Microbiological data

20

20.1 Introduction

Data collected is an important part of microbiology. Each laboratory method generates data in some form or another. Broadly, such data is either qualitative (such as the "pass" or "fail" result from the sterility test) or quantitative (such as a number produced from a bioburden test). This chapter considers some aspects of data capture and data analysis. In total, these are wide ranging areas, and the purpose of the chapter is simply to highlight some of the essential points and provide a base understanding of what microbiological is and how it might be handled. Given that limits setting is doubly important, the chapter explains how microbial test limits might be set, based on a consideration of historical data.

Trending is applicable to most microbiological analyses. Raw monitoring data by itself is of little value, and individual high counts are not often meaningful. Thus, voluminous sets of results need to be appropriately analyzed and presented in order to provide trends and appropriate focus; for example, with the environmental monitoring of cleanrooms.

The reporting and trending of data provides an opportunity for the effectiveness of microbiological control and the appropriateness of the monitoring program to be reviewed and modified. Where good control is demonstrated, there may be opportunities to reduce the level of monitoring, thus reducing cost without compromise to product or patient.

Often microbiological data will contain many zero data points, which can present issues in statistical analysis of the data. It is important to select appropriate analysis tools that do not lead to the masking of significant events or trends.

In whichever way data analysis is undertaken, periodic summary reports should be generated and reviewed by a cross-functional team. In presenting data, a combination of graphical and tabulated formats providing a visual representation with clear supporting summary text is recommended.

20.2 Counting microorganisms

Quantitative data are generated through the counting of microorganisms. One of the important tasks required by pharmaceutical microbiologist is the ability to enumerate microorganisms. Counting is required in order to assess the microbial quality of water, cleanrooms, in-process bioburden samples, of raw materials, and so on. The method used to count microorganisms depends upon the type of information required, the number of microorganisms present, and the physical nature of the sample. An important

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distinction is between total cell count (which counts all cells, whether alive or not) and the viable count (which counts those organisms capable of reproducing).

Total cell counts include direct microscopic examination, the measurement of the turbidity of a suspension (using a nephelometer or spectrophotometer), and the determination of the weight of a dry culture (biomass assessment), adenosine triphosphate (ATP) measurements (typically using the enzyme luciferase which produces light on the hydrolysis of ATP), via fluorescent staining, or electrical impedance. Viable counting techniques include the spread plate, pour plate (through direct plating or an application like the Miles-Mistra technique), spiral plating, and membrane filtration. Such methods were presented in Chapters 1 and 7. To the classic can be added rapid microbiological methods, which often produce more data by being able to address the issue of "unculturability" (see Chapter 17).

20.3 Sampling

The objective of taking a sample is so that the sample taken is representative of the population and by examining or testing the sample, then something meaningful can be inferred about the population. A sample is, therefore, a subset of a population selected by a process. The sample size is the number of items (samples) included. For a water system or an air sample, the sample is a proportion of the total collected at a given time point.

In terms of the numbers of samples taken (the sample design), the sample should be representative. If the sample is not representative or if the sample take is not as intended, then sampling error is said to have occurred (although in practice it is very difficult to know if sampling error has occurred) [1].

In terms of the sample being representative, this means that the sample should be of sufficient volume (such as 200 mL of pharmaceutical grade water) or an appropriate number of samples should be taken in order to produce a representative result (e.g., determining how many samples from a give number of containers of a raw material will give a representative result. There are different statistical tools which can be used for this purpose, the most simple being the square root of the number of containers).

The reason, drawing on the water example above, that the sample size is important since it relates to the distribution of microorganisms. Distribution, as a general principle, is discussed below. In relation to sampling, if water contains 1000 bacteria per liter, this does not mean that each single milliliter will contain one bacterium. However, if a 500-mL sample was taken, then the chance of capturing 50 bacteria is much higher than the chance of capturing one bacterium in a 1-mL sample. In this case, it is more useful to consider the volume required so that a reasonable estimate of the microbial population can be obtained.

