



National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

**Development of a protocol to evaluate
bacterial resistance in response to
household disinfectants**

A feasibility study

RIVM Letter report 2015-0070
M.H.M.M. Montforts et al.



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Colophon

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Development of a protocol to evaluate bacterial resistance in response to household disinfectants

In veel consumentenproducten, zoals schoonmaakmiddelen en hand zeep, zitten stoffen waarmee bacteriën kunnen worden bestreden. De zorg bestaat dat bacteriën resistent kunnen worden tegen dit soort desinfectiemiddelen. Dit zou namelijk ook de resistentie van bacteriën tegen antibiotica kunnen verergeren.

Het is daarom relevant om voordat een middel op de markt komt te weten of het gebruik van dit soort producten daadwerkelijk resistentie bij bacteriën veroorzaakt. Uit een haalbaarheidsonderzoek van het RIVM blijkt dat er momenteel geen methode bestaat om dit vooraf te beoordelen. Het is wel mogelijk om dergelijke testen en scenario's te ontwikkelen, maar dat kost veel tijd en geld. Dat komt doordat deze stoffen in uiteenlopende producten en voor verschillende doeleinden worden gebruikt. Hierdoor is het lastig om in kaart te brengen in welke situatie en bij welke hoeveelheden eventueel resistentie optreedt.

Het RIVM beveelt aan om deze beoordelingsmethodiek internationaal te ontwikkelen. Daarnaast wordt aanbevolen om, nadat een middel op de markt is toegelaten, in de praktijk te controleren of en wanneer resistentie zich voordoet.

Kernwoorden: bacteriële resistentie, desinfectantia, schoonmaakmiddelen, protocol

Synopsis

Development of a protocol to evaluate bacterial resistance in response to household disinfectants

Consumers currently have access to a wide range of antimicrobial disinfectants, including cleaning products and hand soap. There is concern that bacteria may develop resistance against these products. This could also increase bacterial resistance against antibiotics.

It is therefore relevant to investigate if the use of these products actually results in bacterial resistance, and to do so before a product receives marketing authorization. A feasibility study performed by RIVM concludes that there is currently no method available for assessing resistance prior to marketing authorization. Although it is possible to develop tests and scenarios for this purpose, this would be expensive and time-consuming due to the wide range of available substances and products with various intended uses. As a result, it is difficult to identify the circumstances and product quantities that may lead to the development of resistance.

RIVM recommends developing such an assessment method internationally, and recommends post market surveillance to monitor possible development of resistance.

Keywords: bacterial resistance, disinfectants, evaluation protocol

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Summary

An important concern regarding the private use of disinfection products is that bacterial (cross) resistance may arise as a result of this use. On a national level, the Board for the Authorisation of Plant Protection Products and Biocides (Ctgb) has limited the authorisation of disinfection products for private use. However, the European harmonisation of biocides authorizations may alter this policy

During the product authorization process, a validated protocol is necessary in order to appropriately assess the development of antimicrobial resistance. RIVM performed a feasibility study whether such protocols are available or can be developed. This study addresses four questions: 1) is the EFSA guidance on the submission of data in relation to decontamination of foods of animal origin applicable in this situation?; 2) what are the mechanisms underlying the occurrence of bacterial (cross) resistance?; 3) what are the active substances and how are available products used?; and 4) do reliable test protocols or strategies exist?

The EFSA AFC/BIOHAZ panels issued a guidance describing the data requirements for the evaluation of the safety and efficacy. This guidance included only substances intended to remove microbial surface contamination from foods of animal origin intended for human consumption. The current RIVM feasibility study concluded that this guidance can also be used to describe data requirements for the evaluation of the safety and efficacy of substances intended for decontamination of foods of plant origin. And it can be applied to the situation of household application of disinfection products. The conditions for the development of antimicrobial resistance described in this guidance document also apply to the use of household disinfectants. However, the EFSA guidance does not provide testing methodology.

Bacterial resistance to biocides may develop by several mechanisms. In general, the underlying basis of resistance is a decrease in the intracellular concentration of the biocide below the threshold level of damaging the bacterial cell. Resistance mechanisms such as efflux pumps or enzymatic activity may be selected for and proliferate under stress conditions (such as biocide exposure) and be transferred on mobile genetic elements to other individuals (also to/from other species). The resistance mechanisms induced by exposure to non-lethal concentrations of biocides may also result in resistance to other biocides or antibiotics. The level of risk depends on the clinical relevance of the antibiotic (drug) resistance, the local environment, and the organisms involved. Consumer behaviour is a major factor in the spread of antimicrobial resistance. The majority of the products currently on the Dutch market is intended for hand- and skin disinfection, disinfection of spa's and pools, control of algae or moulds on hard surfaces, and disinfection of surfaces. A protocol for the assessment of the risk of resistance development in response to biocides use (including cross-resistance against antibiotics) should therefore consider several consumer exposure scenarios addressing the application and post-application phases, and exposure via treated articles and via the environment. Given the use of a limited number of active substances in

various applications, the risk of aggregated exposure may also need to be considered. It is possible to translate already well-known applications to representative scenarios.

Currently, there are no accepted standard protocols to evaluate the development of antimicrobial resistance in response to the use of disinfectants. The development of meaningful and reliable testing protocols is therefore needed, but will require a large investment of time and money. RIVM recommends establishing post-marketing surveillance to gather information on the development of resistance and on the need for further control. This requires a survey of products currently on the market and their use patterns. In addition, the development of exposure scenarios is recommended, since these will support the development of relevant testing methods and will provide a framework for qualitative risk assessment.

