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Review

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Reviewing the complexities of bacterial biocide susceptibility and in vitro biocide adaptation methodologies

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Decreased bacterial susceptibility to biocides raises concerns due to their influences on antibiotic resistance. The lack of standardized breakpoints, established methods, and consistent terminology complicates this research. This review summarizes techniques for studying biocide resistance mechanisms, susceptibility testing, and in-vitro adaptation methods, highlighting their benefits and limitations. Here, the challenges in studying biocide susceptibility and the need for standardized approaches in biocide research are emphasized for commonly studied biocide classes.

Our reliance on and usage of antiseptics and disinfectants (biocides) has increased to unprecedented levels since the COVID-19 pandemic[1–3], placing increasing pressure on bacterial antimicrobial resistance (AMR) emergence[4] and strain on our global healthcare systems[5]. The World Health Organization (WHO) defines antimicrobial resistance in bacteria as a change in bacteria, where they no longer respond to antimicrobial drugs used to treat the infection they cause. The WHO defines antimicrobials (as antibiotics, antivirals, antifungals, and antiparasitics) as medicines used to prevent and treat infections in humans, animals, and plants. With respect to bacteria, antimicrobials are often broadly defined as any compound(s) that inhibits the growth of and/or kills/eradicates bacteria; based on this definition it can also include biocides. Prior to discussing and defining biocides, it is important to define antimicrobials and antibiotics as these compounds are frequently used as a guideline when studying changes in biocide susceptibilities. According to the WHO, antibiotics are defined as a drug or medicine that is therapeutically used to treat a bacterial infection. However, depending on the profession and field that applies this term, "antibiotic" can refer to the source of the antimicrobial drug or compound. In this instance, "antibiotics" refer to drugs obtained directly from another living organism (e.g. fungal or bacterial) to inhibit or kill cells, and depending on the user or study it may or may not include synthetically derived (non-living/chemistry sources) antimicrobial drugs[6–8]. Regardless of their isolation sources, the term "antibiotics" is typically reserved and suited for referring to therapeutic drug applications such as those most commonly ingested, injected, or topically applied to skin and tissue surfaces[6]. As a result, the term "antimicrobial" is preferred and recommended by the WHO and other organizations that monitor clinical AMR and antimicrobial compounds, so it includes synthetic sources and compounds from living organisms, to define their known therapeutic breakpoints. For this review, we will use the term "antibiotic" to define medicines originating from both synthetic and living organisms as antimicrobial drugs to distinguish them from antiseptic and disinfectant biocides, as antibiotics is a term more easily recognized as a therapeutic intervention.

Given the medical importance of antibiotics, it is understandable that AMR research is primarily focused on antibiotic phenotypes and mechanisms, as they are clinically relevant for optimal treatment outcomes[4]. However, biocides are an important and vital tool in agricultural safety and clinical disinfection regimes[9], as successful antibiotic treatments are highly dependent upon biocide antiseptic and disinfectant practices and efficacy. In contrast to antibiotics, biocides are often overlooked in AMR considerations until they fail[3,10,11]. A growing number of studies show that bacterial biocide exposures and bacterial resistance to low and sub-lethal biocide concentrations can impact antibiotic resistance phenotypes[12–16] as well as heavy metal resistance[17,18]. Therefore, as part of an AMR One Health Approach, which recognizes that human antimicrobial usage extends far beyond the clinical setting to agricultural, industrial, environmental, and even household contamination[19], it is imperative to better understand how biocide resistance, tolerance, and susceptibility emerges in clinically relevant bacterial pathogens and how they intersect with cross-resistance phenotypes for antibiotics and other inhibitory compounds.

Among the many diverse compounds classified as antimicrobials, biocides are some of the least well-defined, differentially regulated across countries, and widely overused. Based on it's Latin origin, a biocide is a compound that kills a living cell i.e. "*bio*" (life) "*-cide*" (a substance that kills). Many organizations, governments, and regulatory agencies in developed countries such as the European Union and National Health Service in the United Kingdom, define biocides as an agent intended to destroy, deter, render harmless, prevent the action of, or exert a controlling effect on any harmful organism by any means other than mere physical or mechanical action[20–22]. This definition is quite broad, where biocides can be categorized differently and depending on the profession or organization that uses them, how they are formulated (mixtures and concentrations added) and applied

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in different practices (e.g. agricultural[23], industrial[24], clinical[25–27], veterinary[28]). As a result, the term "biocide" can encompass antimicrobials, antibiotics, antiseptics, disinfectants, pesticides, and herbicidal compounds. Additionally, some classes and categories of biocides can also be used as preservatives in products, such as antiseptics and disinfectants (eg. Quaternary ammonium compounds). These compounds are often added to extend the shelf life of products and prevent microbial contamination[29,30]. Given these broad applications, biocides are common additives in a wide assortment of products, from cosmetics and household cleaners to clinical/veterinary disinfection[28], and industrial surfactants[1,24,31]. Antiseptics are defined as biocides that are considered safe enough to use on living surfaces to reduce or kill microorganisms, whereas disinfectants, are defined as biocides used on non-living surfaces[30–33]. It is important to note that, antiseptics and disinfectants can occasionally overlap, for example quaternary ammonium compounds (QAC) such as benzalkonium chloride or cetrimide, can be used as either topical skin antiseptic agents or in disinfectant solutions[2,34]. Antibiotics such as polymyxins can be common antiseptic additives to topical antiseptic skin ointments in addition to their therapeutic applications[35,36]. Other biocide classes, such as the disinfectant glutaraldehyde, are only recommended as a disinfectant used on abiotic or non-living surfaces due to their extreme toxicity[37,38]. Therefore, biocides used as antiseptics and disinfectants can be more nuanced when classifying specific compounds and drugs and can be biased by the profession using them (clinical versus food safety versus industrial).

Biocides function as faster-acting broad-spectrum agents when compared to antibiotics, where they target multiple different cell pathways and cell structures to carry out their biocidal actions; the multiple actions of biocides allow them to be effective against bacterial, algal, fungal, and viral microorganisms[31,32,39]. Their widespread clinical applications and multiple cellular targets of action make these compounds a mainstay in routine disinfection practices. Biocides are an essential human and animal safety component often used in agriculture, food production, and food processing sectors, as well as commercial household products[40–42]. Other biocides (including most antiseptics and disinfectants) are used for various industrial applications, in large quantities in chemical, energy sector, food processing, and other industries. They are used as surfactants or added to reduce pipeline contamination and decontaminate biofouling surfaces caused by microbial biofilm contamination[24,43–45]. Biofilm formation represents a common growth physiology of bacteria, where cells grow as sessile, surface-attached communities encased in protective secreted extracellular polymeric substances[46,47]. Biofilms are naturally more resistant to antibiotics and often show reduced susceptibility to the biocides particularly when biofilms are mature and established, as we rely on biocides to disinfect and eradicate microorganisms from surfaces[45,48–50]. Despite their benefits, the ubiquitous overuse of biocides also adds to environmental contamination and gradually increases biocide sub-lethal exposure concentrations by microorganisms, making more instances of decreased biocide susceptibility inevitable[51–53]. Growing evidence supports that not only are decreases in bacterial biocide susceptibility rates rising[11,45,54,55], but biocide resistance determinants (that result in significant minimum inhibitory concentration (MIC) differences) are closely associated with lateral/horizontal gene transfer mechanisms via mobile genetic elements[15,29,39,56]. Additionally, acquired genomic alterations/mutations that produce biocide resistance phenotypes are increasingly co-associated with antibiotic cross-resistant bacterial pathogens[11,14,52,57,58], many recognized by the WHO as AMR critical and high-priority pathogens. Increasing rates of biocide resistance among bacteria, particularly WHO AMR priority and ESKAPE pathogens, threaten to erode the foundations of our public health disinfection practices. While there is limited evidence of bacteria being able to withstand the high in-use working concentrations of disinfectants and antiseptics, the trend of decreasing biocide susceptibility still threatens the ability to use these compounds in the future. Therefore, understanding the selective pressures and mechanisms driving decreased biocide susceptibility and biocide-associated AMR is more important than ever.

As there are many reviews summarizing biocides of importance[3,9,13,32,55,59,60], biocide resistance rates[11,55,61], and mechanisms of biocide resistance[13,16,31,39,52,60], the focus of this review is to delve further into the challenges of current methodologies used to study and identify biocide resistance mechanisms. The lack of bacterial reference strains collected from biocide surveillance and established biocide breakpoints poses significant problems for studying mechanisms of biocide action and resistance. Consequently, antiseptic and disinfectant biocide resistance values according to their minimum inhibitory concentration (MIC) are commonly experimentally measured and characterized using approaches and methods established for antibiotics. These approaches place a greater emphasis on planktonic bacterial growth methods, which have notable benefits and limitations. Biocides have very different modes of action that target numerous cellular components[60,62] when compared to antibiotics[63]. These differences may explain why there are still so few well-characterized biocide-associated molecular mechanisms known. Here, we summarize current approaches to determine biocide resistance value determinations and resistance mechanisms, with an emphasis on clinically relevant AMR priority ESKAPE pathogens. We will also briefly summarize various in vitro experimental techniques to adapt/evolve bacteria to biocides and molecular methods to screen for bacterial biocide resistance genes in systems-based/omics approaches. Ultimately, these are applied to improve biocide-specific resistance mechanism biomarker/gene identifications. We will also discuss biocide susceptibility testing strengths and limitations for measuring biocide resistance and how these insights may shape future studies of biocide resistance mechanisms in the area of AMR research.

