EUROPEAN COMMISSION

Directorate-General for Health & Consumers

Scientific Committee on Consumer Safety

SCCS

Opinion on triclosan

Antimicrobial Resistance

Scientific Committees
on consumer safety
on emerging and newly identified health risks
on health and environmental risks

The SCCS approved this opinion at its 7th plenary of 22 June 2010 after public consultation

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They are: the Scientific Committee on Consumer Safety (SCCS), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and are made up of external experts.

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Scientific Committee members

Jürgen Angerer, Ulrike Bernauer, Claire Chambers, Qasim Chaudhry, Gisela Degen, Gerhard Eisenbrand, Thomas Platzek, Suresh Chandra Rastogi, Vera Rogiers, Christophe Rousselle, Tore Sanner, Kai Savolainen, Jacqueline Van Engelen, Maria Pilar Vinardell, Rosemary Waring, Ian R. White

Contact
European Commission
Health & Consumers
Directorate C: Public Health and Risk Assessment
Unit C7 - Risk Assessment
Office: B232 B-1049 Brussels
Sanco-Sc6-Secretariat@ec.europa.eu

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Dr. J. Davison, Retired, Member of SCHER, FR
Dr. J.-Y. Maillard, Cardiff University, UK
Dr. J.-M. Pagès, University of Marseille, Member of SCENIHR, FR (Rapporteur)
Dr K. Pfaff, BfR, DE
Dr. S.C. Rastogi, Retired, Member of SCCS, DK (Chairman)

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ABSTRACT

Triclosan is a biocide used in many product categories, including cosmetics. The information on environmental concentrations of triclosan in the EU is limited and the bioavailability of the triclosan to bacteria in the environment is not known.

Although the present mandate concerns the evaluation of a possible association between the use of triclosan in cosmetic products and the development of resistance by certain micro-organisms, the SCCS has taken into account all evidence available from all uses of triclosan to perform its assessment.

A number of scientific and technical data gaps about the occurrence and understanding of the resistance profile of triclosan have been identified and should be addressed.

At present, several distinct hazards have been identified: (i) the effect of triclosan on the triggering/regulation of resistance genes in bacteria (ii) the existence of defined mechanisms that can promote resistance and cross-resistance to biocides and antibiotics in bacteria, (iii) high concentrations of triclosan (compared to concentrations known to select for resistance in *in vitro* experiments) have been measured in certain environmental compartments and (iv) bacterial biofilms, which are widespread in the environment and are able to survive exposure to adverse environmental factors. The first two of these hazards have been identified *in vitro*. The presence of resistance genes in soil bacteria should be investigated further.

Based on the six *in situ* studies and the one meta-analysis quoted in this document and recent data from *in vitro* investigations (proteomic and genomic analyses), it is not possible to quantify the risk associated with triclosan (including its use in cosmetics) in terms of development of antimicrobial resistance (i.e. selection for less susceptible population), genetic basis for resistance and dessemination of resistance. In view of the concentrations of triclosan reported to trigger resistance *in vitro*, some of the environmental concentrations found in a number of geographical distinct areas are high enough to suggest that bacterial resistance could be triggered. However, no studies have been conducted on this aspect. The applications of triclosan which contribute to those high environmental concentrations cannot be properly identified nor quantified at present and the presence of other chemicals (e.g. antibiotics, surfactants, other biocides, etc.) in the environment, which may also affect microbial populations, would preclude assessing the effects of triclosan independently. Thus, additional *in situ* information is needed to provide an answer on the level of risk.

EXECUTIVE SUMMARY

Triclosan is a biocide used in many product categories, including cosmetics. The information on environmental concentrations of triclosan in the EU is limited and bioavailability of the triclosan to bacteria in the environment is not known.

Although the present mandate concerns the evaluation of a possible association between the use of triclosan in cosmetic products and the development of resistance by certain micro-organisms, the SCCS has taken into account all evidence available from all uses of triclosan to perform its assessment.

Triclosan is the most studied biocide with respect to bacterial resistance. Such a level of information, notably on its activity against bacteria, the identification of mechanisms of microbial resistance including genomic and proteomic aspects, is commendable and should be extended to other biocides.

Low concentrations of triclosan can trigger the expression of resistance and cross-resistance mechanisms in bacteria *in vitro*. In view of the concentrations of triclosan reported to trigger resistance *in vitro*, some of the environmental concentrations found in a number of

geographical distinct areas are high enough to suggest that bacterial resistance could be triggered. It is however difficult to predict whether microbial resistance would be triggered in these environments. The few *in situ* studies performed to date did not show any bacterial resistance emerging following triclosan exposure. In addition, the presence of other chemicals (e.g. antibiotics, surfactants, other biocides, etc.) in the environment, which may also affect microbial populations, would preclude assessing the effects of triclosan independently.

The emergence of resistance induced/selected by triclosan is related to the genetic control on the resistance gene(s) present on chromosomal and genetic mobile elements. This represents the origin for a hazard about selection and dissemination of cross-resistance with other anti-bacterial molecules including biocides and antibiotics.

Triclosan, like any other biocide, contributes to the selection of less susceptible bacteria in a complex microcosm *in vitro*. The impact of such a selection is unclear, as is the fitness of the "selected" bacterial species following triclosan exposure. The few *in situ* studies investigating long-term triclosan exposure (i.e. at least 6 months) did not indicate changes in the resistance susceptibility in the predominant bacteria selected for monitoring, but the changes in the entire flora were not evaluated. Thus additional *in situ* information is needed to provide a definitive opinion.

There are, so far, no epidemiological data linking outbreaks of antimicrobial resistant human and zoonotic pathogens to exposure to triclosan.

A number of scientific and technical data gaps about the occurrence and understanding of the resistance profile of triclosan have been identified and should be addressed. In particular, where biocides, including triclosan are used intensely, monitoring for emerging resistance in the microbial flora should be conducted. A more detailed research strategy for investigating the antimicrobial resistance effect of biocides is presented in a separate opinion from the SCENIHR (2010).

There is an apparent discrepancy between *in situ* information that suggests the absence of induction of bacterial resistance and cross-resistance triggered by triclosan, and *in vitro* studies describing the mechanistic and genetic aspect of triclosan-resistance in bacteria. A better translation of *in vitro* findings to *in situ* situations is needed, making full use of molecular tools and environmental conditions used in laboratory investigations. Standardized protocols and similar parameters should be applied to both *in vitro* and *in situ* investigations.

Although triclosan resistance was not observed *in situ*, this is not sufficient to conclude that there is no risk. Information is still lacking to provide a risk assessment on the use of triclosan in cosmetic products, including the genetic aspects of resistance, changes in environmental microcosm, maintenance and transfer of virulence and resistance determinants *in situ*.

Due to the limited number of *in situ* studies of resistance induced by triclosan to date, SCCS can only recommend the prudent use of triclosan, for example in applications where a health benefit can be demonstrated. However, conclusions from *in vitro* studies cannot be ignored, notably the role of triclosan (and other biocides) in triggering resistance and in the dissemination (horizontal or vertical transfer of) resistance determinants. Research focused on triggering mechanisms of resistance, maintenance of the gene pool and the transfer of resistance and virulence determinants, and improving the translational application of laboratory results to situations *in situ* are needed. Hence,the SCCS appreciates that research investment from the industry will be maintained to contribute to a better understanding of the potential risks associated with triclosan applications.

1. BACKGROUND

Triclosan (CAS 3380-34-5) with the chemical name 5-chloro-2-(2,4-dichlorophenoxy)phenol or 2,4,4'-trichloro-2'-hydroxy-diphenyl ether has a long history of use as a preservative in cosmetic products. It is currently regulated in Annex VI, entry 25 with a maximum concentration of 0.3%.

An opinion on triclosan (SCCP/1040/06) was adopted by the SCCP at the 9th plenary meeting of 10 October 2006 with the following conclusions to the request:

1. *"On the basis of the available data, the SCCP is of the opinion that there is presently no evidence of clinical resistance and cross-resistance occurring from the use of triclosan in cosmetic products. Information is required on consumer exposure to triclosan from all sources, including cosmetic products.*

2. *For a toxicological assessment of the safe use of triclosan, the SCCP requires a dossier to be submitted in which data is provided to all relevant exposure and toxicological end-points and conforming to currently accepted standards. This should be regarded as a matter of urgency because triclosan has been identified in human milk of some European populations."*

The dossier provided by Industry consists of an update on the bacterial resistance issue (submission III) and of a toxicological dossier for triclosan (submission IV).

Furthermore the Norwegian authority on cosmetics has earlier this year submitted a report "Risk assessment on the use of triclosan in cosmetics; Development of antimicrobial resistance in bacteria – II".

2. TERMS OF REFERENCE

Does SCCS consider a continued use of triclosan as a preservative in cosmetic products as safe taking into account the new provided documentation of resistance development by certain micro-organisms and cross-resistance?

In parallel, the SCCP/SCCS has been asked to assess the toxicological safety of triclosan when used as a preservative with a maximum concentration of 0.3%. This evaluation has been published as opinion SCCP/1192/08.

3. INTRODUCTION

Triclosan is an antimicrobial agent that has been used for more than 40 years as an antiseptic, disinfectant or preservative in clinical settings, in various consumer products including cosmetics, plastic materials, toys, etc. It has a broad range of activity that encompasses many, but not all, types of Gram-positive and Gram-negative non-sporulating, bacteria, some fungi (Jones *et al*. 2000, Schweizer 2001), *Plasmodium falciparum* and *Toxoplasma gondii* (McLeod *et al*. 2001). It has also been shown to be ecotoxic, particularly to algae in aquatic environments (Tatarazako *et al*. 2004). Additionally, it has been shown to interfere with the cycling of nitrogen in natural systems (Fernandes *et al*. 2008, Waller and Kookana 2009).

Triclosan is bacteriostatic at low concentrations, but higher levels are bactericidal (Suller and Russell 1999, 2000). At sublethal concentrations, it acts by inhibiting the activity of the bacterial enoyl-acyl carrier protein reductase (FabI), a critical enzyme in bacterial fatty acid biosynthesis (Heath *et al*. 2002, Zhang *et al*. 2004). At bactericidal concentrations, it is suggested to act through multiple nonspecific mechanisms including membrane damage (Gilbert and McBain 2002).

There are concerns that the widespread use of a low concentration of triclosan in various applications might lead to or select for bacterial resistance to antibiotics. Antibiotic resistance has become an increasingly serious problem worldwide, and the continued use of biocides including triclosan may exacerbate this problem. The main cause of antibiotic resistance remains the use and misuse of antibiotics. During the last decade, antibiotic resistance has increased in bacterial pathogens leading to treatment failures in both human and animal infectious diseases (Harbarth and Samore 2005; for reports see: EARSS Annual Report 2005, WHO 2007).

The safety of continued use of triclosan in cosmetic products has recently been assessed by the EU Scientific Committee on Consumer Products (SCCP 2009). The SCCP emphasised that this risk assessment concerns only the toxicological profile of triclosan and that before a final conclusion on the safety of triclosan in cosmetic products can be reached, the potential development of resistance to triclosan and cross-resistance by certain micro-organisms must be assessed. Earlier evaluations of triclosan, on the basis of available data, EU Scientific Committees concluded that there was no convincing evidence that triclosan poses a risk to humans and environment by inducing or transmitting antibacterial resistance (SSC 2002) as well as there was no evidence of clinical resistance and cross-resistance occurring from the use of triclosan in cosmetic products (SCCP 2006). Further information was sought for an update of these evaluations.

The present evaluation of triclosan is based both on the information submitted by COLIPA[1] to SCCS and on research published in peer-reviewed scientific journals. It aims at determining whether the continued use of triclosan may be associated to the development of resistance in certain micro-organisms. It also aims at identifying additional research needs.