For example, suppose we wish to estimate the microbial population in 1 L sample. We could test the entire liter. This would itself be time consuming and expensive, and if the liter was of value, the sample would be rendered worthless.

If we what to be 95% certain of detecting a reliable count, and we know, from experience, the mean contamination level, then the following formula can be applied:

$$V = \frac{\ln(1-P)}{\mu}$$

where V is the volume of sample to test, P is the probability of detecting an organism, and μ is the mean contamination rate.

Suppose, $P = 0.95$ (as we are seeking 95% confidence, a commonly applied correction factor to most biological data) and the mean contamination rate is 0.022 cfu/mL. Then, the volume required would be 1498 mL (or, in practice, a 1500-mL sample would be tested).

In terms of where the samples are taken from, the general principle is one of the random sampling. This means that the sample is selected in such a way that every sample of the same size has an equal chance of being selected. Random sampling presupposes that the population is well-mixed before sampling takes place. In drawing upon a microbiological example, random sampling would be applied to the sampling of a raw material from several drums.

Random sampling is not desirable in all cases. For sterility testing, for example, the sampling is biased. The bias is so that the samples relate to time and that the samples taken relate to approximately equal moments during the filling of the product (or, in terms of the numbers of containers filled, the samples are taken at equidistant intervals). Here, something different is being measured than if random sampling was used to select the samples. Furthermore, with environmental monitoring, having fixed samples makes trending more meaningful.

Furthermore, an important aspect of the work of pharmaceutical microbiologists is ensuring that the samples taken or submitted to the laboratory have been done so in an aseptic manner and that the containers and storage conditions of the sample have not been adversely affected [2].

20.4 Microbial distribution

The distribution of microbial counts is an important topic for it has a considerable impact upon the trend charts, general statistic, and on the techniques applied for the calculation of alert and action levels. Microbial counts in the environment rarely resemble normal distribution (where a classical bell-shaped curve or binomial pattern is obtained; here the area under the curve is divided into two symmetrical halves). Normal distribution is a phenomenon found in many aspects of physical and biological science (from measurements like human height). Normal distribution is displayed, at its most simples, as a histogram as shown in Figure 20.1.

In practice, the distribution of microorganisms and microbial counts show either Poisson distribution (such as from a water system where microorganisms are distributed randomly) or a marked "skewness," as with counts from a higher-grade cleanroom. Most microbiological monitoring data displays skewness, where the majority

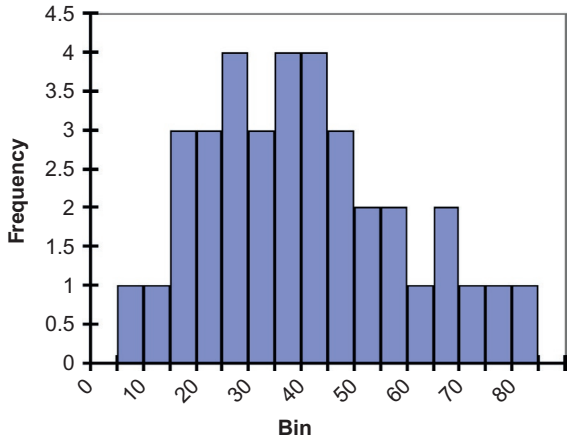

Figure 20.1 A standard histogram, as might be typical for biological data.

of results are zero or low counts, with very few results recording higher counts. Thus, a data plot shows a long, thin tail toward the left of the graph [3].

With Poisson distribution, the frequency of counting "events" over "time" is more random (as with Figure 20.2). Thus, the phenomena of Poisson distribution account for events where a sample may exceed an action level on 1 day, be below it for another 2 days and then be above it again. This situation does not indicate contamination appearing and disappearing, or that one sample has given the correct result and the other has given an unrepresentative one, it merely reflects a distribution across time and space.

