Environmental Science and Ecotechnology 25 (2025) 100557

Contents lists available at ScienceDirect

Environmental Science and Ecotechnology

journal homepage: www.journals.elsevier.com/environmental-science-and-ecotechnology/

Review

Biocides as drivers of antibiotic resistance: A critical review of environmental implications and public health risks

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ARTICLE INFO

Article history:
Received 25 November 2024
Received in revised form
18 March 2025
Accepted 19 March 2025

Keywords:
Adaptive resistance
Antibiotic resistance
Biocides
Biofilms
Cross-resistance

ABSTRACT

The widespread and indiscriminate use of biocides poses significant threats to global health, socioeconomic development, and environmental sustainability by accelerating antibiotic resistance. Bacterial resistance development is highly complex and influenced significantly by environmental factors. Increased biocide usage in households, agriculture, livestock farming, industrial settings, and hospitals produces persistent chemical residues that pollute soil and aquatic environments. Such contaminants contribute to the selection and proliferation of resistant bacteria and antimicrobial resistance genes (ARGs), facilitating their dissemination among humans, animals, and ecosystems. In this review, we conduct a critical assessment of four significant issues pertaining to this topic. Specifically, (i) the role of biocides in exerting selective pressure within the environmental resistome, thereby promoting the proliferation of resistant microbial populations and contributing to the global spread of antimicrobial resistance genes (ARGs); (ii) the role of biocides in triggering transient phenotypic adaptations in bacteria, including efflux pump overexpression, membrane alterations, and reduced porin expression, which often result in cross-resistance to multiple antibiotics; (iii) the capacity of biocides to disrupt bacteria and make the genetic content accessible, releasing DNA into the environment that remains intact under certain conditions, facilitating horizontal gene transfer and the spread of resistance determinants; (iv) the capacity of biocides to disrupt bacterial cells, releasing intact DNA into the environment and enhancing horizontal gene transfer of resistance determinants; and (iv) the selective interactions between biocides and bacterial biofilms in the environment, strengthening biofilm cohesion, inducing resistance mechanisms, and creating reservoirs for resistant microorganisms and ARG dissemination. Collectively, this review highlights the critical environmental and public health implications of biocide use, emphasizing an urgent need for strategic interventions to mitigate their role in antibiotic resistance proliferation.

1. Introduction

Based on the "One Health" concept, which states that "the health of people is connected to the health of animals and the environment," the measures to halt antimicrobial resistance from spreading through the environment are timely and much-needed [1,2]. Biocide pollution contributes to the evolution and dissemination of bacterial resistance among humans, animals, and the environment by creating favourable conditions for selecting resistant bacteria and facilitating the transmission of genetic material across ecological boundaries [3–5]. When released into a certain environment, biocides create a selective pressure favouring bacteria with resistance mechanisms [6,7]. For these reasons, addressing this challenge requires a "One Health" approach, incorporating sustainable biocide use, robust waste management practices, and monitoring of environmental reservoirs [8]. In addition to the

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https://doi.org/10.1016/j.ese.2025.100557

existing monitoring programs that track unnecessary antibiotic prescriptions and misuse, it is essential to include biocides as they may also contribute to resistance spread.

Additionally, the environment should be considered as a potential source of resistance [9,10]. In particular, biocides, which exert their antimicrobial activity through a multi-target mode of action, are essential to control infectious diseases and safeguard animal and human health [11,12]. Demand for biocides is expected to increase in the coming years, driven by their use in various industrial processes and for the disinfection of hospitals and public spaces [13]. A significant increase in pests and hygiene issues is expected because of global warming, climate change, and associated extreme weather events [14]. This requires effective responses to control potential pathogens while ensuring minimal negative impacts on public health and the environment. The societal dependence on biocides for disinfection was remarkable during the SARS-CoV-2 pandemic [15–18].

Understanding the intricate connections between biocides, antibiotics, and antimicrobial resistance is critical for devising strategies to mitigate the spread of resistance across humans, animals, and the environment [19,20]. This review explores the evidence linking biocide exposure to the emergence of resistant microorganisms, focusing on the mechanisms by which biocides drive resistance and the environmental pathways that facilitate the dissemination of resistance genes. This study underscores the urgent need for integrated and sustainable approaches to manage biocide use, protect public health, and preserve ecosystem integrity.

2. Evidence of biocides as drivers of antimicrobial resistance

Biocides are far from innocuous to the environment and public health. They can persist in their target niches for two reasons [21]. Firstly, bacteria elimination by these agents has proven to be difficult, and it has become clear that biocidal efficacy may be questionable in some circumstances. Within this context, bacteria are regularly exposed to sub-bactericidal concentrations, which can lead to bacterial adaption and resistance to in-use biocides [22–24]. Secondly, antimicrobial sub-bactericidal concentrations are present in the environment since some biocides that are not consumed in the reaction of cleaning procedures are freely released in the discharge waters (for instance, oxidative species would be actively quenched by the organic load present and have no legacy effects) [25–27].

For instance, studies have shown that *Pseudomonas aeruginosa* can develop resistance to quaternary ammonium compounds (QACs) such as benzalkonium chloride (BZK) through mechanisms like efflux pump overexpression and alterations in membrane composition [28,29]. This exposure can also lead to cross-resistance to antibiotics like ciprofloxacin, as the same efflux systems expel both biocides and antibiotics from the bacterial cell [28,29]. Moreover, *Listeria monocytogenes* exposed to sub-inhibitory concentrations of BZK in food environments can adapt by altering membrane composition and surface characteristics, leading to increased resistance to BZK and other unrelated antibiotics [29,30]. Concerning triclosan, research has demonstrated that low levels of this biocide, commonly found in personal care products, can induce bacterial resistance through mutations in key enzymes involved in fatty acid synthesis, such as FabI [30]. This resistance can extend to antibiotics like isoniazid, which also targets fatty acid synthesis pathways [30]. For *Acinetobacter baumannii*, sub-lethal exposure to chlorhexidine has been shown to activate efflux pump systems and promote biofilm formation, making the bacteria more resistant to chlorhexidine and antibiotics like carbapenems [30].

One of the possible solutions for this lack of efficiency is an increase in the recommended in-use concentration [11]. In this environment, pollutants are released not only from industrial and hospital discharges but also from a wide range of human activities, including consumer products, personal care items, pharmaceuticals, and their metabolites. Additionally, natural sources, such as compounds secreted by bacteria, fungi, algae, and plants, contribute to the pollution [31–33]. The increased consumption of biocides and the fact that these are poorly biodegradable make them present in the environment as parent compounds or conjugates [31,34–39]. These compounds can be very persistent in the environment, in varying types and concentrations across the globe, since they adsorb strongly to environmental elements such as sludge and sediment, showing low degradation potential [40–45]. Fig. 1 presents a compilation of some studies reporting the world distribution of biocides and antibiotics with high relevance, prevalence, and use worldwide. Wastewater treatment plants (WWTPs), rivers, and basins that receive treated and untreated sewage are important contributors to the dissemination of resistant bacteria and antibiotic-resistance genes (ARGs) [46–49]. The globalization of ecosystems, along with the rising consumption of antibiotics and biocides (such as antiseptics, preservatives, and biocides), contributes to the widespread distribution and accumulation of these biopollutants in ecosystems worldwide [50–53].

Several biocides, such as QACs, are high-production volume chemicals (i.e., production >1000 tons per year) and, in many cases, can also have non-biocidal applications [54]. In 2017, a Chinese study aimed to examine the presence and elimination of nineteen biocides across ten different WWTPs [47]. Additionally, it sought to estimate the per capita usage and emissions of these 19 biocides using a mass balance analysis approach.

The mass balance used was:

$$M_{\text{Influent}} = M_{\text{Effluent}} + M_{\text{Sludge}} + M_{\text{Loss}} \quad (1)$$

where M_{Influent}, M_{Effluent}, and M_{Sludge} (g d^{-1}) represent the mass loads of the target biocide in the influent, effluent, and excess sludge of each WWTP, respectively. M_{Loss} (g d^{-1}) denotes the loss of mass loads of the target biocide during the entire treatment process, primarily due to sorption and degradation.

