Disinfectants and antiseptics: mechanisms of action and resistance

Jean-Yves Maillard[1]*, Michael Pascoe[1]

[1]School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Wales, UK

*e-mail: maillardj@cardiff.ac.uk

ABSTRACT

Chemical biocides are used for infection prevention and control in healthcare, targeted home hygiene, or controlling microbial contamination for various industrial processes including but not limited to food, water and petroleum. However, their use has substantially increased since the implementation of programmes to control outbreaks of methicillin resistant *Staphylococcus aureus* (MRSA), *Clostridioides difficile* and SARS-CoV-2. Biocides interact with multiple targets on the bacterial cells. The number of targets affected, and the severity of damage will result in an irreversible bactericidal effect or a reversible bacteriostatic one. Most biocides primarily target the cytoplasmic membrane and enzymes, although the specific bactericidal mechanisms vary among different biocide chemistries. Inappropriate usage or low concentrations of a biocide may act as a stressor whilst not killing bacterial pathogens, potentially leading to antimicrobial resistance. Biocides can also promote the transfer of antimicrobial resistance genes. In this Review, we explore our current understanding of the mechanisms of action of biocides, the bacterial resistance mechanisms encompassing both intrinsic and acquired resistance, and the influence of bacterial biofilms on resistance. We also consider the impact of bacteria that survive biocide exposure in environmental and clinical contexts.

Table of contents blurb (~50 words max.)

In this Review, Maillard and Pascoe examine the mechanisms of action of biocides, as well as the bacterial intrinsic and acquired resistance to these biocides and its implications in the environmental and clinical settings.

[H1] Introduction

Antimicrobial biocides, also known as microbicides, are distinct from chemotherapeutic antibiotics and they are used in a wide range of applications including disinfection, antisepsis and preservation. Whilst some may be used for either application, the terms disinfectant and antiseptic respectively refer to biocides used on non-living surfaces and living tissues (for example, the skin). The use of biocides has been documented for centuries[1], well before the Germ Theory of Diseases by Louis Pasteur[2] and Koch's postulates[3]. The work of Ignaz Semmelweis represents an important moment in the modern use of disinfection and antisepsis, as it introduced chlorinated lime water for hand desinfection[4], leading to a reduction in the incidence of puerperal fever following births. Most of contemporary biocides were introduced during the 20th century[1], and with improved public awareness about infections and "superbugs", it is now difficult to find consumer hygiene products lacking biocides and claims of antimicrobial activity[5,6].

The COVID-19 pandemic contributed to an escalation of surface, air and skin disinfection. The persistence of SARS-CoV-2 on surfaces, at least for a few hours, not only highlighted the need to improve surface and hand hygiene compliance, but also provided a reason for disinfectant manufacturers to provide longer-lasting antimicrobial protection of surfaces. Footage of disinfectants being sprayed in streets during the pandemic reflects this increase in public awareness. Enhanced control measures during the pandemic were not only limited to the healthcare setting, but also affected domiciliary, transportation, manufacturing, and corporate sectors; global demand for biocides was estimated to increase 600% during this period[7]. Increasing product usage for disinfection and antisepsis means increasing bacterial exposure to biocides.

Many biocide chemistries have been used in disinfectants and antiseptics over the years[1]. The purpose of disinfectants and antiseptics is to kill target microorganisms, effectively reducing their number on skin, surfaces, materials or in water. Unlike chemotherapeutic antibiotics, biocides at their in-use concentration exert bactericidal activity by affecting multiple targets on the bacterial cell. Interactions between biocides and bacterial targets depend on the chemical nature of the biocide, but also on other several factors, some pertinent to application[5]. The poor understanding of manufacturers regarding the different chemistries, including factors that affect efficacy, and inappropriate usage or/and misuse of products (such as incorrect dilution or insufficient contact time) can lead to bacterial survival, potential selection or adaptation. In turn, this may result in bacterial resistance and cross-resistance to unrelated compounds including antibiotics. Decreased bacterial susceptibility to biocides, often referred to as resistance, has been reported since the 1950s and has now been reported for all major types of biocides[8]. In contrast to chemotherapeutic antibiotics, where clinical breakpoints can be used to clearly define 'resistance', the definition of resistance for biocides

is more open to interpretation. Definitions are linked to the protocol used to measure a bacterial change in antimicrobial susceptibility profiles, though these protocols are not standardised[5]. In this Review, the term biocide resistance is used holistically and does not distinguish between decreased susceptibility (a change in susceptibility profile measured by bacteriostasis or growth inhibition), resistance (measured by bactericidal protocols) or tolerance (ability of bacteria to survive a biocide at an in-use concentration).

Bacteria can be naturally tolerant (intrinsically resistant) to a biocide based on innate physiological factors, which may contribute toward an ability to survive — and in some cases thrive — in solutions containing biocides. Some reported outbreaks originated from contamination of specific disinfectant or antiseptic products by intrinsically resistant bacteria; for example, contamination of chlorhexidine solution with *Burkholderia cepacia*[9], benzalkonium chloride solutions with *Serratia marcescens*[10], or alcohol solutions with *Bacillus cereus* spores[11]. Bacteria can also acquire mechanisms leading to resistance through gene exchange or/and genetic mutations (acquired resistance)[12]. Investigations concerning processes where biocides are routinely used, such as endoscope reprocessing, have provided remarkable insights into environmental isolates that are not only resistant to the in-use concentration of high-level disinfectants used in the process, but also to unrelated biocides[13,14]. The clinical implications of these findings, however, remains poorly established and the mechanism of resistance for some isolates remain uncertain[15].

Whilst the use of biocides is an essential cornerstone for infection control and general hygiene, their overuse and misuse may represent a driver for the emergence of antimicrobial resistance (AMR) in bacteria[5,6]. The topic of biocide resistance was comprehensively discussed in a series of reviews across the 1990s and early 2000s.[16-18] More recent reviews on the subject have focused on specific issues posed by particular biocides[19], resistance mechanisms[20], areas of use[21], or provide limited information on the impact on AMR emergence[22].

In this Review, we provide a holistic introduction to the different types of biocide chemistries used in disinfectant and antiseptic products, their applications, mechanisms of action and factors that contribute towards antimicrobial efficacy. We discuss the mechanisms of bacterial resistance to biocides and methodologies used to determine resistance, to understand the practical and clinical implications of recent studies in this area. Finally, we explore existing evidence on the role of biocides in driving antimicrobial resistance development through shared mechanisms of resistance.