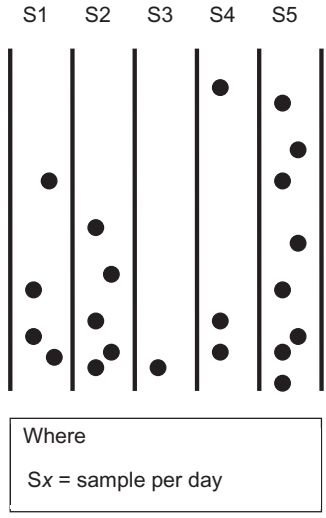

Figure 20.2 Depiction of typical Poisson distribution: a possible distribution of microorganisms in five samples (S*x*) taken from the same water outlet.

Outside of statistical parameters, it should be borne in mind that biological data is varied, and there are reasons outside numerical data itself as to why microbiological data is particularly varied [4]. Variations can arise due to several factors, including:

- the monitoring methods (which are often inherently variable);
- culture media, where variations can arise between different types of media (such as general purpose media or fungal specific media), whether the media contains any additives, such as disinfectant neutralizer, and between manufacturers;
- incubation times;
- incubation temperatures;
- sampling procedures;
- sample size or volume;
- different sample locations;
- different sample times;
- frequency of monitoring or sampling;
- the people undertaking the sampling;
- acceptance criteria (such as the means of establishing alert and action levels).

Imprecision in sampling technique can also add to such effects. This fact alone accounts for why a reasonable number of repeat samples should always be taken in response to an out-of-limits event. Such distributions can also enhance the degree of standard error obtained from plate counting.

20.5 Data trending

The reason for requiring the use of trend charts relates to one of the biggest challenges in pharmaceutical microbiology: that is, the vast quantity of data that is collected and the difficulty in interpreting it; and because a single monitoring event only provides a "snap shot" of what may have been happening on a particular day, which may or may not be representative.

Thus, trend analysis is very important for environmental monitoring and is necessary in order for the microbiologist to see "the big picture." There is no right or wrong approach for selecting a trend analysis system, although there are dangers in selecting a trend system that is inappropriate for the data set. Control charts are useful tools for visualizing a process and provide quick "summaries" of process statistics. Control charts make an assumption that the data plotted is independent, that is, a given value is not influenced by its past value and will not affect future values. Control charts have been used for many years as a part of statistical process control (SPC) systems.

20.5.1 Control charts

The lack of normality and seeming randomness of the distribution is of importance when using control charts. Before constructing a control chart, the collected data should be examined to see if it follows normal distribution. Although, as stated earlier, it is improbable that the distribution of microorganisms as indicated by "counts" from environmental monitoring will follow normal distribution, any statistical analysis that

is based on normal distribution remains the more accurate approach. Therefore, it is incumbent upon the user to demonstrate first if there is normal distribution, then, if possible, to transform the data to approximate normal distribution. Only when this is not possible should alternate methods be used.

Normal distribution is the basis of the most common statistical methods. This is because [5]:

(a) many naturally occurring populations are normally distributed;
(b) the means of large random samples from populations are commonly normally distributed;
(c) many populations can be made to approximate normal distribution through data transformation (see below).

Normal distribution can be assessed visually using a histogram, blob chart or a normal probability plot (where the resultant plot should lie approximately along a straight line).

However, a problem arises because microbiological data is rarely binomial. Binomial refers to the probability of an event occurring where the event has the same probability of occurring on each occasion, such as, a person being male or female [6]. In contrast, microorganisms in a sample follow Poisson distribution and microbial counts from a test tend to follow a skewed distribution, as discussed above. Hence, microbiologists often need to consider data transformation as a preset to running control charts.

20.5.2 Data transformation

Microbiological data that do not follow normal distribution may need to be transformed for certain statistical techniques and charts. Data can also be transformed to make it easier to visualize them. Furthermore, confidence intervals and hypothesis tests will have better statistical properties if the dependent variable is approximately normal with respect to its mean, with constant variance. Here, transformed data can be used in that normal distribution could not be meaningfully deployed.

