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REVIEW
Possible implications of biocide accumulation in the environment on the prevalence of bacterial antibiotic resistance

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The lethality of biocides depends upon their interaction with a number of distinct biochemical targets. This often reflects reactive chemistry for any given agent, such as thiol oxidation. Susceptibility may vary markedly between different target organisms, and changes within the more sensitive targets can alter the inhibitory effect. The multiplicity of potential targets, however, usually dictates against the development of overt resistance to concentrations used for hygienic applications. Similarly, although changes in cellular permeability toward such agents, mediated either by envelope modification or the induction of efflux-pumps may reduce susceptibility, they rarely influence the outcome of treatments at use-concentration. It has recently been proposed that chronic exposure of the environment to biocides used in a variety of commercial products might expose some microbial communities to subeffective concentrations causing emergence of resistant clones. Such resistance might relate to mutational changes in the most susceptible target or to regulatory mutants that cause the constitutive expression of certain efflux pumps. Although selection of organisms with such modifications is unlikely to influence the effectiveness of the biocides, changes in their susceptibility to third-party antibiotics can be postulated. This is particularly the case where a cellular target is shared between a biocide and an antibiotic, or where induction of efflux is sufficient to confer antibiotic resistance in the clinic. Although such linkage has been demonstrated in the laboratory in pure culture, it has not been documented in environments commonly exposed to biocides. In nature, the effects of chronic stressing with biocides are complicated by competition between microbial community members that may result in clonal expansion of naturally insusceptible clones.
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Introduction

Traditionally, biocides have been regarded as distinct from antibiotics because of their lower pharmacological specificity and marked mammalian toxicity. The ideal antibiotic possesses a single biochemical target that is absent in the host organism. The emergence of multiple antibiotic resistance can be largely attributed to indiscriminate and often inappropriate use of antibiotics [30,57]. Antibiotic resistance is related to a number of distinct mechanisms, including alteration of the target, enzymatic neutralisation of the agent and changes in the accessibility of target through reductions in cellular permeability or by the induction of efflux pumps [32,35]. With low therapeutic indices, changes in MIC of 5- to 10-fold are often sufficient to render therapy ineffective. Although similar reductions in susceptibility (up to 50-fold) have been noted for some biocides [12], they rarely result in treatment failure because MICs are not indicative of bactericidal interactions, and in any case, use-levels are generally considerably greater. Indeed, many biocidal molecules have been widely deployed for over 100 years without any apparent loss of effectiveness [8]. For these compounds, small changes in susceptibility, indicated by an altered MIC, can often be generated by sub-lethal exposure and relate to the most susceptible target. This may not necessarily reflect a lethal interaction. Failure of changes in MIC to affect treatment outcomes is therefore a reflection of the multiplicity of biochemical targets possessed by biocides [35].

Although the concentrations of the active agents at the point of application can generally be controlled, sub-lethal exposure to biocide occurs as they become dissipated to the environment. At some distance from the site of use, sub-effective concentrations may exert selection pressures toward resistance development [19]. The widespread incorporation of antibacterial agents into consumer products has compounded the problem, especially for agents with lower chemical reactivity, such as the quaternary ammonium compounds and triclosan. Reactive antimicrobials, that is, oxidising agents such as hypochlorite, will be more rapidly neutralised at the point of use and will therefore not be substantive.

Although it is difficult to conceive that major decreases in biocide effectiveness would result from this type of selection pressure, concerns have been expressed that where targets are shared with third-party therapeutic agents, then resistance towards the latter might emerge [31]. Sub-lethal stress with inimical agents might also induce the expression of general defence mechanisms in microbial cells [37], such as efflux pumps, potentially leading to problems in the clinic. If these selections led to the emergence of constitutively expressed multi-drug efflux mutants, then although the effectiveness of biocides would not generally be affected, multi-drug resistance might become more prevalent.

