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Similarities and differences in the responses of microorganisms to biocides

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Unlike antibiotics, biocides are multi-targeted antimicrobial agents. Several of the damaging effects reported to occur in the most widely studied organisms, bacteria, may also take place to varying degrees in other organisms. Nevertheless, there is considerable variation in the response of different microorganisms to biocides. Bacteria themselves (Gram-positive and Gram-negative vegetative organisms, mycobacteria and spores) respond differently to biocides and this disparity is widened when yeasts, moulds, protozoa and algae are considered. The underlying reasons for these varied responses are poorly understood at present, but the chemical composition of outer cellular layers is likely to be a factor of prime importance. Other possible contributory factors may be differences in stress responses, the presence of efflux pumps and cells occurring within biofilms or algal mats.

Keywords: microbes, microbial response, mechanisms of biocide action, microbial resistance

Introduction

Detailed information (Figure 1) is available about the activity spectra of biocides (antiseptics, disinfectants and preservatives). This provides an important basis for the never-ending attempt to control harmful microorganisms.[1] Comparatively few biocides are bactericidal (including mycobactericidal), sporicidal, virucidal and fungicidal, whereas most are bactericidal (with or without being mycobactericidal), virucidal and fungicidal but do not inactivate spores.[2] Some biocides show activity against protozoa and algae.[3–6] Those factors that affect antimicrobial activity, namely period of contact, concentration, temperature, pH, presence of organic soiling matter, and type of organism, are well documented[7] and influence the manner in which biocides are used for whatever purpose and against a variety of microorganisms.[8]

A particular biocide may thus inactivate (or sometimes inhibit) more than one type of microorganism. With our current levels of understanding of the mechanisms of biocidal action and of microbial resistance, it is pertinent to consider whether it is possible to explain why both similarities and differences in response to biocides occur in microbes that differ widely in their structure and physiology.[9] In some ways, this presents a more formidable task than for antibiotics where a clear knowledge of mechanisms of inactivation and of resistance enables logical conclusions to be reached about specificity and selectivity of action. Thus, as pointed out by Ghannoum & Rice,[10] a comparison between, for example, antibacterial and antifungal resistance to antibiotics is limited. Antibiotics are considered to have one major target site, usually inhibition of a particular biosynthetic process,[11] although other effects may also be known. However, their actions tend to be much clearer-cut than those of biocides, whose effects are highly concentration-dependent.[8] Consequently, delineating the reasons for activity against a range of organisms becomes more difficult with biocidal agents.

Microbial cells

Two types of organisms, prokaryotes (bacteria) and eukaryotes, are considered here. Of the latter, moulds (filamentous fungi) and yeasts (unicellular fungi), protozoa and algae will be discussed. The fungi and algae (except euglenoids) possess rigid cell walls, whereas protozoa lack a 'true' cell wall.

Many different types of microorganism (bacteria, fungi and protozoa) have been associated with serious human infections. Certain filamentous algae may produce thick carpet-like mats in freshwaters. Algal growth causes problems in swimming pools and cooling towers, and their control by algicides is often necessary.[12,13] Food poisoning by micro-algae has been described.[14] Thus, it is important to appreciate not only appropriate chemotherapeutic measures but also suitable procedures, often involving biocides, for controlling or preventing the spread of infections or other hazards caused by a variety of prokaryotic and eukaryotic microbes.

There are obviously considerable differences in the structure and composition of microbial cells and Table 1 summarizes the chemical nature of the outer layers of some of these organisms. There is no consistent theme. There are variations between similar types of organisms, e.g. cocci, Gram-negative bacteria and mycobacteria, whilst spores have coats that are also entirely different chemically from the cell walls of non-sporulating bacteria and indeed from the walls of germinating, outgrowing and vegetative forms produced

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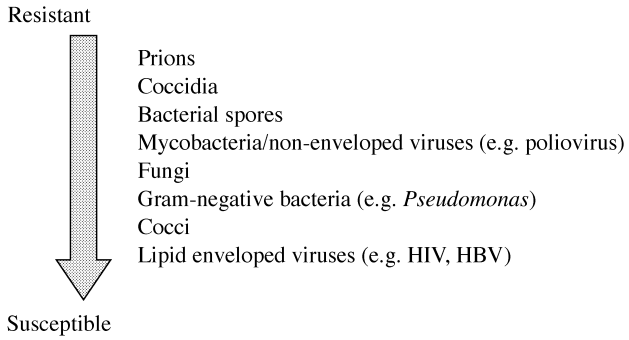

Figure 1. Relative susceptibility of entities (prions, viruses) and microorganisms to biocides. Algae not shown, but likely to be susceptible to at least some biocides.

from spores themselves. Further, variations in chemical composition may occur on growth under different conditions producing altered responses to antimicrobial agents.

It is not surprising, therefore, that microbial susceptibilities to biocides and especially to antibiotics differ greatly. It should be noted, however, that even with antibiotics, some degree of 'cross-activity' occurs. For example, many antibiotics that specifically interfere with the 70S ribosome function in bacteria will also inhibit protein synthesis in mitochondria and chloroplasts. Fusidic acid has some activity against a range of protozoa, including *Giardia lamblia*. Mupirocin is active at low concentrations against staphylococci but has some clinical effect at much higher concentrations against fungi, and chloramphenicol shows a broad spectrum of activity against actinomycetes, mycoplasmas, *Leptospira* species and *Treponema pallidum*.[15] Metronidazole, a 5-nitroimidazole derivative, has activity against anaerobic bacteria and protozoa and interferes with DNA synthesis via a metabolite in which the NO_2 group of metronidazole has been reduced.[16] For their part, biocides usually show a low degree of selectivity in their action against different types of microorganism. Chlorhexidine salts (CHX), quaternary ammonium compounds (QACs), monoaldehydes (formaldehyde), dialdehydes [glutaraldehyde (GTA), *ortho*-phthalaldehyde (OPA)], chlorine-releasing agents (CRAs) and other halogens, organomercurials, phenolics, peroxygens and alcohols all show varying degrees of activity against bacteria, bacterial spores, fungi, viruses and protozoa[1] and at least some have algicidal activity.[3–6]

It is the purpose of this paper to examine the effects of biocides on bacteria, fungi, protozoa and algae in order to determine whether common mechanistic patterns emerge and, if not, the reasons for different responses. Similarities and differences in response, based upon cellular physiology and structure, form the basis of this review. The likely outcomes of biocidal action in practice are thereby not covered, although one particular and important aspect, namely biofilm cultures, will be referred to when necessary. Whilst the actions of biocides on viruses and bacteriophages will not be discussed in detail, it is necessary to consider these effects when appropriate insofar as they shed light on the nature of the interaction between biocides and a particular target site, e.g. protein or nucleic acid.

