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Taking full advantage of microbiological environmental monitoring data

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Abstract

An environmental monitoring (EM) program is required to document, that the manufacturing environment of a product is compliant, with its specifications and performs in an adequate state of control. Due to technical limitations of the conventional culture method and scientific concerns, the microbiological monitoring is not able to prove quantitative information about the sterility assurance of a product. Even an extensive microbiological sampling plan cannot prove the absence of contamination. As a consequence, there is a current shift in the thinking about the microbiological EM, leading away from an approach based on compliance with arbitrary numerical levels to a quality-by-design, risk-based approach. Until now, only *United States Pharmacopeia* chapter <1116> "Microbiological Control and Monitoring of Aseptic Processing Environments" reflects the evolution of guidance documents towards this new direction. Meanwhile, pharmaceutical manufacturers are still concerned about the best way to take full advantage of the huge amount of data generated by their microbiological EM. While inescapable answer at this complex question is well beyond the topic of this whitepaper, some qualitative approaches reflecting current trends towards a parametric product release will be addressed.

1 Introduction

Pharmaceutical companies want to produce safe and effective, high quality products within a set budget. In this aim, the EM program is mainly proactively used as a quality assurance tool. It validates the sanitization program and helps in determining how often cleaning and sanitization is required. It does this by measuring the overall effectiveness of sanitary processes, personnel practices, and operational methods used when a given batch is being manufactured. If the EM is used correctly it can act as an early warning system, by quickly detecting trends and drifts in the manufacturing environment. Globally, the EM program gives relevant information to document that all manufacturing steps were realized in an environment in a coherent, validated state of control. And that is exactly what regulatory authorities want.

In substance, EM consists of an enumeration of viable and non-viable particles suspended in the air, settled on surfaces in the workspace or discharged from the body surfaces of operators. In addition to that, the variation of temperature, humidity, airflow and other environmental parameters must or can be

completed, depending the level-risk of the clean zone and considering some practical aspects.

The most important question is how relevant information can be extracted from this mound of generated data. The concern is even truer concerning data from microbiological EM considering the microbial limits in clean areas specified in both EMA and FDA GMP guidelines (1; 2) and the expectation of documentation of the state of control of the microbiological environment, based on the processes historic values and the ongoing characterization of microorganisms.

Requirements for clean areas classification are based on defined levels of micro-organisms in terms of colony forming units (CFU) tolerated in process areas, with limits near of zero in aseptic areas. Regardless of the CFU enumeration, microbiological EM also documents significant changes in microbial flora. However, conclusions regarding lot acceptability on the basis of sampling results obtained during manufacturing of one given batch is a currently challenged approach.

Given that, trending of microbiological numerical data and qualitative data is emerging as giving more valuable information by documenting the state of control of the manufacturing process. It opens new perspective of the use of microbiological EM as a quality-by-design, risk-based approach for parametric release of manufactured product.

2 THE LIMITATIONS OF MICROBIOLOGICAL MONITORING

Different sampling methods can be used to assess and control the microbiological status of a given controlled environment. Most of them rely on the growth and recovery of microorganisms. The first concern with microbiological monitoring is the poor accuracy and precision of the microbiological method. The second is the relevance of the use microbiological data as a criterion for product release.

2.1 THE MICROBIOLOGICAL METHOD CONCERN

Currently, the (numerical) data collected are still mostly CFU. A CFU is not the same as a bacterial cell but rather a collection of microbial cells, visible to the naked eye when there are enough accumulated cells (generally in the range of 10^7 – 10^8 cells). Due to the broad diversity of physical states (single cells, aggregates associated to particles, microbial cells associated to inert particles, etc.), it is difficult to determine if a biomass of 10^7 cells arises from a single cell, an aggregation of cells, or from other physical states. All that is known is that microbes grew in that spot until there is enough accumulated biomass to be seen with the naked eye. The CFU is therefore, at best an estimate of the numbers of cells, present originally and the plate count (the result) an interpretation of this approximation. This estimate becomes even more imprecise at low numbers of CFU per plate (3). As a consequence, numerical plate counting method has a poor accuracy (the ability of measurements to reflect the true value of the population) and precision (the degree of reproducibility among the measurements).
Moreover, as all quantitative analytical methods, the count plate method has a limit of detection (LOD; 1CFU/plate) and a lower limit of quantification (LOQ = the lower limit of plate counts with acceptable accuracy), under which a value is considered as being noise and not valid for quantitative analysis. Even if CFU can be detected (that is above the LOD of the method), the lower LOQ for the plate count

method has been determined to be from 25 to 30 CFU/plate (4; 5), in any case not less than 20 CFU/plate (6). The FDA Bacterial Analytical Manual (BAM) recommends 25-250 CFU per plate as an acceptable countable range (7). To be compared with regulatory guidance setting action levels as low as single digits, near the LOD. Counts below the LOQ could thus be viewed as being in the noise range of the plate count method and could not be considered as significantly different between each other.