The loss mass fractions ($M_{f,\text{Loss}\%}$) for each biocide were calculated using:

$$M_{f,\text{Loss}\%} = \frac{M_{\text{Influent}} - M_{\text{Effluent}} - M_{\text{Sludge}}}{M_{\text{Influent}}} \times 100\% \quad (2)$$

The mass fractions in effluent ($M_{f,\text{Effluent}\%}$) and excess sludge ($M_{f,\text{Sludge}\%}$) were calculated as:

$$M_{f,\text{Effluent}\%} = \frac{M_{\text{Effluent}}}{M_{\text{Influent}}} \times 100\% \quad (3)$$

$$M_{f,\text{Sludge}\%} = \frac{M_{\text{Sludge}}}{M_{\text{Influent}}} \times 100\% \quad (4)$$

The estimated pollution loads per capita of biocides in the influent, effluent, and excess sludge for each WWTP were given by:

$$\textit{Pollution load per capita} = \frac{M_{\text{Influent/Effluent/Sludge}}}{\textit{Population served}} \quad (5)$$

The back-estimated usage (U, t $year^{-1}$) and estimated emissions (E, t $year^{-1}$) for Guangdong Province or China were determined as:

$$U = L_{\text{Influent}} \times P_{\text{Total}} \times 365.25 \times 10^{-12} \quad (6)$$

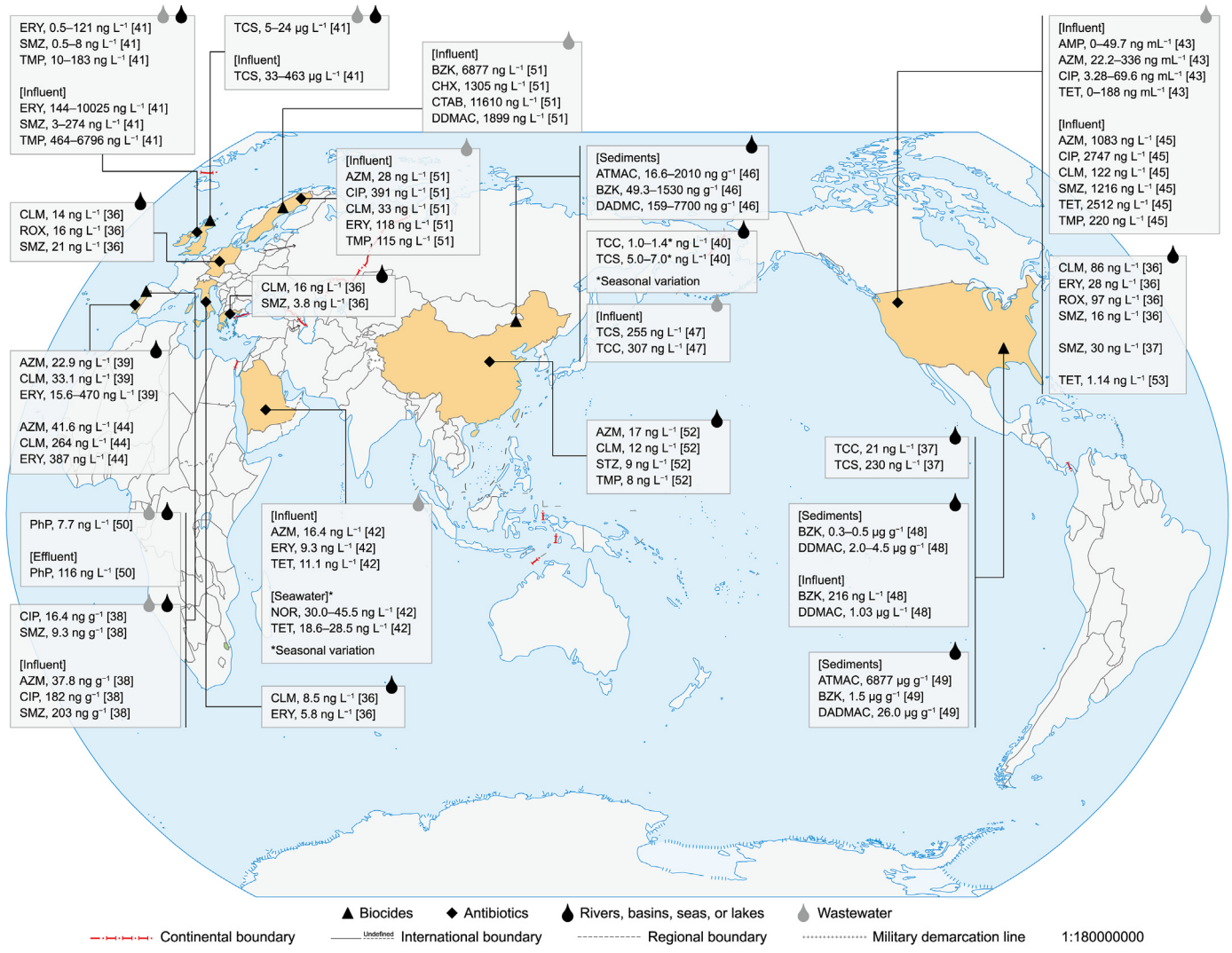

Fig. 1. World distribution of biocides and antibiotics (in ng L^{-1}) in rivers, basins, seas or lakes, and wastewater treatment plants (influent). The investigated biocides and antibiotics were selected due to their high relevance, prevalence, and use worldwide. AMP: Ampicillin; ATMAC: Alkyltrimethylammonium compounds; AZM: Azithromycin; BZK: Benzylalkyldimethylammonium compounds; CHX: Chlorhexidine; CIP: Ciprofloxacin; CLM: Clarithromycin; CTAB: Cetyltrimethylammonium bromide; DADMAC: Dialkyldimethylammonium compounds; ERY: Erythromycin; NOR: Norfloxacin; PhP: Ortho-phenylphenol; ROX: Roxithromycin; SMZ: Sulfamethoxazole; STZ: Sulfathiazole; TCC: Triclocarban; TCS: Triclosan; TET: Tetracycline; TMP: Trimethoprim.

$$E = E_{\text{Rural}} + E_{\text{Urban}} \tag{7}$$

$$E = \left[L_{\text{Influent}} \times P_{\text{Rural}} + \left(L_{\text{Effluent}} + L_{\text{Sludge}}\right) \times P_{\text{Urban}}\right] \times 365.25 \times 10^{-12} \tag{8}$$

where L_{Influent}, L_{Effluent}, and L_{Sludge} (μg d^{-1} $person^{-1}$) are the estimated pollution loads per capita of a target biocide in the influent, effluent, and excess sludge (mean of ten WWTPs). P_{Total}, P_{Rural}, and P_{Urban} are the total, rural, and urban populations. These values are based on the "China Statistical Yearbook" (2015). For Guangdong: $P_{\text{Total}} = 10724 \times 10^4$, $P_{\text{Rural}} = 3432 \times 10^4$, and $P_{\text{Urban}} = 7292 \times 10^4$. For China: $P_{\text{Total}} = 136782 \times 10^4$, $P_{\text{Rural}} = 61866 \times 10^4$, and $P_{\text{Urban}} = 74916 \times 10^4$.

The estimated usage of the biocides under investigation was as high as 453 tons per year, corresponding to 308 μg per day per person (including effluent and excess sludge) [47]. In Minnesota (United States), 1995 metric tons of QACs were sold in 2017 for non-agricultural purposes [48]. A study by Arnold et al. [54] reported that over 450000 metric tons of QACs are produced or imported annually in the United States. In 1997, the total use of QACs in Germany was 12349 metric tons per year by industry and 95.3 metric tons per year by hospitals, while in Great Britain, 28852 metric tons per year are being used [55–57]. Moreover, approximately 75 % of the QACs used each year are discharged into wastewater treatment systems, with the remainder going directly into the environment [58]. Globally, surface water and wastewater effluent contain concentrations of QACs from 1 to 100 μg L^{-1}, this is 0.0000001 %–0.00001 % [weight/volume (w/v)], although influent wastewater sometimes contains 10 times these concentrations [58,59].

Furthermore, the minimum inhibitory concentrations (MICs) of QACs range from 0.005 % to 0.01 % w/v, while the minimum bactericidal concentrations (MBCs) are typically between 0.02 % and 0.05 % w/v [60]. Zheng et al. [61] assessed the bioaccumulation potential of 18 QACs with alkyl chain lengths ranging from C8 to C18. That evaluation was conducted through an *in vitro–in vivo*

extrapolation (IVIVE) model, utilizing data obtained from experiments on human hepatic metabolism and serum protein binding. Of the 18 QACs aimed for detection, 15 were identified in blood, with cumulative QAC concentrations reaching a maximum of 68.6 ng mL^{-1}. Blood samples were obtained from two specific periods: pre-COVID-19 pandemic outbreak (2019; $n = 111$) and during the pandemic (2020; $n = 111$). The cumulative QAC concentrations were notably elevated in samples collected during the pandemic (6.04 ng mL^{-1}) compared to those gathered before (3.41 ng mL^{-1}) [61].

In Europe, the regulation for BZK falls under the purview of the European Commission (EC). Recent regulations in the European market have modified the permissible maximum residual levels of BACs in food products, reducing the limit from 0.5 to 0.1 mg kg^{-1}. In addition, the alterations in legislation, specifically Decision [European Union (EU)) 2016/1950 and the Biocidal Products Regulation (BPR, EU) no. 528/2012 have resulted in the disapproval of BACs for application in various biocidal products. Notably, consumer hand and body wash antiseptics are affected, marking a departure from the prevailing regulations in the United States [62].