There are some general questions that need to be posed when biocidal activity is considered against different types of microbes: (i) are there common pathways of biocide uptake and entry into cells; (ii) are there common target sites; (iii) do target sites change during cell differentiation; and (iv) are there common resistance mechanisms? These and other aspects are discussed below.

Biocide adsorption and uptake into cells

Interaction of a biocide with the whole microbial cell is conventionally measured by determining its adsorption. As a result, five different classes of adsorption are known.[17] These may be summarized as follows: (i) S-shaped pattern, in which the solute molecule is monofunctional, is orientated vertically and meets strong competition from the solvent molecules or by another adsorbed species; (ii) L (Langmuir) pattern in which, as more sites are filled, it becomes increasingly difficult for a solute to find a vacant site; (iii) H (high affinity)

Table 1. Composition of outer cell layers of different microorganisms

Organism	Outer cell layers[a]	Example(s)
Gram-positive cocci	CW: predominantly PTG	staphylococci
Gram-negative bacteria	OM: PL, LPS	*P. aeruginosa*, *E. coli*
Mycobacteria	CW: mycolate of AG, lipid	*Mycobacterium tuberculosis*
Bacterial spores	OSC: alkali-resistant (S-S bonds) ISC: alkali-soluble (acidic polypeptides) cortex: PTG, including spore-specific	*Bacillus* spp.
Yeasts and moulds	CW: chitin + chitosan CW: chitin + glucan CW: glucan + mannan	*Mucor rouxii* *A. niger* *S. cerevisiae*, *C. albicans*
Intestinal protozoa	cysts have thick outer coverings	*C. parvum*
Other protozoa	double-walled cysts containing cellulose (during encystation)	*A. castellanii*
Algae	CW: cellulose + other polysaccharides + other constituents	green (Chlorophyta), brown (Phaeophyta) and red (Rhodophyta) algae

[a]CW, cell wall; PTG, peptidoglycan; OM, outer membrane; PL, phospholipid; LPS, lipopolysaccharide; AG, arabinogalactan; OSC, outer spore coat; ISC, inner spore coat.

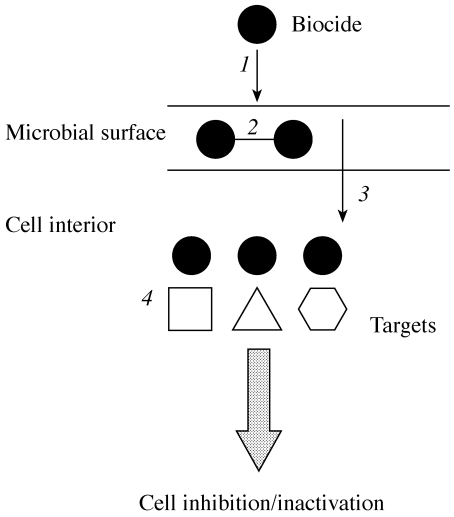

Figure 2. General pattern of biocide entry into different types of microorganisms (for simplicity, no barrier function is envisaged). 1, Adsorption of biocide to cell surface; 2, interaction with outer cell layers; 3, uptake into cell; 4, interaction with target site(s).

pattern, obtained when the solute is almost completely adsorbed; (iv) C (constant partition) pattern, obtained when the solute penetrates more readily into the adsorbate than the solvent; and (v) Z pattern, in which there is a sharp break in the pattern followed by an increased uptake, which is interpreted as being caused by a breakdown of the structure of the adsorbing species with the generation of new adsorbing sites.[17]

Similar adsorption patterns for a test biocide may be shown by different types of microorganisms. For example, the Z adsorption pattern is shown by phenoxyethanol and *Escherichia coli* (but not *Pseudomonas aeruginosa*) and *Candida lipolyica*.[18] Hugo & Newton[19] showed that uptake (adsorption?) of iodine differed between bacteria and yeast. The initial stages followed the H (high affinity) pattern, indicative of a high affinity of iodine for substrates. Thereafter, the shape varied, depending upon the iodine system (solution or iodophor) and the substrate (type of cells).

Biocides and antibiotics must traverse the outer cell layer(s) to reach their target sites, usually present within microbial cells (Figure 1). The information as to how this uptake is achieved is somewhat limited. Figure 2 shows the general pattern of entry of a biocide into microbial cells. It is believed that antibiotics and biocides generally pass through the staphylococcal cell wall by passive diffusion. Little is known about the manner in which biocides enter other Gram-positive bacteria. Enterococci, for example, are generally less susceptible than staphylococci, and the cell wall could act as a barrier to limit intracellular uptake. In Gram-negative bacteria, passage across the outer membrane (OM) depends upon the chemical nature of the inhibitor, hydrophilic antibiotics utilizing the porin channels (hydrophilic route) and hydrophobic antibiotics entering via the hydrophobic route. Generally, large molecular weight hydrophilic molecules (e.g. the polypeptide antibiotic vancomycin) enter Gram-negative bacteria poorly as do relatively hydrophobic antibiotics such as fusidic acid, erythromycin, novobiocin and rifampicin. Self-promoted entry occurs as a result of OM damage induced by cationic agents that include CHX, QACs and the polymyxin antibiotics.[20] Studies with smooth, rough and deep rough strains of *E. coli* and *Salmonella typhimurium*[21–25] have demonstrated that deep rough strains are more susceptible to QACs than wild-type (smooth LPS), but generally of equal susceptibility to CHX. Interesting relationships were also found with a homologous series of esters of *para*(4)-hydroxybenzoic acid.[21]

Few data are available about the uptake of antibiotics and biocides by mycobacteria, fungi or other types of microorganisms.[26–29] The varied composition of the outer cell layers of different types of microorganisms means that only very general conclusions can be reached about uptake into such cells. Virtually all members of the domain Bacteria have cell walls containing peptidoglycan; the *Chlamydia–Mycoplasma* groups lack a cell wall. Eukaryotes, of the domain Eukarya, do not contain peptidoglycan, and cell walls (if present) contain cellulose or chitin (Table 1). Different cell wall types exist in members of the domain Archaea, with the peptidoglycan analogue, pseudopeptidoglycan, or polysaccharide, protein or glycoprotein being present. Thus, it is not surprising that uptake of biocides might differ greatly in such a wide range of organisms in which the composition of the outer cell layers might have a limiting role, albeit for different reasons.

