

## Antimicrobial efficacy and systematic use of disinfectants

K. Steinhauer

Schülke & Mayr GmbH, Research and Development, Robert-Koch-Str. 2, D-22840 Norderstedt, Germany

Microbial contamination is of critical importance when it comes to production processes, especially in the pharmaceutical industry. Avoidance of microbial contamination throughout production processes is therefore crucial. However, what are the essentials of an effective hygiene regime? And what kind of hygiene measures are to be taken in order to be safe and effective? This work demonstrates how a systematic review of monitoring data, sources of contamination, efficacy of biocidal agents and efficacy testing of disinfectants according to European norms can help to set up a customised and effective hygiene regime. Antimicrobial efficacy of biocidal actives will be looked at in more detail, as this provides the data for an appropriate choice of disinfectants in the first place. Furthermore, sources of microbial contamination will be presented in a systematic way and it is demonstrated how these data may be used for a systematic risk assessment of any production site. A systematic review of microbial contamination derived from the monitoring data is provided and it will be demonstrated how monitoring data can be easily used to set up effective hygiene measures. Last but not least, European norms (EN) used for the overall evaluation of antimicrobial efficacy of disinfectants will be discussed.

**Keywords** microbial contamination; cleanroom; hygiene measures; European norm; efficacy testing; disinfectants; EU GMP guideline

### 1. Antimicrobial efficacy of various disinfectant actives

Choosing an effective disinfectant regime that is appropriate to the hygiene requirements in a production plant poses a complex question. In this context, consideration of the antimicrobial efficacy of different biocides is an important decision-making basis. Not all disinfectant actives have the same spectrum of effect. When systematically using disinfectants it is therefore vital to cast a glance at the spectrum of effect of different disinfectants:

Spectrum of action	Bactericidia		Mycobactericidia	Sporicidia	Fungicidia		Virucidia
	Gram+ bacteria	Gram- bacteria	Myco- bacteria	Bacterial spores	Yeasts	Moulds	Viruses
Alcohols	+++	+++	+++	-	++	++	++
Quats	+++	++	-	-	+++	+++	++
Guanidines	+++	+++	-	-	++	++	++
Aldehydes	+++	+++	+++	+++	++	++	++
Active oxygen	+++	+++	+++	+++	+++	+++	+++
Peracetic acid	+++	+++	+++	+++	+++	+++	+++

**Fig. 1: Illustrative presentation of the microbicidal efficacy of biocidal actives.** The microbicidal efficacy of the selected biocides is subdivided into bactericidal, mycobactericidal, sporicidal, fungicidal and virucidal efficacy. +++: good efficacy; ++: moderate efficacy; -: inadequate efficacy

Fig. 1 gives for selected actives an overview of the spectrum of antimicrobial efficacy of different biocides, with a distinction being made between bactericidal, fungicidal, sporicidal, mycobactericidal and virucidal efficacy.

The overview in Fig. 1 shows that alcohols, which act rapidly and effectively against bacteria, mycobacteria, fungi and viruses, do not possess sporicidal efficacy. Quaternary ammonium compounds (quats) likewise have no effect

against bacterial spores, nor are they effective against the mycobacteria that are of such significance as infection pathogens, especially in the medical area.

This is also true of guanidines, which, compared with quaternary ammonium compounds, have rather better efficacy against Gram-negative bacteria, but are rather poorer against yeasts and moulds.