The challenges of defining biocide tolerance, resistance, and susceptibility

Defining biocide tolerance, resistance, and susceptibility in a clinical context becomes somewhat challenging when compared to antibiotics, given biocides have many broad categories and chemistries, they are used at working concentrations that well in excess of their MIC values (10–10,000X), and have more variable applications and standard definitions. For clinicians, many of these definitions understandably rely on medicinal antibiotic definitions which often result in controversy, as few microorganisms have shown resistance to biocides at their high in-use working concentrations[11,55,64–67]. This has led to much debate on how to quantify biocide resistance, tolerance, and susceptibility values given that biocide mechanisms of action can vastly differ from antibiotics. For example, among antiseptic and disinfectant classes, these compounds often act in a concentration-dependent manner, on multiple cellular targets (cell wall, membranes, protein folding, DNA/RNA, reactive oxygen/nitrogen species), and are bactericidal at their recommended working concentrations[68,69]. Due to biocide overuse, dilution, and environmental accumulation, these compounds are showing a steady rise as global pollutants[37,53,70,71], not only in healthcare and household settings[25,53], but in environmental wastewaters and soils[12,72–74]. From a One Health standpoint, there is more urgency to define and quantify the effects of biocides not only at their working concentrations (clinical safety monitoring) but also after their use when biocides dilute environmentally to ascertain the selective pressure they exert on bacterial tolerance, resistance, and susceptibility determinations[70,75].

When defining antibiotic 'tolerance', several reviews define tolerance as the ability of a bacterium to survive transient exposure to antibiotics at concentrations above their MIC value, often at defined time intervals, while 'resistance' is defined as the ability of a bacterium to grow at concentrations above a control or reference strain's MIC value[68,69,76,77]. Defining tolerance and resistance in this manner is comparative and requires knowledge of an established reference strain/isolate to help differentiate distinct and shared mechanisms for each survival strategy between antibiotic exposure time and drug concentrations. For biocides, defining tolerance and resistance is more complicated, as many studies and reviews apply different definitions (some notable examples cited here[11,30,51,75,78–80]). For example, the Maillard et al.[79] study defines biocide "resistance" as a microorganism's ability to survive in-

use/working concentrations of a biocide and recommends the term 'decreased susceptibility' be used when bacteria demonstrate increased MIC or minimal biocidal concentration (MBC) values for biocides[30,79]. From an antibiotic perspective, EUCAST defines "susceptibility" as the likelihood of a compound's therapeutic success when treating a bacterial infection[81]. Furthermore, in the Maillard et al.[79] study, biocide "tolerance" is defined as bacteria exhibiting growth in the presence of low (MIC level) concentrations of a biocide[30,79]. Another example is a recent disinfectant review by van Djik et al., which recommended using antibiotic EUCAST standards for defining tolerance, where tolerance applies to cases where the MBC is much higher than the comparative strain, while its MIC value is unchanged[11]. In the van Dijk et al. review, "resistance" applies to any reduction in susceptibility demonstrated phenotypically by increases in either MIC or MBC from the reference[11]. However, using MIC and MBC values can also have more nuance in definitions. An example of this is the disinfectant review by Cerf et al., who recommended "resistance" be used to describe bacteria that avoid killing based on increased MBC values, and "tolerance" be used to describe biocide adaptation based on increases to inhibitory disinfectant concentrations (i.e. MIC values)[78]. These definitions exemplify the many paradigms for defining biocide *tolerance* and *resistance*, each with logical justifications depending on the applications of the biocide terminology for the studies and descriptions offered. Overall, the Maillard et al.[79] definitions of biocide resistance and tolerance are based on the in-use working concentrations of a biocide, whereas the van Dijk et al. and Cerf et al. reviews base resistance and tolerance on MBC and MIC values as thresholds to determine how bacteria phenotypically interact with a biocide[11,78,79]. These definitions have utility and depend on the context a biocide is being studied toward; for example, if comparing working concentration biocide failure events or if a resistant strain development occurs in environments with lower concentrations of biocide (acting as pollutant) for their affects on microbial biocide susceptibility.

Probing these definitions a bit deeper, based on the Maillard et al.[79] study, using the in-use working concentrations of a biocide would establish practical thresholds for defining organisms as either resistant or tolerant based on clinical and safety recommendation applications. However, these definitions need to be based on the current in-use working concentrations of the biocide antiseptic and disinfectants, which may differ country to country and even by district/province/state, as well as by the biocide manufacturer's product formulation/mixtures; an example of this diversity is the biocide quaternary ammonium compound class[1,2,82]. Defining biocide tolerance and resistance by working concentrations is advantageous as it functions similar to an antibiotic breakpoint value assuming there are set working concentrations. As such, the Maillard et al.[79] definitions are most suitable for diagnostic and surveillance studies as well as programs that need to assess and compare biocide resistance and tolerance to real-world, in-use, working concentrations of a biocide, to accurately assess when disinfection failures occur. These definitions aid and facilitate public health and safety notifications and guide regulatory and policy guidelines and updates. However, these definition terms may be less useful to resolve or identify the often much lower disinfectant/biocide concentrations that may be linked to changes after working concentrations of biocides are used. Lower biocide concentrations around MIC and MBC values are often more relevant and applicable to in vitro experimental biocide and disinfectant mechanistic studies, where small changes of 2–8 fold biocide MIC values can impact antibiotic cross-resistance MICs such as in examples of chlorhexidine and colistin studies[83–85]. In these studies, biocide resistance and tolerance definitions cited by van Dijk et al. and Cerf et al. are more precise and potentially better suited useful for looking at mechanisms of action, as recently shown in the study by Chen et al.[86]. Both the Cerf et al. and van Djik et al. definitions use MIC and MBC values and are analogous to definitions of resistance and tolerance that are used for antibiotics[68]. Despite their differences in defining when a specific MIC and MBC cutoff applies to resistance and tolerance, they are only useful for establishing biocide effects at lower concentrations with bacteria rather than a higher phenotypic breakpoint based on a working concentration[11,78]. As a result, they are less applicable to real-world working biocide applications and clinical safety standard surveillance.

The use of MIC and MBC as biocide definitions by van Dijk et al. and Cerf et al., provides an ability to delve further into biocide mechanisms of actions which include biocide exposure time and concentration effects[11,78], which using only working biocide concentration and mixed biocide manufacturer products might make more challenging based on the Maillard et al.[79] definition of tolerance[79]. Working concentrations of biocides are intentionally fast-acting by design, but after their use and eventual dilution, their actions may become less-concentration dependent and require different exposure times for inhibition/killing. Using the Maillard et al.[79] definitions would mean that all biocide concentrations are well above established MICs, making exposure-time assessments difficult to ascertain during mechanistic studies to determine increased biocide tolerance experimentally. That being said, the biocide exposure time as well as biocide concentration should be considerations in biocide susceptibility measurements and studies. Exposure time represents a knowledge gap in understanding bacterial persistence to biocides, since a better understanding of after-use and sub-lethal biocide exposures are becoming more relevant due to pollution[1,37,53,70,71], which affects the future efficacy of biocides.

Since the main focus of this review is a comparison of methods to study biocide susceptibility and adaptation for mechanistic studies, we define biocide tolerance as the ability to survive transient concentrations of biocides above the MIC for a specified duration based on a control strain under the same durations. We define biocide resistance as the ability to grow at concentrations above a control's MIC value. As we discuss below regarding biocide MIC and MBC values, in the next section, monitoring biocide MIC may be an ideal value to select as MICs are most frequently reported in biocide studies and MBC values are often very close in value (2–4 fold differences) to MIC values[78,87]. Since biocide definitions are contextual and affected by how they are used professionally, future considerations should be made to reconcile the two main biocide definition usages and study paradigms into one cohesive set of definitions. For the purpose of this review, we will use the term "decreased biocide susceptibility" when the context or studies do not pertain specifically to either an MIC based resistance or tolerance or if this was not defined in any cited studies.

Biocide susceptibility testing methods: measuring minimum inhibitory and bactericidal concentrations of biocides

Antimicrobial susceptibly testing (AST), or in the case of biocides, biocide susceptibly testing (BST), is a method vital to quantify a given bacterial strain's ability to withstand a certain concentration of antimicrobial over a predetermined amount of time. Similar to AST methods, BST calculates the minimum inhibitory concentration (MIC) of biocide from an assays that monitors the lowest concentration of biocide that inhibits the growth of a bacterial mono-culture on nutrient agar or broth[78,87,88]. Due to the bactericidal nature of most biocides, it is unclear if cell growth inhibition (i.e. MIC value) is a sufficient means to measure the extent of the biocide susceptibility of a bacterium to a biocide. Since biocide growth inhibition and cell killing (based on measuring cell viability after biocide exposure) often occur at much closer drug concentration value ranges (sometimes within 2-fold ranges) when compared to antibiotics, a minimum bactericidal concentration (MBC) may be more reliable method for assessing bacterial culture viability and cell killing[78,87]. Assays for measuring MBC values assess cell killing based on total viable cell counts recovered by agar plating after an BST[78,87]. Here, cells from BST assays are subcultured by either agar spread plating or in non-selective growth broth media[62,78,87]. In almost all studies we surveyed in this review, viable cell counts for MBC determinations often did not consider or apply methods to neutralize the added biocide that may carry over with cells recovered from BSTs for viable cell count plating. A cell washing step or neutralization buffer may be an important feature to include in future biocide BST-based MBC testing, similar to Biofilm methodologies minimal biofilm eradication concentration (MBEC) drug neutralization cell washing protocols[89,90]. If cell killing and viability are the important factors to

assess and not simply cell growth inhibition by a biocide, MBC values may represent a more reliable method to assess decreased biocide susceptibility rather than MICs[78,87]. Although MBC can give us greater insights into the viability of a culture during exposure to a biocide, this method does not offer insights into the rates or formation of viable but non-culturable cells (referred to as VBNC). VBNCs have been noted for QACs exposed *Listeria* spp.[91] and may be a present but overlooked biocide-induced phenomenon in ESKAPE pathogens when only applying viable cell culturing methods.