1 Introduction

The Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) writes:

“Some mechanisms of resistance are common to both biocides and antibiotics. The selective pressure exerted by biocides may favour the expression of these mechanisms of resistance. The existence of horizontal gene transfer, and, in particular, the presence of mobile genetic elements, and their dissemination as a result of selective pressure create the highest risks for increasing antibiotic resistance. The formation of biofilms contributes to a potential high risk for the development of cross-resistance between antibiotics and biocides” (SCENIHR, 2010).

There are currently no accepted standard protocols for the evaluation of antimicrobial resistance induced or selected by biocides. The SCENIHR strongly recommends the development of standard protocols for the evaluation of the capacity of a biocide to induce/select for antibiotic resistance and for the surveillance of resistance and cross-resistance to monitor the level of resistance and cross-resistance of environmental isolates in all areas of biocide usage.

The same committee wrote a research strategy to address the knowledge gaps on antimicrobial resistance effects of biocides (SCENIHR, 2010). In their opinion they state that: ‘tools need to be developed to define for example the *minimal selecting concentration*: the minimal concentration of a biocide which is able to select or trigger the emergence/expression of a resistance mechanism that will confer clinical resistance to an antibiotic class’. According to Schets et al. (2012) the number of ways to test for the development of resistance by means of laboratory tests is still limited. They recommend the development of protocols for surveillance of resistance and cross-resistance in environmental isolates.

The Board for the Authorisation of Plant Protection Products and Biocides (Ctgb) decided in March 2000 to limit the authorization of disinfection products for private use. This decision was based on the recommendations of the Netherlands Nutrition Centre (Voedingscentrum), the Inspectorates and the Ministry of Public Health, Welfare and Sports (VWS).

This policy decision has been applied for 10 years with the effect that only a very limited number of products has been authorized and marketed.

However, in view of the ongoing European harmonization, the national policy may have to be reconsidered. The Biocides Regulation 528/2012 (EU 2012) that entered into force in September 2013 will further limit the degrees of freedom to follow a national policy for the authorization of disinfection products.

The dominant concern regarding private use of disinfection products is that bacterial cross resistance (resistance against antibiotics, caused by biocides) may arise as a result of this use. There is scientific evidence that biocides select for biocide resistance, but there is, so far, no conclusive evidence that this also determined or will determine an increase in antibiotic resistance (SCENIHR 2009; Oggioni et al. 2013).

In order to facilitate an authorization procedure taking resistance development into account, a risk assessment protocol will have to be made available to applicants (Schets et al. 2012). The Ministry of VWS has commissioned RIVM in 2012 to study the feasibility of a protocol to test for the development of microbial (cross) resistance in response to private use of disinfection products. The results of this study are presented here.

1.1 Scope of this work

This research is intended to outline the feasibility of an evaluation protocol as described above. It is not the intention to start the development of such a protocol here but rather to define the conditions and requirements and judge the availability of information. The availability of the information are scientific publications and guidance documents until 2013. Recommendations for next steps are presented in this report.

1.2 Approach

Four major questions were identified that will help outline the feasibility of an evaluation protocol. These questions are:

- 1) Can the guidance from EFSA, published in 2010, on the submission of data in relation to decontamination of foods of animal origin be applied to the household use of disinfection products?
- 2) What do we know about the mechanisms underlying the occurrence of bacterial (cross) resistance and what can we say about the likelihood of these mechanisms to occur in response to the use of disinfection products?
- 3) What kind of products and applications are currently on the market or can be expected to become available? What kind of active substances are found in available products?
- 4) Are reliable testing protocols available? Do EFSA, SCENIHR or others provide recommendations for relevant testing strategies?

2 Mechanisms underlying the development of resistance

The Scientific Committee on Emerging and Newly Identified Health Risks (SCENHIR) concluded that resistance to biocides is a common phenomenon and considered to emerge following improper use or storage of the formulations resulting in a decrease in the effective concentration (SCENIHR 2009). Although resistance to biocides has been reported in health care settings, most of the evidence on bacterial resistance comes from laboratory-based experiments. In contrast to antibiotics, biocides usually have a broad spectrum of mechanisms of action, i.e. biocides have multiple target sites in microbial cells. Therefore, it is unlikely that bacterial resistance is caused by specific modification of a particular target site or by a specific metabolic by-pass. The basis of bacterial resistance to biocides resides in the mechanisms causing the intracellular concentration of the biocide to stay under the threshold above which it is causing damage to the bacterium. These mechanisms include changes in the cell envelope, changes in cell wall permeability, efflux mechanisms and degradation. These methods may operate synergistically and can have an intrinsic or acquired basis (McDonnel and Russel 1999).

Intrinsic resistance refers to the natural ability of bacteria to counter the effects of biocides. The most described intrinsic mechanism of resistance concerns changes in the permeability of the cell envelope, which can limit the amount of biocide that enters the cell, thereby decreasing the intracellular concentration (Denyer 2002). This includes changes in the outer membrane ultrastructure including lipopolysaccharides (LPS), fatty acid composition, phospholipids and the charge property of the cell surface (SCENIHR 2009). Another intrinsic resistance mechanism is the presence of efflux pumps, which decrease the intracellular concentration of biocides. Although efflux pumps are widespread among bacteria, the importance in terms of bacterial resistance is modest since they usually result in merely an increase in the minimum inhibitory concentration (MIC) rather than resistance to high concentrations of biocides. A third mechanism of intrinsic resistance is enzymatic transformation of biocides. However, like with efflux pumps, this usually results in an increase in the MIC.

With *acquired resistance* there are changes in the genetic composition of the bacterium, leading to resistance. Bacteria can acquire resistance through mutation or by uptake of exogenous genes by so-called horizontal gene transfer from other bacteria. These may include genes encoding for enzymes that modify the antimicrobial compound. Acquired mechanisms of resistance are of particular concerns since previously susceptible bacteria may become insusceptible to biocides (Russel 2002).