The possible role of yeast cell walls in modifying cellular response to CHX has been studied (Table 2).[30] The relative porosity (RP) and thickness of cell walls of *Saccharomyces cerevisiae* and their glucan, but not mannan, composition influence susceptibility to CHX. Decreases in RP and increased wall thickness would be expected to reduce CHX uptake into the cells. The pores in fungal cell walls have been suggested as being too small for the entry of very large molecules,[31] with compounds of molecular weight not greater than about 700 capable of diffusing freely.[32,33]

Table 2. The yeast (*S. cerevisiae*) cell wall and susceptibility to chlrohexidine diacetate (CHX)[a]

Parameter	Possible significance in CHX susceptibility
Cell wall composition	
mannan component	low
glucan component	possible greater significance
Cell wall thickness	increases in cells in older cultures CHX uptake reduced
Relative porosity (RP)	decreases in cells in older cultures CHX uptake reduced

[a]Based on Hiom *et al.*[30]

Table 3. Common target sites for action of some biocides

Biocide[a]	Known or predicted target site(s) in					
	cocci	mycobacteria	G –ves	spores[b]	fungi	protozoa
CHX	CM	CM	IM	membranes?	PM	PM
QACs	CM	CM	IM	membranes?	PM	PM
GTA	$-NH_2$	$-NH_2$	$-NH_2$	elimination of germination	$-NH_2$	?
OPA[c]	$-NH_2$	$-NH_2$	$-NH_2$	elimination of germination	$-NH_2$	?
Phenols	CM	CM	IM	membranes?	CM	?
CRAs	-SH, CM, DNA synthesis	?	-SH, IM, DNA synthesis	IM, cortex	?	?

[a]CHX, chlorhexidine salts; QACs, quaternary ammonium compounds; GTA, glutaraldehyde; OPA, *ortho*-phthalaldehyde; CRAs, chlorine-releasing agents; CM, cytoplasmic membrane; IM, inner membrane; PM, plasma membrane; $-NH_2$, interaction with amino groups in proteins; -SH, alteration of thiol groups.
[b]Only GTA and CRAs are actively sporicidal, OPA to a lesser extent.
[c]Penetration into cell especially important with GTA-resistant *M. chelonae*.

A comparison of the effects of some biocides on different types of microorganisms produces some interesting results. For example, Dychdala[3] considered the biocidal effect of free available chlorine on some algae, bacteria, fungi, protozoa, viruses and bacteriophages. Generally, algal growth was inhibited at low concentrations, whereas considerable variation was observed with bacteria. The two fungal test organisms (*Aspergillus niger* and *Rhodotorula flava*) needed high concentrations for a lethal effect to be achieved, whilst the only protozoon studied (*Entamoeba histolytica* cysts) required a low concentration albeit for a long contact period. It is difficult to come to meaningful conclusions about biocide uptake from these comparisons. Of greater significance, perhaps, is the comparison of inhibitory concentrations of a range of QACs against bacteria, fungi and algae.[5] Gram-positive bacteria were considerably more susceptible than Gram-negative organisms or fungi, with test algae usually being inhibited at still lower concentrations. This suggests, but does not prove, that these algae presented no barrier to the uptake of the QACs. Low molecular weight substances are believed to diffuse freely across the algal cell wall, which is impermeable to larger molecules and to macromolecules.[34] Iodine may not control algae, particularly black algae. Effective algal control in pool water can be achieved by CRAs, QACs and modified copper compounds.[6] Interestingly, relative algicidal concentrations of various compounds may equate to their bactericidal properties.[6]

Bacterial spores present a different type of cell surface to biocides (Table 1). Adsorption (uptake?) of alkaline or acid GTA is greatest to vegetative cell forms of *Bacillus subtilis*, followed by germinating and then by resting spores of this organism.[35] However, *E. coli* cells take up more, and *Staphylococcus aureus* cells less, GTA than *B. subtilis* vegetative cells. *B. subtilis* spores take up considerably more chlorine (from sodium dichloroisocyanurate, NaDCC) and at a much more rapid rate than iodine (from Lugol's iodine). Chlorine is also a much more effective sporicide. In both cases, uptake is increased when coat-deficient spores are used.[36,37] It is likely that the coats act as an efficient barrier especially to the entry of iodine. Uptake of both chlorine and iodine is greater with outgrowing and germinating cells than with spores.[36,37]

In mycobacteria, it has long been known that the cell wall acts as an efficient barrier to the uptake of many biocides and antibiotics,[38,39] as considered later.

The microbial cell surface can thus act as a barrier to the uptake of some, but not necessarily all, types of antimicrobial agents. Impermeability or decreased uptake is a common mechanism for reduced susceptibility to antibiotics and biocides in a variety of microorganisms, notably mycobacteria, Gram-negative bacteria and bacterial spores, but can occur in some types of staphylococci also.[40] This aspect is discussed in more detail later.

Target sites for biocide action

Despite variations in cell structure, physiology and complexity, it is clear that some common target sites (Table 3) might be present in vegetative cells of different species, although most of the published work obviously deals with bacteria. This hypothesis has been examined by considering the antimicrobial activities of a range of chemical agents that are widely employed as biocides.

Aldehydes

Although less important than before, GTA remains a valuable 'chemosterilizer' in endoscopy. It acts on non-sporulating and sporulating bacteria by virtue of its intermolecular cross-linking effects on amino groups in bacterial protein.[41] A consequence of its effects on outer cell layers is that GTA agglutinates bacterial and yeast cells and increases their settling rate.[42] However, few antifungal agents have the fungal cell wall as a primary or major target site but chitin is a potentially reactive site for cross-linking agents (formaldehyde and GTA).[27] The action of GTA on other microbial types is unknown, but the fact that it is used as an electron microscopy fixative suggests that it has similar cross-linking effects on these also. In particular, its interaction with lysine[43,44] is an important aspect of its action, as shown with the capsid proteins of poliovirus.[43] It is likely that similar interactions occur with surface proteins in other microorganisms.

OPA, an aromatic dialdehyde, has to date been studied with Gram-positive bacteria (staphylococci, mycobacteria, spores) and Gram-negative bacteria (*E. coli*, *P. aeruginosa*).[45–47] In all cases, OPA has been shown to be a less effective cross-linking agent than GTA,[41,42–48] its high activity against mycobacteria resulting from its lipophilic nature. GTA does not damage bacterial spore DNA but eliminates the ability of spores to germinate, whereas OPA-treated spores that cannot germinate are not recovered by artificial germinants or by treatment with sodium hydroxide or lysozyme.[49]

The action of OPA on other microbial types has yet to be undertaken.