[H1] Types of biocides and biocide-bacteria interactions

[H2] Main types of biocides commonly used in disinfectant and antiseptic products

Biocides are chemically diverse, with over 900 chemistries available in the European market. Given the importance of establishing efficacy and safety, many markets have enacted specific legislation to regulate their sale. In the European Union, biocides are regulated by the European Chemicals Agency (ECHA) under the Biocidal Products Regulations (BPR) and are differentiated into 22 product types depending on their intended application; in the United Kingdom, the legislation is currently aligned with the European Union BPR, with the Health and Safety Executive serving as the enforcing authority. Similar regulations are also in place in other countries worldwide, for example, the United States (Federal Insecticide, Fungicide, and Rodenticide Act), China (Regulation on the Administration of Pesticides) and Japan (Pharmaceutical and Medical Devices Act).

The type of biocide chemistry used in formulations depends on their application (Table 1). Generally, the impact of formulated biocides (biocide chemistries and excipients) on efficacy is not as well reported as the efficacy of unformulated biocides. Yet, when formulated biocides are studied, for example formulated benzalkonium chloride, their bactericidal efficacy is improved and emerging antibiotic-resistance decreased[19]. Less-reactive, surface-compatible or less toxic biocides such as quaternary ammonium compounds (QAC), biguanides, alcohols and phenolics, may be used on skin and are extensively used on non-porous surfaces in healthcare, food, transport, corporate and domiciliary industries[23].

Because of their wide range of applications, some biocides will enter the environment and impact antimicrobial resistance[24]. In this Review we will discuss some examples, but we will not consider their breakdown products or reaction by-products.

More reactive biocides, such as oxidisers (for example, chlorine or peroxygen-based disinfectants) and alkylating agents (for example, glutaraldehyde) are more efficacious and are used in applications where target microorganisms are considered less susceptible to biocides (FIG. 1), as in the case of bacterial endospores that require high-level disinfection (Supplementary Box 1)[25]. This comes at the cost of increased toxicity, incompatibility with some surface types and reduced residual activity. When appropriately formulated, these biocides are widely applied to disinfect non-living (abiotic) surfaces and liquids, such as drinking water. Product formulation is critical not only for efficacy but also to improve material compatibility and decrease toxicity[26]. The reactivity of biocides refers to their interaction with microbial targets, whether there is a strong interaction with the target through chemical or ionic binding or a weak physical interaction with lipophilic components of the membrane[27].

[H2] Mechanisms of biocide action

At their in-use concentration, biocides exert their bactericidal action by interacting with multiple target sites (FIG. 2). This is in contrast to antibiotics, which acts at specific target sites[23,27]. The number of targets that are affected by the biocide and the severity of the damage imparted to these targets results in bacteriostatic or bactericidal effects (FIG. 2)[8]. It is challenging to determine the exact mechanisms of action due to the non-specific damage caused by biocides. However, an understanding of the underlying chemistry can offer some insight (Table 1). Microbial inactivation by biocides is complex and can be understood by using multiple approaches. These include analysing the effects of biocides on membrane integrity of live cells or vesicles and liposomes through the use of microscopy, the uptake of substrates (for example, fluorescent dyes or particles), and the leakage of cellular components (for example, potassium, ATP and nucleotides or DNA)[28-30]. Additionally, the effects on cellular macromolecules can be evaluated by examining DNA integrity, enzyme activity, lipid or protein modification[31]. Understanding the genotypic and phenotypic determinants that contribute to susceptibility, particularly in the case of sporicides[32,33], is crucial. Computational modelling[34] and changes in metabolism and gene expression, typically following sub-lethal exposure[35,36] are also important. Except for the last example, where viability of the treated population must be maintained, these studies typically use biocides at their in-use concentration; this contrasts with studies concerning antibiotic mechanisms of action. As a rule, biocides must interact with bacteria and reach their target sites in sufficient quantities to exert biocidal effect. For example, the outer membrane of some Gram-negative species can provide intrinsic resistance to quaternary ammonium compounds, by acting as a barrier that prevents interaction with the cytoplasmic membrane. This will be discussed further in following sections. The initial interaction of a biocide with the target bacterial cell is an important determinant of efficacy and can be measured with uptake isotherms[37], which provide information on the nature and strength of the interaction between a biocide and the microorganism[38].

The general mechanisms of action of biocides can be divided into different groups. Alkylating agents (for example, aldehydes and ethylene oxide) act via cross-linking hydroxyl, amino, carboxyl and sulfhydryl groups, impacting on enzyme function and nucleic acid structure, resulting in microbiocidal effects. The extent of crosslinking ability depends on the alkylating agents and does not necessarily impact on efficacy, although this will affect penetration inside the cells. For example, glutaraldehyde interacts with the outer layer of the bacterial cells due it extensive crosslinking ability, whilst ortho-pthalaldehyde, ethylene oxide or formaldehyde penetrate deeper within the cells and can impair nucleic acid and cytoplasmic enzyme functions.

Another group is constituted by oxidising agents such as chlorine, iodine and peroxygens that oxidise various chemical groups (amino, sulfhydryl, thiol) associated with lipids, proteins and

nucleic acids, thus disrupting major cytoplasmic membrane function, enzyme function and DNA synthesis. Chlorine- and iodine-based compounds and peracetic acid have been associated with membrane damage presumably through protein oxidation. The bactericidal efficacy of hydrogen peroxide, however, is likely caused by nucleic acid damage rather than lipid and protein oxidation, although hydrogen peroxide has been shown to interfere with ribosomes preventing protein synthesis. Membrane active agents are very diverse and exert their bactericidal activity through physical damage to the membrane or loss of membrane function. Phenols, quaternary ammonium compounds (QAC) and biguanides will cause potassium leakage, an early indicator of membrane integrity, followed with a change in pH and cytoplasmic enzyme function. Hexachlorophene can inhibit metabolic activity by interfering with the electron transport chain, whereas organic acids and their esters can impact membrane potential, which affects cells proton motive force, resulting in the disruption of active transport and oxidative phosphorylation. Polymeric biguanides such as polyhexamethylene biguanide (PHMB) are also membrane active and interact with the lipopolysaccharide in the outer membrane of Gram-negative bacteria, promoting self-penetration and inducing phospholipids phase separation in the cytoplasmic membrane. The fine interaction of QAC with the membrane depends on the QAC chemistry.

The bactericidal activity of alcohols is probably linked to denaturation of essential membrane proteins, affecting membrane function, as well as cytoplasmic enzymatic functions. The loss of membrane integrity and penetration of some biocides (biguanides, phenolics) into the cell leads to cytoplasm coagulation and further loss of enzymatic functions.

