Potential impact of disinfectants on antimicrobial resistance development

Chief Scientist's Group report

October 2024

Project: SC220008/R

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Dr Robert Bradburne
Chief Scientist

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Executive summary

Antimicrobial resistance (AMR) is a growing global health threat. Bacterial resistance alone is estimated to have directly caused 1.27 million deaths in 2019, globally. The problem of AMR crosses the boundaries of public health into the health of animals as well as the role of the environment in AMR evolution and transmission. Therefore, a holistic 'One Health' approach is needed to tackle it. Exposure to a range of antimicrobial compounds can potentially result in selection for AMR. These include pharmaceuticals such as antibiotics and antifungals as well as plant protection products such as fungicides, and biocides such as disinfectants. The presence of chemicals in the environment as a result of anthropogenic use has the potential to influence the dissemination, selection, and transmission of AMR in the environment. To date much of the focus has been on antibiotics.

This report focusses on disinfectants and discusses their use in the UK, their pathways to and fate in the environment, and their potential for the selection of AMR.

Chemicals authorised (or in the process of being authorised) for use as disinfectant actives in the UK were identified from the lists of authorised actives managed by the Health and Safety Executive (HSE). A total of 123 active substances were identified as of November 2022. This list included a wide range of chemicals including alcohols, aldehydes, biguanides, chlorine-related compounds, and quaternary ammonium compounds (QACs). However, no usage data or sales data are wholly or routinely collected for disinfectants in the UK and thus were unavailable and thus the relative scale of use of the different compounds was not able to be determined.

Disinfectants have a variety of uses including in clinical and health care settings, agriculture, aquaculture and food production settings, household, commercial business, public and industrial settings and disinfection of water for supply and recreational use (e.g., swimming pools).

The diversity of uses of disinfectants means their potential pathways to the environment are complex and numerous including emission during manufacture, use and disposal. The diversity of sources of disinfectants and lack of usage data makes source apportionment in the environment challenging, and it is currently not possible to know which disinfectant use scenario might be of the greatest environmental concern. This is made more complex by the fact a substance may be used in a variety of use scenarios and therefore its presence in the environment could be due to a number of different pathways. The absence of disinfectant use data, and how the quantity used varies between different use scenarios, makes evidence-based identification of key pathways difficult.

An overview of the environmental fate of disinfectants used in the UK shows the variation between the different types of substances. Disinfectant fate and behaviour in the environment are determined by the physicochemical properties of the disinfectant and the characteristics of the environment (e.g., pH, and temperature) in which they are present as well as persistence (biotic and abiotic degradation).

A semi-systematic review of the literature on the development of resistance in microbes following exposure to disinfectants was undertaken for 16 active substances used in disinfectants in the UK. These chemicals were selected based on perceived potential to select for resistance and their persistence in the environment. Across the literature, many methods of determining resistance or interpreting resistance were used, although determination of the minimal inhibitory concentration (MIC) was by far the most used protocol. It was also evident that the term “resistance” is poorly defined and often misleading, with an increase in MIC as low as 2-fold reported as resistance. In addition, studies based on MIC measurements often lack clinical or practical interpretation. Where cross-resistance to antibiotics was investigated, the clinical significance of any susceptibility change was rarely addressed. The most studied mechanisms associated with a change in antimicrobial susceptibility were efflux, change in membrane permeability including change in lipid composition and alteration of porins. Although the clinical or practical impact of sub-MIC exposure to disinfectants was not always addressed, the review of the literature confirmed that bacteria respond to sub-MIC exposure to disinfectants, regardless of the disinfectant used, resulting in changes in antimicrobial susceptibility profile and gene expression.

When *in vitro* disinfectant effect concentrations were compared to measured environmental concentrations from the literature, it was evident that laboratory experiments were conducted with considerably higher concentrations of disinfectant substance than those measured in the environment. Even in cases where relatively low concentrations were studied *in vitro*, i.e., sub-MIC concentrations, these were often still higher than environmental concentrations. As such, there is great uncertainty associated with our understanding of the effect of environmentally relevant concentrations of disinfectants. This represents a significant knowledge gap in understanding the impact of environmental concentrations on development of resistance to disinfectants.

Abbreviations

Ag	Silver
AgNPs	Silver nanoparticles
BCDMH	Bromochlorodimethylhydantoin
BKC	Benzalkonium chloride
BIT	1,2-benzisothiazol-3(2H)-one
CHX	Chlorhexidine
CMIT	5-chloro-2-methyl-4-isothiazolin-3-one
Cu	Copper
CuNPs	Copper nanoparticles
$CuSO_4$	Copper sulfate
DBNAP	2,2-dibromo-2-cyanoacetamide
DCPP	5-chloro-2-(4-chlorphenoxy)phenol
DDAC	Didecyldimethylammonium chloride
MIT	2-methyl-4-isothiazolin-3-one
OPA	Orthophthalaldehyde
PAA	Peracetic acid
PHMB	Polyhexamethylene biguanides
PVI	Iodine (povidone iodine)
QACs	Quaternary ammonium compounds

Definitions

Throughout this report, there will be reference to specific terminology relating to this field of study. These have been defined below. Although there are occasional uses of “antiseptic” throughout this report, for the most part, the term disinfectant will be used to capture both disinfectants and antiseptics.

Antiseptics	Chemical/product with antimicrobial activity which is applied to living tissue [1].
Biocide	A biocide is a chemical substance, mixture, or microorganism intended to control any harmful organism in a way that is not purely physical or mechanical [2].
Disinfectants	Chemical/product with antimicrobial activity which is applied to surfaces and inanimate objects [1].
-cidal	Refers to substances that kill a target microorganism (bacteria, endospores, fungi, viruses): bactericidal, sporicidal, fungicidal, virucidal [3].
-static	Refers to substances that inhibit the growth of the target microorganism (bacteria, fungi): bacteristatic, fungistatic [3]; note the literature mentions sporistatic which refers mostly to the prevention of endospore germination.
Co-selection	Co-selection is the selection for resistance to multiple antimicrobials, and is mainly caused by cross- or co-resistance [4].
Co-resistance	Co-resistance occurs when two or more resistance genes are genetically linked (i.e., in the same genetic location), which may result in one antimicrobial selecting for multiple resistance types [5].
Cross-resistance	Cross-resistance occurs when the resistance mechanism is non-specific and confers resistance against more than one antimicrobial chemical (e.g., an efflux pump) [6].

1 Introduction

Antimicrobial resistance (AMR) is when microorganisms including bacteria, fungi, viruses and parasites, no longer respond to treatment with antimicrobial compounds such as medicines including antibiotics, or biocidal compounds such as disinfectants. AMR is a growing global health threat, with bacterial resistance alone estimated to have directly caused 1.27 million deaths worldwide in 2019 [7]. The problem of AMR is widespread and crosses the boundaries of public health into the health of animals and the role of the environment in its evolution and transmission, thus a holistic, “One Health” approach is needed to tackle it [8]. Antimicrobial resistant microorganisms have been found in many parts of the environment, including soil, surface water, groundwater, and wastewater [9]. Antibiotics and antifungals comprise only a fraction of the manmade chemicals that can select for AMR, with other chemicals including biocides such as disinfectants potentially playing an important role [9]. The release of these chemicals into the environment from anthropogenic sources has been implicated in the dissemination, selection, and transmission of AMR [10].

This report is focused primarily on active substances used in disinfectants which are a type of biocide. Principally, biocides are chemical agents that control or eliminate living organisms, including microorganisms, such as bacteria and fungi [11, 12]. Bacterial resistance to biocides has been reported since the 1950s. The development of resistance to specific biocidal products, including disinfectants, has been well-described, with novel mechanisms still emerging, such as alteration of metabolic pathways [13]. Some genetic and phenotypic evidence indicates that prudent use of disinfectants may be needed to preserve their efficacy and limit the emergence and dissemination of resistant bacteria [13].

The presence of disinfectants can co-select for resistance to other antimicrobials (e.g., antibiotic resistance) within microorganisms [10]. Co-selection can confer resistance against antimicrobials in two ways: cross-resistance and co-resistance. Cross-resistance occurs when the microbial resistance mechanism is non-specific and confers resistance against more than one chemical/chemical compound (e.g., expression and over-expression of efflux pumps that can expel both antibiotics and disinfectants from the cell or can cause changes to cell permeability) [13]. Co-resistance occurs when two or more resistance genes are genetically linked (i.e., in the same genetic location). For example, genes encoding disinfectant resistance and antibiotic resistance can be found on the same plasmid or genetic element (e.g., quaternary ammonium compound (QAC) resistance genes on class 1 integrons) [14]. There is now a body of evidence describing cross-resistance mechanisms to disinfectants and antibiotics, and the role of disinfectant exposure in co-resistance (i.e., selection of antibiotic resistance genes (ARGs)). The development of cross-resistance from exposure to disinfectants is not universal and may depend on the bacterial genus and species as well as the antimicrobial agent. Further information on resistance to disinfectants and co-selection for AMR by disinfectants is covered in Section 6.

There is *in vitro* evidence showing that bacterial mechanisms conferring a decreased susceptibility (i.e., resistance), often defined by an increase in minimum inhibitory concentration (MIC) to a disinfectant lead to resistance to antibiotics [13]. However, there is

now a greater focus on the inhibitory/sub-inhibitory effects of biocides, including disinfectants, as it has been shown that at low concentrations these can lead to the expression of multiple resistance mechanisms (including both biocide and antibiotic resistance) in bacteria [15].

One of the most critical uses of biocides is as disinfectants, which is the focus of this report. Disinfectants are defined by the European Chemicals Agency (ECHA) [16] as "a product that reduces the number of microorganisms in or on […a] matrix – achieved by the irreversible action of a product, to a level judged to be appropriate for a defined purpose". Disinfectants are one of four main biocidal product groups under regulation in the UK with a wide range of uses, for example, for disinfecting hard surfaces, equipment, clothing, and water, in domestic, agricultural, and industrial settings. As a result, they enter the environment via various pathways [9].

The presence of resistance-driving chemicals (including disinfectants) in the environment, may contribute to the persistence of and selection for AMR in rivers, lakes, sediment, coastal environments, soils, and in the microbiomes of wild organisms. Although literature on the extent of transfer of resistance from environmental bacteria to clinical pathogens is limited [17, 18], many studies regard the environment as a source of AMR, clinically relevant or otherwise [19, 20]. The transmission of AMR from the environment to humans who interact with it has been evidenced in multiple studies [21], highlighting the need for further research into the drivers of AMR evolution and transmission in the environment.

The UK Government has published a National Action Plan for AMR titled "Tackling Antimicrobial Resistance 2019–2024" [22]. Although the majority of the AMR National Action Plan focuses on animals and humans and the use of antimicrobials in livestock and clinical settings, the consideration of AMR in the environment is acknowledged and forms part of the Government's proposed ambitions to tackle AMR .The findings from this report will elucidate the role of disinfectants in AMR as a result of their use in the UK and their presence in the environment. Specifically, this report aims to:

- Identify and discuss the active substances authorised for use as disinfectants in the UK, and their potential use scenarios,
- Discuss how these substances can enter the environment and their fate within different environmental matrices,
- Provide an overview of the mechanisms of action of disinfectants against microorganisms,
- Describe the development of microbial resistance to disinfectants and the co-selection of AMR from exposure to disinfectants,
- Compare the selective concentrations of disinfectants within a clinical setting to concentrations present in the environment, indirectly assessing the risk posed by disinfectants in the environment to the development of AMR, and
- Make recommendations and conclusions, which will include any identified evidence gaps and research needs.

2 Overview of mechanisms of action of disinfectants

2.1 General introduction

Active substances (i.e., the substance responsible for the biocidal activity of the product) are formulated into products for use as disinfectants including antiseptics. These active substances are often broad-spectrum and non-specific against microbes, particularly at high-use concentrations. In addition, formulation excipients have an impact on the microbicidal efficacy of the products, and also on the mechanisms of action of the active substance. Notably, studies on microbicidal efficacy are common, yet studies to elucidate the mechanisms of this efficacy are rare.

Broadly, an active substance needs to be in contact with microbial cells to exert its microbiocidal activity. The number and type of targets affected in the cell, and the severity of damage imparted to the target will dictate whether an active substance has a “cidal” or “static” activity. The mechanism of action of disinfectants is often described in terms of the predominant targets in bacterial cells (e.g., membrane active, cytoplasm active or cell wall active). They can also impact membranes and capsid for viruses, targeting cytoplasm for fungi and protozoa and nucleic acid damage against all three.

The damage imparted to a bacterial cell from interaction with a disinfectant is initially reversible but becomes irreversible following longer exposure time or higher use concentrations [23] as a result of damage triggering cell death (Figure 1). An exception to this, are the peroxygens, which continuously damage cell cytoplasm after initial exposure. Although physical damage to the lipid membrane from peroxygens might be limited, they exert their microbiocidal activity by damaging membrane enzymes, cytoplasm components, and nucleic acid. The same principle of reversible/irreversible interactions applies to other microorganisms, although viruses should be considered separately. With fungi and protozoa, damage to the cytoplasmic membrane will also contribute to a change in the pH of the cytosol (pHi) and subsequently cell death. Maintenance of pHi is essential for cell processes (e.g., enzymatic function and proton motive force).

The interaction of a disinfectant with microbial cells depends largely on the physicochemical characteristics of the disinfectant (e.g., ionization constant and lipid solubility). Together with the diverse chemical structures in microbial cells, this can make predicting the precise mechanism leading to a “cidal” activity difficult. Some biocides are known to be highly reactive such as alkylating and oxidising agents, and these will interact strongly with microbial cell structure. Positively charged disinfectants such as QACs, biguanides, and antimicrobial dyes will interact with the negative charges of sugar residues on the microbial cell surface or phosphate groups on the membrane. Disinfectants with long alkyl chains (e.g., QACs) can integrate with the hydrophobic region of phospholipid molecules in the microbial cell membrane, resulting in membrane disruption (i.e., membrane active agents). Other disinfectants, by their weakly acidic or/and lipid solubility properties (i.e., uncoupling

agents), will lead to disruption of the proton motive force resulting in the failure of energy-dependent processes, but also acidification of the pHi affecting cytoplasmic enzyme functions [24, 25].

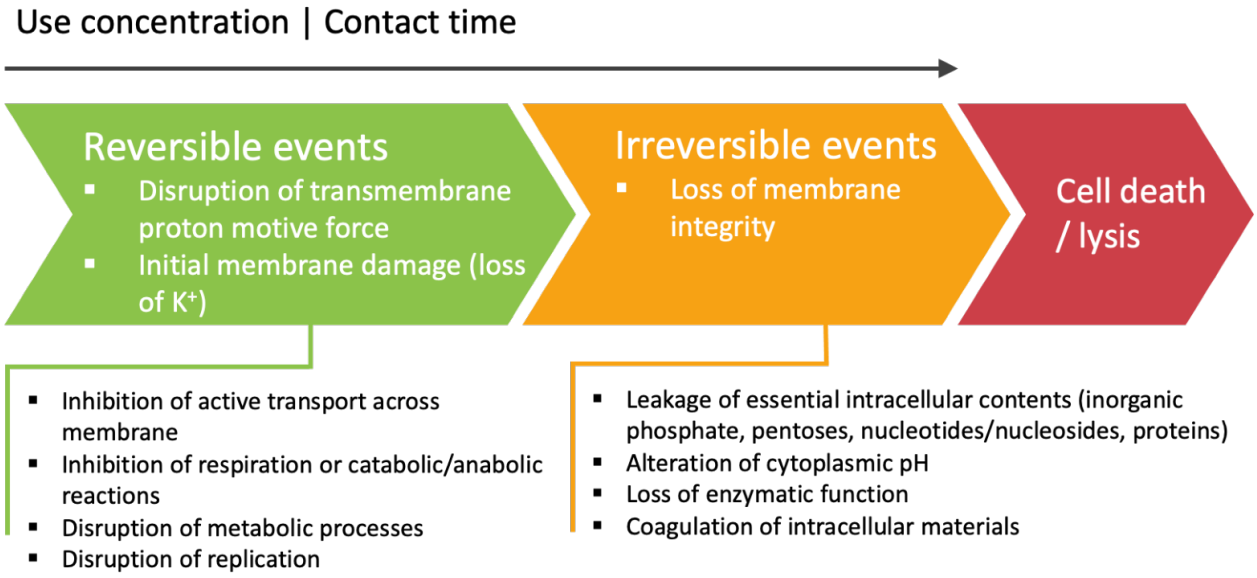

Figure 1 Interactions between disinfectants and bacteria.

The initial interaction of a disinfectant with the target microbial cell is an important determinant of efficacy and can be measured with uptake isotherms [26], which provide information on the nature and strength of the interaction [27].

2.2 Mechanisms of action against bacteria

The different structures between the type of bacteria (e.g., Gram-positive/-negative, Mycobacteria) will impact bacterial susceptibility to a disinfectant (see Section 6.3.1). There are multiple key mechanisms of bactericidal action from disinfectants (Figure 2). A disinfectant will interact with the outer cell layers first, before penetrating deeper within the cell, reaching targets within the cytoplasm. Diffusion within cells can be facilitated by structures such as porins. Penetration is often associated with damage to the outer cell layer. This holds true for most disinfectants. Alkylating agents, however, exert their bactericidal activity by crosslinking proteins within the outer layer of the bacterial cell, limiting their penetration within the cell. The ability to create cross-linkages depends on the alkylating agent. For example, glutaraldehyde produces more cross-links than orthophthalaldehyde (OPA), which allows OPA to penetrate deeper within the bacterial cell structure, and is generally associated with a faster efficacy [24, 25].

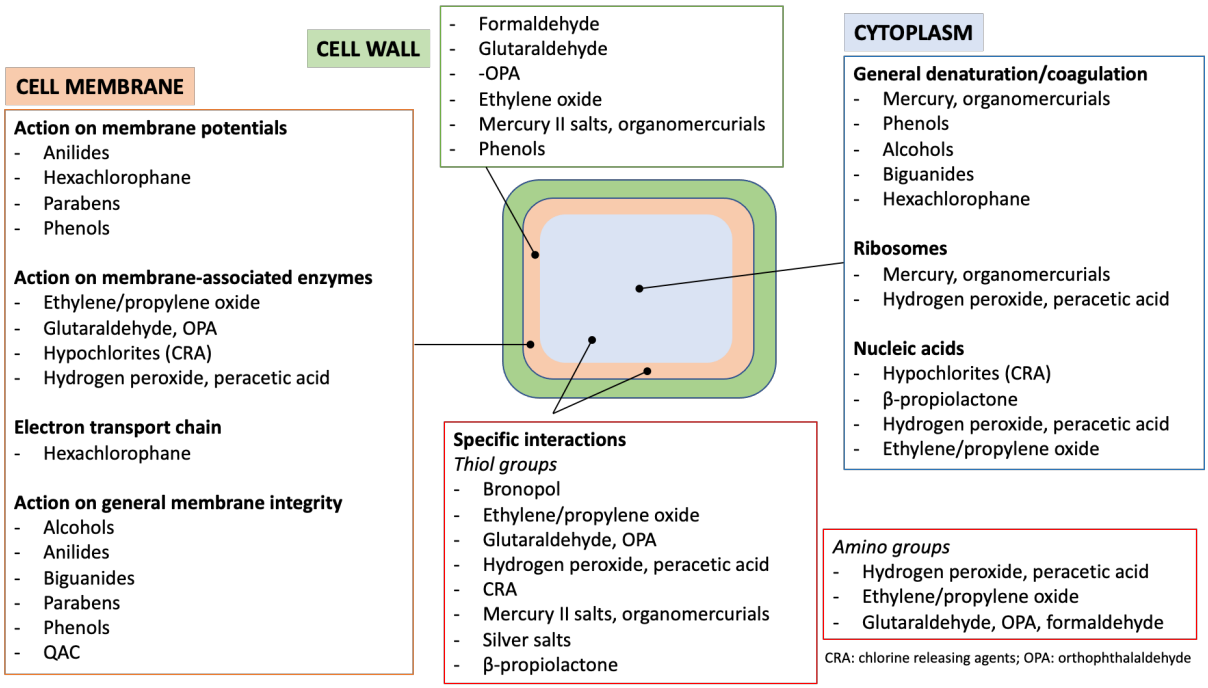

Figure 2 Mechanisms of action of biocides against bacterial cells.

2.3 Mechanisms of action against viruses

There are fewer studies of the mechanisms of virucidal action of disinfectants than those targeting bacteria. Generally, viruses are classed as enveloped and non-enveloped. Enveloped viruses are the most susceptible of all microorganisms to disinfectants (see Figure 7). Disinfectants impact viral viability or infectivity as shown in Figure 3. Interaction or destruction of viral-host receptors, destruction of the lipid envelope or damage to the viral nucleic acid can result in a loss of infectivity, whilst the viral structure and its genome remain intact [28]. Although in some cases (e.g., herpes virus, poxviruses), the genome itself is infectious (Figure 3).

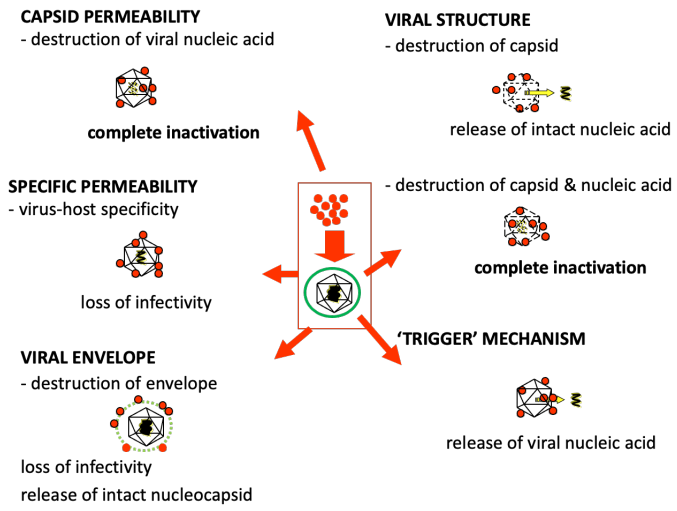

Figure 3 Impacts of disinfectants on enveloped and non-enveloped viruses.

Damage to the viral capsid (for non-enveloped viruses) is key for the virucidal activity of disinfectants and some have been shown to permeate and weaken the viral capsid, enabling penetration and damage to proteins and nucleic acids [29]. Disinfectant interactions with specific viral targets are summarised in Figure 4. For some disinfectants, the interaction has been indirectly shown [30], but no direct evidence is available.

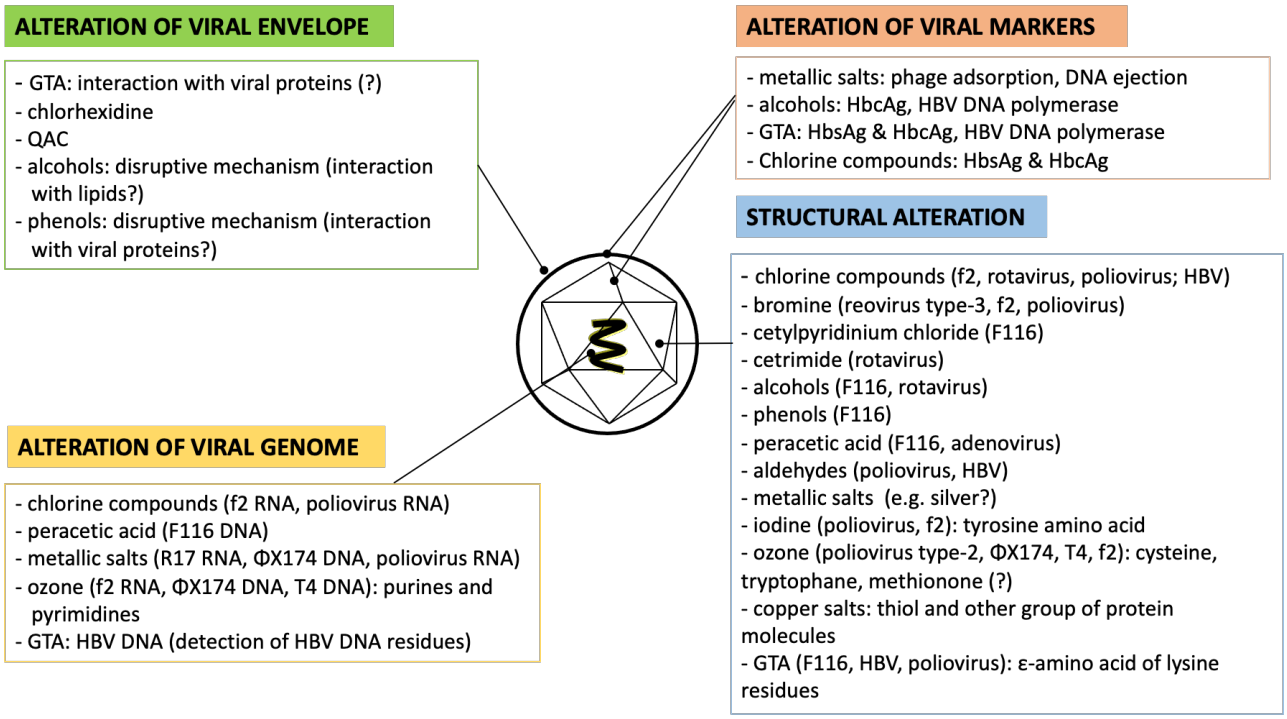

Figure 4 Interactions between disinfectants and viral particles. Some interactions have not been directly identified. GTA: glutaraldehyde; QACs: quaternary ammonium compounds; f2,F116, phi174; T4: bacteriophages; R17: rotavirus; HBV: hepatitis B virus; HBsAg: Hepatitis B virus surface antigen; HBcAg: Hepatitis B virus core antigens.

2.4 Mechanisms of action against fungi

Fungi are microorganisms consisting of moulds and yeasts. Generally, there is little information on the mechanisms of action of disinfectants against them. It is assumed that disinfectants will interact with multiple targets within fungal cells [31]. The mechanisms of action of different chemical disinfectants are thought to be similar to those used against bacteria; for example, membrane active agents will target the cytoplasmic membrane. Some disinfectants can impact fungal metabolic processes (e.g., isothiazolones), resulting in metabolic disruption of key enzymes such as dehydrogenases, affecting critical physiological functions including growth, respiration and energy generation, leading to cell inhibition and cell death [31].

2.5 Mechanisms of action against protozoa

The term protozoa includes a wide variety of unicellular eukaryotic phylogenetically distant species. Most protozoa are found “free-living” in water ecosystems, but some pathogenic species are closely associated with their host in a parasitic lifestyle. The control of free-living amoeba (FLA) with disinfection has been particularly studied [32]. In terms of disinfectant susceptibility, the metabolically active dividing form, the trophozoites, need to be distinguished from the metabolically inactive resistant endocytic form. There have been many publications on the efficacy of disinfectants on diverse FLA [32]. Yet, information on trophocidal mechanism(s) of action remains very limited. It is understood that membrane active agents such as chlorhexidine (CHX) and polyhexamethylene biguanides (PHMBs) will damage the cytoplasmic cell membrane, similarly to the effect of these agents against bacterial cells. For example, both biguanides are associated with pentose leakage in *Acanthamoeba castellanii* [33]. Research suggests that the trophocidal activity results from disinfectant interactions with multiple non-specific targets in the cell [31, 32]. On occasion, more specific damage has been reported. For example, diamidines have been associated with inhibition of S-adenosylmethionine decarboxylase and shown to affect mitochondria and nucleic acid in *Acanthamoeba spp.* [32, 34].

3 Disinfectant usage in the UK

Bioactive chemicals have been used as disinfectants for over a century [35] and are important for the protection of human and animal health from harmful pathogens [36]. This section of the report aims to identify the active substances currently authorised for use in disinfectants in the UK and discusses their potential use scenarios. Determining the usage of disinfectants, including use scenarios and usage volumes, is integral to understanding the wider implications of their use on AMR in the environment.

3.1 Legislation relating to disinfectants

The authorisation and the use of biocidal active substances, such as disinfectants, in Great Britain (GB) (i.e., England, Scotland, and Wales) is regulated under the GB Biocidal Products Regulation (GB BPR) [37]. The GB BPR was translated and amended from the European Union (EU) Biocidal Products Regulation (528/2012) (EU BPR) [38] after the UK left the EU in January 2021. Biocidal regulation in Northern Ireland (NI) is still regulated under the EU BPR.

Disinfectant products are mostly a mixture of active substances but can also be solely composed of a single active substance. The active substance is the component of the product that has a harmful effect on the target organism(s). Active substances can be supplied/sold in solution, in powders, and impregnated into articles such as wipes, depending on their type and intended use. Active substances that are authorised or are in progress for authorisation in GB can be found on the "GB List of Biocidal Active Substances" sheet in the "BPR active substance lists for GB and NI" database on the Health and Safety Executive (HSE) website [39].