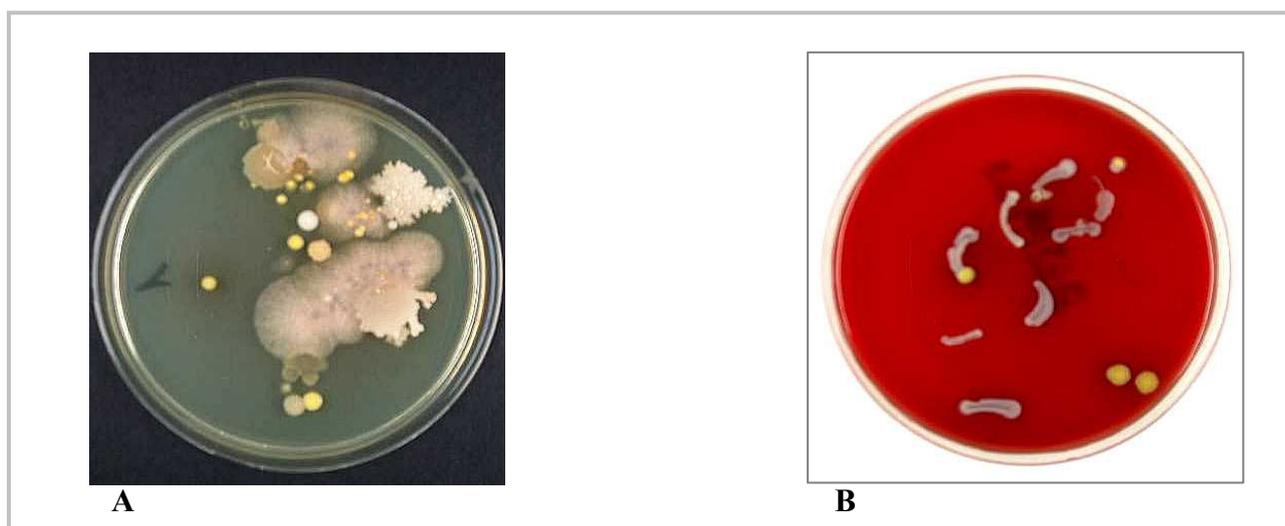
Aldehydes and active oxygen compounds (e.g. perform classic concentrate OXY) and peracetic acid have a broad antimicrobial spectrum of action. However, the fungicidal and virucidal efficacy of aldehydic compounds tends to be rather poorer compared with that of active oxygen compounds and peracetic acid, [1].

## 2. Sources of contamination

In addition to consideration of antimicrobial efficacy, consideration of microbial contamination plays a crucial role when choosing suitable and effective disinfectants in the production plant. Thus a critical look and identification of actual or potential sources of contamination can provide a first indication of necessary disinfection measures:

Fig. 2A shows an example of the microbial contamination of packaging material, which is a typical source of entry for contamination with spore-forming bacteria, moulds and yeasts. In areas where cardboard materials are used extensively, and entry of spore-forming bacteria, yeasts and moulds has been detected during monitoring, for successful hygiene management it is therefore sensible to ensure the use of disinfectants with bactericidal, fungicidal and sporicidal efficacy.

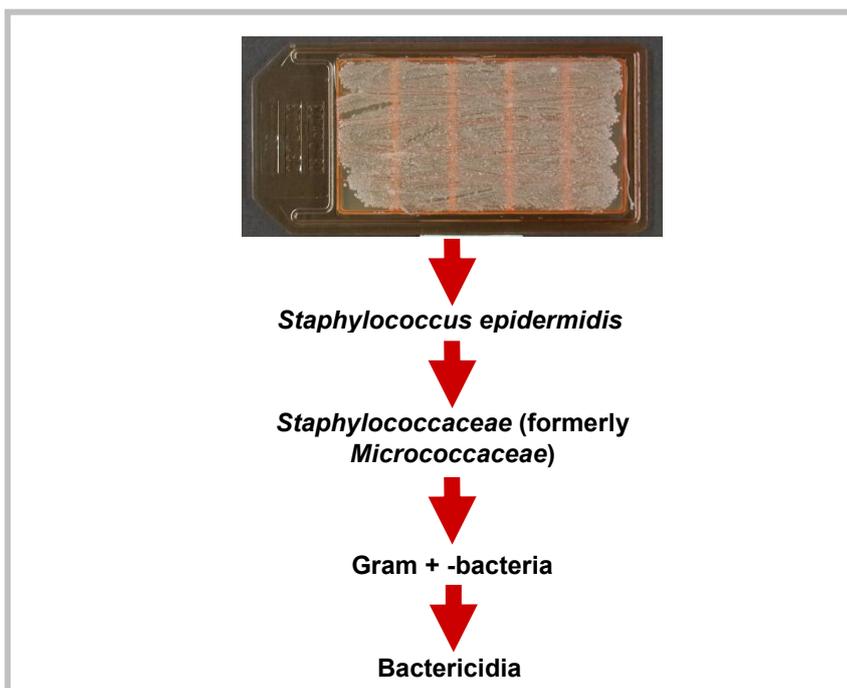
And in the production process the employees themselves are also classic sources of entry of microbial contamination, especially with Gram-positive bacteria such as staphylococci and micrococci, which belong to the resident and transient microbiological flora of humans and other mammals [2]. Fig. 2B shows an example of the colonisation of human hair with *Staphylococcus* spp.