In addition to intrinsic and acquired resistance, the induction of resistance mechanisms following exposure has been described. This can be referred to as *adaptive resistance* and may include over-expression of efflux pumps. Often this is related to the induction of stress-response systems in the bacterium, resulting in decreased growth rates and

altered gene-expression. Stress may involve any condition deviating from the optimal growth conditions. The functional mechanisms of stress-resistance may overlap with mechanisms of resistance to biocides. Therefore, induction of general stress resistance mechanisms by environmental exposure to stress may induce *cross-resistance* to biocides. This may also occur as a result of growth in biofilms and quorum sensing mechanisms.

A specific point of concern with bacterial resistance to biocides is the possibility of cross-resistance and co-resistance. The resistance mechanisms induced by exposure to non-lethal concentrations of biocides may also result in resistance to other biocides or antibiotics. This phenomenon is referred to as *cross-resistance* (Fraise 2002). With *co-resistance* the resistance to a substance (biocide) is genetically linked to the resistance to another substance (antibiotic). This may occur when different resistance genes are localized on the same plasmid or when a gene is transferred or mutated that regulates the expression of resistance pathways of both biocides and antibiotics.

Resistance to antibiotics as a result of exposure to biocides is of public health relevance when it concerns pathogenic bacteria or opportunistic bacteria that can cause serious infections in immune compromised patients. However, 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. The level of risk depends on the clinical relevance of the antibiotic resistance and the organisms involved.

Bacterial species evolve by mutation, and mutations in a bacterial genome happen naturally. Although mutations do not occur fully randomly, it is not predictable when and where in a bacterial genome mutations occur. Strains with natural mutations leading to an increased level of resistance to a biocide will be selected in an environment with sub lethal concentrations of such a biocide. Additionally, the presence of antibiotics and biocides induce bacterial stress. The frequency of mutation is known to increase under conditions inducing stress.

Biocides (like QACs, chlorhexidine) can select for efflux-based resistance mechanisms, if these are present. This depends on the organisms involved. For example for QACs it is known that dozens of efflux determinants can be acquired confer resistance to antibiotics (including fluoroquinolones, tetracyclines, beta-lactams, aminoglycosides, and erythromycin) (Hegstad et al., 2010). Further horizontal gene transfer to pathogens is a function of the local environment. The behaviour of the consumer will be a determinant for infection and further spread of resistant species.

The Minimum Selective Concentration (MSC) is the minimal concentration of a biocide, which is able to select or trigger the emergence/expression of a resistance mechanism concerning an antibiotic class in a defined bacterium (SCENIHR 2009). In the literature, data are available that indicate that resistance is triggered far below the MIC (Gullberg et al., 2011; O'Reilly and Smith, 1999), the lowest concentration that inhibits growth completely. The MIC and MSC

vary between specific micro-organism and with substances. This implies that dilution, diffusion in biofilms, and degradation -by default- lead to a situation where resistance may be favoured.

3 Relevance of existing EFSA guidance

The EFSA AFC/BIOHAZ panels issued a guidance for the evaluation of safety and efficacy of substances for the removal of microbial surface contamination of foods of animal origin intended for human consumption (EFSA 2010). These guidelines have been applied to the evaluation of lactic acid for the decontamination of beef carcasses (EFSA 2011) and to the evaluation of Cecure® (a quaternary ammonium compound) for the decontamination of raw poultry (EFSA 2012).

These guidelines have also been used to describe the data requirements for the evaluation of safety and efficacy of substances for the decontamination of foods of plant origin (by RIVM, commissioned by the Ministry of VWS).

As part of the EFSA guidance (2010) data requirements are described to evaluate the potential emergence of acquired reduced susceptibility to biocides and/or resistance to therapeutic antimicrobials (EFSA (2010) Chapter 8). A summary of this guidance can be found in Appendix I.

Whether or not this guidance is relevant to the situation addressed in the current report is answered by the following statement in the EFSA guidance: *"When no prior knowledge is available concerning a proposed formulated product and its potential for development of acquired reduced susceptibility to biocides and/or resistance to therapeutic antimicrobials, additional tests would be required to address these issues. The use of decontaminating agents in a formulated product may promote the development of acquired reduced susceptibility to biocides and/or resistance to therapeutic antimicrobials as follows (EFSA, 2008a)"*.

Both the efficacy of a biocide and the development of resistance to biocides within a microbiological community depend on a range of factors such as concentration, contact time, temperature, mode of application, the microbial load of the surface and other conditions of application. Especially sub-lethal concentrations of biocides favour the selection of resistant organisms (see Chapter 3). Therefore, before allowing the use of a biocide in a domestic environment, the following points need to be addressed following the EFSA guidance for foods of non-animal origin:

1. A pre-market evaluation including scientific data on the development and dissemination of acquired reduced susceptibility to biocides and/or resistance to therapeutic antimicrobials following exposure to the formulated product at in-use concentration and concentrations that may be lower, for example, when the product is discharged without neutralization.
2. The type and quality of data expected are indicated in section 8.3 (App. I)

3. Target pathogenic and other relevant microorganisms have to be tested for resistance to therapeutic antimicrobials listed in earlier reports (EFSA 2008b,c). In general these antimicrobials are considered appropriate for most pathogens, although account should be taken of differences in the intrinsic resistance of Gram-negative and Gram-positive target pathogenic and indicator organisms to certain antimicrobials.

4. Development of resistance to therapeutic antimicrobials should be tested in:

- Target pathogenic organisms, e.g. *Campylobacter* species, *Salmonella enterica*, *Listeria monocytogenes* and *Staphylococcus aureus*;
- Other relevant organisms which occur in food or can be transferred between humans.