For a biocidal product to be made available on the market and used in GB, the active substances within it must be authorised for use in every product type (PT) category applicable to the biocidal product. The GB BPR follows the ECHA classifications of biocidal products, which come under four main groups: 1) disinfectants, 2) preservatives, 3) pest-control products, and 4) other biocidal products (e.g., antifouling and embalming products) [41]. The focus of this report is on disinfectants, which come under biocidal product group 1. Within the disinfectants group, there are five PT categories:

- Disinfectants used in human hygiene (PT01).
- Disinfectants and algaecides not intended for direct application to humans or animals (PT02).
- Disinfectants used in veterinary hygiene (PT03).
- Disinfectants used in food and feed area (PT04).
- Disinfectants used in drinking water (PT05).

3.2 Disinfectants used in the UK

Information on the disinfectant active substances authorised for use in the UK was collated. This included consideration of all five product types within the disinfectants groups as noted in Section 3.1. To cover all parts of the UK, both the Health and Safety Executive (HSE) and ECHA (Europe) databases (Table 1) were used. This was also necessary as exit from the EU has led to UK-based documents representing a “work in progress” view of authorised disinfectants, with many biocidal products awaiting transfer from EU to UK legislation.

Databases were screened to include only disinfectant PTs 01-05 and active substances/products currently authorised or in the process of authorisation. Active substances/products that were not approved at this time or where approval had expired were not included (see Table 1 for all inclusions and exclusions). Following this screening, active substances included in all five databases were combined and duplicate entries removed to produce a master database (see Figure 5 for the searching, screening and refining process used). The authorised PTs of active substances were also recorded.

In total, 123 active substances authorised or in the process of authorisation for use in disinfectants under PTs 01-05 were identified (Appendix 1, Table S1). Of the 123 active substances, 34 were noted as authorised, with the remaining being in the approval process. The majority of active substances were authorised/in process of authorisation for use in PT02 (104 active substances), followed by PT04 (71 active substances), PT03 (52 active substances), PT05 (28 active substances), and PT01 (26 active substances) (Appendix 1, Table S1). Most active substances were authorised/in process of authorisation for use in multiple product types, with only 45 active substances relating to use in a single product type (Appendix 1, Table S1). Generally, the authorisations found in the HSE and ECHA databases were aligned, with only 15 active substances having a difference in their authorisation status between the two authorising agencies, eight of which were only authorised/in process of authorisation under HSE and not ECHA, and four of which under ECHA and not HSE (Appendix 1, Table S1).

Table 1 Databases and filtering criteria used to identify the active substances authorised/in the process of authorisation for use in disinfectant product types in the UK.

Database	Date last updated	Date data extracted	Filtering inclusions	Filtering exclusions	Weblink
HSE BPR active substance lists for GB and NI; GB Biocidal Active Substances sheet	4th Oct 2022	14th Nov 2022	• Product types: 01-05. • Approval/Assessment status: "Approved", "Open invitation", "Under assessment", "Under review".	• Product types: 06-22, "Blanks". • Approval/Assessment status: "Application withdrawn", "No longer supported", "Not approved", "Notified".	www.hse.gov.uk/biocides/uk-list-active-substances.htm
HSE UK authorised biocidal products; GB Authorised Biocidal Products sheet	3rd Oct 2022	14th Nov 2022	• Product types: 01-05. • Authorisation status: "Current", "Blanks".	• Product types: 06-22, "Blanks". • Authorisation status: "Cancelled", "Expired".	www.hse.gov.uk/biocides/uk-authorised-biocidal-products.htm
ECHA Biocidal Active Substances	8th Nov 2022	14th Nov 2022	• Product types: 01-05. • Approval status: "Approved", "Blanks".	• Product types: 06-22, "Blanks". • Approval status: "Cancelled application", "No longer supported", "Not approved".	www.echa.europa.eu/information-on-chemicals/biocidal-active-substances
ECHA Biocidal Products	11th Nov 2022	14th Nov 2022	• Product types: 01-05. • Authorisation status: "Authorised".	• Product types: 06-22. • Authorisation status: "Expired", "Cancelled".	www.echa.europa.eu/information-on-chemicals/biocidal-products
ECHA Article 95 list (List of active substances and suppliers)	28th October 2022	14th Nov 2022	• Product types: 01-05.	None	www.echa.europa.eu/information-on-chemicals/active-substance-suppliers

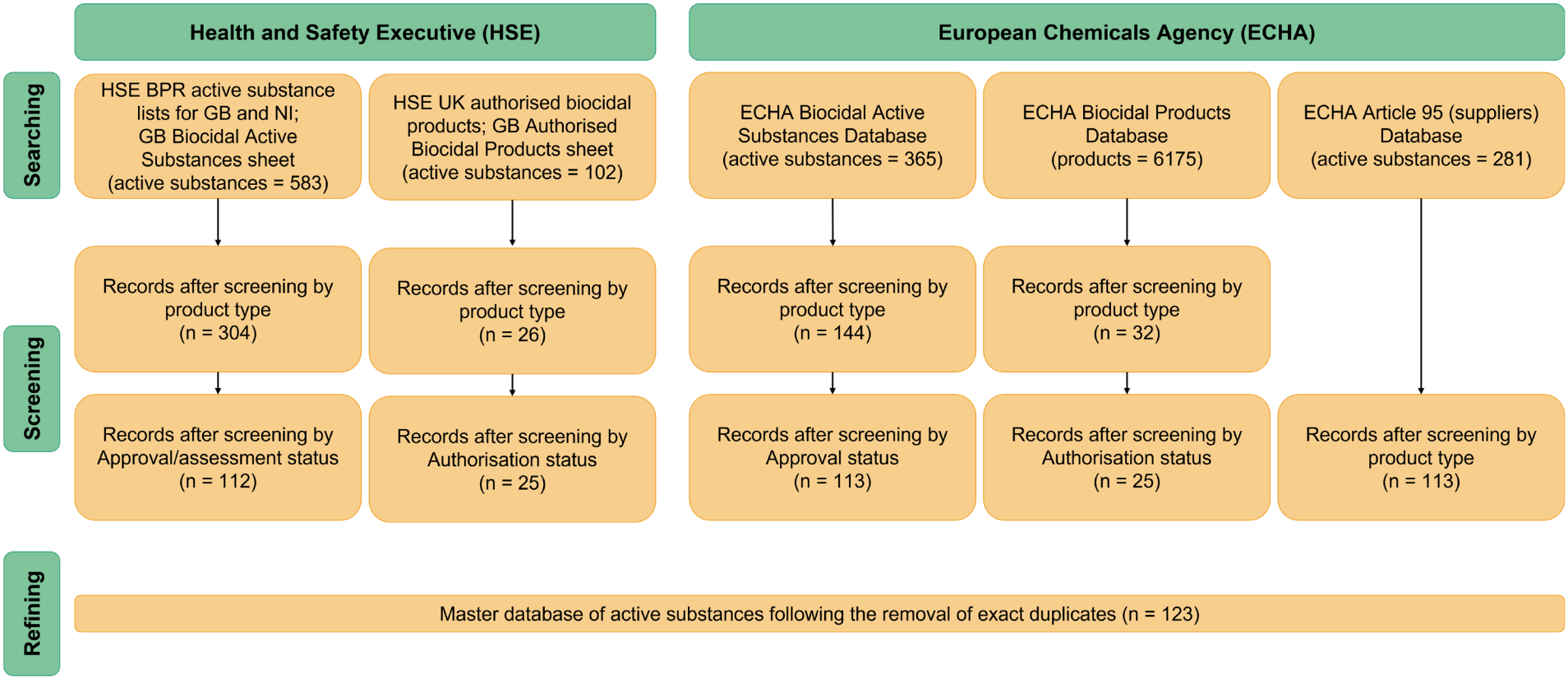

Figure 5 Details of the searching, screening, and refining process to identify active substances used in the UK.

3.3 Scale of use of disinfectants in the UK

To investigate the scale of use of disinfectants in the UK, we contacted relevant parties, including representatives at HSE and academics that may collect or know of relevant data on disinfectant usage. In addition, we compiled a list of trade associations (Table 3), which was informed by advice from a representative at HSE and from internet searches. Internet and literature searches were also carried out to identify any usage data that might be relevant in a UK context.

Table 2 The list of trade associations contacted for data on disinfectant usage in the UK.

Trade association
British Association of Chemicals Specialities (BACS)
British Coatings Federation (BCF)
British Institute of Embalmers (BIE)
British Pest Control Association (BPCA)
British Plastics Federation (BPR)
British Retail Consortium (BRC)
Chemical Hazards Communication Society (CHCS)
Chemicals Business Association (CBA)
Chemicals Industry Association (CIA)
Cosmetics and Toiletry Products Association (CPTA)
European Apparel and Textile Confederation (EURATEX)
European Chemical Industry Council (CEFIC)
European Council of the Paint, Printing Ink, and Artist's Colours Industry (CEPE)
International Association for Soaps, Detergents and Maintenance Products (AISE)
UK Cleaning Products Industry Association (UKCPI)
Water Management Society (WMSoc)

The experts contacted suggested that data on disinfectant usage or sales in the UK are not readily collected or available. Disinfectant usage data are not routinely captured by the BPR and are thus not collected centrally by HSE (HSE, pers. comm.). These data are also not collated by ECHA. This is unlike other antimicrobial agents, such as antibiotics, for which prescribing data (collected by the National Health Service Business Services Authority) [42] and veterinary sales data (collected by the Veterinary Medicines Directorate) [43] are recorded. Occasionally, specific products may have sales data submitted during authorisation, but this is not widespread practice (HSE, pers. comm.).

Of the 16 trade associations we contacted for more information, seven replied. Generally, responses confirmed that they did not collect or hold these kinds of data. Some suggested other options for data retrieval, such as purchasing this information

from market research companies or approaching blenders, manufacturers or suppliers of either the active substances or the finished products.

The results of non-systematic internet and literature searches also confirmed that these data do not wholly exist for the UK. However, the searches revealed that the Scottish Environment Protection Agency (SEPA) require reporting of disinfectants used (by volume) in aquaculture practices in Scotland [44], e.g., formalin usage [45].

The collection of usage data is critical to understand the scale of use of disinfectants in the UK. If available, modelling could be undertaken to investigate use scenarios and potential pathways of disinfectants to the environment. This would enhance our understanding of the risk of contamination of natural environments posed by disinfectants.

3.4 Disinfectant use scenarios in the UK

Disinfectants within PTs 01-05 are used in a variety of everyday settings, including industry, food processing, hospital and care facilities, household settings, livestock rearing, veterinary facilities, and animal housing (e.g., kennels) [12]. Some events such as disease outbreaks can result in an increase in disinfectant use, for example, action taken in response to notifiable diseases in animals (e.g., avian influenza and foot and mouth), and during pandemics [46]. This section will explore the common use scenarios. Where substances are also authorised for use for product types outside those covered by disinfectants (i.e., PTs 01 – 05) discussion of use scenarios will be limited to those falling under PTs 01-05. For example, benzalkonium chloride (BKC) can be used to disinfect surgical instruments (PT01) but it is also authorised under non-disinfectant PTs including as a wood preservative (PT8).

3.4.1 Disinfectants in clinical and healthcare settings

The use of disinfectants in human and animal health settings, such as hospitals, care facilities and veterinary practices, is essential for pathogen and infection control and prevention [47]. Active substances used in these settings often fall under PTs 01 (human hygiene), 02 (disinfections/algaecides not for direct application on humans and animals), and 03 (veterinary hygiene). Disinfectants are used in a variety of ways in these settings. Common use scenarios include disinfection of:

- Frequent contact surfaces, such as handrails and door handles;
- Walls, floors and other hard surfaces;
- Medical equipment (e.g., machines, instruments and patient care devices);
- Implants and invasive devices;
- Clothing and personal protective equipment (PPE);
- Food preparation areas;
- Vehicles (e.g., ambulances);

- Bathrooms and toilet facilities; and
- Human or animal skin and wounds.

The disinfectants used in these scenarios may be general disinfectants, however, some will be use-specific, targeting particular pathogenic microorganisms (e.g., those known to be effective against *Mycobacterium tuberculosis*). Examples of active substances regulated as disinfectants that are commonly used in clinical settings include (but are not limited to) QACs, aldehydes, biguanides, chlorine-releasing agents and peroxygens. For example, walls, floors and other hard surfaces can be disinfected using sodium hypochlorite (commonly used to disinfect surfaces, toilets, and blood spillages [3]), hydrogen peroxide vapour or ozone (whole room disinfecting [35]), and BKC [36]. Skin can also be disinfected using BKC, as well as chlorhexidine gluconate, which is often used in antiseptic handwashing products and oral care [3], and iodine (povidone-iodine (PVP-I), which is used to disinfect skin surfaces before and after operations [48]). Surgical instruments and patient care devices in hospitals, veterinary clinics and other healthcare facilities need a high level of disinfection, and can be disinfected with alkylating agents, such as aldehydes, for example, glutaraldehyde, which can be used to disinfect endoscopes [49], or oxidising agents, such as peracetic acid (PAA), which can be used to disinfect scopes and hemodialysers [3].

3.4.2 Disinfectants in agriculture, aquaculture, and food production

Disinfectants are routinely used in agriculture and food processing. In agriculture, disinfection plays an important and necessary role in biosecurity, limiting the spread of pathogens and infectious diseases, and ensuring animal welfare, food safety and security, and income generation for farmers. In food processing facilities, disinfection is necessary for food safety and to ensure the quality of food reaching the consumer. Active substances used in these settings often fall under PTs 02 (disinfections/algaecides not for direct application on humans and animals), 03 (veterinary hygiene), and 04 (disinfectants used in food and feed area). The range of ways that disinfectants are used include:

- Animal treatments to prevent disease (e.g., teat dips, footbaths, fish egg disinfection);
- Walls, floors and hard surfaces (e.g., animal housing, crop greenhouses, food processing environments);
- Human skin (e.g., handwashing);
- Vehicles and machinery;
- Equipment and clothing; and
- Waste (e.g., contaminated manure [50]).

The disinfectant used in these applications is often determined by environmental factors (for example, some may only be effective work at certain temperatures) and

the receiving matrix (e.g., no prior cleaning of organic matter before use can impact the efficacy of some disinfectants) [51]. Other disinfectants may also be used to target specific diseases and pathogens, for example, Department for Environment, Food & Rural Affairs has approved disinfectants for use against foot and mouth and avian influenza [52]. Generally, the commonly used disinfectants on farms include QACs, phenols, chlorine-releasing agents, aldehydes and peroxygens [51, 53, 54]. Other popular compounds for disinfection can include those containing metals, such as zinc and copper sulphate [55]).

In the case of livestock-rearing and other animal husbandry (e.g., pigs, horses, alpacas, fish, etc.), disinfectants are used for general disinfection, for example, regular handwashing (e.g., BKC), surface disinfection (e.g., BKC chlorhexidine gluconate, etc.), premises, animal housing, abattoir and net disinfection by mopping, spraying and fumigating (e.g., BKC, didecyldimethylammonium chloride (DDAC), PHMBs, formaldehyde foam products, etc.), equipment disinfection by dipping, spraying and wiping (e.g., PAA, BKC, sodium hydroxide, sodium hypochlorite etc.), vehicle and transportation-related disinfection, including cages and crates (e.g., chlorhexidine gluconate, iodophors/iodine, etc.), and clothing and boots disinfection (e.g., QACs) [54, 56, 57]. In the UK dairy industry, disinfectants are commonly used to disinfect milking equipment, teats, and storage tanks (e.g., hypochlorites and PAA [58, 59]), to prevent mastitis and reduce microbial contamination of milk. However, disinfectants are also commonly used for disease and pathogen prevention directly on animals, either regularly or in response to disease outbreaks. For example, for prevention and treatment of foot diseases, dairy cows often walk through footbaths, which frequently contain formalin (an aqueous formaldehyde solution) and copper sulphate, but can also contain PAA, sodium hypochlorite and sodium chloride [55]. Some reports have suggested that around half of UK dairy farmers use formalin footbaths on a weekly basis, with lactating cows walking through footbaths twice a day [55]. Aquaculture practices can see disinfectants released directly into tanks and water. For example, hydrogen peroxide is authorised for veterinary use in aquaculture in the UK and can be released directly into water to prevent parasitic infections in Atlantic salmon [60] or iodine can be used to disinfect fish eggs in the UK [61]. The disinfectants bronopol and formalin are also used in aquaculture in the UK, particularly in Atlantic salmon, to prevent saprolegniosis (caused by *Saprolegnia* fungi) [45].

Similar disinfectant use scenarios as those used in animal husbandry are also seen in crop and other plant production (e.g., ornamental plants). Disinfectants used in these processes can also be used as a biosecurity measure, to prevent the spread of plant pathogens from plant to plant, farm to farm, or farm to next destination (e.g., factories, packaging, markets, shops and garden centres). In parallel with animal-rearing, application can be as sprays, dips or fog treatments, including for disinfecting of vehicles and transport mechanisms (e.g., tractors), processing equipment (e.g., harvesters), clothing (e.g., boots), and housing (e.g., greenhouses and production beds/trays). For example, disinfectant treatment of surfaces such as walkways, trays

and floormats can reduce viral contamination with the cucumber green mottle mosaic virus, which is a highly contagious threat to greenhouse cucumber crops (first described in 1935 in the UK) [62]. Common disinfectants used in the UK include chlorhexidine gluconate, various QACs (e.g., BKC), active chlorines (e.g., sodium hypochlorite), organic acids (e.g., benzoic acid), peroxides (e.g., PAA), peroxygens, alcohols and aldehydes (e.g., glutaraldehyde) [63].

Disinfectant usage continues into food processing. All levels of food processing (i.e., primary (e.g., grading, packaging), secondary (e.g., baking, canning, fermenting), and tertiary (e.g., creating ready-to-eat food, frozen food, and sauces) require cleaning and disinfection protocols. Disinfection in downstream food processing can include the disinfection of factory surfaces, walls and floors, disinfection of equipment, machinery and pipes, disinfection of product transport, disinfecting of clothing and PPE, and even food surfaces. Many of the disinfectants used in food processing mirror those used in food production (e.g., QACs, aldehydes, peroxygens), as they target similar or identical food-based pathogens (e.g., risk of *Salmonella spp.* occurring in poultry and livestock production, but also in milk and chocolate processing) [64]. Other examples include chlorine/chlorine-releasing agents (often as hypochlorous acid and sodium hypochlorite), which can be used to disinfect food-contact surfaces, wash water, and in some cases, directly on to food surfaces [65, 66]. However, the use of chlorine may be hazardous, as it can combine with other chemicals and compounds to produce toxic disinfection by-products (DBPs), such as chloroform and chloramines [67, 68]. Another disinfectant used in food processing is ozone. Similar to clinical settings, some factories in the UK use a gaseous ozone treatment to disinfect hard surfaces [64]. Ozone can also be used in these settings to disinfect equipment and water [67].

3.4.3 Disinfectants in household, commercial business, public, and industrial settings

The use of disinfectants in household, commercial, public and industry settings is essential for public health for effective infectious disease control and food hygiene. Disinfectant practices used in these settings are usually undertaken on hard surfaces, in food preparation areas, and bathrooms. Actives used in these settings will generally fall under PTs 01 (human hygiene), 02 (disinfections/algaecides not for direct application on humans and animals), and 04 (disinfectants used in food and feed area).

Consumer products used for household disinfection commonly include actives such as QACs, chlorine/chlorine-releasing agents, and biguanides [69]. These products are often purchased from shops and supermarkets, and can include wipes, sprays, concentrates, foams, powders and tablets [12, 36]. QACs in particular, can be formulated into sprays and wipes as they do not require post-use rinsing [36](70)(71) (Disinfectant products used in households can be general disinfectants that can be used for a range of purposes or have more specific uses, for example, in laundry

detergents, dishwashing products [72][12] . For example, chlorhexidine digluconate is a common active ingredient in daily-use disinfectant mouthwashes, such as Corsodyl [73].

Disinfectants are used in private business premises (e.g., offices and factories), hospitality premises (e.g., hotels and restaurants), leisure centres and sports venues (e.g., gyms, swimming pools, and stadiums), public spaces (e.g., parks and outdoor areas), public transport (e.g., buses and trains), retail premises (e.g., shops and supermarkets), places of learning (e.g., schools and universities) and places of worship. The formulas of products used in commercial and public spaces are often very similar to household consumer products [12]. However, these products are likely acquired in bulk from commercial manufacturers or stockists. Generally, the disinfection protocols in these settings will involve the disinfection of:

- Frequent contact hard surfaces, such as handrails, door handles, and buttons on machines;
- Walls, floors and hard surfaces, such as countertops;
- Upholstery, such as carpets, beds and seats;
- Food preparation areas;
- Freight and vehicles; and
- Bathrooms.

However, these are use scenarios of disinfection in these settings that are likely to be targeted. For example, in the UK hospitality industry, draught beer lines are disinfected with line-cleaning solution, which can include sodium hypochlorite [74], and tap nozzles disinfected with a range of techniques, including soaking in ozonated water, or using sanitising tablets containing troclosene sodium [75].

During the coronavirus (COVID-19) pandemic caused by the SARS-CoV-2 virus, disinfection practices in commercial and public settings increased, with many businesses, councils and other public-facing bodies globally producing strict cleaning and disinfection guidelines, often following the World Health Organisation (WHO) [76] or national guidance (e.g., from UK Health Security Agency (UKHSA) [77], or the Centre for Disease Control and Prevention (CDC) [78]). Some of this enhanced disinfection guidance included using chlorine-based disinfectants (e.g., WHO and UKHSA guidance), such as sodium hypochlorite.

3.4.4 Disinfection of water

The disinfection of water is vital for public health and many of the processes necessary for everyday life. For example, not only is disinfection of water necessary for drinking, but also for maintaining machinery in manufacturing, including in food and beverages, horticulture, maintaining water systems in healthcare, hospitality and businesses, and for the safe disposal of wastewater. Active substances used in these settings fall under

PTs 02 (disinfections/algaecides not for direct application on humans and animals) and 05 (disinfectants used in drinking water). Disinfection of water is context dependent, with common practices including chlorination, UV treatment, ozone treatment, and the use of chlorine dioxide or sodium hypochlorite [79].

In the UK, water companies are required to meet drinking water quality standards as set out in the Water Supply (Water Quality) Regulations (2016)[80]. These standards include maintaining safe levels of microorganism growth and chemicals, and eradicating pathogenic microorganisms such as *Escherichia coli* and *Pseudomonas aeruginosa* [80]. The choice of disinfection treatment is not specified in water treatment regulations [81], with UV treatment and chlorination being the most widely used practices in the UK [82]. Disinfection with chlorine can create toxic DBPs, therefore some UK water companies also use ozone treatment [83]. Ozone is an effective disinfectant in aqueous forms, and due to its relative instability in water, does not leave long-lasting odours or disinfecting residuals [84]. However, as a result of the unstable nature of ozone, chlorine is used for large-scale disinfection, including water storage and distribution.

Disinfection in the context of wastewater includes the disinfection of municipal wastewater, chemical toilets [85] and industrial wastewater (e.g., food and manufacturing industry waste [86]). Disinfection of municipal wastewater in the UK can occur during tertiary treatment at a wastewater treatment plant (WWTP), particularly if the effluent is entering “sensitive areas” such as bathing waters and eutrophic water bodies. This commonly includes UV treatment [87-89]. However, the use of disinfectants such as ozone is being trialled for the first time in the UK by Severn Trent [90].

In the sports and leisure industry, and in domestic settings, swimming pools, hot tubs and spas waters are often disinfected using chlorination. Popular disinfectant regimes use chlorine (in the form of gaseous chlorine, sodium hypochlorite or stabilised chlorine) to reduce the microbial load in the water and prevent public health issues[91]. However, the use of chlorine-based compounds can have undesired consequences, such as the release of DBPs, as previously discussed [92].

4 Disinfectant pathways to the environment

Due to the wide range of uses and sources of disinfectants, there are many potential pathways by which they could reach the environment. Household, clinical, agricultural and industrial applications of disinfectants can reach the environment through waste water treatment plants (WWTPs), and may also enter the environment via landfill leachate, urban runoff, septic tank leakage, and aquaculture [9]. Additional agricultural uses of disinfectants can lead to residues entering the environment via for example slurry/manure storage leachate following treatment of agricultural buildings (Figure 6). Disinfectants have been detected in a range of environmental compartments, including sediment, groundwater, surface freshwater, coastal waters, and soil [93, 94]. This section gives a holistic overview of common pathways for disinfectants to enter different environmental matrices.

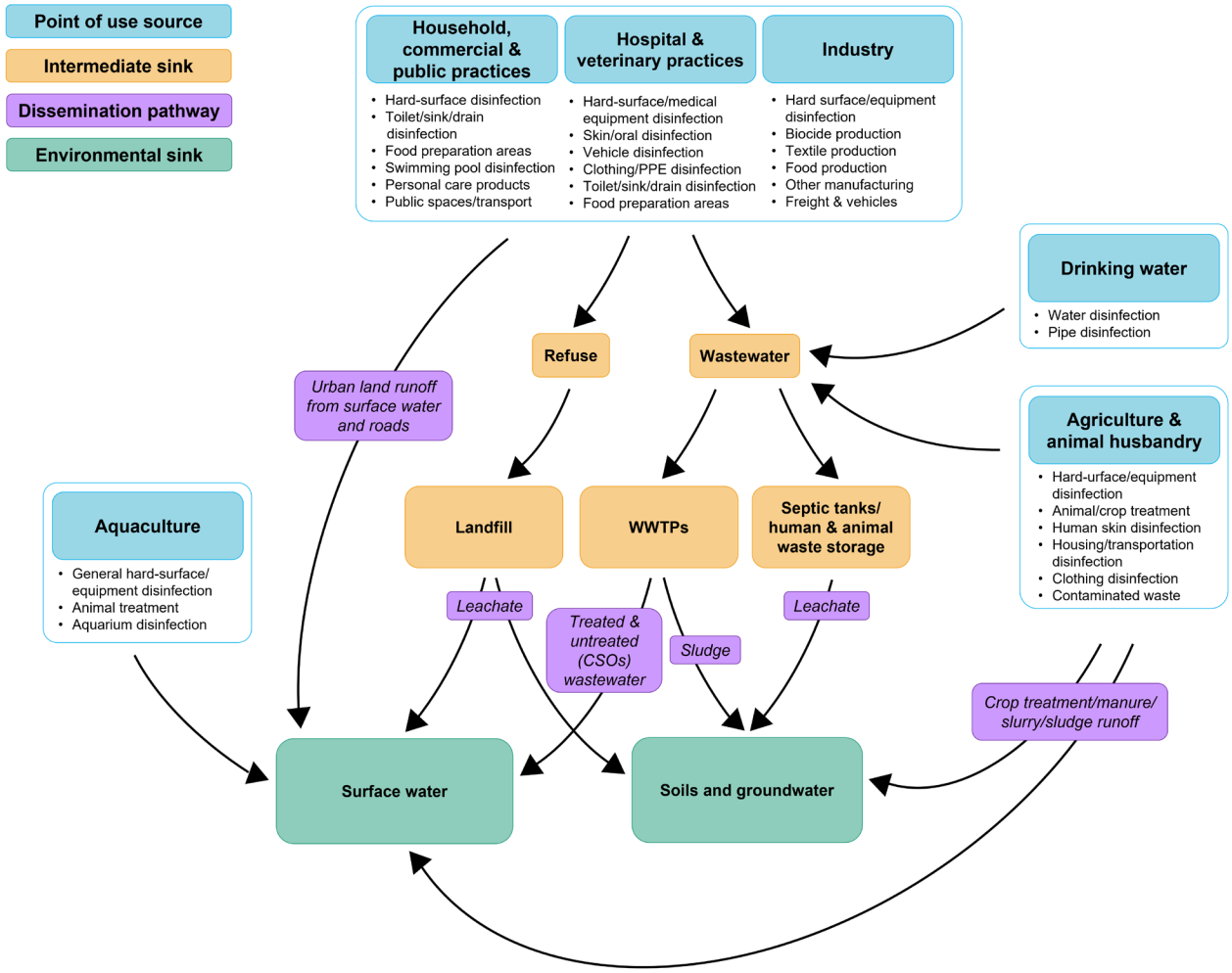

Figure 6 Pathways of disinfectants to the environment.