Several publications have pointed out these limitations. In 2004, Hussong and Madsen (8) emphasized that the microbiological assays used have limits of quantification higher than the customary control limits and are subject to a great deal of variability.

Alternate microbiological methods exist but the requirement for their introduction is that they be at least equivalent to the traditional methods. Due to the important variability between them, it could be difficult to assess.

2.2 The scientific concern

A subsequent article by Farrington (9) highlighted that the relationship between microbiological EM data to finished product quality, was an unproven yet commonly held belief. He argues that the regulatory concern over contamination from environment makes sense, but must be applied with judgment and scientific rigor.

Aseptic processing relies on the exclusion of microorganisms from the process stream and the prevention, of microorganisms from entering into contact, with product during processing. This is reflected by the near zero expectation found in guidance. While the goal of zero is comprehensible and seems logical, it is technically not possible and therefore unrealistic. Firstly, microbial monitoring sample represents only the microorganisms captured, during a narrow length of time and at a particular location. The absence of growth on a microbiological sample only means that growth was not discovered, not that the environment is free of contamination. Some level of microbial contamination is inevitable in any environment, in which human operators are present. Secondly, sterility is, by definition, the complete absence of viable contamination. No analytical method (microbiological, chemical, or physical), regardless of how advanced and sensitive, can measure the complete absence of something. As it would never be possible to use EM to prove sterility, the near-zero condition is not absolutely necessary, for success in aseptic processing (10). Sterilization process could be taken as a comparison. As zero risk doesn't exist, a probability of not more than one viable microorganism in one million sterilized items of the final product (the sterility assurance level (SAL) of 10^{-6}) is currently required for sterilization process (11). As the SAL of the product cannot be measured, the conditions necessary to reach this extremely low probability of contamination are inferred from the general assumption of exponential inactivation kinetics for microorganisms under the influence of antimicrobial effective parameters (that can be measured with accuracy and precision). Because of technical limitations of microbiological monitoring, such a theoretical approach is not available. In other words, there is no correlation between microbiological information collected during the manufacturing process and the sterility of products produced in this process. Microbiological data are therefore useless as quantitative predictors of the system, but valuable as raw data for the determination of trends in the facility as a whole.

All these observations have been taken under consideration by the *United States Pharmacopeia* (USP) in the 2010 revision of USP chapter <1116>: "Both the lack of

precision of enumeration methods and the restricted sample volumes that can be effectively analysed suggest that environmental monitoring is unable of providing direct quantitative information about sterility assurance." (12). It reinforced the official opinion that the microbiological EM program should move more towards a more science-based, qualitative approach.

3 A QUALITY-BY-DESIGN AND RISK-BASED APPROACH

Microbiological data are still often filed, without much consideration as to their further application. Consider on one side the above limitations and on the other side the costs of providing, staffing and running laboratories, to obtain, examine and interpret samples taken, it would be vital to make use of the data generated in a more efficient manner

A quality-by-design and risk-based approach allows to take full advantage of data by allowing a parametric release of the product based on the definition, the monitoring and the assessment of critical process parameters (CPPs) at an early stage rather than on a pragmatic approach consisting of proofing compliance with numerical levels. A well-designed trending of data collected by microbiological EM program could give the assurance that the product is of the intended quality. Regulatory requirements are also satisfied by documenting that the manufacturing process is well understood and that, the facility is running in a state of control.

3.1 TRENDING OF NUMERICAL MICROBIAL DATA

Statistical tools are required to organize and present numerical microbial EM data for the purpose of evaluating it against regulatory action limits and to determine if the environment is in a microbiological state of control. All environment/surfaces are contaminated by some microorganisms, with a degree of variability over time. This reflects the level of control of a given environment/surface. For well-controlled processes based on past experience, variability in values will be low and inherent to the method. It displays common-causes of variation and it can be predicted how the process will vary (within limits) in the future. If the process is unstable, the process displays special cause variation, that is non-random variation from external factors suggesting a new problem in the monitored environment (or a problem in the sampling method). Control charts are simple and robust statistical tools for understanding process variability and to ensure the process is in a state of control. Their main advantage is to allow easier and more quick detection of trends and shifts in a process.

