a perforated cover plate. The air is then blown onto a solid agar media plate, where the particles are impacted. The plates are incubated at an appropriate temperature and time period and the colony forming units (cfus) are counted. Environmental air monitoring in the cleanrooms was performed over a period of eight weeks in areas where little or no manufacturing activities were taking place. Because of the newer device's lower flow rate, for Class A, C, D, and E, 35 liters of air were sampled using the IMD-A. For Class A, C, and D, 1,000 liters of air were sampled using the SAS air sampler. For Class E, 200 liters of air were sampled using the SAS air sampler. Plates from the SAS air samplers were incubated for four days at 37 °C.

Microbial populations in environmental air in an unclassified office space environment were evaluated using the IMD-A and AGI method coupled with ScanRDI analysis. One cubic foot of air was sampled with both the IMD-A and AGI. Then 100 mL of fluid (Fluid A) from the AGI was membrane filtered and processed for ScanRDI analysis in accordance with the manufacturer's instructions. We tested the samples in quadruplicate, with unused Fluid A serving as the negative control for the ScanRDI analysis.

REAL TIME RESULTS POSSIBLE WITH IMD-A

Figure 1A and Table 1 show summary results from the *B. atropheus* 1-m^3 microbial barrier test chamber aerosol challenge study. In general, with the exception of one concentration range tested (concentration 4), the mean biological counts from the IMD-A were equivalent or greater than the mean *B. atropheus* spore counts recovered by the Anderson air sampler. Although at the lower concentration ranges (concentrations 1, 2, and 3) the microbial counts from both instruments were more or less the same, at the higher concentration range (concentration 5) the biological counts

Table 4. Statistical evaluation of 150-m^3 microbial barrier test chamber data from the IMD-A, an Anderson air sampler, and AGI—linear fit of instrument correlation

Instruments compared	r	r^2	ρ
IMD-A (# 1) vs. IMD-A (# 2)	0.997	0.99	0.0001
IMD-A vs. AGI	0.919	0.84	0.0001
IMD-A vs. Anderson air sampler	0.505	0.26	0.0550
AGI vs. Anderson air sampler	0.361	0.13	0.1855

recovered by the IMD-A were substantially higher than those recovered by the Anderson air sampler.

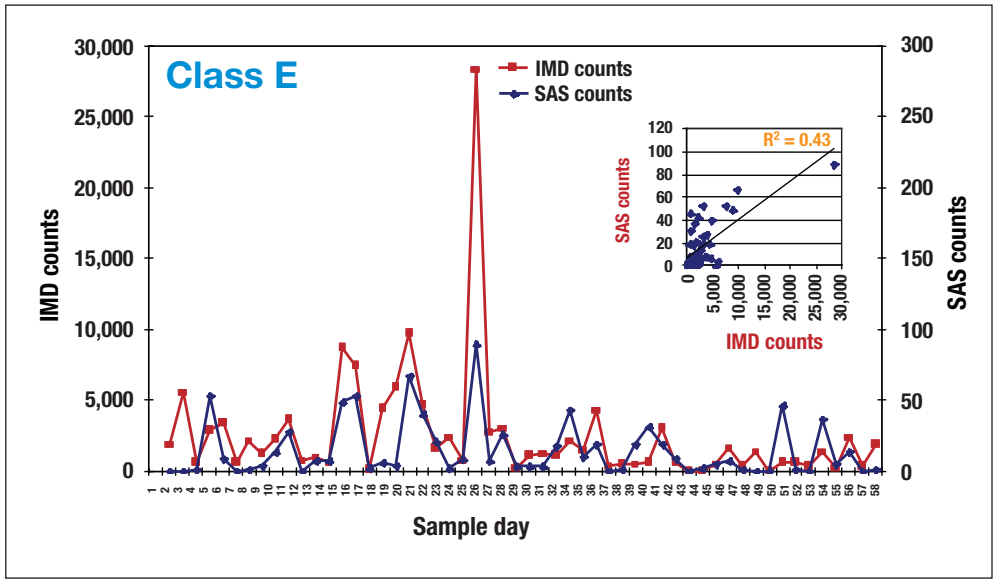
Results from the *S. aureus* 1-m^3 microbial barrier test chamber aerosol challenge study are shown in Figure 1B and Table 2. At both concentrations tested, the recovery of biological counts from the IMD-A were equivalent to or greater than the mean *S. aureus* vegetative cell counts recovered by the Anderson air sampler. The mean biological counts recovered by the IMD-A were 50% and 25% higher than the microbial recoveries of *S. aureus* from the Anderson air sampler.