Some of these chemical compounds end up diluted in what we eat, drink, and breathe [25]. This has triggered concern because, in the environment, biocides persist and bioaccumulate, being harmful not only to humans but also to microorganisms [8]. Biocides can modify the dynamics and action of antibiotics in natural ecosystems, selecting resistant organisms [3,63,64] with increased resistance and co-resistance to other biocides as well as cross-resistance to antibiotics [24,51].

The role of biocides in the selection, spread, and maintenance of resistant bacteria worldwide should be addressed [65], considering that water basins are globally polluted with several classes of biocides [27,66]. Therefore, there is an urgency to understand the mechanisms of action of biocides and resistance and if their presence in the environment, even in reduced concentrations, can cause resistance to antibiotics. This study reviews the phenomena of bacterial resistance to in-use biocides and our current knowledge of how this can trigger antibiotic resistance, with particular emphasis on biocides that are continuously released and accumulated in the environment [51]. The main question remains: Should biocide sub-bactericidal concentrations be considered an important driver of increased antibiotic resistance?

3. Mechanisms of action of biocides and their impact on bacterial resistance

Generally, antimicrobial agents can be classified as any molecule with biochemical properties that kill or prevent the growth of microorganisms, including bacteria, fungi, and algae, on a biotic or abiotic surface [67]. Antimicrobial agents can be divided into antiseptics (agents operating on living tissues), disinfectants (products applied to inanimate objects and surfaces), and preservatives [68,69]. However, they can also be classified according to the type of microorganisms against which they are effective: antibiotics, antivirals, antifungals, and antiparasitics [70,71]. Furthermore, these molecules target crucial processes in cellular metabolism, including the production of biological macromolecules, cellular enzyme activity, and cellular components [71,72].

Consequently, in contrast to antibiotics that are used to treat specific bacterial infections, biocides are a diverse group of antimicrobial compounds that, due to their versatility and broad-spectrum action, are used in personal care products (soaps, handwashes, toothpaste, mouthwashes, and cosmetics) [73–75] and as part of detergent formulations for surface cleaning, the process of removing all foreign material from objects by using water and detergents, and disinfection – elimination of most or all pathogenic microorganisms (except spores) [68,76]. Industrially, biocides are used in high doses to clean hard surfaces – floors, walls, equipment – and food surfaces. Additionally, in most production processes, the water used often has a residual concentration of biocides to ensure its low microbial load [77,78]. In clinical environments, biocides are fundamental for the antisepsis of the patient's skin as well as for the disinfection of water and hard surfaces and sterilization of medical instruments and rooms [77].

Typical biocides used for cleaning and disinfection purposes are QACs, alcohols, aldehydes (glutaraldehyde, ortho-phthalaldehyde [OPA]), bisbiguanides (chlorhexidine), bisphenols (triclosan), diamidines, halogen-releasing agents, halophenols, heavy-metal derivatives and peroxygens (hydrogen peroxide) [68,79]. Many factors affect the efficacy of biocides in both actions – cleaning and disinfection – the stability of the active molecule, the contact time, the concentration of biocide, the age and metabolic status of the microbial community, the existence of adhered bacteria or biofilms, and the presence of organic load and other environmental factors such as temperature and pH [68,80].

Biocides play an important role in limiting bacteria presence and reducing potential sources of contamination and infection and are effective due to their broad spectrum of activity and ability to act on multiple targets [11,68]. Moreover, the majority of biocides disrupt the cytoplasmic membrane [68], dissipating the proton motive force and inhibiting membrane-associated enzymes [81]. Unlike antibiotics, which act selectively against specific cell targets, the mechanism of action of biocides occurs at one or several other sites within the cell [82]. Biocides can interact with the cell envelope targeting, for instance, the cytoplasmic membrane (which is a component of the cell envelope) (e.g., chlorhexidine and QACs) [83]; cross-link with other macromolecules (e.g., glutaraldehyde) [84]; intercalate (e.g. acridines) and interact with deoxyribonucleic acid (DNA; e.g. halogens, silver ions, and oxidizing agents) [81]; and interact with thiol groups in enzymes and proteins (e.g. organomercurials and silver compounds) [81]. Although the outer cell envelope is the first target, as it is the initial point of contact, the inner cell components are also often affected. However, the cell is frequently inactivated through this initial surface interaction [85,86]. Other targets inside the cell, such as nucleic acids, enzymes, and ribosomes, are more difficult to reach since the biocide needs to penetrate the cell [81]. Understanding these interactions is essential for developing effective antimicrobial treatments and addressing the challenge of bacterial resistance. Table 1 presents an overview of various biocides, their mechanisms of action against bacteria, and corresponding bacterial resistance mechanisms. This table highlights how different chemical agents target bacterial cells and the strategies employed by bacteria to resist their effects [68,87].

Biocides are usually applied in high concentrations to improve their efficiency, making it impossible for bacteria to overcome the damage caused and develop resistance [88]. In fact, in hospitals and clinical settings, the antimicrobial in-use concentration is frequently two-to four-fold the MIC of wild-type strains, meaning that the antimicrobials are effective against 99 % of the environmental microorganisms [89,90]. Furthermore, as disinfectants generally contain more than one type of active component, each with a different antimicrobial mode of action, and as they have no specific target, the development of resistance at the level of in-use concentrations is thought to be highly unlikely [68,89]. Nevertheless, two features can contribute to the resistance to biocides and subsequent resistance: (1) the 1 % of bacteria that are not killed by the in-use concentration can present naturally reduced susceptibility due to the presence of resistant genetic information [89]; (2) a cleaning solution at a sub-inhibitory concentration can occur, for example, when organic matter is present (since the organic matter

Table 1
Overview of various biocides, their mechanisms of action against bacteria, and corresponding bacterial resistance mechanisms, focusing on how different chemical agents target bacterial cells and the strategies bacteria employ to resist their effects [68,87].

Biocide	Mechanism of action	Bacterial resistance mechanisms
Quaternary ammonium compounds	Disrupts cell membrane integrity	• Efflux pumps: Expel quaternary ammonium compounds out of the cell • Membrane modification: Reduces permeability
Alcohols (e.g. Ethanol, Isopropanol)	Denatures proteins, disrupts membranes	• Biofilm formation: Limits penetration • Membrane modification: Alters lipid composition
Chlorine/Chlorine-based compounds	Oxidizes proteins, lipids, and DNA	• Enzymatic degradation: Produces enzymes to neutralize oxidative damage • Reduced uptake: Alters porin proteins
Peroxides (e.g. Hydrogen peroxide)	Produces reactive oxygen species, causing cellular damage	• Enzymatic degradation: Catalase and peroxidase break down peroxides • Biofilm formation: Protects against oxidative damage
Phenolics	Disrupts cell walls and membranes denatures proteins	• Efflux pumps: Actively expel phenolics • Biofilm formation: Shields bacteria from penetration
Heavy metals (e.g. Silver, Copper)	Binds to proteins and enzymes, disrupting cellular function	• Membrane modification: Reduces metal ion permeability • Efflux pumps: Actively remove metal ions
Biguanides (e.g. Chlorhexidine)	Disrupts cell membrane, leading to leakage	• Efflux pumps: Expel biguanides • Membrane modification: Alters membrane composition to resist disruption

may physically dilute the cleaning solution, reducing the concentration of active ingredients available to act on microorganisms [91]; can react chemically with disinfectants [91]; and can create a physical barrier that protects microorganisms from direct exposure to the cleaning agent [92]), if insufficient cleaning is applied, or even in the rinsing water [89].

In the environment, we may think both situations happen: water and land accumulate a multiplicity of resistant strains and genetic material, and sub-inhibitory concentrations of biocides are naturally present as persistent organic pollutants [93,94] (Fig. 1).

4. Cross-resistance to antibiotics

Although bacterial recalcitrance to biocides (resistance) was described early in the 1950s or 1960s, it appears to be increasing, and nowadays, we are still debating on the possible emergence of biocide resistance and its association with the emergence of antibiotic recalcitrance [95]. The term "cross-resistance" can be defined as a phenomenon that occurs when a resistance mechanism to a certain antimicrobial enables a specific strain to resist several other antimicrobials with similar mechanisms of action [96]. In the case of co-resistance, the mechanisms conferring resistance to both agents are unrelated but are genetically linked, e.g., located on the same genetic element [97].