At low concentration, some biocides can exhibit specific interactions with the bacterial cell. At a low concentration, o-phenylphenol may interfere with cell wall peptidoglycan synthesis, and triclosan interferes with enoyl acyl reductase, an enzyme involved in fatty acid synthesis and lipid metabolism[39].

The initial interaction of a biocide with a bacterial cell is reversible, triggering adaptation and repair mechanisms and ultimately bacterial survival (FIG. 2). A prolonged interaction would result in severe damage to the bacterial cytoplasmic membrane leading to an irreversible effect and eventually bacterial death[8]. Metabolically inactive bacteria or bacteria with reduced metabolic activity are generally less susceptible to biocides[40,41].

The efficacy of a biocidal product can be influenced by several factors. Some of these factors are inherent to the product, such as its concentration, pH, formulation excipients. Others are related to the application of the product, such as the duration of contact, soiling, and the type of surfaces. There are also factors that are inherent to the microorganisms being targeted (Table 2). Concentration is arguably the most important, as it determines the extent and severity of damage imparted to the bacterial cell[42,43].

[H1] Bacterial resistance to biocides

[H2] Intrinsic resistance

The ability to survive biocide exposure depends on the type of microorganism (FIG. 1) and their intrinsic physiological properties. Intrinsic mechanisms of vegetative bacteria, bacterial endospores and biofilms (multicellular, sessile bacterial communities) may be considered separately (FIG. 3).

Amongst vegetative bacteria, mycobacteria are considered the least susceptible to biocides due to their lipid-rich outer layer of mycolic acids surrounding the cell[44]. In Gram-negative bacteria, the lipopolysaccharide (LPS) layer of the outer membrane, the cytoplasmic membrane lipid composition, and the number, size and substrate specificity of porins may also confer decreased susceptibility to biocides[37]. The importance of the outer membrane in reducing biocide susceptibility can be best exemplified by the use of ethylenediaminetetraacetic acid (EDTA), a metal chelator that disrupts the LPS layer in Gram-negative bacteria to enhance the performance of biocides (FIG. 2)[45].

Bacterial endospores provide the best evidence of biocide resistance derived from intrinsic cell properties. Bacterial endospores are formed through a sporulation process to facilitate survival under adverse conditions[46]. The lack of susceptibility of endospores from the two main spore forming bacterial genera *Bacillus* spp. and *Clostridium* spp. (including *Clostridioides difficile*), have been well reported[47]. The mechanisms of bacterial endospore resistance to biocides have been previously described and can be divided broadly into permeability barriers and nucleic acid protection (FIG. 3)[46].

The intrinsic responses to resistance described thus far are pertinent to individual bacterial cells. However, bacteria in the environment are usually found within multicellular communities (biofilms) which provide additional challenges to biocide efficacy. In addition to the commonly described ‘wet’ biofilms, which are associated with moist environments, biofilms can develop on environmental dry surfaces[48]. These dry-surface biofilms are widespread on surfaces withing healthcare environments[49,50], and are highly resilient to surface disinfection[51]. Biofilms exhibit decreased susceptibility to biocides through several biofilm-intrinsic mechanisms, of which extracellular polymeric substances (EPS) and persister cells are the most described[15,40,41]. EPS consists of secreted nucleic acids, proteins, lipids and carbohydrates. Alongside cellular debris, EPS forms a matrix that acts as a diffusion barrier whilst also quenching the activity of biocides. EPS is the main factor affecting susceptibility of *Pseudomonas aeruginosa* biofilms to peracetic acid and benzalkonium chloride, and its removal through washing yields cells with comparable susceptibility to vegetative bacteria[52]. Cell density and biofilm thickness increase with age, conferring increased protection against biocide exposure[53,54]. The efficiency of diffusion through a biofilm varies between biocides.

For example, peracetic acid reduces *P. aeruginosa* biofilm viability uniformly upon contact, whilst benzalkonium chloride penetrates slowly and directionally[52]. The ability of a biocide to penetrate a biofilm does not entirely explain the differences observed in anti-biofilm performance[55] and the EPS does not fully account for biocide resistance[15], exemplifying the importance of other mechanisms.

Persister cells are characterised by a substantially decreased growth rate and metabolic activity, including protein synthesis[56]. The EPS surrounding persister cells does not solely explain their resistance to biocides, as EPS-free cells retain increased tolerance[40]. Induction of persister phenotypes is driven by stress-induced signals[56] and is partly mediated by the SOS response, which also confers protection against DNA damage[57].

[H2] Acquired resistance

In contrast to intrinsic resistance, acquired resistance involves the acquisition of new properties following gene transfer or mutation. Since biocides interact with multiple targets in bacteria (FIG. 2), reports of mutation(s) responsible for bacterial resistance to in-use concentrations of a biocide are rare. However, the impact of mutations on decreasing susceptibility to biocides, as measured by minimum inhibitory concentration (MIC), is more widely reported[58]. For example, a recent report showed that repeated sub-MIC/MIC exposure to QACs induced mutations in regulators (*acrR, marR, soxR, and crp*), outer membrane proteins and transporters (*mipA and sbmA*), and RNA polymerase (*rpoB and rpoC*) genes in *Escherichia coli*[59]. Owing the nature of biocide interactions with the bacterial cells (FIG. 2), resistance mechanisms are often non-specific, with efflux and alterations in membrane properties being prominent examples (Table 3).

[H2] Efflux

Efflux pumps facilitate the removal of toxic compounds from bacterial cells. Bacterial efflux is a major global resistance mechanism that can be induced by some biocides. Efflux pumps can be categorised into seven major families and superfamilies[60-62]: the drug/metabolite transporter (DMT) superfamily, the major facilitator superfamily (MFS), the ATP-binding cassette (ABC) superfamily, the resistance-nodulation-division (RND) superfamily, the multidrug and toxic compound extrusion (MATE) superfamily, the proteobacterial antimicrobial compound efflux (PACE) family, and the *p*-aminobenzyoyl-glutamate transporter family. Efflux has been widely linked to increases in biocide MIC[63-65], and decreased susceptibility to some antibiotics[66-70]. The *qac* transporter, which belongs to the small multidrug resistance (SMR) family within the DMT superfamily, exports lipophilic cations such as quaternary ammonium compounds and is particularly notable in the context of biocides[71]. Some efflux pumps have broad substrate specificity and can export both biocides and antibiotics[60,61]. For example,