Control chart begins with a time series graph. A central line, the baseline, is added as a visual reference for detecting shifts or trends. Then upper and lower control limits are computed from available data. Finally, specific trend rules are set to track and trend the data in real time and to investigate excursions. These rules recognize patterns on the control chart that could be due to special-causes of variation, like shifts in the process mean and/or in the process variation. There are many different types of control charts and trend rules available. Define the most suitable statistical approach to treat data set is a complex subject well beyond the topic of this paper. However,

it is important that the user understands the differences among the tools available and how they apply to the set of data being evaluated when choosing a method for data trending.

3.1.1 The baseline count

The first step consists to average multiple enumeration collected when the facility is operating under controlled conditions (in a relatively stable manner) over a given period (as long a period as is possible/ practical) to determine a baseline count. At this early stage, the sampling plan should ideally be expansive to allow selection of critical sites for routine monitoring. The statistical rule of thumb consists then to use at least 20-30 results per sampling site to determine the baseline. Their uniformity is important to avoid inappropriately high baseline and consequently alert/action limits. Obvious unusual results obtained during this period should be excluded after investigation.

The data should always be plotted versus date for separate physical location, shift, room, operator, or other parameters to help to identify unusual data points (1). Averaging of results can mask unacceptable localized conditions. The analysis can include totals by month or quarter and ranking within a sort category to document episodic events or any trends that may occur seasonally. Mapping out results on a plant diagram can also be extremely helpful. There is no acceptable recommended value for the baseline because it is mainly different for each facility/site. However, a baseline equal to zero is not acceptable even when zero counts are recorded over a long period of time. This situation often encountered in aseptic environments will be discussed below. Finally, a*s* understanding of processes, proficiency of personnel and experience gained from problem solving grow, will the baseline contamination level change over time and should be calculated again (one or two times per year).

3.1.2 Alert and action levels

Regardless of the re-evaluation periodicity of alert and action levels and the method chosen, the regulatory expectation is that levels are based on historical data, go down with time and that adverse trends are timely detected and addressed.

Optimal test results expected, along with alert and action levels, can then be computed based on this data set. While being a critical step, there is no consensus on the most valuable way to establish action and alert levels but data set, regulatory guidelines, requirements, and risk-benefit analyses of the product should be considered. In any cases, they should ensure time is used appropriately to look for issues that may arise and that action would only be taken when warranted. Alert limit should thus be sufficiently above normal variation in results so that attention is paid to whether or not this was a special- (and undesirable) cause of variation. The response to a value above the alert limit may often be just a notation of the event on a trend analysis, and an assessment that the event was not part of a cluster of abnormal value.

Depending on the data set, control limits could be calculated by using a distribution-based (parametric) approach or by a distribution-free (non-parametric) approach. For example, the use of the means and standard deviation of parameters to set up the control limits preferentially entails that data follow a normal distribution (symmetric around its mean), which is not always the case of microbiological data. One primary reason why microbiological data do not fit a normal distribution is due to spikes. It is common to obtain most values near the mean, but to occasionally have a value which is well above the mean. Such spikes are common, especially when personnel

are involved in the process. Another reason is the frequent occurrence of zero CFU results in aseptic environments. The approach to establish control level is therefore not univocal!

Usually, normal distribution approach suits well with sampling sites with usually high microbial counts (e.g. in ISO 7 cleanrooms). Different control charts could be used, each having pros and cons such as a different ability to quickly detect small or large shifts in the process mean.

The approach is less straightforward for low counts or for counts with (very) high occurrence of zero. When results are not proven normal or are nearly normally distributed, other distributions (Poisson, Negative-Binomial, gamma, etc.) or transformations such as the log transformation or square root transformation are usually considered. A simple alternative is to use the lower and upper percentiles of the historical data to set up the limits. However, there could be scientific limitations with such approaches. Indeed, as a result of the inherent variability of the microbiological method (sampling and analysis), it is not analytically relevant to set an alert level of 1 CFU and an action level of 3 CFU in aseptic environments were the baseline could be equal to zero. As results in this range could not be considered as significantly different to each other, it is neither scientifically valid (and therefore it is a loss of time) to treat them differentially. This led USP to suggest the trending of a frequency distribution by plotting the "contamination recovery rates" (CRR) rather than specific numbers located in the noise range of the microbiological method (12), a methodology presented for the first time in 2004 by Caputo and Huffman (13). CRR is defined as the rate of environmental samples that are found to contain any level of contamination (microbial counts greater than zero) irrespective of the extent of this contamination. It focuses on all samples that have any contamination regardless of colony number. An incident rate of 1% would mean that only 1% of the samples taken have any contamination. The alert and action levels are then defined relative to these percentages. Incident rates in percentage values entails to look historically at least 100 samples back, instead of focusing on just a single current incident, or only on samples showing contamination above action levels