Some studies *in vitro* confirmed this cross-resistance concept (exceptionally clarified in Cândido et al. [98]). Sonbol et al. [99] examined the effectiveness of several antibiotics, such as β-lactams, cephalosporins, macrolides, aminoglycosides, quinolones, sulfonamides, and tetracycline against *Escherichia coli* isolates that have been exposed to sub-bactericidal doses of the biocide triclosan. The outcomes showed that apart from amikacin and trimethoprim/sulfamethoxazole, cells adapted to triclosan became more resistant to the antibiotics tested and remained susceptibility to the biocide, associated with decreased permeability of outer and inner membranes, increased depolarisation of membranes, higher negative net charge of membranes, and higher efflux activity [84]. As triclosan acts as a substrate for bacterial multidrug efflux pumps, *E. coli* [99] and *P. aeruginosa* [100] exposed to triclosan at sub-bactericidal doses displayed a larger operation of efflux pumps, likely associated with cross-resistance. A study by Wand et al. [101] demonstrated that, when exposed to chlorhexidine, *Klebsiella pneumoniae* acquired cross-resistance to the antibiotic colistin. The authors reported that the adaptation of *K. pneumoniae* to chlorhexidine occurs through regulating genes associated with efflux pumps, namely *smvR* and *smvA*. Moreover, the operon *pmrK*, which is responsible for reducing the negative charge of lipid A, was upregulated by chlorhexidine. As a result, a decreased binding affinity of colistin to lipid A occurred, resulting in cross-resistance. However, the resistance to chlorhexidine was not increased by exposure to colistin [101]. A study developed by Curiao et al. [102] showed that mutants of *Salmonella enterica* Typhimurium created after exposure to triclosan, chlorhexidine, and BZK exhibited different phenotypes against a broad range of antibiotics: ampicillin, ceftazidime, ciprofloxacin, erythromycin, gentamicin, chloramphenicol, and tetracycline. Most of the mutants that were confirmed to be resistant to biocides presented an increased susceptibility to molecules whose site of action is the cell wall (β-lactams) or the cell membranes (poly-L-lysine, polymyxin B, colistin, and toxic anions). Moreover, both biocide mutants selected *in vitro* and field isolates with resistance to biocides exhibited overexpression of genes related to cold-shock response (*cpeE*), ribosomal and transcription proteins, efflux pumps (*sugE*), and enzymes of microaerobic metabolism, particularly those of the phosphotransferase system [102]. In Sanchez et al. [103], triclosan proved to selectively induce the expression of the efflux pump *SmeDEF* in *Stenotrophomonas maltophilia*, which in turn triggers a transient low-level resistance to the biocide and cross-resistance. Compared to the wild-type *S. maltophilia*, five out of the 12 triclosan-selected mutants were more resistant to the antibiotics tetracycline, chloramphenicol, ciprofloxacin, tobramycin, and triclosan [103]. Pereira et al. [104] conducted an adaptive laboratory evolution of *E. coli* using constant and sub-inhibitory concentrations of ten widespread biocides: chlorophene, BZK, glutaraldehyde, chlorhexidine, peroxide, povidone-iodine, isopropanol, ethanol, sodium hypochlorite, and peracetic acid. Interestingly, and as a result of evolution, 43 % of strains became resistant to three representative antibiotics with diverse cellular modes of action: ampicillin, chloramphenicol, and norfloxacin. The authors also found mutations in genes encoding for multidrug efflux proteins (*mdfA* and *acrR*), porins (*envZ* and *ompR*), and subunits of *E. coli* RNA polymerase (*rpoA* and *rpoBC*). The biocides chlorophene, BZK, glutaraldehyde, and chlorhexidine were the ones instigating higher antibiotic susceptibility (probably due to the occurrence of mutations in membrane proteins and their regulators, including those responsible for transporting chemicals like antibiotics into and out of the cell), while hydrogen peroxide and povidone-iodine were the ones contributing least to an increase in susceptibility to antibiotics. No evidence of antibiotic cross-resistance for isopropanol, ethanol, sodium hypochlorite, and peracetic acid was observed [104]. *Salmonella* spp. exhibited overexpression of AcrAB-TolC and

underexpression of outer membrane porins after exposure to a quaternary ammonium disinfectant—containing formaldehyde and glutaraldehyde. This phenomenon resulted in antibiotic resistance to ciprofloxacin, chloramphenicol, tetracycline, and ampicillin [105,106].

Tabata et al. [107] reported that the expression level of *OprR* was correlated with the resistance level of *P. aeruginosa* to QACs. A QAC-resistant *P. aeruginosa* strain exhibited a higher expression level of *OprR* compared with a knock-out mutant [107]. Machado et al. [108] demonstrated that *P. aeruginosa* adapted to BZK showed differences regarding the expression of outer membrane proteins (OMPs) compared to a non-adapted strain. Some of these proteins are involved in ciprofloxacin resistance [108].

One example of target alteration was demonstrated by Sheridan et al. [109], where a triclosan-tolerant *E. coli* strain exhibited an amino acid substitution in the FabI protein, where a glycine was replaced by a valine. This point mutation prevents the formation of the FabI-NAD^+ complex, reducing the effect of triclosan in *E. coli* [109]. A stable chlorhexidine-resistant *Pseudomonas stutzeri*, obtained after exposure to chlorhexidine, showed resistance to triclosan and BZK and antibiotics—polymyxin B, gentamicin, and ampicillin [110]. Nevertheless, cross-resistance phenomena do not occur exclusively in cells in the planktonic state, having also been observed in biofilms, as demonstrated in Tabak et al. [111]. The results clarified that the expression of degradative enzymes, changes in the bacterial cell envelope, activation of the efflux pump, and mutations in the enoyl reductase enzyme are all associated with the recalcitrance observed in planktonic *S. Typhimurium* after exposure to triclosan. Additionally, the authors found that, regarding the cells in biofilms of *S. Typhimurium*, their susceptibility to triclosan is associated with the low extracellular matrix diffusion of the substrate, overactivity of efflux pumps, and exopolysaccharide production [111].

In that sense, the underlying message of the previous studies is the same: there is a direct relationship between the presence of biocides in the environment and antibiotic resistance [112]. However, other authors are not certain that this phenomenon can occur, as studies addressing this *in vivo* are still missing [89,97]. The main mechanisms of resistance of conventional classes of biocides and the possible relationship between biocide and antibiotic cross-resistance described in the literature are summarized in Table 2.

Resistance to antibiotics due to exposure to biocides is of public health relevance when it concerns pathogenic or opportunistic bacteria [24]. Co-resistance can also be a problem with non-pathogenic and commensal bacteria when there is a risk of horizontal gene transfer of resistance determinants to (opportunistic) pathogens [113,114]. Moreover, the awareness of risks related to sub-inhibitory biocide concentrations triggering cross and co-resistance in bacteria has substantially increased [97].

In addition, cross and co-resistance appear when the biocide and the antibiotic act on the same cellular target; the biocide and the antibiotic have the same transport mechanism; biocide and antibiotic can be accommodated by the same resistance mechanism, and in situations where genes contributing toward biocide resistance and antibiotic resistance are carried on the same mobile genetic element [22].

Biocides and antibiotics may have similar and common interactions and target sites in bacteria (e.g., efflux pumps, permeability changes, and biofilms), which might express shared resistance mechanisms [93]. When only sub-bactericidal concentrations of biocides are present, minor cell damages can occur, and the expression of multidrug efflux pumps can be induced, selecting clones that confer resistant phenotypes located on the same mobile genetic element such as a plasmid, transposon, or integrons [88,115,116].

Table 3 presents a compilation of the biocides referred to in this study, organized by their chemical category and common applications: QACs [54], bisbiguanides [117], phenolics compounds [118], aldehydes [119], alcohols [120], peroxygens [121], halogen-releasing agents [122], bisphenols [123], heavy-metal derivatives [124], and others (povidone-iodine and diamidines) [125].

5. Mechanisms induced by biocides in the development of resistance

Recent literature [15,84,126,127] provides clear scientific evidence demonstrating that the presence of biocides in the environment can be associated with various types of resistance, namely.

(i) the natural selection of most resistant strains—intrinsic property [15,126];
(ii) the phenotypic adaptation to biocides [84,127];
(iii) the acquisition and/or release of genetic elements from resistant strains; chromosomal gene mutation or genetic material acquisition of (plasmids or transposons) [126];
(iv) the role of biofilm communities in resistance development [127].

In the following sections, we will critically examine these different types of resistance to biocides.

Fig. 2 provides a compilation of the mechanisms by which biocides promote antibiotic resistance, which this study addresses.

5.1. Biocides as natural selectors of resistant strains

Intrinsic resistance is the naturally greater resistance related to certain microbial species than others. Some microorganisms are already genetically predisposed to resist biocides and antibiotics [68,84,128–130] (Fig. 3). A feature that is extensively present within several bacterial species, unrelated to prior antibiotic exposure and unconnected to horizontal gene transfer is referred to as intrinsic resistance [128,131]. Reduced outer membrane permeability and efflux pump activity, namely those associated with multidrug efflux pumps, are the two most frequent bacterial mechanisms resulting in intrinsic resistance [128,132].

