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Anaerobes and the cleanroom operator association: Is there a case for anaerobic environmental monitoring?

Corresponding Author: **Tim Sandle**

BPL

UK

Email: timsandle@btinternet.com

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Abstract

Is anaerobic environmental monitoring necessary? For obligate anaerobes the case for doing so is low. For facultative anaerobes, where aseptically filled parenteral products are involved, the case is far higher. Any such consideration should be based on a quality risk assessment taking into account the interactions between people and exposed product or product components and the opportunity for particle deposition. In many cases, the risk will not be known unless selective environmental monitoring has been performed given the slow growth rate of organisms like *Cutibacterium acnes* and the preferential growth obtained using an anaerobic atmosphere and a blood-based culture medium. The argument of this paper is not so much with whether anaerobic monitoring is necessary for aseptically filled products, but with how often should this be performed?

Introduction

Advances in the understanding of the human microbiome, especially the diversity of organisms found across the ecological niches of human skin, have added to the established limitations with environmental monitoring (poor sensitivity and small sample sizes) in that a smaller diversity of organisms are detected than are shed from the body, thereby highlighting the limitations of recovery using culture-based methods. In addition, such enhanced understanding of the skin microbiome strengthens arguments for effective gown control, as previously made in this journal by the author (1). This improved knowledge of what is probably being deposited on skin particle rafts has also led to the importance of including organisms like Streptococci in the release process for culture media used for environmental monitoring (2). Consideration for widening the test panel used for media growth promotion testing extends further to include areas like disinfectant efficacy testing and sterility test validation (3).

Overall, the human microbiome project has resulted in a shift in our understanding of health and disease (4). High volumes of data have been obtained using culture-dependent (media-based methods) and molecular (genetic sequencing and metabolomic analysis) techniques (5). Understanding the skin microbiome is especially important for pharmaceutical microbiology given that most microorganisms within cleanroom air are carried on particles that originate from desquamated skin (6).

One aspect of the microbiome which has received more limited attention within the cleanroom context is with anaerobic organisms,either obligate or facultative (7). The case for or against anaerobic environmental monitoring in cleanrooms has appeared on and off since the 1960s, with the consensus being that anaerobic monitoring is of limited value given that (obligate) anaerobes in the vegetative form would not survive. But what of spore formers and what of the Propionibacteriaceae (especially *Cutibacterium acnes*)? If we carry Clostridia endospores, why would these not be present? Given all humans host *C. acnes* (8) which accounts for approximately half of the total skin microbiome (typical density of 10^2 to $10^{5–6}$ cm^2) (9, 10) are we confident we can recover this organism. The standard argument is that *C. acnes* is a facultative anaerobe, capable of growing under aerobic conditions. Yet if recovery is straightforward, is the fact that *C. acnes* is rarely recovered from cleanrooms due to good controls or because of limitations with monitoring methods?

This review article considers anaerobic organisms associated, and for the most part residential, with the outermost layers of human skin (11-13) and considers if there is a case for widening the scope of

environmental monitoring where there are human centric operations. The outcome of this review is that a case for periodic monitoring for facultative anaerobes exists in the case of sterile, aseptically filled medicines administered parenterally.

Human skin microbiome

The microbiome of human skin shows phylogenetic diversity and there is a degree of variation between people, notably between younger and older people, between men and women, and according to occupation, personal hygiene, and geographical habitat (14-18). However, there are patterns of microbial species that are relatively common between people, perhaps even universal (19). Universality extends to *C. acnes* (20), a fact that is central to this paper. What is interesting about the human skin – our biggest organ – is that it is formed of different ecological niches. Skin and its microcosms supports life and exerts selective pressures on these organisms. As Marples eloquently wrote in 1974, we can describe the ecology from "… the desert of the forearm, [to] the tropical forest of the armpit, [and] the cool woods of the scalp" (21).

With topography, different regions on the skin influence the types of microorganism that are predominant, including:

- Density of hair follicle,
- Dry areas,
- pH,
- Temperature,
- Sweat glands,
- Sebaceous glands.

