Bioburden determination

7

7.1 Introduction

This chapter is concerned with the examination of products (finished and intermediate) and devices for bioburden. Bioburden is a term used to describe the microbial numbers on a surface (or complete item) or inside a device or from a portion of liquid. In the lexicon of microbiology, the term "bioburden" is somewhat misleading as "burden" implies that the level of microorganisms is automatically a problem or concern; whereas, in practice, the objective of qualitative or a quantitative testing is to ascertain if the types and numbers of microorganisms present are satisfactory or unsatisfactory when compared to predefined acceptance criteria [1].

In some literature, bioburden testing relates to raw materials testing, environmental monitoring, or in-process sample testing. These areas will have a given, even "natural" bioburden. When this bioburden rises above typical levels or ends up in the wrong place, then arguably the appropriate term to use is biocontamination [2]. Biocontamination, therefore, differentiates the population of microorganisms present as a problem, as distinct from the typical bioburden.

While bioburden can apply to the assessment of a number of microbiological attributes, in this chapter, bioburden will be limited to the assessment of nonsterile products, in-process samples, and the assessment of product prior to sterilization. Thus, water testing and environmental monitoring are considered to be separate areas of microbiological monitoring, and they are described in separate chapters (although the reader should note that, in some literature, the term "bioburden" can embrace these types of testing).

In relation to the areas considered, this chapter looks at bioburden testing as a measure of the total number of viable microorganisms: that this, the total microbial count. Some reference is additionally made to the species of microorganism recovered (although assessing whether a species is potentially harmful is the subject of Chapter 8).

7.2 Total microbial count

The term "total microbial count" can refer to the total number of bacteria and fungi present or to the total number of bacteria. This confusion with the term has been enhanced by a pharmacopoeial chapter called the "microbial limits test" (which is harmonized between the European, US, and Japanese pharmacopeia). The chapter describes a "total aerobic microbial count" (TAMC), which refers to bacteria only, and a "total yeast and mould count" (TYMC; which refers to fungi only). The two aspects of the microbial count—TAMC and TYMC—involve testing a sample on different agar and subjecting the tests to different incubation conditions [3]. In past editions of the

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pharmacopeia, the bacterial count and fungal count were added together to produce a total microbial count. However, since 2005, the pharmacopeia requires the two to be reported separately and assessed against specified limits.

Outside of the specifics of pharmacopeia testing, where bioburden is assessed as a quality control test to monitor a product manufacturing process (as with the case of in-process bioburden testing) invariably only one culture medium is used, and a total microbial count is assessed. This is conventionally referred to as either "total microbial count" or "total viable count." The latter term attempting to distinguish between live and dead microorganisms, or, more accurately those microorganisms that are capable of growing on the culture media used under the incubation conditions of the test. Viable is defined as the ability to multiply via binary fission under the controlled conditions. In contrast, in a microscopic evaluation, all cells, dead and living are counted.

Another alternative term is "total viable aerobic count" (or "total viable anaerobic count," depending upon the atmospheric conditions deployed during incubation). To an extent, these terms are interchangeable; what matters foremost is the method used to conduct the test. Knowing this makes the resultant data easier to interpret.

This discussion is not intended to be tautological, more to emphasize that there can be ambiguity in relation to the terms used to describe bioburden testing and clear definitions should be sought from the outset, especially when comparing test results between laboratories.

7.3 Units of measurement

With most methods of bioburden determination, the bioburden quantification is expressed in terms of colony forming units (CFUs). An exception to this is with the most probable number (MPN) method. Furthermore, Chapter 8 discusses the additional testing of samples for the presence or absence of specific microorganisms (this, when so required, and carried out according to the pharmacopeia is conducted as part of the microbial limits test). Here these microorganisms may or may not be enumerated for it may be sufficient, or the test itself is limited to, to note whether a particular microorganism of concern is present or not.

The CFU is an estimate of the number of viable bacteria or fungal cells in a sample. The visual appearance of a colony in a cell culture requires significant growth. With this it is unknown if the progenitor of the colony was one microorganism or several microorganisms. Hence, when counting colonies it is uncertain if the colony arose from one cell or 1000 cells, and importantly CFU is not a direct measure of microbial numbers. Results expressed in CFU are reported to a unit of measurement. Hence, results can be reported as colony-forming units per milliliter (CFU/mL) for liquids, or colony-forming units per gram (CFU/g) for solids to reflect this uncertainty (rather than cells/mL or cells/g). Therefore, an estimation of microbial numbers by CFU will, in most cases, undercount the number of living cells present in a sample [4].