In addition to concentration-dependant viability, MBCs can also be used to measure the viability of a cell culture in a time-dependant manner by subculturing and measuring the viability over time, the minimum duration of killing (MDK) of the biocide at a given concentration can be determined. Unfortunately, far fewer studies report both the MIC and MBC values for a biocide, and even fewer study the broad ranges of biocide class representatives in use. Most biocide studies using BSTs focus on cationic biocides such as QACs (benzalkonium chloride; BZK) and bisbiguanides (chlorhexidine; CHX), followed by anionic biocides such as triclosan (TCS)[11,55,78,92] and halogenated compounds (sodium hypochlorite) and peroxides[93,94]. There are also sparingly few studies[95] that attempt to identify the minimum duration of killing for biocides, which could be much more relevant for practical, in-use working concentrations of biocide disinfection and should be studied further.

There are several organisations worldwide that aim to standardize tests regarding the efficacy of disinfectant biocides, which include organizations like The American Society for Testing and Materials (ASTM International), the Association of Official Agricultural Chemists (AOAC International), the International Committee for Standardization (ISO), and the European Committee for Standardization (CEN)[28]. While the biocide test methods developed and upheld by these organizations are useful for determining if a new biocide is effective as a disinfectant or antiseptic and help determine the in-use working concentrations of these biocides, there remains no organization or significant attempts at standardizing how to study or interpret decreased susceptibility to biocides (MIC and MBC values) in bacteria. These disinfectant regulating organizations also use very different methods to determine the antimicrobial activity of a biocide; for example, CEN sets a 1 h maximum contact time of disinfectant action with a microorganism, but the activity of antimicrobial drugs set by the Clinical and Laboratory Standards Insrtitute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) is at least 18 h. In addition, CLSI[96] or EUCAST[97] have no recommendations for an appropriate AST method(s) or set breakpoints for defining resistance/tolerance to biocides. This is mainly due to many biocide class compounds being insoluble in water and used at excessive, high, final in-use working concentrations (0.01–10% w/v), well above their MIC (10–10,000X), often in mixed chemical compound cleansers, antiseptic solution formulations, and/or added as preservatives. Some of the most commonly used biocides encompass a wide range of compounds such as cationic biocides (QACs, bisbiguanides), anionic (triclosan, sodium dodecyl sulfate-SDS), oxidizers (peroxides), halogenated (hypochlorites/bleach) and iodophors (e.g. povidone-iodine) as summarized in Table 1. Additionally, the reference organisms used in the standard protocols outlined by organizations, including CEN, to determine the efficacy of a given biocide is currently limited, and does not fully encompass biocide effectiveness towards more diverse microorganisms in environment and within diverse food-bourne contexts, including some ESKAPE organisms[98]. Altogether, these factors make BST determinations of decreased biocide susceptibility much more challenging and complicate microbial biocide surveillance efforts.

Biocide susceptibility testing methods: variations in biocide-specific AST methods

Another confounding factor when researching biocide-reduced susceptibility is the differing AST methods with which to measure biocide susceptibility[88,99]. Biocide susceptibility determination has been defined primarily based on AST methods despite biocides having very different uses and mechanisms than antibiotics. Biocide susceptibility tests often involve agar spot dilution plating or agar disk diffusion (Kirby-Bauer) plating as well as 96/384- well broth microplate dilutions testing to calculate MIC/MBC values[100]. Inconsistencies in the type of AST method for biocide testing is a contributing factor limiting breakpoint determination[62,78]. In general, agar plating methods are beneficial for measuring the less soluble biocides (such as phenolics, iodophors, QACs) that require solvents (DMSO, alcohol) and for counting viable cell colony formation/titres for MBC measurements[101,102]. The disadvantage of using agar plate AST methods is that some highly reactive/labile biocide compounds (e.g. oxidizers) may not be accurately measured due to their instability and rapid chemical actions[9]. Additionally, agar plating methods are much more laborious and time-consuming than broth microplate methods, as plates need to be prepared, colonies grown/enumerated, or zones of inhibition measured for disk diffusion assays. Efforts to automate colony counts and zones of inhibition quantification are becoming more commonplace, as are plate imaging techniques, which require specialized plate imagers or image analysis software (e.g. ImageJ)[103,104].

Broth microplate dilution (96–384 well) AST methods are assessed using optical density/absorbance measuring spectrophotometric readers, which relate planktonic cell growth by cell light scatter[88,105]. An added benefit of the broth microplating technique, is that when the assay is finished, the planktonic cell culture liquid can be removed from the microplate wells to measure cells adhered to the wells, which represent cell biofilm biomass. The residual biofilm biomass in the wells can be quantified by staining the extra polymeric substances biofilms secrete in the wells (e.g. crystal violet dye)[102,106]. These AST biofilms are rarely measured in biocide studies, but as biofilm biocide studies gain more importance, they are slowly gaining popularity[100,104]. The benefits of broth microplate AST are their faster setup and ease of use, when compared to agar plating methods. However, broth AST is limited with respect to measurement accuracy, especially when it comes to measuring cell density via optical density and absorbance quantification. Many biocide compounds (iodophors, phenolics, QACs) absorb and scatter light as their concentrations increase or exceed a critical micelle concentration[9,32,107]. Alternatively, qualitative visual assessments for the presence or absence of growth/turbidity can be used to evaluate broth microplate AST, if the quantitative approaches fail. Broth AST methods are also disadvantaged when it comes to measuring less soluble biocides, which require the addition of solvents (dimethyl sulfoxide or alcohol). These solvents may inadvertently influence MIC and MBC determinations due to their own biocidal actions. High-throughput automation of 96 and 384 well microplate preparations with liquid handling robots or even chips and optical reading analysis software tools are becoming routine for microplate AST, particularly in clinical diagnostic labs, but these systems have expensive cost barriers to operate and establish and have not adopted biocides due to a lack of clinical standards. It is noteworthy that the AST techniques discussed above are all limited in their application to real-world, in-use concentrations of biocides. The goals of most biocide susceptibility tests have been to determine MIC or MBCs, which occur at much lower values than the working concentrations of disinfectants (10–10,000X biocide MIC values), for bacteria towards lower concentrations of biocides as would be seen after working biocide concentrations are used such as in polluted environments.

Another confounding factor in biocide studies and testing is the lack of standardized or optimal media. Many biocides have different chemical activities, for example peroxides and hypochlorites are oxidizing, while QACs, bisbiguanides and SDS have strong ionic and detergent-like properities[30,32,59], which can react with or be adsorbed by the complex organic compounds in rich media or the animal fluids they need to disinfect. Hence, the type of medium used can play influence how bioavailable a given biocide is during testing or study. Rich microbial growth media (such as Tryptic soy, Mueller Hinton, Luria Bertani) components may readily adsorb or interact with biocides due to their high protein and organic matter content[82,108,109]. The influence of protein binding can also bias antimicrobial testing, as even CLSI recommended medium Mueller Hinton, can vary from manufacturer to manufacturer and pose reproducibility problems when

Table 1 | A summary of the working concentrations for commonly overused biocide classes and the experimentally determined MIC values for WHO AMR bacterial pathogens representing similar Gram-positive and Gram-negative genera

Biocide chemical	Biocide class	Organism	MIC of WT bacterial strains (μg/mL)	Working concentration ranges (μg/mL)
Chlorhexidine (CHX)	Cationic; Bisbiguanide	*E. coli* *K. pneumoniae* *S. aureus*	1–8[176,186,202] 4–32[84,176] 0.4–2.5[202,203]	5000–40,000[204 a]
Benzalkonium chloride (BZK)	Cationic; QAC	*E. coli* *K. pneumoniae* *S. aureus*	1–18[176,186,202] 16[176] 1–4[202,205]	100–50,000[206 a]
Cetrimide (CET)	Cationic; QAC	*E. coli* *K. pneumoniae* *S.aureus*	30–50[186,207] 32–128[208] 2–4[176,209]	1000–100,000[210]
Didecyldimethyl-ammonium bromide (DDAB)	Cationic; QAC	*E. coli* *S.aureus*	4.5[211] 2[212]	100–50,000[206 a]
Sodium dodecyl sulfate (SDS)	Anionic; Detergent	*E. coli* *S. aureus*	1000[213,214] 40[215]	1000–5000[216 a]
Triclosan (TCS)	Anionic; Phenolic	*E. coli* *K. pneumoniae* *S. aureus*	0.06–64[176,217] 0.5[176] 0.06–64[217,218]	300–10,000[219 a]
Hydrogen peroxide/peracetic acid	Oxidizer	*E. coli* *S. aureus*	250[94] 250[94]	30,000–75,000[220]
Povidone Iodine (PI)	Iodophor	*E. coli* *S. aureus*	12,000–25,000[221a] 2000–10,000[222,223]	25,000–100,000[224 a]
Hypochlorite (Bleach)	Halogenated	*E. coli* *K. pneumoniae* *S. aureus*	500–1000[94,225] 175[226] 128–512[205]	5000–20,000[220]
Ethanol	Alcohol	*E. coli* *S. aureus*	40,000–80,000[227,228] 5000–110,000[227,229a]	600,000–950,000[220 a]
Isopropanol	Alcohol	*E. coli* *S. aureus*	60,000–130,000[202,225] 64,000–128,000[202,230]	700,000[220 a]

This table showcases significant ranges in bacterial MIC values identified against the working in use concentrations of biocides.
QAC Quaternary ammonium compound.
[a]Values were converted % w/v or v/v to μg/mL using the calculation 1% = 10,000 μg/mL.

testing antimicrobials[108,109]. Additionally, if a biocide is oxidizing in action, such as peroxides and hypochlorites, this can change the actual concentration of the biocide that is available to act on cells, as it can react with medium components and reduce in active concentration[110,111]. Other biocides, such as QACs and bisbiguanides, have detergent-like properties that result in critical micellar concentration formations that can also alter biocide bioavailability[32,112]. Minimal media also have problems with some biocides and their counter ions, causing cross-reactions or salt precipitation with increasing concentrations of a biocide, complicating growth testing. Minimal media or even water-based testing protocols may be better suited to reflect real-world biocide disinfectant applications since they are relied on and used on surfaces that lack defined nutrients.