Transformation is a mathematic adjustment applied to data in an attempt to make the distribution of the data fit requirements, each data point, which we will call z_i, is replaced with the transformed value:

$$y_i = f\left(z_i\right)$$

where f is a function.

In statistics, this is sometimes called "power transform."

Considerations for use for microbiological data:

(a) no one type of transformation is ideal for a particular purposes;
(b) transformations for values of 0 need to be adjusted to a minimum value of 1 (a constant will need to be added to every datum). Remember, when performing other statistical calculations, to adjust the data back by subtracting the constant;
(c) for low count data (where the majority of counts are less than 10). The recommendation is to take the square root to transform the data (alternatively squares can be taken);

(d) for high count data (where the majority of counts are greater than 10). The recommendation is to take a logarithm to the base 10. Logarithmic scales are preferred for large variations in counts. This is because by taking the logarithm of numbers, this reduces the increase in count. Logarithms to the base 10 are used simply because easiest to understand.

In Figure 20.3, some bioburden data are presented. There are three counts of very high values (>1000) against a data set where the mean count is 10.

In Figure 20.4, the same dataset has been used. This time the data has been converted to $\log_{10}$. The data is easier to track.

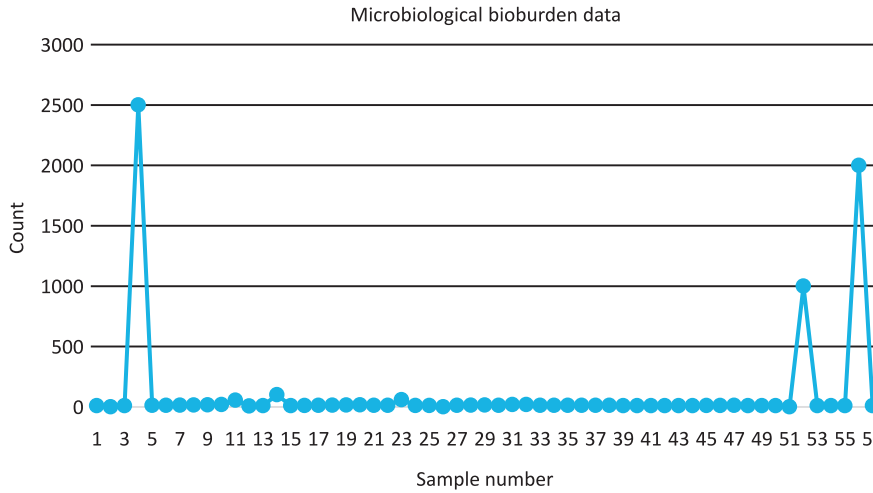

Figure 20.3 Illustrative data, containing high count values.

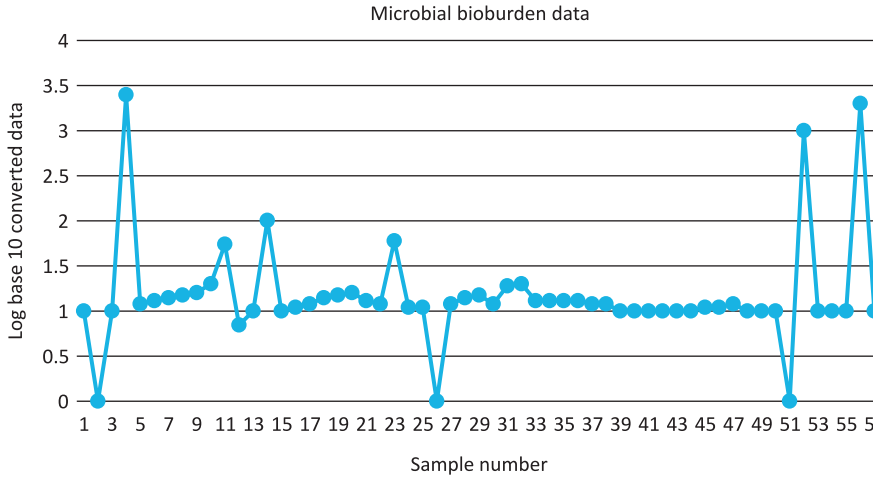

Figure 20.4 Log transformed microbial count data.