Dry skin sites, such as the arms and legs, are dominated by *Micrococcus* and *Staphylococcus* species (22). Moist sites have more variability, with the constantly moist toe web favouring *Corynebacterium* species growth and some Gram-negative organisms like *Acinetobacter* species, while the phalangeal web, which features abundant sweat glands but is generally drier, favouring Staphylococci (23). Invaginations of the skin such as hair follicles, sebaceous glands, and sweat glands create distinct microenvironments and oxygen gradients prompting Propionibacteriaceae (species of *Propionibacterium* and *Cutibacterium*). The highest levels of these organisms are found on the face, the scalp, shoulders, upper chest and back. These are oxygen tolerant anaerobes, although they grow much faster in truly anaerobic environments. This is in contrast to other facultative anaerobes – the Staphylococci - which grow fastest in the presence of oxygen.

How many anaerobes on human skin?

The human skin contains a high proportion of anaerobic organisms, with *C. acnes* being the most populous organism. Most skin anaerobes are members of the family Propionibacteriaceae, although members of the order Clostridiales will also be present, such as organisms in the Peptostreptococcaceae and Clostridiaceae families. Many species of clostridia, including *Clostridioides difficile*, are transient members of the normal flora of the human skin (24). The likelihood of these relates to the presence of wounds, especially skin ulcers (25) and poor personal hygiene.

Propionibacterium and *Cutibacterium* spp. predominate in sebaceous areas and these communities are relatively stable and subject to less variation than other ecological areas of the skin microbiome (26). Sebaceous glands are connected to the hair follicle and form the pilosebaceous unit. The function of these glands is to produce sebum, which is an oily, lipid-containing substance that protects and emolliates the skin and hair (27). The environment of the sebaceous gland is anoxic, and these

organisms have adapted to colonising these regions through the production of lipase which promotes cell adherence to components of the pilosebaceous follicle, such as oleic acid (28, 29).

Longitudinal studies suggest that, in most cases, *Propionibacterium* and *Cutibacterium* bacteria display traits conferring persistency rather than an aggressive response towards the host, although the organisms, *Cutibacterium acnes* in particular, are the primary causes of the pathogenesis of acne, starting with the formation of microcomedones (30). Importantly, for someone with or without acne, the proportion of *C. acnes* is approximately same; it is thought acne arises when other bacteria move out of balance, such as a decrease in the proportion of Staphylococci inhabiting the same regions (31). Conversely, a decrease in the population of these organisms leading to disequilibrium enables the fungi *Malassezia* species to dominant, causing conditions like seborrheic dermatitis (dandruff) (32).

Certain skin conditions can increase the abundance of different anaerobes. Ichthyosis vulgaris, for example, a dry skin condition causing grey or brown discoloration slows down the skin's natural shedding process and causes excessive build-up of keratin; similar effects are seen with filaggrin deficiency. Both conditions may lead to eczema and a large rise in the population of Gram-positive anaerobic cocci, primarily of the genus *Peptococcus* and Peptostreptococcus (33, 34). The most commonly isolated species is *Peptostreptococcus magnus,* followed by *P. asaccharolyticus*, and *P. vaginalis* (35). In crude terms, peptococci are the anaerobic equivalent of staphylococci; and peptostreptococci are the anaerobic equivalent of streptococci (36).

Cleanroom recovered microorganisms and the human association

Various studies have demonstrated that the majority of organisms recovered from a cleanroom environment were residential or transient to people (37). There are of course other origins, notably when water is present or through the transfer of items through the cleanroom cascade. No matter how good a gown, a cleanroom gown will always lead to the release of some skin matter, not least because of the need for operator comfort so a trade-off is required between gown pore size and maintaining suitability for the operator. Cleanroom contamination is enhanced when atypical events occur such as an increase in personnel numbers, unacceptable behaviours (such as rapid movements) or following maintenance (38).