3.1. Scope

Triclosan is used as a preservative in consumer products including cosmetics, where the maximum allowed concentration according to the EU Cosmetics Directive 76/768/EEC is 0.3%. The SCCP has recently performed a risk assessment of the use of triclosan in cosmetic products. Although the present mandate concerns the evaluation of a possible association between the use of triclosan in cosmetic products and the development of resistance by certain micro-organisms, the SCCS has taken into account all evidence available from all uses of triclosan to perform its assessment. This is in line with the SCCP

[1] COLIPA: The European Association of the Cosmetics Industry

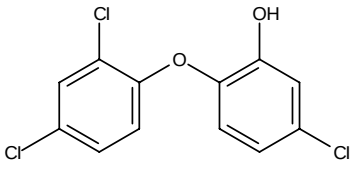
conclusions of 2006 (SCCP/1040/06) and it is scientifically sound as 1) cosmetic uses of triclosan account for most of the total use of this biocide in the EU and 2) it is scientifically impossible at present to assess the use of triclosan in cosmetics only, without taking into account its uses in other applications. In the absence of a clear answer, research needs will be identified. The effect of triclosan on microflora in the environment on the basis of published literature will also be covered, since environmental bacteria represent a pool of antimicrobial resistance genes.

Most of the information provided here relates to bacteria, since studies of the effects of triclosan on other micro-organisms are scarce.

3.2. Physico-chemical properties

INCI Name:	Triclosan
Chemical Name:	2,4,4'-trichloro-2'-hydroxy-diphenylether
Synonyms:	Phenol, 5-chloro-2-(2,4-dichlorophenoxy)-; Ether, 2'-hydroxy-2,4,4'-trichlorodiphenyl; 5-Chloro-2-(2,4-dichlorophenoxy)phenol, Trichloro-2'-hydroxydiphenylether
Trade Names:	Irgasan® DP300, Irgasan® PG60, Irgacare® MP, Irgacare® CF100, Irgacide® LP10, ; Cloxifenolum, Irgagard® B 1000, Lexol 300, Ster-Zac
CAS Reg. No.:	3380-34-5
EC:	222-182-2

Chemical structure:



Empirical formula:	$C_{12}H_7Cl_3O_2$
Molecular weight:	289.5
Physical form:	White crystalline powder

The purity of batches of triclosan used in personal care products since the 1970s is described in the Table 1 (SCCP 2009). These data were provided by COLIPA. The purity and contaminants might be different in triclosan from other sources.

Table 1: Purity specifications for triclosan since 1970

Test Point	Effective from 1970	Effective from 26.09.1985	Effective from 1.1.1990	Effective from 31.12.1994	Effective from 26.6.2000	Effective from 06.11.2003
Triclosan Active Substance[1]	99.0 - 100.0%	99% min	99% min	99.0-100%	97.0-103.0%	97.0 - 103.0%

[1] Analysis by gas chromatography.

Impurities / accompanying contaminants: See Table 2.

Table 2: Impurities in triclosan

Individual related compound (Gas Chromatography)	≤0.1%
Total related compounds (Gas Chromatography)	≤ 0.5%
2,4 Dichlorophenol	≤10 mg/kg
Sum of 3- and 4-Chlorophenol	≤10 mg/kg
2,3,7,8 Tetrachlorodibenzo-p-dioxin	<0.001 µg/kg
2,3,7,8-Tetrachlorodibenzo-furan	<0.001 µg/kg
2,8-Dichlorodibenzo-p-dioxin	≤0.5 mg/kg
1,3,7-Trichlorodibenzo-p-dioxin	≤0.25 mg/kg
2,8-Dichlorodibenzo-furan	≤0.25 mg/kg
2,4,8-Trichlorodibenzo-furan	≤0.5 mg/kg
Ash	≤0.1%
Mercury	≤1 mg/kg
Arsenic	≤2 mg/kg
Antimony	10 mg/kg
Lead	≤10 mg/kg
Cadmium	≤5 mg/kg
Nickel	≤10 mg/kg
Copper	≤10 mg/kg
Chromium	≤2 mg/kg
Sum of heavy metals as lead sulfide precipitation	≤20 mg/kg

Partition coefficient: Log P_{ow} = 4.8

Melting point: 57 ± 1°C

Relative density: 1.55 ± 0.04 g/cm^3

Vapour pressure: 4 x 10^{-6} mmHg (20°C)

pK_a: 8.14 (20°C)

Stability: Triclosan does not decompose under normal storage conditions over 9 years of storage (information from COLIPA).

The solubility of triclosan is described in Table 3.

Table 3: Solubility of triclosan in selected solvents and chemicals

Solvent	Solubility at 25°C (g Triclosan/100 g solvent)
Distilled water (20°C)	0.001
Distilled water (50°C)	0.004
1 N caustic soda	31.7
1 N sodium carbonate	0.40
1 N ammonium hydroxide	0.30
Triethanolamine	>100
Acetone	>100
Ethanol 70% or 95%	>100
Isopropanol	>100
Propylene glycol	>100
Polyethylene glycol	>100
Methyl cellosolve (Union Carbide Corp.)	>100
Ethyl cellosolve (Union Carbide Corp.)	>100
Dipropylene glycol	~40
Glycerine	0.15
n-Hexane	8.5
Petroleum jelly (white, USP)	~0.5
Tween 20 (ICI America Inc.)	>100
Tween 80 (ICI America Inc.)	>100
Triton X-100 (Rohm & Haas)	>100
Olive oil	~60
Castor oil	~90

3.3. Triclosan in biocidal formulations

Biocidal products that contain triclosan as the main antimicrobial are usually complex formulations due to the lack of solubility of this bisphenol. Components of the formulation might affect the activity of triclosan positively (e.g. through synergism) or negatively (e.g. antagonism). There is some information on the effect of formulation components on biocide activity (Alakomi *et al*. 2006, Ayres *et al*. 1999, Denyer and Maillard 2002, Maillard 2005b), but by large this information is restricted due to proprietory restrictions, or the lack of understanding on how formulation components work in term of antimicrobial potentiation.

In the scientific literature, where triclosan acitivity has been reported, there is little reference to the use of formulation. Instead triclosan is often dissolved in a solvent such as DMSO.

3.4. Mode of action

Chemical biocides are generally considered to have multiple target sites against microbial cells, although such interactions are concentration dependent (Russell *et al*. 1997; Maillard 2005a). The bisphenol triclosan is no exception. At a sub-inhibitory concentration, triclosan was found to profoundly affect bacterial growth, indicating a strong interaction with the bacterial targets, despite the high concentration exponent of triclosan (McDonnell and Russell 1999). At higher concentrations, Gomez Escalada *et al*. (2005a) observed that triclosan was both rapid-acting and active at all phases of population growth, although some marked differences in its lethality were observed.

These observations substantiated earlier findings with *Staphylococcus aureus* (Regos and Hitz 1974; Suller and Russell 2000). Inhibition of key metabolic pathway and synthesis (Regos and Hitz 1974; McMurry *et al*. 1998b) might be part of the lethal action mechanisms of triclosan. Indeed, triclosan was found to target specifically fatty acid synthesis with the inhibition of the enzyme enoyl reductase (enoyl-acyl carrier protein reductase, FabI) (McMurry *et al*. 1998a). It acts as a potent irreversible inhibitor of FabI by mimicking its natural substrate (Heath *et al*. 1998; Levy *et al*. 1999) and this inhibition has been described as being slow and competitive (Heath *et al*. 1999). The propensity of triclosan to inhibit fatty acid synthesis in *Plasmodium falciparum* and *Toxoplasma gondii* (McLeod *et al*. 2001) has led to the development of a number of antimalarial and antibacterial pro-drugs based on triclosan (Mishra *et al*. 2008; Freundlich *et al*. 2009).

The rapid action of triclosan at a high concentration might be indicative of membrane damage (Villalain *et al*. 2001) and it is clear that fatty acid synthesis targeting cannot solely explain the lethal effect of triclosan (Gomez Escalada *et al*. 2005b). Triclosan membranotropic effects result in destabilised structures compromising the functional integrity of cell membranes without inducing cell lysis (Villalain *et al*. 2001). Intercalation of triclosan into bacterial cell membranes is likely to compromise the functional integrity of those membranes, thereby accounting for some of triclosan antibacterial effects (Guillén *et al*. 2004).

Recently, the first genome-wide transcriptional analysis of *Staphylococcus aureus* exposed to triclosan (0.05 μM), reported that triclosan down regulated primary metabolism-related and carbohydrate transport, the *cap* operon which is essential for virulence, the *clpB* chaperone-related genes which might trigger the expression of resistant determinants, genes involved in fatty acid production and utilisation (Jang *et al*. 2008).

A number of factors affect the antimicrobial activity of triclosan. These can be divided into intrinsic factors derived from the biocide and its application (e.g. concentration, contact time, pH) and extrinsic factors which derive from the environment during application (e.g. temperature, soiling). Understanding the complex relationship between concentration and contact time (sometimes referred to as CT concept) is crucial to ensure efficacy (Maillard 2005a). The stability of triclosan in particular environments will also influence efficacy.

4. DEFINITIONS

According to the Directive 98/8/EC of the European Parliament and Council of the 16 February 1998, biocidal products are defined as 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, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism by chemical or biological means.

Within the scope of this mandate, the proposition is to apply the following definitions:

- Antimicrobial: biocide or antibiotic.

- Biocide: an active chemical molecule in a biocidal product to control the growth of or kill micro-organisms (including bacteria, fungi, protozoa and viruses). This includes disinfectants, preservatives and antiseptics.
- Antibiotic: an active substance of synthetic or natural origin which is used to eradicate bacterial infections in humans or animals.
- Antimicrobial activity: an inhibitory or lethal effect of a biocidal product or an antibiotic.

The terms employed in the context of this mandate are defined below in order to avoid confusion in the definitions used to describe the level and type of resistance reported.

There are several definitions of resistance to antimicrobials biocides or/and antibiotics and several terms used to describe similar phenomena in the literature. A literal/biological definition of resistance is the capacity of bacteria to withstand the effects of a harmful chemical agent.

The following definitions are based partly on those put forward by Chapman and colleagues (Chapman 1998, Chapman *et al*. 1998), Russell and colleagues (Hammond *et al*. 1987, Russell 2003) and Cloete (2003), and the recent SCENIHR opinion (2009).

The practical meaning of antibiotic resistance is to describe situations where (i) a strain is not killed or inhibited by a concentration attained *in vivo*, (ii) a strain is not killed or inhibited by a concentration to which the majority of strains of that organism are susceptible or (iii) bacterial cells that are not killed or inhibited by a concentration acting upon the majority of cells in that culture.

When non-antibiotic agents (i.e. triclosan or other biocides) are considered, the word "resistance" is used in a similar way where a strain is not killed or inhibited by a concentration attained in practice (the in-use concentration) and in situations (ii) and (iii) described above.

These definitions reflect those given by EFSA whereby "antimicrobial susceptibility or resistance is generally defined on the basis of *in vitro* parameters. The terms reflect the capacity of bacteria to survive exposure to a defined concentration of an antimicrobial agent, but different definitions are used depending on whether the objective of the investigation is clinical diagnostics or epidemiological surveillance" (EFSA 2008)

The term 'Multi-Drug Resistant' (MDR) applies to a bacterium that is simultaneously resistant to a number of antibiotics belonging to different chemical classes by using various mechanisms (Depardieu *et al*. 2007).

The term "co-resistant" is used to denote a strain possessing a biochemical mechanism that inhibits the activity of several antibiotics belonging to the same structural family (e.g. ß-lactamase and ß-lactams). When the transfer of resistance determinants occurs, co-resistance specifically refers to genetic determinants (such as integrons, transposons or plasmids) encoding for unrelated resistance mechanisms, that are transferred in a single event and expressed jointly in a new bacterial host.

The term "cross-resistant" is used to denote a strain possessing a resistance mechanism that enables it to survive the effects of several antimicrobial molecules with mechanism(s) of action that are related or overlap.

Other terms such as "insusceptibility" and "tolerance" have been used in the published literature. Insusceptibility refers to an intrinsic (innate) property of a micro-organism, such as cell layer impermeability in mycobacteria and Gram-negative bacteria. Tolerance denotes a reduced susceptibility to an antimicrobial molecule characterised by a raised minimum inhibitory concentration (MIC), or a situation in which a preservative system no longer prevents microbial growth.

5. PRODUCTION, USE AND FATE OF TRICLOSAN

Triclosan is a broad spectrum antimicrobial used as an antiseptic, disinfectant or preservative in clinical settings, various consumer products including cosmetics, household cleaning products, plastic materials, toys, paints, etc. It is also incorporated on the surface of medical devices, plastic materials, textiles, kitchen utensils, etc., from which it might slowly leach for a long period of time during their use, to perform its biocidal action. A detailed list of products containing triclosan is provided by the US Environmental Protection Agency (EPA) (McMahon *et al*. 2008) and by the Environmental Working Group, a US NGO (http://www.ewg.org/node/26752). According to EU Biocide Directive 1998/8/EC, triclosan is used in product types 1 (human hygiene), 2 (private and public health area), 3 (veterinary hygiene), 7 (film preservative) and 9 (fibre, leather, rubber and polymerised materials preservative).