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Antibiotic resistance and biocide tolerance

Perspectives

A study using a large collection of bacteria, which were archived between 1917 and 1954, showed that antibiotic resistance genes were present during the pre-antibiotic era, although at a significantly lower frequency than today [26]. Antibiotic-resistant enteric bacteria have also been isolated from water samples taken from 2000-year-old Canadian Arctic glaciers. Current problems with antibiotic resistance in the clinic are clearly a consequence of antibiotic overuse. These, combined with a decline in the rate of antibiotic discovery, have raised concerns that our ability to combat bacterial infection might become compromised [23,30]. US$25 million extra funding for the Centre for Disease Control's infectious disease programme has been committed to the surveillance of antibiotic-resistant infections [52], while the World Health Report of 1996 recognised drug resistance as a "huge public health problem."

The pharmacological specificity of antibiotics means that there is a high potential for single mutational events within the target organism to confer resistance [14]. Additionally, cytosolic constituents of an organism, such as glutathione, might chemically quench the activity of thiol interactive biocides. Some enzymes (i.e. aldehyde dehydrogenase and aldehyde lyase) might lead to denaturation of others with consequent marked reductions in their intracellular concentration and apparent effectiveness [11]. Some resistance determinants are likely to be transferred between commensal or environmental bacteria and pathogens *via* transmissible genetic elements [23]. For example, tetracycline resistance can be transferred from intestinal enterococci to pneumococci and erythromycin resistance from *Bacteroides sphaericus* to *Bacteroides fragilis* [16]. Single plasmids conferring multi-drug resistance can be assembled by recombination of several mobile genetic elements (including integrons and transposons) in response to selective pressures [23]. The same general mechanisms of resistance apply to complex microbial communities such as biofilms. In such respects, modification of the target site, chemical and enzymatic inactivation of the agent and reductions in the intracellular concentration of an agent [11,45] are compounded by community effects. Thus, the close proximity of cell clusters that are capable of drug inactivation will confer resistance to adjacent susceptible clones. A difference between the observed resistance of clinical and environmental isolates and that of the corresponding natural communities is that the former is dependent on genotypic change, reflected in properties of the individual cells, whereas the latter also reflects phenotypic heterogeneity and spatial organisation of the cells into biofilm. Spatial organisation of such cells within biofilm communities not only delays achievement of lethal doses within the depths of the community but also, through the imposition of nutrient stress, causes the expression of less susceptible phenotypes [20]. Thus, a large proportion of the community is exposed to a sub-lethal stress resulting in an associated selection/enrichment of genotypes with reduced susceptibility. Where biochemical targets are shared between chemical biocides and therapeutic agents, then cross-resistant forms might be selected by sub-effective biocide treatment [18].

Triclosan

Triclosan is a broad-spectrum bisphenol biocide, which is widely used in household and personal products as an antibacterial agent, as well as having therapeutic applications in the treatment of topical MRSA infection [35]. When triclosan was introduced, it was thought to act similarly to other phenolic agents, through non-specific interaction with the cell membrane causing loss of cytoplasmic materials [47], leakage of protons and an uncoupling of oxidative phosphorylation from respiration [9]. More recently, a number of studies have demonstrated that sub-lethal levels of triclosan select for mutants in the FabI gene of *Escherichia coli*. FabI encodes an enoyl-acyl carrier protein reductase, an essential enzyme involved in synthesis of fatty acids [24]. In this respect, triclosan appears to be a potent inhibitor of the enoyl reductase [29,38,39]. Triclosan shares this target with some current therapeutic agents, suggesting that sub-lethal triclosan exposure could select for resistance to such third-party antimicrobials. Notable in this respect is the enoyl reductase of *Mycobacterium tuberculosis* and isoniazid, currently the most widely used anti-tuberculosis drug [40]. Reductions in the isoniazid susceptibility of *Mycobacterium smegmatis* can be conferred by mutations in InhA, which is a homologue of FabI [39]. However, similar phenomena with *M. tuberculosis* have not yet been documented, and isoniazid-resistant *M. tuberculosis* remains sensitive to triclosan. This suggests that although the two agents share the same target, their interactions with it are distinct. The major clinical use of triclosan relies on its effectiveness against Gram-positives causing skin infections [15]. In this respect, the majority of reported triclosan resistance has been in Gram-negative bacteria. The high degree of homology between the enoyl reductase of *E. coli* and *Staphylococcus aureus*, however, might have implications for potential emergence of triclosan-insusceptible staphylococci. The respective enoyl reductases of these bacteria are functionally interchangeable and genetically engineered mutations in the *S. aureus* FabI can confer triclosan resistance [25]. Concentration of research upon interactions of triclosan with enoyl-reductase enzymes has arguably lost sight of some important properties of this antibacterial and may have led to misleading conclusions being drawn. Although triclosan and possibly hexachlorophane may be unique amongst the chlorinated phenolics in their interactions with a single enzyme at low concentrations, their action at bactericidal levels may well reflect the original understanding of these agents as inflictors of membrane injury. In this respect, Villalain *et al* [60] studied the effects of triclosan on membrane integrity, showing lysis of a variety of oral bacteria at use-concentrations but not at the MIC.