Cationic biocides

CHX and QACs provide the best examples of cationic biocides. They cause significant membrane damage in different types of microorganisms, including Gram-positive and -negative bacteria,[11,17,50] yeasts,[51,52] and the trophozoites and cysts of *Acanthamoeba castellanii*.[53–57] CHX induces K^+ loss from baker's yeast and affects the ultrastructure of budding *Candida albicans* with loss of cytoplasmic constituents.[52] Whilst the composition of the cytoplasmic/plasma membrane in various organisms differs markedly, it is clear that the biocides have similar, possibly phospholipid, target sites (both CHX and QACs are known to combine with phospholipid). The low growth-inhibitory concentrations of QACs versus algae[5] suggest that a similar deleterious effect may apply in these organisms also. As to mycobacteria, little information is available about the specific mode of action of CHX and QACs. However, since low concentrations are mycobacteriostatic, but not generally mycobactericidal, it is likely that the cytoplasmic membrane suffers some injury even if the lack of other effects precludes a cidal effect.[26,48] Some QACs are claimed to be mycobactericidal, but the mechanisms involved are poorly understood.[58]

There is little information about the effects of CHX and QACs on spore membranes.[59] Knott & Russell[60] found that the addition of CHX early in the sporulation process prevented spore formation in *B. subtilis*. It is conceivable that CHX affected membrane permeability during this process. Research has been hampered by the apparent inability of these biocides to traverse the spore coats and possibly the cortex.[61] Coat-less spores have not been fully utilized in this context, being mainly studied from the point of view of reduced spore susceptibility to biocides.

The mechanism of antibacterial action of polymeric biguanides has been extensively studied. They have been shown to have a marked effect on the inner membrane of *E. coli*,[62,63] producing lipid phase separation and domain formation of the acidic phospholipids in the cytoplasmic membrane. These compounds are widely used to control algal growth in swimming pools and it is conceivable that similar damage occurs in these organisms. Although they combine with phospholipids, CHX and QACs do not bring about phase separation and domain formation.[64] Changes in fatty acid composition of *P. aeruginosa* exposed to QACs have been described.[65]

Alcohols

Several alcohols have antimicrobial activity; usually, they are rapidly bactericidal, including in some cases, acid-fast bacteria, but bacterial spores are unaffected even at high concentrations.[1,61]

Ethanol and isopropanol are membrane disrupters, disorganization probably arising from their penetration into the hydrocarbon core of the interior.[66] Ethanol has pleiotropic effects on bacteria, with inhibition of DNA, RNA, protein and peptidoglycan syntheses in *E. coli* being secondary effects that follow membrane damage. Other responses (inhibition of the enzymes involved in glycolysis, fatty acid and phospholipid syntheses and effects on solute uptake) all result directly from an ethanol-induced disruption of membrane structure and permeability.[11,66] Ethanol induces leakage of intracellular material from *S. cerevisiae*[27] and ethanol tolerance is claimed to be associated with reduced leakage.[67] Lipid composition and plasma membrane fluidity also play a role in the susceptibility of yeasts to alcohol.[66] Dermatophytes such as *Microsporum gypsum* spores are less susceptible to ethanol than *C. albicans* and the oocysts of *Toxoplasma gondii* are considerably more resistant than the trophozoites.[68] Aspects of the mechanisms of action of alcohols are considered elsewhere.[69]

Phenylethyl alcohol (phenylethanol, PEA) and phenoxyethanol (POE) also produce membrane disruption, but at low concentrations have more specific effects. POE at low concentrations is an uncoupling agent and causes proton translocation in *E. coli*.[70] PEA inhibits the growth of a range of Gram-negative bacteria,[71] but not *Plasmodium fluorescens* or *P. aeruginosa*, as well as some mycobacteria (*Mycobacterium smegmatis* and GTA-resistant, OPA-susceptible *Mycobacterium chelonae*).[71,72] *S. aureus* was less susceptible. 5-Phenyl-1-pentanol (5-PP), a more hydrophobic aromatic alcohol, is more active than PEA probably because it is taken up to a greater extent by the cells. 5-PP also shows greater activity than PEA against *S. aureus*. POE and PEA also inhibit bacterial DNA synthesis and induce filament formation.[73]

Chlorine compounds

Microorganisms show a wide response to CRAs.[3] However, a variety of concentrations, pH values, temperature and contact periods have been employed, which makes a comparative assessment somewhat difficult. However, mould spores and especially *Cryptosporidium* spp. appear to be more resistant than other microbial types, but reasons for the different responses are unclear.

Available chlorine is a measure of the oxidizing capacity of hypochlorites and is expressed in terms of the equivalent amount of elemental chlorine. Hypochlorites are powerful oxidizing agents with bactericidal, sporicidal and fungicidal activity, hypochlorous acid (HOCl) being the active moiety. They are considered to be multitarget reactors[11,74] that act on cell walls and the amino groups in proteins, but their primary effect is believed to be either or both (i) the progressive oxidation of thiol groups to disulphides, sulphoxides and disulphoxides[17,50,75] and (ii) deleterious effects on DNA synthesis resulting from the formation of chlorinated derivatives of nucleotide bases.[1,76] It is likely that similar types of damage occurs in all types of microorganisms, although bacterial spores do not appear to suffer direct DNA damage.[77]

Chlorine dioxide shows activity against bacteria, fungi, protozoa and algae. Its primary molecular target remains unclear, but inhibition of protein synthesis may be involved as well as membrane damage.[4] Inorganic and organic chloramines and isocyanuric acids are also effective antimicrobial agents[3,78] but the mechanism whereby microbes are inactivated remains obscure.

Iodine and iodophors

Iodine, used as an aqueous (with potassium iodide) or alcoholic solution, is an effective microbicidal agent with rapid lethal effects against bacteria and their spores, moulds, yeasts and viruses.[79]

Iodophors are iodine-carrying complexes in which iodine is solubilized by, usually, surface-active agents. They retain the germicidal action but not the undesirable properties of iodine. The concentration of free iodine in both types is responsible for activity.[79,80] The paradoxical effect of dilution on iodophor activity has been emphasized.[79,80] Here, as the degree of dilution increases, beyond a certain critical point bactericidal activity also increases.