4.1 Wastewater

A frequent and well-studied pathway for disinfectants to reach the environment is via wastewater treatment. Most disinfectants that persist well beyond their point of use are at risk of entering the wastewater system. Many use scenarios lead to disinfectants entering the sewage network, either by washing down hard surfaces , flushing disinfected toilets, washing skin or mouth-rinsing in showers and sinks, or by the carriage of disinfectant residues in human/animal urine and faeces [94-97]. Therefore, wastewater acts as an interface between a wide range of use scenarios for disinfectants and the environment (e.g., influent contains many wastewater streams, such as domestic, hospital, industry, farms, and abattoir sources). Treated wastewater is usually released into receiving waterbodies, which can be rivers/streams or coastal waters. However, WWTP effluent can also be released on to wetlands [98]. In addition to WWTP effluent, sludge and anaerobic digestate can be applied to agricultural fields as a fertiliser [99]. This may lead to disinfectants that have not been removed during

treatment entering soil environments, and subsequently disseminating to aquatic environments via runoff [100]. Although wastewater treatment can have a significant impact on the fate of these chemicals prior to being released into the environment, raw, untreated sewage can enter environments through discharge from combined sewage overflows (CSOs) in combination with urban stormwater runoff [101,[102].

Not all wastewaters will enter a WwTP with premises not connected to the sewer mains using, for example, package sewage treatment plants or septic tanks. Septic tanks can leak, thereby releasing untreated sewage and disinfectants into the immediate environment, which may result in contamination of surface and/or groundwater [103, 104].

4.2 Agri- and aquaculture

Disinfectant pollution can also contaminate environmental compartments from agricultural and farming waste, and spill over from aquaculture [100]. Given the diversity of uses and application methods on farms, it is plausible for disinfectants to enter the environment directly into soil from uses such as disinfectant tractor mats and boot washes, runoff from disinfected animal housing, and from the animals themselves who, like dairy cows, can have regular hoof dips that will leave residues throughout the farm [105]. These disinfectant residues can also be washed, as a result of rainfall, from soil into waterbodies in agricultural runoff [100]. In addition, disinfectants used to treat animal wastes, such as manure, may leach during storage, leading to contamination of the surrounding environment with disinfectant residues [50, 106]. Aquaculture employs numerous disinfectants for the control of pathogens and biofouling [60]. The presence of disinfectant residues on animal skin surfaces, boats, landing docks, and nets/cages may lead to their accumulation in sediment (dependent on the properties of the chemicals/compounds) and transfer via water “downstream” from aquaculture activities (depending on the direction of tide and currents) [44].

4.3 Leachate from landfill

Landfills generate leachate that can contain a broad mixture of chemicals, including those which may be substances used as disinfectants. Landfill leachate can be generated from the percolation of rainwater through contaminated municipal solid waste [108], e.g., containers, such as bottles. Newer and more well-maintained landfills will aim to capture and treat these chemicals [108]. However, even engineered and managed landfills may have a plume of leachate permeating soils and groundwater, risking the contamination of potable groundwater, surface water, and/or the marine environment with disinfectant residues [108].

Multiple sources of disinfectants to the environment makes source apportionment challenging, which in turn leads to uncertainty in understanding what activities are most impactful in terms of the contribution of disinfectants on AMR development. It is likely to be harder than for other antimicrobial agents such as antibiotics because disinfectants are high-volume products used in a much wider variety of ways. Although we know many of the sources of contamination in the environment, we have yet to assign relative and meaningful weight to them.

5 Environmental fate of disinfectants

The fate of disinfectants in different environmental matrices (e.g., soil, rivers) has been described in both the literature and regulatory assessments required for authorisation of active substances. In general, many disinfectants will biodegrade (with the exception of metals), but the rate will vary significantly depending on the environment and the substance. As is typical for most pollutants, biodegradation of disinfectants proceeds much faster in aerobic conditions [109]. Some disinfectant compounds are highly reactive and, therefore, do not persist in the environment (e.g., chlorine and hydrogen peroxide). This section gives an overview of the fate processes for disinfectant residues in different environmental matrices.

5.1 Water environments

5.1.1 Wastewater

Wastewater acts as a pathway between point of use and the natural environment. In many cases, wastewater treatment is the primary method for removal/ destruction of disinfectants. Concentrations of some disinfectants have been shown to be lower in wastewater effluent in comparison to wastewater influent [110-112]. This can result from degradation through the treatment processes (e.g., by biodegradation) or as a result of sorption to the sludge [94]. Therefore, investigating the efficacy of WWTPs and the reduction of disinfectants is critical for understanding environmental inputs.

5.1.1.1 Fate during wastewater treatment

Wastewater treatment can affect the chemical load of disinfectants between influent and effluent, but this is dependent on treatment typeand the chemical properties of the substance. For example, the QACs, BKC and DDAC are readily biodegraded in wastewater (>99.99% removal, according to a continuous activated sludge test) [94], whereas a study investigating removal rates of 5-chloro-2-methyl-4-isothiazolin-3-one (CMIT) in Romanian WWTPs found lower removal rates following treatment (44.6 to 78% removal at different WWTPs [110]).

Several studies have reported measured concentrations of disinfectants in wastewater. Table 4 presents a range of examples of disinfectant concentrations through wastewater treatment. Effluent concentration will be a function of both the influent concentration and the ability of the wastewater treatment processes to remove the chemical. Notably, some active substances used in disinfectants that have been reported here (e.g., copper and silver) have multiple uses that fall outside of the disinfectant PTs (01-05). Therefore, concentrations of these substances will have originated through multiple use pathways and are not wholly from their use in disinfectants. As data for English WWTPs were limited, European countries were

prioritised for their similarity in wastewater treatment technologies, climate, and population demographics.

Table 3 Examples of disinfectant concentrations and removal rates during wastewater treatment. Units are reported as in the publication. Data on removal rate has been reported as presented in the publications except for reference [111] where raw data was available to the authors so removal rates were calculated.

Active substance	Sample details	Influent concentration(s)	Effluent concentration(s)	Removal rate	Reference
BIT	Five Romanian WWTPs	Mean = 20.8 µg/L Max = 36.9 µg/L	Detected range: <LOQ to 7.56 µg/L	63.8 to 79.5% (between different WWTPs)	[110]
BIT	Seine centre WWTP, Paris, France	Min = 210 ng/L Max = 660 ng/L Median = 320 ng/L	Min = 20 ng/L Max = 55 ng/L Median = 24 ng/L	Min = 88% Max = 94% Median = 92%	[113]
BKC C12	Seine centre WWTP, Paris, France	Min = 460 ng/L Max = 5,800 ng/L Median = 1,200 ng/L	Min = 110 ng/L Max = 1,700 ng/L Median = 320 ng/L	Min = -53% Max = 94% Median = 77%	[113]
BKC C14	Seine centre WWTP, Paris, France	Min = 22 ng/L Max = 4,600 ng/L Median = 260 ng/L	Min = <41 ng/L Max = 400 ng/L Median = 55 ng/L	Min = -130% Max = >99% Median = 74%	[113]
BKC C16	Seine centre WWTP,	Min = <29 ng/L Max = 340 ng/L Median = 73 ng/L	Min = <9.8 ng/L Max = 79 ng/L	Min = <-200%	[113]

Active substance	Sample details	Influent concentration(s)	Effluent concentration(s)	Removal rate	Reference
	Paris, France		Median = <41 ng/L	Max = >97% Median = 64%	
CHX	Eleven Swedish WWTPs	Min = 0.335 µg/L Max = 2.368 µg/L Mean = 1.305 µg/L	Min = <LOQ Max = 0.033 µg/L Mean = 0.028 µg/L	Not reported	[112]
CHX	Bromma WWTP, Sweden	Average annual load = 26 kg/year	Average annual load = 0.4 kg/year	Not reported	[114]
CHX	Rya WWTP, Sweden	Average annual load = 164 kg/year	Average annual load = 3.3 kg/year	Not reported	[114]
CHX	Ön WWTP, Sweden	Average annual load = 18 kg/year	Average annual load = 0.2 kg/year	Not reported	[114]
CMIT	Five Romanian WWTPs	Mean = 38.6 µg/L Max = 84 µg/L	Detected range: 5.7 to 18.5 µg/L	44.6 to 78% (between different WWTPs)	[110]
CMIT	Seine centre WWTP, Paris, France	Median = <13 ng/L	Median = <3.7 ng/L	Not reported	[113]
Copper	Eleven Swedish WWTPs	Min = 19.8 µg/L Max = 102 µg/L Mean = 53 µg/L	Min = 0.69 µg/L Max = 10.2 µg/L Mean = 4.41 µg/L	Not reported	[112]
Copper	Ten UK WWTPs	Min = 0.39 µg/L Max = 53.6 µg/L Median = 7.4 µg/L	Min = 0.38 µg/L Max = 24.2 µg/L Median = 3.2 µg/L	Min = -691.77% Max = 97.68%	[111]

Active substance	Sample details	Influent concentration(s)	Effluent concentration(s)	Removal rate	Reference
				Median = 46.79%	
MIT	Seine centre WWTP, Paris, France	Min = 350 ng/L Max = 860 ng/L Median = 620 ng/L	Min = 39 ng/L Max = 350 ng/L Median = 150 ng/L	Min = 55% Max = 89% Median = 78%	[113]
Silver	Eleven Swedish WWTPs	Min = 0.05 µg/L Max = 6.5 µg/L Mean = 0.49 µg/L	Min = <LOQ Max = <LOQ Mean = <LOQ	Not reported	[112]

BIT = 1,2-benzisothiazol-3(2H)-one, BKC = benzalkonium chloride. CHX= chlorohexidine. CMIT = 5-chloro-2-methyl-4-isothiazolin-3-one. MIT = 2-methyl-4-isothiazolin-3-one. LOQ = limit of quantification.

As described above, under some circumstances (such as heavy rainfall), raw wastewater can be discharged directly into the environment without treatment from CSOs, resulting in no chemical removal. One study investigated concentrations of a range of disinfectants in two CSO discharges in Paris, France. The study found that MIT and BKC C16 exceeded concentrations of 0.1 µg/L, with BKC C12 and C14 exceeding concentrations of 1 µg/L in the CSO discharges [101].

5.1.1.2 Fate in sewage sludge

In addition to biodegradation, some disinfectants have the potential to adsorb to sewage sludge during the treatment process which can result in a decreased concentration of these chemicals being released in wastewater effluent. For example, QACs have an affinity for organic matter, which can result in sorption to and accumulation in sewage sludge [94, 115]. The partitioning behaviour of disinfectants can be predicted based on the n-octanol-water partition coefficient (K_{ow}) and the number of and value of the pKa of the parent chemical (if any). A number of studies have measured concentrations of disinfectants in sewage sludge. For example, the isothiazolones BIT and CMIT, registered under PTs 02 and 04, were measured in Romanian sludge at concentrations of 0.53 mg/kg dry weight (d.w.) and 2.63 mg/kg d.w., respectively [110]. Similarly, CHX was detected in every sludge sample tested at eleven WWTPs in Sweden, with concentrations ranging from 2.8 mg/kg d.w. to a maximum of 19 mg/kg d.w [112]. The same study also detected copper and silver (registered under PTs 02, 04, 05 and 01-05, respectively) in 100% of sludge samples,

at a minimum concentration of 110 mg/kg d.w. (copper) and 0.72 mg/kg d.w. (silver) and maximum concentration of 640 mg/kg d.w. (copper) and 3.26 mg/kg d.w. (silver) [112]. These substances however, particularly the metals, have a wide range of uses and sources in addition to use as disinfectants and therefore concentrations detected could be linked to other uses and not solely disinfection.

5.1.2 Surface water

Surface waters include both freshwater (such as rivers and lakes) and salt-water environments (such as coastal and marine water). Some disinfectants have been detected in surface water, for example the QAC, DDAC, was detected in seawater samples from two locations near the mouth of the River Tyne, near Newcastle, UK at concentrations ranging from 0.12-0.27 µg/L [116].

Fate in surface waters is chemical dependent, with factors affecting persistence including photodegradation, biodegradation or environmental matrices characteristics. For example, different forms of chlorine (e.g., chlorine, chlorine dioxide, etc.), which are used for many different disinfection purposes in the UK, do not persist in water environments. Chlorine gas reacts readily with water to form hypochlorous acid, which can further dissociate to hypochlorite ions and hydrogen. In addition, in marine environments, the pH of seawater can result in up to 80% of this hypochlorous acid disassociating [117]. Chlorine also undergoes photodegradation. In water environments that are exposed to sunlight and at pH 8, the half-life is only 12 minutes, compounding the instability of this chemical in the environment [118]. The instability of these chemicals mean that they are often likely to be below the limits of detection in surface water. In contrast, QACs are hydrolytically stable with a >90% recovery after a 33-day experiment [119]. QACs also undergo photolysis in river water, resulting in degradation but this occurs over longer periods of time, resulting in the half-lives of various QACs ranging from 12-94 days [120].

In addition to photodegradation, biodegradation of disinfectants occurs for several compounds. For example, an experiment with a starting concentration of 4,500 µg/L of BIT showed 99% removal over a 168-hour cultivation period with microalgal biodegradation being the primary cause [121]. CMIT is also readily biodegradable [122]. The persistence of some compounds in surface water depends on environmental factors, e.g., salinity, pH and organic matter content. For example, degradation of the disinfectant 2,2-dibromo-2-cyanoacetamide (DBNAP) has shown to be pH dependent [123].

5.2 Soils and sediments

Disinfectants enter soils and sediments via agricultural runoff, and the application of organic wastes containing disinfectants [100]. The fate and behaviour of disinfectants in these matrices depend on a host of physical and environmental factors. Many

studies have examined the biodegradability of disinfectants in aqueous solutions, but few data are available for soil and sediments.

5.2.1 Soils

There are relatively few academic studies on the fate of disinfectants in soils, and one factor that contributes to this lack of studies is that their attributes (e.g., surfactant properties) make them significantly more challenging to recover, meaning that multi-compound environmental screening methods are likely underestimating disinfectant concentrations [124]. As described earlier for WWTPs, compounds partition into sludge as a result of their physicochemical properties (e.g., Kd and pKa) [125]. These same chemical processes will also help to dictate their fate in soil. The cationic surfactant QACs, BKC and DDAC strongly sorb to soils, rendering them immobile due to high partition coefficients [94]. For example, BKC has been shown to persist for 6 months after application [126, 127]. Whilst partitioning behaviours will make it unlikely for these compounds to leach into surface or groundwaters, soil particles can physically move into surface water and subsequently desorb over time. Other compounds, such as glutaraldehyde, have been reported to be moderately mobile across different soil types and even more mobile in sediments [128]. It can also be readily degraded under aerobic conditions in soil [128] (e.g., completely biodegraded in soil within 33–57 days [129]).

Chiral chemicals (i.e., chemicals that cannot be superimposed on its mirror image in any formation) have dramatically different fates [130]. An example of a disinfectant with chiral properties is 5-Chloro-2-(4-chlorphenoxy)phenol (DCPP) which is authorised in the UK under PTs 01, 02 and 04, and is also used as a pesticide. As a disinfectant, DCPP is used in hand soaps, dishwashing detergents, and surface disinfectant products [131]. As a chiral chemical, DCPP has both R- and S-enantiomers. Despite the S-enantiomer being inactive, a mixture of both enantiomers are used in products. These enantiomers behave differently in soil, with the S-enantiomer form persisting longer in silt and sandy loam soils (but not in clay loam soil) [132]. Furthermore, DCPP R-enantiomers are converted into the S-form once present in soils, and persistence of R-form enantiomers doubles when in a mix containing both enantiomers [132]. Generally, the half-life of DCPP in soil can reach up to several weeks, with concentrations around the µg-mg/kg soil range [133]. DCPP is also highly soluble in water, therefore able to readily contaminate aquatic systems through run-off and leaching from soils [133].

5.2.2 Sediments

Literature detecting disinfectants in sediments is sparse, even more so than that of soils and water environments. However, one of the more frequently studied disinfectant groups is QACs. For example, Li et al. (2010) detected BKC-C14 at concentrations up to 8.9 mg/kg in sediment from the Hudson River Estuary, USA [134],

whereas [135] measured combined BKC concentrations of 0.35 mg/kg in sediments downstream from WWTP discharges in Minnesota, USA. Examples of studies on disinfectant fate in sediments are also sparse, however, the fate of QACs in sediments is, again, more frequently studied, for example, *Aeromonas hydrophila sp*. K [136] and microbial communities [137] within estuarine sediment have both been reported to biodegrade BKC. In addition, evidence gathered during the registration of DDAC in Europe found rapid, stable partitioning to sediments across a 120-day experiment [94]. This contrasts with its fate in soils and sewage sludge, in which DDAC showed little degradation [94]. Evidence suggests that rates of biotransformation vary across different environments according to the microbial communities present. For example, the mineralisation of BKC was different in microbial communities isolated from sea sediment samples, compared to those from sewage and sludge samples [138]. Although QACs are more studied, data on other compounds do exist, for example, glutaraldehyde is predicted to be highly mobile in sediments [128]. However, generally, the fate of disinfectants in sediments is still a largely understudied area.

5.4 Environmental fate and mixtures

In the environment, chemicals are rarely found in isolation [151]. Complex mixtures, including intact parent chemicals and degradation products, are commonplace [152]. Knowledge of the fate of chemicals in complex mixtures in the environment is limited, with attempts to model aspects of this resulting in poor predictions [153]. Interactions between chemicals in mixtures can result in additive, synergistic or antagonistic effects [154]. It has been shown that synergistic and antagonistic interactions are common and can substantially impact bioavailability, toxicity and biodegradation rates, for example, glutaraldehyde can affect the biodegradation of other contaminants [129]. There is even the potential for interactions in chemical mixtures to generate novel products. A recent study revealed interactions between glutaraldehyde and DBNPA were responsible for generating new compounds that were less toxic than DBNPA [109]. The reality within the environment is that such mixtures are the norm, thereby making estimations of the fate and effects of chemicals very challenging to predict.

6 General mechanisms of resistance to disinfectants

This section describes the general mechanisms of resistance to disinfectants, to better inform interpretation of data for specific disinfectants and aid our understanding on their role in the development of environmental AMR.

6.1 Definitions relating to resistance

Throughout the literature, on bacterial resistance to disinfectants the term “resistance” and the methodology used to measure “resistance” differs between publications (see Section 6.8). The main terminologies used include “resistance”, “decreased/reduced susceptibility”, “insusceptibility”, “acquired reduced susceptibility” and “tolerance”. The diversity in terminology and how to measure “resistance” to disinfectants reflects a lack of consensus among the scientific community. It has been suggested that disinfectant resistance should reflect the failure of a disinfectant to be biocidal at its use concentration [13]. Most studies define “resistance” as an increase in MIC (as low as a 2-fold increase) despite the fact that i) MICs are well below (i.e., often 1000-fold) the in-use concentration, and ii) when investigated, bacteria with an elevated MIC to a disinfectant are killed by its in-use concentration. The use of the term “decreased/reduced susceptibility” relates to a change in the MIC. The in-use concentration reflects the concentration of the disinfectant product in its concentrated form (on the label) and might not reflect the potential dilution of the product during use. The term “during use” concentration reflects product usage conditions, e.g., potential dilution [13]. Where bactericidal efficacy is investigated, “resistance” is defined as:

(i) a bacterial strain that is not killed by a disinfectant/antiseptic used at the same concentration that eliminates the majority of the same bacterial species, or
(ii) bacterial cells that survive disinfectant/antiseptic exposure at a concentration that kills the majority of the bacterial population.

Overall, this diversity in terminology is problematic when authors do not explicitly define the meaning of “resistance”. In addition, a wide variety of methodologies are used to measure resistance making data comparison between publications difficult (see Section 6.8).

The definition of chemotherapeutic antibiotic clinical resistance is easier and follows established protocols and breakpoints given by established organisations such as the European Society of Clinical Microbiology and Infectious Diseases (EUCAST), the British Society for Antimicrobial Chemotherapy (BSAC) or the International Standard Organisation (ISO). However, it is unfortunate that many publications do not follow

these guidelines resulting in difficulty in comparing results or interpreting the clinical significance of the reported data.

6.2 Resistance to disinfectants

Microbial resistance to disinfectants has been described since the 1950s. Bacteria and fungi express mechanisms that aim to reduce the harmful internal concentration of a disinfectant or enter a metabolically inactive state that can contribute to their survival [23, 155]. Microorganisms have varying susceptibilities to different disinfectants (Figure 7). Their survival depends on the presence of intrinsic resistance mechanisms that enables them to survive even at disinfectant in-use concentrations. Among the least susceptible microorganisms are bacterial endospores. Conversely, the most susceptible microorganisms are enveloped viruses (Figure 7) [155].

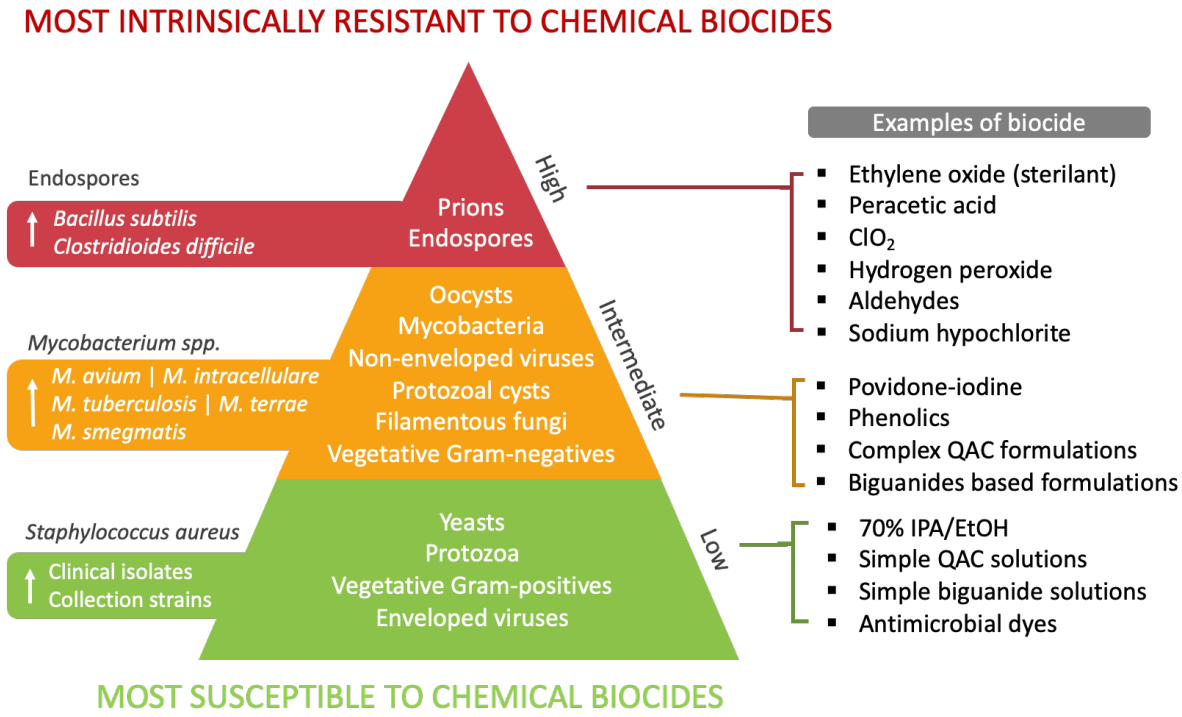

Figure 7 Microbial susceptibility to chemical disinfectants. The spectrum of activity of a given disinfectant depends on its chemical composition. Appropriate formulation of the disinfectants may increase the spectrum of activity. Note that only a limited number of agents are sporicidal. High, intermediate and low refer to the level of disinfection required to kill specific microorganisms.

Despite the presence of existing mechanisms that enable microbial survival, disinfectants – even at concentrations below the MIC – will act as a stressor and will trigger a stress-response from the microbial cell [13, 155]. Microorganisms will use multiple mechanisms to survive chemical disinfectant exposure. Some mechanisms such as efflux and changes in membrane permeability have been widely reported. The

presence/expression of broad mechanisms (e.g., efflux, membrane composition change, dormancy) may confer a change in susceptibility to unrelated antimicrobials (i.e., both disinfectants and chemotherapeutic agents) (see Section 6.4.1). Microbial biofilms need to be considered separately as the nature of biofilms will confer additional resistance mechanisms for bacteria embedded within them (see Section 6.4.4).

6.3 Intrinsic and acquired mechanisms of resistance

Microbial resistance to disinfectants can be intrinsic or acquired. Intrinsic resistance describes an innate property of a microorganism (i.e., a core genomic solution) to resist the inhibitory properties of a chemical, while acquired resistance refers to the acquisition of a resistance gene through gene transfer or mutation [23, 156].

6.3.1 Intrinsic resistance

Different microorganisms have different susceptibilities to chemical disinfectants based on their intrinsic and acquired properties (Figure 7). Bacterial endospores are amongst the least susceptible microorganisms to chemical and physical agents (Section 6.4.2). In vegetative bacteria, intrinsic mechanisms include the composition of the outer cell layer (including the presence of an outer membrane in Gram-negative bacteria composed partly of lipopolysaccharides (LPS)), a mycolic acid layer and lipid-rich outer cell layer in Mycobacteria, porins with specific pore sizes, and efflux [155].

6.3.2 Acquired resistance

Microorganisms can acquire new properties that confer a change in susceptibility following the acquisition of new genes or mutations. Mutations in global regulator genes (e.g., *marA* or *soxS*) will lead to expression or overexpression of a number of resistance mechanisms (see Section 6.4.1) and may lead to a change in metabolic pathways resulting, for example, in a change of membrane lipid composition. The exchange of genetic material between bacteria occurs via horizontal gene transfer (HGT), and has three main mechanisms: conjugation, transduction, or transformation. There is only limited evidence that disinfectant exposure will result in an increase in gene transfer frequency [155]. Bacterial mutations are random by nature and are estimated to occur with a 10^{-6} frequency. Increased mutation rates have been described following exposure to chemotherapeutic antibiotics. However, there is limited evidence on increased mutations reported with exposure to disinfectants. Mutations following triclosan exposure have been reported [156], whereas mutations in the enoyl acyl carrier reductase have been linked to resistance to isoniazid in Mycobacteria [157]. Exposure to QACs has also been shown to induce mutations in global regulator genes such as *acrR, marR, soxR* [158] and *ramA* [159], outer membrane proteins and transporters such as *mipA and sbmA* [159], *sdeS* [160], RNA

polymerase including *rpoA* [159], *rpoB* and *rpoC* [158]. Mutations in *fabI* and *gyrA* have been reported following exposure to an oxidizing- or amine-based formulation [159].

Disinfectants have been shown to select for less susceptible bacteria, whether in a complex microcosm or within a defined single species population [155].

6.4 Bacterial resistance mechanisms to disinfectants

It is recognised that disinfectant interactions with bacteria are not specific, and the number and type of targets affected, and the severity of damage imparted will result in a “cidal” or “static” effect. If a bacterium is not killed, it will have the opportunity to respond by expressing several mechanisms aimed to decrease disinfectant concentrations and repair damage caused to the cell.

In *in vitro* studies, bacterial adaptation to a stressor can be indicated with an extended lag phase, which is then followed by an exponential phase similar to a bacterial population not exposed to the stressor. It has been hypothesised that the extended lag phase corresponds to the expression of resistance and repair mechanisms. This assumes that damage imparted to the cells is reversible [155] (Figure 1).

6.4.1 General mechanisms

6.4.1.1 Outer cell layers and changes in membrane composition

The role of the outer LPS membrane in Gram-negative bacteria and lipid-rich outer cell layers in Mycobacteria in AMR has been well described. In both instances, these layers limit or prevent the penetration of disinfectants within bacterial cells [26]. The importance of the outer membrane as a resistance mechanism has been exemplified indirectly with the use of permeabilisers, such as ion chelators, which restore the bactericidal efficacy of membrane active disinfectants, but also to a lesser extent with the use of spheroplasts and protoplasts (bacteria with partial or no cell walls) [161]. For example, the use of ethylene diamine tetra acetic acid (EDTA) contributes to membrane destabilisation by chelating cations including Ca^{2+} from the outer membrane, enhancing the efficacy of cationic membrane active agents such as CHX and biguanides. Changes in cytoplasmic membrane composition, including proteins, fatty acids and phospholipids have been shown to contribute to a decrease in disinfectant efficacy [23, 155, 162]. The number of porins available or the expression of porins exhibiting a reduced pore size, has been shown to restrict the diffusion of hydrophilic antimicrobials into the bacterial cell and has contributed to decreasing susceptibility to disinfectants.

6.4.1.2 Efflux pumps

Bacteria possess multiple efflux pumps, which are responsible for pumping disinfectant molecules from inside to outside the cell. Efflux pumps contribute to decreasing bacterial susceptibility to disinfectants but are not solely responsible for bacterial resistance at in-use concentrations [23, 162]. Conversely, efflux pumps can be solely responsible for clinical resistance to antibiotics in some cases (e.g., fluoroquinolones [163]). However, overexpression of efflux pumps will decrease disinfectant efficacy measured as MICs. The combination of efflux pump expression and other resistance mechanisms is likely to be responsible for bacterial resistance to disinfectants [164, 165]. Efflux pumps are widespread in bacteria and their effect on disinfectant MICs has been reported in multiple species. To date, five main classes of efflux pump have been reported [165] (Table 5).