Permeability change

The majority of antimicrobial agents must gain access to the cytoplasm to exert their effect [48]. Polycationic agents (e.g. aminoglycosides) gain access to the cell through a self-promoted mechanism [22,58]. In self-promotion, the agent destabilizes cations associated with the cell envelope causing reorganisation of the LPS to facilitate antibiotic entry. It is notable that some biocides, particularly polymeric biguanides [62], share this mechanism of cellular uptake. Adaptations against these agents might therefore demonstrate cross-reaction between biocide and antibiotic.

Efflux

An increasingly observed resistance mechanism is the expression and overproduction of multi-drug efflux pumps [44]. Expression of such pumps is induced, in Gram-negative bacteria, through sub-lethal exposure to a plethora of agents. These include not only small hydrophilic antibiotics but also other reagents such as pine oil, and

salicylate [41,42]. Mutations that increase the expression of such efflux pumps result in elevated levels of resistance. Although efflux pumps are operational in a wide variety of Gram-negative organisms, and may be plasmid- or chromosomally encoded [44], multi-drug efflux pumps qacA-G also contribute to biocide tolerance in *S. aureus* [51]. Maira-Litran *et al* [33] showed that although the efflux system *acr*AB was not required for biofilm resistance, its constitutive expression significantly enhanced the levels of survival within *E. coli* biofilm communities exposed to ciprofloxacin. Moken *et al* [42] and McMurry *et al* [37,39] showed that mutations causing overexpression of *mar*A and *acr*AB are associated with exposure and reduced susceptibility toward triclosan. This is because triclosan is a substrate for this pump, but not an inducer. Similarly, mutations in the MexAB operon of *Pseudomonas aeruginosa* leads to overexpression of this efflux system and triclosan can select for mutants that hyper-express *mex*CD efflux in MexAB-deleted mutants [13].

Many xenobiotics induce the expression of efflux systems. Indeed, under conditions of chronic sub-lethal exposure, such as can be generated in the laboratory, they may also select hyper-expressing mutants. It must be borne in mind that the primary function of energetic efflux is to defend the cell against naturally occurring environmental toxicants [61]. Concentration of research upon induction and selection by antibiotics and biocides neglects the fact that many food substances, including mustard, chilli and garlic can also induce these systems [61]. Also often neglected is that hyper-expressing mutants, although resistant to antibiotics in laboratory cultures, may also pump-out metabolites and be relatively noncompetitive in mixed microbial communities [51]. This is especially the case when the antimicrobial selection pressure is removed or transient.

Cross-resistance

Many biocides have retained their effectiveness over more than 100 years of use. In the case of triclosan, however, recent studies suggest that, assuming *E. coli* is not the exception to the rule, generalised resistance could emerge. Because enoyl reductase is a major target of this compound, then "triclosan abuse" may also jeopardise development of any future agent for which enoyl reductase is a primary target. With respect to other biocides, there exist worrying possibilities that changes in biocide susceptibility may be reflected in antibiotic resistance, especially where a sole antibiotic target is shared with the biocide. Currently, there is insufficient understanding as to whether indiscriminate use of biocides might select resistance toward current antibiotics or hinder the development of new ones. Further urgent investigation is clearly required.