In addition to media, the type of materials used in a BST assay plate are another factor to consider. Most conventional microplate/petri plate materials are now polystyrene (occasionally polypropylene), and this plastic can have cell culture coatings or be untreated, which can bind and sequester charged biocides (cationic and anionic) compounds differentially, causing variations in AST results[113,114]. For example, Bock et al. found a noticeable decrease in MIC/MBC values for *S. aureus* and *E. coli* when exposed to biocides chlorhexidine and octenidine in 96-well microplates made of polypropylene as compared to polystyrene[113]. Research regarding the interactions of biocides with certain materials is also a growing business, as QAC coatings added to plastics are particularly becoming popular as antibacterial treatments. Research exploring the impacts of biocide plastic coating additives for AMR occurrences and/or biocide interactions/adsorption by plastics is still very limited and often focuses on biofilm formation/inhibition[115]. This presents another confounding variable for BST and understanding how materials themselves could influence biocide MICs/MBCs. Standardizing optimal media and assay plate materials for biocide testing will need to account for how the biocide is being applied, its chemical reactivity/binding to biocides, and what factors, such as bacterial growth inhibition or persistence/eradication in liquid media, are to be measured.

Biocide studies considering practical disinfection safety standards at high-concentrations of biocide at their in-use working concentrations may require other testing methods beyond an AST approach to measure how exposure time affects cell survival. As disinfection and safety testing agencies (ASTM, CEN, ISO) deliberately develop formulations to exceed MIC and MBCs, a more philosophical argument for how to establish and determine what to test for (working biocide formulations or lower concentration pollutants) needs to be resolved by regulatory agencies and researchers. Adding alternative tests to AST methods, such as minimum duration of killing (MDK) assays[68] for any suspected bacterial strains/isolates showing decreased biocide susceptibility by an AST method might be an ideal place to start. MDK assays are more time-consuming as they require more time points to ascertain the minimal amount of time needed to inhibit (MIC) and kill cells (MBC)[68,116]. Assays determining MDK values or other time-based assays could be equally appropriate for biocide susceptibility measurements in addition to MIC/MBC values. For example, MDK can measure the effectiveness of biocides in the shortest time and concentration exposures needed if working concentrations need to be considered. Using AST methods that are developed for antibiotics, which act by inhibiting cell growth and replication, require more extended exposure measurement times; CLSI and EUCAST use 18–24 h incubation time minimums for AST. MDK assays and interpretation of MDK values would provide new challenges as well, as there are no established methods for measuring MDK values for biocides and due to a lack of biocide-tolerant reference strains, so controls may be challenging to establish.

AST methods also have problems considering the highly controlled and in vitro nature of its protocol design. In real-world settings, there is a

high degree of chemical and physical parameter inconsistencies and change in an environment, where environmental conditions like pH, organic load, and nutrient availability could vary greatly in short and long periods of time to affect the growth and selection pressure/mutation frequency of bacteria and the effectiveness of the biocide at a given concentration[51]. Environments are also not a monoculture, and mixed communities of microbes could exert additional chemical and physical influences on biocide susceptibility by a given strain/species similar to antibiotics. Ultimately, methods focused on lower-level biocide concentrations to identify decreased biocide susceptibility can elucidate potential increases in AMR cross-resistance and co-resistance[11,55,61,117]. For this reason, these AST-based studies still hold importance from a One-Health perspective, where our release of mass amounts of biocide waste into the environment could be contributing to further development of biocide-resistant organisms[51,70].

Other techniques for predicting bacterial biocide susceptibility, such as mass spectrometry-based Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) or next-generation whole genome sequencing-based techniques are much more limited and further behind when compared to AST predictions for antibiotics[56,118,119]. This is specifically due to the challenges discussed in the sections above regarding the lack of breakpoints and biocide compound surveillance prioritization[11,87]. These techniques rely on the availability of well-characterized, pre-established biocide susceptibility markers (proteins, lipids, cell wall components) and genes that are only more recently emerging amongst ESKAPE and WHO AMR priority pathogens[120–122]. Furthermore, the majority of biocide susceptibility research frequently studies antiseptic biocides, which include bisbiguanides (e.g. CHX)[16,123], specific QACs (e.g. BZK)[13,124], and anionic biocides (e.g. TCS)[125], despite other biocide uses potentially being far greater amongst other classes, especially since the COVID-19 pandemic[53,70,71].

Molecular techniques facilitating the study of biocide resistance mechanisms

In-depth studies into mechanisms of biocide resistance have most successfully focused on molecular techniques to elucidate the relationship between genotype and phenotype of decreased biocide susceptibility. Some of the most promising methods have been transposon insertion sequencing (TN-seq), transcriptomic RNA-sequencing (RNA-seq), gene knockouts, and/or transposon library screening. A brief discussion of each approach with examples of the genes they identified are summarized and discussed below. As noted in section 2 above, the majority of these in-depth molecular studies have concentrated on bisbiguanides (CHX), QACs (BZK) and TCS resistance mechanisms; hence, these biocides will be focused on in the sections below. It is also important to note that within the studies discussed below, researchers often only study one species or a few species/strains of bacteria. Additionally, the effects of a given mutations or gene expression in a specific strain may not necessarily translate to the same phenotypes (or genotype) in an unrelated or taxonomically distant genera, especially between lab reference strains and environmental/clinical isolates.

Single-gene knockouts for verifying biocide-tolerant gene detection

Similar to antibiotic resistance gene characterization strategies, single-gene knockout methods for reconciling biocide-resistant genotypes to phenotypes have been a commonly used method for independently verifying the role of suspected biocide-resistant genes and those identified through other approaches (omics or TN-seq driven). Several techniques can be used to generate precise single-gene knockouts with or without the use of removal selectable markers, such as the λ Red recombinase flippase (FLP)/FRT site homologous recombination system, clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 gene editing tools, and various suicide plasmids[126]. Single-gene knockout mutants using the aforementioned techniques have generated many gene knockout/TN disruption tools and knockout library collections for many of the ESKAPE and WHO AMR priority genera including *Enterococcus faecium*[127], *Staphylococcus aureus*[128,129], *Klebsiella pneumoniae*[130,131], *Acinetobacter baylyi*[132], *A. baumannii*[133], *Pseudomonas* aeruginosa[134–137], and Gram-negative enterics, such as *Escherichia coli*[138–140].

A significant benefit of single-gene knockouts is the ability to confirm and apply a variety of phenotypic assays on the knockout mutant(s) to explore and validate genotypic changes induced by biocide resistance mechanisms. Complementary multiomic approaches including genomics, proteomics, lipidomics, and transcriptomics have all been used to explore biocide resistance. These techniques benefit from and rely on gene knockouts to confirm gene-transcript-protein target associations. However, single-gene knockouts and gene editing tools for investigating biocide resistance mechanisms heavily rely on prior knowledge of a target gene's role. One of the best examples of this is biocide-selective efflux pump genes, where many articles for QAC (*qac*), CHX (*smvA, aceI*), and triclosan (*triABC*)- selective biocide/multidrug resistance pumps review these systems[141–143] and highlight the importance of gene editing/knockouts in confirming phenotypes. Screening knockout collections is also labor intensive, requiring screens of thousands of library mutants exposed to a single biocide exposure condition. Single gene knockouts and gene editing tools have some notable limitations, specifically, when it comes to genomic alterations in response to biocides that may target essential genes or require compensatory or stabilizing mutations in alternate genes[144,145]. Single nucleotide variants and insertion-deletion mutations (indels) in essential genes under a specified condition can be missed or unaccounted for when screening gene-edited or knockout libraries, biasing these techniques towards non-essential gene involvement. Some genes that have been consistently reported as active or mutated in biocide-resistant isolates, such as the example of *qac* genes, which have not consistently conferred phenotypic changes in biocide susceptibility when cloned and expressed in strains[141,146] or when *qac* sequences are detected in bacterial isolate studies[118]. Biocide resistance often manifests with the mutation of multiple genes to withstand the multi-target cellular effects of biocides[29,54], and single gene knockouts or additions may not provide a noticeable resistance phenotype unless additive mutations are generated. Thus, single-gene knockout screening strategies may not always rule out a single gene as a contributor to biocide resistance. AMR databases cataloging biocide resistance genes are emerging as potential tools to decipher the impact of biocide-induced mutations; some examples are BacMet and Comprehensive Antimicrobial Resistance Databases (CARD)[147,148]. However, more research is needed to expand biocide-resistant and susceptible gene knowledge beyond efflux pump-based biocide susceptibility mechanisms. Additionally, the genes featured in these databases have not always been studied thoroughly and are predicted but not confirmed to contribute to biocide resistance. The following sections below discuss other tools and techniques for addressing in-depth genetic characterization and their roles in molecular biocide resistance mechanism determination.