Other distribution-free approaches have then been suggested for analysis of these “non-zero” values in extremely low level microbial counting environments, such as the most probable number (MPN) methodology (14). MPN is a method to estimate the concentration of viable microorganisms in a sample by means of replicate liquid broth growth in ten-fold dilutions. The idea is to use the fundamental statistics as if only a single dilution were being considered. Even if the weakness of “non-zero” trending methodologies is that there is no allowance for recognizing repeated occurrence of an unusually large CFU count, it is considered that in many cases, the magnitude of an individual excursion is less informative than the frequency with which contamination occurs. However, if CRR are adopted as a way to analyse microbial contamination, USP <1116> emphasizes that for an ISO 5 cleanroom, any excursion of >15 CFU should also be investigated (12).

An interesting case study using CRR is presented on the PDA website (15). The interested reader can also refer to the recent paper of Bar R. (16) describing a simple and straightforward construction of control charts of individual microbial counts as they are or of contamination rates derived from them irrespectively of the type of the parent data distribution and without the need to transform the data into a normal distribution.

3.1.3 Control chart rules

Special causes of variation are detected on control charts by using rules to identify certain types of patterns that appear. The simplest pattern observed is a value beyond the control limits. Runs of points in a row on one side of the average line could also be interpreted as a signal of some change in the process. Recognizing patterns in the process is one key to quickly detect special cause of variation. As long as the all ongoing data are within the control levels and there are no patterns, only common causes of variation are present in the process and it is said to be "under control".

3.1.4 Investigation of excursion

In any cases, microbiologists should use practical scientific judgement in their approach to excursions. USP <1116> states that all recoveries should be investigated and include the identification of the organism recovered (12). In the case of isolated single excursion, only general corrective measures can be considered because it is not judicious to suggest a root cause for which there is no scientific evidence. Except for uncontrolled facilities, each sample site has its own baseline, so a result outside control limits may not be addressed as a collective event but as an independent event.

Excursions than more than 15 CFU recovered from a single ISO 5 sample (airborne, surface, or personnel) should occur very rarely, indicate of a significant loss of control and should induce a careful and thorough Investigation. Plotting alert and action levels for excursions rates could therefore be useful to trend and document such events. The interested reader can refer to the paper of Huang H. describing an example of multivariate control chart constructed for simultaneous monitoring of frequency and magnitude of microbiological excursions (17).

3.2 Trending of microbial flora

The value of microbiological data is greatly reduced if the microorganisms isolated are not characterized to some degree. The qualitative analysis of microbiological EM data gives a different but complementary information than the numerical data analysis. Microorganisms recovered by a sampling can originate from various sources and have been carried across segregation barriers by a number of different vectors (the by far most important one being cleanroom personnel). The nature of microorganism(s) isolated may often inform about the source of contamination, the pathway of contamination across the segregation barriers and the vector involved while the number of microorganisms reflects the degree of efficiency of all combined segregation measures used to prevent the microbial contamination.

Characterization of microbial isolates is of equal or higher importance than collection of numerical data of CFU. For example, an excursion of the expected level of microorganisms in a manned aseptic environment carries very different significance if the isolated microorganisms are composed of gram-positive cocci, gram-negative rods or spore forming gram-positive rods (18). As for numerical data, the qualitative trending of microbiological data is the approach giving the most valuable information.

Firstly, it allows demonstrating the level of compliance to regulatory EM requirements. Recovered microorganisms should be characterized to a level of identification sufficient to meet trending needs, whether to gram stain, genus, or species level. Basically, it is advised to identify one sample of air and surface per sampling period for a full year to get the normal flora of the room and then to perform the identification on a regular basis to

document that the flora is still conform to product specifications. The identification frequency should be higher than once a month to enable the manufacturer to have a sufficient understanding of the normal microbial flora that could be present in the production area (19). Anything over the alert and action limits should be identified. Multiple occurrence of microorganisms, even below the action level, should be considered as a trend which require investigation and corrective action.