Field studies

Although an association between chronic sublethal exposure of monocultures in the laboratory has been unequivocally demonstrated to be associated with changes in susceptibility, this phenomenon remains to be demonstrated in the real world. In real-life situations, individual species of bacteria are in competition with their congeners and their competitive fitness determines their survival. Arguably, the clinic represents an environment where biocide usage has been extreme. Accordingly, a number of studies have been carried out to evaluate whether clinical or environmental isolates that show reduced susceptibility to biocides also exhibit resistance to antibiotics. The results of these studies have been ambiguous. Thus, Stickler and Thomas [55] assessed MICs toward a range of antiseptics, disinfectants and antibiotics, of Gram-negative bacteria isolated from a hospital environment, and found that approximately 10% of the isolates (mainly *Pseudomonas*, *Proteus* and *Providencia*) exhibited some reduced susceptibility to chlorhexidine and cetrimide and were also generally more resistant to multiple antibiotics. Similarly, Reverdy *et al* [49] showed that antibiotic-sensitive *S. aureus* and other staphylococci for which elevated MICs toward various antiseptics were recorded, were also less susceptible, albeit below a resistance threshold, to a wide variety of antibiotics. Increased MICs for methicillin-resistant *S. aureus* (MRSA) strains have been reported for some biocides, including chlorhexidine, cetrimide, benzalkonium chloride (HAC), hypochlorite, triclosan, parahydroxybenzoates and betadine [10,43,51,59]. Bamber and Neal [5], however, found that none of 16 MRSA isolates exhibiting low-level mupirocin resistance had increased MICs toward triclosan. Similarly, Suller and Russell [56] found that a series of MRSA clinical isolates showed some degree of reduced susceptibility to a range of biocides that included chlorhexidine, cetylpyridinium chloride (CPC), benzalkonium chloride and triclosan, relative to methicillin-sensitive isolates. Most of the strains described in the above studies remained equally susceptible to bactericidal concentrations bearing testimony to the multiplicity of target sites implicated in the bactericidal action of biocides. Many other studies fail to observe any changes in MIC. Thus, Stecchini *et al* [54] showed that, despite widespread antibiotic resistance in strains of *Enterobacteriaceae* isolated from mince meat, these were not resistant to the bactericidal activity of an amphoteric Tego™ disinfectant. Similarly, Femandez-Astorga *et al* [17] isolated psychrotrophic non-fermenting Gram-negative strains from vegetables and showed that antibiotic-resistant strains were susceptible to the bactericidal action of QAC and hypochlorite disinfectants.

Baillie *et al* [3] compared the chlorhexidine sensitivity of 33 clinical isolates of *Enterococcus faecium*, sensitive to vancomycin and gentamicin, with that of 12 vancomycin- and 7 gentamicin-resistant strains. The results showed no increase in resistance to chlorhexidine as indicated by MIC. Interestingly, a study of 67 ciprofloxacin-resistant isolates of *P. aeruginosa* yielded four isolates which were hypersensitive to chlorhexidine, whereas none were found amongst 179 ciprofloxacin-sensitive isolates [4].

Andessen *et al* [2] determined the susceptibilities of vancomycin-resistant and -sensitive enterococci (VRE and VSE) to various concentrations of commonly used hospital disinfectants, including quaternary ammonium compounds, phenolics and an iodophor, at recommended use-dilutions and extended dilutions using the suspension test. They concluded that there was no relationship between vancomycin resistance and resistance to disinfectants at use-dilution. This was confirmed by Suller and Russell [56], who showed that a series of VRE and VSE clinical isolates showed no significant difference in sensitivity to chlorhexidine, CPC and triclosan when evaluated both by bacteriostatic (MIC) and bactericidal assays.

Bamber and Neal [5] determined the MIC of 186 isolates of MRSA and MSSA. Published data for triclosan state that the expected MICs for staphylococci should be between 0.01 and 0.1 ppm. They found 14 isolates (7.5%) with MICs greater than 1.0 ppm, but these were equally distributed between MRSA and MSSA strains.