The cytoplasmic membrane is probably the major target for most classes of biocides, and since different microorganisms have different membrane structures, it is easy to understand that the activity of biocides varies between different types of microorganisms and even between different strains of the same species [81,128,133,134]. Based on the membrane structure, among vegetative bacteria, mycobacteria are considered the most resistant to biocides, followed by Gram-negative bacteria, and vegetative Gram-positive the most susceptible [81,135]. Some bacterial species are also innately more resistant to biocides than others due to the presence of a low level of efflux pump systems that decrease intracellular concentration, reduce biocide penetration, enzymatic degradation of biocides, and physiological, structural, and metabolic changes [84,136–139]. In addition, it is conveniently accepted that most of the in-use biocides, at sub-inhibitory concentrations, are naturally selecting bacteria according to their susceptibility and intrinsic resistance [130,140,141] (Fig. 3).

The specific growth rate is another significant factor associated with antimicrobial resistance as it is a determinant factor of how bacterial populations respond to molecules, influences the expression of resistance mechanisms, and can affect the outcome of bacterial treatment [142–144]. Rapidly growing bacteria are often more susceptible to molecules that target active cellular processes, such as cell wall synthesis and DNA replication, because these cells can experience more mutations in a given period due to more

Table 2
Compilation of studies establishing a possible connection between several biocides and their resistance mechanisms with cross-resistance to antibiotics in various bacterial strains.

Biocides	Antibiotics	Microorganism	Mechanism of resistance/Cross-resistance	References
Triclosan	Amikacin Ampicillin Ampicillin/ sulbactam Azithromycin Cefaclor Cefazoline Cefepime Cefotaxime Chloramphenicol Ciprofloxacin Erythromycin Gentamicin Imipenem Lomefloxacin Tetracycline Trimethoprim/ sulfamethoxazole	*Escherichia coli* clinical isolates	The cells adapted to triclosan exhibited extended susceptibility to the biocide and recalcitrance to the investigated antibiotics associated with: • Decreased permeability of outer and inner membranes • Increased depolarisation of membranes • Higher negative net charge of membranes • Higher efflux activity	[99]
Triclosan	–	*Pseudomonas aeruginosa*	Triclosan is a substrate for *Pseudomonas aeruginosa* efflux pumps, namely MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY, associated with cross-resistance to as antibiotics	[100]
Chlorhexidine	Colistin	*Klebsiella pneumoniae* clinical isolates	The adaptation of *Klebsiella pneumoniae* to chlorhexidine occurs through the regulation of genes associated with efflux pumps, namely *smvR* and *smvA* The operon *pmrK* is upregulated by chlorhexidine, resulting in a decreased binding affinity of colistin to lipid A, associated with cross-resistance	[101]
Benzalkonium chloride Chlorhexidine Triclosan	Ampicillin Ceftazidime Chloramphenicol Ciprofloxacin Erythromycin Gentamicin Tetracycline	*Salmonella enterica* Typhimurium	Resistance to biocides and cross-resistance is associated with the expression/overexpression of: • Genes related to cold-shock response (*cpeE*) • Ribosomal and transcription proteins • Efflux pumps (*sugE*) • Enzymes of microaerobic metabolism	[102]
Triclosan	Chloramphenicol Ciprofloxacin Tetracycline Tobramycin Triclosan	*Stenotrophomonas maltophilia*	Triclosan selectively induces the expression of the efflux pump *SmeDEF*, which in turn triggers a transient low-level resistance to the biocide and cross-resistance to antibiotics Compared to the wild-type, five out of the twelve triclosan-selected mutants were more resistant to antibiotics	[103]
Benzalkonium chloride Chlorhexidine Chlorophene Ethanol Glutaraldehyde Isopropanol Peracetic acid Peroxide Povidone-iodine Sodium hypochlorite	Ampicillin Chloramphenicol Norfloxacin	*Escherichia coli*	43 % of strains became resistant to the antibiotics Resistance and cross-resistance with antibiotics is associated with the expression of: • Multidrug efflux proteins (mdfA and acrR) • Porins (envZ and ompR) • Subunits of *Escherichia coli* RNA polymerase (rpoA and rpoBC) For isopropanol, ethanol, sodium hypochlorite, and peracetic acid, there was no evidence of antibiotic cross-resistance	[104]
Quaternary ammonium compounds disinfectant containing formaldehyde and glutaraldehyde	Ampicillin Chloramphenicol Ciprofloxacin Tetracycline	*Salmonella* spp.	After exposure to a quaternary ammonium disinfectant containing formaldehyde and glutaraldehyde, *Salmonella* spp. exhibited overexpression of AcrAB-TolC and underexpression of outer membrane porins, resulting in resistance to ciprofloxacin, chloramphenicol, tetracycline, and ampicillin	[105,106]
Quaternary ammonium compounds	–	*Pseudomonas aeruginosa*	A Quaternary ammonium compound-resistant *Pseudomonas aeruginosa* strain exhibited a higher expression level of *OprR* compared with a knock-out mutant, showing that the expression level of *OprR* was correlated with the level of resistance to quaternary ammonium compounds.	[107]
Benzalkonium Chloride	Ciprofloxacin	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa* adapted to benzalkonium chloride showed differences regarding the expression of outer membrane proteins when compared with a non-adapted strain, and some of these proteins are involved in ciprofloxacin resistance	[108]
Triclosan	–	*Escherichia coli*	A triclosan-tolerant *Escherichia coli* strain exhibited an amino acid substitution in the FabI protein, where a glycine was replaced by a valine, preventing the formation of the FabI-NAD $^{+}$ complex and reducing the effect of triclosan in *Escherichia coli*	[109]
Chlorhexidine	Ampicillin Gentamicin Polymyxin B	*Pseudomonas stutzeri*	A chlorhexidine-resistant *Pseudomonas stutzeri*, obtained after exposure to chlorhexidine, showed resistance to triclosan, benzalkonium chloride, polymyxin B, gentamicin, and ampicillin	[110]
Triclosan	–	*Salmonella enterica* Typhimurium	The recalcitrance of planktonic cells of *Salmonella enterica* Typhimurium after exposure to triclosan is associated with: • The expression of degradative enzymes • Changes in the bacterial cell envelope • The activation of the efflux pump	[111]

(*continued on next page*)

Table 2 (*continued*)

Biocides	Antibiotics	Microorganism	Mechanism of resistance/Cross-resistance	References
			• Mutations in the enoyl reductase enzyme The susceptibility of biofilms of *Salmonella enterica* Typhimurium to triclosan is associated with: • The substance's low extracellular matrix diffusion • Overactivity of efflux pumps, • Exopolysaccharide production	

Table 3
Compilation of the biocides referred to in this study, organized by their chemical category and common applications.

Category	Biocides	Common Applications	References
Quaternary ammonium compounds	Benzalkonium chloride Cetyltrimethylammonium bromide Alkyltrimethylammonium compounds Dialkyldimethylammonium compounds	Disinfection in healthcare, personal care products, and industrial cleaning	[54]
Bisbiguanides	Chlorhexidine	Hospital antisepsis, dental care, and household cleaning	[117]
Phenolics compounds	Triclosan Ortho-phenylphenol	Personal care products (e.g., soaps, toothpaste, cosmetics)	[118]
Aldehydes	Glutaraldehyde Ortho-phthalaldehyde	Industrial disinfection, hospital sterilization	[119]
Alcohols	Isopropanol Ethanol	Hand sanitizers, surface disinfectants	[120]
Peroxygens	Hydrogen peroxide	Surface disinfection, water treatment	[121]
Halogen-releasing agents	Sodium hypochlorite Peracetic acid	Water disinfection, industrial cleaning, and surface sterilization	[122]
Bisphenols	Triclosan Triclocarban	Antimicrobial agents in personal care products	[123]
Heavy-metal derivatives	Silver compounds Povidone-iodine Diamidines	Medical devices and surface coatings Antisepsis in healthcare, personal hygiene products.	[124] [125]