Table 4 Efflux pump families in bacteria.

Family	Example efflux pumps	Example substrates	
		Disinfectants	Antibiotics
Drug/metabolite transporter superfamily (MATE)	NorM	Cationic biocides	Aminoglycosides, fluoroquinolones
Major facilitator superfamily (MFS)	QacA	Acriflavine, QACs, CHX	Not described
Multidrug and toxic compound extrusion family (SMR)	QacC	QACs	Fluoroquinolones
Resistance-nodulation-division (RND)	AcrAB-TolC	QACs, CHX	Fluoroquinolones, aminoglycosides
ATP-binding cassette (ABC) superfamily	LmrA	QACs, CHX	Fluoroquinolones, aminoglycosides

Environmental and clinical isolates found in environments where antimicrobials, including disinfectants, have been heavily used, have been shown to harbour multiple efflux genes (e.g., *qacA/B*, *norA*, *nor B*, *smr*). These isolates, when studied, have decreased susceptibility to disinfectants. Although not all disinfectants can activate efflux pumps, efflux expression can be associated with cross-resistance to diverse antimicrobials (Table 5).

6.4.1.3 Enzymatic degradation

Enzymatic degradation can contribute to decreasing disinfectant activity, although it may not provide bacterial resistance at in-use concentrations. A well-described degradation mechanism is the expression of catalase and superoxide dismutase in bacteria, which confer resistance to oxidising agents, particularly hydrogen peroxide [23, 155]. Other enzymatic degradation mechanisms have been reported for parabens, aldehydes, and metallic ions.

6.4.1.4 Change in metabolism

Metabolically inactive bacteria are less susceptible to antimicrobials, including disinfectants. This is particularly the case with bacteria embedded in biofilms (Section 6.4.4). Conversely, bacteria with high metabolism have been shown to be more susceptible to antimicrobials [155, 162]. Change in metabolic pathways following exposure has been documented for several disinfectants, including CHX and QAC [155, 162]. Changes in metabolic pathways may contribute to a change in membrane lipid composition and help cell repair. This is consistent with disinfectants having non-specific interactions with the cell. Several studies have described the expression of a "defence network", which triggers the expression of multiple mechanisms within the bacterial cell [155, 162]. However, the full impact of metabolic changes on disinfectant resistance is not fully understood.

6.4.1.5 Bacterial response to stressors

Even at low concentrations (sub-MIC), disinfectants act as stressors which can select for reduced susceptibility (even within the same population) and trigger a bacterial response in surviving bacteria (Figure 8). The impact of disinfectant-driven stress on the expression of global gene regulators, such as *marA* and *soxS,* has been described. Whilst the selection for resistant bacteria displaying specific mutations might lead to a narrow, albeit permanent, response, the adaptation of bacteria following exposure with a stressor might lead to a more global, but transient, response (Figure 8). Disinfectant concentration and exposure duration are key factors in determining bacterial response (Figure 1). Notably, even at low sub-MIC levels, disinfectants can act as a stressor, even to bacteria intrinsically resistant to that disinfectant [155].

Disinfectant exposure can lead to the selection of less susceptible bacteria while killing the most susceptible ones, and a general stress response or an SOS response where bacterial DNA is affected. General stress response and SOS response have been shown to trigger "emergency" RNA polymerase which will introduce codon mutations. Although this has been demonstrated following antibiotic exposure, this has not been investigated with disinfectants. Resulting mutations can be specific when a structural gene is affected, or more global when mutation occurs in a regulatory gene. General stress responses will trigger a change in the expression of regulatory genes, which impact the expression of a number of specific resistance mechanisms including efflux, porin change, repair mechanisms, etc. [155].

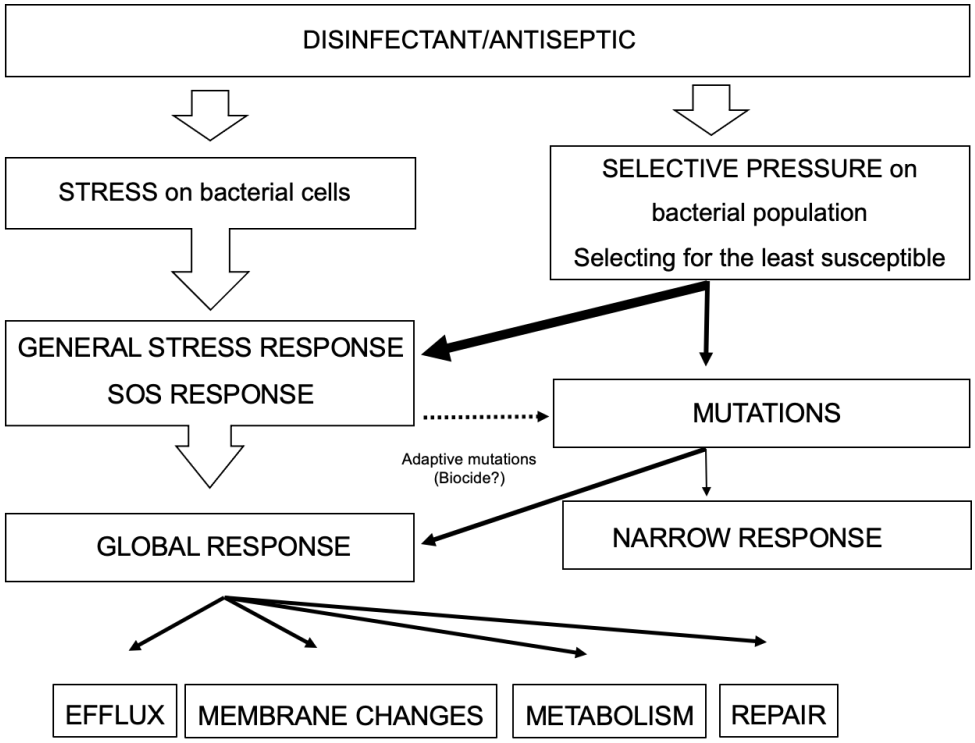

Figure 8 Bacterial responses to non-lethal concentrations of a disinfectant/antiseptic.

6.4.1.6 Cross-resistance to unrelated antimicrobials

Cross-resistance describes the process whereby the disinfectant resistance mechanisms expressed will also confer resistance to unrelated antimicrobials including chemotherapeutic antibiotics [13, 23, 155]. The mechanisms mentioned throughout Section 6.4, such as changes in membrane composition, efflux expression and metabolism, are non-specific mechanisms that will also contribute to decreasing efficacy of unrelated compounds. There are many *in vitro* studies showing disinfectant exposure causes a change in antibiotic susceptibility, leading to an increase in the concentration of disinfectants required to kill the bacteria (see BKC discussions below, for example). However, most studies rely on an experimental protocol which does not represent bacterial exposure to disinfectants in the environment (Section 6.8). In addition, the clinical significance of a change in antibiotic susceptibility profile is often difficult to ascertain owing to the use of non-standardised protocols (Section 6.8). Nevertheless, bacteria have the ability to express mechanisms that will aid their survival and by doing so, reduce the efficacy of other antimicrobials [162]. Bacterial isolates exhibiting disinfectant resistance (at their in-use concentration) have been isolated from the environment. Some isolates have also been shown to be cross-resistant to unrelated disinfectants. For example, glutaraldehyde (2% w/v) resistant Mycobacteria have also been found to be resistant to chlorine-releasing and oxidising agents, and vegetative *Bacillus subtilis*, resistant to in-use chlorine dioxide

concentrations, has been found to be resistant to other oxidising agents such as PAA and hydrogen peroxide.

6.4.1.7 Disinfectants and maintenance of resistant genes

The role of disinfectant exposure on gene maintenance is difficult to ascertain in comparison to understanding susceptibility profiles and gene carriage. Many studies have shown clinical isolates with reduced susceptibility to commonly used disinfectants in healthcare settings, such as QACs, can harbour many efflux determinants, notably *qac* and *smr* genes [155]. However, it is difficult to show that QACs are solely responsible for the maintenance of these genes, as many other antimicrobials, such as antibiotics, are used in high volumes in these environments [155].

6.4.2 Bacterial endospores

Bacterial endospores are among the least susceptible microorganisms to chemical and physical agents (Figure 7). The reasons for spore resistance are the absence of metabolic activity, the physical barrier to penetration caused by spore complex structure (Figure 9), and additional protection to the bacterial genome within the spore core [166]. The spore coat, cortex, and particularly, the highly compressed inner membrane, prevents penetration of a disinfectant, whilst the small acid-soluble proteins protect spore DNA from oxidising damage. It is worth noting that spores from different genera and species, will exhibit different susceptibilities to any given disinfectant [166].

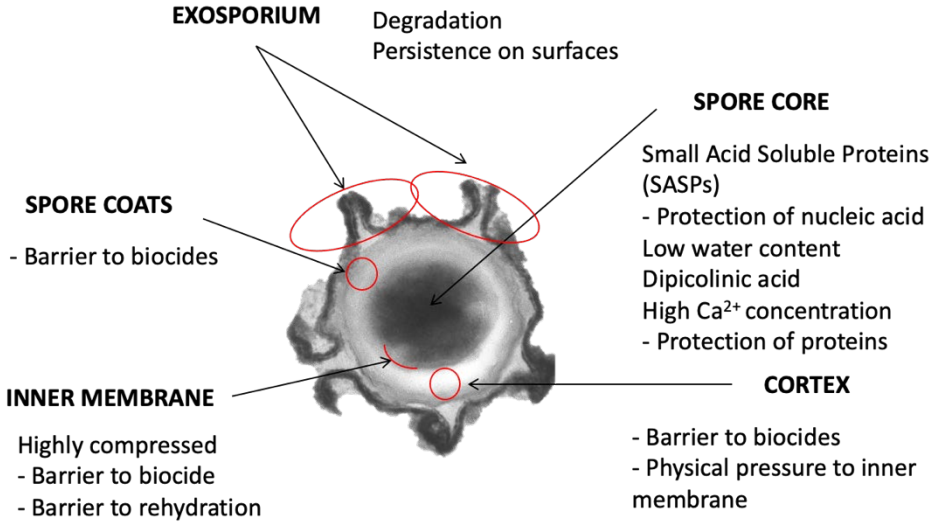

Figure 9 Intrinsic resistance mechanisms of bacterial endospores.

6.4.3 Pleiotropism (polymorphism)

The ability of bacteria to change shape as a result of disinfectant exposure has not been widely investigated, despite the fact that such a change can be associated with a decreased susceptibility [167, 168]. For example, in response to chlorination, *Vibrio cholera* can form shorter round cells, which are associated with increased biofilm formation and a decreased susceptibility to chlorine. This effect is transient and in the absence of selective pressure, bacteria revert to their original curved-rod form [167]. In addition, small colony variants in *Staphylococcus aureus* can be induced from triclosan exposure and have been associated with a decreased susceptibility to antimicrobials [168]. Small colony variants in *S. aureus* and other bacteria, including Gram-negatives, such as *E. coli* and *P. aeruginosa*, have been associated with persistence in the host and antibiotic resistance [169].

6.4.4 Biofilms

Biofilms are single or multiple species communities of bacteria that form on surfaces. They can be divided into wet and dry biofilms. Environmental dried surface biofilms (DSB) were first described in 2012 and to date have only been studied in healthcare settings [170]. Bacteria embedded in biofilms have been shown to be significantly less susceptible to antimicrobials. Several biofilm-associated mechanisms have been associated with such resistance [171]:

i) Disinfectant concentration diffusion gradient through extracellular polymeric substances (EPS) effectively reducing the concentration at the target organism,
ii) Mopping up of highly reactive disinfectants such as oxidising agents by reactions with EPS and lysed bacterial cell (mechanical inactivation),
iii) Decreased bacterial metabolism and bacterial dormancy (described as persister cells),
iv) Increased expression of bacterial resistance mechanisms such as efflux and disinfectant degradation, and
v) Increased gene exchange within the biofilm via HGT.

Complex biofilms are rarely studied for bacterial adaptation to disinfectants *in vitro*, as protocols to study complex biofilms are prone to high variability. Exposure of artificial complex microcosms transplanted from a drain to disinfectants has shown a change in biofilm composition, with the most susceptible species eliminated [172]. Overall, bacterial species in biofilms become less susceptible (based on MIC data) but the practical or clinical significance is not clear.

6.5 Fungal resistance mechanisms to disinfectants

Mechanisms conferring a decreased susceptibility in yeasts are mainly associated with decreasing effective disinfectant concentrations within the cell because the chitin

cell wall acts as a barrier to penetration [31]. The expression of efflux pumps can also contribute to decreasing antimicrobial concentrations. However, these mechanisms are unlikely to cause resistance to the high in-use concentrations of disinfectant products. Yeasts do not necessarily present a challenge to disinfection (Figure 7), although the electric charge of the membrane might affect the efficacy of cationic disinfectants [31].

Moulds are different and more challenging. Moulds produce fungal spores, although these are not as difficult to eliminate via disinfection compared to bacterial endospores. The actively growing part of the mould is the mycelium, which has a high metabolic activity and does not present a challenge to disinfection. However, the presence of fungal pigments decreases mould susceptibility, particularly to oxidising agents [173].

6.6 Viral resistance mechanisms to disinfectants

Enveloped viruses do not present a challenge to disinfectants (Figure 7), as the exposed lipid envelope is fragile and can easily be damaged. Damaging the lipid envelope will reduce infectivity, but the viral capsid and nucleic acid may remain intact (Figure 3). Small non-envelope viruses (picornavirus) are usually considered less susceptible to disinfectants compared to large non-enveloped viruses. Capsid composition might account for the difference in susceptibility between viruses, but the interaction between disinfectants and viral capsid is usually poorly understood [13]. Aside from their structure, viruses can survive disinfectant exposure by the formation of viral aggregates. Viruses embedded inside the aggregate are less exposed to the disinfectant. The formation of aggregates is not dissimilar to a penetration barrier. Some disinfectants, particularly cationic ones, can trigger the formation of viral aggregates in solution [13]. Overall, disinfectant interactions with viral particles and their mechanisms of resistance have not been well studied. Some stepwise protocols have been performed whereby viruses were exposed to increasing concentrations of a disinfectant between propagation [174]. Such experiments have shown that virus susceptibility can be decreased up to a point at which all resistance is lost. Such adaptation occurs only in a small subset of the viral population following propagation and is thought to result from a conformational change of the capsid [13, 174].

6.7 Protozoan resistance mechanisms to biocides

The main resistance mechanism in protozoa is the formation of endocysts. When environmental conditions are not favourable, amoebae trophozoites can form cysts through the process of encystation. Cysts can remain dormant but viable in the environment for years [32]. The resistance of FLA cysts to chlorination has been described for 40 years [32]. The encystment process is rapid and similar to that of sporulation, as it occurs in different stages: i) induction (degradation of cellular components), ii) immature cyst (cell wall synthesis) and iii) mature cyst (synthesis of

the second cell wall). The process leading to mature cysts is associated with a gradual decreased susceptibility to disinfectants, such as cationic disinfectants and oxidising agents [32]. The resistance of cysts is attributed to their double cell wall, but also to low or absent metabolism within the cyst [32]. The outer ectocyst wall is mainly composed of protein and lipid materials, and is fibrillar in appearance, whilst the inner endocyst wall contains cellulose, and its structure appears as fibrils embedded in a granular matrix [32]. The composition and appearance of the cyst wall may vary between FLA species, possibly explaining differences in disinfectant susceptibility [32]. Several disinfectants induce FLA encystment, including diamidines (e.g., diminazene aceturate and pentamidine isethionate) and CHX. Endocysts are more susceptible to disinfectants than bacterial endospores (Figure 7) and many disinfectants are cysticidal at their in-use concentration [31, 32].

6.8 Measuring disinfectant resistance in bacteria

The use of the appropriate protocol is essential to support the assertion of bacterial resistance and cross-resistance. The majority of the literature on disinfectant resistance is based on measuring MICs. The appropriateness of using MICs to determine resistance has been questioned, since in practice the concentration of disinfectant used can be >1000 fold higher than a MIC. The term “decreased susceptibility” where MICs are measured is preferred. Decreased susceptibility has been argued to be an indication of bacterial change. However, many studies define resistance as a change in MIC as low as a 2-fold increase [15]. There are many protocols to measure MICs. The most common one is using the microdilution broth, such as the Clinical and Laboratory Standards Institute (CLSI) standard microbroth dilution. The use of a standardised test facilitates the comparison of results between studies [13, 23]. Determining changes in the minimum biocidal concentration (MBC; minimal concentration that will kill a target bacterium) may be more appropriate as it indicates a change in the lethality of the disinfectant. Yet MBCs are also below the in-use concentration of a disinfectant [13, 15]. Occasionally, the bactericidal efficacy of a disinfectant at its in-use concentration has been investigated. Single time points and inactivation kinetics have been undertaken. Such studies are time-consuming but yield practical information. When measuring inactivation kinetics, a tailing-off indicates either a depletion of the active or the presence of a non-susceptible sub-population.

As for antibiotics, measuring the change in antimicrobial susceptibility profile should be based on internationally accepted standard protocols such as those given by CLSI, EUCAST, ISO and BSAC. Following these protocols enables the comparison of results between studies and enables the determination of the clinical significance of results. Unfortunately, these protocols are rarely used for disinfectants and the clinical significance of antimicrobial susceptibility changes is rarely addressed [13]. Several methods have been used to expose bacteria to a disinfectant and to measure a change in antimicrobial susceptibility. *In vitro* studies can be divided into those investigating:

i) Environmental/clinical isolates from an environment where disinfectants are regularly used,
ii) Complex biofilms transplanted in laboratory fermenters reproducing environmental conditions,
iii) Repeated exposures to a sub-MIC concentration,
iv) Repeated exposure to increasing concentrations, starting with a sub-MIC (stepwise training protocol),
v) Co-exposure studies where two antimicrobials are used at the same time, measuring reduced “cidal’ efficacy of one of the antimicrobials, and
vi) Protocols that mimic bacterial exposure to during-use concentrations of a disinfectant product

There are also *in situ* studies that analyse bacterial diversity and susceptibility profiles following use of selected disinfectant products.

The most common methods are stepwise training and repeated exposure to sub-MIC concentrations. Stepwise training involves repeated exposure of a microorganism to increasingly higher concentrations of the chemical or compound, usually starting at sub-MIC. Stepwise protocols are used jointly with a determination of MIC. Although academically interesting, since these studies help identify reasons for a change in susceptibility profile, they do not reflect a realistic disinfectant exposure and have limited or no practical and clinical impact [13, 15]. Studies of clinical/environmental isolates that present a change in susceptibility profile compared to their standard culture counterpart have identified multiple ARGs and at the time specific mechanisms responsible for a decreased susceptibility profile (e.g., efflux). It is often difficult to conclude that the change of susceptibility observed is caused by the usage of a specific disinfectant due to the presence of a mixture of chemicals. The type of isolates used in test protocols is important to consider. Environmental isolates are often considered less susceptible than culture collection strains. In addition, response to disinfectant exposure differs between environmental isolates and their culture collection counterpart [156].

6.8.1 Susceptibility determination protocols

Throughout the literature on bacterial resistance to disinfectants, most studies measured a change in MIC, sometimes MBC using standard efficacy protocols, notably the CLSI microdilution broth method (Figure 10). The efficacy of CHX has mainly been measured by deriving MICs using CLSI standards and not standard microbroth dilution protocols, whilst more diverse MIC determination protocols have been used with BKC (Figure 10a & b). Whilst standard MIC protocols are used with BKC and CHX, it is not the case for other disinfectants (Figure 10c). The term “resistance” is defined as an increased MIC in many studies, as a low as a 2-fold increase in 40% of studies based on BKC data. It has been argued that a <10-fold increase in MIC is marginal [3, 13], yet 85% of papers on BKC, and 75% of papers on CHX, defined “resistance” as a <10-fold MIC increase. Only 53% (117/219) of BKC

studies investigated the antimicrobial susceptibility profile of isolates. Of those, 73% used accepted standardised antimicrobial protocols such as those recommended by CLSI, BSAC or the use of e-test; that number increases to 82% with CHX studies (Figure 11a & b).

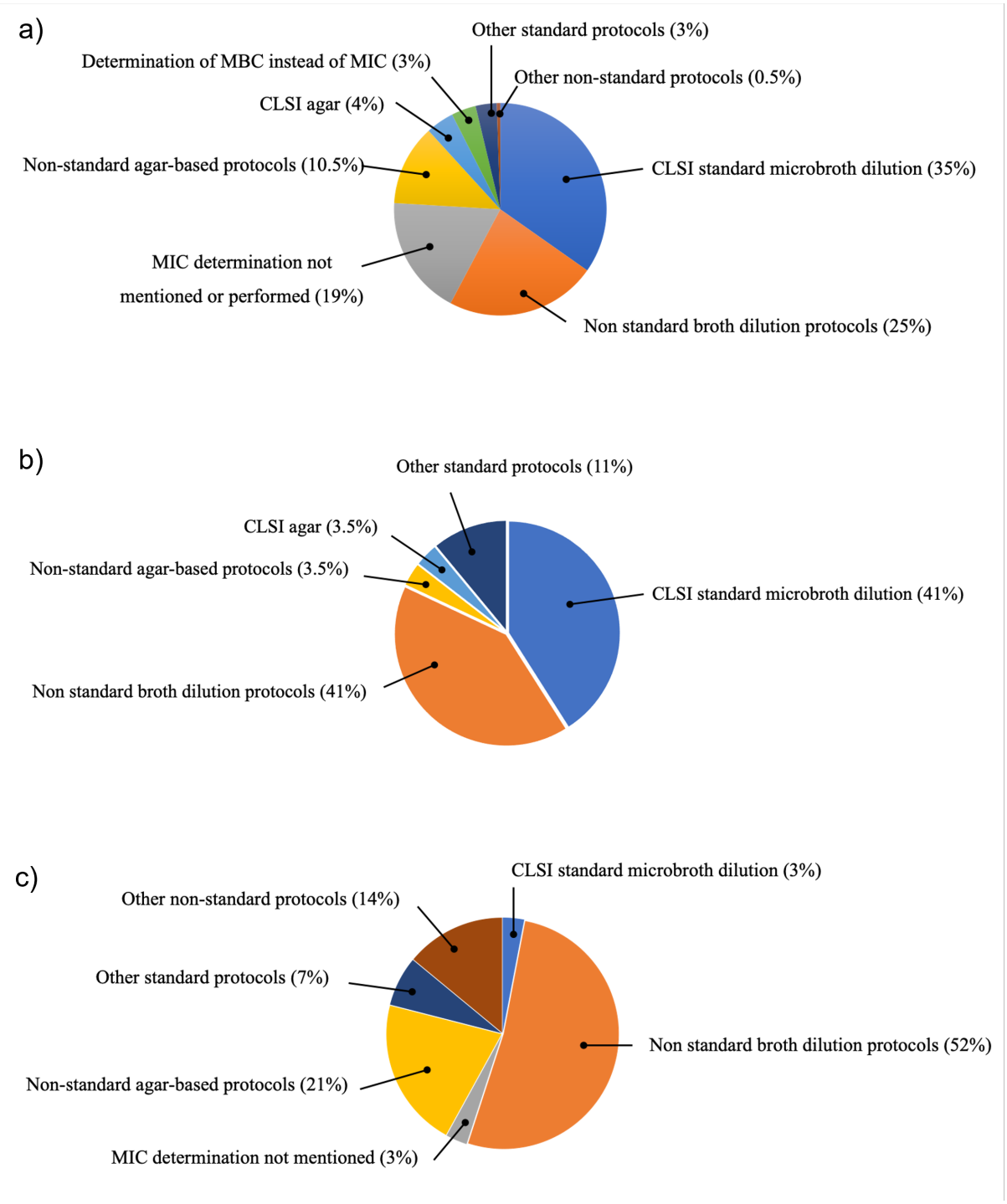

Figure 10 Protocols used to measure a decreased susceptibility to disinfectants. a) based on BKC data (Adapted from Maillard 2022), b) CHX data and c) other biocides (chlorocresol, DCPP, isothiazolones, DDAC, phenylphenol, bronopol, DBNPA, silver, copper).

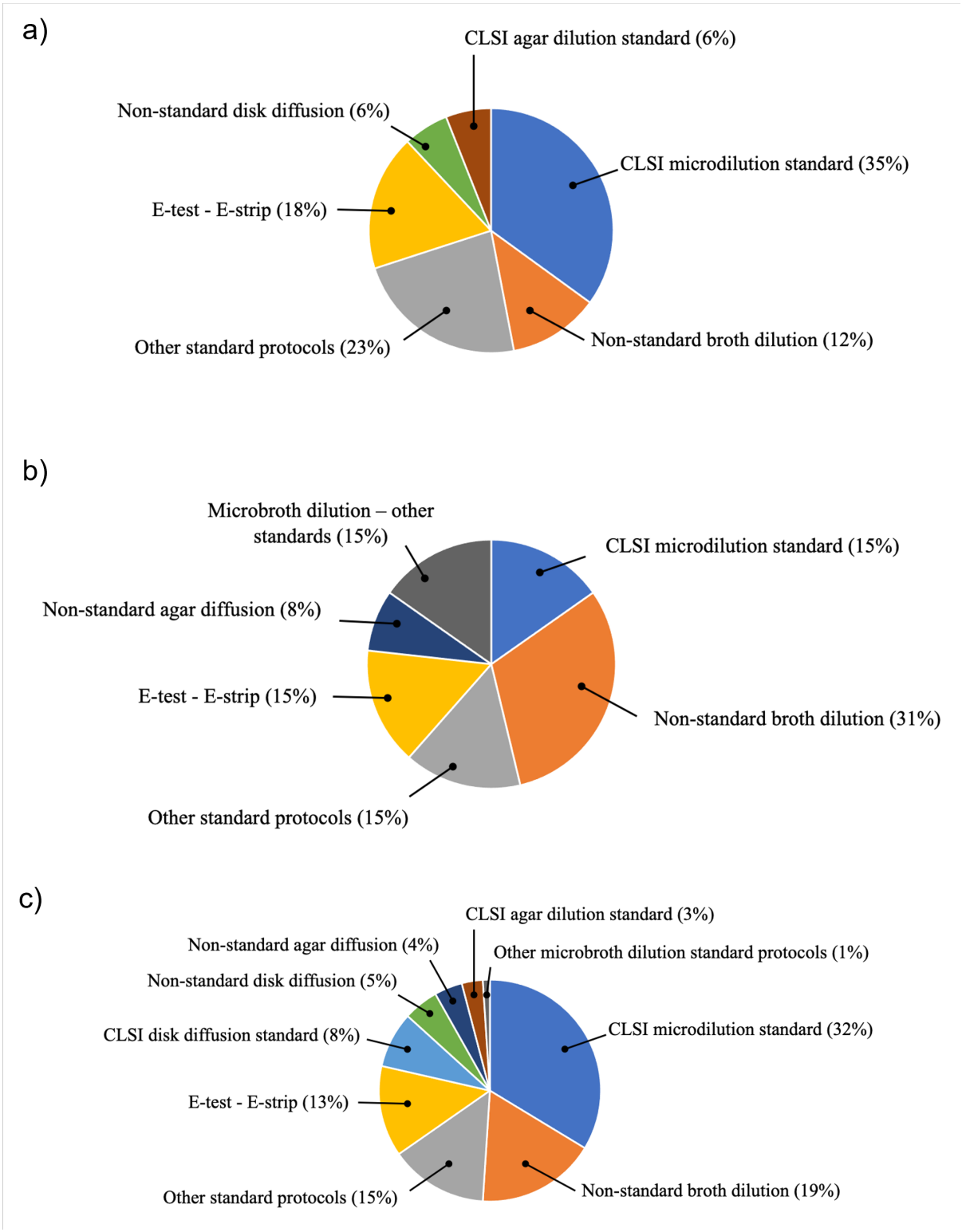

Figure 11 Protocols used to measure a change in antimicrobial susceptibility profile. a) based on BKC data (Adapted from Maillard 2022), b) CHX data and c) other biocides (chlorocresol, DCPP, isothiazolones, DDAC, phenylphenol, bronopol, DBNPA, silver, copper).

7 Bacterial resistance to disinfectants used in the UK

This section of the report aimed to collate and assess available literature on the role of a select list of disinfectants used in the UK in the development of AMR in bacteria. To achieve this, a semi-systematic review of the literature from select publication databases was performed. Fungi, viruses and protozoa are also considered briefly in Section 9. Bacterial spores were not included as they are intrinsically resistant to disinfectants and are not prone to develop resistance following exposure.