Inaccuracies can also occur with the act of plate counting. Due to the size of the agar plate, there will be an optimal counting range, and errors will occur where microbial

numbers are above an upper countable limit (due to confluence or overcrowding) or below a lower limit (due to statistical error in relation to accuracy of the count, particularly where dilutions have been performed) [5]. A further source of error can arise with rounding up or down or through averaging. For a comprehensive review of plate counting errors, Sutton has produced a seminal paper on the subject [6].

Another source of inaccuracy is due to many microorganisms in the environment being unculturable. By this, "unculturable" means, there are viable microorganisms present (and capable of renewed metabolic activity) but of a type that cannot grow on culture media (or at least the culture media and incubation conditions provided for by the test) [7]. The term can also apply to microorganisms that might, under a different set of conditions, grow on the medium, but they cannot because they have undergone physiological stress (such as starvation, elevated osmotic concentrations, exposure to white light and so forth), or they have been rendered sublethally damaged; conditions that prevent them from growing [8]. For these microorganisms, terms such as "viable but non-culturable" or "active but non-culturable" are deployed [9]. Numerous bacteria, both Gram-positive and Gram-negative, pathogenic and nonpathogenic, can enter such a state [10].

The count obtained is also dependent upon the extent that culturable microorganisms can grow under the test conditions. This is a factor of the type of culture media used, the atmospheric conditions, the temperature of incubation, and the time that the cultivated medium is incubated for. Importantly, no testing scheme can detect everything. Thus, tests and test results have the objective of providing the best indicator possible of the microbial bioburden *but not* the absolute bioburden. In many cases, test regimes are biased toward aerobic, mesophilic microorganisms. This is because such organisms are common to the environment; they will often be a problem should they contaminate the product since they are the most likely to grow; and because most human pathogens fall within this grouping [11].

Inaccuracies and error can occur with colony counting. The counting of colonies manually is normally carried out using an artificial light source, such as a colony counter. As an alternative, several automated colony counters are available. Automated systems can be difficult to validate and can experience problems when attempting to differentiate plates with colonies from a range of different microbial species [12].

7.4 Nonsterile products and microbial limits testing

The microbiological quality of the finished product is determined by the quality of the starting materials; materials with a known low bioburden should be purchased whenever possible. These are, in most cases, "nonsterile" materials or products. Such materials are either used to manufacture more complex pharmaceutical products (such as tablets, creams, or ointments) or used in the preliminary stages of what will become sterile products.

Nonsterile products are assessed, according to the harmonized pharmacopeia, for total count (a separate bacterial and fungal count is required). In addition, for some

products, additional testing is required for indicator microorganisms. This is covered in Chapter 8 (the chapter extends the discussion of prescribed indicator microorganisms to any additional ones that are considered to be "objectionable" based on a risk assessment).

With the pharmacopeia, if a material monograph requires a test for microbial limits, then Ph. Eur 2.6.12 or USP <61> is applied (what is termed the microbial limits test) [13]. With such products or constituent raw materials that are used for the manufacture of sterile products, the microbial limit for these materials must not exceed 10^2–10^3 microorganisms per milliliter (as set out in the harmonized pharmacopeia, with the USP this is chapter <1111> and with the European pharmacopeia this is Chapter 5.1.4). Raw materials are defined as those substances that can be brought into a manufacturing unit either for further processing or to aid in such processing. In the microbiological control of pharmaceutical raw materials, there is one primary aim—to exclude any microorganism that may subsequently result in deterioration of the product or may harm the patient.

The compendial microbial limits test is made up of two parts [14]:

- TAMC. This is an estimation of viable aerobic mesophilic microorganisms that can be derived from a general purpose medium (the pharmacopeia recommends soya bean casein digest medium).
- TYMC. This is an estimation of mesophilic aerobic fungi (yeast like and filamentous, and those that are dimorphic). The test uses a general purpose fungal medium (the pharmacopeia recommends Saboraud dextrose agar).

