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Assessment of degree of risk from sources of microbial contamination in cleanrooms; 2: Surfaces and liquids

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The degree of risk from microbial contamination of manufactured products in healthcare cleanrooms has been assessed in a series of three articles. The first article discussed airborne sources, and this second article considers surface contact and liquid sources. A final article will consider all sources and give further information on the application of the risk method.

The degree of risk to products from micro-organisms transferred from sources by surface contact, or by liquids, has been assessed by the means of fundamental equations used to calculate the likely number of microbes deposited (NMD) onto, or into, a product. The method calculates the likely product contamination rate from each source and gives a more accurate risk assessment than those presently available. It also allows a direct comparison to be made between microbial transfer by different routes, i.e. surface, liquid and air.

Key words: Risk assessment, degree of risk, source, surface contact, contamination, micro-organisms, microbes, MCPs.

Introduction

The requirements for minimising microbial contamination in pharmaceutical cleanrooms are outlined in regulatory documents published by authorities that include the European Commission[1] and the Food and Drug Administration in the USA[2]. These authorities also suggest the use of risk management and assessment techniques to identify and control sources of microbial contamination[3,4]. Risk assessment and management methods have been investigated by the authors of this article[5–9] and other approaches are discussed by Mollah *et al*[10].

Risk assessment methods are used to calculate the degree of risk to the product from microbial sources in a cleanroom. Factors that influence risk are determined and assigned descriptors of risk, which are of the 'high', 'medium', and 'low' type that act as surrogates for actual numerical values. Numerical scores are assigned to these descriptors and the scores combined, usually by multiplication, to obtain a risk assessment for each source of contamination. However, a risk assessment carried out in this manner may not be accurate, for the following reasons.

- Assigning risk descriptors and risk scores is subjective.
- The way the risk scores are combined may not reflect the actual mechanism of contamination.
- Differences between the transfer mechanisms of air, surface contact and liquid make it difficult for these types of risks to be compared.

It would be beneficial if a risk assessment method was available that avoided these short comings, and could calculate the contamination rate of products from the various sources in a cleanroom. A previous article by Whyte and Eaton[11] discussed the application of such a method to airborne sources of microbe-carrying particles (MCPs). This article considers the application of the method to surface and liquid sources.

Calculation of microbial deposition onto a product

Risk is defined[12] as the product of the 'severity' (also known as 'criticality') of harm and the 'probability' of occurrence, and its magnitude can be determined by multiplying together values assigned to these two variables.

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Equation 1

Degree of risk = severity of harm × probability of harm

In the context of microbial contamination of products in a cleanroom, 'severity' can be considered as the product of the concentration of microbes in, or on, a source of contamination, and the likelihood that these microbes will be transferred to a product. The 'probability' can usually be considered in (a) airborne contamination as the time the product is exposed to contamination, (b) surface contamination as the number of contacts, and (c) liquid contamination as continuous.

Fundamental risk factor equations have been derived by Whyte and Eaton[7,11] to calculate the number of microbes that deposit onto, or into, a product from air, surface contact, or liquids. These equations calculate the number of microbes deposited (NMD) onto, or into, one product unit, and typically give a numerical value well below one. The NMD in this article uses the format 1 x 10^{-6} but it can be alternatively given as a product contamination rate of 1 in 10^6, or 1 in a million units. It is important to make sure that the units of measurement are consistent in the risk equations, and those mainly used in this article are centimetres and seconds, although metres and seconds are also used.

Equation 2 has been derived by Whyte and Eaton[7, 11] to calculate the NMD_A from air sources.

Equation 2; Airborne

$$NMD_A = c*p*a*t*s_v$$

Where, NMD_A = number of airborne MCPs deposited onto a single product, c = concentration of microbes in the airborne source, p = transfer coefficient of MCPs transmitted from source to product, a = area of product exposed to microbial deposition, t = time of exposure to airborne deposition, and s_v = settling velocity of MCPs through air.

The settling velocity (s_v) is the rate that MCPs fall through the air, and has been discussed and used in the previous article[11]. Microbes do not normally exist in the air as single cells. They are mainly dispersed on skin particles by personnel and have an average aerodynamic diameter of about 12 μm[13,14], with an average deposition velocity of about 0.46 cm/s[15].

Equation 3 can be used to calculate the NMD_{SC} from surfaces.

Equation 3; Surface contact

$$NMD_{SC} = c*p*a*n$$

Where, NMD_{SC} = number of MCPs deposited onto a single product by surface contact, c = concentration of MCPs on the surface of a source, p = transfer coefficient of MCPs from donating to receiving surface, a = area of contact, and n = number of contacts.

Equation 3 is used to calculate the NMD_{SC} onto a product by surface contact. Much of the information required to solve Equation 3 will be known, or can be measured. The proportion of microbes on the donating surface, which are transferred to a receiving surface or product, is known as the transfer coefficient. Whyte and Eaton[16] have carried out experiments using skin-derived MCPs to obtain transfer coefficients and the following average values were obtained: gloves to stainless steel = 0.19, stainless steel to stainless steel = 0.10, and clothing to stainless steel = 0.06. The contact between stainless steel and glass is between hard surfaces and assumed to have a similar value to that between stainless steel and stainless steel. As these coefficients have similar values, and for simplification, a worst case transfer coefficient of 0.2 was used in all surface transfers considered in this paper.

Equation 4 can be used to calculate the NMD_L from liquid sources.

Equation 4; Liquid

$$NMD_L = c*p*v$$

Where, NMD_L = number of liquid-borne microbes deposited into a single product, c = concentration of microbes in a liquid source, p = transfer coefficient of microbes from source to product, and v = volume of liquid deposited into product.