290 *oqxAB* expression in *E. coli* promotes increased resistance to benzalkonium chloride,
291 triclosan, SDS and a variety of common antibiotics[72]. However, efflux is unlikely to confer
292 resistance to in-use product concentration. The decreases in biocide susceptibility conferred
293 by efflux remain modest, with 2 to 10-fold increases in MIC typically reported[63,70,73]; biocides
294 are typically applied at concentrations exceeding 100 to 1000-fold greater than the MIC. One
295 notable exception is the reported expression of TriABC pump conferring *P. aeruginosa*
296 resistance to triclosan (> 1mg/mL)[74].
297 Efflux pumps also play an important role in biofilm formation[75-77]. The expression of efflux
298 pumps in biofilms has been reported as one of the mechanisms responsible for biofilm
299 resistance to antimicrobials, particularly antibiotics[78], and studies have shown that efflux pump
300 expression is upregulated in biofilms[76].
301
302 *[H2] Porins*
303 As is the case of efflux pumps, changes in porin expression may confer increased resistance
304 to biocides. Porins facilitate the transport of hydrophilic solutes, including nutrients and
305 xenobiotics, across the cytoplasmic membrane (influx). General diffusion porins, such as
306 OmpC, allow a wide range of substrates to traverse the membrane, whilst others may exhibit
307 a higher degree of substrate specificity. Porins can be an intrinsic resistance mechanism, for
308 example in decreasing QAC susceptibility in *P. aeruginosa*[79], but generally the literature
309 reports modified porin expression conferring decreased susceptibility to biocides. For
310 example, decreased expression of Msp-type porins in mycobacteria results in increased
311 resistance to glutaraldehyde and ortho-phthalaldehyde and a number of antibiotics including
312 rifampicin, vancomycin, clarithromycin and erythromycin[80]. Msp-type porins constitute over
313 70% of all porins in some *Mycobacterium* species and provide a route of entry for antibiotics[81].
314 In *E. coli,* mutations in the porin regulators OmpR and EnvV following sublethal exposure to
315 chlorophene and povidone-iodine has been associated with changes antibiotic susceptibility
316 in vitro[82].
317
318 *[H2] Other mechanisms contributing towards resistance*
319 Other acquired resistance mechanisms have been reported (Table 3). For example, in the
320 case of ionic silver, decreased susceptibility can result from multiple mechanisms (such as
321 those encoded by *silA-S* genes) that encompass efflux, reduced penetration, and
322 neutralisation and reduction of ionic silver to its inactive metallic form[83]. A change in surface
323 charge has been implicated in reduced benzalkonium chloride efficacy in *P. aeruginosa*[67].
324 The ability of bacteria to repair damage following exposure to a biocide has generally received
325 little attention[84-86], yet repair is essential to bacterial survival (FIG. 3). The impact of repair on
326 bacterial survival is better considered in the food industry, where bacterial ability to repair

injuries inflicted with chemical and physical agents is important to evaluate potential food contamination post-processing[87].

Another mechanism of resistance rarely considered is pleomorphism, the ability of a bacterium to change shape. For example, *Vibrio cholerae* cells can form shorter, round, rugose (wrinkled) variants which are associated with enhanced biofilm formation and decreased susceptibility to chlorine[88].

Emerging small colony variants (SCV) following antibiotic[89,90] or biocide exposure[91] is driven by mutations[92,93]. SCV are associated with several survival advantages, including intracellular persistence and reduced antimicrobial susceptibility, and are implicated in disease[94]. Reduced antimicrobial susceptibility of SCV phenotype relies on reduced growth rate[95], reduced transmembrane potential driven by alteration of the electron transport chain[96] and persistence within host cell, decreasing antimicrobial exposure. The SCV phenotype is also associated with biofilm formation[97].

[H2] Coordinated expression of multiple resistance mechanisms

Single mechanisms conferring bacteria resistance have been described so far. However, it is now clear that bacteria can use a combination of mechanisms to survive biocide exposure as part of a global response, for example a combination of efflux and changes in membrane properties[66,74,98,99]. The alteration of metabolic pathways is part of this global response[66,98,100-103]. Sub-lethal exposure to biocides may indirectly induce oxidative stress response regulators such as *marA* and *soxS*[104-106]. This can impact the expression of small regulatory RNA[107], which may also confer resistance to a range of chemotherapeutic antibiotics[108,109]. Mutations in global regulators can also impact bacterial susceptibility to biocides and promote cross resistance to antibiotics. It has been reported that mutations in the two-component regulator *phoPQ* and a putative Tet repressor gene (*smvR*) lead to chlorhexidine adaptation in *Klebsiella pneumoniae* via an efflux mediated mechanism[110]. Whether caused by stress or mutation, a change in the expression of these global regulators can induce a cascade of events resulting in phenotypic changes (FIG. 3). Several publications referred to these global networks as ‘triclosan resistance network’ when investigating response from *Salmonella enterica* serovar Typhimurium to triclosan[100], or ‘complex cellular defence network’ describing the genetic response of *S. enterica* serovar Typhimurium to chlorhexidine[101]. Metabolic changes following biocide exposure has sometimes been associated with a change in antibiotic susceptibility, for example aminoglycoside resistance in *Listeria monocytogenes*[111], or isoniazid resistance in *Mycobacterium smegmatis*[112], both following triclosan exposure.

[H2] Measuring acquired biocide resistance

Whilst antibiotic resistance may be clearly defined by clinical breakpoints[119-121], similar definitions for 'biocide resistance' are lacking and there is little consensus as to what it should be and how it should be measured[5]. In addition, whilst antibiotic resistance is linked to clinical practice, there is no such concept with biocide resistance. One proposed definition is based on the failure of a product at its in-use concentration to kill bacteria[5].

Whilst there are no clinical breakpoints for biocides, evaluation of biocide resistance inadequately aligns with tests designed for determining antibiotic efficacy, which principally measure the minimum inhibitory concentration (MIC); this test measures bacterial growth in medium with various concentrations of a biocide and over a period of 24 hours[5,19]. The efficacy of biocides may be substantially affected by growth medium composition and even the type of plastic used in the assay plate[122]. Similarly, minimum bactericidal concentration (MBC), the minimum concentration required to inactivate bacteria, is typically ascertained following 24 hours of contact. MBC are often determined following the use of a MIC determination protocol and rarely use a neutralisation step that inactivate the biocide. Quenching the activity of a biocide is paramount for evaluating the efficacy of a biocide and failing to do so can result in overestimation of biocide efficacy[25,123].