Transposon (TN) insertion sequencing (TN-seq) for identifying biocide-resistance genes

TN-seq uses the properties of transposons to randomly insert a single time into the genome, allowing the generation of a transposon mutant library (TN-library)[149–151]. This library is composed of a population of cells with randomly inserted transposons covering various coding and non-coding regions of their genomes to generate a library of mutants[150,151]. By subjecting the mutant TN-library cultures to a given biocide exposure condition (typically prolonged sub-lethal concentrations), the fitness of a specific gene(s) can be determined within the TN-library via next-generation sequencing (or PCR amplification in past methods) of the transposons. Mapping the TN locations to a sequenced reference genome helps determine the prevalence of each gene disruption within the library's mutant population[150]. Commonly, TN-seq is useful for determining conditional gene essentiality[152], as essential genes would lack or have fewer transposon insertions while the non-essential genes would be more frequently disrupted under a particular condition[153]. In the context of AMR, if a gene has higher or lower frequencies of transposon insertion under biocide stress relative to the unexposed

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Table 2 | A summary of genes and MIC values identified from lab-evolved/adapted biocide methods to characterize biocide resistance based on antimicrobial susceptibility tests

Adaptation method used	Genus species	Biocide tested	Biocide affected genes identified[f]	MIC fold changes	References
Test tube gradual adaptation (20 passages)	*Enterococcus*[a], *Enterobacter*[b], *Staphylococcus*[c] and *Bacillus*[d] species isolated from organic foods	CET (range of concentrations)	Efflux pump genes: *emeA* *norC* *acrB* *efrB* Antibiotic resistance genes: *ant(4)-Ia*	All species >100-fold increase from WT strain	Gadea et al.[184]
Test tube gradual adaptation (20 passages)	*Enterococcus*[a], *Enterobacter*[b], *Staphylococcus*[c] and *Bacillus*[d] species isolated from organic foods	CHX (range of concentrations)	Efflux pump genes: *sepA* *qacH* *acrB* *efrB* Antibiotic resistance genes: *mecA, mphA, ant(4)-Ia, aph(2)-Ic*	Between 4 to 20-fold increase from WT strain	Gadea et al.[184]
Test tube gradual adaptation (10 passages)	*Escherichia coli* K 12	BZK (range of concentrations in 2 µg/ml steps)	*Proteomic & Transcriptomic analyses* Multiple antimicrobial resistance regulator: Mar regulon	7-8 fold change from WT strain	Bore et al.[200]
Test tube adaptation (time kill exposure)	*Escherichia coli* K-12 MG1655	BZK (40.7 µg/ml)[g]	LPS modifying genes: *lpxM, lpxL* Periplasm genes*: opgG and opgH* General stress*: rssB* Motiliy and chemotaxis*: cheW, cheA, notB, motA, flhC*, Biofilm*: csgD*	ND	Nordholt et al.[187]
Test tube gradual adaptation (32 passages)	*Escherichia coli* K-12	CHX (range of concentrations starting at 0.4 µg/ml)	Outer membrane lipid transporter system: *mlaA*	4–8 fold increase from WT strain	Gregorchuk et al.[186]
Test tube serial dilution adaptation (2–3 days)	*Listeria monocytogenes* (6 unique ribotypes)	BZK (1–10 µg/ml)	*Serotyping & Fatty acid profiling only*	2–4 fold increases from WT strains	To et al.[183]
Test tube nutrient- deprived nasal saline solution (1–16 months)	*Burkholderia cepacia* complex	BZK (53 µg/ml)[g]	ND	No difference from reference isolates	Tavares et al.[231]
Microplate adaptation (500 generations)	*Escherichia col*i MG1655 (40 bioreplicates)	BZK (4 µg/ml)[g] CHX (0.83 µg/ml)[g]	Antibiotic resistance genes*: mlaA gshA, rrfF* Intergenic mutations: upstream of *mdfA*) *rpoB, rpoC, ymfE* upstream of *cdgI, pgpA*, upstream of: *isrC, flu*	ND	Pereira et al.[54]
Microplate gradual adaptation	*Salmonella* serovar Typhimurium (clinical isolates)	BZK (range of concentrations)	Multidrug efflux pump resistance (MDR) regulator: *ramR*	Between 1.3 and 3.2 fold increase from WT strain	Wu-Chen et al.[188]
Agar plate adaptation (24 h)	*Listeria monocytogenes EGD-e*	BZK (4 µg/ml and 6 µg/ml)	Iron Enterobactin efflux pump transcriptional regulator: *fepR* *fepA* Guanidinium-Selective Efflux pump: *sugE*	2–4 fold increases from WT strain	Schulz et al.[192]
Agar plate forming spontaneous mutants (1 day passage)	*Acinetobacter baumannii*	BZK (32 µg/ml)	Ribosomal protein genes: 50S *UTR* *L23* *L24* *S11* *secY* *A1S_1648*	2-fold increase from WT	Knauf et al.[154]
Agar plate method (paper disk diffusion and biocide gradient)	*Escherichia coli*, and *Pseudomonas aeruginosa*	DDAC (20 000 µg/ml)	ND	Between 2-to-9-fold increase from WT	Walsh et al.[193]

Table 2 (continued) | A summary of genes and MIC values identified from lab-evolved/adapted biocide methods to characterize biocide resistance based on antimicrobial susceptibility tests

Adaptation method used	Genus species	Biocide tested	Biocide affected genes identified[f]	MIC fold changes	References
Agar plate method (biocide gradient)	*Acinetobacter baumannii* MBRG165.1 *Escherichia coli* ATCC 25922 *Staphylococcus aureus* ATCC 6538 *Pseudomonas aeruginosa* ATCC 9027 *Enterococcus faecalis*	BZK (100-fold gradient) CHX (100-fold gradient) DDAC (100-fold gradient)	ND	≥4-fold BZK increase for *E. coli*, *P aeruginosa* and *A. bumanii*[a]; ≥4-CHX fold increase *P. aeruginosa*[e]; ≥4-fold DDAC increase *E. faecalis*[e]	Cowley et al.[190]
Bioreactor adaptation (3 years, ~3000 generations)	River sediment (selective for *Pseudomonas aeruginosa*)	Chemical variants of BZK (60:40 mix: 50 µg/ml)	Efflux pump systems: *sugE-A*, *sugE-B*, ATP-binding cassette, resistance nodulation division (RND)	4-fold increase from WT strain	Kim et al.[57]
Bioreactor adaptation (5 days)	Sewage samples (mixed/polymicrobial populations)	BZK (1, 10, and 100 µg/ml)	Antimicrobial resistance genes *qacF, qacH, qacH, mdtG, msrE, mtrD*	ND	Yang et al.[198]

BZK Benzalkonium chloride, *UTR* untranslated region, *WT* wild-type, *CET* cetrimide, *CHX* chlorhexidine, *DDAC* Didodecyl-dimethyl ammonium chloride, *ND* not determined.
[a]*Enterococcus: E. casseliflavus UJA49b, UJA49c, UJA53e, Enterococcus* sp. UJA76g, UJA53t, UJA59t, UJA61a, UJA81m, *E faecium* UJA43a, UJA43b, UJA43c, UJA44b, UJA44d, UJA44e, UJA49e, *E. faecalis* UJA69t, UJA80f.
[b]Enterobacter: E. cloacae UJA81f, E. ludwigii UJA81g, Enterobacter sp. UJA51k.
[c]*Staphylococcus: S. saphrophyticus* UJA47j, UJA69g, UJA69h, UJA72j, *Staphylococcus* sp. UJA69f, UJA73p, UJA76f, UJA76i.
[d]*Bacillus: B. cereus* UJA62p, UJA67p, UJA72g, UJA82t, *B. licheniformis* UJA55q, *Bacillus sp.* UJA49u and UJA55u.
[e]Only included organisms that exhibited stable resistance, i.e. exhibited resistance after growth in media without antimicrobial.
[f]Genes stated were identified and reported as being significantly altered or impacted (overexpressed, mutated) in the resulting adapted isolates using the specified method as compared to the wildtype or parental strain controls within each study.
[g]Converted from original published µM or w/v % value.

control, that gene is considered important to the survival of the cells in that condition[149–151].

TN-seq and its related techniques have been important in elucidating several genetic mechanisms or genes associated with biocide resistance. For example, a TN-seq approach to screen for genes associated with BZK resistance in *Acinetobacter baumannii*, revealed that protein aggregation related to ribosomal gene mutations (L23, L24), 50S ribosome untranslated regions, and *secY* were most frequently associated with BZK exposure[154]. Another TN-seq study of *A. baumannii* exposed to sub-inhibitory concentrations of BZK revealed that efflux mechanisms were responsible for phenotypes, where significant reductions in TN insertions were identified in multi-drug efflux pump operon *adeABC*[155]. Reduced TN insertions within activator genes *adeRS*, and over 100-fold decreases in *adeB* insertions were also noted suggesting that these efflux pump and regulatory genes were important to *Acinetobacter* survival in BZK[155]. Other studies exploring triclosan resistance in *E. coli* associated with fatty acid synthesis gene *fabI*[156–158] used a modified TN-seq technique to include an outward-directed promoter feature within the transposon, called transposon-directed insertion site sequencing (TraDIS)[159]. TraDIS increases the utility of TN-seq by allowing researchers to upregulate the expression of nearby genes that may be associated with biocide resistance, to determine the gene fitness of both essential and non-essential genes[149,159].

Although TN-seq methods are powerful screening tools for screening one-to-two genes at a time, as with gene editing and gene deletion method limitations, it is challenging for TN-seq methods to identify multiple point mutations that may be needed for a stable biocide-resistant phenotype. TN-seq methods also do not replicate the same adaptive selective pressures as biocide exposure and may identify less relevant biomarkers, since only particular TN insertions may be favored under the conditions of testing for a biocide-resistant phenotype, but it may not necessarily be an occurrence that would be identified in environmental or clinical isolates. Additionally, both TN-seq and gene knockouts/editing methods require a substantial understanding of the organism being studied, making them less applicable to understudied organisms or novel strains lacking sequenced genomes.