Release of microorganisms from people poses either a direct risk to exposed product (or product contact materials) or a longer-term risk to the cleanroom, should the organisms survive. In terms of survival, this depends on when the organisms are deposited. Longer term the diversity of released organisms diminishes and only hardy, robust organisms survive in oligotrophic cleanroom environments (39), such as *Micrococcus luteus* (40) or those organisms capable of forming endospores (41). Propionibacteriaceae are not among the longer-term survivors. These microbes most probably die off shortly after being shed from the skin of their human host, their natural ecosystem, and exposed to oxygen. Nonetheless, the risk of product deposition remains; Clostridia spores are among the potential survivors, although the conditions for germination are unlikely to be present so an atypical act of transfer would need to occur.

In the case of Clostridia, we cannot expect environmental monitoring to detect anywhere near the actual types and populations of organisms present. To do so would require a range of culture media and incubation conditions in the case of those organisms that are culturable and assessing the likelihood of ‘viable but non-culturable’ organisms cannot be extended beyond the theoretical expectation informed by molecular biological investigations. Therefore, by identifying all organisms found within an aseptic processing facility and trending the recovered species, the proportion of skin-associated organisms and potentially those endospore forming bacteria that will be recoverable using a conventional environmental monitoring regime (*Bacillus* species grown on tryptone soya agar), a

reasonably reliable indicator of the state of control can be obtained in conjunction with the operation of cleanroom protective parameters such as pressure, air changes, airflow, temperature and so on. Hence, the inability of recover Clostridia spores that may occasionally be present is not significant.

In terms of attempting to recover other organisms, there is no value in attempting to isolate Peptostreptococcaceae since collection methods, transportation, and specimen cultivation are challenging and the required techniques are unlikely to be found within the typical pharmaceutical microbiology laboratory (42).

Another type of organism requires consideration: Microaerophilic organisms. According to the United States Pharmacopeia chapter on aseptic processing, microaerophiles may be observed in aseptic processing. A microaerophile is a microorganism that requires environments containing lower levels of dioxygen than are present in the atmosphere. These contrast to facultative anaerobes, which are anaerobic organisms that can switch to aerobic respiration when required. USP <1116> does not address the question of facultative anaerobes, despite these organisms being more likely than microaerophilic organisms. Those organisms most commonly used as exemplars of microaerophiles - *Campylobacter* species and *Helicobacter pylori* – would require a highly improbable and complete breakdown of operator hygiene to be present. Therefore, the focus needs to remain with the facultative anaerobes.

Monitoring strategy

On the basis of microbiological risk, pharmaceutical products can be divided into two groupings:

- Non-sterile products
- Sterile products.

The presence of anaerobes is largely irrelevant to non-sterile products. The anaerobes most likely to be present, should they survive, are either not objectionable or they would not be found in sufficient numbers to pose a patient or consumer health risk.

With sterile products, those products subjected to terminal sterilisation are less likely to be at a risk. The possibility of anaerobic spore forming organisms being present in a high number as to adversely challenge a validated sterilisation cycle is very low. With aseptically filled products, however, a risk exists should anaerobic organisms be deposited into the pharmaceutical product. Should a product capture and retain an organism like *C. acnes*, a subsequent administration of the product into the blood could cause deleterious effects. *C. acnes* is associated with several types of foreign-body infections. associated with blood, bone cells and cerebrospinal fluid infections (43).

Controls can be built around people, but since most microorganisms are introduced to cleanroom facilities by humans (44), despite various rigorous and stringent control measures (45), it would be logical to make an assessment of the risk posed by the most ubiquitous of residential skin organisms (46). As to whether *C. acnes* will be recovered from aerobic environmental monitoring using standard agar, it is possible that some will be recovered but this cannot be guaranteed, and absence of evidence does not mean evidence of absence. This is because the growth rate of facultative anaerobes, like *C. acnes*, is supressed under aerobic conditions, leading to a decreased or fully supressed growth rate (47, 48). The weakness of conventional culture media and incubation regimes was demonstrated in a study where molecular viability assay based on propidium monoazide (PMA) was conducted. PMA is able to mask DNA from dead microbes with permeable cell structures and free extracellular DNA after light activation, for downstream PCR based analysis (49). The NASA-led study showed significantly

higher detection of *Propionibacterium* and *Cutibacterium* species within cleanrooms, following PMA treatment, in comparison with data from standard aerobic environmental monitoring (50).