According to the information provided by COLIPA, the quantity of triclosan used within the EU reached approximately 450 tons (as 100% active) in the year 2006. Dye *et al*. (2007) estimated triclosan production in the EU to be 10-1,000 tonnes per year. It is not clear whether the above information on use or production of triclosan includes the amounts of triclosan which may be imported in the EU or exported from the EU via finished products, such as medical devices, toys, textiles, etc. In the EU, about 85% of the total volume of triclosan is used in personal care products, compared to 5% for textiles and 10% for plastics and food contact materials (usage data reported by COLIPA in 2007).

The Danish EPA performed a survey of the use of triclosan in Denmark for the period 2000-2005 (Borling *et al*. 2005). This survey showed that the amount of triclosan in products on the Danish market had decreased from approx. 3.9 to 1.8 tonnes (54%) in the period 2000-2004. Cosmetics were the largest contributor to the amount of triclosan on the Danish market (99% of the total reported amount in the survey). However, this might not be respresentative for the whole EU, as similar data for comparison is not available for the EU as a whole or for any of its Member States.

5.1. Triclosan in cosmetics

Triclosan was listed in 1986 in the European Community Cosmetics Directive (76/768/EEC) for use as a preservative in cosmetic products at concentrations up to 0.3%. The recent risk assessment performed by the EU Scientific Committee on Consumer Products (SCCP) concluded that, although its use at a maximum concentration of 0.3% in toothpastes, hand soaps, body soaps/shower gels and deodorant sticks was considered safe on a toxicological point of view in individual products, the magnitude of the aggregate exposure to triclosan from all cosmetic products is not safe. Any additional use of triclosan in face powders and blemish concealers at this concentration was also considered safe, but the use of triclosan in other leave-on products (e.g. body lotions) and in mouthwashes was not considered safe for the consumer due to the resulting high exposures[2]. Inhalation exposure to triclosan from spray products (e.g. deodorants) was not assessed.

In a Danish EPA survey (Borling *et al*. 2005), the highest amount of triclosan in cosmetics was found in products for dental hygiene, including toothpaste. In this group, the amount had decreased by 37% during 2000-2004. Deodorants were the group of cosmetics with the greatest decrease in amount of triclosan (79%). A recent Danish EPA survey revealed that 15% of the most commonly sold deodorants in the Danish market contained <0.3% triclosan (Rastogi *et al*. 2007).

Triclosan being non-ionic, it can be formulated in conventional dentifrices. However, it does not bind to the oral surfaces for more than a few hours, and therefore does not deliver a sustained level of anti-plaque activity. To increase uptake and retention of triclosan by oral

[2] SCCP opinion on triclosan COLIPA n° P32, SCCP/1192/08

surfaces for the improvement of plaque control and gingival health, triclosan/polyvinylmethyl ether maleic acid copolymer and triclosan/zinc citrate and triclosan/calcium carbonate dentifrice are used (Williams 1998, Davies *et al*. 2004, Brading *et al*. 2004, Davies 2007).

5.2. Triclosan in healthcare and medical devices

Triclosan has been effectively used clinically to eradicate micro-organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA) (Brady *et al*. 1990; Cookson *et al*. 1991; Webster *et al*. 1994; Zafar *et al*. 1995), notably with the recommendation to use 2% triclosan bath. Triclosan is employed as surgical scrubs, and it is widely used in hand washing (Boyce and Pittet 2002) and as a body wash to eradicate MRSA from carriers prior to surgery (Wilcox *et al*. 2003).

Triclosan is used in a number of medical devices, for example ureteral stents (Knudsen *et al*. 2005), surgical sutures (Ford *et al*. 2005; Justinger *et al*. 2009) and might be considered to prevent graft infection (Cakmak *et al.* 2009). Bojar et al. (2009) did not observe a difference in colonisation between triclosan-coated sutures and regular multifilament suture, although their work concerned five bacteria and is only based on the determination of the zone of inhibition. In ureteral stents, triclosan has been shown to inhibit the growth of common bacterial uropathogens and to reduce the incidence of urinary-tract infections and, potentially, catheter encrustation (Chew *et al*. 2006, Cadieux *et al*. 2009). Wignall *et al*. (2008) have recently demonstrated synergistic effects of triclosan and relevant antibiotics on clinical isolates comprising seven uropathogenic species, and they support the use of the triclosan-eluting stent when necessary, along with standard antibiotic therapy in treating complicated patients. In some further developments, the use of triclosan in urinary Foley catheter was suggested since triclosan successfully inhibited the growth of *Proteus mirabilis* and controlled encrustation and blockage of the catheter (Stickler *et al*. 2003, Williams and Stickler 2008). Recently, Darouiche *et al*. (2009) demonstrated synergistic, broad-spectrum and durable antimicrobial activity of the catheters coated with a combination of triclosan and DispersinB, an anti-biofilm enzyme that inhibits and disperses biofilms (Kaplan *et al*. 2004, Itoh *et al*. 2005).

5.3. Triclosan in household and other consumer products

The broad-spectrum antimicrobial activity of triclosan has led to its incorporation in an extended range of product formulations intended for home use such as liquid soaps, detergents, chopping boards, children's toys, carpets and food storage containers (Bhargava and Leonard 1996, McBain *et al*. 2003, Yazdankhah *et al*. 2006, Gilbert *et al*. 2007). A detailed list of consumer products containing triclosan is provided by the US Environmental Protection Agency (EPA) (McMahon *et al*. 2008) and by the US NGOs "Environmental Working Group" (http://www.ewg.org/node/26752) and "Beyond Pesticides" (http://www.beyondpesticides.org/antibacterial/products.htm).

An increasing number of clothing articles are treated with biocides. Triclosan is one of the finishing agents for the production of such textiles (Orhan *et al*. 2009).The fabrics finished with triclosan are treated with cross-linking agents to provide durable antibacterial properties. On the basis of the available information, 17 products from the Danish retail market were analysed for the content of some selected antibacterial compounds: triclosan, dichlorophen, Kathon 893, hexachlorophen, triclocarban and Kathon CG. Five of the products were found to contain 0.0007% - 0.0195% triclosan (Rastogi *et al*. 2003).

Aiello *et al*. (2007), in the first systematic review assessing the benefit of soaps containing triclosan, evaluated 27 studies published between 1980 and 2006. One of the key findings is that soaps that contained less than 1% triclosan showed no benefit from non-antimicrobial soaps (the EU limit is 0.3%). Studies that used soap contaning > 1% triclosan showed a significant reduction in bacterial levels on hand, often after multiple applications. The apparent lack of relationship between the use of soap containing triclosan and reduction in

infectious illness was difficult to ascertain in the absence of identification of the biological agents responsible for the illness symptoms. Two recent US studies (Fischler *et al*. 2007, Fuls *et al*. 2008) demonstrated that hand washing with antimicrobial soap containing triclosan (0.46%) reduced bacterial load and transfer of bacteria from hands, compared to handwashing with a non-antimicrobial soap.

5.4. Triclosan in food and feed

5.4.1. Triclosan in food production

Triclosan was evaluated by the Scientific Committee on Food (SCF 2000) and the European Food Safety Authority (EFSA 2004) for use in food contact materials and classified in SCF List 3[3] with a restriction of 5 mg/kg of food. The evaluation was referred to the use of triclosan as surface biocide i.e. as substance intended to inhibit the growth of bacteria on the surface but which is not intended to have an antimicrobial effect on the food itself. Potential uses beyond household articles like cutting boards, kitchen utensils and food storage containers exist (e.g. conveyor belts, machinery, work surfaces and transport containers used in food processing). However, in April 2009 the petitioner has withdrawn the application for these uses. According to a March 2010 Commission Decision[4] triclosan shall not be included in the positive list of additives to Directive 2002/72/EC and cannot be used in the manufacture of plastics intended to come into contact with food.

In Germany, the use of triclosan in food contact plastics is banned since September 2009. BfR supports the ban on triclosan in food contact materials (BfR Opinion N°. 031/2009, 12 June 2009).

Triclosan has been identified in drinking water in certain places (Stackelberg *et al*. 2004, Boyd *et al*. 2003). Kantiani *et al*. (2008) found methyl triclosan (12 µg/L) in one of the 22 drinking water samples from Barcelona.

5.4.2. Triclosan as disinfectant in food and feed production

Triclosan is not notified in the framework of the European regulations on biocides (Directive 98/8/EC) for use as disinfectant in food and feed production.

5.4.3. Triclosan as food preservative

Triclosan is not approved as food preservative in Europe. Food preservatives are regulated by Directive 95/2/EC on food additives other than colours and sweeteners. In Annex III of this Directive on the permitted preservatives and restrictions for their use, triclosan is not listed. As a result, the use of triclosan in so-called "active food contact materials and articles" is not allowed. Regarding substances released from such materials in order to extend the shelf-life of food, the Regulation (EC) 1935/2004 on food contact materials refers to the authorisations applicable to their use in foods.

[3] Substances for which an Acceptable Daily Intake or Tolerable Daily Intake could not be established, but where the present use could be accepted.

[4] Commission Decision of 19 March 2010 concerning the non-inclusion of 2,4,4'-trichloro-2'-hydroxydiphenyl ether in the Union list of additives which may be used in the manufacture of plastic materials and articles intended to come into contact with foodstuffs under Directive 2002/72/EC *(notified under document C(2010) 1613)*

5.4.4. Triclosan in animal husbandry

Triclosan is notified in the framework of the European regulations on biocides (Directive 98/8/EC) for use in veterinary hygiene biocidal products.

5.4.5. Triclosan as feed preservative

According to Regulation (EC) 1831/2003 on additives for use in animal nutrition the use of triclosan as preservative in feed is not authorised. The substance is not listed in the corresponding Community Register of Feed Additives (2004/C 50/01).

5.5. Triclosan in the environment

The widespread use of triclosan results in the discharge of this compound to wastewater. Incomplete removal of triclosan from wastewater treatment plants (WWTPs) as well as spreading the triclosan laden biosolids into soils, leads to triclosan being distributed in soils and surface waters.

Triclosan has been widely detected (see Table 4) in influents, effluents and biosolids of WWTPs, in lakes, rivers and sea water in various countries in Europe (Paxeus 1996, Lindström *et al*. 2002, Adolfsson-Erici *et al*. 2002, Kanda *et al*. 2003, Bester 2003, Sabaliunas *et al*. 2003, Samsø-Petersen *et al*. 2003, Xie *et al*. 2008, Singer *et al*. 2002, Tixier *et al*.2002, van Stee *et al*. 1999, Kantiani *et al*. 2008, Dye *et al*. 2007), in the USA (McAvoy *et al*. 2009, Coogan *et al*. 2007, Coogan *et al*. 2008, US EPA 2009, Cha and Cupples 2009, Fair *et al*. 2009, Halden and Paull 2005, Chalew and Halden 2009, Kumar *et al*. 2010), in Canada (Hua *et al*. 2005), in Australia (Ying and Kookana 2007, Fernandes *et al*. 2008), in Japan (Okumura and Nishikawa 1996) and in Hong Kong (Chau *et al*. 2008).

5.5.1. Fate of triclosan in the environment

Bacteria are able to survive triclosan exposure by activating specific or general genetic cascades (see 6.2.4). The environmental concentrations of triclosan may affect bacterial activities. Consequently it is important to evaluate the fate of triclosan in the environment such as in WWTPs, rivers, effluents, etc.