Whole genome sequencing for identifying biocide-resistance genes

Whole genome sequencing has revolutionized AMR studies by providing a means to predict antibiotic susceptibilities by combining the knowledge derived from decades of experimental molecular characterization associating AMR phenotypes to well-characterized genetic markers and mutations[160–162]. WGS methods are rapidly evolving[163] and are posed to become standard practice in national AMR surveillance programs, aiding in typing strains with changes in susceptibility for epidemiological purposes[164]. Briefly, next-generation WGS tools currently consist of short-read (PacBio-, Illumina-based) and long-read (Oxford Nanopore) approaches which amplify, read, and assemble a library of DNA sequences into organized contiguous segments (Contigs) that eventually can be resolved to produce a final completed genome sequence map. WGS methods are ideally suited for identifying single nucleotide variants, indels, and genetic rearrangements selected for among biocide tolerant/resistant phenotypes. Some examples of studies utilizing WGS in biocide resistance can be found in Table 2. These next-generation sequencing tools have also been modified and applied to metagenomic analyses, which identify and sequence genetic material from mixed/heterogenous communities/populations of microbes without culturing cells individually[165]. Metagenomic approaches have the advantage of identifying many well-known biocide resistance gene associations (again only known biocide mechanisms such as efflux pump genes) to known antibiotic and metal resistance genetic markers, across a variety of AMR One Health environments which typically lack frequent biocide surveillance[165,166].

Both WGS and metagenomic approaches both have disadvantages in that these tools are only as useful as to the extent of what biocide-resistant/susceptible gene knowledge is at the time of study. When compared to antibiotic resistance genes, biocide resistance gene discovery and validation are ongoing, and more experimental validation will be needed to realize the

full power of next-generation sequencing for reliable biocide susceptibility prediction. Machine learning and artificial intelligence (AI) tools may provide key advancements in biocide-susceptibility and resistant gene predictions[167], but AI still requires well curated datasets to strengthen their predictive reliability. Combining these tools with other omics experimental validation will be useful in advancing microbial biocide susceptibility studies. Many of these approaches are discussed in the following sections.

Transcriptional RNA sequencing (RNA-seq) for identifying biocide-resistance

Transcriptomic RNA-seq analysis consists of monitoring transcript (mRNA, total RNA) changes from bacterial cultures exposed to sub-lethal levels or short exposure to lethal levels of a biocide(s) under a variety of physiological conditions[168]. Briefly, extracted cellular RNA is purified and converted into complementary DNA (cDNA) for further analysis. This extraction process can involve additional steps such as selective targeted mRNA amplification and/or the removal of ribosomal (rRNA) or transfer (tRNA). The converted transcript cDNA sequencing requires library generation and often involves short-read next-generation sequencing techniques for library generation. The selection and identification of one or more housekeeping genes for comparing gene expression values across exposure conditions is also crucial for determining gene expression patterns attributed to biocide exposure. The relative abundances of transcript cDNAs are then analyzed and cross-referenced with a reference genome; those cDNA with altered abundances as compared to control conditions identify altered transcript response to antimicrobial exposure[168].

RNA-seq permits genome-wide coverage of transcriptome changes to be identified from a given biocide or multiple biocide exposures and can be a valuable screening tool for identifying expression changes by biocide-associated genes. For example, CHX-exposed *A. baumannii* RNA-seq studies were able to discover and confirm the up-regulation and involvement of a novel CHX-selective efflux pump, *aceI* representing a new family of biocide-selecting pumps in Enterobacterales genera[169,170]. However, another RNA-seq study of sub-lethal CHX exposure to *Streptococcus mutans*, showed alterations to 675 genes related to various pathways involving nucleotide synthesis, transport systems, biofilm formation, and oxidative stress responses[171].

Other studies of transcriptomic changes, such as those determined for *P. aeruginosa* due to sub-lethal exposure to another biocide, isothiazolone, a compound known as Kathon, which is widely used as an antimicrobial preservative, were less clear. In this study only RNA-seq methods were used to explore *P. aeruginosa* Kathon biocide exposure, and it identified over 1500 genes with differential expression caused by this biocide, many related to nitrogen metabolism and oxidative phosphorylation pathways[172]. Without an added method/technique to filter out or enrich for key gene involvement, RNA-seq only studies always benefit from additional experimental techniques to identify particular biocide-resistant genes, pathways, and mechanisms. Examples of these studies include the combination of TN-libraries with RNA-seq approaches are the Knauf et al. 2018 study, which examined BZK-exposed *A. baumannii*[154]. The Knauf et al. 2018 study verified specific roles for RNA-seq transcripts with TN-seq to identify ribosomal and protein folding systems (*secY*) involvement in BZK resistance[154]. A similar approach by Pereira et al. 2020 examined *E. coli* MG1655 exposed to 10 different commonly used antiseptics and disinfectants. This study revealed that transcriptome patterns amongst groups of these biocides showed the common involvement of either a dominant efflux pump component *acrA*, zinc starvation transport systems (*znuABC* and *zupT*), and biofilm formation genes (*zinT* and *zur*) which could be verified using an *E. coli* single gene knockout library[173]. A review of these studies and other QAC transcriptome studies involving *Listeria*[174], *Salmonella*[175] and *Klebsiella*[176] offers a more in-depth discussion of these findings from these QAC-focused transcriptome analyses[177].

Methods for in vitro bacterial biocide adaptation studies

Since robust and frequent AMR surveillance does not include or prioritize biocides in their screening panels, very few established ESKAPE and WHO AMR priority pathogen isolates with verified biocide-resistance are available for study[3,11]. To overcome these gaps, in vitro lab-based biocide adaptation methods have been used to further research towards connecting biocide resistance phenotypes to genotypes. The sections below discuss various laboratory-directed evolution (or gradual adaptation) methods to 'adapt' or 'evolve' bacteria to known quantities and types of biocides. This experimental strategy has been used successfully for antibiotic resistance studies[178–181], and has also been applied to biocide resistance. Some of the challenges with lab-adaptive experiments are the many techniques that can be used for the research: test tubes, microplates, agar plating, and continuous-culture/bioreactor-based approaches. These adaptation methodologies have strengths and limitations and will be discussed in more depth below.

Test tube and microtitre plate biocide adaptation techniques

Among the many biocide-adapted test tube approaches, slight differences in how test tube adaptations are applied make assessments of findings even by the same species to the same biocide highly variable. The core experimental design of the test tube adaptation method consists of growing a nutrient broth mono-culture stock of a species/strain selected for adaptation (which also serves as the unadapted control) by repeatedly subculturing that stock in test tubes supplemented with sub-lethal sub-MIC values of a biocide in broth. Typically, the tube with the highest growth turbidity (either by optical density or visual assessment) at the highest drug concentration is selected for reinoculation into broth culture containing either the same biocide concentrations or slight increases in biocide concentration from the previous set of cultures (Fig. 1A). Most adaptation studies have used one or more variations of this basic test tube adaptation technique design, and they are summarized in Table 2. Some test tube methods adapted their strain(s) to the lowest biocide concentration that showed growth for further subculture adaptation rounds[182]. Other methods shortened or substantially extended the number of adaptation subculturing performed from 1 or 3 days[183] to 10[164], 20[184,185] and even 32 subcultures[186], while some studies used a single-time kill test tube exposure assay[187]. A few published examples adapted a collection of isolates representing different genera collected from contaminated foods/environmental sources[184,185] or similar strains of clinical isolate collections to a single biocide using the adaptation technique[183]. In some studies, the 'adapted' final isolates from these tests were subjected to further AST to other selected biocides or to antimicrobials to assess the final isolate's cross-resistance profile[184–186].

In general, there does not appear to be any correlation between adaptation subculturing durations/repetitions or even starting drug concentrations to the final adapted isolate's fold change increase in MIC value as shown in Table 2. In the few studies that examined the antimicrobial cross-resistance of their final adapted isolates, there also was no consistency in MIC values or cross-resistance profiles, even for the same species and biocides used for test tube adaptation (see QAC and CHX examples Table 2), making it challenging to compare findings[184–186]. This could be due to the large variation in genes that can be altered in response to biocides and their cellular targets but it may also be confounded by the use of different growth methods (media type, incubation time, biocide concentration used) and strains in the various studies, all of which could have an impact on the genes mutated and resulting susceptibility changes. With respect to test tube biocide-adapted methods for identifying genetic alterations in their final biocide-adapted isolates compared to the wild type or parental strains, a variety of molecular techniques were used to identify genes (WGS, TN-library, multiomics); few unrelated studies identified the same genes for the same adapted biocide species tested, showcasing the variety of different genes that can be altered when bacteria adapt to biocides (Table 2). Regardless, many common themes were noted with respect to the functional roles of genes identified among the different biocide-adapted isolates. Many genes identified had prior associations with established AMR genes and their peripheral mechanisms, suggesting their implication in biocide resistance. However, the presence of these genetic mutations does not necessarily

