Settle plate exposure under unidirectional airflow and the effect of weight loss upon microbial growth

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Settle plates play an important part in the environmental monitoring programme and for the assessment of microbial settlement at key locations within cleanrooms, particularly when situated within unidirectional airflow devices. It is important that the exposure time of the settle plate is assessed to ensure that the proportion of weight loss (through the loss of moisture) does not result in a loss of growth-promoting properties. A second important concern is with avoiding cracks in the agar which might render reading sections of the exposed plate impossible. This paper outlines a case study to assess the exposure time through microbial growth promotion.

Key words: Environmental monitoring, cleanrooms, unidirectional airflow, settle plates, agar, microorganisms, microbial recovery.

Introduction

Environmental monitoring forms an important part of the bio-contamination control of pharmaceutical facilities. One element of this concerns the assessment of airborne microorganisms. Monitoring the airborne contamination level of unidirectional airflow (UDAF) devices used for aseptic filling is an important part of environmental monitoring. There are two ‘standard’ methods to assess the microbiological quality of the air: active (volumetric) air samplers and settle plates. Both methods, although seeking to assess microbial airborne contamination, assess something different. An active air sampler estimates the number of microorganisms, free-floating or carried on particles within a given size, within a cubic metre of air. Whereas the settle plate provides an indication of any microorganisms which might settle out of the air due to gravitational effects. Here, most microorganisms are associated with physical particles which are large enough to settle out of the air-stream due to gravity (refer to Whyte, 1986). Additionally, settle plates can provide information about interventions into the unidirectional cabinet, provided that the plate is in a representative location, as the person working within the critical zone could potentially deposit microorganisms into the air stream.

Settle plates typically consist of Petri dishes filled with a culture medium, such as tryptone soya agar (TSA), which is a general purpose medium. The amount of culture medium in a 90 mm diameter settle plate is typically 20–30 mL. Sometimes plates of a 140 mm diameter are used in order to provide a larger surface area. The work summarised in this paper refers to 90 mm diameter plates only.

Agar is a polymer made up of subunits of the sugar galactose, and is a component of the cell walls of several species of red algae (Class Rhodophyceae, of which the species Gelidium is the preferred choice of agar manufacturers) that are usually harvested in eastern Asia and California. Laboratory agar has a gelatinous appearance and the gel is maintained at room temperature. Agar is typically used in a final concentration of 1–2% for solidifying culture media, although different agars have different gel strengths. Agar has traditionally been used to grow bacteria rather than gelatine because the majority of bacteria will not degrade the agar, as would be the case with gelatine-based media. Specifications for bacteriological grade agar include good clarity, controlled gelation temperature, controlled melting temperature, good diffusion characteristics, absence of toxic bacterial inhibitors and relative absence of metabolically useful minerals and compounds.

TSA is a medium which contains enzymatic digests of casein and soybean meal, which provides amino acids and other nitrogenous substances making it a nutritious medium for a variety of organisms. To this, dextrose is added to provide the energy source and sodium chloride to maintain the osmotic equilibrium, whilst dipotassium phosphate acts as a buffer to maintain pH. These ingredients are added to agar which acts as the gelling agent.

Settle plates are exposed in aseptic filling zones, at determined monitoring locations, ideally positioned and exposed either side of the main activity in the room or UDAF cabinet, where the lids of the dishes are removed. The settling rate depends partly on the characteristics of the particles and

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on the airflows. Larger particles will tend to settle faster (due to gravitational effects) and settling is facilitated by still airflows (which should not occur within a correctly designed UDAF zone). Smaller particles have a lower tendency to settle due to air resistance and air currents. The principle behind settle plates is that most microorganisms in air are in association with particles. Generally the ‘complete particle’ (microorganism in association with the ‘carrier’) is 12 µm diameter or larger.Outside of unidirectional air, such as a cleanroom, there is a greater degree of turbulence. The amount of air turbulence is proportional to the amount of time that particles remain suspended in the air. Thereby, the greater the amount of air turbulence the longer the particles will remain suspended in the air. This can influence the reliability of the settle plate; however, the additional use of active air samplers provides a further monitoring method for assessing the cleanroom cleanliness.