Iodine interacts with thiol groups in enzymes and proteins[11,17] and this is believed to be responsible for its bactericidal, sporicidal and fungicidal actions. Iodine causes extensive morphological changes to the poliovirus structure by affecting the viral capsid rather than RNA.[81] Because -SH groups are widely found in proteins, Gottardi[80] considers the action of iodine to arise mainly from its oxidation of these groups of the amino acid cysteine. Iodination of phenolic and imidazole groups of tyrosine and histidine and lipid interactions in lipid-enveloped viruses[82] and phospholipids in bacteria[83] also contribute to its lethality.

Peroxygens

The most important peroxygens are hydrogen peroxide (H_2O_2), peracetic acid (PAA; CH_3COOOH) and ozone (O_3).[84,85]

Hydrogen peroxide is a powerful oxidizing agent that acts via the formation of hydroxyl radicals (·OH) which oxidize thiol groups in enzymes and proteins. [50,74,75] However, other effects are also known, including dissociation of 70S ribosomes into ribosomal subunits, cell surface changes and cleavage of the DNA backbone.[86,87] Peroxide-treated spores maintain their permeability barrier and it is unlikely that significant damage occurs to the spore inner membrane,[88] although Shin *et al.*[89] found that peroxide sensitizes spores to thermal injury. According to Melly *et al.*,[88] usual targets for peroxide attack on membranes are polyunsaturated acids, which occur at only very low levels in spores. Damage to spore DNA has been described.[89] Anti-protozoal activity of peroxide against the cysts and trophozoites of *Acanthamoeba* spp. is known,[90] but the mechanisms have not been elucidated.

PAA is the most potent peroxygen.[91] It is bactericidal, fungicidal and sporicidal but fungi such as *A. niger* are less susceptible to PAA than yeasts or non-sporulating bacteria.[84] Reduced transition metal ions sensitize spores to PAA.[92] The major targets for its action are believed to be the free radical oxidation of enzyme and protein thiol groups. [11,50]

Although the mechanisms of action of these two peroxygens have not been widely studied against other microorganisms, their multi-targeted effects suggest that similar mechanisms might be responsible for microbial inactivation.

Ozone is a powerful bactericidal, sporicidal and fungicidal agent,[85] although yeasts and moulds are less susceptible than bacteria.[93] It will also inactivate protozoan oocysts, with *Cryptosporodium parvum* being most resistant. However, Rennecker *et al.*[94] have pointed out that variations between protozoan strains and oocyst purification techniques could at least partially account for these differences. Nevertheless, ozone is considered to be superior to any of the halogens against *Cryptosporidium* oocysts or *E. histolytica* and *Giardia* cysts.[95] *G. lamblia* is at least an order of magnitude more resistant than *E. coli*, and some four times more so than *Giardia muris*.

The mechanisms of ozone action are unknown. It is considered to disrupt cellular enzyme activity by reacting with thiol groups, but also modifies purine and pyrimidine bases in nucleic acids.[96] These broadly based effects would account for its wide range of activity against a variety of organisms.

Phenols

Several phenols are used for disinfectant or preservative purposes.[1,97] Phenol induces progressive loss of intracellular constituents from treated bacteria and produces generalized membrane damage with intracellular coagulation occurring at higher concentrations.[17] The plasma membrane of fungi is also damaged,[1,27] although *A. niger* and *C. albicans* are less susceptible than bacteria.[98] Low concentrations are claimed to lyse growing cultures of *E. coli*, staphylococci and streptococci,[99] but this effect has not been examined in detail. Fentichlor, a chlorinated phenol, acts as an uncoupling agent against susceptible bacteria.[100]

Bacterial spores are very resistant even to high concentrations of phenol, but germination is inhibited by low phenol concentrations.[1,11,61] Mycobacteria may be inactivated by phenolics, the damage being presumably membrane-orientated.

Phenylether (triclosan)

The phenylether, triclosan [5-chloro-2-(2,4-dichlorophenoxy) phenol], is a broad-spectrum antimicrobial agent.[101] Triclosan is predominantly bacteriostatic or fungistatic in action, with low concentrations inhibiting the growth of many Gram-positive (including some mycobacteria) and -negative bacteria. Much higher concentrations are bactericidal, but *P. aeruginosa* and certain other bacteria are highly intrinsically resistant. Yeasts and moulds tend to be much less susceptible than *S. aureus* and bacterial spores are unaffected.

Triclosan is membrane-active,[102] but studies[103] have also indicated that its growth-inhibitory properties against *S. aureus*, *E. coli* and other bacteria arise from its blocking lipid biosynthesis by specifically inhibiting NADH-dependent enoyl-acyl carrier protein (ACP) reductase, FabI, or its homologue, InhA, in *M. smegmatis*.[104] FabI is a single chain polypeptide in plants and bacteria (including mycobacteria), but is part of a complex polypeptide in animals and fungi.[105] Triclosan also inhibits the enoyl-ACP reductase of *Plasmodium falciparum*.[105–107]

Some bacteria possess triclosan-resistant enoyl-ACP reductase homologues (FabK) and both triclosan-susceptible and -resistant enzymes can be found in *P. aeruginosa.*[108,109] At lethal concentrations, triclosan induces K^+ leakage.[110] Interestingly, triclosan shows a Z-pattern adsorption,[111] which implies the breakdown of a structure (presumably the cytoplasmic membrane) and the generation of new adsorbing sites.

Organic acids and esters

Benzoic and sorbic acids are active against Gram-positive and Gram-negative bacteria and yeasts, and inhibit spore germination but are not sporicidal.[78] They are most effective at acid pH and the activity resides in the undissociated form, although the anion is also believed to contribute to the overall effect. They affect membrane activity by an action on the proton-motive force (ΔpH component) whereby transport is inhibited.[112,113]

The most widely used esters are the parabens [methyl, ethyl, propyl and butyl esters of *para*(4)-hydroxybenzoic acid]. They are predominantly bacteriostatic and fungistatic and at high concentrations affect the cytoplasmic/plasma membrane causing leakage of intracellular constituents. At lower concentrations, there is a selective

inhibition of ΔpH across the membrane and transport is inhibited.[112,113]