In developing any type of environmental monitoring strategy there are some key elements to consider, such as monitoring locations, time for monitoring, optimal culture media to promote recovery and so on. These are examined below.

Location

The detection of microbial diversity and abundance within the cleanroom is strongly correlated with the applied sampling and detection methods. In terms of selected methods, physical processes affect the movement of particles and most microorganisms, including the facultative anaerobes, which will be carried on particles formed of skin detritus or dust. This places an emphasis upon air monitoring, both active (volumetric) and settle plates. The movement of microbial carrying particles will relate to the complexities of air currents and deposition will be influenced by both particle size and air movement (51). A given cleanroom microbiome is also strongly influenced by both the architecture and control parameters (such as humidity, temperature, airflow, ventilation) of a particular facility. Airflow visualisation is a useful tool for helping to determine monitoring sites within unidirectional airflow, especially with the positioning of settle plates where a risk of deposition could occur in relation to exposed product, product contact materials or surfaces.

As established above, obligate anaerobes are killed when exposed to an oxygen atmosphere for as brief a time as 10 minutes. Pharmaceutical cleanrooms are continuously swept by filtered air, and surfaces are smooth and clean; consequently, there should be few opportunities for anaerobes to survive. However, there may be pockets where such organisms could exist, such as grease, or in oil sumps. While these lubricants should not normally be exposed there may be circumstances of higher risk, such as following maintenance of conveyor belts or where panels are opened. Here, a surface assessment for anaerobes might be prudent.

Culture media and incubation

Many types of anaerobic organisms are challenging to recover. Anaerobic microorganisms require the absence of oxygen and usually a low redox potential to initiate growth (52). Strict anaerobes require enrichment cultures, and these organisms generally grow better in liquid media. This is not practical for environmental monitoring and adds to the earlier discussion of there being little value in screening environments for obligate anaerobic organisms. A different situation arises where compressed gases are used to ensure an oxygen-free headspace atmosphere during processing. Under these circumstances a case exists for running additional aseptic process simulations using a liquid medium like fluid thioglycolate broth (53). This medium, which contains a redox-sensitive dye such as resazurin, is used in the sterility test for the recovery of obligate anaerobes, facultative anaerobes and aerobic organisms, where the location of growth within the medium corresponds to the type of organism (an obligate anaerobe, for example, will grow at the bottom of the medium). More specialised compressed gas monitoring may also be required, either in relation to filling or with processes like freeze-drying, focusing on both aerobic and anaerobic organisms Endospore formers are most likely to be the survivors in compressed gases although vegetative organisms may thrive depending on the levels of oil and water.

To recover facultative anaerobes, the question that arises is whether these organisms – as represented by *C. acnes* – will grow on tryptone soya agar (TSA) under aerobic conditions or whether anaerobic monitoring is required. It follows that if anaerobic monitoring is deemed necessary, is the default environmental monitoring culture medium – TSA – the most suitable to promote recovery?

The answer to the most appropriate medium will be evidenced by experiments, although laboratory studies indicate that recovery on TSA is weak and that Propionibacteriaceae (including *C. acnes*) are optimally recovered using a defined synthetic medium with glucose as the main carbon-energy source, at 33 to 37^{o}C (54, 55) (pH range 4.5 to 8.0) (56). Even where TSA proves suitable, *C. acnes*, as an environmental isolate, grows relatively slowly, typically requiring up to 14 days, around the double the incubation time detailed in environmental monitoring protocols. For example, one study on the growth rate of *C. acnes* found the bacterium to possess a median growth of 7 days (with a maximum of 11 days) (57). However, environmental isolates that replicate at a slower rate due to low metabolism will require longer - up to 14 days, should they be recoverable (58).

Where literature indicates the use of media other than TSA, this is either blood agar, which supplies the nitrogenous substances and amino acids necessary for the growth of anaerobic bacteria, or Schaedler agar (59), as a suitable alternative to blood agar. This medium contains cysteine hydrochloride and glucose as reducing substances (60). Cysteine is a specific growth promoting agent for *C. acnes*. Supplementing the medium with Vitamin K and sodium succinate provides essential growth factors for many other anaerobes. Plates for anaerobic incubation should be incubated as soon as possible to prevent loss of viability (61). Where subculture or enrichment is required back in the laboratory, the recommended broth is thioglycolate.