Description of cleanroom studied

In the first article of this series, Whyte and Eaton[11] described a method of calculating the NMD_A from airborne sources of microbes, and illustrated it with a pharmaceutical cleanroom used to aseptically fill batches of pharmaceutical products in a unidirectional air flow (UDAF) workstation. The same example will again be used to calculate the NMD from surfaces and liquids.

Cleanrooms that control microbial contamination use a variety of designs and manufacturing methods. Increasing regulatory expectations are leading to designs of pharmaceutical cleanrooms for aseptic filling that include an isolator or restricted access barrier system (RABS). However, to illustrate the wider application of the risk assessment method to more traditional cleanroom designs found in other types of healthcare rooms, the following cleanroom and manufacturing method is used as an example.

1 Vials are aseptically filled with 2 cm^3 of aqueous solution, and sealed with sterile closures. This is carried out in batches of 4000, which take about 4 hours to process.
2 Eight litres of an aqueous solution of the active ingredient is prepared in an adjacent preparation cleanroom and piped from the preparation vessel through a sterilised, sterilising-grade filter, and into the filling workstation. An aseptic connection is made in the workstation with the product-filling equipment before filling starts.
3 The vials are sterilised in a depyrogenation tunnel from which they exit, and are conveyed through a UDAF workstation (*EU Guidelines to Good Manufacturing Practice* (GGMP) Grade A), which is

known in this article as the 'filling workstation'. The vials, which have an inner neck area of 2 cm^2, are automatically filled in the filling workstation and closed by a stopper. The vials are open in the filling workstation to airborne contamination for 600 s.

4 The filling workstation is situated in a non-unidirectional airflow cleanroom (EU GGMP Grade B) which is known as the 'filling room'. The filling room has a volume of 300 m^3 and an air supply of 3.33 m^3/s of HEPA-filtered air (40 air changes per hour).

5 Two people work in the filling cleanroom and one of these attends to the filling operation within the workstation. Access into the filling workstation is through plastic-strip curtains that hang round the perimeter and down to just above the floor. Interventions may occur when there are problems with the filling line, and these are normally corrected by sterilised long forceps.

6 Vial stoppers are held in a hopper that has a capacity of 1000 stoppers, and replenished every hour.

7 Personnel wear cleanroom clothing consisting of a one-piece polyester coverall with full hood, overboots and mask. Sterilised, latex, double sets of gloves are worn over disinfected hands.

8 Hard surfaces, which do not come into contact with the product containers or closures, are disinfected. Hard surfaces, such as pipework that contacts the product solution, and product-contacting surfaces, such as the sterile vial closures, storage hopper, forceps, and track-ways, are sterilised.

Degree of risk from sources of surface and liquid microbial contamination in a cleanroom

Shown in **Figure 1** are the main surface and liquid sources of microbial contamination of a product, along with methods of controlling microbial concentrations and transfer. The source of most, if not all, of microbes in a cleanroom is people, who are considered the prime source. Also included are sources external to the cleanroom that may be the cause of contamination in the primary product and containers. The sources which directly contact product are within the UDAF workstation and are known as primary sources. The floor is also included as it is within the workstation, but it is not a primary source, as microbes on the floor's surface are firstly dispersed into the air by walking, and then transferred by air to product. Also given in **Figure 1**, for the sake of completeness, are methods of controlling airborne transfer of MCPs to surfaces.

Not shown in **Figure 1**, or considered in this article, are secondary sources, e.g. walls, doors, trolleys, tables, disinfectant cans, etc., whose surface microbes do not directly contact the product but do so through an