Many studies define 'biocide resistance' as a change in MIC, as low as a 2-fold increase (Supplementary Box 2). As the concentration of biocide within disinfectant products are typically 100- to 1000-fold higher than the MIC, and the goal is typically to kill microorganisms within a short contact time rather than prevent their growth, MIC-based protocols have been criticised poor markers of biocide resistance: such small increases in MIC are unlikely to lead to disinfection failure[5,43]. The use of MIC distribution to determine a biocide cut off value, in analogy to the definition of epidemiological cut off (ECOFF) values of antibiotic susceptibility[124], has been explored[58]. However, the benefit of trying to establish an association between reduced susceptibility to biocide and antibiotic resistance is not certain, even if a large MIC data set is used[125]. Therefore, relying on MIC measurement to define 'biocide resistance' is inappropriate in any context of biocide application[5]. It should not be used for regulatory or intellectual property recommendations.

Overall, it is difficult to predict the impact of biocide exposure on emerging resistance and cross-resistance to unrelated antimicrobials[5,111] (Supplementary Box 3). The use of different protocols to induce bacterial resistance following biocide exposure yields divergent results, as protocols that mimic realistic exposure conditions fail to isolate resistant bacteria[19,126]. Stepwise training protocols that involve initial exposure of bacterial suspensions to increasing sub-MIC concentrations, contribute to a better understanding of antimicrobial resistance mechanisms[67,104,127], but do not accurately reflect product usage[5,19]. Although the MIC of a biocide may increase to levels close to those used in practice[67], this reduced susceptibility may be readily counteracted by excipients present in formulated products[128].

The in-use concentration of a biocide can be reduced during product application, through dilution, interaction with organic soils like dirt, surface abrasion or, in the case of antimicrobial handwash, when entering drains. A lowered concentration attained following product application, referred to as the 'during use' concentration, has been proposed as an appropriate concentration for challenging bacteria in AMR predictive assays[129]. For example, it has been reported that the concentration of chlorhexidine left on surfaces were within the MIC–MBC range (0.002–0.01 mg/mL) for *Escherichia coli* up to 168 hours post-application of 2% chlorhexidine[98]. Exposure to these concentrations resulted in stable changes in antibiotic susceptibility profile, clinical resistance to ampicillin, amoxicillin and clavulanic acid, ciprofloxacin, cefpodoxime, cephalotin, and a 32- to 62-fold increase in MIC and MBC to chlorhexidine. There have been other approaches to determine changes in biocide resistance by examining contact times necessary to achieve a reduction threshold. Such approaches may provide insights that are more readily applicable to real-world scenarios[130].

[H1] Implications of biocide exposure

The impact of bacterial resistance to biocides remains a fundamental question within infection control that has no easy answers, since most of the evidence comes from in vitro studies that are mostly based on observing MIC increases. However, it is important to note that these concentrations typically fall below the in-use concentration of the biocide. Yet, bacterial survival in biocidal products and their clinical implications have been reported.

[H2] Examples of biocidal product contamination leading to outbreaks and pseudo-outbreaks

Over the years, there have been many reports of outbreak or pseudo-outbreak the later corresponding to an increase in identified organisms but without evidence of infection resulting from bacterial contamination of disinfectants[131,132]. Bacterial survival in biocidal products may be the result of contamination with an intrinsically resistant bacteria, as in the case of *Bacillus cereus* spores contaminating ethyl alcohol solution[11], with bacteria that acquired resistance, as in the case of *Serratia marcescens* contaminating a 2% aqueous chlorhexidine solution[133], or because an ineffective biocide concentration was used following inappropriate usage of a biocidal product[134-137].

Biocidal product usage can also lead to the selection of resistant bacteria. One of the earliest examples where the use of an antiseptic led to the selection for resistant bacteria was the introduction of wound dressings containing 0.5% silver nitrate to combat *P. aeruginosa* infection[138]. Although silver nitrate was successful in eliminating most *Pseudomonas* infections, *Pseudomonas* strains with a silver nitrate MIC > 0.5% were isolated in a few instances, resulting in treatment failure[138]. Further analysis of the patients' wound highlighted

a change in microbiota diversity. Whilst *Pseudomonas* was mostly controlled, the use of silver nitrate enhanced the abundance of other species, particularly bacteria normally associated with the gastrointestinal tract (coliforms)[138].

Another study reported an outbreak of *Mycobacterium massiliense* in 38 hospitals in the state of Rio de Janeiro, Brazil, that occurred between August 2006 and July 2007 following video-assisted surgery[139]. The strains responsible for the outbreak were clinically resistant to ciprofloxacin, cefoxitin and doxycycline, but also resistant to glutaraldehyde (2% w/v) which was used for endoscope disinfection at the time, although the origin of the outbreak was not confirmed.

[H2] Impact of biocide exposure on emerging resistance and cross-resistance to unrelated antimicrobials

The emergence of biocide and antibiotic cross-resistance varies depending on biocide type. It has been observed that, amongst 10 biocides tested, antimicrobial resistance selection in *E. coli* was greatest in those exposed to chlorophene and benzalkonium chloride[82]. A smaller but still notable number of resistant mutants were isolated from those exposed to glutaraldehyde, chlorhexidine hydrogen peroxide and povidone-iodine. In contrast, no resistant mutants were isolated from groups treated with alcohols (isopropanol, ethanol), sodium hypochlorite or peracetic acid[82]. The ability of a non-intrinsically resistant bacteria to survive biocide exposure at in-use concentration is not confined to less reactive biocides but has also been reported with chlorine dioxide[14] and glutaraldehyde[13]. Remarkably, bacterial isolates were observed to be cross-resistant to unrelated biocides. For example, vegetative *Bacillus subtilis* isolated from endoscope washer disinfector were resistant to chlorine dioxide (0.03%) but also to peracetic acid (2.25%) and hydrogen peroxide (7.5%), whilst comparable counterpart strains were killed (>99.99% reduction in viability within 30 seconds) in 0.03% chlorine dioxide[14]. A *Mycobacterium chelonae* isolate from endoscope washer disinfector was resistant to 2% glutaraldehyde, sodium dichloroisocyanurate (NaDCC) and Virkon®[13]. These findings suggest that mechanisms allowing bacterial survival may also confer resistance to chemically unrelated biocides. Unfortunately, neither study assessed changes in clinical antibiotic susceptibility profiles. Oxidising agents appear to be less capable of inducing resistance, which may imply a wider variety of potential targets, enhanced self-promoted uptake, or a smaller number of potential adaptations to counteract biocide effects without significantly compromising reproductive fitness.