Figure 2 and Table 3 show results from the *B. atropheus* 150-m^3 microbial barrier test chamber aerosol challenge study. At four of the concentrations tested, the biological counts recovered by the IMD-A instruments (IMD-A 1 and IMD-A 2) were equivalent. At all concentrations tested, the mean recoveries from the AGI method were higher than recoveries observed from the IMD-A instruments and the Anderson air sampler. The microbial recoveries from the Anderson air sampler varied when compared with the results from the IMD-A instruments and the AGI at each of the four concentrations tested. We observed a high degree of correlation for microbial recoveries between the two IMD-A instruments and between the IMD-A instruments and the AGI, as shown in Table 4. The degree of correlation between IMD-A and Anderson air sampler and between the AGI and Anderson air sampler were substantially lower.

Microbial recoveries from environmental air in cleanroom environments Class E (ISO 9), Class D (ISO 8, Class 100,000 at operational and static), and Class C (ISO 7/8, Class 10,000 at static and 100,000 at operational) are shown in Figures 3, 4, and 5 respectively. In all three classified areas, the microbial recoveries from the SAS air sampler were lower than the biological counts recovered by the IMD-A. Correlations (r^2) between the IMD-A and the SAS air sampler for Class E, Class, D, and Class C were 0.51, 0.42, and 0.32, respectively. Of the several replicated tests done in the Class A (ISO 5) environment, no biological counts were detected using either the IMD-A or the SAS air sampler (data not shown).

We also assessed microbial recoveries from environmental air in an unclassified office space environment using the IMD-A and AGI methods coupled with ScanRDI (Figure 6). Mean microbial recoveries from the environmental air were nearly identical between IMD-A and AGI method coupled with ScanRDI.

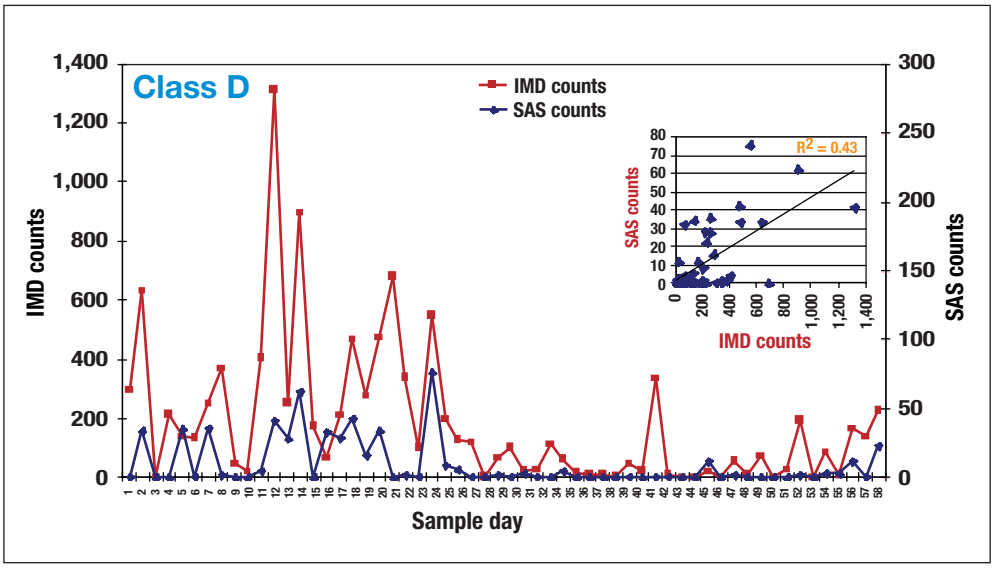
Figure 3. Evaluation of microbial counts in environmental air in a pharmaceutical cleanroom (Class E) environment using an IMD-A and an SAS air sampler



THE FUTURE OF IMD-A MONITORING

Results for the controlled microbial barrier test chamber studies demonstrate

Figure 4. Evaluation of microbial counts in environmental air in a pharmaceutical cleanroom (Class D, Class 100,000) environment using the IMD-A and an SAS air sampler



that the microbial recoveries obtained from the IMD-A units were comparable to or higher than the conventional culture based air sampling methods and indicate that the IMD-A has the potential to reliably evaluate microbial populations in environmental air. Mean microbial recoveries from the IMD-A were slightly lower than, but in the same order of magnitude as, those recovered by the AGI. On the other hand, microbial recoveries from the Anderson air sampling method were substantially lower than those recovered using the IMD-A or the AGI method.