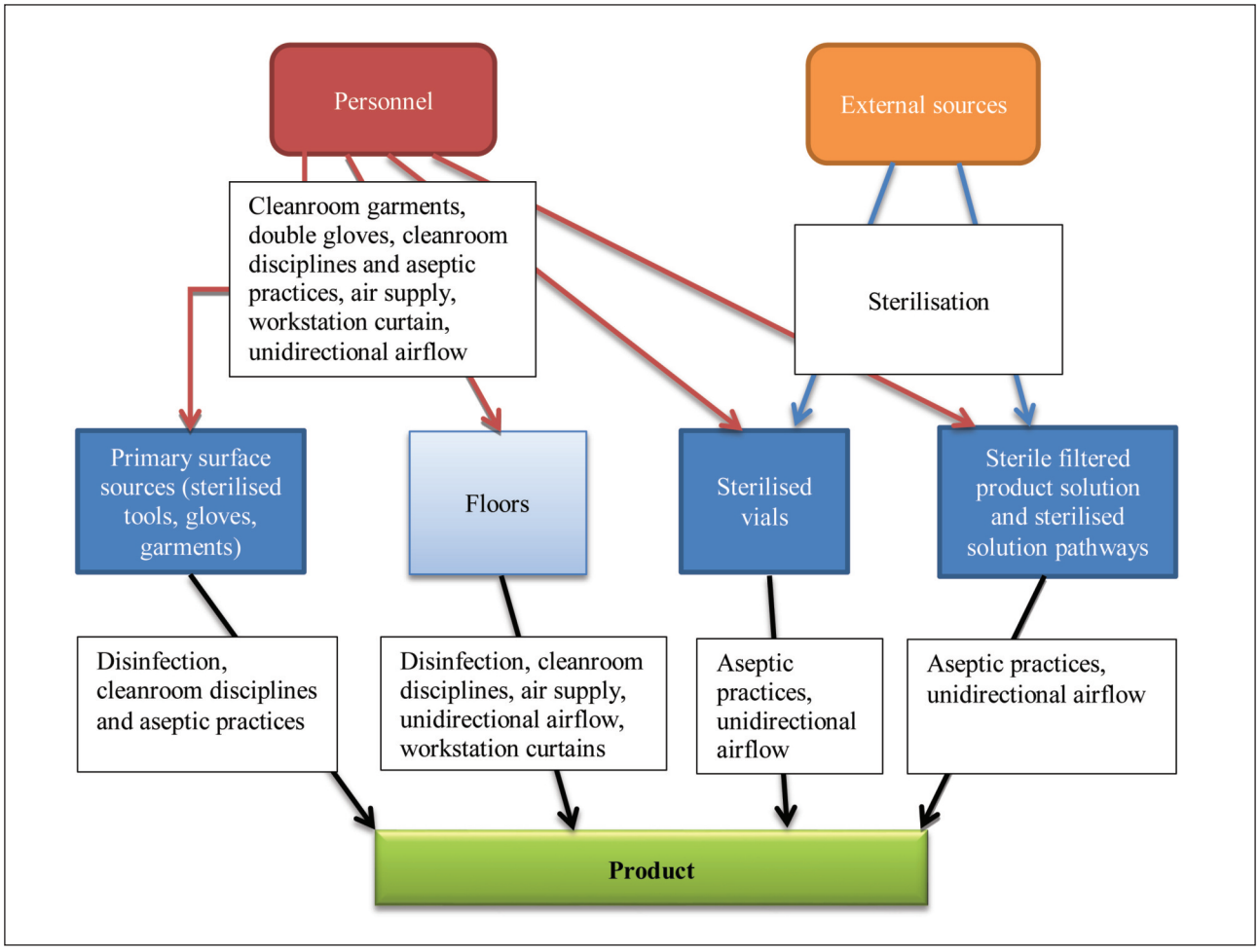

Figure 1. Risk diagram showing sources of surface and liquid microbial contamination along with control methods.

intermediate vector. These secondary sources are too numerous to be considered, but are usually less important than primary sources as their microbes are subject to an additional transfer step associated with an intermediate vector. However, the last vector which contacts the product will be one of the primary sources, with gloves the main one, and the degree of risk can therefore be indirectly ascertained by a risk assessment of the primary sources, particularly gloves.

Should it be thought necessary, the NMD_{SC} from secondary sources can be calculated. Equation 2 can be used to calculate the number of source microbes deposited onto an intermediate vector surface, and the surface concentration on the vector can then be used to calculate the NMD_{SC} onto product. The NMD can also be calculated for mixed routes of transfer, such as surface contact and liquid transfer, or surface contact and air transfer, as demonstrated in the "Pipework, filling tubes and needles" and the "Microbial dispersion from cleanroom floor" sections, respectively.

The microbial sources shown in **Figure 1** are tools, gloves, cleanroom garments, product solution, solution pathways (pipework, filling tubes and needles, etc.), containers, and floor, and their degree of risk is now assessed.

Tools

Sterilised tools include items such as long-length forceps used to correct product vials that are displaced or fall over, and the forceps may contact the product. The tools will be sterilised and, as demonstrated in Annex A, the surface concentration of microbes following sterilisation and prior to use is negligible, and can be ignored. However, the forceps may be contaminated by airborne deposition, or by touching other contaminated surfaces and, if they contact the vulnerable inner neck of the vial, microbes may be transferred to product. The NMD_{SC} can be calculated as follows.

Risk factor	Assessment
1. Microbial concentration on forceps surface (number/cm^2)	The microbial concentration on sterile surfaces was determined by sampling after completion of manufacturing and will, therefore, represent the worst case concentrations. From 38,062 samples, one microbe was recovered. The forceps had a contact area with the sampling media of 1.2 cm^2, which gives a surface concentration of 2.2 x 10^{-5}/cm^2
2. Transfer coefficient	As discussed in the "Calculation of microbial deposition onto a product" section, the transfer coefficient between stainless steel and glass surfaces is assumed to be 0.2
3. Area of contact (cm^2)	The area of the forceps that makes contact with the inner neck of the vial was measured and found to be 0.3 cm^2
4. Number of contacts	At worst, the internal neck area is contacted 10 times per 4000 containers, which is a frequency of 2.5 x 10^{-3}
Using Equation 2, the NMD_{SC} is: $NMD_{SC} = c^*p^*a^*n$ = 2.2 x 10^{-5} * 0.2 * 0.3 * 2.5 x 10^{-3} = **3.3 x 10^{-9}**	

Gloves

In the cleanroom example, personnel wear disposable double latex gloves. These gloves have been sterilised by gamma radiation and, as demonstrated in Annex A, the risk from surface microbes on unused gloves can be ignored. However, there is a low possibility of skin microbes being on glove surfaces because of punctures[17]. Glove surfaces may also be contaminated when donned, and by touching various surfaces during the cleanroom manufacturing activities, as well as deposition of airborne contamination. Gloves are routinely disinfected during manufacture with sterile 70% isopropyl alcohol (IPA) to control the level of surface contamination. Personnel are instructed never to contact product with gloves, but it is useful to consider what may occur if the vulnerable inner neck area of vials is touched by gloves, and the NMD_{SC} can be calculated as follows.