The bioburden test is either one or both of the compendial TAMC or TYMC methods, or an alternative. For the examination of the microbial count, there are four recommended methods. These methods are outlined below. The methods described are the variants according to the pharmacopeia, which are required to claim that the microbial limits test has been conducted. Where the pharmacopeia is not required to be followed (such as for in-process bioburden testing, as discussed later), then variations to these methods can be conducted if appropriately justified.

7.4.1 Membrane filtration

This is the method of choice and should be applied to samples that contain antimicrobial substances. With the method, the sample is passed through a membrane filter with a pore size of 0.45 µm or less. Filters about 50 mm across are recommended, but other sizes may be used. Usually, the test measures two test fluids of 10 mL each, passing each sample through a separate filter. It is important to dilute the pretreated test fluid if the bacteria concentration is high, so that 10–100 colonies can develop per filter. After filtration, each filter must be washed three times or more with an appropriate liquid such as phosphate buffer, sodium chloride-peptone buffer or fluid medium. The volume of the washings should be about 100 mL each. If the sample includes lipid, polysorbate 80 or an appropriate emulsifier may be added to the washings.

After filtration, for bacteria detection, the two filters must be placed on a plate of soybean–casein digest agar medium, and for fungi detection, an antibiotic is added to

the medium and placed onto a plate of one of the Sabouraud glucose agar. Plates are incubated for at least for 5 days at 30–35 °C for bacteria detection and at 20–25 °C for fungi detection. At the end of the incubation period, the number of colonies is counted.

7.4.2 Direct plating methods

There are two direct plating methods: pour plate and spread plate. Of the two, the pour plate is preferential because of a greater theoretical accuracy.

7.4.2.1 Pour plate method

With the pour plate method, Petri dishes of 9–10 cm in diameter are used, with two agar media used for each dilution. For the test:

- Take 1 mL of the test fluid or its dilution into each Petri dish aseptically, add to each dish 15–20 mL of sterilized agar medium, previously melted and kept below 45 °C, and mix (45 °C is just above the point of solidification to minimize heat-induced cell death).
- For bacteria detection, use soybean–casein digest agar medium and for fungi detection, use Sabouraud glucose agar media, to which antibiotic has previously been added.
- After the agar solidifies, incubate at least for 5 days at 30–35 °C for bacteria detection and at 20–25 °C for fungi detection. If a large number of colonies develop, calculate viable counts based on counts obtained from plates with not more than 300 colonies per plate for bacteria detection and from plates with not more than 100 colonies per plate for fungi detection.

7.4.2.2 Spread plate method

With the spread plate method:

- Place 0.05–0.2 mL of the test fluid on the solidified and dried surface of the agar medium and spread it uniformly using a spreader.
- Proceed under the same conditions as for the pour plate method, especially with regard to Petri dishes, agar media, incubation temperature and time, and calculation method.
- A variant, not described in the pharmacopeia, is the drop-plate method (or Miles and Misra method), wherein a very small aliquot (usually about 10 µL) of sample from each dilution in series is dropped onto a Petri dish [15].

7.4.3 Most probable number method

The MPN method (alternatively, the method of Poisson zeroes) is a method of obtaining quantitative data on concentrations of discrete items from positive/negative (incidence) data. The method involves taking the original solution or sample, and subdividing it by orders of magnitude (frequently 10× or 2×) into culture broth, and assessing the presence/absence in multiple subdivisions. The major weakness of MPN methods is the need for large numbers of replicates at the appropriate dilution to narrow the confidence intervals [16]. The MPN is only effective of the examination of bacteria, as it does not provide reliable results for the enumeration of fungi.

To determine the accuracy and sensitivity of the test methods used for microbial limit testing, 10 g or 10 mL samples of the test material are examined. It is also important, when conducting these tests, to ensure that the testing method does not either introduce bacteria into the test sample or kill bacteria in the test sample [17]. Furthermore, the dilution of microbial challenges needs to be as precise as possible [18].

7.4.4 Method verification

Method verification is an important step. While the total count method is a "compendia test," which means that, by convention, the test itself does not require validating, the suitability of each material must be qualified to show that the test method is not inhibitory and that any microorganisms present can be recovered. This assessment is particularly important for samples that have antimicrobial activity.

When test samples have antimicrobial activity or when they include antimicrobial substances, these antimicrobial properties must be eliminated by dilution, filtration, neutralization, inactivation, or other appropriate means. The tests should be conducted for samples prepared by mixing multiple portions randomly chosen from individual ingredients or products. When samples are diluted with fluid medium, the tests must be conducted quickly. Due attention must be paid to the effective quality control and the prevention of biohazard.