The strong correlation (Table 4) in microbial recoveries between the IMD-A and the AGI suggests that the IMD-A reliably detects microbial populations in environmental air. This is further supported by the fact that the mean microbial recoveries from environmental air were nearly identical between the IMD-A and the AGI method coupled with ScanRDI analysis (Figure 6). The slightly higher microbial recoveries attained with the AGI method in the microbial barrier test chamber can be explained by the fact that the impingement method is known to disperse microbial aggregates more effectively than other methods used to sample environmental air, while the IMD-A would potentially undercount microbial aggregates.

However, since the IMD-A can simultaneously detect whether a particle is biological or not and determine its size, any potential microbial aggregates larger than individual cells can be readily flagged by the IMD-A. The lower microbial recoveries from the Anderson air samplers compared with the IMD-A or the AGI are not unexpected, since centrifugal, filtration, and impinger methods are thought to provide better recoveries and suggested to be more suitable methods for microbiological air monitoring than are impaction methods such as Anderson air samplers.[4] Additionally, the desiccation effects[5] created during impaction can limit the recovery of microorganisms on media plates. It is also well recognized that microorganisms in air may remain viable but lose the ability to form colony forming units, so that culture-based methods may underestimate microbial populations in environmental air.[6]

In the classified cleanroom environments examined, microbial recoveries in environmental air as assessed by the IMD-A were substantially higher than those from the SAS air sampler, which recovers microorganisms by the impaction method. Reasonable correlation of microbial recoveries were observed between the IMD-A and the SAS air sampler in cleanroom environments (Figures 3, 4, and 5) Class E, Class, D, and Class C (r^2 = 0.51, 0.42, and 0.32, respectively). Microbial recoveries from the IMD-A were approximately two orders of magnitude higher than those observed from the culture-based SAS method. Because of its slower flow rate, the IMD-A was operated for a longer time than the SAS air sampler. The higher recoveries may be partly explained by the fact that the IMD-A device could have captured microorganisms from activities in the cleanrooms subsequent to the end time of testing using SAS.

Additionally, in natural environments, microorganisms can exist in viable, nonvi-

able, or viable but nonculturable (VBNC) states, the latter of which is the predominant form.[7–9] This is partly due to the fact that culture-based methods are very selective in their ability to recover microorganisms that often exist in symbiotic or syntrophic relationships in natural environments. There is little or no published information available about the VBNC phenomenon as it occurs in controlled cleanroom environments. However, it is quite conceivable that cleanroom environments could have substantial levels of nonviable microorganisms, because of the controlled environmental conditions in cleanroom environments that could be captured by the IMD-A, especially if the microorganisms recently lost their viability but still have substantial pools of metabolic cofactors such as NADH that persist and are detected by the IMD-A sensor.

Results for the controlled microbial barrier test chamber, the ScanRDI study, and the cleanroom environment studies suggest that the IMD-A has the potential to provide a reliable evaluation of the microbial populations in environmental air and can detect variations in environmental microbial counts in cleanroom environments that are usually detected by conventional air sampling methods. While the microbial counts from the environmental air detected by the IMD-A in cleanroom environments were substantially higher than the conventional air sampling method, the ability of the IMD-A to simultaneously detect microorganisms in real time will prove the IMD-A to be a valuable analytical tool once its baseline for cleanroom environments is established.

IMMEDIATE APPLICATIONS

The IMD-A system can be valuable in monitoring environmental air in pharmaceutical manufacturing areas because of its ability to evaluate microbial quality of the environmental air in real time. There are several areas where we believe the IMD-A has potential immediate benefits:

1) Investigations: If conventional environmental monitoring methods show unexpected elevated levels of microbiological contamination, or if media fill results in an aseptic area yield one or more positive vials, the IMD-A system would be a valuable tool to immediately assess the environment. Assuming the microbiological contaminants were still present 5–7 days later (which is not always the case), the IMD-A could help locate the contamination and ascertain its cause to facilitate corrective action. The IMD-A can also check the effectiveness of corrective actions during investigations in real time before operations are resumed.

2) Reduction of shutdown time: Currently, conventional environmental monitoring methods verify that an area has returned to its validated state after being shut down for repairs or renovations. Such environmental monitoring data determine when to restart the operation. In an aseptic environment, they are used to determine when to perform media fills. The IMD-A could

Figure 5. Evaluation of microbial counts in environmental air in a pharmaceutical cleanroom (Class C, Class 10,000) environment using the IMD-A and an SAS air sampler