Risk factor	Assessment
1. Microbial concentration on glove surface (number/cm^2)	The post-manufacture measurement of the microbial concentration of five finger tips gives an average of 3.9 x 10^{-3}/cm^2. The five finger tips have a total surface area of approximately 7.5 cm^2, and so the glove surface concentration is 5.2 x 10^{-4} /cm^2
2. Transfer coefficient	As discussed in the "Calculation of microbial deposition onto a product" section, the transfer coefficient between gloves and vials is assumed to be 0.2, and all contamination transferred enters the product
3. Area of contact (cm^2)	The area of contact between a single glove tip and the inner neck of the vial was measured and estimated to be 0.5 cm^2
4. Number of contacts	At worst, the glove tip might accidently contact the internal neck area 1 per 4000 containers, which is a frequency of 2.5 x 10^{-4}
Using Equation 2, the NMD_{SC} can be calculated; $NMD_{SC} = c^*p^*a^*n$ = 5.3 x 10^{-4} * 0.2 * 0.5 * 2.5 x 10^{-4} = **1.3 x 10^{-8}**	

Cleanroom garments

Garments are sterilised by radiation prior to use and, as shown in Annex A, they will be effectively free of microbes when unused. However, their surface may be contaminated when donned, touched by contaminated surfaces during manufacturing, or from microbes depositing from the air. It is expected that the use of long-length sterilised tools and good aseptic practices will prevent garments contacting product. However, if they accidently contact the vial, it is useful to know the degree of risk, and the NMD_{SC} can be calculated as follows.

Risk factor	Assessment
1. Microbial concentration on garment surface (number/cm^2)	The forearms and chest of garments are sampled after manufacturing using RODAC plates and an average concentration is 2.7 x 10^{-2} per 24 cm^2, which is a concentration of 1.1 x 10^{-3}/cm^2
2. Transfer coefficient	As discussed in the "Calculation of microbial deposition onto a product" section, the transfer coefficient between the garment and the vulnerable inner neck area of the vial is assumed to be 0.2. All contaminants were assumed to enter the product
3. Area of contact (cm^2)	The area of contact between a garment and the vulnerable neck area of the container was measured and estimate to be about 0.5 cm^2
4. Number of contact	At worst, the contact of the garment with the internal neck area of the container is assumed to be 1 contact per 4000 containers, which is a frequency of 2.5 x 10^{-4}
Using Equation 2, the NMD_{SC} can be calculated; $NMD_{SC} = c^*p^*a^*n$ = 1.1 x 10^{-3} * 0.2 * 0.5 * 2.5 x 10^{-4} = **2.8 x 10^{-8}**	

Filtered aqueous product solution

The solution of primary product is a potential source of microbial contamination and is filtered through a sterilised, sterilising-grade filter of the membrane type. Pre- and post-use integrity testing of the filter is carried out using an automated test unit that measures the rate of diffusive gas flow. This measurement is directly related to a bacterial challenge test performed by the filter manufacturer, where the filter is challenged with *Brevundimonas diminuta*, with a size of approximately 0.3 μm. Filters are required[2] to retain a challenge of 1 x 10^7 bacteria per cm^2 of filter area. The number of microbes deposited (NMD_L) in product can be calculated as follows.

Risk factor	Assessment
1. Microbial concentration in the product solution (number/cm^3)	The maximum concentration, prior to sterile filtration, is determined experimentally to be 10/cm^3
2. Transfer coefficient	The filter has a total filtration area of 1000 cm^2, and required to retain a challenge of 10^{10} bacteria. The transfer coefficient across the filter is, therefore, 1 x10^{-10}. Although there may be deposition of microbes throughout the pipework from filter to filling point, this will be very small compared to the removal efficiency of the filter, and is ignored
3. Volume of product solution dispensed into vial (cm^3)	2 cm^3
Using Equation 4, the NMD_L can be calculated; $NMD_L = c^*p^*v_c$ = 10 * 1 x 10^{-10} * 2 = **2.0 x 10^{-9}**	

Pipework, filling tubes and needles

The product solution is transferred from the sterilising-grade filter to the filling point through a flexible transfer pipe that is connected to filling needles. The flexible pipe and needles are decontaminated and steam sterilised at 121°C. The internal surface of all these items prior to sterilisation has been determined experimentally to have 14 microbes and, as calculated in Annex A1, the number of microbes likely to survive steam sterilisation is 10^{-19}. If all of these microbes are washed off the pipework by the passage of 8000 cm^3 of product solution, the concentration of microbes in the product solution will be 1.3 x 10^{-23} per cm^3. Such a low concentration can be ignored.

When the flexible pipe is connected to the filter, or needles fitted into the filling machinery, the opening of a pipe or needle surface may touch a glove, and microbial transfer may occur. Any microbes transferred are assumed to mix with product solution and be subsequently dispensed into the vials. The area of the glove that contacts with the pipework is likely to be different from that of a needle opening. However, to avoid multiple calculations, the area of 0.5 cm^2, previously used in the "Gloves" section when a glove touches vials, is again used.

The NMD is calculated in two stages, namely, glove to pipework or needles, and then from pipework or needles to product.

Risk factor	Assessment
1. Microbial concentration on glove surface (number/cm^2)	The post-manufacture measurement of the microbial concentration of five finger tips gives an average of 3.9 x 10^{-3}/cm^2. The five finger tips have a total surface area of approximately 7.5 cm^2, and so the glove surface concentration is 5.2 x 10^{-4} /cm^2
2. Transfer coefficient	As discussed in the "Calculation of microbial deposition onto a product" section, the transfer coefficient between gloves and pipework or needles is assumed to be 0.2
3. Area of contact (cm^2)	An area of 0.5 cm^2 was assumed
4. Number of contacts	The frequency of contact is unlikely to exceed 1 contact per filling batch of 4000 vials, which is a frequency of 2.5 x 10^{-4}
Using Equation 2, the NMD_{SC} onto the pipe or needle opening can be calculated as follows: $NMD_{SC} = c^*p^*a^*n$ = 5.2 x 10^{-4}* 0.2 * 0.5 * 2.5 x 10^{-4} = **1.3 x 10^{-8}**	
The calculation in the row above shows that 1.3 x 10^{-8} MCPs are transferred to the pipe and needle openings and these are assumed to enter the product solution. The number that will enter a product by liquid transfer can now be calculated	
5. Microbial concentration in product solution (number/cm^3)	The product solution passes through the internal areas of pipework and needles, all microbes are assumed to be washed and mixed into the product solution, and the concentration in the solution of 4000 cm^3 is 1.3 x 10^{-8} ÷ 4000 = 1.6 x 10^{-12}/cm^2
6. Transfer coefficient	All microbes introduced by contact are assumed to be swept by product solution into the containers and the transfer coefficient is 1
7 Volume of product solution dispensed into vial (cm^3)	2 cm^3
Using Equation 4, the NMD_L can be calculated; $NMD_L = c^*p^*v$ = 1.6 x 10^{-12} * 1 * 2 = **3.3 x 10^{-12}**	