Triclosan is transported through the domestic waste stream to WWTPs. Municipal wastewater treatment helps to achieve average removal efficiencies in the range of 58-99%, depending on the technical capabilities of sewage treatment systems (McAvoy *et al*. 2002, Kanda *et al*. 2003, Bester 2003, Singer *et al*. 2002, Federle *et al*. 2002, Lishman *et al.* 2006, Lindström *et al*. 2002, Lopez-Avila and Hites 1980, Thomson *et al*. 2005, Ternes *et al.* 2004). However, mass balance studies have demonstrated that triclosan also exhibits significant persistence, partitioning and sequestration in biosolids (by-product of wastewater treatment). Approximately 50 ± 19% of the incoming mass of triclosan was observed to persist and become sequestered in biosolids produced by a conventional WWTPs employing activated sludge treatment in conjunction with anaerobic biosolid digestion (Heidler and Halden 2007). Thus, important pathways of biocide release into the environment include WWTP effluent discharge into surface waters and the land application of biosolids. Effluent from WWTPs contains a complex mixture of anthropogenic and natural compounds. Soil samples from ten agricultural sites in Michigan previously amended with biosolids, collected over two years, revealed triclosan concentration 0.16-1.02 µg/kg (Cha and Cupples 2009). 90 to 7060 µg/kg triclosan was found in biosolids from 3 Michigan wastewater treatment plants.

Triclosan, along with many other compounds, may have multiplicative or synergistic effects on micro-organisms including bacteria.

Environmental concentrations of triclosan reported in the published literature are described in Table 4.

Table 4: Environmental concentrations of triclosan (data from worldwide sources)

Environmental matrix	**Triclosan concentration**	**Reference**
Surface water Lake/river/streams with known input of raw wastewater	1.4 ng/L-40000 ng/L	Kolpin *et al*. 2002, Lindström *et al*. 2002, Lopez-Avila and Hites 1980, Singer *et al*. 2002, Remberger *et al*. 2002, Kolpin *et al*. 2004, Bendz *et al*. 2005, Glassmeyer *et al*. 2005, Zhang *et al*. 2007, Halden and Paull 2005, Chau *et al*. 2008, Coogan *et al*. 2007, Coogan and La Point 2008
Wastewater Influent	20-86161 ng/L	Lindström *et al*. 2002, Samsø-Petersen *et al*. 2003, Singer *et al*. 2002, Remberger *et al*. 2006, McAvoy *et al*. 2002, 2009, Halden and Paull 2005, Lishman *et al*. 2006, Waltman *et al*. 2006, Heidler and Halden 2007, Kantiani *et al*. 2008, Fair *et al*. 2009, Kumar *et al.* 2009
Effluent	23-5370 ng/L	Lindström *et al*. 2002, Samsø-Petersen *et al*. 2003, Bester 2003; Kanda *et al.* 2003; Sabaliunas *et al*. 2003, Bendz *et al*. 2005, Halden and Paull 2005, Thompson *et al*. 2005, Ying and Kookana (2007), Fair *et al*. 2009, Kumar *et al*. 2009
Sea water	<0.001-100 ng/L	Xie *et al*. 2008, Okumura and Nishikawa 1996, Fair *et al*. 2009
Sediment Lake/River/other surface water	<100-53000 µg/kg d.w.	Fjeld *et al*. 2004, Remberger *et al*. 2002, Singer *et al.* 2002; Morales *et al.* 2005; Miller *et al.* 2008
Marine	0.02-35 µg/kg d.w.	Okumura and Nishikawa 1996, Fjeld *et al*. 2004
Biosolid from WWTP	20-133000 µg/kg d.w.	Svensson. 2002; Remberger *et al*. 2002, 2006; Bester 2003; Morales *et al.* 2005; Kinney *et al.* 2006; Chu and Metcalfe 2007, US EPA 2009, Cha and Cupples 2009, Ying and Kookana 2007
Activated/digested sludge	580-15600 µg/kg d.w.	McAvoy *et al*. 2002, 2009, Singer *et al*. 2002, Chu and Metcalfe 2007, Kumar *et al*. 2010
Pore water	0.201-328.8µg/L (calculated)	Chalew and Halden 2009

d.w.: dry weight

Photodegradation of Triclosan

Despite its high chemical stability, being extremely resistant to high and low pH, triclosan is readily degraded in the environment via photodegradation. Eight photoproducts were tentatively identified, including chlorinated phenols, chlorohydroxydiphenyl ethers, 2,7- and 2,8-dichlorodibenzo-p-dioxin, and a possible dichlorodibenzodioxin isomer or dichlorohydroxydibenzofuran (Tixier *et al*. 2002; Sanchez-Prado *et al*. 2006a, 2006b, Canosa *et al.* 2005; Lores *et al.* 2005; Aranami and Readman 2007, Prada *et al*. 2004, Latch *et al*. 2005, Ingerslev *et al. 2003*). Some of these products show enhanced toxicity compared to triclosan but have been shown to be degraded in the environment by bacteria such as *Pseudomonas, Burkholderia* and *Sphingomonas* (Field *et al*. 2008a and 2008b). The end products are CO_2 and chlorine with chlorocatechols as intermediates. Recently, Son *et al*. (2009) demonstrated that TiO_2-photocatalytic degradation of triclosan is mainly achieved by radicals, and these radicals can further degrade dioxin-type intermediates once they are produced in photocatalysis. The presence of hydrogen peroxide enhanced the oxidation (Yu *et al*. 2006).

Triclosan is hydrolytically stable under abiotic and buffered conditions over the pH 4-9 range based on data from a preliminary test at 50°C. Photolytically, triclosan degrades rapidly under continuous irradiation from artificial light at 25°C in a pH 7 aqueous solution, with a calculated aqueous photolytic half-life of 41 minutes. One major transformation product was identified, 2,4-dichlorophenol, which was a maximum of 93.8-96.6% of the applied triclosan 240 minutes after treatment.

Hydrolysis is not expected to be an important environmental fate process due to the stability of triclosan in the presence of strong acids and bases. However, triclosan is susceptible to degradation via aqueous photolysis, with a half-life of <1 hour under abiotic conditions, and up to 10 days in lake water. An atmospheric half-life of 8 hours has also been estimated based on the reaction of triclosan with photochemically produced hydroxyl radicals. Additionally, triclosan may be susceptible to biodegradation based on the presence of methyl-triclosan following wastewater treatment.

Degradation in chlorinated water

Triclosan addition to chlorine spiked ultra-pure water or to chlorinated tap water led to the formation of two tetra- and one penta-chlorinated hydroxylated diphenyl ether, as well as 2,4-dichlorophenol. Chlorination of the phenolic ring and cleavage of the ether bond were identified as the main triclosan degradation pathways (Canosa *et al*. 2005). Free chlorine mediated oxidation of triclosan leads to the formation of chloroform and other chlorinated organics (Rule *et al*. 2005, Fiss *et al*. 2007).

Ozone treatment

Treatment with ozone during municipal sewage treatment was efficient at removal of triclosan (Suarez 2007; Wert *et al*. 2009; Dodd *et al*. 2009). The degradation products were however not identified.

Biodegradation

Aerobic bacterial hydrolysis plays an important role in triclosan degradation. A consortium of bacteria able to partially degrade triclosan was isolated and one consortium member was shown to be a *Sphingomonas*-like micro-organism (Hay *et al*. 2001). In a different study, two strains of *Pseudomonas putida* TriRY and *Alcaligenes xylosoxidans* subsp. *denitrificans* TR1 were shown to utilise triclosan as sole carbon source (Meade *et al*. 2001). Zhao (2006) also isolated one strain of triclosan-degrading bacteria (*Sphingomonas* or *Sphingopyxis*) from activated sludge. Zhao also found that *Nitrosomonas europaea,* an important nitrification bacterium in wastewater treatment plants, has the ability to degrade triclosan

through co-metabolism. Triclosan and its chlorinated degradation products can also be degraded by bacteria (*Pseudomonas, Sphingomonas, Burkholderia*) under aerobic conditions.

Very little is known of the biochemistry of the biodegradation of triclosan and nothing is documented in the Minnesota biodegradation database (http://umbbd.msi.umn.edu/). There is a data gap on the degradation pathway of triclosan and its intermediary products.

Under anaerobic conditions and in the dark, triclosan is quite stable. Due to its low water solubility, triclosan is readily adsorbed to particles and tends to accumulate in sediments. Digested sludge concentrations of triclosan ranged from 0.5 to 15.6 µg/g (dry weight), where the lowest value was from an aerobic digestion process and the highest value was from an anaerobic digestion process. These results suggest that triclosan is readily biodegradable under aerobic conditions, but not under anaerobic conditions (McAvoy *et al*. 2009).

The limited data available indicate that effect levels of triclosan on activated sewage sludge micro-organisms vary depending on the level of acclimation. A concentration of 2 mg/L inhibited activated sludge micro-organisms that had not been acclimated to triclosan; however, the same concentration had no effect on acclimated organisms. Laboratory-derived IC_{50} values range from 20-239 mg triclosan/L based on carbon dioxide (CO_2) evolution and glucose utilisation.

Triclosan (≥2 mg/L) had a slight effect on chemical oxygen demand removal under laboratory conditions, but had a major inhibitory effect on the nitrification process. Anaerobic sludge digestion was significantly inhibited at a concentration of 10 mg/L. A NOEC for sewage microbes was not available (NICNAS 2009).

5.5.2. Effect of triclosan on micro-flora and toxicity of metabolites

Inhibitory effects on micro-organisms were shown to begin at concentrations ranging from 25 to 80,000 µg/L for triclosan (Federle *et al*. 2002, Samsø-Petersen *et al*. 2003, Sivaraman *et al*. 2004, Neumegen *et al*. 2005, Stasinakis *et al*. 2007, Farre *et al*. 2008, Stickler and Jones 2008). It should be noted that the upper range minimum inhibitory concentrations (MICs) reported are well in excess of published solubility limit for triclosan. MIC threshold values for micro-organisms are exceeded by environmental levels of triclosan in several sediments, biosolids, and activated sludge. Lawrence *et al*. (2009) observed a change in the structure and composition of a river biofilm microcosm following exposure to triclosan (10 µg/L) over a 8-week period.

Waller and Kookana (2009) studied the effect of triclosan on selected microbiological activity and biochemical parameters in Australian soil. Substrate-induced respiration and nitrification, plus activities of four enzymes relevant for carbon turnover (acid and alkali phosphatase, 3-glucosidase, and chitinase) were measured. The effect of triclosan on enzymatic activity was minimal even at a high concentration (100 mg/kg). Likewise respiration was not affected. However, the study demonstrated that triclosan at concentrations below 10 mg/kg can disturb the nitrogen cycle in some soils.

McBain *et al*. (2003) showed that long-term exposure of domestic-drain biofilms to sublethal levels of triclosan (2-4 g/L, four times daily) did not affect bacterial viability or significantly alter antimicrobial susceptibility. This lack of effect may reflect the biofilm phenotype present in the microcosm, the presence of intrinsically tolerant bacteria and degradation of triclosan by the drain biofilm consortium. However, microbial diversity after exposure to triclosan was profoundly affected.

Studies reporting on the effect of repeated exposure of triclosan against complex oral microcosms failed generally to show an increase in resistance determined either by an increase in MIC or in Minimal Bactericidal Concentration (MBC) (Sullivan *et al*. 2003; McBain *et al*. 2004). In addition, McBain *et al*. (2004) did not observe any cross-resistance to other biocides or to some antibiotics (tetracycline and mitrodinazole) in a number of bacterial

species such as *Streptococcus sanguis*, *Sterptococcus oralis* and *Prevotella nigrescens* with a decreased susceptibility to triclosan resulting from exposure to the bisphenol. However, these results contrasted with those obtained with *E. coli*, for which repeated exposure to increasing concentrations of triclosan led to a 400-fold increase in resistance (MBC from 0.2 to 39.1 mg/L) (McBain *et al*. 2004). Moreover, bacteria inside biofilms resist better to biocidal agents. For example, reduced susceptibility to triclosan was observed in *Salmonella* (Tabak *et al*. 2007) and *Proteus/Providencia* (Stickler and Jones 2008, Williams and Stickler 2008).

5.6. Triclosan in the human body

Triclosan enters the human body orally through toothpaste, mouthwashes and dental treatments. In humans, triclosan is rapidly and completely absorbed from the gastrointestinal tract, while a lower rate of absorption occurs dermally. It has been found in human blood, plasma and milk (Allmyr *et al*. 2006, 2008, Adolfsson-Erici *et al*. 2002) in Sweden and Australia. In the USA it was found in human urine (Calafat *et al*. 2008). A volunteer study in Sweden (Sandborgh-Englund *et al*. 2006) showed that the accumulated urinary excretion varied between the subjects, with 24 to 83% of the oral dose being excreted during the first 4 days after exposure.