Oxidising agents that degrade nucleic acids may also reduce the opportunity for horizonal gene transfer via DNA uptake in the environment. However, exposure to subinhibitory concentrations of sodium hypochlorite has been associated with decreased susceptibility to a

range of antibiotics in Gram-negative species, including *Salmonella* spp. and *P. aeruginosa*[117,140].

Emerging antimicrobial resistance following biocide exposure in vitro is not limited to clinical strains. The release of biocides into the environment has been shown to result in the selection of resistant phenotypes. The discharge of detergent-containing wastewater into riverine ecosystems has been linked to the dissemination of class-1 integrons, which increased tolerance to QACs and multiple antibiotics in environmental *E. coli* isolates[141]. Repeated exposure of *Salmonella enterica* serovar Typhimurium to farm disinfectants was associated with acquired low-level multiple drug resistance (MDR) and decreased susceptibility to antibiotics, including ciprofloxacin, in vitro[142]. However, these MDR strains, which exhibited up-regulation of AcrAB efflux pump, were not able to disseminate in chickens compared to the isogenic parent strain, nor did they show a competitive advantage when chickens were treated with ciprofloxacin[142].

There is limited evidence of the impact of biocidal products on emerging antimicrobial resistance in situ. In a randomised trial, clinical and environmental samples were collected from two distinct groups: individuals who used domestic biocidal products and individuals who did not use them (with the exception of specific items like mouthwash and toilet bowl cleaner); the authors found no evidence of differences in biocide and antibiotic cross-resistance between groups[143]. However, increased prevalence of potential pathogens was observed in the non-user group. Another study, a longitudinal double-blind, randomized clinical trial, explored the impact of biocide products (QAC- and triclosan-based) usage on change in antimicrobial susceptibility profile[144]. After 1 year of product usage, the authors reported differences between the group that used antibacterial products and the group that did not. An association was observed between high QAC MIC and antibiotic resistance in the product 'user' group. Bacterial isolates with a high QAC MIC were likely to show a high triclosan MIC and resistance to one or more antibiotics.

All the in vitro studies mentioned so far are based on the principle of pre-exposure, whereby bacteria are exposed or pre-exposed to a biocide concentration and changes in susceptibility are then investigated. Co-exposure refers to exposing bacteria to two antimicrobials (for example an antibiotic and a biocide) at the same time. Although this scenario might not often occur in practice, it nevertheless can provide interesting observations. A study investigating co-exposure of benzalkonium chloride (1-4 mg/L) and gentamicin in *Acinetobacter baumannii* reported a decreased gentamicin bactericidal activity and an increased bacterial mutation frequency with decreased aminoglycoside susceptibility linked to a decreased intracellular antibiotic accumulation[145].

[H2] Biocide exposure and antimicrobial gene maintenance and dissemination

There are many examples of studies that report clinical isolates carrying multiple resistance genes with an increased biocide MIC. An increasing number of studies are reporting multiple resistance gene carriage in clinical and environmental isolates from settings where biocides are regularly used. A previous study analysed gene carriage of efflux determinants in 53 *Staphylococcus aureus* clinical isolates[146] and reported that 83% of isolates carried plasmids encoding *qacA/B* and 77% carried *smr*. Many isolates carried multiple efflux genes: 53% carried *qacA/B* and *smr*, 11% carried *qacA/B*, *smr* and also *qacH*. These isolates were clinically resistant to the antibiotic mupirocin and showed an elevated MIC to chlorhexidine (> 4 µg/mL). Multiple gene carriage, particularly of genes encoding efflux pumps, have been reported in ESKAPE pathogens, including *S. aureus*[146-148], *K. pneumoniae*[149], *A. baumannii*[150,151], *P. aeruginosa*[69,151-154], and *Enterobacter spp.*[154]. In these studies, the implication of biocide usage in increasing gene carriage, and specific efflux genes, was not ascertained although clinical isolates showed an increased MIC to various biocides.

Although multiple antimicrobial resistance gene carriage in environmental and clinical isolates is well documented, the impact of biocide use on antimicrobial resistance gene dissemination has not particularly been well investigated. A correlation between increased MIC to copper and the incidence of antibiotic resistant phenotypes in *Salmonella* isolated from the feed and faeces of pigs has been observed[155]. Resistance to the antibiotics seemingly occurred independently of the carriage of the copper efflux gene *pcoA*, indicating that other co-selective mechanisms may have contributed towards their observations. However, in cases where isolates originate from an environment where both antibiotics and biocides are used, it becomes difficult to conclude the impact of biocides alone on gene dissemination. In studies that have investigated bacterial clone clusters and lineages displaying an elevated biocide MIC[156,157], or the presence of *qac* genes on class-1 integrons[158] along with reduced antibiotic susceptibility, the role of the biocide in gene dissemination was not explored.

Co-location of resistance determinants within the same mobile genetic element will facilitate co-selection and acquisition of new properties following biocide exposure[159,160].

In biofilms, the microenvironment promotes plasmid stability and may facilitate the transmission of mobile genetic elements encoding resistance genes, such as QAC efflux pumps (for example, *qacA*B)[75,161]. The selective pressures exerted by biocide exposure may accelerate the acquisition of antibiotic resistance genes (for example the sulfonamide resistance gene *sul1,* and the β-lactamase gene bla_{TEM}) biofilms[162].

[H1] Conclusion

The use of biocidal products for preservation, antisepsis and disinfection is the corner stone of infection prevention and control in healthcare[163], the food industry[164] and home hygiene

settings[165]. The use of biocidal products to reduce infection risk is an integral element of combatting the spread of AMR[166,167]. The bactericidal effectiveness of a biocide depends on many factors (Table 2) and failure to understand these will contribute to bacterial survival, outbreaks and potential antimicrobial resistance. The role of biocide usage on AMR continues to be less well studied compared to that of chemotherapeutic antibiotics which remains the driver for emerging AMR. In addition, the study of biocide effects on AMR still suffers from several drawbacks, including a lack of cohesion on the definition of resistance, an inappropriate use of MIC determination to measure biocide resistance, a lack of proper protocols that reflect product usage to study resistance emergence and a lack of practical or clinical significance on in vitro studies. Yet, our understanding of biocide impact on AMR has progressed in the last 20 years. Considering a comprehensive AMR review published in 1999[17], the principles for and mechanisms of intrinsic and acquired resistance remain broadly the same. However, the use of new research tools has allowed us to understand that biocide effects can be transient and biocide-led cross-resistance to different chemistries, including chemotherapeutic antibiotics, might not be associated with a deceased susceptibility to the biocide. We have gained a better understanding of the remarkable ability of bacteria to respond to biocide exposure, notably by coordinating the expression of multiple resistance mechanisms. Yet the potential risks posed by rising biocide usage remains to be addressed, particularly in biofilms. There is still plenty of scope for research investigating the role of biocides in increasing antibiotic resistance genes carriage and dissemination, fitness cost associated with expressing multiple resistance genes and mutation rate driven by biocide exposure and its impact on AMR.