For these investigations, reference strains of target pathogenic and other relevant organisms should be included.

According to EFSA (2010), a post-market evaluation is recommended if a product is discharged without neutralization as this will lead to environmental concentrations lower than intended. If the effect of such lower concentrations is already included in the pre-market evaluation, a post-market evaluation is not necessary.

In conclusion, the EFSA (2010) guidance is fully relevant to the application of disinfection products in household settings.

4 Product usage and areas of application

4.1 Definition of the scope

The data inventory on usage and areas of applications has been restricted to the Biocides Regulation (EC) 528/2012 Product Types 1 and 2 (PT01 and PT02 as presented in Scheepmaker et al. (2012), for all currently authorised biocides see the Ctgb website). Several other product types, treated articles, and borderline applications are not considered.

- Consumer products intended to fight higher organisms (insects, rodents etcetera) are not considered here, since there is no direct link to cross-resistance against antibiotics.
- Biocides for wood or textile preservation (as well as articles made of treated materials) are not considered. Research on the mechanisms of fungal resistance against triazole compounds was performed by the European Centre for Disease Prevention and Control (ECDC, 2013). The Dutch Ministry of Infrastructure and the Environment commissioned a research into the triazole use in biocides in the Netherlands (Schoep and Sterenberg, 2013).
- Several active substances have a wide range of applications in plant protection products, cosmetics, medicinal products or medical devices. Examples are: - quaternary ammonium compounds to fight moss on terraces (since algae and mosses occur together, such products might have dual authorization) and disinfectants in glasshouses or storage; - triclosan in toothpaste, disinfectants for open skin or with a medical claim, and in disinfectants for medical devices. These uses are not assessed since they are out of the legal scope of the Biocides Regulation (EC) 528/2012. However, this knowledge does raise the awareness that aggregate exposure should be considered when assessing the risks of biocide use (see e.g. Skovgaard et al. (2013)).
- Several ingredients used in consumer products, such as cationic surfactants and fragrances, possess antimicrobial properties. Surfactants have an intrinsic antibacterial activity (anionic, non-ionic, organic acids [active against Gram-positive bacteria] and compounds with alkyl chains [active against both Gram positive and negative bacteria]) and may increase the overall bactericidal activity of the associated products when used in combination. These uses are not assessed since they are out of the legal scope of the Biocides Regulation (EC) 528/2012. Like with the above mentioned products, this points at the need for the assessment of aggregate exposure.

4.2 Available products and relevant areas of use

Recent data of registered and illegal biocides in the Netherlands¹ show that the majority of products on the market target hand- and skin disinfection (PT01), and disinfection of spa's and pools, control of algae or mould on hard surfaces, and disinfection of surfaces (PT02). Under the Biocides Regulation 528/2012 (EU 2012), treated articles will also be regulated. The use of (nano-)silver and triclosan in consumer products like textile and cutting-boards are typical examples to be considered.

The list of the active substances used in these biocides for consumer use (households), or in consumer products, can be classified on their mode of action as suggested by the SCENIHR (2009). This is presented in Table 1. Biocides like aldehydes (formaldehyde, glutaraldehyde) and ethylene oxide are considered to be restricted to professional use only, and are not listed.

Table 1 List of active substances in biocidal consumer products and their mode of action (SCENIHR, 2009)

Biocide	Usage/areas of applications	General mode of action
Quaternary ammonium compounds (QACs)	Algicide (outside terrace), smellicide (textile), surface disinfection	Membrane destabiliser, at a high concentration produce cytoplasmic protein aggregation (loss of tertiary structure)
Biguanides	Surface and skin disinfection	Chlorhexidine specifically inhibits membrane-bound ATPase
Phenols/cresols	Smellicide (textile), surface disinfection (including treated articles)	Triclosan: enoyl acyl reductase at a low concentration. Dinitrophenol collapses membrane energy (ATP synthesis). A low concentration of fenticlor and triclosan inhibits energy-dependent uptake of amino acids. A low concentration of triclosan discharges membrane potential in <i>E. faecalis</i>
Alcohols ethanol, isopropanol, n-propanol	Surface and skin disinfection	Inhibition of DNA and RNA synthesis, cell wall synthesis (secondary effect). Low concentration of phenoxyethanol induce proton translocation in <i>E. coli</i>
Organic acids	Surface disinfection, preservatives	Dissipation of proton motive force; Inhibition of uptake of amino acids
Iodophors	Skin disinfection	Covalent binding to thiol groups (povidone iodine)
Metals, Metallic salts	Surface disinfection (including	Interactions with thiol-group ((nano-)silver, copper)

¹ www.ctgb.nl accessed 9 November and 16 November 2012; Scheepmaker et al. (2012)

	treated articles)	
Isothiazolinones	Preservatives in many household products	BIT (benzisothiazolinone) affects active transport and oxidation of glucose in <i>S. aureus</i> , activity of thiol containing enzymes , ATPases, glyceraldehyde-3-phosphate dehydrogenase
Peroxides	Surface, water, and skin disinfection	Oxidising agents
Chlorine compounds and other halogens	Surface and water disinfection	Oxidising agents
Etheric oils	Preservative, fragrance, disinfection	Unknown membrane interaction

An inventory was made of disinfection products available in the Netherlands for non-professional use. The number of products available is listed in Table 2. This list includes illegal products, i.e. products that have not been authorized or registered. These products do give an indication of favoured applications.

Table 2 Number of disinfection products available in the Netherlands for non-professional use.

Status	PT01	PT02	PT01 + PT02
Authorised ¹	2	111	-
Registered ²	32	7	10
Illegal ^{3, 4, 5}	3	83	-

¹ and ² based on data obtained from Ctgb, November 2012 (most recent)

³ Distinction between non-professional and professional uses is not feasible for illegal products, as this distinction is made at the official authorization/registration by Ctgb.