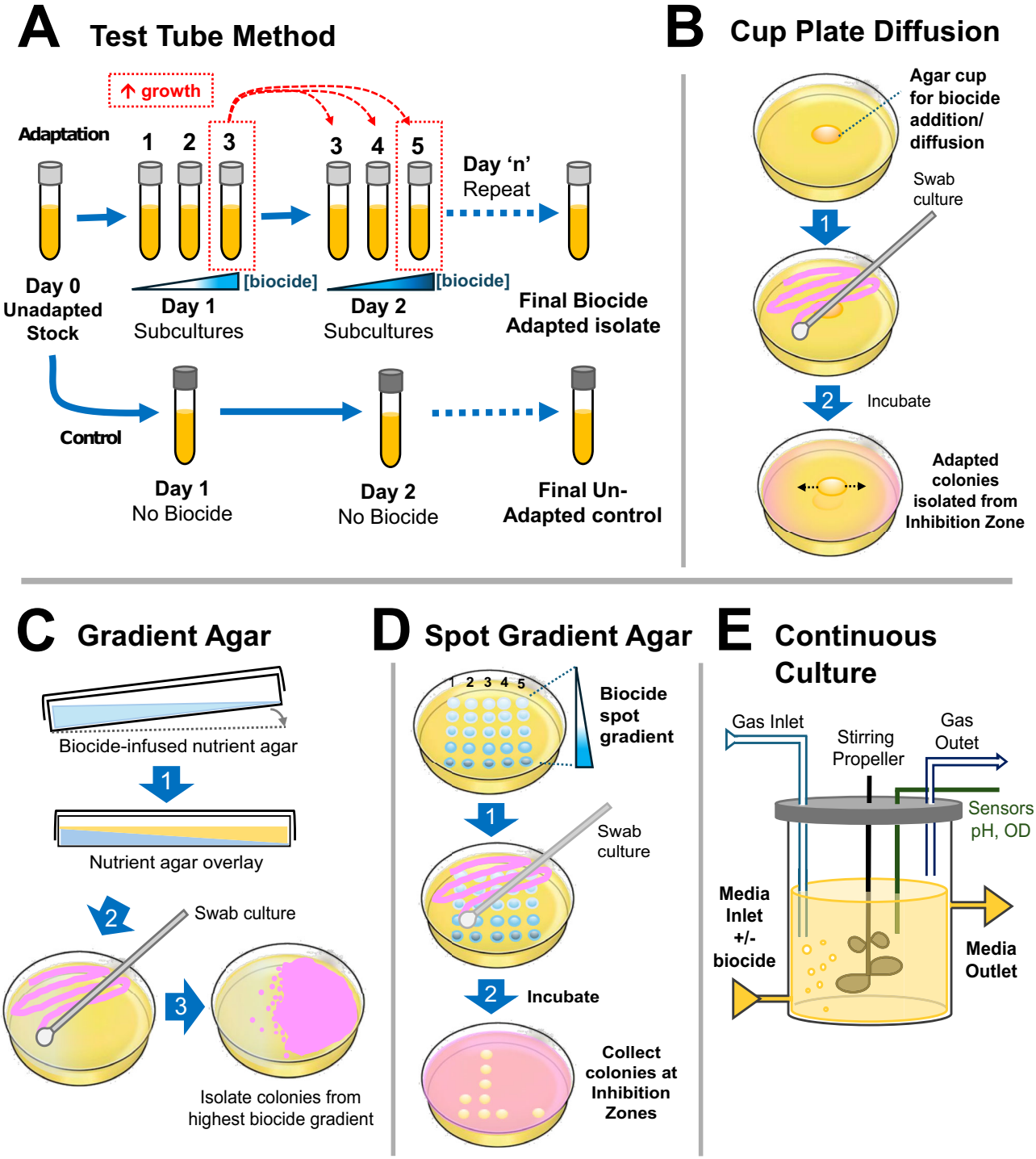

Fig. 1 | A summary of the various techniques used for in vitro biocide adaptation of bacteria. In all panels, a right-angle triangle represents biocide concentration gradients, where the increasing biocide (blue) intensifies as its concentration increases. Bacterial colonies (panels **B**–**E**) are colored in pink for clarity. **A** The test tube biocide adaptation experiment core design is shown based on previously published methods listed in Table 2. Numbers above Day 1 and Day 2 subcultures represent gradually increasing arithmetic dilutions of a given biocide and red boxes show an example of a culture with the highest measured growth. **B** Cup plate dilution agar experimental design; black dotted arrows shown on the agar plate after the step 2 represents the zone of biocide growth inhibition[193]. **C** The gradient agar biocide adaptation method is shown[193]. **D** The spot gradient biocide agar technique at step 1, requires a ≥1 h time incubation for spotted biocides to adsorb into the agar[190,194,195]. **E** A continuous culture biocide adaptation technique is shown. Continuous culture designs can be machine-automated or operated manually with peristaltic pumps[196]. The duration of the culture incubation period and when to sample adapted isolates are determined by the operator (ranges from days-years). The images shown in this diagram were generated using Inkscape v. 1.4 graphic design tools.

imply causation as some identified gene functions in biocide resistance were not confirmed by separate experimentation or validation (Table 2). However, the repeated identification of the same gene by the same biocide exposure between different adaptation studies was not shown (Table 2). Similar to agar plating methods, preparation of test tube adaptions is laborious, requiring dozens of test tubes for a single bioreplicate and then added time to grow and subculture or transfer cells to measure optical densities. The larger culturing volume capacity of test tubes offers added

benefits for direct cryopreservation of intermediate and final isolates, in addition to optical density measurements throughout each subculturing round of an adaptation process, which is a benefit noted in a few biocide adaptation studies[186]. Cryopreservation and genetic analysis of intermediate isolates during a biocide adaptation may help refine the optimal duration to expose and adapt isolates towards a given biocide class.

Lastly, a similar approach to test tube biocide adaptation in vitro is by using lower volume and higher throughput microtitre plating methods. The same experimental design conditions described for test tube adaptations above are applicable to microtitre plate adaptations, and the lower volumes and greater well options (96/384) make microplate arrays an ideal system to expose far more strains, and their bioreplicated cultures, to more biocide conditions in a high throughput manner at each desired adaptation subculturing stage (Fig. 1B). In contrast to test tube methods, microplate adaptations are far less represented in in vitro biocide adaptation studies (Table 2). Pereira et al. applied this approach to a collection of 10 biocides to lab evolve *E. coli* MG1655 in a 96 well plate system[54]. In this study, four bioreplicates of each *E. coli* biocide-exposed isolate were grown in 12 h microplate subculture intervals for a total of 500 generations. Among the final 40 biocide-adapted isolates, 17 demonstrated significant increases in resistance to a collection of clinically relevant antibiotics[54]. Other studies involving dairy farm isolated *P. aeruginosa* and *Salmonella enterica* sv. Typhimurium used the 96 well microplate adaptation method to study BZK[188,189]. Both methods inoculated strains that grew in the highest biocide concentration in a fresh well with higher concentrations of BZK[188,189]. This method offers researchers an opportunity to test multiple biocides on the same plate and increase the number of generations and bio-replicates. As discussed for test tube adaptations above, the identification overlap of biocide-resistant genes was not noted; however, AMR functional roles were a dominant theme (Table 2). A notable disadvantage of microplate adaptation techniques is due to their lower volumes, which may easily evaporate in the well over longer exposures/growth incubations, which can concentrate the biocide (and if required, its solubilizing agent). Evaporation can magnify the biocide exposure effect more than intended, potentially yielding false negative adaptation outcomes.

As so many variations in core test tube/microplate adaptation techniques have been used in past studies, it is difficult to confidently determine what factor(s) may contribute to increased MIC/MBC values or their genetic alterations. There may be different biocide resistance mechanisms responsible for or relied upon by different planktonically growing genera in broth, such as the microliter to ml volumes used which influence factors that include nutrient availability, oxygenation, or cell density/biofilm surface accumulation. Variability in test tube adaptation findings may also highlight the numerous differences between biocide mechanisms of action and their cellular targets[9,190,191]. Ideally, future studies will benefit from ensuring their experimental test tube adaption design considers factors that include what biocide to select/prioritize, the minimum-maximum drug concentration ranges to test, and what starting culture strains/genera/species to prioritize, in addition to the optimal number of adaptation subculture rounds. These factors may help narrow in on biocide resistance genes and mechanisms across genera for a given biocide.

Agar plating techniques for in vitro bacterial biocide adaptation

Agar plating techniques for in vitro biocide adaptation have also been used to identify biocide resistance mechanisms. In contrast to test tube/microplate broth adaptation methods, various experimental designs have been used to test different biocide concentrations in agar plates (Fig. 1B–D). The most straightforward biocide adaptation agar plating design example that is most similar to the test tube adaptation method was used by Schulz et al. 2023. In this study, a fixed concentration of sub-inhibitory QAC biocides (BZK or Cetyltrimethylammonium bromide; CTAB) was added to nutrient agar plates to culture-streaked *Listeria monocytogenes* isolates[192]. Colonies that grew on the highest biocide concentration as compared to a susceptible wildtype, were selected for and re-streaked repeatedly (2–4 subculture rounds), each time on agar plates with gradually increased QAC concentrations[192]. WGS of the adapted *L. monocytogenes* isolates revealed the involvement of multidrug efflux pump and ciprofloxacin resistance regulator gene *ramR*[192].

Other agar plating methods have employed different experimental design strategies (Table 2). In a study by Walsh et al., various plating techniques such as paper disk diffusion, cup plate diffusion, and biocide gradient agar plates were used to adapt *E. coli*, *S. aureus*, and *P. aeruginosa* to various QAC including didecyldimethylammounium chloride (DDDMAC or DDAC)[193]. To perform the disk diffusion technique, paper disks were soaked in the desired biocide concentration and then were added to the surface of agar swabbed with bacteria and incubated for colony lawn formation. The surviving colonies closest to the zone of inhibition around the paper disk were isolated and analyzed again with disc diffusion AST[193]. For the "cup plate diffusion" technique, an agar plate pre-swabbed with culture inoculum had a defined volume of biocide was placed in a well in the agar, where a zone of growth inhibition was determined and surviving colonies at the inhibition halo were assessed further by AST[193] (Fig. 1B). For the gradient agar plates tested by Walsh et al. 2003, this experiment used plates prepared with a single fixed concentration of biocide-infused nutrient agar that was allowed to solidify on an angle then another layer of nutrient agar only was overlaid and allowed to solidify on a 180° surface (Fig. 1C). These biocide gradient agar plates were swabbed to produce a lawn of colonies, and the colonies capable of growth at the highest concentration of biocide on the plate were collected for further AST[193]. Out of these three plating techniques by Walsh et al., only the biocide disk diffusion and the biocide gradient plate methods (2/3 methods) resulted in phenotypically stable QAC-resistant mutants[193] Table 2). It is important to highlight these findings suggest that the development of stable biocide resistance phenotypes to their respective biocides at their recommended working concentrations may be more difficult to achieve than using lower biocide concentrations around its MIC and MBC values.