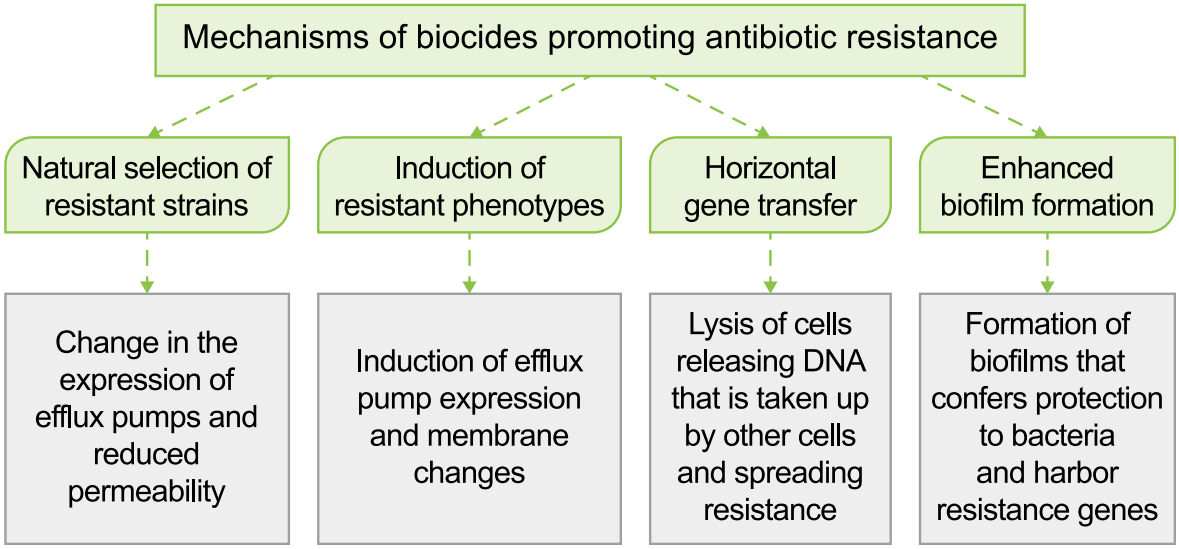

Fig. 2. Compilation of the mechanisms by which biocides promote antibiotic resistance addressed in this study [15,84,126,127].

frequent DNA replication [142,145,146]. In contrast, slow-growing or dormant bacteria may evade, leading to tolerance and the development of resistance mechanisms, like persister cells [147,148]. Slow-growing or dormant cells within a bacterial population can act as highly tolerant persister cells. These cells can survive the treatment and then resume growth once the molecule is removed [147,149]. In addition, slow-growing or stressed bacteria may activate stress-response pathways, which can lead to an increased mutation rate and the acquisition of resistance elements [147,150]. The specific growth rate can also modulate bacterial resistance mechanisms through the expression of resistance genes and affect the evolution of resistance [151,152].

5.2. Biocides as inducers of resistant phenotypes

The concentrations of biocides in natural environments are unlikely to be sufficient to kill large numbers of bacteria [25]. Moreover, chronic sub-bactericidal exposure to biocides can cause

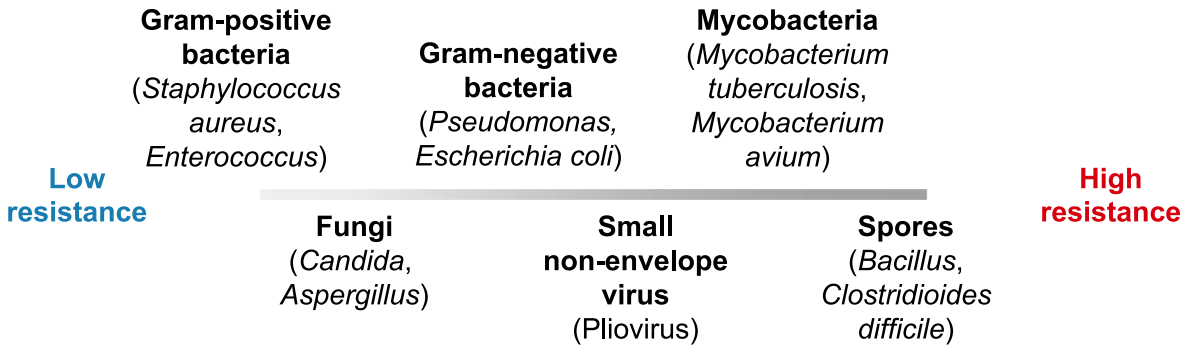

Fig. 3. Ascending order of resistance to biocides and antibiotics (based on McDonnell and Russell [68]).

transient mutations in these microorganisms [25,153,154]. Such mutations are usually not enough to kill the cell, but more likely, they just cause a minor phenotypic change in the bacteria that will make it more or less fit for its environment and more or less susceptible [155]. These phenotypic changes are temporary, most of the time, but can sometimes become permanent when mutants arise [156,157]. These responses have evolved to permit bacteria to adapt rapidly to environmental stresses and may drive selective enrichment of antimicrobial-resistant strains of bacteria [156,158]. The induction of such bacterial resistance mechanisms is often linked with the over-expression of efflux pumps [159], the over-expression of multigene and protein systems [128], and altered exopolysaccharide production [151]. Also, alterations in surface charge that result in less affinity for cationic antimicrobials and alteration in lipid content that complicates the diffusion of lipid-soluble antimicrobials across the cellular membrane [160]; reduced expression of porins, which limits antimicrobials uptake [161]; high expression of efflux pumps that expel a wide range of toxic molecules reduces antimicrobials accumulation inside the cell [162]. This topic has been comprehensively reviewed recently and was graphically summarized in Fig. 4 [130,159,163,164].

Furthermore, alterations in membrane properties can lead to QAC resistance and cross-resistance to membrane-active antibiotics [29,84,165,166]. In *E. coli*, spontaneous acquisition of resistance to cetyltrimethylammonium bromide (CTAB) was reported to be due to alteration in membrane lipopolysaccharide composition and reduction in *OmpF*, which reduces bacteria permeability. These changes also conferred resistance to a wide variety of antibiotics [167].

Concerning the role of OMP expression associated with QAC resistance, some studies [105,168,169] showed that resistance to QACs was accompanied by reduced levels of *OmpC*, *OmpF*, and *OmpA*. In *E. coli*, the loss of *OmpF* and *OmpC* genes makes cells resistant to some antibiotics, namely to hydrophilic antibiotics, such as β-lactams [85,170]. Macrolides, aminoglycosides, rifamycins, novobiocin, fusidic acid, and cationic peptides diffuse across the lipid bilayer, so alterations in lipid content can be related to resistance to these antibiotics [170].

One of the main controversial issues in antimicrobial research is the fact that *in vitro* conditions and laboratory findings do not mimic perfectly the environmental concentrations of such compounds and so, adaptive resistance and its role in cross-resistance to antibiotics must be studied and reported with some precaution [171,172]. The majority of susceptibility tests are based on bacterial inoculum, which is prepared in a very rich medium, and it is very unlikely that bacteria *in situ* have such readiness of nutrients [171].

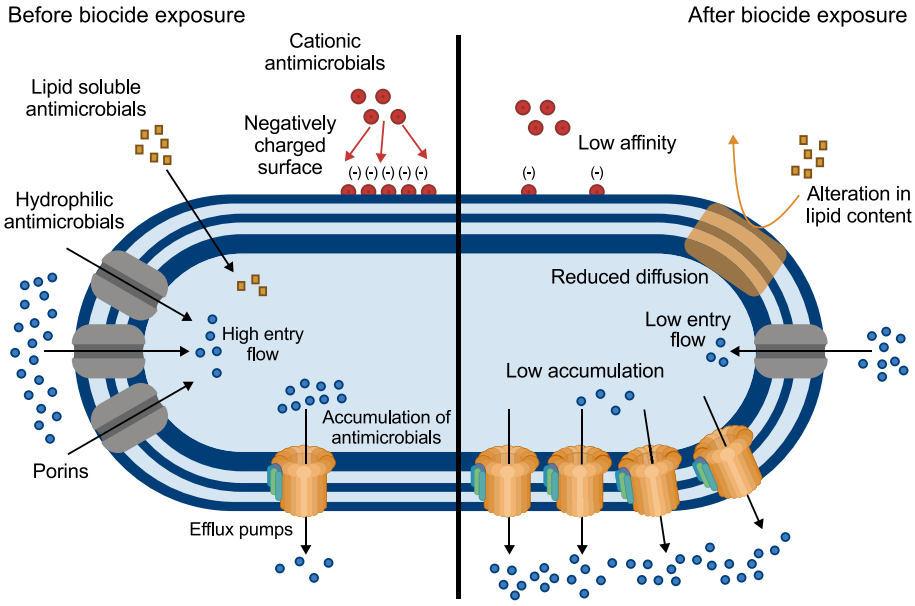

Fig. 4. Non-specific structural and functional alterations in the bacterial outer layers following biocide exposure, leading to resistance to a broad spectrum of antimicrobials, evolving the over-expression of efflux pumps, the over-expression of multigene and protein systems, altered exopolysaccharide production, alterations in surface charge and in lipid content, and reduced expression of porins (based on Lorusso, Carrara [130], Huang, Wu [159], De Gaetano, Lentini [163], Chetri [164]).