One of the main limitations of biocide resistance is that generalisation of bacterial AMR response to a given biocide exposure might be difficult to ascertain. The use of predictive protocols[129] can provide practical and clinical relevance reflecting a biocide in-use condition, despite being mainly based on MIC determination.

With the rising utilization of biocides across various environments, such as clinical, domestic, veterinary, and food settings, it is fundamental that future studies address the many knowledge gaps regarding the contribution of biocides to AMR. This will ensure that biocides remain effective in controlling bacterial pathogens and contaminants without adding to the AMR problem.

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Sentences highlighting key references added underneath selected references.

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Competing interests

J.Y. Maillard is the Director of Biocide Consult Ltd. M. P. declares no competing interests.

Supplementary information

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Related links

ECHA: https://echa.europa.eu/information-on-chemicals/biocidal-active-substances

ECHA, Biocidal Product Regulation: https://echa.europa.eu/regulations/biocidal-products-regulation/legislation

Display items

Table 1. Mayor types of biocides and their mechanisms of action

Types	Mechanism of action	Examples of chemistry	Application and areas of use
Highly reactive biocides - strong interactions through chemical or ionic binding			
Alkylating agents	Reacts with amino acids to form cross links and fix proteins.	Glutaraldehyde, formaldehyde, ortho-phthalaldehyde (OPA)	Disinfection of surfaces, materials, equipment Disinfection of materials and surfaces associated with the housing or transportation of animals
Oxidising agents	Oxidation of macromolecules (proteins, lipids and nucleotides), whilst causing non-specific damage to the cytoplasmic membrane	Sodium hypochlorite, peracetic acid, hydrogen peroxide, ethylene oxide	Disinfection of surfaces, materials, equipment Disinfection of materials and surfaces associated with the housing or transportation of animals. Disinfection of drinking water
		Povidone-iodine	Disinfection of skin, scalps, surfaces, materials, equipment
Less reactive biocides - weak physical interaction			
Cationics	Positively charged, hydrophilic region interacts with negatively charged cell surface. Hydrophobic region partitions into membrane, disrupting intermolecular bonds and leading to loss of intracellular contents	Quaternary ammonium compounds (for example, benzalkonium chloride)	Disinfection of skin, scalps Disinfection of surfaces, materials, equipment Incorporated in textiles, tissues, mask, producing treated articles with self-disinfecting properties
		Biguanides (for example, chlorhexidine, polyhexamethylene biguanide)	Antisepsis of skin, scalps. Disinfection of surfaces, materials, equipment, swimming pools.
		Diamines, amine oxides	Disinfection of surfaces, materials, equipment
Phenolics	Protonophore which targets the cytoplasmic membrane, causing loss of membrane potential. At low concentrations, triclosan inhibits fatty acid synthesis	Triclosan	Disinfection of surfaces, materials, equipment Incorporated in textiles, tissues, mask, producing treated articles with disinfecting properties
Alcohols	Permeabilization of the cytoplasmic membrane; denaturation of proteins; dehydration of exposed bacteria	Ethyl alcohol (ethanol), isopropyl alcohol	Disinfection of skin, scalps Disinfection of surfaces, materials, equipment
Weak organic acids	Uncoupling of proton motive force; Acidification of bacterial cytoplasm, leading to inhibition of	Citric acid, benzoic acid	Disinfection of skin, scalps Disinfection of surfaces, materials, equipment

	enzyme activity and biosynthesis whilst exerting osmotic stress		
Metal ions	Redox active. Interacts with thiol groups and generates reactive oxygen species which damages macromolecules	Silver, copper	Antimicrobial surfaces, textiles, wound dressings
Antimicrobial dyes	Intercalation with DNA. Production of singlet oxygen (photosensitisers)	Methylene blue, toluidine blue, crystal violet	Wound dressings, photodynamic therapy (photosensitisers)

Table information based partly on[21,27]

Table 2. Extrinsic factors affecting the performance of biocides

Biocide properties	Mechanism of action	Spectrum of activity determined by chemistry underlying biocide-microbe interaction
	Use concentration	Concentration correlates with speed of effect
	Formulation and product composition	Excipients, co-actives and pH may affect biocide reactivity, interaction with bacterial cells (for example, EDTA destabilisation of outer membrane), drying time (formulation to wipe ratio) and surface wettability (surfactants)
Application factors	Contact time	Level of inactivation partially determined by time (disinfection kinetic)
	Presence of organic soils (Has the surface been cleaned?)	Organic matter may react with biocides and reduce performance
	Surface type	Performance may be affected by target surface (for example, polyvinyl chloride (PVC) versus stainless steel)
	Environmental temperature	Increased temperature increases rate of reaction
	Method of delivery (for example, vaporisation, spraying, wiping)	Efficacy of a biocide will change if it is a in a liquid or gas form. The method of delivery will also impact on the overall efficacy of the formulation.
	Interactions between biocide and applicator	Some biocides may interact with applicator (for example, wipe material), reducing effective concentration
	Concentration upon subsequent dilution and abrasion	Reduction in concentration during use may reduce biocidal efficacy
Target organism	Endospores	Metabolically inactive structures of *Bacillus* spp. and *Clostridioides* spp. highly tolerate biocide exposure (FIG. 3)
	Bacterial type (for example, mycobacteria, Gram-negative species)	Intrinsic factors may affect resistance to specific biocides (for example, outer membrane and quaternary ammonium compounds (QACs))
	Metabolic activity	Reduced metabolism associated with decreased susceptibility

Lifestyle (BOX 1)	Microbial communities (biofilms) exhibit reduced susceptibility to antimicrobials

EDTA, ethylenediaminetetraacetic acid.

Table 3. Mechanisms of acquired biocide resistance and biocide-induced cross-resistance to antibiotics.