6. MECHANISMS OF RESISTANCE TO TRICLOSAN

6.1. General considerations on biocide resistance in bacteria

Unlike antibiotic resistance, the issues relating to biocide resistance in the healthcare environment are considered to have a very low profile and priority (Cookson 2005). Despite the widespread use of disinfectants and antiseptics in healthcare settings, acquired resistance to biocides in bacteria isolated from clinical specimens or the environment is not routinely characterised. Emerging bacterial resistance to biocides has been well described *in vitro*, but evidence in practice is still lacking (Russell 2002b, Cookson 2005, Maillard and Denyer 2009).

It is widely accepted that biocides have multiple target sites against bacteria (Denyer and Maillard 2002, Lambert 2002, Maillard 2002, Maillard 2007, Poole 2004, Stickler 2004, Gilbert and Moore 2005, Maillard 2005b)with their efficacy depending on a range of intrinsic and extrinsic factors, (Reuter 1984, 1989, 1994, EFSA 2008, SCENIHR 2009). Thus, the emergence of general bacterial resistance is likely to arise from a mechanism/process causing the decrease of the intracellular concentration of a biocide, under the threshold that is harmful to the bacterium (Tattawasart *et al.* 2000a, Tattawasart *et al.* 2000b; Braoudaki and Hilton 2005; Maillard 2005a, Maillard and Denyer 2009). Several mechanisms based on this principle (mode of action) have been described including change in cell envelope, change in permeability, efflux and degradation (Table 5). Bacteria in biofilms are also less susceptible to biocides because of a number of factors. It is likely that some of these mechanisms operate synergistically although very few studies investigating multiple bacterial mechanisms of resistance following exposure to a biocide have been performed.

Bacterial resistance to biocides is not a new phenomenon and it has been reported since the 1950's (Adair *et al.* 1971; Russell 2002b; Chapman 2003). To date, bacterial resistance has been described for all the biocides that have been investigated. Resistance often occurs following an improper usage of the formulated biocide, leading notably to a decrease in active concentration (Sanford 1970, Prince and Ayliffe 1972, Russell 2002b).

Table 5: Mechanisms of bacterial resistance to biocides at the cell level

Mechanisms		References
Change in cell permeability	Decrease in concentration (that reaches the target sites)	Russell 1990; Russell *et al.* 1997; Denyer and Maillard 2002; Lambert 2002; Cloete 2003, Hawkey 2004; Champlin *et al.* 2005;
	Spores (layers: cortex, spore envelope)	
	Gram-negative (outer membrane)	Munton and Russell 1970; Ayres *et al*. 1998; McDonnell and Russell 1999; Tattawasart *et al.* 2000a, b; Denyer and Maillard 2002; Fraud *et al.* 2003; Stickler 2004; Braoudaki and Hilton 2005
	- Lipopolysaccharides	
	- Proteins (porins)	
	- Fatty acid	
	- Phospholipids	Gandhi *et al.* 1993; Brözel and Cloete 1994; Winder *et al.* 2000 Jones *et al.* 1989; Méchin *et al.* 1999; Guérin-Méchin *et al.* 1999, 2000 Boeris *et al.* 2007
	Mycobacteria mycoylarabinagalactan	McNeil and Brennan 1991; Broadley *et al*. 1995; Russell, 1996; Russell *et al*. 1997; Manzoor *et al.* 1999; Walsh *et al.* 2001; Lambert, 2002
Change in surface properties	Decrease binding and interaction between biocide and cell surfaces	Bruinsma *et al.* 2006
	Surface charge	
Efflux mechanisms	Decrease intracellular concentration of a biocide - Small multidrug resistance (SMR) family (now part of the drug/metabolite transporter (DMT) superfamily) - Major facilitator superfamily (MFS) - ATP-binding cassette (ABC) family - Resistance-nodulation-division (RND) family - Multidrug and toxic compound extrusion (MATE) family	Nikaido, 1996; Paulsen *et al.* 1996; Schweizer 1998, 2001; Brown *et al.* 1999; Putman *et al.* 2000; Borges-Walmsley and Walmsley, 2001; Poole, 2001, 2002a, b; Levy 2002; Chuanchuen *et al*. 2003; McKeegan *et al*. 2003; Piddock 2006
Enzymatic modification	Decrease intracellular and exocellular concentration of a biocide	Demple 1996; Kummerle *et al*. 1996; Valkova *et al*. 2001; Cloete 2003;
Target mutation	FabI mutation in *Mycobacterium smegmatis*	McMurry *et al*. 1999;
By-pass metabolic blockage	Increase in pyruvate synthesis and fatty acid production via an altered metabolic pathway (expression of 'triclosan resistance network')	Webber *et al*. 2008b

It is worth noting that some mechanisms (e.g. efflux, target protection, degradation) can be horizontally transferred to other bacteria (Poole 2002a, Quinn *et al*. 2006, Roberts and Mullany 2009, Yazdankhah *et al*. 2006; Hawkey and Jones 2009, Juhas *et al*. 2009). In addition, Pearce *et al*. (1999) showed that some biocides, at a sub-lethal concentration, may increase or decrease the frequency of gene transfer by conjugation and transduction.

6.2. General considerations on the study of triclosan

Triclosan is described as a broad spectrum biocide. However, some bacteria are intrinsically resistant to triclosan, notably *P. aeruginosa* (Lear *et al*. 2002) and triclosan is not active against bacterial endospores. This is likely due to the structure of the Gram-negative bacteria and particularly the outer membrane, preventing triclosan to penetrate through the bacterium to reach its target sites.

Bacterial resistance mechanisms to triclosan have been widely studied. However, most studies have considered resistance as an increase in MIC and not necessarily as an increase in MBC. Using MICs to measure bacterial resistance is arguable, since much higher concentrations of biocides have usually been used in practice and, therefore, failing to achieve lethality because of elevated MICs is unlikely. Some studies have shown that bacterial strains showing a significant increase in MICs to some biocides, such as cationics, were nevertheless susceptible to higher (*in use*) concentrations of the same biocide (Thomas *et al.* 2005) or triclosan (Lear *et al.* 2006). MRSA showing a 40-fold increase in MIC to triclosan remained susceptible to 1 mg/L (Suller and Russell 1999). Concentration is central to the definition of resistance in practice (Maillard and Denyer 2009). Hence, bacterial resistance based on the determination of MIC does not reflect accurately the *true* resistance profile of biocides, including triclosan.

Concentration is one the most important factors that will affect the activity and efficacy of a biocide (Russell and McDonnell 2000, Maillard 2005a, b 2007). Biocides with a high concentration exponent (Russell and McDonnell 2000) such as triclosan are particularly affected by dilution since a small decrease in concentration will profoundly affect efficacy. Hence, it might not be surprising that products with a low concentration of a phenolic biocide or other biocides with a high concentration exponent (e.g. alcohols) are less effective and might be prone to bacterial contamination and growth.

Most laboratory studies have been performed with triclosan dissolved in a solvent such as DMSO, and in some cases alcohol, and did not investigate commercially available formulations. Differences between laboratory (*in vitro*) investigations and situations in practice have not been addressed to date (Maillard and Denyer 2009). Hence, emerging bacterial resistance to triclosan investigated *in vitro* conditions might not necessarily reflect such development of resistance *in situ*. Components of the formulations might have a potentiation effect (or not) on the activity of triclosan, and their role on emerging bacterial resistance to triclosan has not been studied.

6.3. Mechanisms of bacterial resistance to triclosan

Bacterial resistance against triclosan involves both intrinsic and acquired mechanisms (Yazdankhah *et al*. 2006), and include: mechanical barrier (altering intracellular concentration), change in target site (mutation of the target site) (Heath *et al*. 1998), efflux, and by-pass of metabolic pathway (Webber *et al*. 2008b). These mechanisms have been also described to confer antibiotic resistance (Davin-Regli *et al.* 2008).

Change in enoyl acyl carrier reductase

At sub-lethal concentrations, triclosan has been shown to affect specific bacterial targets. Triclosan interacts specifically with an enoyl-acyl reductase carrier protein (ENR) at a low concentration (Heath *et al*. 1999; Levy *et al*. 1999, Roujeinikova *et al*. 1999, Stewart *et al*. 1999). Triclosan was found to inhibit fatty acid synthesis by targeting FabI in *E. coli* (Heath *et al*. 1998) and *S. aureus* (Heath *et al*. 2000), and InhA in *M. smegmatis* (McMurry *et al*. 1999) and *M. tuberculosis* (Parikh *et al*. 2000).

Triclosan resistant mutations in *fabI* decrease the interaction of triclosan with the ENR-NAD^+ binding. Mutation in *fabI* in *E. coli* was shown to confer a 60-fold decreased susceptibility to triclosan (Heath *et al*. 1998). Mutation in *fabI* has led to an increase in triclosan MIC in a number of bacterial genera (McMurry *et al*. 1998a, Parikh *et al*. 2000, Health *et al*. 2000, Slater-Radosti *et al*. 2001, Massengo-Tiassé and Cronan 2008, Webber *et al.* 2008b). In *Acinetobacter baumannii* high-level triclosan resistance could be explained by a Gly95Ser mutation of FabI, whilst wild-type *fabI* was observed to be overexpressed in low-level resistant isolates (Chen *et al*. 2009). Likewise in *Ps. aeruginosa*, high-level resistance to triclosan has been shown to be associated with FabV (Zhu et al. 2010).

McMurry *et al*. (1998b) postulated that mutations at *mar* and *sox* in *E. coli* only conferred a 2-fold increase in resistance presumably by a modest overexpression of AcrAB. This expression is unlikely to decrease the efficacy of triclosan. However such a mutation, together with mutations at other loci such as *fabI* (increasing resistance to 90-140-fold) could be more significant

Efflux of antimicrobials

Triclosan is a substrate of AcrAB efflux pump in *E. coli*, of MexAB-OprM and MexCD-OprJ, MexEF-OprN, MexJK-OprH multidrug efflux pumps in *P. aeruginosa*, of AcrB in *S. enterica* serovar Typhimurium, and CmeB in *Campylobacter* spp. (Piddock 1996; McMurry *et al*. 1998; Chuanchuen *et al*. 2001, 2002, 2003; Schweizer 1998). These efflux pumps are similar to other efflux pumps in other Gram-negative pathogens (Piddock 2006) and as such, it is likely that triclosan is a substrate of such pumps in other Gram-negative bacteria.

In *S. enterica* serovar Typhimurium, active efflux via AcrAB-TolC conferred decreased susceptibility to triclosan. The triclosan resistant mutants (MIC ≥32 mg/L) did not lose any fitness when compared to wild-type strains (Webber *et al*. 2008a). The pump efflux system of *P. aeruginosa* has been shown to confer a high level of intrinsic triclosan resistance (Mima *et al*. 2007). In addition, mutants of *E. coli*, and *S. enterica* which overexpress the AcrAB–TolC efflux system, have decreased susceptibility to various agents, including triclosan, demonstrating that triclosan is a substrate for efflux pumps (Webber *et al*. 2008a).

As previously reported for antibiotics, the presence of active efflux pumps is required for the acquisition of target mutations, which in turn increase the level of resistance (Webber *et al.* 2008b). In *Acinetobacter baumannii*, although active efflux did not appear to be a major reason for triclosan resistance, the acquisition of resistance appeared to be dependent on a background of intrinsic triclosan efflux (Chen *et al*. 2009).

By-pass of metabolic blockage

The proteomic analysis of *S. enterica* serovar Typhimurium triclosan-resistant mutants showed a set of proteins with commonly altered expression in all resistant strains. This "triclosan resistance network" included 9 proteins involved in production of pyruvate or fatty acid and represents a mechanism to increase fatty acid synthesis by an alternative pathway (Webber *et al*. 2008b). In addition to the expression of this "network", these mutants showed specific patterns of protein expression leading to the conclusion that triclosan resistance was multifactorial and potentially involved a number of mechanisms acting synergistically to attain high-level resistance (≥32 mg/L) (Webber *et al*. 2008b). In *S. aureus*, a modification of the membrane lipid composition associated with the alteration of the expression of various genes involved in the fatty acid metabolism were observed in triclosan resistant strains (Tkachenko *et al*. 2007).

Seaman *et al*. (2007) studied the appearance of small colony variants in MRSA following exposure to triclosan *in vitro*. The small colony variants displayed reduced susceptibility (23-60 fold; 1.5-4 mg/L from 0.063 mg/L) to triclosan and resistance to penicillin and gentamicin. Bayston et al. (2009) noted that prolonged exposure (i.e. 72 h) to triclosan-

impregnated silicone resulted in the induction of small colony variants and a 67-fold increase in triclosan MIC.