Taking the logarithms of numbers also makes the characteristics of data fit better and overcomes the problems associated with non-normal distribution. To assess whether normality has been achieved, a graphical approach is usually more informative than a formal statistical test. For instance, a normal quantile plot is commonly used to assess the fit of a data set to a normal population. Alternatively, rules of thumb based on the sample skewness and kurtosis have also been proposed, such as having skewness in the range of −0.8 to 0.8 and kurtosis in the range of −3.0 to 3.0. A more in-depth explanation is provided by Sandle [7].

20.6 The use of alert and action levels and the setting monitoring limits

Many microbiological methods have specifications, particularly those contained within the pharmacopeia. However, for environmental monitoring, it is typical for the user to set alert and, for nonsterile products, action levels. With environmental monitoring, such levels are not specifications. They are indicators of change and are used for trending purposes and for initiating investigations as required [8].

Alert and action levels can be defined as:

- *Alert level*: a level, when exceeded, indicates that the process may have drifted from its normal operating condition. This does not necessarily warrant corrective action but should be noted by the user.
- *Action level*: a level, when exceeded, indicates that the process has drifted from its normal operating range. This requires a documented investigation and corrective action.

It is important to note that for environmental monitoring, water testing, and other bioburden determinations, we are using the term "level" rather than "limit." Limit is a more absolute term implying pass or fail, accept, or reject. This is appropriate for the sterility test, but meaningless for environmental monitoring where the trend is of prime importance rather than an individual count (which is rarely of significance, due to the variability in microbial counting and the effect of standard error. This is increasingly important when the action level values are small).

There are two approaches for setting alert and action levels. The first is the long established approach of having fixed values where sample results below the value are considered to be satisfactory and sample results at or above the value are considered, as set by both the US Food and Drug Administration (FDA) and within EU good manufacturing practice (GMP), to be excursions. For example, the action level for Grade B/ISO class 7 active air-samples is 10 colony-forming units (cfu)/m^3. Ignoring trend monitoring for the time being, if our results are below the value of 10 they are satisfactory. If our results are at or above 10, they are considered to be unsatisfactory, and an "action" is expected (normally an investigation). For nonsterile facility environmental monitoring, such an action level would need to be set, and for both sterile and nonsterile area environmental monitoring, alert levels need to be calculated.

There are no preset rules for the calculation of alert and action levels. The precise techniques and quantities of data to be used will depend upon several factors, which may include:

- the length of time that the facility has been in use for;
- how often the user intends to use the limits for (i.e., when the user intends to re-assess or re-calculate the limits. Is this yearly? Two yearly? And so on);
- custom and practice in the user's organization (e.g., is there a preferred statistical technique?).

The principles behind the calculation of alert and action levels are:

- they be calculated from an historical analysis of data. The quantity of historical data to be used is something the user will need to define. This can be time based or for a set minimum number of samples. To look at data meaningfully, a reasonably larger number of observations is also required in order for the data set to be representative. So, it is recommended that any analysis is, as a minimum, 1 year or 100 results;
- this should use some type of statistical technique. Statistical techniques are commonly divided into parametric and nonparametric techniques. The difference here is that parametric refers to a procedure that sets out to test a hypothesis about a parameter within a population described by a certain distributional form, which is typically normal distribution. Therefore, parametric methods only really apply to data sets that are normally distributed. An example of a parametric technique is Student's *t*-test;
- three statistical techniques for assessing monitoring levels are common: percentile cut-off, the normal distribution, and negative exponential distribution approaches.