Metal ions

Metal ions such as mercury (Hg^{2+}) and silver (Ag^{+}) interact strongly with thiol groups in bacterial enzymes and proteins.[114,115] Depending upon various factors, e.g. concentration, period of contact, it may be possible for this reaction to be reversed by the addition of -SH compounds.[11] Mercuric salts have also been shown to react with ribosomes.[74] Silver salts produce structural changes in the cell envelope of *P. aeruginosa*, an organism that is very susceptible to Ag^{+}. Ag^{+} ions act preferentially on bases other than phosphate groups in DNA.[116] Bacterial spores are unaffected by Ag or Hg, but organomercury compounds inhibit outgrowth, whereas inorganic mercury compounds inhibit germination.[61] Interaction of Ag^{+} with spore DNA has, however, been described as well as with phage DNA.[117]

Silver salts and other heavy metals act by binding to key functional groups of fungal enzymes.[118] Silver nitrate causes marked inhibition of *Cryptococcus albidus* and is deposited as granules in vacuoles and the cell wall.[119]

Alkylating agents

Depending on concentration and under appropriate conditions of use, ethylene oxide (EtOx), β-propiolactone and formaldehyde combine with amino, carboxylic, sulfhydryl (thiol) and amino groups in proteins and enzymes.[1] EtOx also interacts at N-7 guanine moieties in DNA. They are all capable of inactivating bacteria and spores, fungi and viruses and it is likely that that this inactivation is brought about in a similar manner in each type of organism. Interactions of formaldehyde with viral nucleic acids have been described.[120]

Microbial differentiation and changes in biocide response

The bacterial spore cycle and encystation and excystation in the simpler forms of protozoa provide excellent tools for associating morphological and biochemical changes in cells with susceptibility to antimicrobial agents, both antibiotics and biocides (Table 4).[57]

Members of the genera *Bacillus* and *Clostridium* have complex life cycles with the two extremes of dormant (spore) and metabolically active (vegetative cell) forms. Seven stages have been identified in the sporulation of *B. subtilis*, with stages IV (cortex development), V and VI (coat development) being the most relevant in relation to reduced susceptibility to biocides. Small acid-soluble proteins (SASPs) exist in the spore core as two types.[77,121] These are (i) α,β-types associated with DNA and synthesized at about the third hour of sporulation, (ii) γ-types, also synthesized at around t_3 but not associated with any macromolecules. Susceptibility to biocides decreases as the synthesis of the cortex and inner and outer spore coats proceeds, with late development of comparative insusceptibility to lysozyme and especially to GTA[59] In addition, it has been shown that spores (α^{-} β^{-}) of *B. subtilis* deficient in α,β-type SASPs are much more susceptible to hydrogen peroxide but not to iodine, GTA or OPA than wild-type spores.[122,123] Taken as a whole (Figure 3), these findings indicate that (1) impermeability to biocides increases as sporulation proceeds, (2) saturation of DNA with α,β-type SASPs protects the DNA from attack by hydroxyl radicals.

Despite being metabolically dormant, the spore contains a number of enzymes that act on the corresponding substrates in a very short period of time during germination. For example, 3-phosphoglycerate (3-PGA) is catabolized during the first few minutes to generate ATP, and germination endoprotease (GPR) is responsible for the degradation of SASPs to amino acids during the first 30 min.[77,121] Profound degradative changes occur during germination whereas biosynthetic processes take place during outgrowth. During germination phase I, cation and DPA (dipicolinic acid) release, partial core hydration and SASP degradation occur, with cortex hydrolysis and further core

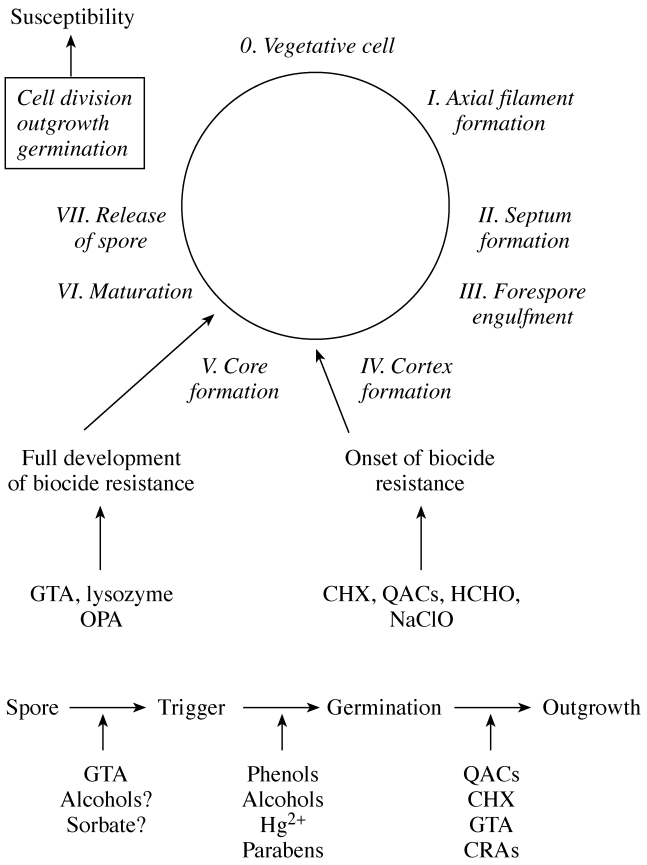

Figure 3. Sporogenesis and susceptibility and resistance to biocides. For role of SASPs, see text. GTA, glutaraldehyde; CHX, chlorhexidine; HCHO, formaldehyde; NaClO, sodium hypochlorite; QACs, quaternary ammonium compounds; CRAs, chlorine-releasing agents.

Table 4. Microbial differentiation and changes in biocide responses

Organism	Form	Biocide response
B. subtilis	vegetative cell	usually susceptible
	spore	much less susceptible
	germinating cell	susceptibility usually increases
	outgrowing cell	susceptibility usually increases
C. albicans	yeast form	susceptible
	mycelial form	less susceptible
A. castellanii	trophozoites	usually susceptible
	cysts	less susceptible

hydration during germination phase II, followed by biosynthetic processes, escape from spore coats and eventual cell division.[124]

During periods of stress, trophozoites of *Acanthamoeba* spp. undergo a cell differentiation process (encystment), resulting in the formation of dormant cysts. Extensive changes occur during encystation in *A. castellanii*, with the development of acid-insoluble protein-containing ectocyst wall and an alkali-insoluble cellulose endocyst wall. Resistance to several biocides commences with the synthesis of the cellulose-containing wall, implying that a physical barrier is responsible for this decreased susceptibility rather than it being a consequence of a metabolically dormant cyst.