Recent evidence highlighted that bacterial swarming motility might confer some resistance to triclosan (5 mg/mL in *B. subtilis* and 0.1 mg/mL in *E.coli*) when compared to no swarming bacteria. The mechanism(s) by which swarming might confer some resistance is unknown, but is unlikely to be caused by efflux (Lai *et al*. 2009).

Involvement of multiple mechanisms

At bactericidal concentrations, triclosan seems to act against multiple and various targets, causing disruption of the bacterial control of cell wall permeability (Villalain *et al*. 2001; Guillén *et al*. 2004). One study in particular, investigated the role of both the permeability barrier and efflux in increase resistance to triclosan in *E. coli*. The MIC of triclosan-resistant *E. coli* mutants (MIC >1000 mg/L) was reduced to 10-25 mg/L when treated with both ethylene diamine tetra-acetic acid (EDTA; a chelating agent enhancing outer membrane permeability) and carbonyl cyanide m-chlorophenylhydrazone (CCCP; a proton motive force uncoupler), as compared to a MIC of 0.1 mg/L in sensitive *E. coli* strain, indicating that potentially both permeability and efflux worked together to provide the high level resistance to triclosan. However, neither CCCP nor EDTA reduced the susceptibility of *P. aeruginosa* to triclosan (Gomez Escalada 2003). In *Acinetobacter baumannii*, triclosan-resistant isolates were characterized by antibiotic susceptibility, clonal relatedness, *fabI* mutation, *fabI* expression, and efflux pump expression (Chen *et al*. 2009). Yu *et al.* (2010) described a multiple mechanism response in *E. coli* following exposure to triclosan. The involvement of a number of mechanisms was shown to confer triclosan resistance up to 80 mg/L.

Bacterial biofilms

Generally, bacteria are attached to surfaces and associated in a community (termed biofilm) and are rarely present as single cells (planktonic). Bacterial biofilms have been shown to be highly resistant to antimicrobials compared to planktonic cultures. A biofilm-associated phenotype has been described (Brown and Gilbert 1993, Ashby *et al*. 1994, Das *et al*. 1998; Gilbert *et al*. 2003). The mechanisms of resistance involved in a bacterial biofilm include decreased metabolism, quiescence, reduced penetration due to the extracellular polymeric matrix (Pan *et al*. 2006), enzymatic inactivation of biocides (Sondossi *et al*. 1985) Giwercman *et al*. 1991, Huang *et al*. 1995), and the induction of multi-drug resistant operons and efflux pumps (Maira-Litran *et al*. 2000). Bacterial biofilm resistance to triclosan has been poorly studied.

One study reported that the tolerance to triclosan of *Salmonella* in biofilm was attributed to low diffusion through the extracellular matrix, while changes of gene expression might provide further resistance both to triclosan and to other antimicrobials (Tabak *et al*. 2007). McBain *et al*. (2003) investigated the fate of a complex bacterial biofilm exposed to sub-lethal concentrations of triclosan (2–4 g/L) over a 3 month period. The authors identified a change in the composition of the biofilm and an increase in resistance of the complex population as measured by MIC. Interestingly, the composition of the biofilm changed, with a decrease of species diversity. The triclosan tolerant species such as Pseudomonads and Stenotrophomonads were still present, but other triclosan tolerant species (*Achromobacter xylosoxidans*) demonstrated a clonal expansion. Most importantly, the authors noted that the antibiotic susceptibility profile was not affected.

A study investigating the effect of triclosan in the development of bacterial biofilms on urinary catheters highlighted the selectivity of triclosan. While triclosan inhibited *P. mirabilis*, it had little effect on other common bacterial pathogens (Jones *et al.* 2006). In addition, the control of *P. mirabilis* by triclosan resulted in emerging triclosan-resistant strains *in vitro*. While most of these strains were still susceptible to the triclosan concentration used in the urinary catheter, one strain (MIC = 40 mg/L) was not (Stickler and Jones 2008). Smith and Hunter (2008) showed that recommended concentrations of

three biocidal products used in healthcare (one containing benzalkonium chloride 10 g/L, one containing chlorhexidine gluconate 40 g/L and one containing triclosan 10 g/L), were ineffective in eliminating hospital-acquired MRSA or *P. aeruginosa* biofilms, highlighting differences in susceptibility between planktonic and biofilm bacteria.

It is however interesting to note that Tabak et al. (2009) observed a synergistic action of sequential treatment of triclosan (500 mg/L) followed by ciprofloxacin (500 mg/L) against biofilm of *S. enterica* serovar Typhimurium. There is little information in the literature about the potentiation of activity between a biocide and an antibiotic and such a study is important and describes an interesting application/effect of triclosan.

6.4. Mutation rates and transfer of resistance

The development of bacterial resistance through acquired mechanisms such as mutation and the acquisition of resistant determinants are of concern since a bacterium that was previously susceptible can become insusceptible to a compound or a group of compounds (Russell 2002a). In *S. enterica* serovar Typhimurium, mutation frequency following exposure to triclosan was low (5×10^{-9}), lower than mutation frequency observed following antibiotic exposure (Birošová and Mikulášová 2009).

Cookson *et al*. (1991) isolated MRSA strains exhibiting triclosan resistance (2-4 mg/L) from patients using mupirocin and triclosan baths. Although in this study the resistance was shown to be transferable in association with the plasmid encoding for mupirocin resistance, this could not be confirmed subsequently by other studies. The transfer of a plasmid encoding for mupirocin resistance to a triclosan sensitive *S. aureus* strain failed to increase resistance to triclosan (Suller and Russell 2000). Other studies investigating clinical *S. aureus* isolates resistant to mupirocin also failed to observe this association (Bamber and Neal 1999). Although various genetic mobile elements have been described to be involved in the dissemination of cross-resistance towards biocides-antibiotics (Roberts and Mullany 2009, Schlüter *et al*. 2007) no specific genetic mobile element has been associated with triclosan resistance.

6.5. Induction of resistance

There are two types of induction. The first corresponds to the trigger of genes governing the genetic cascade (global regulation) which promotes the expression of efflux pumps and/or down regulates membrane permeability (porin synthesis). The second corresponds to the direct activation of the promoter region (local regulation) for example controlling efflux genes (Davin-Regli *et al*. 2008).

The induction of bacterial resistance mechanisms following exposure to a low concentration of a biocide has been reported in a number of studies for a number of biocides (SCENIHR 2009). In some occasions, a specific mechanism has not been established and a phenotypic change leading to the emergence of resistance to several unrelated compounds *in vitro* has been reported following exposure to a low concentration of a biocide (Moken *et al*. 1997).

It is possible that triclosan induces a stress response followed by, or in addition to, the expression of mechanisms that reduce the deleterious effect of the biocide (McMurry *et al*. 1998b; Gilbert *et al*. 2002). A decrease in growth rates in *E. coli* and *P. aeruginosa* has been described following exposure to sub-lethal concentrations of triclosan, which indicates the generation of a stress to the organism (Gomez Escalada *et al*. 2005).

Triclosan induces bacterial resistance through the over-expression of efflux pumps via activation of *mar* and *ram* (Randall *et al.* 2007; Webber *et al*. 2008a; Bailey *et al.* 2009), over-expression and mutagenesis of *fab1*, expression of regulatory genes involved in the control of antibiotic resistance cascades (activator of drug efflux, decrease of membrane

permeability) and fatty acid metabolism in a number of bacterial genera (Jang *et al*. 2008, Webber *et al*. 2008b, Bailey *et al*. 2009). These genes are involved in resistance to triclosan, but also in possible cross-resistance and multi-resistance to different antibiotic and biocide classes. In *Stenotrophomonas maltophilia*, the overexpression of an efflux pump (SmeDEF), involved in antibiotic resistance, was demonstrated in several triclosan-selected mutants (Sánchez *et al*. 2005). In *E. coli*, overexpression of *acrAB* or *marA* or *soxS* (positive regulator of *acr*AB) decreased susceptibility to triclosan 2-fold. Deletion of the *acr*AB locus increased susceptibility to triclosan approximately 10-fold. It was observed that clinical isolates overexpressing *acrAB* showed enhanced resistance to triclosan. A clinical strain overexpressing *marA* had a triclosan MIC of 0.27mg/L as compared to susceptible strain with an MIC of 0.090 mg/L. In *S enterica* serovar Typhimurium overexpressing AcrAB and *C. jejuni* overexpressing CmeB, triclosan MIC increased to 32 mg/L (Pumbwe *et al*. 2005; Buckley *et al*. 2006). Moken *et al*. (1997) described the induction of the MDR phenotype in *E. coli* and its relevance to cross-resistance between pine oil, triclosan and multiple antibiotics. Jang *et al*. (2008) reported that, in *S. aureus,* exposure to triclosan (0.015 mg/L) resulted in down-regulation of the *clpB* chaperone-related genes, which might trigger the expression of resistant determinants. A recent study demonstrated that triclosan activates the expression of several groups of genes in *E. coli* and *S. enterica* (Bailey *et al* 2009). Transcriptome analyses (including microarray and RT-PCR experimental approaches) of bacteria exposed to triclosan (0.12 mg/L for 30 minutes) indicated an induction of the expression of various genes involved in drug efflux (e.g. *acrB*), in the genetic activation of resistance genes (e.g. *marA*), in the control of oxidative and drug response (e.g. *soxS*), and in the control of membrane permeability (e.g. *ompR*). Despite some differences in the response level observed between the two bacterial species, triclosan was shown to induce a rapid and adaptative response including the activation of several regulatory and structural genes involved in antibiotic resistance (Bailey *et al.* 2009).

McBain *et al*. (2004), however, failed to demonstrate a biologically significant induction of drug resistance in a number of bacterial species exposed to sub-lethal concentrations of triclosan, suggesting that triclosan-induced drug resistance is not generally readily inducible nor is it transferred across bacterial species.

6.6. Bacterial cross-resistance to triclosan and antibiotics

6.6.1. General considerations

The possibility that the mechanisms involved in triclosan resistance may contribute to reduced susceptibility to clinically important and structurally unrelated antimicrobials is of major concern. It is important to note that antibacterial actions from antibiotics and biocides show some similarities in their mechanisms of action, behaviour and clinical aspects (Poole 2007).

Among the similarities, we can mention (i) the penetration/uptake through bacterial envelope by diffusion, (ii) the effect on the membrane integrity and morphology, (iii) the effect on diverse key steps of bacterial metabolism (replication, transcription, translation, transport, various enzymes). Faced with this chemical aggression and stress, bacteria mobilise similar defence mechanisms conferring resistance against structurally non-related molecules (Walsh and Fanning 2008).

6.6.2. Triclosan and cross-resistance

A number of (but not all) laboratory studies have demonstrated an association between triclosan resistance and resistance to other antimicrobials. However, this link has not been

confirmed in the limited number of *in situ* studies that have been performed to date. A number of bacterial mechanisms potentially conferring cross-resistance has been identified in laboratory investigations (see Table 6).

Table 6 Bacterial mechanisms inducing potential cross-resistance

Mechanism	Nature	Level of susceptibility to other biocides[1]	Cross-resistance
Change in bacterial envelope	intrinsic (acquired)	no	yes
(over)Expression of efflux pumps	intrinsic/acquired	reduced	yes
Enzymatic modification	acquired/intrinsic	reduced	no[2]
Mutation (target site)	acquired	reduced	no[3]
Phenotypic change	Following exposure	reduced	yes

[1] to other biocides - level of susceptibility defined according to the concentration of biocides
[2] in the case of acquired resistance, co-resistance has been described
[3] triclosan cross-resistance with specific antibiotics (e.g. isoniazid) acting against enoyl acyl carrier proteins (e.g FabI) has been described.