Product vials

Following decontamination in an automated washing unit, the vials are transferred to the filling workstation through a depyrogenation tunnel, where they are sterilised. The possibility that microbes can survive within the vial after sterilisation can be calculated using the method given in Annex A.

The maximum microbial concentration on the inner, product-contacting surface of each vial, following decontamination and prior to depyrogenation, was determined experimentally to be 10. As calculated in Annex A2, a dry heat sterilisation cycle of 170°C for 2 hours would reduce this to a concentration of about 1×10^{-119}. However, the depyrogenation cycle uses a temperature of 250°C for 30 minutes and this additional heat will decrease the microbial concentration to about 1×10^{-300000} per vial. As these microbes are within the vial, and there is no transfer coefficient to be considered, the NMD_{SC} will remain at about 1×10^{-300000}.

Microbial dispersion from cleanroom floor

The transfer of microbes from cleanroom floor to product occurs in two stages. MCPs are dispersed into the air by contact of shoes with the floor, and then transmitted through the air to the product, where they may deposit. The concentration of airborne microbes in the air of the filling cleanroom and filling workstation that have been dispersed from a floor is calculated in Annex B, and can now be used to calculate the NMD_A.

Filling cleanroom

The concentration of MCPs dispersed into the cleanroom air from the floor is calculated in Annex B. Assuming a microbial concentration on the floor of $1.2 \times 10^{-4}/cm^2$, and two people walking about for half of the total manufacturing time, the number of MCPs dispersed into the air in the filling cleanroom is calculated. These MCPs mix with room air, and the airborne concentration of microbes in the filling cleanroom that is derived from the floor has been calculated to be $6.3 \times 10^{-6}/m^3$ ($6.3 \times 10^{-12}/cm^3$).

For the airborne MCPs in the filling cleanroom to reach a product, they have to be transmitted across the curtains and the UDAF within the filling workstation, and deposited into a container. People may work through the curtain, or enter the workstation to attend to containers and machinery. Movement through the curtain and within the UDAF allows airborne MCPs from the filling cleanroom to be transmitted to product. Experiments carried out by Ljungqvist and Reinmuller[18] have shown the proportion of airborne particles released outside the workstation that reached the product when personnel were working, was about 1×10^{-4}; this proportion is the transfer coefficient. The NMD_A dispersed from the floor of the filling cleanroom and deposited into the vial by the airborne route can be calculated as follows.

Risk factor	Assessment
1. Concentration of airborne MCPs dispersed from the filling cleanroom floor (no/cm^3)	The airborne concentration of MCPs in the filling cleanroom derived from the floor is $6.3 \times 10^{-12}/cm^3$ (see calculation in Annex B)
2. Transfer coefficient of MCP from filling cleanroom to product	The transfer coefficient is assumed to be 1×10^{-4}
3. Area of product exposed (cm^2)	The inner neck area of vial is 2 cm^2
4. Time of deposition (s)	The proportion of time that a person works in the filling workstation is 0.1 of the total time, and therefore the time for the transfer of contamination from the filling room (normally 600 s) is reduced to 60 s
5. Deposition velocity through air of MCPs (cm/s)	The average setting velocity of MCPs through the air and into the vial is assumed to be 0.46 cm/s (see the "Calculation of microbial deposition onto a product" section).
Using Equation 1, the NMD_A can be calculated to be as follows: $NMD_A = c^*p^*a^*t^*s = 6.3 \times 10^{-12} * 1 \times 10^{-4} * 2 * 60 * 0.46 =$ **3.5×10^{-14}**	

Filling workstation

In the filling workstation, the mechanism of dispersion of MCPs from floor to air by walking is the same as the filling cleanroom. However, the walking activity is reduced, as is the microbial concentration on the floor, and this information is used in Annex B to calculate the dispersion rate from the floor. Because of the downward flow of UDAF, the MCPs dispersed from the floor will not mix with all of the air in the filling workstation, but only with air close to the floor. The airborne concentration above the floor has been calculated in Annex B to be $2.3 \times 10^{-7}/m^3$ ($2.3 \times 10^{-13}/cm^3$).

It is now necessary to consider the transfer of the airborne MCPs from the area near to the floor to vials at the filling location. The experiments carried out by Ljungqvist and Reinmuller[18] did not investigate this exact situation but found that the proportion that reached a closures hopper from the floor area was about 1×10^{-3}. It may seem surprising that the proportion of MCPs that reaches the closures or vials against the downflow of air may be greater than that transmitted across the airflow (found to be about 1×10^{-4}). However, machinery can disrupt the downward airflow and produce a turbulent wake where particles can flow in the opposite direction to the overall flow. The transfer of contamination in such conditions can be complicated, and it is best determined experimentally in the individual situation. However, it has been assumed that in the worst condition, the transfer coefficient is 1×10^{-3}.