**Fig. 2: Illustration of microbial contamination. A: Microbiological contamination of packaging material.** Spore-forming bacteria and also yeasts and moulds are often carried into the production process by packaging material. Depicted is the microbial contamination of a carton. **B: Human hair after incubation on blood agar.** Human hairs are naturally colonised with microorganisms, and especially *Staphylococcus* spp. can be detected on human hairs.

In Fig. 3 the result of a swab test on a ring are shown. Here, the inner side of the ring that comes into contact with the skin was swabbed with a sterile cotton wool swab. The swab was then streaked out onto a sterile nutrient medium and incubated. The result shows in an impressive manner the extent to which a human being can function as a potential source of contamination. Here too, with *Staphylococcus epidermidis*, it is a species that belongs to the resident flora of the human skin. The data also show that jewellery can play an essential role as a source of contamination. Employees who are involved in critical process steps should therefore be urged to remove jewellery in the interests of production hygiene.

As shown in Fig. 3, *Staphylococcus epidermidis* is a Gram-positive bacterium that is killed by disinfectants with proven bactericidal efficacy.



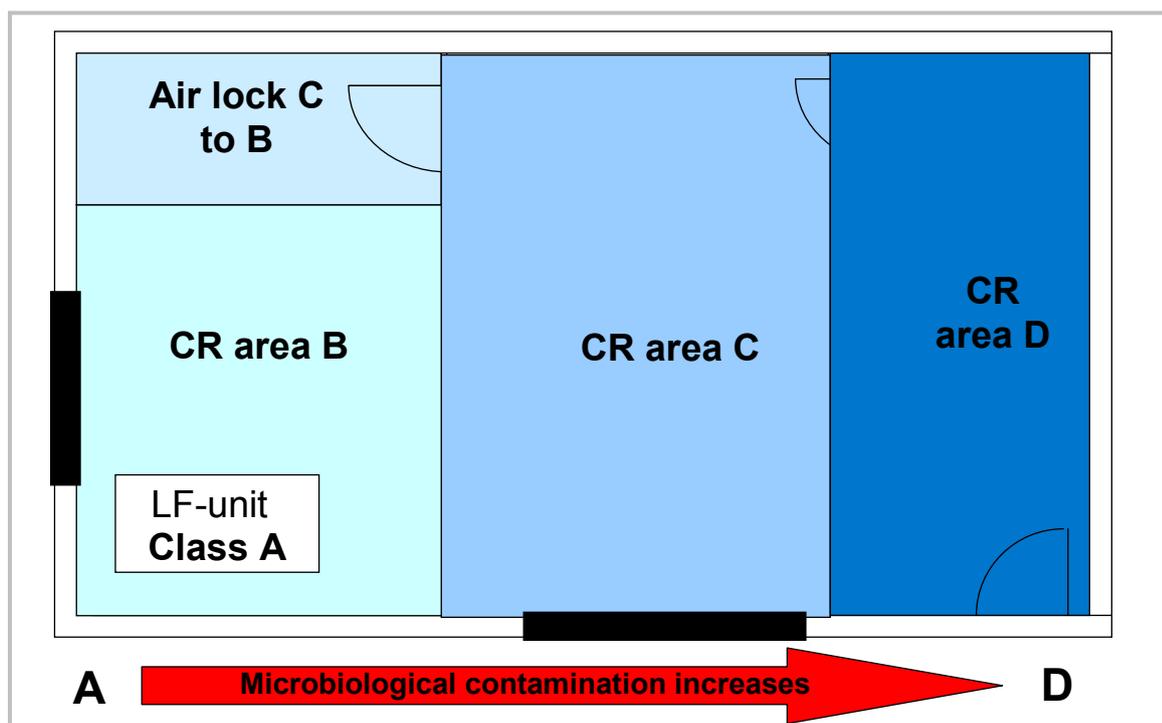
**Fig. 3:** Detection of *Staphylococcus epidermidis* on the inner side of a ring that comes into contact with the skin. Shown is the microbiological contamination of a ring as a result of the intensive skin contact, constituting a potential contamination danger in the production process. *Staphylococcus epidermidis* is a Gram-positive bacterium, and can be effectively combated by the use of bactericidal disinfectants.