General mechanism	Organism	Biocide (test concentration)	Change in biocide susceptibility	Antibiotic resistance	Specific mechanism	Reference
Efflux						
	Mixed waterborne community	Copper (8-500 mg/L)	N/A (environmental isolates only)	Clarithromycin; Tetracycline	CusA, CusB CusS, CutE	[113]
	A. baumannii	Triclosan (128 mg/L)	2- to 32-fold increase in MIC	Trimethoprim	FabI, AdeIJK	[114]
	P. aeruginosa	Benzalkonium chloride (BZC) (12.5 mg/L)	12-fold increase in MIC	Ampicillin; Cefotaxime; Ceftazidime	MexAB-OprM; MecCD-OprJ	[115]
	Campylobacter spp.	BZC; Chlorhexidine; Cetylpyridinium chloride	2- to 4-fold increase in MIC	Erythromycin; Ciprofloxacin	Not established (confirmed with efflux inhibitors)	[116]
	P. aeruginosa	Sodium hypochlorite (100 mg/L)	circa 2.5-fold increase in MIC	Ampicillin; Tetracycline; Chloramphenicol Kanamycin	MuxABC-OpmB*	[117]
Porins						
	M. chelonae	Glutaraldehyde (0.2-2%)	>6 $\log_{10}$ survival of resistant strain in 2% glutaraldehyde	Rifampicin, Vancomycin, Clarithromycin, Erythromycin	Msp	[80]
	E. coli	Chlorophene (0.5-2.49 mM) Povidone-iodine (67-111 µg/ml)	Increased growth in 2- to 5-fold higher concentrations of biocide after 500 generations	Ampicillin; Chloramphenicol; Norfloxacin	OmpR; EnvZ	[82]
Metabolic changes						
	E. coli	Hydrogen peroxide (200 µM)	Increased growth in circa 2-fold higher concentration after 500 generations	Ampicillin; Chloramphenicol	RNA polymerase (*rpo*)	[82]

	M.smegmatis	Triclosan (0.8-1.6 mg/ml)	4- to 6-fold increase in MIC	Isoniazid	Lipid metabolism (InhA)	[112]
	Listeria monocytogenes	Triclosan (1-4 µg/ml)	No change in MIC	Aminoglycosides	Heme metabolism *(hemH/hemA)*	[111]
Modification of surface charge						
	P. aeruginosa	BZC (50-1600 mg/L)	7 to 25-fold increase in MIC	Polymyxin B	*pmrB*	[67]
Extracellular metal-binding protein						
	Klebsiella pneumoniae	Silver (≤64 µM)	N/A (clinical isolates only); resistance to silver based on literature values.	Beta lactams, fluoroquinolones, aminoglycosides (plasmid-encoded)	SilE	[118]

*Induction of SOS response and antioxidant enzymes also noted

N/A, not applicable; MIC, minimal inhibitory concentration.

Figure 1. Susceptibility of microorganisms to biocides. Biocide efficacy depends partly on the type of microorganisms being targeted. High, intermediate and low refer to level of disinfection required to render a contaminated surface safe and depends on the expected microbial contaminant. The least susceptible organisms, such as bacterial endospores, require high level disinfection delivered by reactive oxidising and alkylating agents. Prions are the agents responsible for mad cow disease and new variant Creutzfeldt-Jakob disease. Their proteinic nature makes them less susceptible to conventional high-level disinfectants. Some microorganism types including enveloped viruses, and to some extent vegetative Gram-positive bacteria, are usually more susceptible to biocides and will be killed by quaternary ammonium compound (QAC) formulations, biguanides, antimicrobial dyes and phenolics. Enveloped viruses are particularly susceptible to membrane active agents including both biocides and detergents. Multidrug antibiotic resistant clinical isolates are not necessarily less susceptible to biocides when used at their in-use concentration, though some isolates can exhibit increased tolerance to dilute solutions of biocide, depending on the mechanism of resistance. Environmental isolates, however, can be less susceptible to biocides at their in-use concentration. Vegetative referred to bacteria that can actively divide and cause an infection as opposed to bacterial endospores which are a form of bacterial survival and are dormant (see main text). ClO_2, chlorine dioxide; IPA, isopropyl alcohol; EtOH, ethyl alcohol.

Figure 2. Mechanisms of action of disinfectants and antiseptics. The mechanisms of action of biocides depends on the main bacterial structures targeted[23,27]. On the left, major bacterial targets of biocides. On the right, the inactivation of bacterial cells by biocides is a time and concentration-dependent process which follows a series of reversible and irreversible events. Reversible events include initial release of intracellular potassium (a), which causes a depletion of membrane potential and loss of protonmotive force (PMF) necessary for ATP biosynthesis (b). This leads to an arrest of active transport (c), normal metabolic processes (d) and replication (e). Continued exposure to the biocide eventually leads to irreversible damage, including changes to cytosolic pH (f), which cascades into disruption of enzymatic function and coagulation of intracellular material (g). If the cytoplasmic membrane becomes significantly damaged, cytoplasmic constituents including proteins, nucleotides, pentoses and other ions may be lost from the cell (h). Whilst not considered a biocide, ethylenediaminetetraacetic acid (EDTA) disrupts the outer membrane of Gram-negative bacteria, potentiating biocidal effects.

QAC, quaternary ammonium compound; PAA, peracetic acid, H2O2, hydrogen peroxide; PO_4^{3-}, phosphate; K^+, potassium ion.

Figure 3. Intrinsic factors governing microbial resistance and tolerance to biocides. a) in a vegetative bacterium, the outer surface of some species may act as an impermeable barrier, preventing biocide diffusion into the cytoplasmic space. Penetration of biocides can be moderated by the density and substrate specificity of porins. In some cases, biocide–cell surface interactions are modulated by surface properties, such as charge and fatty acid composition. Pigments, including melanins and carotenoids, can quench the activity of both cationic and oxidising biocides. Biocides that reach the cytoplasmic membrane, periplasm or cytoplasm may be actively exported from the cell by efflux pumps, reducing their effective concentration. b) In the case of endospores, damage to nucleic acids can be substantially reduced by a variety of DNA protection mechanisms. c) in sessile biofilms, extracellular polymeric substances may substantially interfere with microbicidal activity, whilst metabolic changes and enhanced SOS response induction protects against insults; cell–cell communication and horizontal gene transfer are enhanced within biofilm communities. Non-specific mechanisms of resistance may confer cross-resistance to a range of antimicrobial agents, including antibiotics. SASPs, small acid-soluble proteins; VBNC, viable but non-culturable, DAP–CA^{2+}, Dipicolinic acid bound to calcium.