Studies on *S. enterica* and *Stenotrophomonas maltophilia* described the effect of triclosan on emerging bacterial cross-resistance. In *S. enterica,* Karatzas *et al*. (2007) reported that a triclosan-resistant strain overexpressing an efflux pump was less susceptible to antibiotics than the wild type original strain. Another study described the survival of *S. enterica* serovar Typhimurium following exposure to various disinfectants at a low concentration on the resulting changes in antibiotic profile (Randall *et al*. 2007). The authors concluded that growth of *Salmonella* with sub-inhibitory concentrations of biocides favours the emergence of strains resistant to different classes of antibiotics. In *Stenotrophomonas,* Sanchez *et al*. (2005) analysed the effect of triclosan on the selection of mutants overexpressing the efflux pump SmeDEF involved in both intrinsic and acquired resistance to antibiotics. The authors demonstrated that triclosan was able to select 5 mutants overexpressing this pump, out of a total of 12 mutants. This overexpression conferred resistance to a number of antibiotics such as tetracycline, chloramphenicol and ciprofloxacin.

Similar results have been reported with *S. enterica* and *E. coli* (Braoudaki and Hilton 2004). *E. coli* O157 strains, involved in the "hamburger disease", acquired high- levels of resistance to triclosan after only two sublethal exposures and when adapted, repeatedly demonstrated decreased susceptibilities to various antibiotics, including chloramphenicol, erythromycin, imipenem, tetracycline, and trimethoprim, as well as to a number of biocides. Bailey *et al*. (2009) showed that triclosan triggered the expression of a number of genes (e.g. encoding for efflux pumps, porins) directly involved in antibiotic resistance, and regulatory genes involved in the control of the antibiotic resistance gene cascade (activator of drug efflux, decrease of membrane permeability). Alteration in InhA in *M. smegmatis* following exposure to triclosan resulted in resistance to isoniazid (McMurry *et al*. 1999). Likewise, exposure of *M. tuberculosis* to triclosan led to mutation in *inhA* causing cross-resistance to isoniazid. However, isoniazid-resistant mutants were still susceptible to triclosan (Parikh *et al*. 2000).

Pycke et al. (2010) observed that triclosan exposure of the environmental α-proteobacterium *Rhodospirillum rubrum* led to an increase in triclosan MIC. The extent of this increase as well as the generation of different antibiotic susceptibility profiles was triclosan-concentration dependent, indicating the expression of distinct resistance mechanisms.

However, direct linkage between triclosan usage and bacterial resistance to other biocides and antibiotics might not be universal. Cottell *et al*. (2009) investigated the antibiotic

susceptibility of triclosan tolerant *S. aureus*, *E. coli* and *Acinetobacter johnsonii* and reported that these strains remain susceptible to antibiotics used in clinical settings. In addition, triclosan-tolerant *E. coli* were found to be significantly more susceptible to aminoglycosides (Cottell *et al*. 2009). Likewise, triclosan resistant mutants in *S. aureus* did not show an altered antibiotic susceptibility profile compared to their parent strains (Suller and Russell 2000). Lear *et al*. (2006) demonstrated that environmental isolates with an increased MIC to triclosan remained susceptible to other biocides and antibiotics. Birošová and Mikulášová (2009) reported that continuous exposure of sub-inhibitory concentrations of triclosan did not increase emerging antibiotic resistance in *S. enterica* serovar Typhimurium but helped to maintain antibiotic-resistant bacteria in the population, notably those showing a *mar* phenotype. A short-term exposure to triclosan (30 min at 0.5 MIC, i.e. 0.098 mg/L) did not result in the selection of antibiotic resistant mutants.

6.7. Triclosan resistance in bacteria *in situ*

Triclosan has been the most studied biocide with respect to its anti-bacterial activity. However, investigations concerned mainly laboratory experiments and only very few studies are available to date on bacterial resistance to triclosan *in situ*. Furthermore, in most *in vitro* studies, resistance to triclosan has been measured as an increase in MIC. As mentioned in section 6.2 above, the measurement of resistance based on MIC only, might have little bearing on bacterial survival to concentrations found *in situ*.

Ledder *et al*. (2006) investigated acquired high-level triclosan resistance in a number of distinct environmental isolates and reported that a relatively small number of strains showed a decrease in triclosan susceptibility (*E. coli*, *Klebsiella oxytoca*, *Aranicola proteolyticus* and *S. maltophilia*) while the susceptibility of the remaining 35 species remained unchanged. They concluded that repeated exposures to triclosan did not systematically produce high-level triclosan resistance in all bacteria. Furthermore, among the strains with decreased susceptibility, there was no change in antibiotic susceptibility or susceptibility to other biocides. Similarly, another study by the same group on repeated exposure of dental bacteria to triclosan resulted in the same conclusions (McBain *et al*. 2004).

Cole *et al*. (2003) collected 1238 isolates from the homes of users and non-users of antibacterial product and were unable to demonstrate any cross-resistance to antibiotic and antibacterial agents in target bacteria. In addition, this study showed an increased prevalence of potential pathogens in the homes of non-users of antibacterial products. However, in this study, the isolates were selected based on their antibiotic resistance and were then tested for their insusceptibility to biocides. With our current state of knowledge, it is generally accepted that antibiotic resistance in clinical isolates is not necessarily associated with resistance to biocides. Sullivan *et al*. (2003) studied the effect of triclosan in toothpaste on some bacterial species from the oral flora of 9 human volunteers over a 14-day period. Triclosan usage contributed to a decrease in lactobacilli although this decrease had no clinical significance. Furthermore, the antibiotic susceptibility profile of the oral streptococci investigated did not change following the use of triclosan containing toothpaste. Aiello *et al*. (2004) did not find any statistical significance between elevated triclosan MICs and antibiotic susceptibility in bacterial isolates taken from the hands of individuals using antibacterial cleaning and hygiene products for a 1-year period. Earlier studies reported no change in the ecology of the oral flora or resistance to triclosan following the use of triclosan-containing toothpaste. Jones *et al*. (1987) reported no change in the predominant plaque flora in 13 volunteers following the use of triclosan (2 g/L) for seven months. The authors did not observe any increase in triclosan MIC in these bacteria. Similar conclusions were reported by Walker *et al*. (1994) who reported no changes in the microbial flora in 144 patients following the use of 3 g/L triclosan-containing toothpaste. A meta-analysis of 16 clinical studies of the long-term effect (at least 6 month) of using triclosan toothpaste showed reduction in dental plaques and gingivitis (Davies *et al*. 2004).

7. TRICLOSAN BIOAVAILABILITY AND FORMULATION EFFECTS

The concentration of triclosan that comes in contact with a micro-organism governs the subsequent effect on that micro-organism (e.g. inhibitory, lethal, adaptation, selection). Hence the bioavailability of triclosan is paramount.

As described in Chapter 5, triclosan present in various environmental media is susceptible to degradation by oxidation by ozone, chlorine and sunlight, and to biodegradation by micro-organisms. The main route of exposure to soil is expected to be via the application of sewage sludge to agricultural soil. The bioavailability will depend on the sorption, mobility and degradation in soil under various physical conditions. Triclosan is released into surface waters via effluents from WWTP, and the bioavailability of the triclosan to micro-organisms in these media will depend upon sedimentation by binding with the particulate matter and stability of the compound during the exposure period.

The US EPA (2008) states on stability of triclosan in the environment that:

"*Triclosan is hydrolytically stable under abiotic and buffered conditions over the pH 4-9 range based on data from a preliminary test at 50°C.*

Photolytically, triclosan degrades rapidly under continuous irradiation from artificial light at 25°C in a pH 7 aqueous solution, with a calculated aqueous photolytic half-life of 41 minutes.

Triclosan degrades rapidly in aerobic soils maintained in darkness at 20 ± 2°C, with calculated half-lives of 2.9-3.8 days.

In aerobic water-sediment systems maintained in darkness at 20 ±2°C, triclosan degraded with calculated nonlinear half-lives of 1.3-1.4 days in the water, 53.7-60.3 days in the sediment, and 39.8-55.9 days in the total system.

In soil, triclosan is expected to be immobile based on an estimated Koc of 9,200.

Triclosan is not expected to volatilize from soil (moist or dry) or water surfaces based on an estimated Henry's Law constant of 1.5 x 10-7 atm-m^3/mole.

Triclosan partially exists in the dissociated form in the environment based on a pKa of 7.9, and anions do not generally adsorb more strongly to organic carbon and clay than their neutral counterparts.

In aquatic environments, triclosan is expected to adsorb to suspended solids and sediments and may bioaccumulate (K_{ow} 4.76), posing a concern for aquatic organisms.

Hydrolysis is not expected to be an important environmental fate process due to the stability of triclosan in the presence of strong acids and bases. However, triclosan is susceptible to degradation via aqueous photolysis, with a half-life of <1 hour under abiotic conditions, and up to 10 days in lake water. An atmospheric half-life of 8 hours has also been estimated based on the reaction of triclosan with photochemically produced hydroxyl radicals.

In the laboratory, triclosan degraded via aerobic soil metabolism and aerobic aquatic metabolism, with half-lives of <4 days in soils and half-lives of <1.5 days (water layer) and up to 60 days (sediment and total system) in water-sediment systems."

Samsøe-Petersen *et al*. (2004) have described that half-life of triclosan for three experimental soils was calculated to be in the range of 17.4 to 35.2 days

Some observed concentrations of triclosan in the environment (e.g. Kumar *et al*. 2010) are high enough to induce changes in the microbial population. However, the bioavailability of triclosan in these environments (WWTP effluents, sludges, sediments, etc.) has not been

determined. It is therefore important that the concentration effects of bioavailable triclosan are measured during the exposure period under study.

The presence of other chemicals (e.g. antibiotics, other biocides, surfactants…) in the environment may also affect the microbial population. Therefore it may be difficult to assess the effect of triclosan alone against microbial populations in the environment.

Triclosan-containing products are complex formulations since triclosan is poorly soluble in water. The role of the formulations is important to ensure the bioavailability of triclosan. Formulations might also enhance biocidal activity and/or reduce microbial aggregation, improving the biocidal activity of the product. The bioavailability of triclosan in surfaces or textiles, etc., is product dependent. Some manufacturers claim that triclosan does not leach out of their product.

8. MEASUREMENT OF RESISTANCE AND CROSS-RESISTANCE

Concentration is central to the definition of bacterial resistance in practice (McDonnell and Russell 1999, Maillard and Denyer 2009). Therefore, the measurement of bacterial lethality rather than the measurement of bacterial growth inhibition is paramount. The determination of the lethality of the in-use concentration of a biocide will indicate, by comparison to a reference strain, whether a bacterial strain is insusceptible (i.e. intrinsically resistant) or has acquired resistance to a biocide or not.

The determination of *minimum bactericidal concentrations* (MBCs) is also an appropriate methodology that allows the comparison of lethality between a reference strain and "resistant" clinical/environmental isolates. Here, the reference strains represent the population of bacteria which is normally susceptible to the biocide. In addition the determination of the lethality of a biocide must involve the use of a neutralising agent or the removal of the biocide. Failure to do so will provide an over-estimation of the lethality of the biocide.

The determination of bacterial growth kinetics in the presence of a low concentration of a biocide can also provide indications to a change in bacterial phenotype (Thomas *et al.* 2004; Gomez Escalada *et al.* 2005a; Maillard 2007), but it does not indicate whether bacteria will become resistant to the biocide and cross-resistant to unrelated compounds or not.

Likewise, a number of protocols have been used to measure antibiotic susceptibility in bacterial isolates showing resistance, tolerance or increased insusceptibility to biocides or vice versa. The variety of protocols used contributes to the variability of the results reported on antibiotic "resistance". For example, some studies based a change in antibiotic susceptibility profile on measurement of zone of inhibition (Tattawasart *et al.* 1999; Thomas *et al.* 2005). More meaningfully studies used standardised antibiotic susceptibility methodologies such as those given by the British Society for Antimicrobial Chemotherapy (BSAC) or Clinical and Laboratory Standards Institute (CLSI) to measure a change in antibiotic susceptibility profile. However a limited number of studies have looked at a decrease in antibiotic susceptibility that would be associated with treatment failure (Lear *et al.* 2006; Cottell *et al*. 2009).The effect of biocides on antibiotic susceptibility in bacteria has been measured indirectly, whereby a bacterial population is treated first with a biocide and the surviving bacteria then investigated for their susceptibility to antibiotics. However, there are currently no well-referenced criteria or standard protocols for the evaluation of the capability of a biocide to induce or select for resistance to antibiotics. Therefore, 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 in a defined bacterium (SCENIHR 2009).

Since cross-resistance can be conferred by a number of distinct mechanisms, it is important to evaluate the propensity of a bacterium to express these mechanisms. Advances in

modern genetic methods (e.g. PCR, -omics) and the development of an assay using specific chemosensitizers or markers (e.g. efflux pumps inhibitors) might allow the development of routine tests to identify resistance mechanisms.