Suci & Tyler[125,126] investigated the effect of CHX on yeast and filamentous forms in an early stage *C. albicans* biofilm and found that a portion of the yeast cells germinated to produce filamentous forms. The rates of propidium iodide penetration were substantially higher in filamentous forms when exposed to CHX.

Stress adaptation

Applied stress is (i) any deviation from the optimum growth condition that produces a reduced growth rate, (ii) exposure to an environmental situation that produces damage to cellular components in the absence of a cellular response, or (iii) a situation that stimulates the expression of genes known to respond to a specific environmental condition.[127]

Stress adaptation refers to the ability of bacteria or other microorganisms to adapt to a chemical or other applied stress. Gould[128] pointed out that vegetative bacterial cells react homeostatically to stress in a variety of ways; these include the activation and expression of latent groups of genes following exposure to oxidative stress. Oxidative stress and the SOS response in *E. coli* and *Salmonella* are intrinsic defence mechanisms conferring tolerance to stress by hydrogen peroxide and involve the production of an array of neutralizing enzymes to prevent cell damage and of exonucleases to repair lesions in DNA.[129] Peroxide-induced stress proteins overlap with heat-shock proteins (HSPs).[130] A regulated adapted response in growing *E. coli* cells exposed to hydrogen peroxide results in the cells becoming resistant to normally lethal doses of peroxide and the synthesis of around 40 new proteins.[131]

When *E. coli* is subjected to nutrient limitation or to antimicrobial agents, the growth rate is depressed and gene expression is markedly altered. This is essential for long-term survival of the cell and is partly mediated by alternative sigma factors. Programmed cell death (PCD) is a programmed suicide mechanism, with persisters being defective in PCD and using the exudate from lysed cells as a source of nutrient.[132] It has been postulated[133] that the metabolic imbalance following biocidal or other type of stress leads to free radical production and self-destruction. Highly metabolic cells, which are more susceptible to biocides,[134] can be readily differentiated from stationary phase cells by this phenomenon.

The adaptational network of *B. subtilis* involves the induction of stress proteins[135] and the production of SASPs. Stress response proteins are induced when sporulating cells are heat-shocked.[136] Nutritionally-limited cells expressing starvation phenotypes are more resistant to biocides than 'normal' cells.[137]

Stress adaptation responses are also known in yeasts.[138] Yeast cells have evolved a wide range of responses to many different types of stresses, both physical (e.g. heat, starvation) and chemical (such as oxidative stress, ethanol). These responses are (i) intrinsic (constitutive) and depend on growth phase and the stage of an organism in its life cycle, or (ii) inducible.[139] The main toxic effects of the superoxide anion (O_2^-) and hydrogen peroxide results from their ready conversion into the highly reactive hydroxyl radicals (·OH). These can damage cellular nucleic acids, proteins and lipids. The main defence systems in *S. cerevisiae* involve (i) degradation or detoxification of the reactive oxygen species, (ii) maintenance of metal ion homeostasis to prevent free metal ions generating ·OH, and (iii) repair of damage.

As with vegetative bacterial cells, yeast cells can adapt to a subsequent dose of hydrogen peroxide. During this adaptation, several polypeptides are induced, some of which are unique to peroxide treatment with others also being produced following heat shock. Catalase and possibly glutathione play a role in this adaptive response.[140,141] A 'heat shock response' is also produced in *C. albicans* following exposure to ethanol.[142]

Stresses such as heat, oxidative stress and pH shock on *Acanthamoeba* trophozoites have been studied.[143] Unstimulated pathogenic *Acanthamoeba* (*castellanii*, *cilbertsoni*) had relatively high levels of HSPs 60 and 70, whereas unstimulated trophozoites of free-living *A. rhysodes* had the lowest background levels of these HSPs and were the most affected by the stresses.

General aspects of resistance mechanisms to biocide action

Resistance to biocidal agents has been widely studied in bacteria[11] and to some extent in fungi,[27] with some useful information beginning to emerge with some types of protozoa (Table 5).[53–57] In non-sporulating bacteria, the major mechanisms of resistance are reduced uptake (impermeability and/or efflux),[11] with possible mutation, transferable resistance and biocide degradation worthy of consideration.[144] In addition, biofilm formation is a major reason for the refractory response of many organisms to biocides.[145] There could also be different target site affinities for biocides in different types of microorganisms, although this aspect has been less widely studied.

Cellular impermeability as a resistance mechanism

Gram-negative bacteria, and especially *P. aeruginosa*, *Proteus* spp., *Providencia* spp. and *Serratia marcescens*, generally show reduced susceptibility to biocides compared with the Gram-positive cocci.[11] A major reason for this reduced susceptibility resides in the OM acting as a permeability barrier, so that uptake into the cell is reduced, as described earlier. This aspect has been considered in greater detail elsewhere.[11,146] VRSA possess thickened cell walls as well as altered peptidoglycan[147] and thus might limit the uptake of biocides. This could prove to be a worthwhile investigation. In terms of their biocide susceptibility, mycobacteria occupy an intermediate position between bacterial spores and other bacteria.[26] There is no evidence that efflux plays a role in this. The major reason for their recalcitrance to biocide activity is the lipid-rich, waxy cell wall which limits intracellular uptake of many biocides.[26,48]

Bacterial spores tend to be much less susceptible to biocidal agents than non-sporulating bacteria. An obvious reason is to be found with the nature and composition of the spore coats and possibly cortex (Table 1) which present an effective permeability barrier to the entry of many biocides.

Antibiotic resistance in yeasts is known to occur via target site mutations and reduced uptake (impermeability and efflux). Examples occur with fluconazole (modification in the quantity and quality of 14α-demethylase, leading to reduced azole affinity and uptake) and polyenes (membranes with modified sterols that have lower affinity for nystatin binding).[148] The antibacterial antibiotic, rifampicin, is

Table 5. Common resistance mechanisms to biocide action

Resistance mechanism	Type of organism	Actual or proposed mechanism[a]
Cellular impermeability	staphylococci	thickened CW
	G –ve bacteria	OM barrier
	mycobacteria	waxy overcoat
	bacterial spores	OSC and ISC
	fungi	CW
	protozoa	
	cysts	reduced uptake
	trophozoites	unlikely
	algae	unlikely to be a problem?
Mutation	bacteria	
	G +ve, G –ve	modified enoyl reductase and TC
	other organisms	?
Overproduction of target	bacteria	
	G +ve, G –ve	overproduction of enoyl reductase and TC
	other organisms	?
Enzymatic degradation of biocide	bacteria	several biocides: relevance to 'in-use' concentrations?
Efflux of biocide	staphylococci, G –ves	low-level resistance to CHX, QACs
	yeasts	organic acids
	other types of microorganisms	not found?