A further typical source of contamination is raw materials. For example, when raw materials of plant origin are used, entry of Gram-negative bacteria such as *Enterobacteriaceae* or spore-forming *Clostridium* spp. can occur. If a risk of entry of spore-forming *Clostridium* spp. or the likewise spore-forming *Bacillus* spp. is identified, appropriate sporicidal disinfection measures are necessary. However, if the risk can be excluded, use of sporicidally effective disinfectants is not necessary.

### 3. Microbiological monitoring

Analysis of the data obtained during regular microbiological monitoring is a further essential cornerstone for establishing a systematic disinfection regime.

In Fig. 4 the different cleanroom areas (A-D) are depicted schematically. The permissible microbiological burden increases from cleanroom class A to D. Cleanroom class D can therefore be considered to be the area in which in a correctly run operation the greatest microbiological burden is to be expected, and as an example is to be considered more closely.



**Fig. 4: Schematic representation of different cleanroom areas (A-D).** The permissible microbiological contamination increases from A to D. Abbreviations: LF – Laminar-Flow; CR – cleanroom.

In Table 1 the microbiological findings obtained from various sampling sites in a class D cleanroom over a period of several years are given as examples. The findings of the microbiological monitoring in Table 1 show that the detected microbial contamination is due essentially to the following microorganisms:

- micrococci
- spore-formers
- moulds

In the following, as a basis for the choice of a suitable disinfection regime, the microorganisms detected in the microbiological monitoring are to be considered more closely and classified according to their taxonomic group:

Viewed taxonomically, micrococci belong to the *Micrococcaceae*. The “coccus” part of the name indicates the spherical shape of the bacterial cells. *Micrococcaceae* are Gram-positive bacteria, and occur widely in nature, including on the skin and mucous membranes of mammals. Representatives of the *Micrococcaceae* family are e.g. *Kocuria rhizophila*, or *Micrococcus luteus* [2]. The genus *Staphylococcus* was also classified as part of the *Micrococcaceae* family for a long time on account of its morphological characteristics [3]. However, according to more recent studies based on analyses of the DNA, the staphylococci have been classified as constituting their own family, the *Staphylococcaceae* [2]. Both *Micrococcaceae* and *Staphylococcaceae* belong to the large group of Gram-positive bacteria that is in contrast to the Gram-negative bacteria. Gram-positive bacteria and Gram-negative bacteria differ essentially in their cell wall structure. With regard to susceptibility to biocides, among themselves Gram-positive bacteria and Gram-negative bacteria consequently each behave similarly. However, when compared directly with each other, on account of their different cell wall structures Gram-positive bacteria and Gram-negative bacteria differ in their susceptibility to biocides. Therefore, in tests of disinfectants for their bactericidal efficacy according to European norms, both Gram-positive and Gram-negative bacteria are always used in the testing.

**Table 1:** Example of microbial contamination detected in cleanroom class D during a regular microbiological monitoring inspection.  
\*[4] Annex 1 EU GMP Guidelines (2008 rev.)