Consequently, assessing bacterial resistance is essential for understanding bacterial responses to drugs, disinfectants, and other treatments. Several well-established methods are available for evaluating bacterial resistance *in vitro* [173–175]. The disc diffusion method (Kirby-Bauer test) involves spreading a bacterial suspension on an agar plate and placing antibiotic-impregnated paper discs on the surface. After incubation, zones of inhibition (areas where bacterial growth is prevented) are measured to assess susceptibility [176,177]. A more quantitative approach, the broth microdilution method, involves culturing bacteria in microplates containing varying concentrations of antibiotics or antimicrobials. The MIC is determined as the lowest concentration that inhibits visible bacterial growth [175,178]. The E-test (Epsilometer Test) utilises a gradient strip infused with an antibiotic placed on a bacterial lawn, with the MIC being identified at the point where bacterial growth intersects the strip [179]. Molecular techniques are also instrumental in identifying resistance markers. Polymerase chain reaction (PCR) amplifies specific resistance genes to confirm their presence [180,181], while whole-genome sequencing provides comprehensive genomic data, enabling the detection of both known resistance genes and novel mutations [182]. Additionally, automated systems such as VITEK 2 and BD Phoenix efficiently determine bacterial susceptibility by analysing growth parameters in the presence of antibiotics [183–185].

Moreover, most studies involving biocide resistance and cross-resistance to antibiotics are based on planktonic cells. Little is known about the behaviour of cells in the biofilm community when exposed to biocides. Besides, information is lacking on whether biocide-resistant cells are more likely to form biofilm communities [186].

5.3. Role of environmental biofilms in resistance

Biocides can enhance biofilm formation under certain conditions [127]. While biocides are designed to inhibit or kill microorganisms, sub-lethal concentrations or improper usage can act as environmental stressors, triggering bacteria to form biofilms as a protective mechanism [12,127]. Biofilms provide a collective defence, shielding bacteria from biocides and other antimicrobial agents [187]. Moreover, biocides may induce the overexpression of efflux pumps in bacteria that not only expel biocides but also play a role in biofilm formation by enhancing bacterial adhesion and resistance to environmental threats [188,189]. In addition, bacterial exposure to biocides may modify the composition of the cell membrane, making it easier for bacteria to adhere to surfaces [190,191].

Biofilm communities are present in the most diverse environments—industrial and hospital surfaces, food processing lines, home surfaces, and naturally in the environment—being continuously exposed to sub-inhibitory concentrations of molecules [192–195]. In addition, biofilms represent an ideal setting for acquiring and spreading antibiotic resistance determinants and thus be considered suitable biological indicators of anthropogenic pollution [195,196]. The abundance of resistance determinants through bacterial adaptation and phenotypic plasticity may also indicate specific types of environmental contamination [197]. In particular, biofilms can be considered specific niches of resistant genetic material and microorganisms with increased resistance to antibiotics [197,198]. Environmental biofilms are potential hotspots for resistant bacteria and ARGs. Biofilm communities that harbour resistant bacteria and genes may be influenced by the input of biocides and antibiotics from urban wastewater treatment plant effluents and even rivers, lakes, and basins [199,200] (Fig. 1). Understanding the ecology of biofilm communities and their resistome is much needed, since these micro niches can contribute to resistance spreading [197,201].

Biofilms can be 10–1000 times more resistant than their planktonic counterparts [202], which renders antimicrobials ineffective at the in-use concentrations [203]. The high antimicrobial resistance of biofilms results from the combination of innate and induced mechanisms of resistance: innate resistance is related to biofilm physiology itself, whereas induced resistance occurs with the interaction between the antimicrobial and the bacteria [204,205]. One example of innate resistance is the extracellular matrix of biofilms, constituted by polysaccharides, DNA, and proteins that prevent the antimicrobials from reaching their target either by diffusional limitations, chemical reaction, or by the production of specific metabolites like alginate that trap antibiotics [206–208]. Negatively charged exopolymers bond to positively charged molecules such as aminoglycosides or other cationic antimicrobials like QACs, delaying their penetration into the biofilm [205,209]. In *P. aeruginosa* biofilms, resistance to QACs and peracetic acid can be partly explained by diffusional limitations through the biofilm structure [210]. However, reduced diffusion due to the extracellular matrix is not the only resistance mechanism, as other resistance mechanisms should be implicit, such as when total penetration of the antimicrobial was observed together with antimicrobial resistance [211].

Establishing microenvironments within the biofilm, with reduced nutrients and oxygen, also leads to slow bacterial growth. This “dormant” state can be a way of defence since antibiotics typically act upon rapidly growing bacteria [187,212,213]. *P. aeruginosa's* antibiotic resistance against tobramycin and ciprofloxacin was partly due to oxygen limitation and low metabolic activity [214]. Likewise, dormant variants of regular cells called “persister cells” are highly tolerant to antimicrobials to help biofilm protection [208,215]. Simões et al. [216] investigated the physiology and behaviour of *Pseudomonas fluorescens* after treatment with OPA in both planktonic and sessile states. Biofilm cells were resistant to the action of the biocide OPA compared to their planktonic counterparts. Biofilm cells were also able to recover after treatment with OPA, while planktonic cells remained inactive after 48 h. This example elucidates that biofilms have persister cells that can resist antimicrobial treatment [216].

Stream biofilms act as environmental reservoirs of antibiotic-resistant bacteria (ARB) and ARGs. However, there are not enough studies reporting how bacteria within biofilms face spatial and temporal alterations of stream physicochemical properties and how these changes can alter the abundance of ARGs within these biofilms [197]. Biofilms act as particular reservoirs within the environment, and since biofilm mass is always regenerating through sloughing-off events, these sessile structures retain suspended particles, including chemical pollutants and ARG, but also release them as a function of external stresses. As such, the presence of such drivers of resistance may have a profound impact on the composition and function of stream biofilms and the ecosystems in their surroundings [197]. Pagedar et al. [217] showed that *E. coli* strains increased the biofilm formation potential in response to sub-bactericidal residual levels of BZK, suggesting a role of the food chain in the emergence of antimicrobial resistance. When in the presence of BZK, biofilms tend to be more dense and cohesive than biofilms formed without those compounds [186,217].

Sessile microorganisms are also present in drinking water distribution systems (DWDS), where they are continuously exposed to trace concentrations of emerging contaminants and disinfectants, typically chlorine. Gomes et al. [218] showed that *Stenotrophomonas maltophilia* biofilms isolated from a DWDS became more resistant to sodium hypochlorite after being exposed to trace concentrations [218]. Zhang et al. [219] tracked the detachment of several ARBs with resistance to tetracycline, sulfamethoxazole,

clindamycin, and norfloxacin from biofilms formed on the pipes of a DWDS. Acinetobacter, Sphingomonas, and Bradyrhizobium-resistant strains were higher in the outlet water than in inlet water, even in chlorinated systems. This was verified during the one-year sampling period, with high cell counts attributed to the detachment of biofilm cells [219].

WWTP effluents can be a source of ARGs in environmental biofilms. The prevalence of resistance-associated genes of the main antibiotic families [beta-lactams (bla CTX-M), fluoroquinolones (qnrS), sulphonamides (sul I), and macrolides (ermB)] were found in WWTP [195]. These ARGs modify the physical chemistry and biological characteristics of the receiving streams and can be a driver for the spread of antibiotic resistance in microbial benthic communities. The highest concentration of ARGs was observed in biofilms collected downstream of the WWTP discharge points, favouring the increase and spread of antibiotic resistance among environmental biofilms [195]. Roberto et al. [197] demonstrated that ARG abundances co-vary about the extent of urbanization within a watershed over time and that differences in abundances are reflected in taxonomic community structure changes due to stream physicochemical properties. The abundance of resistance determinants through bacterial adaptation and phenotypic plasticity may indicate specific types of environmental contamination [197].

The antimicrobial resistance of environmental complex biofilm communities may be maintained, disseminated, and evolved due to exposure to environmental contaminants [220–222]. Biofilms, due to their diverse composition, can harbour antibiotic-resistant bacteria, representing great risks to public health [198,223,224].

Testing biofilm resistance is critical for understanding chronic infections and environmental resistance, though standardised protocols are still being developed [223]. The minimum biofilm eradication concentration (MBEC) assay is increasingly recognized as a standard method for determining the concentration of an antibiotic or antimicrobial required to eradicate an established biofilm in laboratory investigations [225,226]. In addition to MBEC, other methods are employed to evaluate biofilm resistance. The crystal violet assay quantitatively measures biofilm formation by staining the biofilm biomass. Although it does not directly assess resistance, it is frequently used to gauge the capacity of bacteria to form biofilms, a key factor in biofilm-associated resistance [227,228]. The resazurin assay measures metabolic activity within biofilms, utilizing a colour change in the resazurin dye to indicate cellular respiration and biofilm viability following antimicrobial treatment [229,230]. Biofilm dispersal assays assess the ability of a molecule to disrupt biofilm integrity, with confocal microscopy or scanning electron microscopy (SEM) providing visual insights into biofilm structure before and after treatment [231]. Lastly, PCR can be employed to detect biofilm-associated genes, such as *icaA* in *Staphylococcus aureus* and *pelA* in *P. aeruginosa*, which play a role in biofilm formation. Identifying these genes helps determine whether a bacterial strain possesses a genetic predisposition for biofilm production, often linked to increased resistance levels [232,233].