[a]CW, cell wall; OM, outer membrane; OSC, outer spore coat; ISC, inner spore coat; TC, triclosan; CHX, chlorhexidine salts; QACs, quaternary ammonium compounds.

ineffective versus fungi. However, when used in combination with the polyenic antifungal drug, amphotericin B (which combines with fungal membrane sterol), it shows activity against several fungal species. This has led to the suggestion that increased uptake of rifampicin occurs as a consequence of amphotericin action and that membrane sterols pose a barrier to its entry. CHX and QACs cause damage to the yeast plasma membrane;[51,52] it is not, however, known whether this interaction is reduced by the presence of membrane sterols which could effectively limit further uptake into the cell interior.

The outer layers of protozoal cysts are likely to act as a barrier to some biocides. The outer shell of *Cryptosporidium* oocysts renders them more resistant to biocides.[29] In the protozoon, *A. castellanii*, resistance to biocides is likely to result from the cellulose content of the outer layers.[54,56]

Target site

Biocides are considered to be multitargeted chemical agents.[1] However, as pointed out earlier, the growth-inhibitory properties of triclosan involve inhibition of enoyl reductase. Mutation in the target enzyme or its overproduction can lead to considerable increases in MICs. With alcohols, the lipid composition and plasma fluidity play a role in the susceptibility of yeasts.[149]

Efflux as a resistance mechanism

Efflux is a major mechanism for the resistance shown by bacteria to antibiotics.[150–152] Efflux of biocides is known and has been the subject of several authoritative reviews.[152,153] In bacteria, several classes of efflux pumps have been described.[153] The silver resistance determinant from a hospital burn ward *Salmonella* plasmid encodes a periplasmic silver-specific binding protein (SilE) plus two parallel efflux pumps. One of these is a P-type ATPase (SilP) and the other a membrane potential-dependent, three component cation/proton antiporter (SilCPA).[154]

Efflux of antifungal antibiotics has also been described.[10] Efflux has been shown to play a role in the response of some strains of yeasts to organic acids as an inducible preservative elimination system,[155] but there is no evidence to date that low-level resistance to cationic biocides occurs by active efflux pumps in yeasts.

Biofilms

The mechanisms of reduced susceptibility to biocides and antibiotics of bacterial cells present within biofilms have been the subject of considerable experimentation and debate.[154,156] These mechanisms include (i) reduced access of biocide molecules to bacterial cells, (ii) chemical interactions between biofilm and biocide, (iii) modulation of the micro-environment, producing nutrient- and oxygen-limited and starved cells, (iv) production of degradative enzymes that might be effective at lower biocide concentrations within the biofilm, (v) genetic exchange between cells, (vi) quorum sensing, (vii) presence of persisters and of pockets of surviving organisms, (viii) adaptation and mutation within the biofilm, and (ix) biocide efflux.

In nature, it is likely that biofilms will consist of mixed populations of different types of microorganisms. Many types of bacteria and yeasts interact with protozoa, e.g. the co-evolution between *Legionella* and *Acanthamoeba* and other protozoa, and between *Cryptococcus neoformans* and *Acanthamoeba*.[157] This co-evolution of bacteria and lower order eukaryotes has equipped the organisms for environmental survival as well as virulence towards higher order

eukaryotes. *Legionella pneumophila* within *Acanthamoeba* cysts are protected from the action of chlorine.[158]

With algae, the presence of mats equates to biofilms and constant dosing with biocides may be needed to prevent algal recontamination.[157]

Other factors

An additional factor in bacterial spores, but not in other micro-organisms, is the presence of α,β-type SASPs (referred to earlier). These can coat spore DNA, thereby protecting it from damage by enzymes and antibacterial agents. They thus play an important role in determining spore susceptibility to antibacterial agents.[122,123] Other factors also need to be considered, namely the reduced water content in the core and the ability or otherwise to repair DNA damage during germination.

Overall comments and conclusions

It is important to understand the reactions of different types of micro-organisms to biocidal agents. This is useful from the point of view of cell structure and physiology but also provides valuable information about (i) the mechanisms of action of biocides, (ii) the mechanisms whereby microorganisms resist biocide action, and (iii) the improved usage of biocides in clinical and environmental situations. With the emergence of new pathogenic organisms[159] and the current level of concern about microbes used as bioterrorism weapons, it is increasingly important to understand the actions and effects of biocidal agents on as wide a range of organisms as possible and of how organisms might resist those actions. Additionally, yeasts and fungi are more closely related to mammalian cells than originally thought and can be used as screening tools to elucidate the mechanisms of action of anti-neoplastic agents.[160]

It is clear that antibiotics generally are very selective for the type of organism against which they are used. This is not true for biocides which generally 'attack' most types of microorganisms. This implies that different organisms, despite their varied structures, have similar target sites, although with algae, for example, so many different types are known that it is impossible to generalize.

In particular, when considering the mechanisms of antimicrobial activity of biocidal agents, compounds that interact with proteins, enzymes or nucleic acids are likely to be effective against a wide range of microorganisms probably as a result of the same basic actions.

The reasons for the variations in (non-)susceptibility arising between different types of microorganisms can then be ascribed to: (i) the considerable differences in adsorption by and uptake into cells resulting from the dissimilarities in chemical composition and architecture of the outer cell layers—this is an area where much additional information is needed, but is clearly of considerable importance. Concentration is a key issue in biocide activity[1] and is particularly relevant to the present discussion; (ii) possible slight or marked differences in the actual target site(s) so that the affinity of the site(s) for a biocide is modified; (iii) possible differences in the amounts of available target site(s); (iv) the presence within some types of cells of protective chemicals such as the spore-specific SASPs that protect against DNA damage; (v) stress responses, i.e. the manner in which cells respond to a harmful agent. For example, an SOS response, an efflux pump safety mechanism, or biocide degradation (actually unlikely at in-use concentrations of biocide, although one claimed mechanism for the reduced susceptibility of biofilm cells) in different types of microbial cells must be considered; and (vi) the presence of a biofilm (an increasingly important field of study) or, in the case of algae, a mat, that is responsible for the recalcitrance shown by cultures to biocides.

Further investigations on the relative responses of different types of microbes to biocides should build on current knowledge with the ultimate overall aim of achieving greater understanding of the action and resistance mechanisms involved and of the control of microbial inactivation processes.

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