7.1 Disinfectants of interest

Active substances authorised (or in the process of authorisation) for use in disinfectants in the UK or Europe (Appendix 1, Table S1) were refined to further investigate the specific role they may play in the development and maintenance of AMR. These active substances are also the focus of Section 8, which investigates their potential to select for AMR at their MECs, by comparison to experimental selective concentrations. Therefore, this refined list includes active substances that are: a) relevant to UK disinfectant use scenarios; b) likely to be selective for AMR; and/or c) likely to exist in measurable concentrations in the environment (i.e., persistent or slow biodegradation) (Table 6).

Table 5 Disinfectant active substances examined for their role in AMR.

Active substance	Authorising agency	Use in product types
Biguanides		
Chlorhexidine (chlorhexidine digluconate)	HSE & ECHA	PT01, PT02, PT03
Polyhexamethylene biguanide (PHMB)	HSE & ECHA	PT02, PT03, PT04
Isothiazolones		
1,2-benzisothiazol-3(2H)-one (BIT)	HSE & ECHA	PT02
Mixture of 5-chloro-2-methyl-2H- isothiazol-3-one and 2-methyl-2H-isothiazol-3-one (CMIT/MIT)	HSE & ECHA	PT02, PT04
Metallic salts		
Copper	HSE & ECHA	PT02, PT05
Silver, Silver nanoparticles (AgNPs) & other formulations	HSE & ECHA	PT01, PT02, PT03, PT04, PT05
Phenolics		
5-chloro-2-(4-chlorphenoxy)phenol (DCPP)	HSE & ECHA	PT01, PT02, PT04
Biphenyl-2-ol	HSE & ECHA	PT01, PT02, PT03, PT04
Chlorocresol	HSE & ECHA	PT01, PT02, PT03
Quaternary ammonium compounds (QACs) and amines		
Alkyl dimethylbenzyl ammonium chloride (ADBAC/BKC)	HSE & ECHA	PT01, PT02, PT03, PT04
Benzalkonium saccharinate	HSE & ECHA	PT02, PT04
Didecyldimethylammonium chloride (DDAC)	HSE & ECHA	PT01, PT02, PT03, PT04
Others		
2,2-dibromo-2-cyanoacetamide (DBNPA)	HSE & ECHA	PT02[a], PT04
Bromochlorodimethylhydantoin (BCDMH)	HSE & ECHA	PT02

Active substance	Authorising agency	Use in product types
Bronopol	HSE & ECHA	PT02
Iodine	HSE & ECHA	PT01, PT03, PT04

[a] – only authorised/in authorisation process for this product type under ECHA

7.2 Literature search

7.2.1 Search strategy

A semi-systematic review of the literature from select publication databases was performed. The databases searched were PubMed, Web of Science and Google Scholar.

Search terms used included the names of the active substances for the disinfectants of interest, “resistance” and when appropriate “bacteria”.

7.2.2 Inclusion criteria

Articles of interest were then screened at the abstract and title level and, subsequently, at the full text. Inclusion and exclusion criteria for both levels of screening can be found in Table 7.

Table 6 Searching criteria for semi-systematic review of the literature.

Topic	Include	Exclude
Disinfectant	Disinfectant of interest (specified in Table 6) mentioned	Disinfectant of interest (specified in Table 6) not mentioned
Resistance	Discusses resistance/decreased susceptibility	Does not mention any terminology that relates to resistance
Methodology	Detailed summary on methodology in publication on how resistance/change in susceptibility was measured	1. No mention of the methodology used to undertake this 2. Limited details on methodology used to undertake this
Microorganism	Study investigates relevant microorganism (bacteria, fungi, viruses, protozoa)	Studies which investigate irrelevant microorganisms (e.g., bacterial spores as these are intrinsically resistant to disinfectants and are not prone to become more resistant following disinfectant exposure)

7.2.3 Search constraints

Only peer-reviewed scientific articles were considered. The literature search encompassed literature published between January 1st 1980 and 9th November 2022 unless otherwise stated. Only articles in English were included.

7.2.4 Data analysis

For all the articles retained (see Table 8), detailed analysis and collation of the test protocols used (MIC/MBC determination, microbicidal efficacy protocols, antimicrobial susceptibility assay), mechanisms of resistance, and maintenance of resistance genes, were performed. The results of this are presented in a comprehensive literature review, below.

Table 7 Literature search results using search engines. Numbers represent the number of articles published in an initial screen.

Active substance	Keywords	Search results (keywords + resistance)				
		WoS		GS	PubMed	
			+ bacteria			+ bacteria
Biguanides						
Chlorhexidine	Chlorhexidine + resistance	1,675	626	1,260	1,865	1,426
Polyhexamethylene biguanide (PHMB)	Polyhexamethylene biguanide or PHMB + resistance	597	128	21	146	98
Isothiazolones						
1,2-benzisothiazol-3(2H)-one (BIT)	1,2-benzisothiazol-3(2H)-one or BIT + resistance	265,962	-	-	-	-
	1,2-benzisothiazol-3(2H)-one or benzilthiazolone + resistance	10	4	583	-	-
Mixture of 5-chloro-2-methyl-2H- isothiazol-3-one and 2-methyl-2H-isothiazol-3-one (CMIT/MIT)	Methylchloroisothiazolone + resistance	3	-	-	-	-
	2-methyl-2H-isothiazol-3-one or MIT + resistance	24,617	-	1	12	-
	Methylisothiazolone + resistance	14	7	106	20	-
Metallic salts						
Copper	Copper + resistance	26,538	2,014	13,700	8,021	2,626
Silver, Silver nanoparticles (AgNPs)	Silver or AgNPs + resistance	262,780	12,895	-	-	-
	Silver + resistance	-	-	4,130	8,571	3,385
	AgNPs + resistance	-	-	37	294	178
Phenolics						

Active substance	Keywords	Search results (keywords + resistance)				
		WoS		GS	PubMed	
			+ bacteria			+ bacteria
5-chloro-2-(4-chlorphenoxy)phenol (DCPP)	5-chloro-2-(4-chlorphenoxy)phenol or DCPP +resistance	171	8	0	6	-
Biphenyl-2-ol	Biphenyl-2-ol or phenylphenol + resistance	46	1	2	23	-
Chlorocresol	Chlorocresol	13	3	1	13	-
Quaternary ammonium compounds (QACs) and amines						
Alkyl dimethylbenzyl ammonium chloride (ADBAC/BKC)	Alkyl dimethylbenzyl ammonium chloride or benzalkonium chloride or ADBAC + resistance	4,322	577	15	491	403
Benzalkonium saccharinate	Benzalkonium saccharinate + resistance	0	-	0	0	-
Didecyldimethylammonium chloride (DDAC)	Didecyldimethylammonium chloride or DDAC + resistance	261	47	3	55	46
Others						
2,2-dibromo-2-cyanoacetamide (DBNPA)	2,2-dibromo-2-cyanoacetamide or DBNPA + resistance	42	11	5	6	-
Bromochlorodimethylhydantoin (BCDMH)	Bromochlorodimethylhydantoin or BCDMH + resistance	18	4	0	1	-
Bronopol	Bronopol+ resistance	13	7	3	8	-
Iodine	Iodine or PVI + resistance	74,302	1,242	274	3,536	89

WoS = Web of Science; GS = Google Scholar.

7.3 Bacterial resistance to biguanides

7.3.1 Polyhexamethylene biguanides (PHMBs)

Of the 128 publications screened, only three papers investigated PHMB-driven AMR [175-177]. In addition, other publications screened but not deemed relevant investigated PHMB compatibility with various materials and efficacy of PHMB. Two of the relevant papers investigated *E. coli* [176, 177], whereas the other used a complex community microcosm from a domestic drain [175]. All studies pre-exposed the target microorganisms to PHMB and found a change in susceptibility measured by MIC. However, whilst Cuzin et al. (2021) and Moore et al. (2008) observed an increased PHMB MIC (20-fold and above), Henly et al. (2018) observed a decrease in PHMB MIC (2-fold) [175-177]. Cuzin et al. (2021) investigated PHMB pre-exposure of *E. coli* sedimentation biofilm and found a clone with stable clinical resistance to gentamicin and trimethoprim [177]. However, the gentamicin-resistant clone had a different growth rate characteristic (i.e., lag phase, exponential phase) compared to the parent strain, questioning the clone fitness. Henly et al. (2018) investigated six uropathogenic clinical isolates of *E. coli* and two laboratory strains. Following stepwise training using a gradient plate (12 passages), they isolated a number of clones all showing a decreased susceptibility to PHMB (measured as MIC, MBC). However, when grown as a biofilm, an increase in MBC of PHMB (29.2-fold) was observed. Only two clones showing stable clinical resistance to trimethoprim-sulfamethoxazole (CFT073) or gentamicin (EC26) were observed. In addition, increased pathogenicity was measured with the *Galleria mellonella* model following PHMB stepwise training (0 vs. 12 passages) and biofilm biomass, depending upon the isolate studied [176]. Moore et al. (2008) exposed a drain microcosm to PHMB for six months and measured changes in diversity and susceptibility of specific bacterial isolates before and after exposure. PHMB did not decrease the overall viability of the microorganisms but significantly altered the diversity by reducing the Gram-positive cocci and increasing the abundance of Pseudomonads. This resulted in the altered microcosm surviving exposure to other cationic biocides, CHX, QACs as well as PHMB. Some changes in MIC (>2-fold) were observed in individual bacteria. Changes in antibiotic susceptibility profile were not measured [175].

Overall, these studies showed that repeated exposure of bacteria to PHMB can lead to some clinical antibiotic resistance in *E. coli*, but the fitness of these antibiotic resistant bacteria is unclear. Henly et al. (2018) questioned the use of biocide-impregnated catheters as this may lead to isolates with different properties, including possible antibiotic resistance, increased pathogenicity and biofilm formation [176]. The study by Moore et al. (2008) provides evidence that repeated exposure to PHMB changes the composition of a complex lab-grown microbial community, which in turn became less susceptible to other cationic biocides [175].

7.3.2 Chlorhexidine (CHX)

As a result of the large number of potential articles (1,426) investigating CHX resistance in bacteria, initially, the analysis was confined to the last three years. From this, 27 articles out of 223 were retained for detailed analysis.

These studies relate to healthcare-clinical (56%), laboratory (33%) and environmental (11%) settings. The majority of studies investigated *P. aeruginosa* (7/27), *E. coli* (5/27), *Klebsiella pneumoniae* (5/27) and *Staphylococcus aureus* (5/27). Other bacteria included *Enterococcus* spp., *Streptococcus mutans*, *Acinetobacter baumannii* and *Proteus mirabilis*. Eight of the 27 studies investigated emerging resistance following a stepwise training protocol, whereby bacteria were passaged in increasing concentrations of CHX, whilst three studies repeatedly exposed bacteria to the same concentration of CHX. Only one study [178] investigated the bactericidal efficacy of CHX based on inactivation kinetics and MBC using an appropriate neutralisation step. Another five studies investigated MBC but without the use of a neutraliser which casts doubts over what is being measured [179-183].

The majority of studies reported on either multiple mechanisms of resistance (44%) or efflux alone (38%) (Figure 12). Multiple mechanisms included changes in membrane porins, upregulation of efflux, flagella protein, membrane permeability, peptidoglycan synthesis, LPS or metabolic activity [184-189]. One study mentioned possible repair mechanisms expressed following CHX exposure (0.3 mg/L) in *S. mutans* [190]. Sub-MIC exposure impacted bacterial regulatory functions by affecting sRNA expression, leading to changes in gene expression [180] likely associated with changes in multiple metabolic functions [178, 184, 187, 191, 192].

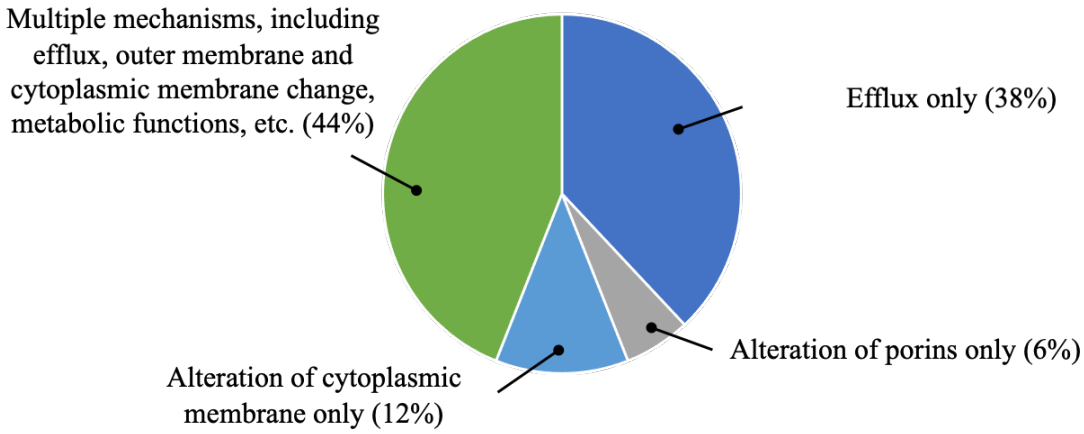

Figure 12 Reported mechanisms of bacterial resistance to chlorhexidine (Based on 27 peer-reviewed studies from 2019-2022).

7.3.2.1 CHX and cross-resistance

From the literature, it is uncertain whether there is a potential association between CHX exposure and colistin resistance. Zhang et al. (2019) showed a colistin MIC increased from 0.25 to 32 mg/L following *K. pneumonia* stepwise training in CHX (final CHX MIC: 128 mg/L) [193]. Efflux (overexpression of *cep*A) and point mutation in PmrB (lipid A change) were likely to be associated with the observed phenotype. Likewise, Singkham-In et al. (2022) observed an increase in colistin MIC (from 0.5 to 4 mg/L) in *P. aeruginosa* exposed to sub-MIC CHX. Notable changes in metabolic pathways were identified following CHX exposure, including changes that could lead to lipid A and outer membrane composition changes [187]. Hashemi et al. (2019) observed an 8 to 32-fold increase in colistin MIC in *K. pneumoniae* following repeated exposure to sub-MIC CHX [184]. However, when investigating a large collection of *E. coli* clinical isolates, Royer et al. (2021) did not report any correlation between CHX decreased susceptibility and colistin resistance. They identified several mutations in isolates with increased CHX MIC, but none of these mutations were related to colistin resistance [194]. Likewise, Morante et al. (2021) did not find a correlation with colistin resistance when investigating 59 *K. pneumoniae* clinical isolates [195]. Lescat et al. (2021) did not observe a change in colistin susceptibility in *K. pneumoniae* following stepwise training in CHX [185].

The impact of CHX on resistance to other chemotherapeutic antibiotics has been reported. Morante et al. (2021) reported a positive correlation between CHX MIC and resistance to trimethoprim/sulfamethoxazole in *K. pneumoniae* [195]. Pelling et al. (2019) reported a decreased susceptibility to polymyxin B and fosfomycin in *P. mirabilis* following stepwise training in CHX. The adapted strains showed increased efflux expression and changes in LPS [186]. Perreira et al. (2021) observed resistance to ampicillin and chloramphenicol in *E. coli* following repeated CHX exposure [189]. Tag ElDein et al. (2021) noted a change in susceptibility profile to cefepime, ciprofloxacin and meropenem in *P. aeruginosa* following stepwise training in CHX [188]. Susceptibility changes likely resulted from a change in efflux, porins and membrane permeability. Likewise repeated exposure to CHX resulted in *Porphyromonas gingivalis* with decreased susceptibility to azithromycin [196].

7.3.2.2 CHX and fitness

Bacterial adaptation to CHX following stepwise training or repeated exposure to a CHX sub-MIC did not change growth characteristics in CHX-free broth compared to the parent strain [189, 193, 197, 198]. Singkham-In et al. 2022 showed that repeated exposure of *P. aeruginosa* to CHX increased MIC by 2-fold and changed some antibiotic susceptibility profiles, but did not affect virulence with no change in fibroblasts or macrophage response, or difference in wound infection in a mouse model [187].

7.3.2.3 CHX and biofilms

Several studies investigated the impact of CHX on bacterial biofilms. In some papers, CHX did not impact biofilm formation in *S. aureus* [198], *P. aeruginosa* [199], or *Streptococcus oralis* selected from a complex oral biofilm following CHX treatment [181]. Uzunbayir-Akel et al. (2020) showed that CHX impacted on biofilm formation in *P. aeruginosa* possibly by affecting expression of quorum sensing [200]. Yet, Singkham-In et al. (2022) reported that CHX-adapted *P. aeruginosa* following bacterial growth in sub-MIC CHX concentrations [187]. CHX at 1 mg/L was shown to induce adhesion and colonisation of *P. aeruginosa* and *Rhodococcus eythropolis* to surfaces [201]. One study investigated the removal of CHX from a complex biofilm sludge reactor [202]. CHX at 1 mg/L impacted biofilm composition selecting for *Luteolibacter spp.* (4.3–10.1-fold change) and *Runella spp.* (6.2–14.1-fold change) expressing active efflux, whilst reducing core members (Comamonadaceae and Flavobacteriaceae) of activated sludge, potentially affecting contaminant removal and floc formation directly associated with the performance of WWTPs.

7.3.2.4 CHX and gene exchange

Jutkina et al. (2018) investigated the impact of CHX on HGT and reported that CHX 24.4 μg/L (a concentration 200 times below the MIC) significantly increased the frequency of HGT from a complex sewage microcosm to an *E. coli* recipient strain [203]. Wesgate et al. (2020) showed that the impact of CHX on gene exchange by conjugation was concentration dependent. At 0.05 mg/L, CHX did not impact ampicillin gene transfer by conjugation in *E. coli* whilst 2 mg/L CHX prevented conjugative transfer; both concentrations were at sub-MIC level [178].

7.3.2.5 CHX and practical considerations

Hardy et al. (2018) investigated 160 clinical *S. aureus* isolated over different periods from 1928 to 2014. CHX MIC increased 3-fold between 1928-1953, when CHX was not heavily used in healthcare settings, but did not significantly increase between 2002-2012 and 2013–2014, periods for which CHX was heavily used. Around 11% of isolates carried *qacA/B* and these isolates had a CHX MBC greater than 4 mg/L. *qacA/B* carriage was highest in isolates from 1954-2001 during which little CHX was used. The authors concluded that the clinical impact of CHX in decreasing the efficacy of the decolonisation regimen was unclear. This was particularly pertinent where the small increase in MIC remained substantially lower than the concentration used in practice (20-40 g/L) [179]. Yet, Wesgate et al. (2020) showed that following the deposition of 20 g/L CHX on a glass surface, only 0.47 to 0.75 mg/L remained. These concentrations remain bactericidal with a five-minute contact time against *E. coli*. Yet survivors were shown to have a change in antibiotic susceptibility profile, presumably following changes in metabolic pathways impacting efflux and membrane composition [178]. Zheng et al. (2022) observed that 32 of 294 *P. aeruginosa* clinical isolates had an elevated MIC (>50 mg/L). These isolates were associated with hospital length of

stay, ICU admission, length of stay in ICU, invasive procedures, duration of mechanical ventilation, CHX usage, and occurrence of nosocomial pneumonia [204]. Pereira et al. (2022) observed that *Enterococcus faecalis* isolates from the food chain and human samples expressed higher CHX tolerance. The clinical *E. faecalis* CHX MIC from humans was significantly higher among those associated with infection [183].

7.3.2.6 Limitations

Only the peer-reviewed literature from 2019 onwards was analysed. Out of 253 papers, 27 were retained for analysis. There were many CHX papers from 1980-2019 (>1,400) that would warrant examining to provide a more thorough understanding of CHX impact on emerging bacterial resistance.

7.4 Bacterial resistance to phenolics

7.4.1 Biphenyl-2-ol (phenylphenol)

Of the search results, two papers of relevance were identified and analysed further [205, 206]. Thorrold et al. (2007) did not look at the impact of phenylphenol on emerging bacterial resistance, but at the susceptibility of antibiotic resistant and antibiotic susceptible isolates to a phenylphenol-based product [206]. Lambert (2004) provided a retrospective study on the difference in disinfectant (including phenyphenol) susceptibility of isolates of methicillin-resistant *S. aureus* (MRSA), methicillin-sensitive *S. aureus* (MSSA) and *P. aeruginosa* isolated in 1989 or 2000. There were no details about how MIC values to disinfectant or antibiotics were obtained. The findings showed that clinical isolates isolated in different years did not show a change in susceptibility. The hypothesis that antibiotic resistance was driven by the use of disinfectants in healthcare settings, based on increased disinfectant usage between 1989 and 2000 was not supported [205].

7.4.2 Chlorocresol

Only one paper investigating the effect of chlorocresol on microbial resistance was relevant. Roedel et al. (2021) investigated 93 *E. coli* isolates from broiler farms and only one isolate showed increased MIC to chlorocresol (1,000 mg/L). Most isolates harboured efflux genes. No conclusion between chlorocresol use and decreased susceptibility or gene carriage could be drawn [207].

7.4.3 5-chloro-2-(4-chlorphenoxy)phenol (DCPP)

Only one paper investigating the effect of DCPP against *Pseudomonas putida* was found [208]. Exponentially growing *P. putida* was exposed to DCPP for 2 hours and changes in proteome were investigated. DCPP did not induce a heat shock response

and only catalase/peroxidase HPI as an oxidative stress response. Differential protein expressions were measured, and expression of proteins, such as multidrug transporters, probably resulted from the need to remove toxic, lipophilic compounds from the membrane.

7.5 Bacterial resistance to quaternary ammonium compounds and amines

7.5.1 Benzalkonium chloride (BKC)

The literature search for BKC is up to date as of 25/01/2023. Of the results, 219 publications relating to "BKC resistance" were relevant. These studies relate to food (31%), healthcare-clinical (29%), laboratory (25%), animal-health (9%) and environmental (6%) settings. A wide range of bacterial pathogens was investigated including *Staphylococci spp.* (mostly *S. aureus*), *Listeria monocytogenes*, Pseudomonads (mostly *P. aeruginosa*), *E. coli*, *Salmonella enterica*, *K. pneumonia* and *A. baumannii*. In addition, only a few studies investigated Enterococci, *Burkholderia* spp., *Stenotrophomonas* spp., *Serratia marcescens*, *Enterobacter* spp., *Campylobacter* spp.

7.5.1.1 BKC exposure and bacterial response

In vitro studies investigating the effect of BKC exposure to low (sub-MIC) concentrations usually aimed to understand resistance mechanisms [158, 180, 191, 209-244]. As such, these studies are not based on mimicking product use in practice. For example, 22/34 studies reporting an increase in BKC MIC following BKC exposure, are based on stepwise training, which relies on increasing BKC concentration in a stepwise manner after 24 hours of growth in BKC (1 passage); the initial concentration being at the sub-MIC level.

One study reported a BKC MIC of 100,000 mg/L in *S. marcescens* following stepwise training [209]. This indicates that *S. marcescens* shows resistance to in-use BKC concentrations. Such levels of resistance have not been reported elsewhere, with *S. marcescens* or any other bacteria studied. The majority of studies (85%; Figure 13) reported a less than 10-fold increase in MIC. Only 5% of studies reported >50-fold increase in MIC. Among these, Knapp et al. (2015) reported a 100-fold increase in BKC MIC in *S. enterica* serovar Typhimurium following exposure to during-use concentrations (0.015 mg/L or 0.004 mg/L) [226]. Apart from Chaplin (1951) [209], the highest MIC recorded was 460 mg/L in a complex microcosm [227].

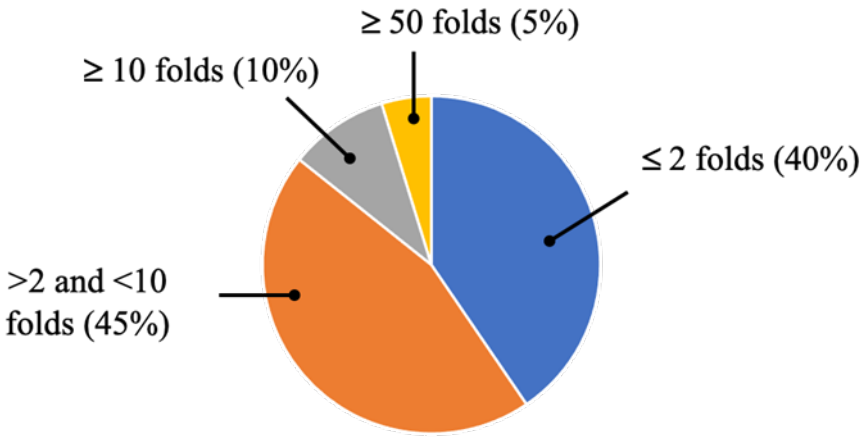

Figure 13 Fold increase in BKC MIC reported in the literature (Adapted from Maillard 2022).

Sub-MIC BKC concentrations have been shown to induce a change in the chemotherapeutic antibiotic susceptibility profiles of exposed bacteria. This has been reported in Gram-negative bacteria such as *P. aeruginosa* [210-212, 233, 235, 245, 246], *E. coli* [158, 189, 217, 224, 228, 229, 240, 247, 248], *Burkholderia* spp. [225, 226, 249], *Salmonella* spp. [215, 218, 221, 229], *K. pneumoniae* [228], *A. baumannii* [250], *L. monocytogenes* [220, 238, 242, 251] and *Campylobacter* spp. [244]. In addition, changes in chemotherapeutic antibiotic susceptibility profiles have been observed in Gram-positive bacteria such as *Staphylococci spp*. [239, 247, 252], or against diverse isolates from food sources [253] and wastewater effluents [227].

Overall, only four studies reported on the clinical impact of antibiotic susceptibility changes following BKC exposure [189, 225, 240, 254] and five studies investigated the persistence of this change [225, 235, 238, 244, 254].

The literature was conflicting at times. For example, some studies reported no change in antibiotic susceptibility profile following BKC exposure [215, 226, 255]. Likewise, increased BKC MIC, change in antibiotic susceptibility profile and clinical antibiotic resistance were often reported to be unstable in the absence of BKC. Other studies, however, found increased BKC MIC to be either stable, as in the cases of *S. enterica* [215, 216, 218] and *P. aeruginosa* [235] or transient as in the cases of *S. aureus* [256] and *Burkholderia lata* [225]. Voumard *et al.* (2020) reported that pre-exposure of *P. aeruginosa* to BKC (40-70 mg/L; i.e. 50% or 88% MIC) showed no change in clinical susceptibility to antibiotics despite a stable increase to BKC MIC to 150 mg/L [235]. Clinical susceptibility changes for ceftazidime, imipenem and ciprofloxacin in *B. lata* isolates following exposure to BKC were reported to be random occurring only in 50% of the experimental replicates [226].

The impact of bacterial adaptation following BKC exposure on fitness cost has been investigated. Whilst some studies reported no impact on fitness cost [222, 228, 231,

255], a recent study investigated *E. coli* repeated growth in BKC at a concentration of 4 mg/L for 500 generations and reported higher fitness of the adapted strain (showing altered porins and efflux pumps) in competition assay in comparison to the wild-type strain [189].

The impact of BKC exposure on persisters has been investigated. In *L. monocytogenes* formation of viable but non-culturable cells (VBNC) is linked to persistence. Noll et al. (2020) reported VBNC state in *L. monocytogenes* exposed to 0.008 mg/L BKC, with decreased antibiotic susceptibility to ceftriaxone, gentamicin, linezolid, tetracycline, and trimethoprim/sulfamethoxazole, but only a 2-fold increase in BKC MIC [236]. Nordholt et al. (2021) reported phenotypically tolerant *E. coli* subpopulations to BKC exposure, with a better growth rate in the presence of antibiotics. These persisters had reduced cell surface charge and mutations in the *lpxM* locus, which is involved in lipid A biosynthesis [257].

7.5.1.2 BKC exposure and bacterial stress response

BKC exposure likely leads to a stress response in bacteria. Yet only a few studies have explored expression of stress responses. Ceragioli et al. (2010) investigated gene expression in *Bacillus cereus* exposed to 0.5 to 7.0 mg/L BKC concentrations during mid-exponential growth and reported genes involved in the general and oxidative stress responses [258]. In *L. monocytogenes*, short exposure to BKC at 50 mg/L resulted in elevated c-di-GMP levels [259]. Exposure to 10 mg/L of BKC resulted in enriched bacterial genomes with genes involved in alkaline and oxidative stress in *L. monocytogenes* [251]. Overexpression of *sigB* (a gene involved in bacterial stress response) in *L. monocytogenes* was associated with a decreased susceptibility to 20 mg/L of BKC for five minutes [260]. Cpx envelope stress response system was shown to play a role in response to BKC exposure in *K. pneumoniae* [261].