20.6.1 Percentile cut-off

For low count data (such as EU GMP Grade A/ISO 14644 class 5 environmental monitoring, or from a water-for-injection system), percentile cut-off approach is most suitable (and the easiest to apply). Percentiles are sets of divisions that produce exactly 100 equal parts in a series of continuous values. In order to calculate percentiles, the data must be collected, sorted, and ranked from lowest to highest.

In selecting percentile cut-off values, typically, the warning level is set at the 90th or 95th percentile, and the action level set at the 95th or 99th percentile. Thus, if the 90th percentile is selected, this means that any result above the 90th percentile is 90% higher than values typically collected over the past year (or whatever the data selection period was). There is no easy guide as to the appropriate percentile to select. In this author's experience, it is more common to use the 95th percentile for the alert level and the 99th percentile for the action level. This selection is based on the level of risk that the user wishes to build into the system. A further consideration is whether the microbiologist wishes to round up or down to the nearest zero or five. By doing so, this may make it easier for those using the levels to implement them. Laboratory staff might find it easier to recall and recognize alert and action levels of 10 and 20, as opposed to 8 and 22.

Where data are of a broad range, an alternative approach is to group the data into categories using frequency distribution, for example, 0–10, 11–20, 21–30, and so on. When the category closest to the percentile cut-off is selected, either the mid-point of

the category or the upper value of the category can be selected. For example, if the 21–30 category represents the 95th percentile, the action level selected maybe 25 or 30 depending upon the predefined criteria adopted.

To calculate the 95th and 99th percentile, the best way is to copy the data into MS Excel and to use the "PERCENTILE" function. This is:

$$= \text{PERCENTILE}(\text{array}, p)$$

20.6.2 Standard deviations/negative exponential distribution

For higher count data (such as active air-sample counts at Grade D), either standard deviations (if there is normal distribution) or negative exponential distribution (for skewed data) is employed. Unlike the percentile cut-off approach, this technique uses the mean count and observes the spread (or variance) of the different observations. For these approaches, the alert level is equivalent to two standard deviations and the action level to three standard deviations of the mean.

Where the data appears normally distributed, or if a successful data transformation step such as taking the square root or logarithm has been employed, standard deviations can be used to calculate monitoring levels (although there is a danger that inaccuracies can creep in). A common mistake is to produce a histogram and use the second (for the alert level) and third (for the action level) standard deviations. This is incorrect because in doing so this approach has an inherent two-tail probability built into it.

Negative exponential distribution (a term for negatively skewed data) provides reasonable approximation of normal distribution. By multiplying the mean by 4.6, an approximation of the 99th percentile is produced and by multiplying the mean by 3.0, an approximation of the 95th percentile is produced.

20.6.3 Frequency approach

An alternative approach is to use a frequency cut-off approach. This is often applied for aseptic filling operations. The rationale for this approach is that setting alert levels of <1 or 0 cfu, with action levels at 1 or 2 cfu is scientifically incorrect because neither air-sampling technologies nor current (or anticipated) microbiological methods support these requirements. For instance, there are no standard methods for air sample collection, and variability is comparatively high, based on the metrology and analytical capability of the method. Moreover, there is no data on limit of detection of environmental sampling methods (zero does not mean absence of contamination, it merely means below the level of detection at that point in time) [9].

Another complication is that, at very low recovery levels, there is no agreed way to establish alert or action levels statistically, because the counts are simply too low to make statistical analysis useful. On this basis, it a count of 1 cfu is not significantly different from a count of 10 cfu (indeed some scientific literature suggests that ± 0.5 log is a reasonable assumption of variability).

To overcome this, the second approach to assess areas on the basis of the frequency of distribution of counts and for an "action" to be set if the level of

incidences (or "contamination events") exceeds a certain level. With sterile processing areas, the frequency is based on the acceptable level of nonzero counts. Thus, there is an expectation that contamination rate events for aseptic processing should be infrequent.