⁴ based on Scheepmaker *et al.*, 2012. The overview of illegal products is most likely an underestimation.

⁵ Database/Report Scheepmaker *et al.*, 2012 was focused on P02. Occurrence of PT01 products will be underestimated.

These products have been analysed for intended use and active substance. The result of this is presented in Table 3. A number of main application areas can be identified as well as frequently present active substances. These results are found to be largely in line with the data provided by the SCENIHR.

Table 3 Main areas of application and the actives found in products.

Area of application	Active substances found
hand and skin	propanol, chlorhexidine digluconate, ethanol, iodine, polyvinylpyrrolidone iodine, QACs, triclosan
pool and spa	calcium- and sodium hypochlorite, sodium dichlorisocyanurate, trichlorisocyanuric acid, QACs
algae and moulds	didecyldimethylammoniumchloride, QACs

surfaces and appliances	hydrogen peroxide, silver chloride, chlorhexidine digluconate, ethanol, propanol, QACs
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4.3 Information requirements for the assessment of resistance development

A protocol for the assessment of the risk of resistance against biocides and of cross-resistance against antibiotics should consider several consumer exposure scenarios. Scenarios should address the application phase, the post-application phase, exposure via treated articles, and the exposure and distribution to the environment. At this stage, the discharge to the environment (via waste) is not considered. The environmental route also applies to non-consumer use of biocides and issues like aggregate exposure are deemed to be too complex to develop a useful protocol at this stage.

These consumer exposure scenarios should be designed based on criteria that reflect the likelihood of frequent exposure to concentrations in the range of the Minimum Selective Concentration (MSC) of specific microbial pathogens and the relative importance of this exposure. These criteria involve the exposure conditions, the scale of use, the microbial species involved at the target site, and the occurrence of environmental factors that may favour the selection of resistance.

Next to these criteria that refer to the exposure of a microbial community to one or more biocides, there are criteria that refer to the exposure of the consumer to this microbial community, and to other risk factors that e.g. speed up horizontal gene transfer, such as contact with large reservoirs of resistance genes (e.g. soil, or clinical settings). The consumer groups may even act as this transmission mechanism. Here we are concerned with the frequency, intensity and duration of exposure to microbial communities, and the potentially vulnerable consumer groups involved. A special case is the event that the targeted microbial community is present on the consumer. In this event the exposure of the consumer to the biocide coincides with that of the consumer to the microbial community (biocides in PT01, or used in textile or cosmetics).

In reality, the consumer is exposed via various pathways (including aggregate exposure) to both active substances and to affected microbial communities. Aggregate exposure is the exposure to one single chemical via all exposure routes (dermal, oral and inhalatory) and from different sources (for example several different consumer products and/ or in combination with food). For example silver may be present in biocides, but also in cosmetics like body wash, in bandages, and in consumer products like textile. To develop and select representative exposure scenarios, data must be collected and assessed on the four identified major areas of use, in terms of the criteria specified. Label claims on areas of use will guide this process. Data should be quantified as far as possible, in order to parameterize the values in the exposure scenarios.

Table 4. Cross table of areas of use of biocides and criteria to select representative exposure scenarios. Examples are given in the cells.

Criteria Areas of use	Number of products	Large scale of use	Frequency & pathways of exposure to microbes or biocide	Aggregate exposure	Vulnerabl e groups of consumer s	Pathogen * of concern and risk factors
Hand and skin	>50	Ethanol	Skin	Pool, surface		<i>S. aureus</i>
Spa and pool	>50	Chlorine	Skin, water	QACs		<i>S. aureus</i> , <i>Legionella</i>
Hard surfaces disinfection	>50, treated articles	All compounds	Skin, food, ingestion	QACs to clean cutting boards and other surfaces	Exposure of toddlers via toys (QACs)	<i>Salmonella</i> <i>Listeria</i> . Risk factors: dilution at edges, biofilms
Algae and moulds	>50	QACs against algae on outside terrace	Skin, aerosols	QACs against algae on outside terrace		<i>Listeria</i> ; Outside: source of resistance
Textile	Treated articles	Silver, QACs, triclosan	Skin	QACs, triclosan		<i>S. aureus</i>

* Including communal organisms relevant for horizontal gene transfer

From the preliminary data in Table 4 it may be concluded that scenarios should be developed both for specific areas of use, regardless of the active substance, and for specific active substances, in view of their potential for aggregate exposure.

It should be noted that not only the risk of resistance against the active substance (affecting efficacy and the risk that a desired level of protection against harmful organisms is not reached) should be assessed. Clearly the risk that cross-resistance against clinically relevant antibiotics could develop in either pathogens or communal organisms should be addressed.

With the information specified in Table 4 the most relevant situations can be identified, based on the combination of the intensity of use, the exposure of vulnerable groups, the proximity of pathogens, and the likelihood that conditions are present that select for resistance. This would at least provide for a framework to qualify the risk of resistance development and subsequent impact.

4.4 Parameters and conditions for the development of methodology

In order to develop exposure scenarios for each of the relevant areas of use, that also allow for aggregate assessments:

- the situation of use (including intended concentrations) and exposure, and of consumer behaviour in a household setting should be specified.
- related to this situation, details are needed on the precise locations (e.g. edges, cuts, biofilms) that should be assessed, the pathways via which the biocide dissipates, and on pathways that lead to exposure to potentially resistant microbes.
- relevant information regarding spatial and temporal scale, ambient conditions (temperature, pH etcetera) should be collected.
- contact time, transfer coefficients, and other human exposure parameter information should be gathered.
- Risk factors for horizontal gene transfer (HGT), including the presence of (opportunistic) pathogens, the presence of a reservoir of resistance genes, and environmental (stress) factors that favour HGT.
- For aggregate assessment scenarios should be grouped based on the consumer behaviour (what activities can be expected to be undertaken in such a way that an aggregate assessment is warranted?) and on active substances (is there a possibility that identical or complementary selection processes occur?).