To create the necessary anaerobic conditions, commercial kits that use a mixture that reacts with water to chemically bind all or part of the atmospheric oxygen in a given volume of air (anaerobic jar or special incubation bag), while simultaneously releasing carbon dioxide, are commonplace. The resulting growth environment is rich in carbon dioxide and either devoid of oxygen (anaerobic) or oxygen deficient.

Time and frequency of monitoring

Should monitoring for facultative anaerobes be undertaken, it is rational to perform this monitoring in real time and with a focus on direct product deposition. The key question becomes ‘how often?’ One of the arguments for transitioning to a single culture medium for monitoring aseptic processing, especially where Restrictive Access Barrier Systems (RABS) are used is that it reduces the number of manipulations that would otherwise be present if two different culture media were used. An assessment for facultative anaerobes could be carried out selectively to assess the risk presented by personnel. The worst case challenges presented by an aseptic process simulation is an example. Alternatively, targeted monitoring could be performed such as in relation to isolator and RABS setup, interventions within RABS devices, or during freeze-dryer loading.

Discussion

This review paper is designed to act as a stimulus, in reconsidering the appropriateness of anaerobic monitoring in cleanrooms used for pharmaceutical processing. The central argument is that monitoring for obligate anaerobes should not be necessary. Organisms in the vegetative state will not survive and occasional spore formers will be outnumbered by other bacteria that can be detected through conventional monitoring. Hence, by reacting to trends the microbiologist gains an insight into the degree of cleanroom and personnel control. With the monitoring of facultative anaerobes, there does not appear to be a case for non-sterile pharmaceuticals unless there is a specific anaerobe of concern that is presented as an objectionable microorganism for a given product and patient group. For product treated in the final container to a terminal sterilisation cycle, monitoring for anaerobes should not be necessary unless an unduly high number were suspected. There are many studies into bacterial spore inactivation by heat that show species of Clostridia to be less resistant (by a factor of

50%) than the biological indicators of *Geobacillus stearothermophilus* used in qualification studies (62, 63). In cases where, for some reason, a concentration of anaerobes could occur, the pre-terminal sterilisation bioburden could include a test for anaerobes.

With aseptically filled products, where there is a degree of human activity in close proximity to the product, then a risk of transfer exists as with any other skin microorganism. Given that *C. acnes* occurs in such high numbers, the isolation of the occasional Gram-positive coccus makes the potential deposition of *C. acnes* just as likely. It can be argued that the areas where *C. acnes* is found in the highest numbers – on the face, especially on the forehead, hair follicles, and around the nose (64) - do not represent the most common areas of human interaction with the aseptic core, compared with arms for example. To counter this, many filling operations still require a human to enter Grade A and *C. acnes* is recoverable from the arm. In addition, an important variable will be with dermatological conditions (65), which influences the homeostasis of the host and the microorganisms found on the skin (66). Rates of skin shedding are increased through excessive movement and by skin conditions, such as dry skin (67).

It can also be argued that the sterility test includes a test for anaerobic bacteria. While this is so, the insensitivity of the sterility test has been well-documented, starting with Bryce in 1956 who pointed out the limitations of sample sizes and extended with our understanding of viable but non-culturable organisms (68). As an aside, the sterility test can be strengthened by including *C. acnes* in the method suitability test and as a release organism, as previously argued by this author in this journal (2). However, it is not possible to rely on the test for sterility alone.