The compendial chapters outline useful information for the development process as well:

- if the product contains antimicrobial activity, this should be neutralized;
- if inactivators are used, their efficacy and their absence of toxicity for microorganisms must be demonstrated;
- common neutralizing agents and methods include the addition of polysorbate or lecithin, and/or dilution methods.

7.5 In-process material bioburden assessment

The environmental and process bioburden should be monitored to ensure that they are both within acceptable limits. Environmental monitoring is addressed in Chapter 16; the focus here is the bioburden monitoring of intermediate product as the product moves through the manufacturing stages ("in-process" monitoring). The requirement for this step is outlined in the Code of Federal Regulations. 21 C.F.R. 211.110 (a)(6), which states that bioburden in-process testing must be conducted pursuant to written procedures during the manufacturing process of drug products.

EU good manufacturing practice (GMP) does not specifically address in-process sampling. Nonetheless, selecting and examining samples for bioburden determination using a total viable count method is commonplace. With bioburden testing, an appropriate test method should be selected. Here either membrane filtration (using

a 100 mL sample) or the pour plate (using a 1 mL sample) are the most common methods. The pharmacopoeial methods, as outlined above, do not need to be followed (e.g., it is more common to use one general purpose agar, such as soybean–casein digest medium, rather than two different agars and to incubate samples between 20 and 35 °C). Whichever method is adopted, it is important that the method is assessed as suitable. Here, as with the microbial limits test, an assessment must be made of the presence of any antimicrobial substances.

In establishing an in-process bioburden regime, appropriate limits should be set (in the form of "alert" and "action" levels). The levels can be defined as [19]:

- Alert level: a level that, when exceeded, indicates a process may have drifted from its normal operating condition. Alert levels constitute a warning, but do not necessarily warrant corrective action.
- Action level: a level that, when exceeded, indicates a process has drifted from its normal operating range. A response to such an excursion should involve a documented investigation and corrective action.

These limits should decrease as the process moves downstream. While limits will relate to specific processes, a general guideline is 100 CFU for the start of the process and 10 CFU near the end of the process (per 1 mL of per 100 mL). With sterile products, where a liquid is subject to a terminal sterilizing filter, within Europe there is a requirement for the liquid to contain no more than 10 CFU/100 mL.

When setting "alert" and "action" limits, it is good practice to:

- base levels on historical data;
- use means and/or standard deviations;
- perform continued trend analysis and data evaluation to determine if the established levels remain appropriate;
- watch for periodic spikes, even if averages stay within levels.

In addition to the monitoring of in-process samples, steps should be taken to ensure that control is maintained. Where known risks are apparent, specific process steps should be included to reduce these potential risks to a level consistent with the level of control required. Keeping control means that alert and action level excursions should be investigated. At the alert level, the following items could be considered, although generally no action or investigation is required:

- numbers and types of routine bioburden trends (product and environment);
- identification of recovered microorganisms;
- evaluation of microorganism for resistance to the sterilization process;
- production personnel impact (e.g., proper training or new personnel);
- manufacturing process changes;
- sampling and testing procedures changes;
- evaluation of laboratory controls and monitors;
- additional testing;
- thorough cleaning of production area;
- modification of sampling plan;
- raw materials and supplier changes;
- water-source contamination.

At the action level, the above-mentioned items are usually required in addition to the following:

- root cause analysis/investigation;
- determination of potential impact on sterilization specifications.

7.6 Presterilization bioburden assessment

For sterile products, bioburden assessment is a key requisite prior to sterilization. This is necessary of terminally sterilized products and products that are to be aseptically filled.

7.6.1 Terminally sterilized products

With terminally sterilized products, understanding the bioburden is necessary because the extent of the treatment of a sterilization process is a factor of the typical bioburden on or in the product; the resistance of the microorganisms that make up the bioburden; and the sterility assurance level required [20]. The test is important because an underestimation of the bioburden population could result in a miscalculation of the sterilizing requirements for a given product; in contrast, an overestimation could result in excessive exposure to the sterilizing agent, which in turn could affect the quality of the product.