An alternative spot dilution gradient agar biocide adaptation technique was used in a number of studies, where a set of biocide dilutions were spotted directly onto a pre-solidified nutrient agar plate, then allowed plates to dry to adsorb the biocides before applying a bacterial culture overtop[190,194,195] (Fig. 1D). The spot-gradient agar adaptation technique was used to adapt numerous genera to biocides and antimicrobials. Biocide-adapted bacterial colonies were isolated from the highest biocide gradient region of the plate were re-streaked onto spotted biocide gradient plates, and grown for 4 days[190,194,195]. The process was repeated until a total of 10–14 passages/re-streaking rounds was completed. One study using this technique, reported that the AST findings for >9 genera were 11-fold lower for both MIC and MBC value for formulated biocides than non-formulated pure chemical biocide stocks[190]. Additionally, most adapted bacteria-to-biocide isolates generated by the approach produced 2–8-fold change increases in MIC and MBC values[190].

The advantages of agar plating adaptation techniques, particularly the "in-agar" biocide gradient agar plating adaptation method of Walsh et al., may be their potentially faster and more straightforward timeframes to generate biocide-adapted isolates from a single experiment[193]. However, for spotted biocide gradient agar adaptation studies, these methods required multiple (10–14) rounds of agar plate adaptations before any AST were performed and often reported 4-fold increases in MIC and MBC values of their final adapted isolates[190,193]. It's also unclear if the biocide-adapted isolates on agar plates represent planktonic physiologies or biofilms[101], and how their biocide resistance phenotypes may differ in liquid biofilms versus colonies formed at the air surface interface on agar surfaces. Disadvantages of agar plating techniques are similar to those described for biocide susceptibility agar testing methods. Some specific limitations of these adaptation methods are due to the reliability of biocide drug solubility and penetration/diffusion in the agar, which may differ between techniques and biocide classes applied. Other challenges for these techniques are concerns of plates drying out during longer incubations. For example, some agar

plating methods required 2–4 day incubations before colonies could be visibly detected[190,194].

Continuous culturing techniques for in vitro biocide adaptation studies

Bioreactors are often used as microbial culturing vessels to generate controlled environmental conditions for cells to continuously grow in culture and can be referred to as chemostat cultures. The simplest experimental designs have a culture growth chamber fed by an inlet reservoir of fresh nutrients and an outlet to remove and stabilize culture volumes in the main vessel during the incubation process[196] (Fig. 1F). More automated chemostat bioreactor systems where flow rates and growth conditions are electronically monitored and automated and these tools have been applied to antibiotic resistance studies[197] as well as biocides. Biocide adaptation bioreactor studies have very different applications than the static techniques discuss above in previous sections. For example, one study by Kim et al.[57] highlights the power of this technique for growing and sustaining polymicrobial/mixed species communities from a single environmental sample. Here, bioreactors were filled with nutrient broth (50:50 dextrin: peptone) and inoculated with a BZK-contaminated river sediment microbial sample, then maintained in a continuous culture for 1 year. This 1 year continuous culture source was used as an inoculum for another bioreactor fed the same nutrient broth with added QAC mixture (60:40 of benzyldimethyldodecylammonium chloride and benzyldimethyltetradecylammonium chloride at 50 μg/ml) and grown for 3 years. After 3 years of mixed QAC exposure the initial inoculum and QAC-adapted communities were assessed using 16S rDNA and metagenomic sequencing analyses to compare genomic changes amongst the initial and QAC adapted genera enriched in 3 year continuous population. As few biocide-specific resistance genes are well known, most findings by Kim et al. identified established AMR stress induced, regulators, and efflux pump gene alterations, but many other genes with undefined functions were identified[57].

Not all continuous culturing experiments required years of study. A shorter 5-day continuous adaptation study by Yang et al.[198] directly incubated a sewage sludge sample in a bioreactor where sterile water with different concentrations of BZK (0, 1, 10 μg/L) were aerobically added[198]. Samples were also incubated for 5 days in the dark but aerobically to avoid abiotic light degradation of the QAC. After metagenomic sequencing of the BZK-treated sludge before and after adaptation, many sequenced microbes with known AMR and biocide resistance genes as well as increased detection of mobile genetic elements were detected as compared to the BZK untreated sludge communities[198]. Other bioreactor methodologies explored bacterial monocultures. A study by Mc Cay et al.[199] examined a continuous monoculture of *P. aeruginosa*, which was treated with BZK (at 50% of its known MIC value) after 6 generations of growth, where additional BZK was added to the culture again after optical density measurements showed the culture turbidity recovered to 70% of its initial value[199]. High biocide selective efflux pump activity when compared to the unadapted culture was noted in these studies and mutations to *gyrA* associated with ciprofloxacin resistance, as well as efflux pump regulatory genes *nfxB* and *mexR*[199].

The benefits of continuous culturing techniques are their ability to adapt mixed or mono-culture populations of microbes in defined growth conditions or in samples directly isolated from an environment[57,198,199]. It has the ability to maintain, monitor and sample microbes exposed to a biocide condition over extremely long periods of time, in growth conditions much closer to natural environments. Although longer incubation times can be a benefit, these may also pose a disadvantage, as even manual peristaltic pump driven bioreactor/chemostat experimental tools can be costly and experimental set-ups are labor intensive and time-demanding to maintain. The findings from these techniques are also only impactful if there is sufficient pre-existing biocide resistance mechanism/gene knowledge available, as well as completed and well-annotated open reading frame curated microbial genomes for reference. Furthermore, when continuous culture adaptations are coupled with WGS and metagenomic approaches, there can be many genetic alterations identified, making it very difficult to distinguish which alterations are directly linked to a given biocide exposure/ phenotype. Lastly, as for all the in vitro methods discussed above, this lab-derived method does not fully replicate the pressures and variable conditions acting on natural samples. This can lead to false positive biocide-resistant gene identifications and result in unstable fitness, and short-lived mutations impacting biocide-tolerant phenotypes. For example, in two studies by Gadea et al.[185] and Gadea et al.[184], both identified mutations acquired from gradual exposure to biocide in the test tube model resulted in unstable biocide-tolerant phenotypes, resulting in adapted isolates with transient biocide susceptibility[184,185]. It should be noted that not all test tube adaptation methods have reported biocide adaptation phenotypic instability for final their adapted isolates[200,186]. Hence, biocide phenotype stability testing of cultures, where adapted isolates are subjected to AST after days of subculturing without added selection, is necessary in adaptation design workflows to ensure the final adapted isolate has a stable biocide resistant phenotype.

Conclusions and future considerations

Overall, biocide susceptibility testing and resistance mechanism research has combined a variety of molecular tools and in vitro study designs to identify new biocide resistance genes and mechanisms of resistance. These studies are limited in their scope due to a lack of standardization in resistance breakpoints and a lack of reference strains identified from biocide-focused microbial surveillance initiatives. Similar to antibiotic-specific techniques, various omics, library-screening, and drug adaption approaches can be combined with different in vitro experimental designs (broth-based, agar plate, bioreactor) to develop even more biocide-associated methods. Despite all the excellent research devoted to biocide resistance mechanisms and biocide susceptibility testing so far, many knowledge gaps should be addressed to glean more meaningful results from future biocide-focused AMR studies and experimental designs. These questions/ gaps include:

i. What is the minimal or optimal duration of biocide exposure for experiments? Are MDK assays a valuable metric to add to biocide MIC and MBC values?
ii. What is the optimal concentration range of biocide exposure to start testing? Should it include working concentrations set by antiseptic/ disinfectants regulatory agencies or focus on biocide MIC and MBC concentrations that may be more representative of pollutants?
iii. How should we prioritize biocide studies? Should the focus be on those only of clinical importance for disinfection practices and product safety, or those that are frequently overused or detected as global pollutants of concern?
iv. Can and should all biocides be studied with the same technique(s)? For example, disinfectant working concentrations of biocides require different standards than biocides at lower concentrations after they dilute in an environment.
v. Should resistance, tolerance, and susceptibility definitions be different for high concentration working solutions of antiseptics, disinfectants, pesticides/herbicides and should different terms be applied to biocides studied at lower MIC and MBC concentrations?
vi. As biocides classes differ greatly in their inherent chemical properties, do some of these properties make certain biocide methods less useful or comparative to other biocides? Do some biocides need separate AST methods (eg. for less water soluble/insoluble biocides)?
vii. Should biocide research and surveillance focus on WHO priority and ESKAPE pathogens only or take a more holistic AMR One Health approach and include commensal and environmental isolates?

This review clearly shows that there is still a long way to go in resolving knowledge gaps associated with biocide resistance. As more biocide susceptibility testing, biocide surveillance initiatives, and biocide resistance breakpoint determinations are established, greater advancements in biocide susceptibility research and gene discovery will be achieved. Evidence-based biocide resistance data is urgently needed to help better establish and guide policy reforms to regulate biocide overuse, maintain disinfection efficacy, and reduce biocide resistance impacts on AMR. Biocide policy reforms can

be powerful, as evidenced by the 2016 US Food and Drug Administration's ban on 19 hand sanitizers and soaps that contain biocides additives that included QACs and triclosan[201].

Data availability

No datasets were generated or analyzed during the current study.

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Competing interests
The authors declare no competing interests.

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