Rutala *et al* [53] and Payne *et al* [46] showed that a series of antibiotic-resistant clinical and environmental isolates, including

P. aeruginosa, *Klebsiella* spp., *E. coli*, *S. aureus* and *Staphylococcus epidermidis*, were no less susceptible to the bactericidal activity of disinfectants, including a phenol and quaternary ammonium disinfectant, chloroxylenol, cetrimide and povidone iodine.

The variable nature of the observable links between changes in biocide and antibiotic susceptibility suggests that there is no single underlying cause and that realistic assessments of the implications can only be made by better understanding their physiological basis.

Fitness implications of resistance mutations

Many studies have shown that adaptation to resistance is associated with decreased fitness of the organism. Mutations that confer resistance may generate functional deficits in certain cellular processes [21,27]. Decreased fitness at the simplest level may be recognised in terms of reduced competitiveness in pure or mixed culture, or in the decreased virulence of pathogens. For example, carriage of resistance plasmids has been associated with decreased growth rate [27], whereas isoniazid resistance in *M. tuberculosis* and virulence were negatively correlated [6]. In this respect, it is arguable that FabI mutants, selected by triclosan, would be less efficient at fatty acid biosynthesis, and that efflux hyper-expressing mutants will invest comparatively more energy than a wild-type bacterium in maintaining efflux operation. With respect to more thoroughly studied antibacterials, resistance has indeed been associated with decreased rates of replication which, in the absence of the drug, may serve as a selective advantage for the wild-type strain [21].

Evolution operates by gene substitution under selection pressure and the most competitive clones or species will theoretically prevail. One could therefore envisage that in the absence of the selection pressure, sensitive revertants would proliferate and outcompete resistant clones. Accordingly, any mutation that alters the fitness of a bacterium may alter the ecological balance of a community. In this respect, climax biofilm communities are remarkably resistant to further colonisation [1,34]. To propagate within a community, a newly selected clone should possess enhanced competitive fitness. Considering the high prevalence of naturally resistant species, it is highly likely that, rather than selection of resistant mutants, triclosan use will cause clonal expansion of pre-existing resistant flora. In this respect, many natural biofilm communities are dominated by species that are refractory to a wide range of antimicrobial compounds. For example, in biofilm material removed from a household kitchen sink drain that had been exposed only to oxidising biocides, considerable numbers of bacteria belonging to the family *Pseudomonadaceae* were cultured, of which many were intrinsically multi-drug resistant, including *Stenotrophomonas maltophilia* and *P. aeruginosa* [36].

A more worrying possibility is that compensatory mutations may occur that ameliorate the fitness burden of the original mutation [50]. Mutations such as these have been documented [7]. Levin *et al* [28] ran computer simulations, *in vitro* experiments, and DNA sequencing with low frequency rpsL (streptomycin-resistant) mutants of *E. coli*, with and without fitness-engendering compensatory mutations. In these studies, intermediate fitness-resistant clones with compensatory mutations, rather than high fitness revertants prevailed, due to the higher frequencies of compensatory mutations, against revertants. Björkman *et al* [7] used multi-drug resistant *Salmonella typhimurium in vivo* to show a negative correlation between resistance and virulence. In this study, however, the avirulent-resistant mutants rapidly accumulated various compensatory mutations during repeated passage, which restored virulence without associated increases in susceptibility. In fact, only 4 out of 26 compensatory mutants regained susceptibility.

Conclusions

Although triclosan interacts with specific targets and upregulates efflux systems in laboratory strains of *E. coli*, the implications remain unclear. These investigations have almost exclusively been carried out with pure cultures. In terms of studying the possible propagation of mutant clones in the environment, most experiments have utilised serial batch cultures to measure fitness or simple animal models to investigate virulence. Fitness has been considered mainly in comparison to the parent strain. For fitness-impaired resistant mutants to ascend in the environment, they must survive through competitiveness during treatment, and persist after treatment has finished. Experiments with pure cultures should therefore be interpreted with care, because they may not necessarily be truly representative. Studies should therefore be carried out to properly investigate the implications of biocide misuse in microbial communities, either in the environment or in laboratory microcosms.

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