Indirectly, deletion of *sigB* affected the ability of *L. monocytogenes* to survive the lethal concentration of 40 mg/L of BKC [262] or the viability of planktonic and sessile *L. monocytogenes* exposed to 20 mg/L of BKC for 15 minutes [263].

Jia et al. (2022) observed increased stress response expression following BKC-adapted *E. coli* strains following stepwise training protocol. However, upregulated gene expressions were reversible when QACs stresses were removed [158]. Schmidt et al. (2022) reported a significant expression of stress response and SOS response following exposure to BKC in *E. coli* [264].

Apart from a stress response, BKC exposure leads to a change in bacterial gene expression. In *A. baumannii*, exposure to 6 mg/L of BKC resulted in the expression of 227 genes involved in bacterial fitness and 335 genes involved with cell envelope maintenance, drug efflux, proteostasis, and oxidative stress defence [232]. Exposure of *Desulfovibrio vulgaris* Hildenborough to 1.25 mg/L of BKC during mid-log growth phase led to 103 genes upregulated and 95 genes down regulated, mostly genes

involved with cell envelope biogenesis, outer membrane, metabolism, energy production and conversion, translation, ribosomal structure and biogenesis [265]. Pereira et al. (2020) reported the overexpression of several chaperones and cochaperonins (e.g., *dnaK*, *dnaJ*, *groL*, *groS*, *htpG*, *hscA*, *cpxP* and *clpB*) in *E. coli* following exposure to 3.63 mg/L of BKC for 30 minutes [266].

7.5.1.3 Efflux and decreased susceptibility to BKC

Efflux is a primary mechanism in decreasing susceptibility to BKC measured by MIC (Figure 15). In particular, 51 out of the 126 papers mentioned *qac*-based efflux genes. Efflux pump gene carriage is widespread in Gram-positive bacteria including staphylococci [223, 239, 250, 255, 267-281], *Lactococcus* spp. [282], *Enterococcus* spp. [250, 279, 282], *Lactobacillus* spp. [282] and *Bacillus* spp. [282], but also in Gram-negative bacteria such as *K. pneumoniae* [250, 283, 284], *E. coli* [158, 240, 248, 282, 283, 285-287], *P. aeruginosa* [288-292], *Enterobacter* spp. [250, 282, 283, 293], *Helicobacter* spp. [282], *P. mirabilis* [14], *L. monocytogenes* [241, 242, 260, 294-304], *Citrobacter freundii* [305], *Stenotrophomonas maltophilia* [283, 305], *A. baumannii* [283, 306-309], *Salmonella* spp. [250] and *Flavobacterium* spp. [283].

The use of bacterial constructs with specific efflux pumps resulted in decreasing BKC MIC [160, 189, 257, 307, 310-321].

Of note, several mechanisms working together, such as efflux and change in membrane property can be responsible for a decreased in BKC MIC [189, 217, 230, 244, 322, 323].

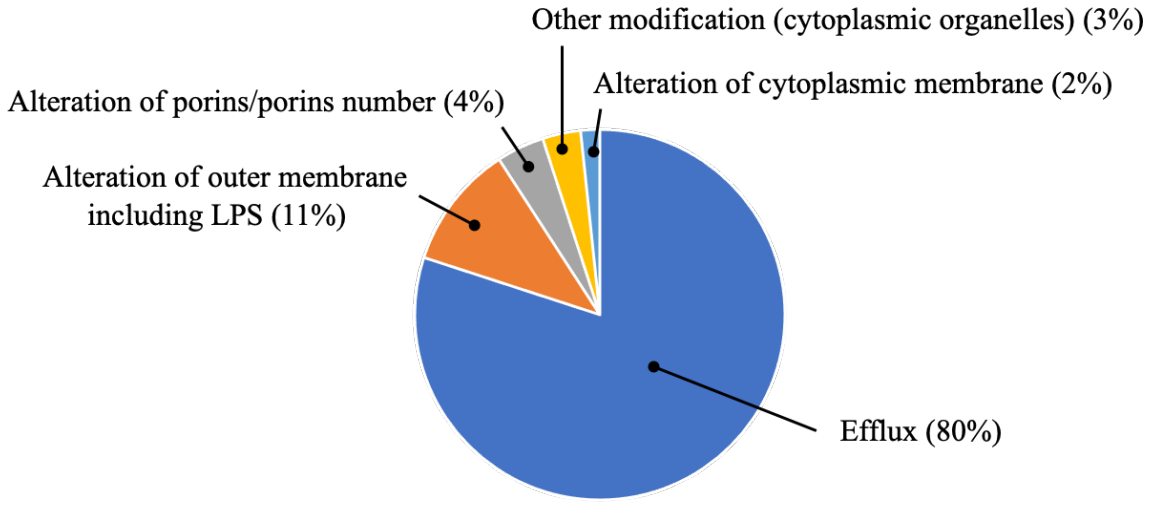

Figure 14 Mechanisms involved in reducing susceptibility to BKC (Adapted from [15]).

The role of efflux in decreasing antibiotic susceptibility in bacteria exposed to BKC has been reported in Gram-positive bacteria, for example, in staphylococci [239, 270, 272,

273, 324], *Lactococcus* spp. [282] and *Lactobacillus* spp. [282], and in Gram-negative bacteria including *E. coli* [189, 240, 282], *Enterobacter* spp. [293], *P. aeruginosa* [288, 290, 292], *Helicobacter* spp. [282], *Campylobacter* spp. [244], *A. baumannii* [309, 315] and *L. monocytogenes* [242, 296, 304].

7.5.1.4 BKC exposure and induced resistance gene expression

Morita et al. (2003) indirectly showed the induction of efflux in *P. aeruginosa* which was able to grow in the combined presence of both 6 mg/L of BKC and 1 mg/L of norfloxacin, but when *P. aeruginosa* was exposed to 1 mg/L of norfloxacin only, in the absence of BKC, growth was inhibited [245].

Galluzzi et al. (2003) showed *S. aureus* exposure to 1 mg/L of BKC repressed the expression of QacR, an efflux pump gene repressor [325]. A very low concentration of BKC (12 ng/mL) was able to induce such repression [326]. Grkovic et al. (2003) showed that 1 mg/L of BKC was a moderate inducer of QacA [327]. However, Braga et al. (2011) observed that efflux gene expression (*qacZ*) in *E. faecalis* was not inducible by BKC, although *qacZ* expression was associated with a decreased susceptibility [317]. Chittrakanwong et al. (2021) observed that the addition of 2 to 8 mg/L of BKC during the exponential growth phase of *S. maltophilia* resulted in derepressing MfsR activity [321]. Overexpression of smeDEF in *S. maltophilia* was associated with a 2-fold decrease in BKC MIC and a reduced susceptibility to quinolones and chloramphenicol [231].

7.5.1.5 BKC and dissemination of efflux genes

Any role of BKC in efflux genes dissemination remains speculative. Morissey et al. (2004) found no association between an elevated BKC MIC and the presence of *qac* genes *qacI*, *qacE* and *qacK* in 3,319 clinical isolates of bacteria from a range of genera [250].

7.5.1.6 BKC and gene transfer

Only two recent studies purposefully investigated the effect of BKC exposure on HGT. Schmidt et al. (2022) showed that BKC did not have an impact on the rate of HGT by conjugation or transformation [264]. Similarly, Bolten et al. (2022) concluded that HGT either did not occur or did not yield *L. monocytogenes* isolates with enhanced BKC tolerance [241].

Other studies investigated the occurrence of QAC resistance harboured on plasmids in environmental isolates [14, 287, 328], specific gene abundance (e.g. *sul1* and bla_{TEM}) in complex microcosm [329] or the HGT of *qac* genes [272]. Kücken et al. (2000) observed BKC MIC in *E. coli* could be increased from 20 to 80 mg/L following transformation with a plasmid containing an integron *qacE*. However, there was no correlation between increased BKC MIC and the presence of *qacE* or qac*EΔ1* [305].

Katharios-Lanwermeyer et al. (2012) showed that cadmium (a heavy metal) and BKC resistance could be co-selected during conjugation with *Listeria* spp. harbouring the BKC-resistance cassette *bcrABC*. The paper mentioned that BKC "resistant" strains have a higher ability for conjugation, but the MIC protocol is not described and "resistant" strains are not defined [330].

7.5.1.7 BKC and mutations

The impact of BKC exposure on mutation rates in bacteria was reported in several studies. Following stepwise training of *L. monocytogenes* in BKC, Bolten et al. (2022) identified nonsynonymous *fepR* mutations in 48/67 adapted isolates. *fepR* is a local repressor of the MATE family efflux pump FepA. These mutations were likely responsible for the increase in BKC MIC in the adapted strains. However, these adapted isolates remain as susceptible (4.48 $\log_{10}$ reduction) to the bactericidal efficacy of BKC (300 mg/L for 30 seconds) than the parent strains [241]. Douarre et al. (2022) identified 37 SNPs and three deletion sequences in 18 genes as well as ten SNPs and two insertion sequences in 11 intergenic regions in 16 adapted *L. monocytogenes* exposed to BKC (0.6 mg/L) or DDAC (0.31 mg/L). Eight mutations were observed in the strains adapted to BKC. The number of mutations in each strain varied from one to seven mutations depending on the isolate. These mutations were predicted to lead to overexpression of the efflux pump responsible for ciprofloxacin-enhanced resistance in these isolates [242]. Jia et al. (2022) showed that *E. coli* exposure to BKC (following a stepwise training protocol for 60 days) resulted in mutations that were likely to cause overexpression of efflux and increase MIC to both BKC and antibiotics [158]. However, Schmidt et al. (2022) did not observe a change in mutation in *E. coli* exposed to BKC [264].

7.5.1.7 Other mechanisms involved in decreased BKC susceptibility

Whilst efflux is a primary mechanism involved in reducing susceptibility to BKC, other mechanisms have been described including a change in bacterial cytoplasmic membrane composition [235, 236, 258, 323, 331-334], alteration of outer membrane [211, 212, 257, 259, 335, 336] or change in outer membrane protein [189, 214, 336, 337].

In *A. baumannii*, Knauf et al. (2017) suggested mutations in ribosomal protein resulted in protecting bacteria against BKC-induced protein aggregation [232].

7.5.1.8 Biofilm/microbial community exposure to BKC

The effect of BKC on biofilms is often contradictory but may be associated with the use of different BKC concentrations. In some studies, BKC was shown to induce biofilm formation in various bacteria including *Staphylococcus epidermidis* [252], *K. pneumoniae* [338], *L. monocytogenes* [339, 340] and *E. coli* [266]. Other studies showed an increase in biofilm biomass [341-343]. However, Chaieb et al. (2011)

observed that 1 mg/L of BKC exposure induced biofilm formation in *S. epidermidis*, whereas higher concentrations (2 to 5 mg/L) decreased biofilm formation [344].

Some studies investigated the impact of BKC on complex microcosms. Gray et al. (2020) reported that bacterial isolates from WWTPs that were able to grow on 250 to 500 mg/L of BKC accounted for 0.2% of the overall culturable communities and were mainly *Pseudomonas* spp. [345]. Tandukar et al. (2013) observed a decrease in diversity of a wastewater effluent microcosm following exposure to BKC 50 mg/L for four years, but only an increased BKC MIC of less than 2-fold [227]. Bastian *et al*. (2009) reported a change in a bacterial microcosm exposed to a product containing 10 to 25% BKC over a three-year period [346].

Few studies have explored the effects of sub-MIC concentrations of disinfectants within bacterial communities. One study exposed a sewage bacterial community to 8mg/L of BKC in a seven-day evolution experiment and tracked changes in community composition, antibiotic (ARGs), metal and biocide resistance genes [347]. BKC exposure significantly affected community composition but did not select for increased relative abundance of ARGs, while metal and biocide resistance gene relative abundance significantly decreased under BKC exposure. Though selection/co-selection was not observed, this may have been due to enrichment of a small number of intrinsically resistant species and exclusion of species with only reduced susceptibility to BKC. Solely focusing on heritable resistance genes from incomplete databases may also explain the lack of selection/co-selection observed.

Housekeeping genes have also been suggested to play a role in disinfectant, specifically biocide, resistance. A recent study found that functional metagenomic libraries generated from bacterial communities isolated from environments exposed to QACs contained multiple hits for UDP-4-galactose epimerase (*galE*) -like genes, with their phenotype confirmed as reducing susceptibility to BKC (based on MIC determination) and a selection of antibiotics, particularly, sulfamethoxazole and trimethoprim [348]. It was hypothesised the *galE*-like genes played a role in biofilm and/or LPS synthesis, and that these genes were potentially mobilisable, being co-located with transposons in some cases.

Forbes et al. (2017) observed an enrichment of bacteria, particularly *Pseudomonads* spp., following daily exposure of a domestic drain biofilm to BKC-containing products (100 mg/L of BKC) for six months. A decrease in antibiotic susceptibility profile was observed, but no clinically relevant resistant bacteria emerged [349]. Bacteria with reduced BKC susceptibility were still susceptible to higher BKC concentrations. Exposure to 10 mg/L of BKC for 96 hours decreased the alpha diversity values and expanded the relative abundance of Alphaproteobacteria in a complex microcosm from a domestic WWTP [350]. A significant increase in the *qacE*/*qacEΔ1* genes was observed with the change in bacterial community diversity.

Jiang et al. (2022) observed that *L. monocytogenes* strains following stepwise training in BKC for 10 passages demonstrated significantly increased biofilm biomass compared with their parent strains. In addition, they demonstrated that the Agr system rather than the Lux system was involved in the adaptation process that led to a 2-fold increase in BKC MIC and increased biofilm biomass. The BKC-adapted strains also showed increased virulence gene expression (*prfA*, *plcA*, *mpl*, *actA*, and *plcB*) by 2-fold, but a 2-fold decrease in haemolysis-associated gene (hly) expression [243].

Zeng et al. (2022) studied soil microcosm exposed to sulfamethazine (SMZ) (10 mg/kg) and/or gradient concentrations of BKC (0−100 mg/kg) for 28 days. BKC was responsible for a change in microbial diversity, with higher concentration (100 mg/kg) having a more profound impact than 1 or 10 mg/kg BKC. The authors investigated the abundance of antimicrobial resistance genes (ARGs) and eight mobile genetic elements (MGEs) between BKC or/and sulfamethazine-exposed soil and unexposed soil. Overall, 85 unique ARGs and 8 MGEs were identified in all samples. The highest number of ARGs detected were in soil combining both treatments (SMZ 10: BKC 100 mg/kg). However, BKC-treated soil alone increased ARG abundance with increasing concentrations. Where 1 and 10 mg/kg BKC was used, BKC imparted stress was the main factor in soil ARG spectrum changes. The authors observed that high BKC concentrations correlated to higher co-occurrence of ARG and MGE incidences concluding that BKC accumulation in soil might be associated with an increased occurrence and spread of ARGs [254].

7.5.1.9 BKC in situ investigations

Only one study investigated the impact on household microflora following the use of BKC-containing products and triclosan handwashing soap for one year [351]. After one year of use, bacterial isolates with high BKC MICs were more likely to have high MICs for triclosan and be resistant to one or more antibiotics. The change in antibiotic susceptibility profile was random and remains unclear whether the change in antibiotic susceptibility profile was driven by the BKC-products only.

7.5.1.10 Impact of product formulations containing BKC

Formulated disinfectants (i.e., active substances when in a product ready for commercial distribution) are not as well studied for their impact on bacterial adaptation as non-formulated ones. With BKC as an example, only 15% of studies investigated formulated BKC. When BKC formulations were studied, lower MIC and MBC were observed in comparison to unformulated BKC even following repeated exposure [352]. However, formulated BKC has been reported to increase BKC MIC and MBC, and isolates with high formulated BKC MIC have been shown to harbour efflux gene determinants [281, 301]. Likewise, formulated BKC was found to impact bacterial diversity in a complex community microcosm [227, 346, 349]. Of note, studies reported that bacterial isolates with elevated BKC MIC remain susceptible to the in-use concentration of formulated BKC [219, 221, 280, 349, 353, 354].

7.5.2 Didecyldimethylammonium chloride (DDAC)

Eleven papers were retained overall. Five of these conducted stepwise training experiments to artificially increased DDAC MIC in the test strains [158, 355-358]. One paper exposed the test strain to DDAC concentration of 0.31 mg/L [242]. The main bacteria investigated included *E. coli* [158, 264, 356, 357, 359, 360], Enterococci [356, 360, 361], *Salmonella* spp. [357, 362], *Pseudomonas* spp. [355], *Acinetobacter* spp. [264], *L. monocytogenes* [242, 357], *S. epidermidis* [358], *B. subtilis* [264] and *Campylobacter coli* [357].

Ten papers measured DDAC MIC and only one bactericidal efficacy using a test based on EN1276. Of the 9 papers measuring MIC, either the test protocol was not mentioned, or the MIC protocol used was not standard. Of the 8 papers that measured a change in antibiotic susceptibility profile, only 3 use a standard microdilution broth protocol.

Studies that use stepwise training reported various increase in MIC. From 4.5 to 9 mg/L after 21 days and 18 mg/L after 43 days in *E. coli* K12 [158], from 1.4 to 21.9 mg/L in Enterococci [356], from 32 to 128 mg/L in *Salmonella* [362], and a 3 folds increase in *E. coli*, *L. monocytogenes* and *Salmonella* isolates [357]. One study reported a 180-fold increase in DDAC MIC enabling *S. epidermidis* strains to potentially grow in products containing 50 mg/L of DDAC [358]. Increases in antibiotic MIC have been reported following bacterial adaptation to DDAC using stepwise training protocol, but only one study reported on clinical significance of a change in antibiotic MIC. One study reported a change in clinical resistance of one out of six *S. epidermidis* isolates. Although the breakpoints were based on EUCAST and ISO, the antibiotic MIC determination protocol was not reported [358].

Other studies investigated environmental/clinical/veterinary isolates' susceptibility to both DDAC and antibiotics [359-361]. Buffet-Bataillon et al. (2011) studied 153 *E. coli* clinical isolates (DDAC MIC range 2 to 64 mg/L) and reported an association between low DDAC MIC (less than 8 mg/L) and susceptibility to cotrimoxazole. Importantly the authors reported no correlation between DDAC MIC and clinical characteristics of bacteraemia (e.g., source, type, severity of bacteraemia or clinical outcome) [359]. Wieland et al. (2017) investigated 438 *E. coli* and 120 Enterococci veterinary isolates (*E. coli* DDAC MIC range from ≤ 0.36 to 3.6 mg/L) and reported some correlation between *E. coli* DDAC MIC and elevated MIC for piperacillin, sulfamethoxazole/trimethoprim, chloramphenicol and florfenicol. Clinical significance of increased antibiotic MIC is not addressed [360]. Bischoff et al. (2012) analysed 585 *E. faecalis* isolates from various sources. One *qacA/B* carrier had an elevated DDAC MIC (2.45 to 3.5 mg/L) compared to wildtype *qac* gene carriers (DDAC MIC of 1.05 mg/L). Antibiotic susceptibility profile was variable depending on the isolate [361].

The majority of the studies linked increased DDAC MIC with efflux [242, 264, 355-358, 361], although Sinwat et al. (2021) reported that efflux genes were less prevalent in

Salmonella isolates [362] and Wieland et al. (2017) reported that DDAC MICs did not correlate with the presence of the integron marker *qacEΔ1* in *E. coli* isolates [360]. Other studies reported other mechanisms linked to DDAC-induced stress in *E. coli* [158, 264], impacting gene regulators in *L. monocytogenes* [242], and resulting in a change in fatty acid composition in *S. epidermidis* or an increase in biofilm formation for some but not all *S. epidermidis* isolates [358].

Schmidt et al. (2022) investigated DDAC-induced mutations and gene transfer in *E. coli.* Exposure to 20 mg/L of DDAC increased mutation rates by 2.7-fold but this concentration did not affect conjugation or transformation. In addition, stress response did not seem to be correlated with increased mutation rate [264].

Overall, DDAC seems to cause stress to cells which may result in an increased MIC following stepwise training protocol. The clinical change in antibiotic susceptibility profile associated with an increased DDAC MIC is difficult to ascertain, although elevated antibiotic MIC has been correlated with increased DDAC MIC in some studies. Only one study relying on stepwise training reported that the increased DDAC MIC in *S. epidermidis* would enable the bacterium to potentially grow in products containing 50 mg/L of DDAC.

7.5.3 Benzalkonium saccharinate

There were no relevant papers on benzalkonium saccharinate and microbial resistance in the literature.

7.6 Bacterial resistance to isothiazolones

Six papers were retained for the isothiazolones (MIT, CMIT and MIT-CMIT combination). Some papers investigated mechanisms of decreased susceptibility of various bacterial isolates to MIT-CMIT. Brözel & Cloete (1994) identified a protein T, which, when absent, provided a decreased susceptibility in *P. aeruginosa* to MIT-CMIT. They established that the growth of *P. aeruginosa* in sub-MIC of MIT-CMIT resulted in higher MIC because of protein T disappearance and not mutations [363]. Frenzel et al. (2011) showed that porins were essential in the susceptibility of *Mycobacterium smegmatis* to isothiazolones [364]. In *Enterobacter gergoviae*, a common cosmetic product contaminant that can result in infections, Périamé et al. (2014) showed that efflux was likely responsible for industrial isolates' decreased susceptibility to isothiazolones and other disinfectants [365]. Growth of MIT-CMIT-adapted *E. gergoviae* in MIT-CMIT-adapted isolates with elevated MIC showed decreased mobility and increased biofilm formation. A concentration of 0.0075% MIT-CMIT was inhibitory but bacterial growth resumed albeit slowly when MIT-CMIT was removed, indicating some bacterial damage [366].

Stepwise training of *E. gergoviae* in increasing concentration of MIT-CMIT resulted in differences in MBC only compared to the parent strain, as well a change in antibiotic susceptibility profile to some antibiotics [367], however, no cross-resistance with antibiotics and elevated MICs was observed. A number of adaptative mechanisms were suggested, including overexpression of detoxifying enzymes, flagellin, and modification of membrane structure/function.

Rushton et al. (2013) investigated stepwise training of *B. lata* in MIT, BIT and CMIT which resulted in 2 to 8-fold increases in MIC. A longer lag phase of adapted MIT and M-CMIT 383 isolates of *B. lata* was observed. A change in antibiotic susceptibility profile was observed particularly to ciprofloxacin. The change in ciprofloxacin susceptibility was not caused by mutations in the quinolone resistance determining region (QRDR). Overall, the change in antibiotic susceptibility profile in the adapted strain was less than in the wild-type strain. Efflux was deemed responsible for the change in MIC observed [368].

Overall, these studies showed that various bacteria can be trained to become less susceptible to isothiazolones. The increase in MIC was low (<8-fold), and a change in antibiotic susceptibility profile was not recorded as clinically significant. A number of mechanisms were associated with a decreased MIC in isothiazolones. The mechanisms differed between bacterial genera. Efflux was involved in *Burkholderia* spp. and *E. gergoviae*, whilst porins seem to be important in Mycobacteria. Of interest are the loss of motility and increased biofilm formation in *E. gergoviae* as a result of exposure to MIT-CMIT.

7.7 Bacterial resistance to metallic salts

7.7.1 Silver and silver nanoparticles (AgNPs)

Five studies that were relevant for the analysis of 377 original papers published in 2022-2023. The majority (three of five articles) concerned laboratory settings [369-371], with one concerning food isolates [372] and one investigating multiple environments (clinical, environmental and veterinary isolates) [373]. The bacteria investigated were diverse, encompassing Gram-positive and Gram-negative species. One study included stepwise training whereby bacteria were exposed to increasing concentrations of silver nitrate (starting at 1 mg/L) [370], and three studies relied on repeated exposure or growth of bacteria in the presence of silver nitrate or silver nanoparticles (AgNPs) [369-371].

7.7.1.1 Environmental isolates and silver resistance

Two observational studies were found investigating heavy metal or silver resistance in a large number of bacterial isolates. Gufe et al. (2022) investigated 157 bacterial isolates from fish samples and observed that a high proportion of these isolates (from

43% for *Proteus* spp. to 88.5% for *Aeromonas* spp.) were able to grow in the presence of 100 mg/L silver nitrate (100mg/L) and reported clinical resistance to at least one systemic antibiotic (particularly lincomycin and rifampicin) among these isolates [372]. The other one (Vilela et al. 2022) reported the presence of efflux genes in 80 *Salmoella infantis* isolates but did not report antimicrobial susceptibility profile [373].

7.7.1.2. Mechanisms associated with silver resistance

Efflux was reported in three of the five studies. Vilela et al. (2022) investigated AMR gene carriage in 80 isolates of *S. infantis* and observed high prevalence of efflux: *gol*S in 98.75%, *mdf*A in 58.75% and *tet(A)* in 37.5% of isolates and *sil*ABCDEFPRS (a complete *sil* operon) in 36.25% of isolates [373]. Following stepwise training or repeated exposure to silver nitrate, MIC of silver nitrate reached over 512 mg/L in some *K. pneumoniae* strains [370]. Although the high MIC could be reached in strains lacking the *sil* operon, strains with a *sil* operon showed a mutation in *silS* and a greater expression of *sil*A. Strains with *cus*S and *omp*C mutations showed increased *cus*A expression (over 250-fold) and decreased *omp*C expression (over 30-fold). Wu et al. (2022) reported AgNPs exposure affected gene expression in *E. coli*. A high concentration 480 mg/L of AgNPs selected for mutations in *cus*S, *cus*R and *omp*R, whilst repeated exposure to a lower concentration (60 mg/L) increased MIC by 2.5-fold and induced mutations in *cus*S, *cus*R, and *arc*A [371]. Detoxification is also a mechanism that has been reported to help bacterial survival to silver exposure [369-371]. Metryka et al. (2022) reported that the level of expression of antioxidant systems (catalase, peroxidase, superoxide dismutase) depended on the type of heavy metal and their concentration [369].

7.7.1.3. Silver exposure and antibiotic resistance

Only one study investigated the antibiotic susceptibility of environmental isolates [372] and did not report any correlation between the ability of isolates to grow in the presence of silver nitrate (100 mg/L) and clinical resistance to antibiotics.

7.7.1.3 Limitations

The analysis of silver was limited, as it only concerned papers published in 2022-2023 as a result of resource and time limitation. It does not provide a global and informed analysis of the impact of silver, AgNPs and bacterial cells. The keyword choice did not always identify manuscripts of potential interest, in this case, 337 papers of potential interest based on keywords were identified but only 5 were selected for analysis and there are no other intuitive keywords to use. Most papers (2022-2023) concerned silver-compound combinations, synthesis and antimicrobial evaluation. Several papers evaluated the antimicrobial efficacy of different forms of silver as an adjunct to antibiotics.

In addition, several forms and combinations of silver with other molecules/compounds are being reported in the peer-reviewed literature. This is an additional parameter to consider for understanding the impact of different forms of silver including different types of AgNPs.

7.7.2 Copper

Thirteen peer-reviewed articles were found to be relevant for analysis out of 273 papers published in 2022-2023. The majority (9 out of 13) concerned environmental settings whilst the remaining ones were laboratory investigations. When the efficacy of copper was investigated, no papers used a standard efficacy test. Only four papers [374-377] investigated antibiotic susceptibility profile, and only two of these four used standardised methodologies [375, 377]. It was not always clear what copper compounds were used. Whilst some papers mentioned copper sulphate or copper nitrate, some only mentioned copper or copper ions. One study investigated copper nanoparticles (CuNPs) [369]. Three studies investigated mechanisms of resistance of specific isolates for bioremediation purposes [376, 378, 379]. Five studies concerned complex environmental microcosms either exposed to set copper concentrations or where heavy metal pollution was reported [380-384].

7.7.2.1 Impact of copper on complex microcosms

Gao et al. (2022) exposed activated sludge samples from WWTPs to either increasing or decreasing concentrations of copper to investigate the abundance of ARGs and MGEs. Copper had an effect on the distribution of bacterial community but ARGs abundance increased particularly with samples exposed to increasing copper concentrations [380]. Similarly, Gupta et al. (2022) investigated ARG abundance in water and sludge samples which were contaminated with heavy metals. A strong positive correlation was observed between copper and other heavy metals, and number of metal resistant bacteria (MRB) and antibiotic resistant bacteria found. In addition, the authors reported that copper triggered the co-selection of all MRB [381]. Whist a correlation between the presence of heavy metals, including copper, and the presence of MRB and antibiotic resistant bacteria was observed in sediments, this was not the case with water samples because these contain low heavy metal concentrations [382]. Liu et al. (2022) reported the presence of a high number of ARGs (145) and MRGs (312) in manure from broilers, dairy cows or fattening pigs. Copper, among other heavy metals such as zinc, are commonly used in animal feed. The abundance of ARGs encoding for aminoglycoside, sulfonamide, chloramphenicol and multidrug resistance was high in manure from broilers or pigs. The abundance of MRGs from the different manure origins was similar. The abundance of copper resistance genes was positively correlated to tetracycline, aminoglycoside and multidrug resistance genes [383]. Santas-Miguel et al. (2022) exposed soil samples to 1000 mg/kg of copper for 42 days at 22°C in the dark to investigate the tolerance of soil microcosm to copper. Soil bacterial communities exposed to high concentrations

of copper showed a co-tolerance to tetracycline, oxytetracycline, and chlortetracycline, although no correlation was established between copper-driven damage and antibiotic co-tolerance [384].