In summary, the rationale presented in this paper (as per Figure 1) is:

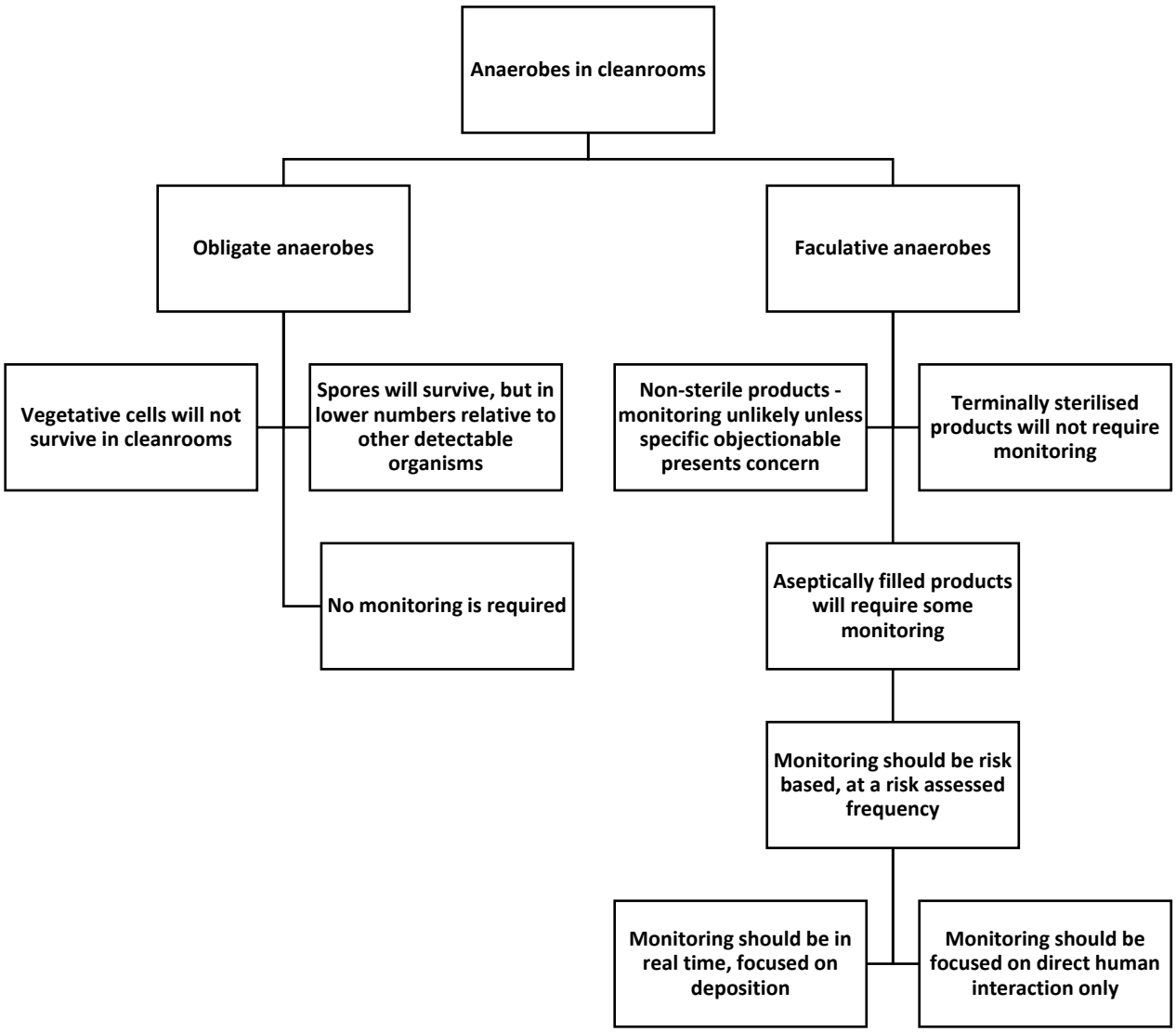

Figure 1: Anaerobic monitoring rationale, as presented in this paper

Whether environmental monitoring for facultative anaerobic bacteria is necessary will be dependent on a facility assessment as part of the contamination control strategy. Such a risk assessment must look at the controls around people. Even where a risk outcome is considered low, the challenge remains that if organisms like *C. acnes* have not been purposefully looked for using an appropriate agar and a suitably long incubation time, what is this low risk based on, especially where an isolator is not in use? The rationale outcome of this review paper is that for those involved in the production of aseptically filled parenteral medicines, some form of assessment at a risk-based frequency should be undertaken and the results used to reconsider the pre-existing controls around people as necessary.

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