5.4. Biocides as drivers of resistant genetic material

Acquired resistance can occur due to mutations, horizontal gene transfer, acquisition of plasmids or transposons, upregulation of efflux pumps, membrane structure/alteration, formation of endospores, and biofilm formation [134,234].

Efflux pumps, such as the SMR pumps and MFS QacA/B efflux pumps, in Gram-positive bacteria are plasmid-encoded. Meanwhile, it is common for efflux pumps to be chromosomally encoded in Gram-negative bacteria and multidrug pumps [134,234]. Plasmids are important in disseminating genes that confer resistance to QAC and other biocides [15,58]. The acquisition of genes encoding for efflux pumps, generally found in mobile genetic elements [58], is particularly important in cross-resistance to antibiotics [106,235]. However, the literature does not describe resistance mechanisms for some biocides. The mode of action of biocides is generally non-selective and multi-factorial. Thus, many resistance mechanisms, such as efflux pumps and changes in porin expression, are not specific to any biocide. Moreover, only a few cases have been described in which resistance mechanisms are chemistry-specific [84]. For instance, triclosan resistance can spread via horizontal gene transfer and exert selective pressure to drive the spread of resistance [236]. This is problematic since the gene *FabI* is a promising target for new-narrow-spectrum antimicrobials against drug-resistant *S. aureus* [237]. For *K. pneumoniae* that underwent adaptation to chlorhexidine, mutations leading to the upregulation of the efflux protein SmvA and the two-component regulator PhoPQ were linked to resistance against this biocide [104]. Resistance to BZK, on the other hand, was associated with alterations in the membrane component TolC and the regulation of OmpF porin levels [104]. In addition, the development of hydrogen peroxide resistance was mediated by a set of enzymes, not exclusively peroxidases [104].

Another interesting point that should be addressed is the possibility of genetic material released to the environment from the disruption of bacterial cells after chemical treatments with disinfectants being able to remain intact and be internalised by other bacteria. Moreover, as biofilms age, they undergo structural and compositional changes, experiencing reduced stability due to nutrient depletion, waste accumulation, or other environmental stressors [187,238]. During the detachment of the biofilm, its microbial cells and matrix may be released into the environment, including extracellular DNA (eDNA) and intracellular DNA from lysed cells (a common occurrence in ageing biofilms) [239,240]. Free DNA in the environment can be taken up by other microorganisms, potentially facilitating the transfer of genetic genes, such as the ones responsible for antibiotic resistance or virulence factors expression [3,20,241].

This phenomenon is relevant in the context of horizontal gene transfer (HGT), especially regarding the potential spread of antibiotic resistance genes (ARGs) [242,243]. The persistence of extracellular DNA, particularly in environments where bacteria can form biofilms or disinfectants that are not completely effective in degrading DNA, provides opportunities for horizontal gene transfer, including the spread of ARGs [223,239,244]. These events highlight the importance of considering the trajectory of genetic material during disinfection processes and its potential impact on microbial evolution and public health [245,246]. Consequently, the potential for eDNA to remain intact and be taken up by other bacteria is of significant concern, especially regarding the spread of antibiotic resistance in healthcare settings (where disinfectants are widely used to control infections and the DNA from resistant bacteria can be transferred to other bacteria, exacerbating the problem of multidrug resistant infections) [247] and agriculture and aquatic systems (where disinfectants are used to manage bacterial populations and resistant bacteria can release their genetic material into soil and water systems, leading to the spread of antibiotic resistance across different ecosystems) [248,249].

Evidence has demonstrated that eDNA can remain intact after chemical treatments with disinfectants, depending on the type of disinfectant, the environmental conditions, and the bacteria involved [248,250,251]. While disinfectants such as chlorine, ethanol, or QACs are highly effective at killing bacterial cells, they do not always fully degrade or break down the DNA released from these cells [29,59,68]. For example, research has shown that

chlorine can kill bacteria effectively, but plasmid DNA and chromosomal DNA from these cells can remain intact and potentially available for uptake by other microorganisms in their environment [252–254]. Moreover, in biofilms, DNA from lysed cells can become incorporated into the matrix, shielding it from disinfectant degradation and enabling eDNA to remain available for horizontal transfer over time [187,255]. Cells in biofilms release eDNA as part of their extracellular matrix, and this phenomenon plays a significant role in the resistance of biofilms to disinfectants, especially oxidative agents like hydrogen peroxide and sodium hypochlorite [127,256,257]. Studies have shown that bacterial cells in biofilms release more eDNA under stress from disinfectants, helping protect them against chemical attacks [127,239,258]. Biofilms also limit the accessibility of disinfectants to eDNA by forming small protective pockets within the matrix, making the eDNA more resistant to degradation (e.g., by DNases) compared to planktonic (free-floating) bacteria [255,259,260]. Additionally, the resistance of biofilms can be heightened in multispecies biofilms. For instance, species like *E. coli* have been shown to protect *Salmonella* in dual-species biofilms when exposed to chlorine [261,262]. This suggests that interactions between different bacterial species can influence how effectively disinfectants degrade eDNA and kill bacteria [261,262].

It is also known that some bacteria from diverse genera, known as naturally competent bacteria, can naturally uptake free plasmid DNA or chromosomal DNA from their environment through a process called transformation [263,264]. This mechanism allows them to internalize genetic material, including genes that could confer beneficial characteristics, such as antibiotic resistance [265].

In a different scenario—water treatment plants where disinfectants such as chlorine or UV are used—researchers have observed that antibiotic resistance genes can persist in the environment even after bacterial cells are killed since released DNA can be uptaken by competent bacteria in the system, facilitating the spread of resistance genes in treated water systems [266,267]. The same reasoning is valid for hospital wastewater systems and agricultural settings, where disinfectants are regularly used to manage microbial populations [268,269].

Besides what was said before, the stability of eDNA and the likelihood of its internalization by other bacteria is influenced by several factors like temperature, pH, organic matter, and the presence of DNases (enzymes that degrade DNA) [270,271]. DNA bound to surfaces such as soil particles or biofilm matrices is more resistant to enzymatic degradation and can persist longer, increasing the chances of bacterial uptake [272]. Moreover, the effectiveness of disinfectants in degrading DNA depends on their concentration and exposure time, as some studies have shown that suboptimal concentrations of disinfectants may not degrade DNA sufficiently, allowing it to persist in the environment [273,274].

6. Conclusions and future perspectives

The emergence and dissemination of bacterial resistance in the environment is a growing issue affecting people's and the environment's health worldwide. It is recognized by clinicians, researchers, policymakers, and the public as crucial to circumvent new pandemics caused by resistant microorganisms. Resistance development can be due to residual concentrations of biocides and antibiotics, particularly in water ecosystems. Identifying anthropogenic inputs contributing to the spread of resistant bacteria and resistance genes will aid in developing strategies to combat antibiotic resistance. Moreover, the increase in the concentration of biocides can be caused by the overuse and misuse and increased production of these agents as antiseptics, detergents, and other disinfectants in anthropogenic activities, possibly responsible for the emergence of antibiotic-resistant bacterial strains. Also, over the years, there has been an inevitable increase in the in-use concentration of these products to maintain their effectiveness, accompanied by the bioaccumulation of resistance-associated biopollutants, that are increasingly present in these ecosystems. One of the reasons for the lack of surveillance is the lack of standardised testing methodology and definitions for resistance. The majority of studies have utilised MICs; however, the methodologies differ. This technique is less relevant for antiseptics, where the lethal concentration, rather than the inhibitory concentration, needs to be measured, and MBC testing is deemed more meaningful. Due to the absence of a standardised testing methodology, there has yet to be a national or international agreement regarding an appropriate cutoff value for defining resistance. Thus, a standard definition and protocol to assess resistance to biocides *in vitro* is needed to reach a better understanding of published scientific data. Since using this class of compounds is as inevitable as employing antibiotics due to their vast application and efficiency in reducing the microbial load on the surface, it is imperative to reduce their fate in the environment. Although several measures have already been adopted in the European legislation to control the emissions and increase the WWTP's efficiency in retaining these chemical compounds, new measures should be adopted before the levels of these chemicals become toxic to the biomes.

CRediT authorship contribution statement

Mariana Sousa: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Idalina Machado:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Lúcia C. Simões:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Manuel Simões:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Data availability

All data are available from the corresponding author upon reasonable request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by national funds through FCT/MCTES (PIDDAC): LEPABE, UIDB/00511/2020 (DOI: 10.54499/UIDB/00511/2020) and UIDP/00511/2020 (DOI: 10.54499/UIDP/00511/2020) and ALiCE, LA/P/0045/2020 (DOI: 10.54499/LA/P/0045/2020); by Project InnovAntiBiofilm (ref. 101157363) financed by European Commission (Horizon-Widera 2023-Acess-02/Horizon-CSA); Mariana Sousa's PhD scholarship (2023.00337.BD) is provided by The Foundation for Science and Technology (FCT).

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