Settle plates are typically exposed for periods of up to 4 hours. An exposure time of 4 hours is recommended in the EU Guidelines to Good Manufacturing Practice, although individual settle plates may be exposed for less than 4 hours (and for pharmaceutical operations subject to other regulatory requirements, alternative exposure times may be considered.) The origin of the 4-hour time period is not recorded; although the reasoning will have included some attempt to quantify the deposition rate, as with x microorganisms being detected at a given location per hour(s) of exposure. Thus, the results from settle plates are normally recorded in terms of the number of colony forming units (CFU) settling per 4 hours of exposure.

Guidelines, such as the EU Guidelines to Good Manufacturing Practice, express recommended levels as CFU/4 hours. It is important to note that a CFU, as defined, can consist of one microorganism, or a pair, chain or bunch of microorganisms. Therefore, with the interpretation of settle plate data, the actual ‘value’ obtained, such as 1 CFU on a settle plate, could represent one microorganism or several microorganisms that were carried on a raft of skin detritus. Such uncertainty restricts the interpretative value of the settle plate.

Another major variable is the performance of the agar medium as this is likely to be affected by water loss (desiccation) over a 4-hour exposure period. The process of desiccation can be considered in terms of water loss or in terms of reduced access to moisture due to the formation of a ‘skin layer’ on the agar surface. Reduced access to moisture will reduce the growth-promoting properties of the culture medium leading to a failure of the plate to grow some or all of the microorganisms which might settle onto it. This can lead to an underestimate of the number of microorganisms through loss of culturability or viability, because different species respond to the sampling stress differently. Desiccation tolerance in relation to microbial survival is linked to a switch to a metabolically inactive state as well as the ability to repair protein oxidation and DNA damage upon rehydration. Recovery of such organisms is also influenced by the quality of the agar used to revive them.

It is incumbent upon each user of settle plates to assess the impact of agar desiccation. This will vary depending upon how the medium is manufactured (and the consistency of the manufacturing process); how the plates are stored before use; the agar fill volume; as well as the environment in which the plate is exposed. The weight loss tends to be greater when plates are exposed under a UDAF cabinet compared with exposure within a turbulent flow cleanroom. This difference is a result of the air velocity from the unidirectional environment. A related factor is particle bounce, which, although a more significant factor with active air samplers, can also accelerate the ‘drying’ of agar.

The type of agar and the incubation conditions will also affect the types of microorganisms recovered. Importantly, there is no set of ‘universal’ conditions, although the microbiologist can take steps to ensure that the agar is suitable for the types of microorganisms expected from cleanroom environments, especially in relation to aseptic processing. Research suggests that such microorganisms are typically Gram positive, with human skin commensals, such as the Staphylococci and Micrococci, representing the overwhelming majority of isolates. The incubation conditions are related to whether the culture medium used in the settle plate is designed to capture both bacteria and fungi or whether two plates are to be used containing a general agar for bacteria and a selective agar for fungi. Where a single culture medium is used, a two-step incubation is often required, such as 20–25˚C for 2–5 days followed by 30–35˚C for 2–5 days. This is an important area that requires assessment.

This paper presents a possible approach in examining the effect of desiccation for settle plates exposed within a UDAF device and summarises a study that was undertaken to assess the weight loss of settle plates.

### Study to examine the effect of weight loss

The purpose of the assessment was to show if settle plates retain the ability to support microbial growth after the maximum exposure time (which, as indicated above, is 4 hours). When designing a validation test protocol to examine the impact of weight loss, there are a number of factors which can be considered.

a) The type of culture medium.

b) The use of neutralisers in the culture medium (this may or may not be a factor depending on the application of the plates).

c) The placement of plates (locations and schedule).

d) The hydration state of the medium and the impact of this on the rate of desiccation.

e) The metabolic and physical state of any microorganism that may be deposited onto the plate surface.

f) The length of the exposure time.

g) The environment used (for example, exposure under a UDAF unit).