Risk factor	Assessment
1. Concentration of airborne MCPs derived from filling workstation floor (no/cm^3)	The airborne concentration of MCPs just above the floor that is derived from the workstation floor by walking is 2.3 x 10^{-13} /cm^3
2. Transfer coefficient of MCP from around the floor to vial	The transfer coefficient is assumed to be 1 x 10^{-3}
3. Area of product exposed (cm^2)	The inner neck area of vial is 2 cm^2
4. Time of airborne deposition (s)	The proportion of time that a person works in the filling workstation is 0.1 of the total time, and therefore the time for the transfer of contamination from the filling room (normally 600 s) is reduced to 60 s
5. Settling velocity of MCPs through air (cm/s)	The settling velocity through air and into a vial is 0.46 cm/s
Using Equation 1, the NMD_A can be calculated to be as follows: $NMD_A = c^*p^*a^*t^*s$ = 2.3 x 10^{-13} * 1 x 10^{-3} * 2 * 60 * 0.46 = **1.3 x 10^{-14}**	

Relative importance of sources of contamination in a typical pharmaceutical cleanroom

The NMDs from surface contact and liquid routes found in the example cleanroom are given in **Table 1**.

Discussion and conclusions

The method of ascertaining the degree of risk from sources of microbial contamination in a cleanroom is carried out by calculating the number of MCPs deposited (NMD) into, or onto, a product by means of equations presented in the introduction. A previous article[11] has considered the degree of risk from airborne sources and this article ascertains the risk from surface and liquid sources.

To illustrate the method, a pharmaceutical cleanroom is used in which batches of vials are aseptically filled in a UDAF workstation. Cleanrooms used for aseptic filling are now being designed with isolators and RABS but the cleanroom used in the example allows the demonstration of the risk assessment in a wider spectrum of cleanroom design and manufacturing methods. However, if a different cleanroom or manufacturing process is to be considered, the risk assessment must be carried out for that cleanroom.

The equations used to calculate the NMD from surface contact or liquids are fundamental, and if the input into the equations is correct then the result will be exact. Some of the equations variables (risk factors) will be known, e.g. the horizontal area of product exposed to airborne contamination, and others may need an additional collection of information, such as the concentration of MCPs on surfaces and in liquids. The transfer coefficient is a more difficult variable to ascertain, as information is not readily available. The values of the airborne transfer coefficient used in this article are obtained from the results of Ljungvist and Reinmuller[18], but we recommend further experiments to extend this knowledge. The surface transfer coefficients are based on our experimental results[16], which determined that in the worst case situation the surface transfer coefficient was unlikely to be greater than 0.2. The values of the transfer coefficients are, therefore, reasonable estimates. However, if the required risk variables cannot be obtained, and estimates based on an informed estimate, the resulting risk assessment is almost certain to be more accurate than a risk assessment based on descriptors and risk scores.

It can be seen in **Table 1** that the highest degree of risk from surface contact and liquid sources in the cleanroom example occurs if the vulnerable area of the product is touched by the gloves or garments worn by the cleanroom personnel. Personnel are trained to avoid such contact but the calculation shows what can occur if mistakes are made, and the NMD_{SC} is in the region of 10^{-8}, i.e. one product in every 10^8 may be contaminated by microbes. However, if contact is made with an

Table 1. Importance of sources of surface contact and liquid contamination in a pharmaceutical cleanroom.

Risk importance	Source of microbial contamination	NMD from surface contact and liquids
1	Contact of product with cleanroom garments*	2.8 x 10^{-8}
2	Contact of product with double gloves*	1.3 x 10^{-8}
3	Contact of product with 'sterile' tools, e.g. forceps with container neck	3.3 x 10^{-9}
4	Filtered aqueous product solution	2 x 10^{-9}
5	Liquid contamination through contact of gloves with pipework and filling needles*	3.3 x 10^{-12}
6	Floor in the UDAF filling workstation EU GGMP grade A	1.3 x 10^{-14}
7	Floor in the non-unidirectional airflow filling room EU GGMP grade B	3.5 x 10^{-14}
8	Sterilised product containers	1 x $10^{-300000}$

*Under normal control conditions, the risk will be much smaller. However, it is useful to determine the degree of risk when normal control measures have been breached and these contamination rates relate to this.

inanimate item, such as a sterilised tool or ancillary item, e.g. forceps, the NMD_{SC} will be about 10^{-9}. If the personnel's gloves make contact with vulnerable areas of pipework or needle assembly, during the set-up of the filling equipment, the NMD_{SC} is likely to be about 10^{-12}. When the primary solution of product is filtered by a single sterilised sterilising grade filter, the NMD_L is likely to be less than about 10^{-9}. The NMD from the floor in both the filling cleanroom and the filling workstation is negligible and about 10^{-14}. The risk from sterilised (depyrogenation cycle) containers is infinitely low (1×10^{-300000}).

In a previous article, Whyte and Eaton[11] discussed and calculated the NMD_A from airborne sources. A further article will consider all sources of microbiological contamination in various types of cleanrooms, i.e. those transferred by air, surface contact, and liquid routes. Also discussed will be methods used to reduce the degree of risk, where it is considered too high.

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Annex A: Calculation of the reduction of surface microbial concentrations by sterilisation

In the main body of this article, it has been assumed that surfaces of microbial sources, such as gloves, tools and garments, which are unused and sterilised by steam, dry heat and radiation, have no surface micro-organisms, or such an extremely small number that it will make no significant contribution to microbial contamination of product. The justification of this assumption is contained in this annex. With knowledge of sterilisation kinetics, the number of microbes likely to survive sterilisation can be calculated for the three sterilisation processes.