Site	Sample	Limit value*	2005	2006	2007	2008	2009
Clean-room Cl. D	A	500 cfu/m <sup>3</sup>	<b>138</b> cfu/m <sup>3</sup> ( <i>spore-formers</i> )	<b>40</b> cfu/m <sup>3</sup> ( <i>spore-formers, moulds</i> )	<b>10</b> cfu/m <sup>3</sup> ( <i>micrococci</i> )	<b>0</b> cfu/m <sup>3</sup>	<b>20</b> cfu/m <sup>3</sup> ( <i>micrococci, moulds</i> )
Clean-room Cl. D	B	40 cfu/h	<b>3</b> cfu/h ( <i>micrococci</i> )	<b>2</b> cfu/h ( <i>spore-formers</i> )	<b>0</b> cfu/h	<b>0</b> cfu/h	<b>2</b> cfu/h ( <i>moulds</i> )
Clean-room Cl. D	C	300 cfu/75 cm <sup>2</sup>	<b>24</b> cfu/ 75 cm <sup>2</sup> ( <i>spore-formers, micrococci</i> )	<b>8</b> cfu/ 75 cm <sup>2</sup> ( <i>spore-formers, micrococci</i> )	<b>48</b> cfu/ 75 cm <sup>2</sup> ( <i>spore-formers, micrococci</i> )	<b>9</b> cfu/ 75 cm <sup>2</sup> ( <i>spore-formers, micrococci</i> )	<b>4</b> cfu/ 75 cm <sup>2</sup> ( <i>spore-formers</i> )

Under hostile environmental conditions spore-forming bacteria are able to form endospores. Endospores are extremely resistant survival forms that are intrinsically inert in the face of many environmental influences, and can also survive temperatures > 100°C undamaged. The genus *Bacillus* with more than 100 species includes the best characterised representatives among the aerobic spore-forming bacteria. In addition to *Bacillus subtilis*, mention must be made in this connection of species such as e.g. *B. megaterium*, *B. anthracis*, *B. cereus*, *B. licheniformis* or *B. thuringiensis*, some of which have a considerable pathogenic potential [5].

As saprophytes, aerobic spore-forming bacteria are ubiquitous in the environment, and are present both in the ground and in water.

The ability to form endospores, which are resistant to drying out, also results in the extensive dissemination of endospore-forming bacteria by the air [5]. Thus Rahman *et al.* (2006) in their examination of eye drops were able to detect *Bacillus* spp. among others as contaminants in the containers that were in use [6].

A further important genus among the endospore-forming bacteria is the genus *Clostridium*, which combines aerotolerant and anaerobic spore-forming Gram-positive bacteria. Clostridia, which like *Bacillus* spp. are ubiquitous in the environment, are also to be found as a component of the intestinal flora, and as pathogenic bacteria are frequently associated with a number of serious infections [7]. *Clostridium botulinum*, as a result of its ability to produce highly potent neurotoxins, is the most important of these pathogens, being the most lethal [8]. With regard to food poisoning, *Clostridium perfringens* is one of the most significant pathogens: in an investigation of cases of food poisoning in England and Wales between 1992 and 2000, Adak *et al.* (2002) identified *C. perfringens* as the second most lethal food-poisoning pathogen after *Salmonella* [9].

*Clostridium sporogenes* is closely related to the proteolytic strains of *C. botulinum*, although the endospores of *C. sporogenes* are about 5 times more resistant [8].

Viewed taxonomically, moulds belong to the fungi. At the same time, in the technical area a differentiation is frequently made between the filamentous fungi, to which mycelium-forming fungi belong, and the non-filamentous yeast fungi. This differentiation on the basis of morphological characteristics with regard to mycel formation is also reflected in the testing of efficacy of biocidal substances: thus a differentiation is made between levurocidal and fungicidal efficacy of biocides, with levurocidia indicating efficacy against yeasts, and fungicidia efficacy against yeasts and filamentous fungi (therefore against moulds). In the case of testing for fungicidia, the conidiospores of the filamentous mould fungi as well as the vegetative cells of the yeast are used in the test of efficacy [10].

Mould fungi are ubiquitous and are subject to mainly aerogenic dissemination throughout the world [11]; [12]. The spectrum of the different mould fungus species is subject to seasonal oscillations: in their study of the presence of different fungal species in the air, increased numbers of *Cladosporium* spp. and *Alternaria* spp. were detected by Fröhlich-Nowisky *et al.* (2009) in summer and autumn, but in winter and spring they detected increased numbers of *Penicillium* spp. and *Blumeria graminis* [13].