7.7.2.2 Mechanisms of bacterial survival to copper

Several studies reported on the mechanisms of bacterial resistance to copper. Boyd et al. (2002) investigated the effect of repeated growth of *E. coli* in the presence of copper sulfate ($CuSO_4$) (75 mg/L). After 37 days, the copper-evolved *E. coli* tolerated growth in copper concentrations ranging from 8 to 62 mg/L. Tolerance was associated with: upregulation in ABC transport and ATP binding; and downregulation in bacterial flagellum, iron transport, and cell division. The copper-evolved *E. coli* had a reduced ability to grow in the presence of some antibiotics including bacitracin, sulfonamide, rifampicin, and chloramphenicol [374]. Liu et al. (2023) reported on changes in oxidative stress response and membrane permeability in *E. coli* grown in the presence of 20 mg/L of $CuSO_4$ for 48 hours. Isolates showed an increase in MIC to either ampicillin or cefalexin which was associated with an increase in oxidative stress or/and membrane changes rather than an expression of *amp*C (no change in abundance observed after $CuSO_4$ exposure) [377]. Xu et al. (2022) studied emerging *Pseudomonas fluorescens* resistance to copper using repeated growth cycle/deposition on a copper surface. Following repeated exposure, MIC only increased from 518 to 598 mg/L, but the evolved strain was able to survive contact killing on copper surface for 60 minutes. The copper-evolved strain showed a lowest growth rate and lower ability to form biofilms. However, the authors estimated that the mutation rate was about five times greater when $CuSO_4$ (319 mg/L) was added into growth medium [376].

Metryka et al. (2022) was the only study that investigated CuNPs. Growth of bacteria in the presence of CuNPs (1/2 IC_{50} – 90 mg/L for *E. coli*, 56 mg/L for *S. epidermidis* and 26 mg/L for *B. cereus*) decreased *kat*E and *sod*A gene expression in *E. coli* and *S. epidermidis*, but a 159-fold increased *katE* expression in *B. cereus*. Catalase activity increased however in *E. coli* and *B. cereus* and decreased in *S. epidermidis*. Bacterial response to CuNPs is dependent on the type of bacteria and CuNPs concentration [369].

The study of potential bacterial isolates for copper bioremediation yielded some information about mechanisms of resistance. Cheng et al. (2022) investigated the response to copper. Growth of *Planococcus sp.* O5 in 80 mg/L of copper affected oxidative response (increase in superoxide dismutase and glutathione reductase activity) and membrane composition (fatty acids formation) [378]. Likewise, exposure of *B. cereus* T6 to both 51 and 510 mg/L copper increased oxidative stress response and fatty acid metabolism as well as iron metabolism, and denitrification pathway [379].

7.7.2.3 Copper, gene expression and gene exchange

Liu et al. (2022) reported that heavy metal facilitated HGT and co-selection of ARGs in animal manures. The authors also reported that the co-selection of ARGs by heavy metal was more prominent in Proteobacteria [383].

However, Palm et al. (2022) reported a 100-fold decrease in conjugation transfer of the IncF conjugative plasmid in *E. coli* in the presence of 320 mg/L of $CuSO_4$ [385].

7.7.2.4 Limitations

The analysis was very limited as it only concerned papers published in 2022-2023. It does not provide a more global and informed analysis of the impact of copper and CuNPs on bacterial cells. The keyword selection did not allow to select directly manuscripts of potential interest for this review. 273 papers of potential interest based on keywords were identified but only 13 were selected for analysis.

Most papers (2022-2023) examined copper mixtures with other antimicrobials including antibiotics. When mentioned, several forms of copper are being reported in the peer-reviewed literature, an important parameter to consider when researching further the impact of copper.

7.8 Bacterial resistance to bronopol

One paper by Pan et al. (2019) exploring bacterial resistance to bronopol was retained for analysis. The authors investigated the impact of six weeks exposure of bronopol on the microbial composition (bacteria:fungi ratio) of complex soil microcosm at a concentration of 2 mg/L. The authors speculated that bronopol exposure selects for less susceptible bacteria but did not provide any data to support this hypothesis [386].

7.9 Bacterial resistance to Bromochlorodimethylhydantoin (BCDMH)

There were no relevant published articles on bromochlorodimethylhydantoin (BCDMH) and bacterial resistance in the literature. Papers on BCDMH concerned efficacy mostly against biofilms and role of biofilms in decreasing efficacy of BCDMH.

7.10 Bacterial resistance to 2,2-dibromo-2-cyanoacetamide (DBNPA)

There were four relevant papers DBNPA. Pereira et al. (2021) showed that within a complex microcosm from water from oil fields, sulfate-reducing bacteria were less susceptible to DBNPA [387]. No real conclusion can be drawn as susceptibility measurements were based on an ad hoc MIC protocol. The impact of DBNPA on the

metabolic function of the microcosm was not studied. Grobe et al. (2002) investigated whether *P. aeruginosa* biofilms were less susceptible to DBNPA due to the slow penetration of the disinfectant into the biofilm [388]. Alhajjar et al. (2022) showed that DBNPA exposure (at 45 mg/L) can increase the MIC of *E. coli* from 45 to 1200 mg/L after ten repeated passages. The increased MIC was likely caused by efflux [389]. Campa et al. (2019) showed that DBNPA impacted microbial diversity in complex hydraulic fracturing (HF) surface water biofilm community. HF surface water biofilm community tolerated DBNPA better than non-HF communities, based on gene abundance measurements [123]. No studies showed the impact of DBNPA on a change in the antimicrobial susceptibility of bacteria.

7.11 Summary

Reviewing the literature surrounding the development of bacterial resistance following disinfectant exposure (both to disinfectants and in combination with other antimicrobials) resulted in thousands of hits, mostly focused on silver (including AgNPs), copper (including CuNPs), chlorhexidine and benzalkonium chloride.

The term “resistance” was poorly defined in the literature and often misleading. The literature mostly defined “resistance” as an increase in MIC, often as low as a 2-fold increase. This has been criticised for reflecting reduced susceptibility, which would not impact the efficacy of products in practice [13, 390]. The contamination of products at their in-use concentration was documented and resulted mainly from contamination with an intrinsically resistant microorganism, often a bacterial (endo)spore. Product contamination also resulted from an inappropriate preparation of the product resulting in a decrease in the in-use concentration allowing vegetative bacteria to grow.

Where a change in antimicrobial susceptibility was investigated as a result of disinfectant exposure, the clinical significance of the results was often not addressed. In addition, the use of internationally accepted protocols to measure bacterial resistance to antibiotics was often lacking.

Several papers investigating the stability of MIC change in the absence of disinfectants, observed a transient change in antimicrobial susceptibility profile, whereby an increased susceptibility was observed following bacteria passaging in the absence of a disinfectant. However, some studies, reported a stable change in susceptibility profile, indicative of mutations.

When bacteria were exposed to MIC or sub-MIC concentrations of a disinfectant, a change in MIC was often reported, together with a change in antibiotic susceptibility profile, regardless of the disinfectant investigated. The most commonly studied mechanisms associated with a change in antimicrobial susceptibility were efflux, change in membrane permeability including change in lipid composition and alteration of porins. When gene expression was studied, exposure to sub-MIC concentrations had a profound effect, regardless of the disinfectant studied. Also, sub-MIC

concentrations were seen to increase biofilm formation and impact the diversity of complex microcosms. The clinical or practical impact of sub-MIC exposure on individual bacteria or complex biofilms was rarely addressed. However, it was clear from *in vitro* studies that bacteria, regardless of the species, respond to sub-MIC exposure to disinfectants, regardless of the disinfectant used, resulting in a change in MIC and in gene expression. It was also clear that bacteria respond by expressing multiple mechanisms, enabling bacterial survival and/or the decrease of the concentration that produces a stress response.

Overall, it is not possible to predict whether exposure to a defined disinfectant at sub-MIC concentrations will result in a change in antimicrobial susceptibility in bacteria or the expression of certain mechanisms. Such change needs to be measured experimentally and some *in vitro* protocols have been proposed.

Investigations of products containing several disinfectants on microbial resistance have been rarely reported. Based on the literature used for this report, the use of a disinfectant mixture (not a product) on microbial resistance has not been studied. In addition, very few papers examine the effects of disinfectant and other antimicrobial mixtures on the development/co-selection and maintenance of AMR, with the majority of studies assessing co-selection of antibiotic resistance focussing on CHX or BKC. The reality within the environment is that mixtures are the norm, therefore whether these mixtures will have additive, synergistic or antagonistic effects on AMR in the environment is unknown.

8 Potential for environmental concentrations of disinfectants to select for resistance in bacteria

Comparing concentrations of disinfectants that are likely to select for resistance, or impact bacterial communities (e.g., affecting biofilm biomass) to concentrations of disinfectants found in the environment is important to understand the risk posed by disinfectants entering the environment from anthropogenic pollution. If concentrations found within environmental niches are significantly higher than those at which an effect is observed, it is important to employ mitigation strategies to limit the development of AMR. Therefore, this section aimed to compare effect concentrations with measured environmental concentrations (MECs) of disinfectants.

Concentrations of disinfectants at which changes in bacterial antimicrobial susceptibility, changes in microbial diversity in complex microcosms, or induction of specific mechanisms leading to cross-resistance, such as efflux, were derived from the results of the semi-systematic literature review described in Section 7. Studies based on stepwise training have been excluded from this further analysis, since the artificial increase of concentrations is unrealistic in environmental matrices [13, 15].

To compare *in vitro* selective concentrations with those found in the environment, a non-systematic literature review was performed to identify example MECs for each active substance in the refined list of disinfectants of interest (refined list in Table 6). If UK MECs were not available, MECs from European countries were recorded. The results of this literature review are presented in Table 9. Only MECs in aquatic environments were included, as this allowed for better comparison to *in vitro* concentrations (i.e., used in solution, often given as mass per litre).

Table 8 Example measured environmental concentrations (MECs) for disinfectant active substances of interest.

Active substance	Sample type	Concentration (µg/L)	Measurement type	Country	Reference
Biguanides					
Chlorhexidine	WWTP effluent	* ; 0.031; 0.028; 0.033	Min; Med; Mean; Max	Sweden	[112]
Polyhexamethylene biguanide (PHMB)	No relevant concentrations found in the literature.				
Isothiazolones					
1,2-benzisothiazol-3(2H)-one (BIT)	River water (upstream WWTP)	* - 47.5	Range of means	Romania	[110]
	River water (downstream WWTP)	40.8 - 81.1	Range of means	Romania	[110]
	WWTP effluent	* - 7.57	Range of means	Romania	[110]
	WWTP effluent	0.005; 0.03; 0.06	Min; Med; Max	France	[113]
	CSO effluent	0.02; 0.02; 0.06	Min; Med; Max	France	[113]
CMIT	River water (upstream WWTP)	2.36 - 112	Range of means	Romania	[110]
	River water (downstream WWTP)	55.3 - 144	Range of means	Romania	[110]

Active substance	Sample type	Concentration (µg/L)	Measurement type	Country	Reference
	WWTP effluent	5.7 - 18.5	Range of means	Romania	[110]
	CSO effluent	* ; 0.01; 0.16	Min; Med; Max	France	[113]
MIT	WWTP effluent	0.04; 0.15; 0.35	Min; Med; Max	France	[113]
	CSO effluent	0.01; 0.07; 0.29	Min; Med; Max	France	[113]
	River water	0.05 - 0.30	Range	France	[391]
Metallic salts					
Copper	River/surface water	0.12; 1.95; 7.15; 740	Min; Med; Mean; Max	UK	Dissolved and total Copper from EA Water quality Archive WIMS database (2019-2023) [392]
	Seawater	0.24; 0.75; 1.10; 5.85	Min; Med; Mean; Max	UK	Dissolved and total Copper from EA Water quality Archive WIMS database (2019-2023) [392]
	WWTP effluent	0.69; 4.03; 4.41; 10.2	Min; Med; Mean; Max	Sweden	[112]
	WWTP effluent	0.38; 3.23; 5.19; 24.2	Min; Med; Mean; Max	UK	UKWIR National Chemical Investigation

Active substance	Sample type	Concentration (µg/L)	Measurement type	Country	Reference
					Programme 2020-2022 Vol. 1 [111]
	WWTP effluent	1.10; 6.00; 9.38; 680	Min; Med; Mean; Max	UK	Dissolved and total Copper from EA Water quality Archive WIMS database (2019-2023) [392]
	CSO effluent	16.0; 19.0; 19.0; 22.0	Min; Med; Mean; Max	UK	Dissolved and total Copper from EA Water quality Archive WIMS database (2019-2023) [392]
Silver	River water	* ; 0.0061; 0.0681	Min; Mean; Max	UK	[393]
	WWTP effluent	0.0085; 0.028; 0.078; 0.409	Min; Med; Mean; Max	UK	[394]
Silver nanoparticles (AgNPs)	River water	0.002 - 0.07	Range	Germany	[395]
	River water (downstream WWTP)	0.001 - 0.002	Range	Germany	[395]
	WWTP effluent	* ; 0.0053; 0.0062; 0.0127	Min; Med; Mean; Max	UK	[394]
Phenolics					
5-chloro-2-(4-chlorphenoxy)phenol (DCPP)	River water	* - 0.01	Range	Poland	[396]
	Seawater (Baltic Sea)	0; 0.0002; 0.0003; 0.0028	Min; Med; Mean; Max	Germany/ Baltic Sea	[397]

Active substance	Sample type	Concentration (µg/L)	Measurement type	Country	Reference
	Seawater (German Bight)	0; 0.0001; 0.0002; 0.0014	Min; Med; Mean; Max	Germany/ Baltic Sea	[397]
Biphenyl-2-ol (2-Phenylphenol)	River water	* - 0.0028	Range	Spain/ Morocco	[398]
	River water	* ; 0.0064; 0.0066; 0.01	Min; Med; Mean; Max	Portugal	[399]
	River water	0.009; 0.017; 0.032; 0.091	Min; Med; Mean; Max	Portugal	[399]
	Seawater	* ; 0.0025; 0.0027; 0.0049	Min; Med; Mean; Max	Portugal	[399]
	WWTP effluent	0.044; 0.116; 0.110; 0.162	Min; Med; Mean; Max	Portugal	[399]
	River water	* - 0.047	Range	Germany	[400]
	WWTP effluent	* - 0.015	Range	Germany	[401]
	WWTP effluent	* - 0.00826	Range	Spain	[402]
Chlorocresol (4-Chloro-3-methylphenol)	River/surface water	* ; * ; 0.0171; 0.92	Min; Med; Mean; Max	UK	EA Water quality Archive WIMS database (2019-2023) [392]
	River/surface water	* - 0.00246	Range	Denmark	[403]

Active substance	Sample type	Concentration (µg/L)	Measurement type	Country	Reference
	River/surface water	* - 0.14	Range	Germany	[404]
	WWTP effluent	*	Range	Germany	[404]
Quaternary ammonium compounds (QACs)					
Benzalkonium chloride (BKC) C10	WWTP effluent	* ; 0.002; 0.002; 0.003	Min; Med; Mean; Max	Sweden	[112]
Benzalkonium chloride (BKC) C12	WWTP effluent	* ; 0.033; 0.066; 0.31	Min; Med; Mean; Max	Sweden	[112]
	WWTP effluent	0.11; 0.32; 1.70	Min; Med; Max	France	[113]
	CSO effluent	1.30; 2.60; 5.80	Min; Med; Max	France	[113]
Benzalkonium chloride (BKC) C14	WWTP effluent	* ; 0.024; 0.03; 0.084	Min; Med; Mean; Max	Sweden	[112]
	WWTP effluent	* ; 0.06; 4.60	Min; Med; Max	France	[113]
	CSO effluent	0.42; 0.96; 2.10	Min; Med; Max	France	[113]
Benzalkonium chloride (BKC) C16	WWTP effluent	* ; 0.012; 0.012; 0.013	Min; Med; Mean; Max	Sweden	[112]
	WWTP effluent	* ; * ; 0.08	Min; Med; Max	France	[113]
	CSO effluent	* ; 0.19; 0.38	Min; Med; Max	France	[113]

Active substance	Sample type	Concentration (µg/L)	Measurement type	Country	Reference
Benzalkonium saccharinate	No relevant concentrations found in the literature.				
Didecyldimethylammonium chloride (DDAC) (unspecified)	Seawater	* - 0.27	Range	UK	[116]
Didecyldimethylammonium chloride (DDAC) C10	River water (upstream WWTP)	0.022 - 0.15	Range	Austria	[405]
	River water (downstream WWTP)	0.015 - 0.081	Range	Austria	[405]
	WWTP effluent	0.024 - 0.85	Range	Austria	[405]
	WWTP effluent	* ; 0.012; 0.013; 0.022	Min; Med; Mean; Max	Sweden	[112]
Didecyldimethylammonium chloride (DDAC) C12	River water (upstream WWTP)	* - 0.022	Range	Austria	[405]
	River water (downstream WWTP)	* - 0.019	Range	Austria	[405]
	WWTP effluent	* - 0.16	Range	Austria	[405]
Didecyldimethylammonium chloride (DDAC) C14	River water (upstream WWTP)	*	Range	Austria	[405]

Active substance	Sample type	Concentration (µg/L)	Measurement type	Country	Reference
	River water (downstream WWTP)	*	Range	Austria	[405]
	WWTP effluent	* - 0.26	Range	Austria	[405]
Didecyldimethylammonium chloride (DDAC) C16	River water (upstream WWTP)	* - 0.05	Range	Austria	[405]
	River water (downstream WWTP)	* - 0.05	Range	Austria	[405]
	WWTP effluent	* - 0.17	Range	Austria	[405]
Didecyldimethylammonium chloride (DDAC) C18	River water (upstream WWTP)	* - 0.083	Range	Austria	[405]
	River water (downstream WWTP)	* - 0.19	Range	Austria	[405]
	WWTP effluent	* - 0.21	Range	Austria	[405]
Others					
2,2-dibromo-2-cyanoacetamide (DBNPA)	Industrial effluent	4.20	Single measurement	Spain	[406]
Bromochlorodimethylhydantoin (BCDMH)	No relevant concentrations found in the literature.				

Active substance	Sample type	Concentration (µg/L)	Measurement type	Country	Reference
Bronopol	No relevant concentrations found in the literature. This is likely because it undergoes rapid hydrolysis and biodegradation into transformation products, thus is unstable [407, 408].				
Iodine	No relevant concentrations found in the literature.				

“ * ” denotes that the substance was either not detected, or that concentrations were below the limit of detection or below the limit of quantification.

A comparison of MECs to selective *in vitro* concentrations can be seen in Table 10. For several disinfectants, *in vitro* experimental information and/or environmental concentrations could not be identified (Table 11). In addition, for CHX, the experimental data on resistance is limited to the last three years, whereas for silver (including AgNPs) and copper (including CuNPs), it is limited to the last year (2022 onwards).

Table 9 Effect concentrations of disinfectant active substances that produce a bacterial response vs. concentrations found in the environment. Substances listed in the table are those for which information on both experimental *in vitro* data for bacteria and environmental concentrations have been documented.

Active substances	Range of concentration used in antimicrobial assay (µg/L)[1]	Mechanisms of resistance	Range of concentrations found in the environment (µg/L) [2]
Biguanides			
Chlorhexidine	0.05 – 1,220	• Metabolic change • Membrane permeability change • Porins • Efflux • Increased biofilm formation	0.031(med)[3]-0.033(max)
	500-1,000	• Change in microcosm composition	
Isothiazolones			
CMIT-MIT	3,500-75,000	• Change in OMP • Increased biofilm formation	0.01(min)-144(max)
Quaternary ammonium compounds (QACs)			
BKC	250-400,000	• Outer membrane permeability change • Efflux • Porins • Increased biofilm formation • Increased virulence[4]	0.002(min)-5.80(max)[5]
	0.1-10,000	• Increase antibiotic gene abundance in complex microcosm	
	50,000 µg/L 1,000-100,000 mg/kg	• Change in microcosm composition • Increase antibiotic gene abundance in complex microcosm	

Active substances	Range of concentration used in antimicrobial assay (µg/L)[1]	Mechanisms of resistance	Range of concentrations found in the environment (µg/L) [2]
DDAC	310	• Efflux	0.012(med)-0.85(max)
Phenolics			
DCPP	2,551	• Efflux	0.0001(med)-0.01(max)
Metallic salts, including nanoparticles			
Silver, AgNPs	3,400-480,000	• Increased catalase activity • Upregulation of *kat*E and *sod* gene expression[6] • Efflux	0.001(min)-0.409(max)
Copper, CuNPs	4,000-320,000	• Efflux • Membrane transport • Membrane permeability change • Increased catalase activity[6] • Upregulation of *kat*E gene expression	0.12(min)-740(max)
	1,000 mg/kg	• Co-tolerance of microcosm to tetracycline, oxytetracycline, and chlortetracycline	
Others			
DBNPA	45,000	• Efflux • Mutations	4.20
	125,000	• Change in microcosm composition	

[1] Lowest and highest concentration used in *in vitro* experiments regardless of the bacterial species investigated.
[2] Concentrations range found in diverse environments, including WWTP effluent, river water (downstream WWTP), CSO effluent, river water (upstream WWTP), sea water, river/surface water.
[3] Concentration range: min: minimum; med: medium; max: maximum.
[4] Only one study showed increased expression of virulence genes in *L. monocytogenes.*
[5] BKC concentrations regardless of chain length (C10-C16).
[6] Downregulation of *kat*E and *sod* genes in *S. epidermidis.*

When experimental and environmental concentrations are compared, regardless of the bacterial species or the type of environment (Table 10), it is evident that effect

concentrations found bacterial exposure in *in vitro* experiments far exceed concentrations found in the environment by 10,000-100,000 times or more (except CHX). The concentrations used in *in vitro* experiments are based on the MIC and particularly sub-MIC level (often half MIC). This means that MICs, regardless of the bacterial species, far exceed concentrations found in the environment. When complex microcosms were studied, disinfectant concentrations used for repeated exposure exceeded environmental concentrations by at least 200-10,000 times for BKC, 30,000 times for CHX and DBNPA, but only by 540 times for $CuSO_4$. At the concentrations tested against complex microcosms, a change in microcosm diversity was observed. Jutkina et al. (2018) used a CHX concentration 200 times lower than that of the MIC to investigate the impact of CHX on HGT and showed an increased frequency of HGT from a complex sewage microcosm to an *E. coli* recipient strain [203].

Based on the literature analysed, it is difficult to conclude the impact of concentrations over 10,000 times lower than the MIC. The impact of different sub-MICs on bacterial cells has not been well documented. Wesgate et al. (2020) showed that different sub-MICs had different impacts on *E. coli*. Exposure to CHX at concentrations of 4.7 or 7.5 mg/L resulted in amoxicillin/clavulanic acid or amoxicillin/clavulanic acid and cefoxitin resistance depending on the *E. coli* isolate investigated. Exposure to CHX at concentrations of 0.05 mg/L resulted in active efflux. Exposure to CHX at concentrations of 2 mg/L had a greater impact on metabolism than exposure to concentrations of 5 mg/L. In addition, different sub-MICs had different impacts on gene transfer by conjugation in *E. coli*; whilst conjugation still occurred in the presence of 0.05 mg/L of CHX, 2 mg/L of CHX prevented conjugative transfer in an *E. coli* phenotype [178].

Environmental concentrations might have effects on bacterial cells, but the extent of the effect in relation to AMR, resistance gene abundance and maintenance needs to be investigated. In addition, chemicals and compounds exist in the environment in complex mixtures, thus the impacts of disinfectant and other antimicrobial mixtures at environmentally relevant concentrations on the co-selection of AMR needs to be investigated. Currently, there are too few data on the effects of environmentally relevant concentrations of disinfectants to draw conclusions on their relevance to AMR in the environment.

Table 10 Disinfectant active substances for which information on concentration is missing either from *in vitro* experimental data and disinfectant analysis or documented environmental concentrations.

Active substances	Comments
BCDMH	No relevant environmental concentrations found in the literature.
Benzalkonium saccharinate	No relevant information on the effect of exposure on resistance **AND** no relevant environmental concentrations found in the literature.
Biphenyl-2-ol (Phenyl phenol)	No relevant information on the effect of exposure on resistance.
Bronopol	No relevant environmental concentrations found in the literature. This is likely because it undergoes rapid hydrolysis and biodegradation into transformation products, thus is unstable [407, 408].
Chlororesol	No relevant information on the effect of exposure on resistance.
Iodine	No relevant environmental concentrations found in the literature.
Isothiazolones (BIT)	Literature search on resistance not yet performed due to high number of potential relevant publications.
PHMB	No relevant environmental concentrations found in the literature.

9 Fungal, viral and protozoan resistance to disinfectants used in the UK

This section of the report aimed to signpost the available literature from scientific databases relating to the role of a select list of disinfectants used in the UK in the development of AMR in fungi, viruses and protozoa. The disinfectants of interest are the same as those used in Section 7 and represent the refined list of the active substances authorised (or in the process of authorisation) for use in disinfectants in the UK or Europe. The refined list includes active substances that are: a) relevant to UK disinfectant use scenarios; b) likely to be selective for AMR; and c) likely to exist in measurable concentrations in the environment (i.e., persistent or slow biodegradation) (Table 6).

9.1 Literature search

9.1.1 Search strategy

The databases searched were PubMed and Web of Science. The keywords used are the commercial and chemical names for the biocides of interest, “resistance” and “virus”, “fungi” or “protozoa” (Table 11). This is intended to be a rapid preliminary screening of the peer-reviewed literature. When the number of “hits” was low, the relevance of the article was checked by screening the abstract only.

9.1.2 Limits

There were no limits of time or geographical location imposed on this search.

9.1.3 Data analysis

For this section, no formal data analysis was undertaken as this was to be a scoping exercise of the literature found. Instead, the number of search results and potentially relevant articles are presented in Table 11.

Table 11 Literature search on "resistance" of viruses, fungi or protozoa to biocides. Numbers represent the number of potential articles published in an initial screen.

Active substance	Keywords	Search results (keywords + resistance)					
		WoS			PubMed		
		+ fung*	+ virus	+ protozoa	+ fung*	+ virus	+ protozoa
Biguanides							
Chlorhexidine	Chlorhexidine + resistance	45(6)	14 (0)	9(4)	61	23	17
Polyhexamethylene biguanide (PHMB)	Polyhexamethylene biguanide or PHMB + resistance	25(3)	21(2)	8(3)	6	3	11
Isothiazolones							
1,2-benzisothiazol-3(2H)-one (BIT)	1,2-benzisothiazol-3(2H)-one or BIT + resistance	51	102	16(0)	7(0)	14(0)	1(0)
	1,2-benzisothiazol-3(2H)-one or benzilthiazolone + resistance	11(1)	4(0)	0	2(0)	0	0
Mixture of 5-chloro-2-methyl-2H- isothiazol-3-one and 2-methyl-2H-isothiazol-3-one (CMIT/MIT)	2-methyl-2H-isothiazol-3-one or MIT + resistance	119	237	2(0)	74	197	44
	Methylchloroisothiazolone + resistance	0	0	0	0	0	0
	Methylisothiazolone + resistance	0	0	0	0	0	0
Metallic salts							
Copper	Copper + resistance	1,048	164	19(4)	263	93	40

Active substance	Keywords	Search results (keywords + resistance)					
		WoS			PubMed		
		+ fung*	+ virus	+ protozoa	+ fung*	+ virus	+ protozoa
Silver, Silver nanoparticles (AgNPs)	Silver or AgNPs + resistance	4,721	1,795	314	209	268	86
Phenolics							
5-chloro-2-(4-chlorphenoxy)phenol (DCPP)	5-chloro-2-(4-chlorphenoxy)phenol or DCPP +resistance	0	0	0	0	0	0
Biphenyl-2-ol	Biphenyl-2-ol or phenyl phenol + resistance	6(1)	0	0	9(0)	8(0)	2(0)
Chlorocresol	Chlorocresol	0	1(0)	0	3(1)	0	0
Quaternary ammonium compounds (QACs) and amines							
Alkyl dimethylbenzyl ammonium chloride (ADBAC/BKC)	Alkyl dimethylbenzyl ammonium chloride or benzalkonium chloride or ADBAC + resistance	135	126	9(2)	20	19	4(3)
Didecyldimethylammonium chloride (DDAC)	Didecyldimethylammonium chloride or DDAC + resistance	29(1)	13(0)	1(1)	2(0)	2(0)	1(1)
Others							
2,2-dibromo-2-cyanoacetamide (DBNPA)	2,2-dibromo-2-cyanoacetamide or DBNPA + resistance	0	0	0	0	0	1(0)

Active substance	Keywords	Search results (keywords + resistance)					
		WoS			PubMed		
		+ fung*	+ virus	+ protozoa	+ fung*	+ virus	+ protozoa
Bromochlorodimethylhydantoin (BCDMH)	Bromochlorodimethylhydantoin or BCDMH + resistance	0	0	0	0	0	0
Bronopol	Bronopol + resistance	3(1)	0	1(0)	0	0	0
Iodine	Iodine or PVI + resistance	528	471	52	38	59	28

WoS = Web of Science.