Considering these factors, there are different approaches which can be taken when designing a study. Three such approaches are as follows.
Approach 1
This approach is centred on the question: “should settle plates be exposed first and then inoculated with a microorganism post-exposure?” This condition demonstrates whether a plate retains the ability to support growth during the incubation period. The advantage with this approach is that exposing plates and inoculating them after 4 hours is a greater challenge because a microorganism is more likely to be deposited onto the surface of a settle plate some time during exposure rather than at the start of the exposure time. Furthermore, at the end of 4 hours the plates will have undergone maximum weight loss.

Approach 2
This second approach examines the question: “should settle plates be inoculated with a microorganism and then exposed?” The disadvantage with this approach is that it does not assess the ability of the plate to recover microorganisms at the end of the exposure time. Furthermore, inoculating the plates at the start of the incubation could potentially result in microorganisms being carried from the surface as the moisture evaporates, leading to an inaccurate challenge.

Approach 3
The third approach considers the question: “should plates be exposed first, then incubated for the maximum incubation time, and then inoculated with a microorganism to assess growth?” This is a variation of the first approach. Although it is of interest, it has too many variables to give meaningful data. For example, is the study an examination of settle plate exposure or of incubation time?

Weighing up the advantages of the different approaches, the first approach presents the greatest challenge because it accounts for physiological effects that occur during exposure and as a result of post-exposure incubation. For the example presented in this paper, the first approach was adopted.

Example study
The paper summarises a study that was undertaken at a cleanroom facility located in South-East England. The study was carried out using TSA settle plates of 90 mm diameter (approximate internal area 64 cm²), with a 25 mL fill. The plates weighed 17 g prior to the agar fill. The plates were pre-poured commercially and subject to irradiation. Prior to use, the plates were stored at 20–25°C for 5 days and were within the expiration date recommended by the manufacturer.

For the study, a combination of standardised type cultures were used (sourced from the American Type Culture Collection (ATCC)). These were the organisms recommended by the culture media manufacture for the assessment of growth promotion. These organisms were used to assess the suitability of each incoming batch. It was, therefore, deemed appropriate to use the same panel of organisms to assess the media after it had been subject to the exposure study. Moreover, these organisms generally presented the types that could be found within cleanrooms. In addition, two Gram-negative rods were added because settle plates can be used to monitor wash bays. The Gram-negative organisms selected were those described in the European Pharmacopoeia for media testing, representing Pseudomonad and non-Pseudomonad type Gram-negative organisms. Furthermore, two fungi were included. These represented the taxonomic groups filamentous fungi and yeast-like fungi, as well as being indicative of cleanroom fungi. In addition to type cultures, two environmental isolates, isolated from cleanrooms in a manufacturing facility, were included.

The microorganisms were selected to be representative of different contamination sources: people, equipment and water. The microorganisms (bacteria and fungi) used in the study were as follows.

1. Bacillus subtilis (ATCC 6633)
2. Candida albicans (ATCC 10231)
3. Staphylococcus aureus (ATCC 6538)
4. Escherichia coli (ATCC 8739)
5. Aspergillus niger (ATCC 16404)
6. Pseudomonas aeruginosa (ATCC 9027)
7. Staphylococcus epidermidis (environmental isolate)
8. Ralstonia pickettii (environmental isolate)

The inoculation level used was designed to be of a low level (that is less than 100 CFU). This is a standard microbial challenge for the assessment of growth promotion.

The first part of the study was to measure the weight loss. Under a UDAF cabinet, four plates were exposed in the approximate locations shown in Figure 1. The plates were exposed on the surface of the cabinet (at ‘working height’). The measured air velocity at the location of the plates was 0.49 m/s (this was within the range recommended in the EU Guidelines to Good Manufacturing Practice: 0.35 to 0.54 m/s).

The exposure time was 242 minutes. One set of four plates was exposed in preparation for testing against each of the eight microorganisms. Each microorganism was assessed on three occasions. Therefore, during the course of the study, 24 test sessions were performed and 72 test plates were exposed, incubated and challenged with microorganisms.