This information will be integrated to qualify and, as far as possible, quantify

- the conditions that could select for resistance in specific (to be identified) micro-organisms
- the risk of transferring this resistance between species of micro-organisms.

This will provide the benchmark to test for resistance development in a suitable realistic worst-case reference situation, or to extrapolate *in vitro* (laboratory) results or *in loco* field results on resistance development to this benchmark.

Information on consumer behaviour and consumer exposure parameters is –in general- available. Approaches to assess hot spots *in loco* are available from other frameworks such as medical devices. Data on consumer related pathogens, commensals, and on risk factors for HGT, are available. The scenarios should address the intended use, as well as situations that can be accepted as foreseeable misuse by consumers. Label claims and user instructions provide for such conditions (environmental factors) that should be considered for proper use and efficacy. Not observing such conditions could enhance the development of resistance and conversely give information on environmental conditions.

In conclusion, it is possible to develop scenarios and derive from these the conditions that will have to be implemented in resistance testing in order to make the testing protocol meaningful. The efforts to do this are considerable, and depend both on the degree of involvement of experts and stakeholders, and on data availability (see also Maillard et al. (2013)).

5 Testing methods for resistance

There are currently no accepted standard protocols for the evaluation of antimicrobial resistance induced or selected by biocides. The SCENIHR strongly recommends the development of standard protocols for the evaluation of the capacity of a biocide to induce/select for antibiotic resistance and for the surveillance of resistance and cross-resistance to monitor the level of resistance and cross-resistance of environmental isolates in all areas of biocide usage.

Reproducible testing of the frequency of occurrence of genetic processes such as mutations, and horizontal gene transfer is apparently difficult. . "The design of efficacy test protocols for **biocides** is complex notably because of the number of factors that need to be controlled. These factors can be divided into those depending upon the micro-organism (e.g. test strain, preparation of inocula, detection and count of survivors) and those depending upon the test method (e.g. quenching antimicrobial activity, physical parameters" (SCENIHR, 2009).

One of the major limitations of efficacy test protocols is their reproducibility and robustness. In addition, practical tests conducted in laboratory conditions that aimed to simulate conditions in the field, might sometimes be too rigid and do not allow much flexibility which impinge on the ability to set parameters reflecting conditions found in practice. On the other hand tests *in loco* are costly and difficult to standardize since parameters cannot be controlled accurately in the field. These tests remain poorly reproducible and their outcomes might be contentious, although they would provide key information on the **antimicrobial** efficacy of **biocides** to the manufacturers and end users.

Schets et al. (2012) recommend developing protocols for surveillance of resistance and cross-resistance in environmental isolates, before and after authorization of the product(s). This surveillance will also require further method development. One advantage of this approach lies in the possibility to collect samples *in loco* and compare samples taken before and after application of the biocidal product. The study of Skovgaard et al (2013) provides an example on triclosan. In this way, the large number of possible variables in the application of the products does not need to be considered in the experimental set up. Given the current availability of disinfection products, establishing post-marketing surveillance is the quickest way to gather information on the development of resistance and on the need to for further control.

Meaningful and reliable testing protocols are not sufficiently available at this moment in time. The development of such protocols is critical for the design of a risk assessment approach. A large number of parameters and a high complexity will have to be considered. The RIVM holds the expertise required to contribute to method development. However, development of appropriate methodology, for example a framework as outlined by Maillard et al (2013), requires an international effort and a time investment that should be expressed in years.

6 Conclusions and recommendations

6.1 Conclusions

- The EFSA guidance describing the data requirements for the evaluation of safety and efficacy of substances for the removal of microbial surface contamination of foods of animal origin intended for human consumption can be applied to the situation of household application of disinfection products.
- The mechanisms of emerging resistance to biocides and therapeutical anti-microbials are well understood. The risk of emerging (co-)resistance is higher for some biocides than for others.
- Indications for dominant application areas in household use can be based on currently available products (considering legal and illegal offer).
- Resistance to biocides is a common phenomenon. The risk that the substances predominantly found in known products specifically induce cross resistance to therapeutic antimicrobials remains to be assessed. Given the use of a limited number of active substances in various applications, the risk of aggregate exposure needs to be considered.
- It is possible to translate currently known applications to scenarios that represent situations of concern and that define the conditions and parameters for testing.
- Meaningful and reliable testing protocols are not sufficiently available at this moment in time.
- The RIVM holds the expertise required to contribute to method development. However, development of appropriate methodology requires an international effort and will take several years.

6.2 Recommendations

It is recommended *not* to formulate a research project to develop a regulatory evaluation protocol. The unavailability of reliable and meaningful testing methods forms the bottleneck to proceed with an evaluation protocol.

The development of reliable and meaningful testing methods (both *in vitro* and *in loco*) should be initiated, preferably in an international context. Relevance is strongly determined by knowledge about gene exchange within microbial communities, about composition of such communities in consumer environments, about environmental conditions of such communities, and about consumer behaviour.

The development of scenarios (explicit descriptions of consumer use situations) is recommended, since this will drive the development of relevant testing methods and will provide for a framework for qualitative risk assessment.

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Appendix I Summary of the EFSA guidance

[Note: this text is taken from EFSA (2010) Chapter 8. The citations in this text are not copied and the reader is referred to the original text]

Decontamination treatments involve the application of a substance in order to reduce the microbial contamination. Therefore there are three main aspects to be considered when assessing the substances: i) safety of the intended substance itself, ii) its effect as to the development of antimicrobial resistance, and iii) the efficacy i.e. does the use of the substance in practice decrease the level of contamination of pathogenic microorganisms.