9.2 Publications identified

Overall, using the keywords above, the largest number of hits was for silver and copper, then 2-methyl-2H-isothiazol-3-one (likely because of the use of “MIT” as a keyword), iodine (PVI) and BKC. Perhaps surprisingly based on product usage, there were fewer hits for the biguanides, particularly CHX. Looking at the abstract of the papers on biguanide resulted in a much fewer number of articles relevant to this document.

It is possible that some publications on viruses, fungi and protozoa have been missed in this preliminary search and more hits would be generated with specific microorganism names.

The results of this initial search serve as a scoping view of the research landscape relating to the effects of disinfectants on fungi, viruses, and protozoa. Extracting and analysing the data from the articles found, as done with bacteria in Section 7, is an important future step for understanding how resistance in these organisms develops and the effects of disinfectants on other microorganisms in the environment.

10 Conclusions and recommendations

AMR is a growing public health threat of global proportions and crosses the boundaries from public health to the health of animals and the role of the environment in AMR evolution and transmission. Therefore, a holistic 'One Health' approach is required to tackle the AMR. The selection and persistence of AMR are driven by a range of antimicrobial chemicals, including antibiotics, metals and biocidal products (e.g., disinfectants). This report aimed to provide an overview of the scientific understanding on the use of disinfectants in the UK and their potential for the development of AMR in the environment. To achieve this, the report provides a background on disinfectants and their relation to AMR; describes their use in the UK; considers their pathways to and fate in the environment; and compares the *in vitro* selective concentrations to those measured in the environment. The conclusion of this report lists knowledge gaps and a set of recommendations, as described below.

10.1 Disinfectant usage in the UK

UK and EU databases were used to determine the number of active substances authorised, or in the process of authorisation, for use in disinfectants (product types 01-05) in the UK. From this, 123 active substances were identified for use in disinfectants, as of 14th November 2022. This list included alcohols, aldehydes, biguanides, chlorine-related compounds, QACs, and more. Disinfectant use (including their comprising active substances) was found not to be recorded centrally. There are some instances of reporting use volumes to public bodies, for example, fish farms reporting disinfectant usage in aquaculture to SEPA. Disinfectants have a wide range of uses in the UK including in clinical, food production, household and public environments. An overview of potential use scenarios and pathways to the environment was provided.

10.1.1 Limitations and knowledge gaps

The main limitations relating to disinfectant usage in the UK are the lack of usage, sales and manufacturing data. Due to this, only example use scenarios relating to UK usage were discussed, with the volumes of these substances used in these everyday scenarios remaining uncertain.

The lack of available data on disinfectant usage in the UK is a principal knowledge gap limiting our understanding of the potential pressure disinfectant usage may be having on selection and persistence of AMR in UK environments. Disinfectant usage data by industry and concentrations of disinfectant substances used by use case are essential elements of a life-cycle assessment for disinfectants that would inform pathways and receptor models for environmental risk assessments.

10.2 Disinfectants in the environment

The diversity of uses of disinfectants means their pathways to the environment are complex and numerous. For example, disinfectants can enter the environment through wastewater, runoff, spillover from food production processes (e.g., agri- and aquaculture) and as leachate from landfill. The presence of disinfectants (both parent and degraded compounds) through such pathways, allows them to exert selective pressure on microorganisms, potentially leading to the development and/or maintenance of AMR in intermediate sinks (e.g., during the wastewater treatment process) and in environmental sinks (e.g., receiving water bodies). A review of the literature (focused on disinfectants used in the UK), revealed that the environmental fate of disinfectants is variable. Fate depends on the specific chemical or compound (e.g., metals are persistent, whereas other compounds, such as hydrogen peroxide, are so reactive they may not make it to the environment following their use), and multiple environmental factors, including the type of environment (i.e., behaviour is often different in aquatic matrices compared to solid matrices, such as soils, sludge and sediment), pH, and temperature. These varying disinfectant fates can include persistence, biodegradation, photodegradation, possible breakdown into degradation products, sorption to organic matter, interaction with other chemicals and compounds, and generation of new products.

10.2.1 Limitations and knowledge gaps

The multiple sources of disinfectants to the environment can make source apportionment challenging, which in turn leads to uncertainty in understanding what activities are most impactful in terms of AMR development. In the absence of usage data, better surveillance of disinfectants substances throughout pathways and in receptor environments would aid our understanding of the relative importance of different pathways to the environment.

10.3 Evidence for the selection of resistance by disinfectants

A semi-systematic review of the literature surrounding the development of bacterial resistance following disinfectant exposure (both to disinfectants and other antimicrobials) was performed for a select group of active substances used in disinfectants in the UK. This list was compiled of 16 substances of interest, chosen for their relevance to UK settings, perceived potential to select for resistance, and presence in the environment. Searching resulted in thousands of hits, mostly focused on silver (including silver nanoparticles), copper (including copper nanoparticles), chlorhexidine and benzalkonium chloride.

Across the literature, many methods of determining resistance or interpreting resistance were used, including measuring changes in antimicrobial (antibiotic (MIC) and/or disinfectant (MBC)) susceptibility, changes in gene expression, and changes in biofilm or microbial community structure. MIC determination was by far the most commonly used protocol, with an increase in MIC as low as 2-fold reported as resistance. Analyses of the results showed the most studied mechanisms associated with a change in antimicrobial

susceptibility were: efflux, change in membrane permeability including change in lipid composition and alteration of porins. Although the clinical or practical impact of sub-MIC exposure to disinfectants was not always addressed, the review of the literature confirmed that bacteria, regardless of the species, respond to sub-MIC exposure to disinfectants, regardless of the disinfectant used, resulting in changes in antimicrobial susceptibility profile and gene expression. Throughout the literature, most of the studies assessing co-selection of antibiotic resistance involved chlorhexidine or benzalkonium chloride.

10.3.1 Limitations and knowledge gaps

Limitations

There are several limitations associated with the literature search. Due to time constraints, and the high number of potential articles of interest, the literature search was limited to the last 3 years (2019-2023) for chlorhexidine, or the last year (2022-2023) for copper and silver. As these substances have been studied since the 1950-60s, the limited years investigated here will have impacted the in-depth analysis of chlorhexidine and the metallic salts and their nanoparticles and antimicrobial resistance, including co-resistance (i.e., the co-selection of antibiotic and biocide resistance genes).

Further, due to time and resource constraints, this report only investigated bacterial resistance to disinfectants, and did not expand on other microorganisms (e.g., fungi, viruses, protozoa). However, potential papers of interest, based on keywords and partial abstract reading for some disinfectants, have been identified. From this initial search, it is apparent that the literature reporting the effects of disinfectants on other microorganisms than bacteria is more limited. Therefore, the same semi-systematic review approach presented here should be carried out for other microorganisms, including fungi, viruses and protozoa.

Knowledge gaps

The literature on disinfectant resistance is rich and mainly focused on changes in susceptibility profiles based on MIC determinations. However, when MICs were investigated, the studies were often observational, and lacked clinical or practical interpretation. Drawing conclusions on emerging resistance based solely on MIC determinations may be unsuitable. Where cross-resistance to antibiotics was investigated, the clinical significance of an observed change in susceptibility was rarely addressed. Further, the literature investigating changes in complex microcosms following disinfectant exposure described changes in microcosm diversity, but the environmental impact of such changes was rarely addressed. This is compounded by the different interpretations of “resistance” and the diversity of methods used throughout the literature. Following the review, it was evident that the term “resistance” was poorly defined and often misleading, with many studies defining “resistance” as an increase in MIC which was often as low as a 2-fold increase. Overall, this diversity in terminology can be problematic when authors do not explicitly define the meaning of “resistance.” In addition, a wide variety of methodologies are being used to measure resistance making data comparison between publications difficult.

Much of the literature investigating the development of bacterial resistance to disinfectants relied on *in vitro* stepwise training, which is based on increasing concentrations stepwise after 24 h growth in broth, starting with a sub-MIC concentration (usually ¼ or ½ MIC). This protocol provides a better understanding of the mechanisms conferring resistance (measured by MIC) that enable bacterial survival at higher concentrations (although still below the in-use concentration). The use of a more realistic exposure protocol is needed to provide a better understanding of the realistic impact of a disinfectant during use.

Bacteria responded to sub-MIC exposures to disinfectants by expressing multiple mechanisms to reduce the disinfectant concentration to a level that does not trigger a stress response. However, the lowest concentration of a disinfectant that induced a stress response was often based on a concentration just below the MIC. With this in mind, understanding the impact of disinfectant concentrations found in the environment on bacterial expression and change in susceptibility is difficult to determine.

Many studies reported on an increase in AMR gene carriage, particularly genes encoding efflux pumps. However, these studies were generally observational and failed to show a link between biocide exposure and increased gene carriage. Thus, the clinical or environmental impact was not addressed.

The majority of *in vitro* studies focused on single disinfectant chemistry, and very few investigated complex formulations (products) containing more than one disinfectant chemistry. The impact of complex chemistries on AMR remains to be investigated. Where complex formulations were studied (e.g., benzalkonium chloride-based products), the product was more efficacious at killing bacteria and less likely to promote AMR. More investigations on the impact of disinfectant products (i.e., fully formulated) is needed to provide a more practical impact on emerging AMR to disinfectants. Likewise, there is limited research on combinations of disinfectants and other antimicrobials, with the majority of co-selection studies focusing on chlorhexidine or benzalkonium chloride.

10.4 The potential for environmental disinfectant concentrations to select for resistance

A review of *in vitro* disinfectant effect concentrations was performed using data gathered from the semi-systematic review of the literature on the development of resistance following disinfectant exposure. Again, this focused on a refined group of active substances used in disinfectants in the UK. These concentrations were then compared to example data gathered from the literature on the measured environmental concentrations of these substances. *In vitro* effect concentrations were related to changes in metabolism, membrane permeability, porins, efflux, biofilm formation, virulence, gene abundance and expression, and community composition. The *in vitro* literature showed that many experiments were conducted using considerably higher concentrations of disinfectant substance, usually based on the MIC, than those measured in the environment. Even in cases where sub-MIC concentrations were investigated, these were mostly higher than environmental concentrations, therefore, making comparisons of *in vitro* effect concentrations and environmental concentrations difficult. Only a limited number of papers on benzalkonium

chloride and chlorhexidine reported use concentrations in par with the highest concentrations found in some environmental matrices.

10.4.1 Limitations and knowledge gaps

Limitations

As the results of the semi-systematic literature review were used, the same limitations exist relating to time constraints, i.e., analysis of the experimental data for chlorhexidine was limited to the last three years, whereas data for silver (including silver nanoparticles) and copper (including copper nanoparticles) were analysed from the last year (2022 onwards). In addition, some disinfectant active substances were not included in the comparison, if they either lacked data on *in vitro* effect concentrations or if no relevant environmental concentrations could be found. This included polyhexamethylene biguanides, 1,2-benzisothiazol-3(2H)-one, benzalkonium saccharinate, chlorocresol, biphenyl-2-ol, bronopol, povidone iodine and bromochlorodimethylhydantoin. Further, this comparison was only performed for bacteria, and did not include other microorganisms such as fungi, viruses and protozoa.

Knowledge gaps

Based on the literature from the semi-systematic review, the concentrations used in experimental *in vitro* studies were based on bacterial MICs and often far exceeded (>10,000 times) those reported in the environment. The only study investigating several low sub-MIC chlorhexidine concentrations, reported different effects on the bacterial cells. It is possible that disinfectants at the concentration found in the environment might have some effect on bacterial cells, but the extent of the effect in relation to AMR, resistance gene abundance and maintenance needs to be investigated. Approaches to determine this may include, for example, determining minimum selective concentration (MSC) ranges for disinfectants.

Different disinfectant chemistries can be found at the same time in the environment. The impact of the sum of the concentrations of the different chemistries, or the impact of the highest concentration of a specific chemistry in relation to the other disinfectants found needs further study. Likewise, as chemicals and compounds exist in the environment in complex mixtures, the impact of disinfectant and other antimicrobial mixtures on the co-selection of AMR needs to be investigated. This would also aid understanding of whether concentrations of disinfectants in the environment, when combined with other stressors, would have additive, synergistic or antagonistic effects on the development of AMR.

10.5 Recommendations

Based on the findings of this review, recommendations for future research and policy needs include:

1. Oversight by regulatory bodies of the volumes of disinfectants used and/or sold as a function of industry-type: food production, industrial, household, and clinical environments. This would allow for modelling efforts to predict concentrations that

may enter the environment. It would also allow for interventions to be drafted for source reduction and monitoring, for the purpose of regulating discharges to the environment.

2. Increased monitoring (of both the volume of studies and variety of substances) of disinfectant residues in different environmental matrices will allow for an assessment of disinfectants on the selection and persistence of AMR in the environment.
3. Expanding the reviews undertaken here, for example to include other microorganisms, such as fungi, viruses and protozoa, will bring greater confidence to our understanding of the current state of the literature on the effects of disinfectant exposure on AMR.
4. Increase understanding of the behaviour and ecotoxicity of complex mixtures that include disinfectants. Generating data on realistic environmental chemical mixtures is important to understand the fate of disinfectants in environmental matrices and role in the development of AMR.
5. Realistic *in vitro* exposure protocols need to be developed to provide clearer environmental and clinical significance to findings.
6. Realistic *in vitro* biofilm protocols need to be developed to better understand the impact of disinfectants on resistance gene abundance and/or maintenance.
7. The definition of the term “resistance” and how resistance should be measured *in vitro* needs to be harmonised.
8. The impact of environmental concentrations of disinfectants, and mixtures including disinfectants, on the development and maintenance of AMR needs to be determined (for example, by determining MSCs).

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Appendix 1

Table S1 Active substances approved or in the process of approval for use in disinfectant product types (01-05) in the UK.

Active Substance	Approved for use in product type	
	HSE	ECHA
(+)-Tartaric acid	PT02, PT03, PT04	N/A
1,2-benzisothiazol-3(2H)-one (BIT)	PT02	PT02
2,2-dibromo-2-cyanoacetamide (DBNPA)	PT04	PT02, PT04
2-bromo-ethanoic acid (Bromoacetic acid)	PT04	PT04
2-Phenoxyethanol	PT01, PT02, PT04	PT01, PT02, PT04
2-Propenoic acid, 2-methyl-, butyl ester, polymer with butyl 2-propenoate and methyl 2-methyl-2-propenoate (CAS nr: 25322-99-0)/ Polymeric quaternary ammonium bromide (PQ Polymer)	N/A	PT02
4-chloro-3-methylphenol (Chlorocresol)	PT01, PT02, PT03	PT01, PT02, PT03
5-chloro-2-(4-chlorphenoxy)phenol (DCPP)	PT01, PT02, PT04	PT01, PT02, PT04
6-(phthalimido)peroxyhexanoic acid (PAP)	PT01, PT02	PT01, PT02
Active bromine generated from sodium bromide and calcium hypochlorite	PT02	PT02
Active bromine generated from sodium bromide and chlorine	PT02	PT02
Active bromine generated from sodium bromide and sodium hypochlorite	PT02	PT02
Active bromine generated from sodium bromide by electrolysis	PT02	PT02
Active chlorine generated from chloride of ambient water by electrolysis	PT02	PT02
Active chlorine generated from magnesium chloride hexahydrate and potassium chloride by electrolysis	PT02	PT02

Active Substance	Approved for use in product type	
	HSE	ECHA
Active chlorine generated from sodium chloride and pentapotassium bis(peroxymonosulphate) bis(sulphate)	PT02, PT03, PT04, PT05	PT02, PT03, PT04, PT05
Active chlorine generated from sodium chloride by electrolysis	PT02, PT03, PT04, PT05	PT01, PT02, PT03, PT04, PT05
Active chlorine generated from sodium N-chlorosulfamate	PT04	PT04
Active chlorine released from calcium hypochlorite	PT02, PT03, PT04, PT05	PT02, PT03, PT04, PT05
Active chlorine released from chlorine	PT02, PT05	PT02, PT05
Active chlorine released from hypochlorous acid	PT01, PT02, PT03, PT04, PT05	PT01, PT02, PT03, PT04, PT05
Active chlorine released from sodium hypochlorite	PT01, PT02, PT03, PT04, PT05	PT01, PT02, PT03, PT04, PT05
Alkyl (C12-16) dimethylbenzyl ammonium chloride (ADBAC/BKC (C12-C16))	PT01, PT02, PT03, PT04	PT01, PT02, PT03, PT04
Alkyl (C12-18) dimethylbenzyl ammonium chloride (ADBAC (C12-C18))	PT01, PT02, PT03, PT04	PT01, PT02, PT03, PT04
Alkyl (C12-C14) dimethyl(ethylbenzyl)ammonium chloride (ADEBAC (C12-C14))	PT01, PT02, PT03, PT04	PT01, PT02, PT03, PT04
Alkyl (C12-C14) dimethylbenzylammonium chloride (ADBAC (C12-C14))	PT01, PT02, PT03, PT04	PT01, PT02, PT03, PT04
Amines, N-C10-16-alkyltrimethylenedi-, reaction products with chloroacetic acid (Ampholyt 20)	PT02, PT03, PT04	PT02, PT03, PT04
Benzoic acid	PT03, PT04	PT03, PT04
Biphenyl-2-ol	PT01, PT02, PT03, PT04	PT01, PT02, PT03, PT04
Bromoacetic acid	N/A	PT04
Bromochloro-5,5-dimethylimidazolidine-2,4-dione (BCDMH/Bromochlorodimethylhydantoin)	PT02	PT02

Active Substance	Approved for use in product type	
	HSE	ECHA
Bronopol	PT02	PT02
Calcium dihydroxide/calcium hydroxide/caustic lime/hydrated lime/slaked lime	PT02, PT03	PT02, PT03
Calcium magnesium oxide/dolomitic lime	PT02, PT03	PT02, PT03
Calcium magnesium tetrahydroxide/calcium magnesium hydroxide/hydrated dolomitic lime	PT02, PT03	PT02, PT03
Calcium oxide/lime/burnt lime/quicklime	PT02, PT03	PT02, PT03
Chlorine dioxide	PT02, PT03, PT04, PT05	PT02, PT03, PT04, PT05
Chlorine dioxide generated from sodium chlorate and hydrogen peroxide in the presence of a strong acid	PT02, PT05	PT02, PT05
Chlorine dioxide generated from sodium chlorite by acidification	PT02, PT03, PT04, PT05	PT02, PT03, PT04, PT05
Chlorine dioxide generated from sodium chlorite by electrolysis	PT02, PT03, PT04, PT05	PT02, PT03, PT04, PT05
Chlorine dioxide generated from sodium chlorite by oxidation	PT02, PT03, PT04, PT05	PT02, PT03, PT04, PT05
Chlorine dioxide generated from Tetrachlorodecaoxide complex (TCDO) by acidification	PT02, PT04	PT02, PT04
Cinnamaldehyde / 3-phenyl-propen-2-al (Cinnamic aldehyde)	PT02	PT02
Citric acid	PT02	PT02
Copper	PT02, PT05	PT02, PT05
Copper sulphate pentahydrate	PT02	PT02
Cyanamide	PT03	PT03
D-gluconic acid, compound with N,N″-bis(4-chlorophenyl)-3,12-diimino-2,4,11,13-tetraazatetradecanediamidine(2:1) (CHDG)	PT01, PT02, PT03	PT01, PT02, PT03
Dialuminium chloride pentahydroxide	N/A	PT02
Didecyldimethylammonium chloride (DDAC (C8-C10))	PT01, PT02, PT03, PT04	PT01, PT02, PT03, PT04
Didecyldimethylammonium chloride (DDAC)	PT01, PT02, PT03, PT04	PT01, PT02, PT03, PT04
Dimethyloctadecyl[3-(trimethoxysilyl)propyl]ammonium chloride	PT02	PT02

Active Substance	Approved for use in product type	
	HSE	ECHA
Disodium peroxodisulphate (Sodium persulphate)	PT04	PT04
Ethanol	PT01, PT02, PT04	PT01, PT02, PT04
Ethylene oxide	PT02	PT02
Formaldehyde	PT02, PT03	PT02, PT03
Formic acid	PT02, PT03, PT04, PT05	PT02, PT03, PT04, PT05
Free radicals generated in situ from ambient air or water	PT02, PT03, PT04, PT05	PT02, PT03, PT04, PT05
Glutaral (Glutaraldehyde)	PT02, PT03, PT04	PT02, PT03, PT04
Glycolic acid	PT02, PT03, PT04	PT02, PT03, PT04
Glyoxal	PT02, PT03, PT04	PT02, PT03, PT04
Hydrochloric acid	PT02	PT02
Hydrogen peroxide	PT01, PT02, PT03, PT04, PT05	PT01, PT02, PT03, PT04, PT05
Hydrogen peroxide released from sodium percarbonate	PT02, PT03	PT02, PT03
Iodine (Polyvinylpyrrolidone iodine)	PT01, PT03, PT04	PT01, PT03, PT04
Lactic Acid	PT01, PT02, PT03, PT04	PT01, PT02, PT03, PT04
Lavender oil	PT02, PT03, PT04	N/A
Magnesium monoperoxyphthalate hexahydrate (MMPP)	PT02	PT02
Mecetronium ethyl sulphate (MES)	PT01	PT01
Mixture of 5-chloro-2-methyl-2H- isothiazol-3-one (EINECS 247-500-7) and 2-methyl-2H-isothiazol-3-one (EINECS 220-239-6) (Mixture of CMIT/MIT)	PT02, PT04	PT02, PT04
Monochloramine generated from ammonia and a chlorine source	PT05	PT05
Monochloramine generated from ammonium hydroxide and a chlorine source	PT05	PT05
Monochloramine generated from sodium hypochlorite and an ammonium source	PT05	PT05

Active Substance	Approved for use in product type	
	HSE	ECHA
Monolinuron	PT02	PT02
N-(3-aminopropyl)-N-dodecylpropane-1,3-diamine (Diamine)	PT02, PT03, PT04	PT02, PT03, PT04
n-Decanoic acid (Decanoic acid)	PT04	PT04
n-Octanoic acid (Octanoic acid)	PT03, PT04	PT04
Nonanoic acid (Pelargonic acid)	PT02	PT02
Ozone generated from oxygen	PT02, PT04, PT05	PT02, PT04, PT05
Pentapotassium bis(peroxymonosulphate) bis(sulphate)	PT02, PT03, PT04, PT05	PT02, PT03, PT04, PT05
Peppermint oil	PT02, PT03, PT04	N/A
Peracetic acid	PT01, PT02, PT03, PT04, PT05	PT01, PT02, PT03, PT04, PT05
Peracetic acid generated from 1,3-diacetyloxypropan-2-yl acetate and hydrogen peroxide	PT02	PT02
Peracetic acid generated from tetraacetylethylenediamine (TAED) and hydrogen peroxide	PT02	N/A
Peracetic acid generated from tetraacetylethylenediamine (TAED) and sodium percarbonate	PT02, PT03, PT04	PT02, PT03, PT04
Peracetic acid generated from tetraacetylethylenediamine and hydrogen peroxide		PT02
Performic acid generated from formic acid and hydrogen peroxide	PT02, PT04	PT02, PT04
Poly(oxy-1,2-ethanediyl), α-[2-(didecylmethylammonio)ethyl]- ω-hydroxy-, propanoate (salt) (Bardap 26)	PT02, PT04	PT02, PT04
Polyhexamethylene biguanide hydrochloride with a mean number-average molecular weight (Mn) of 1415 and a mean polydispersity (PDI) of 4.7 (PHMB (1415;4.7))	PT02, PT04	PT02, PT04

Active Substance	Approved for use in product type	
	HSE	ECHA
Polyhexamethylene biguanide hydrochloride with a mean number-average molecular weight (Mn) of 1600 and a mean polydispersity (PDI) of 1.8 (PHMB (1600;1.8))	PT02, PT03, PT04	PT02, PT03, PT04
Polymer of N-Methylmethanamine (EINECS 204-697-4 with (chloromethyl) oxirane (EINECS 203-439-8)/Polymeric quaternary ammonium chloride (PQ Polymer)	PT02	PT02
Propan-1-ol	PT01, PT02, PT04	PT01, PT02, PT04
Propan-2-ol	PT01, PT02, PT04	PT01, PT02, PT04
Pyridine-2-thiol 1-oxide, sodium salt (Sodium pyrithione)	PT02	PT02
Pyrithione zinc (Zinc pyrithione)	PT02	PT02
Quaternary ammonium compounds, benzyl-C12-18-alkyldimethyl, salts with 1,2-benzisothiazol-3(2H)-one 1,1-dioxide (1:1) (benzlkonium saccharinate)	PT02, PT04	PT02, PT04
reaction mass of N,N-didecyl-N-(2-hydroxyethyl)-N-methylammonium propionate and N,N-didecyl-N-(2-(2-hydroxyethoxy)ethyl)-N-methylammonium propionate and N,N-didecyl-N-(2-(2-(2-hydroxyethoxy)ethoxy)ethyl)-N-methylammonium propionate (Redefined from: Poly(oxy-1,2-ethanediyl), .alpha.-[2-(didecylmethylammonio)ethyl]- .omega.-hydroxy-, propanoate (salt) (Bardap 26))	N/A	PT02, PT04
Reaction mass of peracetic acid and peroxyoctanoic acid	PT02, PT03, PT04	PT02, PT03, PT04
Reaction mass of titanium dioxide and silver chloride	N/A	PT01, PT02
Reaction products of aluminium trihydroxide and hydrochloric acid and aluminium and water	N/A	PT02
Reaction products of para-formaldehyde and 2-hydroxy-propylamine (ratio 1:1) / α,α′,α″-trimethyl-1,3,5-triazine-1,3,5(2H,4H,6H)-triethanol (HPT)	PT02	PT02

Active Substance	Approved for use in product type	
	HSE	ECHA
Reaction products of paraformaldehyde and 2- hydroxypropylamine (ratio 3:2) / 3,3′-methylenebis[5-methyloxazolidine] (Oxazolidin/MBO)	PT02	PT02
Reaction products of: glutamic acid and N-(C12-C14-alkyl)propylenediamine (Glucoprotamin)	PT02, PT04	PT02, PT04
Salicylic acid	PT02, PT03, PT04	PT02, PT03, PT04
Silver	PT02, PT04, PT05	PT02, PT04, PT05
Silver borophosphate glass	PT02	PT02
Silver chloride	PT01, PT02, PT04	PT01, PT02, PT04
Silver copper zeolite	PT04	PT04
Silver nitrate	PT01, PT02, PT03, PT04, PT05	PT01, PT02, PT03, PT04, PT05
Silver phosphate glass	PT02, PT04	PT02, PT04
Silver phosphoborate glass	PT02	PT02
Silver sodium hydrogen zirconium phosphate	PT04	PT04
Silver zeolite	PT04	PT04
Silver zinc zeolite	PT02, PT04	PT02, PT04
Sodium dichloroisocyanurate dihydrate	PT02, PT03, PT04, PT05	PT02, PT03, PT04, PT05
Sodium N-chlorobenzenesulphonamide (Chloramine B)	PT02, PT03, PT04, PT05	PT02, PT03, PT04, PT05
Sulfur dioxide generated from sulfur by combustion	PT04	PT04
Symclosene	PT02, PT03, PT04, PT05	PT02, PT03, PT04, PT05
Tosylchloramide sodium (Chloramine T)	PT02, PT03, PT04, PT05	PT02, PT03, PT04, PT05
Troclosene sodium	PT02, PT03, PT04, PT05	PT02, PT03, PT04, PT05
α,α',α''-trimethyl-1,3,5-triazine-1,3,5(2H,4H,6H)-triethanol (HPT)	N/A	PT02

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