Steam sterilisation

The number of microbes that survive steam sterilisation can be calculated by means of the following equation[19].

Equation A1

$$\text{Log } B = \text{Log } A - (F_0 / D)$$

Where, A = number of microbes at the start of sterilisation, B = number of microbes at the end of sterilisation, F_0 = equivalent exposure time, and D = D-value

The values of F_0 and D are ascertained as follows.

F_0: For steam sterilisation, 121°C is the reference temperature used to calculate the effectiveness of sterilisation at other temperatures, and calculated by Equation A2:

Equation A2

$$F_0 = L \times t$$

Where, L = lethal rate, and t = sterilisation time.

At 121°C, the lethal rate (L) has a value of 1 and, therefore, for sterilisation at 121°C for 20 minutes, the F_0 value is 20 minutes.

D value: The D-value is the time required, at a specified temperature, to reduce the microbial population by one logarithmic value (90% reduction). The D-value varies according to the type of micro-organism but at 121°C, most microbes die instantly. However, bacterial spores have a much greater thermal resistance, and a D-value of 1 minute is often assumed[19]. This is a reasonable value, as spores isolated in cleanrooms are likely to be the mesophilic type that is more susceptible to heat treatment and are likely to be less than 5% of the microflora found in cleanrooms.

If appropriate values of F_0 and D are used in Equation A1, the number of surviving organisms can be calculated. For example, if the number of microbes in the internal surfaces of pipework and needles is 14, the number of surviving microbes is 10^{-19}.

Dry heat sterilisation

The number of microbes remaining after dry heat sterilisation can be calculated by means of the following equation[20].

Equation A3

$$\text{Log B} = \text{Log A} - (F_H/D)$$

Where, F_H = equivalent exposure time in dry heat.

For dry heat sterilisation, 170°C is the reference temperature from which the effectiveness of sterilisation at other temperatures can be calculated by means of Equation A4.

Equation A4

$$F_H = L \times t$$

For dry heat sterilisation at 170°C, the lethal rate (L) is 1. Therefore, using Equation A4, the F_H value for a cycle of 120 minutes at 170°C is 120. At a dry heat temperature of 170°C, a D-value of 1 minute is assumed[20].

Using Equation A3, the number of surviving micro-organisms, when the maximum number on the internal surface of an object such as a container is 10, can be calculated to be 10^{-119}. However, containers that are subjected to the depyrogenation conditions of 250°C for 30 minutes will have an increased lethal rate at this temperature that can be calculated from use of Equation A5.

Equation A5

$$L = 10^{[(To-Tb)/Z]}$$

Where, T_O = sterilisation temperature utilised, Tb = base temperature, and Z = z value.

The z-value is the temperature coefficient of microbial destruction and is the number of degrees Centigrade required to cause a 10-fold increase in the sterilisation rate, and is assumed to be 20°C[20]. Utilising a T_b value of 170°C, the lethal rate at 250°C is calculated to be 10^4. The F_H value for a 30 minute cycle at this temperature is then calculated by Equation A4 and found to be 3×10^5. Under these conditions, the number of surviving microbes in each container can then be calculated using Equation A3. As an example, if the D-value at a dry heat temperature of 250°C for 1 minute is considered with a microbial concentration on the internal surface of a vial of 10, the number of surviving organisms is $10^{-299999}$.

Radiation sterilisation

Cleanroom garments are normally sterilised by gamma radiation, using a minimum radiation dose of 25 kGy. The number of microbes on a cleanroom garments prior to sterilisation can be determined by immersing and agitating the garment in liquid, filtering the liquid, and incubating the filter. A one-piece coverall is the item of cleanroom clothing with the largest area and, therefore, the highest bioburden, and shown to have a bioburden prior to sterilisation of 190 microbes.

The radiation dose required to achieve a given sterility assurance level up to 1×10^{-6}, for a range of average bioburdens of microbes with a standard distribution of resistance against radiation, is given in table 5 of ISO 11137-2[21]. For a bioburden of 190 microbes, this is represented graphically in **Figure A1**. By extrapolation of the graph, it can be seen that the number of surviving microbes after exposure to 25 kGy is approximately 1×10^{-7}. As the one-piece coverall has an external surface area of about 16,000 cm^2, and hence a

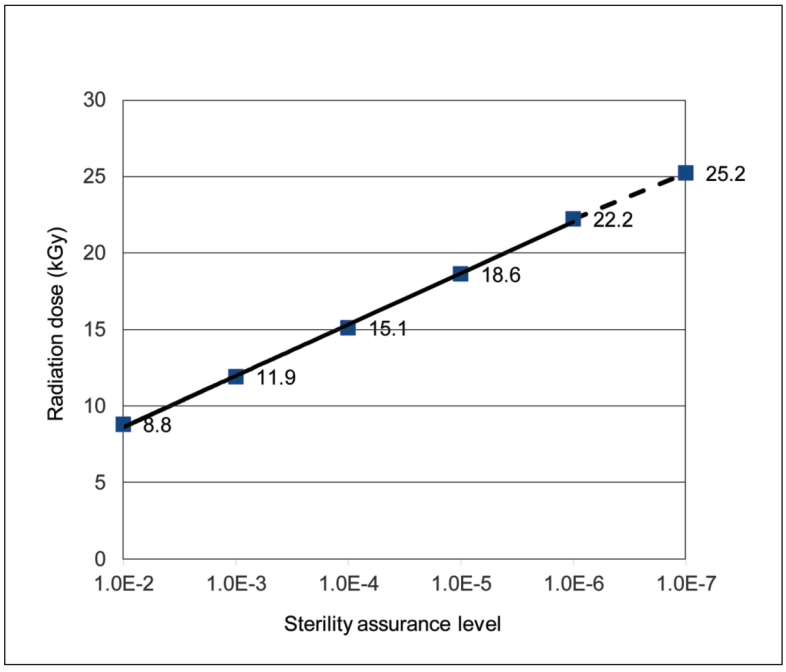

Figure A1. Radiation dose required to achieve a sterility assurance level for an average bio-burden of 190, extrapolated for a dose of 25 kGy.

total internal and external area of about 32,000 cm^2, the concentration of surviving micro-organisms on the garment surface can be assumed to be $1 \times 10^{-7} \div 32{,}000 = 3.1 \times 10^{-12}/cm^2$.