INFORMATION NECESSARY FOR THE EVALUATION OF THE POTENTIAL EMERGENCE OF ACQUIRED REDUCED SUSCEPTIBILITY TO BIOCIDES AND/OR RESISTANCE TO THERAPEUTIC ANTIMICROBIALS

In cases where the formulated product has already been in use previously as "processing aid" in food products or as a food additive and it does not appear that such usage has led to the development of, or selection for acquired reduced susceptibility to biocides (other than the compound to be tested) and/or resistance to therapeutic antimicrobials, the applicant may apply for approval based on the history of apparent safe use. If data are available from application of the product for uses other than removal of food surface contamination, they could be submitted for consideration.

When no prior knowledge is available concerning a proposed formulated product and its potential for development of acquired reduced susceptibility to biocides and/or resistance to therapeutic antimicrobials, additional tests would be required to address these issues.

The use of decontaminating agents in a formulated product may promote the development of acquired reduced susceptibility to biocides and/or resistance to therapeutic antimicrobials as follows (EFSA, 2008a):

1. Cross-resistance: (i) selection for genes encoding resistance to both the formulated product and one or more antimicrobial classes or (ii) change the physiological response of the bacterium to become less susceptible to both formulated product and antimicrobials.
2. Co-resistance: selection for clones or mobile elements also carrying acquired reduced susceptibility to biocides and/or resistance to therapeutic antimicrobials.
3. Indirectly select for clones that are resistant to antimicrobials other than those related to the formulated product.
4. Enhance DNA uptake by e.g. activating a SOS response in microorganisms.

In the generic context of a potential selection for acquired reduced susceptibility to biocides and/or resistance to therapeutic antimicrobials through the use of the formulated product it is necessary to be aware of these potential ways of resistance development (selection and dissemination). The evaluation of untested formulated products will entail a case-by-case approach.

In order to assess the potential emergence of acquired reduced susceptibility to biocides and/or resistance to therapeutic antimicrobials, studies will be required to investigate if the use of the formulated product leads to development of resistance to such antimicrobials.

Following submission of the dossiers, the results of these studies will be evaluated by expert bodies. In most cases the interpretation will be based on experimental studies, supporting information and published data. When a formulated product is taken into use, the contribution to the overall level of resistance to therapeutic antimicrobials is expected to be negligible. Awareness should be high if acquired reduced susceptibility to biocides and/or resistance to therapeutic antimicrobials develops due to the use of the formulated product.

The evaluation is divided into pre-market and post-market evaluation. A plan for the post-market evaluation should be provided when an authorization for a decontaminating agent in a formulated product is sought.

Pre-market evaluation

The following points have to be addressed:

- i. The pre-market evaluation should include scientific data on the development and dissemination of acquired reduced susceptibility to biocides and/or resistance to therapeutic antimicrobials following exposure to the formulated product at in-use concentration and concentrations that may be lower as, for example, when the product is discharged. As indicated above, existing information may be considered.
- ii. The type and quality of data expected are indicated in the section 8.3.
- iii. Target pathogenic and other relevant microorganisms have to be tested for resistance to therapeutic antimicrobials listed in earlier reports (EFSA 2008b,c,e). In general these antimicrobials are considered appropriate for most pathogens, although account should be taken of differences in the intrinsic resistance of Gram-negative and Gram-positive target pathogenic and indicator organisms to certain antimicrobials.
- iv. Development of resistance to therapeutic antimicrobials should be tested in:
 - Target pathogenic organisms, e.g. *Campylobacter* species, *Salmonella enterica*, *Listeria monocytogenes* and *Staphylococcus aureus*;
 - Other relevant organisms.

For these investigations reference strains of target pathogenic and other relevant organisms should be included.

If the formulated product is neutralised before discharge of wastewater, then tests about development and dissemination of acquired reduced susceptibility to biocides and/or resistance to therapeutic antimicrobials of environmental microorganisms are not required.

In the absence of neutralisation, environmental indicator microorganisms isolated from sediment and wastewater treatment plants should be examined, taking into account the possible intrinsic resistance of such strains. In such cases, a sampling procedure should be performed in order to specifically address the microbial flora upstream and downstream of the waste water efflux, preferably also from sediments and wastewater drains. These samples should be tested by viable counts of microorganisms in the presence of the concentrations of the formulated product and/or degradation products which leave the processing environment.

Post-market evaluation

Development of resistance to therapeutic antimicrobials in pathogens or indicator microorganisms in the food or processing environment should be examined simultaneously with verification of efficacy of the formulated product through HACCP.

If the product is released into the environment without neutralisation, a post-market monitoring and evaluation is recommended to determine the long-term effects of using the formulated product on selection and dissemination of acquired reduced susceptibility to biocides and/or resistance to therapeutic antimicrobials. The following points have to be addressed, if the formulated product is not neutralised before discharge:

- i. Any novel scientific information about the formulated product should be taken into account.
- ii. A statistically significant number of environmental samples should be collected in the wastewaters and both upstream and downstream of the point of discharge. The sampling strategy should take into account seasonal changes and characteristics of the effluent.
- iii. From the environmental samples taken, relevant indicator microorganisms should be isolated, identified and used for monitoring of acquired reduced susceptibility to biocides and/or resistance to therapeutic antimicrobials as described above. All experimental data should be provided.
- iv. These examinations should be performed in a structured follow-up during a minimum of three years in line with EMEA (2006).