#### 4. Microbiological reports – benefits for estimation of the risk

On the basis of taxonomic affiliation, conclusions can be reached that are helpful in the selection of suitable disinfectants. For this, individual reports of the efficacy (e.g. EN 1276, EN 1650, EN 13704, EN 13697) of the biocides under consideration serve as a basis for an evaluation.

The efficacy tests according to EN are divided into different phases that depict the similarity of the tests to practical conditions. Phase 1 quantitative suspension tests form the basic test, and are carried out without organic loads (EN 1040 (bactericidia) [14]; EN 1275 (fungicidia) [15]; EN 14347 (sporicidia) [16]). These tests are the furthest from use in practice. The tests in phase 2 simulate practical conditions more closely, and phase 2 consists of 2 stages: quantitative suspension tests, phase 2 / step 1, which are carried out in the presence of organic load (EN 1276 (bactericidia) [17], EN 1650 (fungicidia) [10], EN 13704 (sporicidia) [18]) and quantitative microorganism carrier tests, phase 2 / step 2, in which in addition to the presence of organic load practical use on standardised surfaces is also simulated (EN 13697 (bactericidia / fungicidia) [19]). EN 13697 can be modified by the use of bacterial endospores (e.g. *Bacillus subtilis*) so that testing for sporicidal efficacy is possible (EN 13697 mod.).

In the case described (see Table 1) spore-forming bacteria, *Micrococcaceae* and moulds were detected. Thus, for effective hygiene management in this example disinfectants must be selected for which sporicidal, bactericidal and fungicidal efficacy has been proven according to the appropriate EN tests.

Using the example of efficacy against spore-forming bacteria, in the following it should be seen how the results from the monitoring, together with the specific tests of efficacy of a disinfectant in accordance with European norms (ENs) can be used for a systematic analysis of the risks:

As verification of the sporicidal efficacy of disinfectants tests according to EN 14347 (phase 1), EN 13704 (phase 2 / step 1) and EN 13697 (phase 2 / step 2) can be used.

The test for sporicidia according to EN 13704 is a quantitative suspension test (phase 2 / step 1), in which the sporicidal efficacy of a disinfectant is tested in the presence of an organic load. In addition, the test on surfaces simulating practical conditions (EN 13697) can be modified and also used to prove sporicidal efficacy. In both cases *Bacillus subtilis* ATCC 6633 is the standard test organism. In addition, however, *Bacillus cereus* ATCC 12826 or *Clostridium sporogenes* 51 CIP 7939 may also be used.

Fig. 5 shows how the basis for a systematic risk analysis can be achieved by means of the taxonomic classification of the microorganisms detected in the monitoring:

In the monitoring, spore-forming bacteria were detected. The spore-forming bacteria with hygienic relevance as described above are limited to the genera *Bacillus* and *Clostridium*. *Bacillus subtilis* ATCC 6633 is used in EN 13704 as an obligatory test organism in the form of endospores, and can also be used in EN 13797 mod. as a standard test organism in testing for sporicidia.

Our own laboratory tests have also shown that the resistance of *Bacillus subtilis* endospores to various biocides is greater than that of *Clostridium sporogenes* endospores (Steinhauer, K. *et al.* (2010) unpublished).

Thus the efficacy of a disinfectant tested according to EN 13704 and / or EN 13697 mod. against the relevant spore-formers detected in the microbiological monitoring (see Table 1) is systematically confirmed.

As a result of the reduction of at least  $10^3$ - $10^4$  microorganisms per test surface (3-4 log steps) required in the efficacy test EN 13697 (phase 2 / step 2), with regard to practical use there is also a considerable safety margin in the case of disinfectants tested according to EN 13697: thus the recommended limit values for microbiological supervision (cleanroom class D) are 50 cfu (colony forming units) / plate (diameter 55 mm; surface 23.8 cm<sup>2</sup>, [4]).

However, with a surface disinfectant that was tested under simulated practical conditions according to EN 13697, a reduction in the microbial count of 3-4 log steps was achieved on a surface of diameter 20 mm (3.1 cm<sup>2</sup>) [20].

Example:

Detection of spore-forming bacteria in the monitoring:

-> hygienically relevant spore-forming bacteria are *Bacillus* spp. or *Clostridium* spp.