During the course of the study, plates were weighed at the following time intervals: pre-exposure (time zero); after 2 hours exposure, after 4 hours exposure and then at the end of incubation. The incubation conditions were 5 days at 20–25°C followed by 2 days at 30–35°C. This was undertaken because it conformed to the standard environmental monitoring regime applicable to the test laboratory. The weight of the test plates was compared with unexposed, but incubated, control plates.

Results
Data from one session, pertaining from one organism, is displayed in Table 1. The weight of the test plates was compared with unexposed, but incubated, control plates.
Table 1. Weight loss assessment for plates prepared for testing against one microorganism. The data relates to three control plates and three sets of four plates.

<table>
<thead>
<tr>
<th>Test</th>
<th>Plate reference</th>
<th>Weight (g)</th>
<th>Percentage weight loss*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Zero hours</td>
<td>2 hours</td>
</tr>
<tr>
<td>Control</td>
<td>A</td>
<td>40.8648</td>
<td>40.3368</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>41.4935</td>
<td>40.9675</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>40.2479</td>
<td>39.6236</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>40.87</td>
<td>40.31</td>
</tr>
<tr>
<td>Test 1</td>
<td>A</td>
<td>40.8656</td>
<td>37.6864</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>41.7061</td>
<td>38.4254</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>41.2053</td>
<td>39.3663</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>40.3667</td>
<td>38.8215</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>41.04</td>
<td>38.57</td>
</tr>
<tr>
<td>Test 2</td>
<td>A</td>
<td>40.6046</td>
<td>39.5601</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>40.2326</td>
<td>35.9606</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>41.8610</td>
<td>38.5509</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>42.7275</td>
<td>39.8943</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>41.36</td>
<td>38.49</td>
</tr>
<tr>
<td>Test 3</td>
<td>A</td>
<td>42.1058</td>
<td>39.2699</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>41.0372</td>
<td>38.1036</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>41.0017</td>
<td>38.1627</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>40.9041</td>
<td>38.9069</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>41.26</td>
<td>38.61</td>
</tr>
</tbody>
</table>

* Calculated from: \[
\frac{\text{Post-incubation}}{\text{Zero hours}} \times 100 - 100
\]
The data is summarised as the mean of all 72 exposed plates in the graph shown in Figure 2.

The graph demonstrates an initial rapid weight loss and then a steady reduction through to the end of the 4-hour exposure. A further reduction, albeit of a far smaller volume, occurred during incubation. Although the temperature and time post-incubation will vary across different laboratories, the data presented here suggests that it is not a major contributor to weight loss within the temperature range 20–35˚C over a 7-day period.

The data indicated that the typical weight loss was approximately 6 g (on average) from initial exposure to the end of the incubation. The minimum and maximum values and the standard deviations indicated that the values did not vary greatly and that most plates underwent a similar level of weight loss. In subtracting the highest recorded mean weight from the lowest mean weight (42.15–35.72 g), the maximum mean weight loss recorded was 6.43 g (or approximately 16% of the agar). The greatest weight loss from an individual plate was 20.2 g and the lowest weight loss recorded was 10.9 g. This was a relatively wide range.

The second part of the study involved testing the plates for microbial growth promotion after exposure. Following the inoculation of the plates with the microbial challenge, the plates were incubated for 3 days for plates challenged with bacteria and for 5 days for plates challenged with fungi. The microbial recovery from the test plates was compared with control plates, which were not subject to exposure. The acceptance criteria, using the US Pharmacopeia validation recommendation for microbiological examinations (chapter <1227>), was that the test plates had to recover ≥70% of the challenge. The study was repeated for each microorganism on three occasions.

The results of the study are summarised in Table 2. (The microbial counts are the mean counts across the replicate studies for illustrative purposes.) The microbial recovery data indicates that all the microbial challenges from the test plates were recovered within 70% of the control plates, with the lowest range of recoveries being recorded for the environmental isolates. This is unsurprising given that these bacteria would be in a relatively greater stressed state compared with laboratory cultures. With the successful recovery of all the microorganisms, it was demonstrated that settle plates can lose approximately 6 g (or 16%) of their weight and retain their ability to support growth.