9. DATA GAPS ON SCIENTIFIC KNOWLEDGE

In the course of this work, several important gaps were noted. These can be divided into scientific and technical gaps:

9.1. Scientific gaps:

1. Environmental studies focussing on the identification and characterisation of resistance and cross-resistance to antibiotics following use of triclosan.
2. *In vitro* studies to demonstrate whether triclosan, used at sub-lethal concentrations, triggers the emergence of antibiotic resistance and/or select bacteria resistant to antibiotics. This has only been demonstrated in a limited number of bacterial genera. Further information for other genera should be obtained.
3. Despite *in vitro* evidence of the effect of triclosan on the emergence of antibiotic resistance and on the selection of bacteria resistant to antibiotics, epidemiological data indicating public health relevance are lacking.
4. There is no information available on the maintenance and transferability of resistance and virulence markers in the presence of triclosan.

9.2. Technical gaps:

1. Standardisation of methodologies to measure resistance and cross-resistance is needed.
2. Information on production, use volumes is required to assess the exposure of bacteria to triclosan in various matrices.
3. Data on the fate and bioavailability of triclosan in the environment are sparse. Information on environmental concentrations, contact time, microbial population present in the field and bacterial exposure, is insufficient to determine whether expression of resistance actually occurs *in situ*.
4. No validated methodologies are available for the determination of the dose-response relationships and of the threshold triggering the emergence of antibiotic/biocide resistance and/or the selection of resistant bacteria.
5. The role of bacterial biofilm in resistance to triclosan has been shown. Furthermore, bacterial biofilms are very common in the environment. Yet, most laboratories are not using biofilm tests to assess the efficacy of biocides (Cookson 2005). There are, currently, no European standards for the testing of disinfectants against biofilms for health care applications.

A more detailed research strategy for investigating the antimicrobial resistance effect of biocides is presented in a separate opinion from the SCENIHR (2010).

10. RISK ASSESSMENT

Triclosan is the most studied biocide with respect to antimicrobial resistance. Such a level of information, notably on its activity on bacteria, the identification of mechanisms of microbial resistance, including genomic and proteomic aspects, is commendable. However, in spite of this level of information on mechanisms, information on the interaction between triclosan and microbial cells/communities including data on exposure and bioavailability *in situ* is lacking. Thus, a full risk assessment of triclosan cannot be performed. However, a number of points can be made:

- a hazard has been identified concerning the effect of triclosan on the regulation of resistance genes in bacteria

- mechanisms which can promote resistance and cross-resistance to biocides and antibiotics in bacteria have been identified

- high concentrations of triclosan (compared to concentrations known to select for resistance in *in vitro* experiments) have been measured in certain environmental compartments, however a link with cosmetic or other specific product uses could not be made.

- bacterial biofilms are widespread in the environment and are able to survive exposure to adverse environmental factors.

10.1. Limitation in activity

Bacteria can be classified according to their intrinsic resistance to biocides. Bacterial endospores are considered to be most resistant, followed by mycobacteria, Gram-negative bacteria and Gram-positive bacteria (Maillard 2005a). Triclosan is not sporicidal. It is not bactericidal against certain bacteria such as *P. aeruginosa* and *Burkholderia* sp. (Rose *et al*. 2009).It might also have limited activity against certain mycobacteria as these micro-organisms are considered to be less susceptible to biocides than Gram-negative bacteria.

10.2. Genetic and bacterial point of view

Recent laboratory studies indicate that, during short exposures of mid-logarithmic growth phase to MIC concentrations (30 min at 0.12 mg/L), triclosan can trigger a genetic response in Gram-negative bacteria (e.g. *E. coli, S. enterica*) inducing expression of genes involved in biocide and antibiotic resistance. In addition, in *Listeria monocytogenes* triclosan concentrations of 19 mg/L to 150 mg/L activate the expression of virulence factors (Kastbjerg *et al*. 2010).

Concerning the genetic aspects; genetic mobile elements play an important role in bacterial resistance response since they contain resistance genes (coding for pump, enzyme, qnr factors, etc) which can confer resistance to different drug families. The gene pool encoding for various mechanisms that confer resistance to antimicrobials has been shown to be present in soil bacteria (Dantas *et al*. 2008). Although exposure to some biocides (such as quaternary ammonium compounds) favours the dissemination and maintenance of such genetic mobile elements in bacteria and subsequently may facilitate the exchange of key genes between bacterial species (Paulsen *et al*. 1998, Pearce *et al*. 1999, Sidhu *et al*. 2001 2002, Bjorland *et al*. 2001, Noguchi *et al*. 2002), this has not been reported for triclosan.

10.3. Environment point of view

Several recent studies have clearly demonstrated the widespread presence of triclosan in the environment, especially in wastewater, in wastewater treatment plant effluents, in rivers and in sediments. However, there is limited information from the EU. The reported concentrations range from less than 0.001 ng/L (seawater) to 133 mg/kg (biosolids from WWTP) (see Table 4). The following information is also necessary for the risk assessment:

a) The bioavailability of triclosan in these environments,

b) The microflora in contact with triclosan in these environmental compartments,

c) Whether this microflora contains bacterial species in which triclosan is able to trigger a genetic response. If not, could the environmental bacteria in contact with triclosan transfer genetic elements (containing resistance genes) to "target" bacteria?

Regulation cascades and corresponding resistance genes are present in the soil bacteria. These bacteria may both serve as original source/reservoir of genetic mobile elements (horizontal transfer) and as genetic manipulator (exchange between chromosomal and mobile genes) of resistance genes in the presence of a selective pressure.

10.4. Biofilm formation in specific environmental conditions

Bacterial biofilms are widespread in the environment including waters, plants, etc. They deserve a special attention because of three main characteristics: the decrease in bioavailability of antibacterial agents within the biofilm, the presence of dormant/persister bacteria, and in complex biofilms the presence of various bacterial species in close contact that facilitate exchange of genetic material.

11. CONCLUSIONS

Triclosan is the most studied biocide with respect to bacterial resistance. Such a level of information, notably on its activity against bacteria, the identification of mechanisms of microbial resistance including genomic and proteomic aspects, is commendable and should be extended to other biocides. This information allows better understanding of triclosan interactions with bacterial cells and should be applied to ensure that its use is sustainable for human health. Based on the available scientific information, it is not possible to quantify the risk of development of antimicrobial resistance induced by triclosan applications, including its use in cosmetics. However, there are environmental concentrations in a number of geographically distinct areas high enough to suggest that triggering of bacterial resistance could also occur in the environment. The applications of triclosan which contribute to those high environmental concentrations cannot be properly identified nor quantified at present. This should be taken into account when considering the current and future uses of triclosan in all applications so as to ensure that the demonstrable benefits for human health in certain applications are not compromised.

Low concentrations of triclosan can trigger the expression of resistance and cross-resistance mechanisms in bacteria *in vitro*. Some environmental concentrations reported in a number of geographically distinct areas are high enough to give plausibility to this scenario occurring outside of the laboratory and warrant further investigation. The presence of other chemicals (e.g. antibiotics, surfactants, other biocides, etc.) in the environment, which may also affect microbial populations, would preclude assessing the effects of triclosan alone.

The emergence of resistance induced/selected by triclosan is related to the genetic control on the resistance gene(s) present on chromosomal and genetic mobile elements *in vitro*. This represents the origin for a hazard about selection and dissemination of cross-resistance with other anti-bacterial molecules including biocides and antibiotics.

Bacterial biofilms are widespread in the environment including waters, plants, etc. They deserve special attention because of three main characteristics: the decrease in bioavailability of antibacterial agents within the biofilm, the presence of dormant/persister bacteria, and in complex biofilms the presence of various bacterial species in close contact that facilitates some genetic exchange.

Triclosan, like any other biocide, contributes to the selection of less susceptible bacteria in a complex microcosm *in vitro*. The impact of such a selection is unclear, as is the fitness of the "selected" bacterial species following triclosan exposure. The few *in situ* studies

investigating long-term triclosan exposure (i.e. at least 6 months) did not indicate changes in resistance susceptibility in the predominant bacteria selected for monitoring but the changes in the entire flora were not evaluated.

There is so far no epidemiological data linking outbreaks of antimicrobial resistant human and zoonotic pathogens following exposure to triclosan from cosmetics and other products.

When used appropriately, biocides, including triclosan, have an important role to play in disinfection, antisepsis and preservation. Information on the expression/triggering of bacterial resistance mechanisms should be considered to (re-)assess the uses of triclosan in order to preserve its efficacy.

Where biocides, including triclosan, are used intensely, monitoring for emerging resistance in the microbial flora should be conducted.

12. OPINION

Does the SCCS consider a continued use of triclosan as a preservative in cosmetic products as safe taking into account the new provided documentation of resistance development by certain micro-organisms and cross-resistance?

At present, several distinct hazards have been identified: (i) the effect of triclosan on the triggering/regulation of resistance genes in bacteria (ii) the existence of mechanisms which can promote resistance and cross-resistance to biocides and antibiotics in bacteria, (iii) high concentrations of triclosan (compared to concentrations known to select for resistance in *in vitro* experiments) have been measured in certain environmental compartments and (iv) bacterial biofilms are widespread in the environment and are able to survive exposure to adverse environmental factors. The first two of these hazards have been identified *in vitro*. The presence of resistance genes in soil bacteria should be investigated further.

The six *in situ* studies and the one meta-analysis quoted in this document have failed to demonstrate an increase in antibiotic resistance following triclosan use. While these results are at first sight reassuring, the differences of methodologies used to measure "resistance" and to analyse the data make it premature at this stage to conclude that triclosan exposure never leads to developing microbial resistance. These studies were state-of-the art at the time they were performed but they did not have the modern tools (e.g. proteomic or genomic analysis) available today to investigate the complete bacterial population and the bacterial response to biocides. These useful *in situ* studies do not provide information on expression of genes involved in resistance, maintenance of resistance and virulence genes and transfer of resistance determinants. Thus the SCCS strongly recommends performing additional *in situ* studies looking at these aspects and bacterial phenotypes where known concentrations of triclosan have been found in the environment.

This opinion concerns the safety of triclosan in terms of microbiology, i.e. generation of bacterial resistance harmful for human health. Based on the available scientific information including recent data from *in vitro* investigations (proteomic and genomic analyses), it is not possible to quantify the risk associated with triclosan (including its use in cosmetics) in terms of development of antimicrobial resistance (i.e. selection for less susceptible population), genetic basis for resistance and dessemination of resistance. In view of the concentrations of triclosan reported to trigger resistance *in vitro*, some of the environmental concentrations found in a number of geographical distinct areas are high enough to suggest that bacterial resistance could be triggered. However, no studies have been conducted on this aspect. The applications of triclosan which contribute to those high environmental concentrations cannot be properly identified nor quantified at present and the presence of other chemicals (e.g. antibiotics, surfactants, other biocides, etc.) in the environment, which may also affect microbial populations, would preclude assessing the effects of triclosan independently.

Due to the limited number of *in situ* studies of resistance induced by triclosan to date, SCCS can only recommend the prudent use of triclosan, for example in applications where a health benefit can be demonstrated. However, conclusions from *in vitro* studies cannot be ignored, notably the role of triclosan (and other biocides) in triggering resistance and in the dissemination (or lack of) resistance determinants. Hence, the SCCS appreciates that research investment from industry will be maintained to contribute to a better understanding of the potential risks associated with triclosan applications. Research in triggering mechanisms of resistance, maintenance of the gene pool and the transfer of resistance and virulence determinants, and improving the translational application of laboratory results to situations *in situ* are needed.

13. COMMENTS RECEIVED DURING THE PUBLIC CONSULTATION

A public consultation on this opinion was opened on the website of the EU non-food scientific committees from 29 March to 26 May 2010. Information about the public consultation was broadly communicated to national authorities, international organisations and other stakeholders.

In total, 10 contributions were received of which 5 were from public authorities, 3 from industry and two from individuals with professional links to this issue.

Each submission to the public consultation was carefully considered by the Working Group and responses were formulated for each.The opinion has been revised to take account of all the relevant comments and the literature has been updated with relevant publications. The scientific rationale and the opinion were clarified and strengthened in certain respects. The overall opinion, however, remains unchanged.

14. MINORITY OPINION

None

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