20.6.4 Problems with limits setting

There are a number of problem areas associated with the calculation of alert and action levels. These include:

- selection of an insufficiently small set of data so that the norm of the process was not captured;
- the set of data was large but for different reasons special causes resulted in it not being typical over a longer period of time;
- whether levels should be lowered or increased following each review if the data set indicates a change in direction. Here it needs to be firmly established if any change in the historical data is due to a special cause or a common cause. If the conclusion is special cause, and these have been corrected, the monitoring levels should probably *not* be changed. Whereas, if changes are due to a common cause the monitoring levels should probably be changed. Even if a common cause is established, it may be prudent not to change unless the trend appears over 2 years. The purpose of reviews should not be to drive limits upwards or downwards without very strong reasons.

It must not be forgotten that by setting monitoring levels based on the premise that 95% and 99% of the data falls within and that 5% and 1% of the data falls without. Therefore, occasional excursions from these levels are to be expected for the data that is gathered and trended over the next year, and some action level excursions will always be expected *if* the data set used for the calculations was truly representative.

It should be noted that for several practitioners, the relevance of having an alert limit is questioned, especially when applied to nonsterile manufacturing. In many instances, if an excursion occurs it will usually go from a "normal level" to action without an increased trend to the alert level.

20.6.5 The need to set monitoring limits

In contrast to sterile manufacturing, here are no major regulatory standards for the setting of limits for nonsterile manufacturing.

The setting of monitoring limits for nonsterile manufacture may involve an assessment of:

- the chemical composition of the product;
- the production process;
- the route of application;
- intended use of the product;
- the delivery system of the product;
- the type of anti-microbial preservative.

When setting limits, many of the principles for sterile manufacturing are relevant.

20.7 Data reporting

Whichever method of reporting is selected, microbiological data must be presented, interpreted, and summarized so that senior management can understand the trend and the "big picture." This data needs to be presented at the correct frequencies (that is not too often or too infrequently). There is no right or wrong way to present data. However, a clear and simple approach is often the most useful. This can include:

- the use of graphs and tables. This allows the trend of one area to be compared with another and for informed questions to be asked;
- each test should be reported separately. Multiline graphs and the use of more than one scale on a graph are generally confusing;
- focus on each filling room or main operation separately. It is often useful to compare different areas, but it is confusing to attempt this on one graph;
- include all of the available data. It is important to select the time period over which the data should be collected and plotted (typically, this will be monthly, quarterly, yearly or, occasionally, over a longer term). Once a time period has been selected, data must never be excluded;
- include warning and action limits on graphs. A trend can sometimes be misleading. It is important to understand how a trend relates to the monitoring levels applied;
- include appropriate information with tables and graphs. This helps to identify patterns and possible reasons for a given trend. Such information includes:
  1. locations,
  2. dates,
  3. times,
  4. identification results,
  5. changes to room design,
  6. operation of new equipment,
  7. shift or personnel changes,
  8. seasons,
  9. heating, ventilation, and air conditioning (HVAC) problems (e.g., an increase in temperature).

20.8 Conclusion

The chapter has considered the variables associated with microbiological data and has outlined how such data is of a skewed distribution and how it does not lend itself to straightforward statistical analysis. Knowing this variation is essential for the interpretation of data relating to microbial counts. This is also necessary for running control charts. There are three general approaches for control charts: histograms, cumulative sum charts, and the Shewhart charts [10].

The chapter has then taken this distribution concept and applied it to data trending and to assigning microbial limits (where no compendial or regulatory limits exist.) Data trending is necessary for microbial count interpretation because individual results from microbiological monitoring are rarely of significance when examining the totality of data gathered. This approach is of importance for exercises such as

environmental monitoring or water testing. What is of importance is the direction of the trend that the data is taking over time and the alert levels applied to indicate deviations from the norm.

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