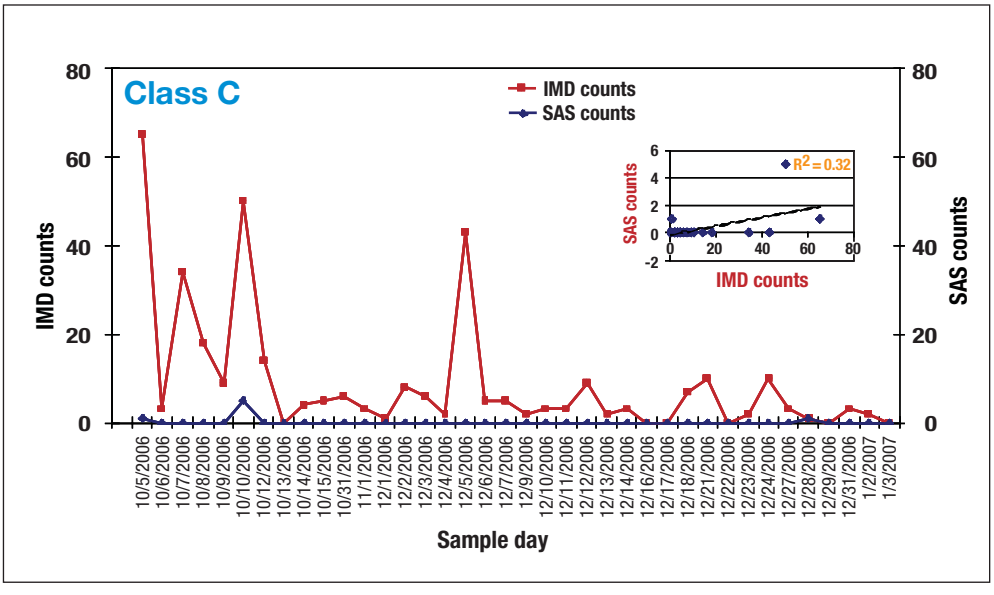
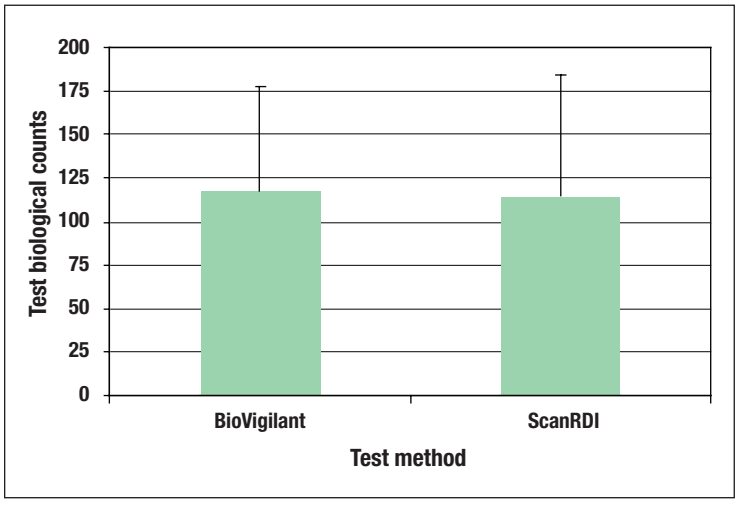


Figure 6. Evaluation of microbial counts in environmental air in an unclassified office space environment using an IMD-A and the AGI method coupled with ScanRDI



supply information about the environment rapidly, reducing the risk of performing media fills before the environment has returned to normal. In combination with conventional environmental monitoring and media fill data, the IMD-A could provide additional information to collectively assess the risk of resuming operations faster than with competing methods.

3) Training: The IMD-A's ability to provide immediate feedback can be helpful to assess how an operator's movements in an aseptic environment could affect the product. This feedback can provide information about whether or not an operator is ready to be trained using conventional environmental monitoring data and media fills.

4) Modification of aseptic process: The IMD-A could also be used to determine how a change might affect the environment. For example, if we want to change the way an intervention to clear a blockage in the fill line is performed, the IMD-A can assess which method generates the least risk to the process, thereby reducing the risk of failure when the intervention is performed in media fills before use in routine operations.

In the examples provided above, we have been careful to point out that the IMD-A can be used for additional information, but that it is not the official system for environmental air monitoring. At Bayer, we are exploring the use of the IMD-A for these purposes while pursuing additional studies and the question of what will it take to replace environmental monitoring of air with the IMD-A. Answering this question promises the biggest future benefit—the ability to determine when an aseptic environment is not performing acceptably and using real-time data to make immediate decisions about product manufacturing. We feel that the IMD-A will have positive effects on our industry, helping us continue to provide safe and effective products.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Shayna Zucker, Peter Nilsson, Jerry Ho, and Alan Chen for assistance with studies in the cleanroom environment and Lisa Yonocruz for assistance with ScanRDI analysis. The authors would like to acknowledge Nelson Laboratories and the US Army's Dugway Proving Ground for their microbial barrier test chambers and for their assistance during this study. ◆

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