At the stage prior to terminal sterilization, bioburden can be regarded as the sum of the microbial contributions from a number of sources including raw materials, manufacturing of components, assembly processes, manufacturing environment, assembly/manufacturing aids (such as compressed gases, water, and lubricants), cleaning processes, and packaging of finished product.

All of this matter because during the assessment of the suitability of the sterilization process, each product will have been assessed against a particular sterilization method (such as dry heat, most heat, radiation, or gas) for a particular dose (such as temperature or radiation dose) and for a given time. This assessment will have been made either by testing various representative items of the product for bioburden or using biological indicators of a known population, species and resistance. If the bioburden on or in a given product exceeds the bioburden assessed during the initial qualification then, theoretically, some microorganisms might survive.

Whether assessing the bioburden alone is a sufficient control measure is something the microbiologist must decide. If by designing the manufacturing process, the presterilization bioburden is kept consistently and reliably at very low numbers, a direct bioburden-based cycle may be a possible approach. Bioburden-based cycles are where the bioburden is assessed prior to each individual sterilization cycle being run. This is as a replacement to the use of biological indicators [21].

7.6.2 Aseptically filled products

With aseptic processing, one of the most important samples is taken from the bulk material prior to transfer through a sterilizing grade filter in preparation for aseptic filling. The filters used are generally of a pore size of 0.22 µm, configured in a

series [22]. The "sterilizing filter" was defined in 1987 by the US Food and Drug Administration (FDA) on the basis of its retaining a minimum of 1×10^7 CFUs of *Brevundimonas diminuta* per square centimeter of effective filtration area (EFA).

Within Europe, there is a requirement for the challenge liquid to contain no more than 10 CFU/100 mL. In other territories, the permitted number of microorganisms is determined by risk assessment.

While bioburden assessment is important, aseptic processing carries continued risks. The sterile filtered liquid must subsequently be dispensed into sterile containers under a protective airflow. At this stage, contamination can be introduced if controls are not properly maintained.

7.6.3 Medical devices

A third area for bioburden determination prior to sterilization is in relation to medical devices. As with terminally sterilized pharmaceutical products, the objective is to ensure that the presterilization bioburden is below that used to qualify the sterilization process. Bioburden testing for medical devices made or used in the United States is governed by Title 21 of the Code of Federal Regulations and worldwide by the standard ISO 11737.

Bioburden control with medical devices, as described in ISO 11737, consists of five key steps. These are:

1. sample selection;
2. the process of removing any microorganisms from the sample;
3. transfer of microorganisms to recovery solutions;
4. enumeration of microorganisms;
5. data interpretation (with the application of correction factors, where necessary).

7.7 Alternative methods of bioburden assessment

As with many established fields of microbiology, rapid and alternative microbiological methods are available. Alternative methods include the use of polymerase chain reaction (PCR) assays, where deoxyribonucleic acid (DNA) can be extracted and examined using specific primers. The advantages conferred by PCR are specificity toward target microorganisms and faster time-to-result [23]. A different approach is with fluorescent-based technology, where cells are labeled and fluorescence detected through imagers. The reaction requires active microbial metabolism for enzymatic cleavage of a nonfluorescent substrate. Once cleaved inside the cell, the substrate liberates free fluorochrome into the microorganism cytoplasm. As fluorochrome accumulates inside the cells, the signal is naturally amplified. The cells are then exposed to the excitation wavelength of the fluorescent dye in a reader so that they can be visually counted. A variation upon this is with the use of digital imaging technology that automatically enumerates microcolonies earlier than the traditional visual plate counting methods allow. Such systems capture the native fluorescence (autofluorescence) that is emitted by all living cells.

7.8 Conclusion

This chapter has considered the subject of bioburden (the degree of microbial contamination or microbial load; the number of microorganisms contaminating an object) and bioburden testing. As the chapter has discussed, "bioburden" is not a straightforward concept. Complications arise in relation to the recovery of microorganisms and the fact that the "CFU" is a mere estimation of the numbers of microorganisms present.

Bioburden testing is applied to materials and products as per the internationally harmonized pharmacopeia under the guidelines pertaining to the microbial limits test. Here methods and agars, along with incubation conditions, are precisely defined. Beyond the pharmacopoeial test, bioburden assessments are required for intermediate product (in-process material) and, with sterile products, at the point closest to sterilization. Thus, bioburden estimation stands as an important and necessary part of pharmaceutical microbiology.

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