A visual examination was made of each plate to look for evidence of cracks being formed across the agar surface. None were detected. Agar is generally resistant to shear forces; however, the addition of additives can cause chemical reactions that reduce the strength of agar. None of the plates used in the study contained additives like disinfectant neutralisers.

**Conclusion**

This paper has described a method which can be used to assess the weight loss from settle plates. Such a study is an important one for microbiologists who use settle plates to assess the environment within a UDAF setting. This is because a high weight loss may lead to the failure to grow microorganisms. Moreover, this is likely to be something that the microbiologist will need to demonstrate to a regulatory agency.

With the study presented, the air velocity remained relatively constant under the UDAF cabinet and the initial weights of the plates were within a relatively narrow range. However, the final weights of the plates were more varied, with the difference between the plate showing the greatest weight loss and the plate showing the least weight loss being around 12 g, suggesting that as the agar sets the solidification process is subject to biological variation.

Although the process of preparing agar and its use as a culture medium are much better understood, the process is still prone to variation. Culture media manufacturers will, by design (or sometimes accident), produce agars of different characteristics. Examples include processing with either a low or high temperature gelling or practising low syneresis (moisture loss). What is important is that the media has a constant gel strength to ensure good colonial growth. Different characteristics suit different purposes. For example, high gel strength media will grow small colonies because the flow of nutrients and removal of toxins is reduced; whereas low gel strength media will allow the growth of larger colonies, but can be difficult to streak. Something midway is important for settle plates – robust enough to capture colonies, however, soft enough to allow colonies to be removed for subculturing and

### Table 2. Microbial recoveries.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Test plate mean count (CFU) [A]</th>
<th>Control plate mean count (CFU) [B]</th>
<th>Percentage difference [A÷B × 100]</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus subtilis</td>
<td>52</td>
<td>64</td>
<td>81%</td>
<td>Satisfactory</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>31</td>
<td>33</td>
<td>94%</td>
<td>Satisfactory</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>44</td>
<td>43</td>
<td>102%</td>
<td>Satisfactory</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>27</td>
<td>31</td>
<td>87%</td>
<td>Satisfactory</td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>45</td>
<td>44</td>
<td>102%</td>
<td>Satisfactory</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>62</td>
<td>70</td>
<td>89%</td>
<td>Satisfactory</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>24</td>
<td>32</td>
<td>75%</td>
<td>Satisfactory</td>
</tr>
<tr>
<td>Ralstonia pickettii</td>
<td>19</td>
<td>25</td>
<td>76%</td>
<td>Satisfactory</td>
</tr>
</tbody>
</table>
eventual identification.

Ensuring adequate fill volume is similarly important and it is most likely that 90 mm plates prepared for use as settle plates will be designed to have a larger fill volume than those used to take personnel finger dabs or those that remain within the laboratory for plating out.

Despite the variation in weight demonstrated, each of the plates was able to recover successfully each microbial challenge and at a level high enough to meet the requirements of the acceptance criteria.

An important point with the study outlined is that of a case study. Such a study can only be performed by the microbiologist within a given facility because different types of culture media (different agars may have different gel strengths or degrees of stiffness) and the presence of additives (such as neutralisers, needed when plates of a similar design are used as finger plates) are important variables. In addition, different incubation regimes, and different unidirectional or cleanroom environmental conditions are further variables and ones that can only be demonstrated locally.

In the event that a study indicates that microbial growth is not recovered at a sufficiently high level then either the environmental conditions should be examined (such as rate of air velocity or the temperature and humidity of the environment). In most cases, these cannot (and probably should not) be adjusted since they serve a contamination control purpose. This brings attention to the way that the plate is prepared, including an assessment of the volume of agar added to the Petri dish.

There are some scenarios which might require a follow-up study. A further reassessment might be required if the type of culture media changes (such as the introduction of a dual media monitoring regime); or if the supplier of the media changes (which could result in a different fill volume); or if there is a major change to the design of the unidirectional air space.

References