*Taxonomic classification of Bacillus* spp.:

Bacteria; Firmicutes; Bacilli; Bacillales; *Bacillaceae*; *Bacillus*

**Test organisms in EN tests**

EN 13704 (phase 2 / step 1), quantitative suspension test with organic load

EN 13697 mod. (phase 2 / step 2), quantitative surface test

- *Bacillus subtilis* ATCC 6633 (obligatory)
- *Bacillus cereus* ATCC 12826 (additional)
- *Clostridium sporogenes* 51 CIP 7939 (additional)

**Fig. 5:** Example for a systematic risk analysis on the basis of the taxonomic classification of the microbial contamination detected in the monitoring (in this example they are spore-forming bacteria that were detected (see Table 1)).

The test for bactericidia according to EN 1276 is a phase 2 / step 1 quantitative suspension test, and like the test on surfaces simulating practical conditions according to EN 13697 requires *Staphylococcus aureus* ATCC 6538 as the obligatory test organism. As already stated in the previous section, *S. aureus* is a member of the *Staphylococcaceae* family and was previously classified as a member of the *Micrococcaceae* family. Both the staphylococci and the micrococci are Gram-positive bacteria, which can be regarded as equal with regard to their susceptibility to disinfectants. Thus efficacy of a disinfectant tested according to EN 1276 and EN 13697 against the micrococci detected in the microbiological monitoring (see Table 1) is systematically and reliably verified.

An analogous procedure can be followed in the case of fungicidia. Here the mould fungus *Aspergillus brasiliensis* (formerly *A. niger*) ATCC 16404 and the yeast *Candida albicans* ATCC 10231 are used as standard test organisms in the EN tests (EN 1275 (phase 1), EN 1650 (phase 2 / step 1), EN 13697 (phase 2 / step 2)).

If mould fungi are detected in the monitoring or if a risk of mould fungi in the production process is identified it is advisable to fall back on disinfectants with proven fungicidal efficacy. If there is no problem of contamination with filamentous fungi (moulds), but there is a danger of contamination with yeasts, fungicidally effective disinfectants are not necessary. In this case it is sufficient to use a disinfectant that is effective against yeasts that is characterised by the concept of levurocidal efficacy. This is documented by the exclusive use of the yeast *Candida albicans* as the test organism in tests of efficacy, and then the result is the claim of levurocidal efficacy, which is limited to efficacy against yeasts. As stated above, this can be perfectly adequate if there is no risk of contamination by mould fungi.

## 5. Summary

Avoidance of microbiological contamination in the course of the production process is an essential condition for the production of safe products in the pharmaceutical industry. Appropriate and effective measures can be taken if information with regard to the general spectrum of efficacy of biocides, the potential sources of contamination, information from microbiological monitoring and expert reports on the microbiological efficacy of disinfectants are systematically brought together and evaluated. As shown above, the risks can be deduced systematically from this information, forming the basis for an individual hygiene regime adapted to the operational requirements.

Regular microbiological monitoring is of crucial importance in this: on the one hand the monitoring findings form an important decision basis for the selection of suitable disinfection measures, and on the other hand the monitoring has a decisive control function: by means of the microbiological monitoring the efficacy of the disinfectants used is repeatedly checked. In this way any changes in the microbiological spectrum are detected immediately and in turn form the basis for appropriate adaptation of the disinfection regime. For example, exceeding of the microbiological warning values (limit values) in combination with a changed microbiological spectrum of the contamination is a clear indication of altered conditions, which must be taken into account in a systematic adaptation of the hygiene regime. An example of this would be the sudden occurrence of mould spores as a result of a newly installed composting facility in the proximity of the production plant, although mould fungi have never previously played a role in the production site.

The microbiological monitoring is also of essential importance with regard to checking that the disinfection measures are being applied correctly. The microbiological monitoring enables a regular check of whether the selected hygiene regimen is also being followed correctly, and this is documented by the warning limit (action limit) being within the specified limits when the microbiological spectrum is unchanged. If, however, the warning limit (action limit) are exceeded when there has been no change in the microbiological spectrum of the contaminants, it is reasonable to suppose errors in the application of the disinfection measures used, an immediate response to which must be made e.g. by training of those responsible.

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