Type and quality of data

- i. The methods used should be reproducible and validated with the necessary controls and samples included. If available, standardised methods should be used.
- ii. The data should be suitable for risk assessment and if possible quantitative.
- iii. Susceptibility testing methods for therapeutic antimicrobials should be done using the most recent updated standardised methods (e.g. ISO and CLSI standards) for determination of the Minimal Inhibitory Concentration (MIC).
- iv. Susceptibility testing methods for biocides should be performed using the most recent updated methods. Determination of MBC should be performed according to a standard efficacy test (e.g. CEN standard).
- v. Information on the conditions of application of the formulated product must be documented, including the minimum concentration of the decontaminating agent in a formulated product achieved at the point of application, presence and nature of organic load, minimum exposure time, temperature, type of surfaces.
- vi. The interpretative criteria used to determine the level of resistance to therapeutic antimicrobials should be based on published recommendations from EUCAST and EFSA (EFSA 2008b, c, e).
- vii. The interpretative criteria used to determine the level of susceptibility to biocides should be based on MBC population distributions of the bacterial species in question.

DEFINITIONS

ANTIBIOTIC

A substance produced by, or derived (chemically produced) from a micro-organism that selectively destroys or inhibits the growth of other micro-organisms (ECDC, EMEA, EFSA, SCENIHR, 2009).

ANTIMICROBIAL

An active substance of synthetic or natural origin which destroys microorganisms, suppresses their growth or their ability to reproduce in animals or humans, excluding antivirals and antiparasites (ECDC, EMEA, EFSA, SCENIHR, 2009).

ANTIMICROBIAL ACTIVITY

The inhibitory or lethal effect of a decontaminating agent in a formulated product or an antibiotic.

ANTIMICROBIAL RESISTANCE

The ability of micro-organisms of certain species to survive or even to grow in the presence of a given concentration of an antimicrobial that is usually sufficient to inhibit or kill micro-organisms of the same species (ECDC, EMEA, EFSA, SCENIHR, 2009). Of primary concern is the

emergence of resistance to therapeutic antimicrobials, defined as antimicrobials used for treatment of diseases in humans and animals.

ACQUIRED REDUCED SUSCEPTIBILITY TO BIOCIDES

The situation when a bacterium develops tolerance to higher bacteriostatic or bactericidal concentrations than phenotypically related bacteria of the original or "wild type" strain (EFSA, 2008a).

BIOCIDES

Active substances and preparations containing one or more active substances, put up in the form in which they are supplied to the user, intended to destroy, deter, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism by chemical or biological means.

CO-RESISTANCE

Genes conferring resistance are frequently contained in larger genetic elements such as integrons, transposons or plasmids, and as such may be linked to other, unrelated resistance genes. In such cases, multiple resistance genes may be transferred in a single event. When two or more different resistance genes are physically linked, this is termed "co-resistance". Consequently, selection for one resistance attribute will also select for the other resistance gene(s), termed co-selection (ECDC, EMEA, EFSA, SCENIHR, 2009).

CROSS-RESISTANCE

It is the tolerance to a usually toxic substance as a result of exposure to a similar acting substance. Antimicrobials are a diverse group of molecules, commonly ordered in classes with similar structure and mode of action. Within a class, the target in the bacterial cell and the mode of action of the antimicrobial is the same or similar in each case. Some mechanisms of resistance will confer resistance to most or all members of a class, i.e. cross-resistance (ECDC, EMEA, EFSA, SCENIHR, 2009).

DECONTAMINATING AGENTS

Substances applied to remove or reduce surface contamination of food. When decontaminants are used on food, the substance is considered a processing aid if removed following the application. If the substance is not removed, it will be classified as a food additive (it remains present in the food and has a technological effect, e.g. a preservative action; a food additive can also be applied on the surface of food e.g. glazing agents).

DISINFECTION

The reduction, by means of chemical agents and/or physical methods, of the number of microorganisms in the environment, to a level that does not compromise food safety on suitability.

ECOTOXICOLOGICAL RISK

The ecotoxicological risk is the risk linked to the hazards (substances discharged in the environment) characterized by toxicological studies on different representative environmental species and the exposure of these species depending on the chemical and physical properties of the substance, environmental characteristics, duration and route of

exposure. The use of bio monitors is frequent for the routine surveillance.

ECOTOXICOLOGY

Science dealing with the fate and effects of pollutants on ecosystems.

FOOD ADDITIVES

Any substance not normally consumed as a food in itself and not normally used as a characteristic ingredient of food, whether or not it has nutritive value, the intentional addition of which to food for a technological purpose in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food results, or may be reasonably expected to result, in it or its by-products becoming directly or indirectly a component of such foods.

FORMULATED PRODUCT

The ready-to-use product for which authorisation is sought.

MULTIDRUG RESISTANCE

This term is used when a bacterial strain is resistant to more than one antimicrobial or antimicrobial class. There is no standard definition, which makes the term problematic and comparisons difficult. It is therefore important to define multidrug resistance in any document referring to 'multiple resistance'. Traditionally multidrug resistance is regarded as resistance to at least three different chemically-unrelated classes of antimicrobials, and is frequently transmissible. Strains exhibiting such resistance are termed 'multidrug-resistant' (MDR) (ECDC, EMEA, EFSA, SCENIHR, 2009).

PROCESSING AIDS

Any substance which (i) is not consumed as a food by itself; (ii) is intentionally used in the processing of raw materials, foods or their ingredients, to fulfil a certain technological purpose during treatment or processing; and (iii) may result in the unintentional but technically unavoidable presence in the final product of residues of the substance or its derivatives provided they do not present any health risk and do not have any technological effect on the final product;

RESIDUE

One or more of the substances present in a biocidal product which remains as a result of its use including the metabolites of such substances and products resulting from their degradation or reaction.

THERAPEUTIC ANTIMICROBIALS

Antimicrobials used for treatment of diseases in humans and animals