Annex B: Number of MCPs dispersed from cleanroom floor

To calculate the risk to product from MCPs dispersed by personnel walking on a floor, it is necessary to know the concentration of MCPs in the air of a clean zone that are derived from the floor. These are calculated in this annex.

The number of MCPs dispersed from a floor by walking has been investigated by Whyte *et al*[22] who showed it to be dependent on the total number of steps per second taken by all of the personnel in the room, the shoe area, and the 'redispersion fraction' (R_F), which is the fraction of MCPs on the floor surface that is dispersed by one step. The dispersion rate can be calculated as follows.

Equation B1

$$D_F = C_F \times A_S \times R_F \times N \times W \times P$$

Where, D_F = microbial dispersion rate, C_F = concentration of microbes on floor surface, A_S = area of shoe in contact with floor, R_F = redispersion fraction, N = number of people in room, W = walking rate (number of steps/s), and P = proportion of time spent walking.

Knowing the dispersion rate of MCPs from the floor by walking, the airborne concentration of MCPs in both the filling cleanroom and filling workstation can be calculated as follows.

Filling cleanroom

When MCPs are dispersed from the cleanroom floor, they will mix with the air in the non-unidirectional airflow filling cleanroom to give a reasonably constant concentration across the room. The airborne concentration of MCPs can be calculated by Equation B2 derived by Whyte *et al*[22] to take account of dilution by the air supply to the cleanroom and the loss by gravitational re-deposition onto the floor.

Equation B2

$$\text{Airborne concentration of floor-derived MCPs/m}^3 = \frac{D_F}{Q+(V_D \star A)} = \frac{C_F \star A_S \star R_F \star N \star W \star P}{Q+(V_D \star A)}$$

Where, Q is the rate of air supply volume (m^3/s), V_D is the deposition velocity of MCPs (0.0046 m/s), and A is the deposition area (m^2) in the room (normally the floor).

The meaning and the value of the deposition velocity of MCPs, which is 0.0046 m/s, is discussed in Part 1 of these articles[11] and the experiment to determine the redispersion factor, which was 0.0012, is described by Whyte *et al*[22].

In the cleanroom example being studied, the following is assumed: two people walk about the filling cleanroom for a proportion of 0.5 of the time, at a rate of 1.5 steps per second, and have shoes with a contact area of 110 cm^2 (0.011 m^2). The redispersion fraction is 0.0012, the air supply rate is 3.33 m^3/s, and the floor area is 100 m^2 with a microbial surface concentration of $1.2/m^2$. The airborne concentration of MCPs in the filling cleanroom in the steady-state condition during manufacturing (C) is, therefore, as follows.

$$C = \frac{1.2 \star 0.011 \star 0.0012 \star 2 \star 1.5 \star 0.5}{3.33+(0.0046 \times 100)} = 6.3 \times 10^{-6}/m^3 = 6.3 \times 10^{-12}/cm^3$$

This concentration is used in the *"Filling cleanroom"* subsection of the "Microbial dispersion from cleanroom floor" section to calculate the NMD_A when the source is the filling cleanroom floor.

Filling workstation

In the filling workstation, the mechanism of dispersion of MCPs from the floor into air is the same as the filling cleanroom, and the number of MCPs dispersed per second can also be calculated by Equation B1. However, only one person attends to the filling line, and spends a smaller proportion of their total time (0.1) working and walking in the filling workstation. Their walking rate is again 1.5/s with a shoe area of 0.011 m^2. The microbial concentration on the floor of the filling workstation is lower than the filling room, and $0.42/m^2$. The microbial dispersion rate (D_F) is therefore as follows.

$$D_F = C_F \times A_S \times R_F \times N \times W \times P =$$
$$0.42 \star 0.011 \star 0.0012 \star 1 \star 1.5 \star 0.1 = 8.3 \times 10^{-7}/s$$

Because of the downward UDAF in the filling workstation, the MCPs dispersed from the floor will not mix with all of the air in the workstation but only with the air above the floor. The area of the air supply filters is 3 m x 3 m, and air is discharged from the filters at a velocity of 0.4 m/s; there is, therefore, an air supply volume of 3.6 m^3/s. As the UDAF does not pass through the floor, the UDAF will change to non-unidirectional airflow above the floor and turbulently mix with the dispersed MCPs. The concentration of MCPs close to the floor can, therefore, be calculated in the steady-state condition by use of Equation B2 but, as the floor area is so small, the MCP deposition on the floor area is ignored.

$$\text{Airborne concentration of floor-derived MCPs close to floor} = \frac{\text{microbial dispersion rate from floor/s}}{\text{air supply volume rate (m}^3\text{/s)}} = \frac{8.3 \times 10^{-7}/s}{3.6 m^3/s}$$

$$= 2.3 \times 10^{-7}/m^3 = 2.3 \times 10^{-13}/cm^3$$

The NMD_A is calculated in the *"Filling workstation"* subsection of the "Microbial dispersion from cleanroom floor" section from this concentration.