Secondly, ongoing characterization of microflora can quickly reveal changes and give early warning of developing problems or system/procedures failures (cleaning or gowning practices, migration of microorganisms, condensation, etc.). Therefore, it gives information about the efficiency of processes (cleaning, sanitization, gowning, etc.) and helps to determine proactive improvements or the best frequency and intensity of cleaning, disinfection and training measures.

Qualitative microbiological trending is also a paramount support for investigations. As numerical isolated excursions in EM data are difficult to evaluate, the type of microorganisms isolated at a given time and in the recent monitoring history should therefore always be taken into consideration. Investigations for excursions and changes in microbial flora should be thorough with an emphasis on determination of the root cause. Here are some common root causes for microflora patterns (20):

- Gram-positive cocci (*Staphylococcus*, *Streptococcus*, *Micrococcus*, etc.): the most typical flora detected. Primarily associated with human skin (personnel habits or gowning problems) or from respiratory tract
- Gram-negative rods (*Pseudomonas*, *Serratia*): associated with water: condensation, leaking, aerosols and possible hygiene problem
- Gram-positive non-sporulating bacilli (*Actinomyces*, *Corynebacterium*, *Listeria*): poor air conditioning inducing uncomfortable work conditions and gowns discharge from sweating personnel
- Spore forming species (*Bacillus*): dust or dirt sediment on soil and resuspended by floor traffic or less usually from air handling
- Molds: influx of unfiltered air (often when construction works near the site), mold from street clothing or contaminated cardboard, possible water reservoir
- Yeast: possible outdoor air influx or clothing-borne (especially in late summer/fall), possible human contaminant

These information helps to determine the pathways of contamination and the product impact, to defend deviations and to decide on necessary corrective actions.

4 Conclusion

Current guidance documents about microbial limits for clean areas are based on defined levels of micro-organisms in terms of CFU tolerated in processing environment. This approach has undoubtedly the advantage of being easy to audit as CFU measured are either over or under the regulatory limits. As a consequence, the use of collected data to demonstrate that microbiological conditions during the manufacture of one single batch were compliant with guidance is still the common practice. However, there are some concerns with this approach. Firstly, microbiological monitoring cannot recover all microorganisms present in an environment, nor on a surface. A continuous monitoring still cannot ensure sterility. Secondly, regulatory expectations are based on data generated by a

method recognized as having poor accuracy and precision. In aseptic environments, the sampling will commonly give counts of zero, with worries expressed if the count is close to three, a threshold than the accuracy of the method cannot sustain. As processes, have improved and zero results have become the norm, the regulatory reaction has been to multiply the tests and in-process workload which, while intuitively logical, is scientifically inappropriate and valueless.

These limitations of the microbiological monitoring have been taken into account by the USP in the 2012 revision of USP chapter <1116> that recalls that "The real value of a microbiological monitoring program lies in its ability to confirm consistent, high quality environmental conditions at all times" (12). Regulators are actually looking after a a documentation that the manufacturing process is well understood and that the facility is running under a state of control. Management is expected to know what EM data mean, what the issues have been, what organisms are present in the environment and to show that procedures for preventing contamination are effective. Therefore, environmental monitoring should be considered as a process indicator, a tool to understand and achieve a stable and predictable process and not as a product release criterion. This reflects the evolution of microbiological EM towards a new direction, leading away from arbitrary numerical levels to a more qualitative trending methodology.

Trending is emerging as the most effective use of microbiological EM generated data. It allows trends and shifts in the process environment to be detected and visualized. As a result, the environment can be observed to ensure it is still under control through current and historical data values.

Control charts allow to organize, present, analyse and interpret the large amount of numerical data that EM generates. The choice of a control chart depends upon the data set (and how results are interpreted), certain expected conditions that arise when the process is out of control, as well as the sensitivity desired for detecting such conditions (in agreement with the risk assessment approach). There is no way to establish alert or action levels as statistically significant at very low recovery levels. Therefore emphasis should be on incidents, even those having just 1 CFU. Alternative approaches are regularly addressed, as the CRR methodology recommended by USP <1116> (12). None of these are yet adopted and enforceable by EMA, FDA or any other government agency but their use is generating a positive debate concerning clarification about values for microbial limits.

Microbiological EM data monitoring should be considered as a science-based process to document that the manufacturing environment is understood, under control and compliant with regulatory expectations. It is a multidisciplinary approach that involves a broad array of stakeholders, well beyond microbiologists and quality assurance people.

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6 AUTHORS

- Guillaume Henry, PhD industrial pharmacist ghenry@nalys-group.com