ANSES Opinion
Request No 2016-SA-0238

The Director General

Maisons-Alfort, 7 November 2019

OPINION
of the French Agency for Food, Environmental and Occupational Health & Safety

on "Resistance to antimicrobial biocides"

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It provides the competent authorities with all necessary information concerning these risks as well as the requisite expertise and scientific and technical support for drafting legislative and statutory provisions and implementing risk management strategies (Article L.1313-1 of the French Public Health Code).

Its opinions are published on its website. This opinion is a translation of the original French version. In the event of any discrepancy or ambiguity the French language text dated 7 November 2019 shall prevail.

On 9 November 2016, ANSES issued an internal request to conduct the following expert appraisal: Internal request on bacterial resistance to antimicrobial biocides.

1. BACKGROUND AND PURPOSE OF THE REQUEST

Regulation (EU) No 528/2012 of 22 May 2012, which was implemented on 1 September 2013, governs the making available on the market and use of biocidal products. Its purpose is to improve the free movement of biocidal products within the European Union while ensuring a high level of protection of both human and animal health and the environment. Within the framework of this Biocides Regulation, it is necessary to ensure that each biocidal product placed on the market is effective against the claimed target organisms, has no unacceptable effects on humans and non-target organisms, and no unacceptable effects on target organisms, in particular resistance or cross-resistance.

There are currently no guidelines on the assessment of unacceptable effects such as resistance. An approach for assessing this resistance phenomenon therefore needs to be proposed, in order to help both the competent authorities and the applicant meet the requirements laid down in various points of the Biocides Regulation.

ANSES therefore issued an internal request on 9 November 2016 with a view to proposing methods for assessing the emergence of resistance/cross-resistance, i.e. assessing the capacity, level and maintenance of any resistance that could be developed by bacteria following exposure to biocidal substances and products. Resistance management strategies were also to be proposed where appropriate.

This internal request focuses solely on antimicrobial biocides with antibacterial action. These are used in a wide range of fields, mainly human hygiene, veterinary area, industry and water treatment, both as disinfectants and preservatives.

This internal request does not address the development of bacterial resistance to antibacterials in the environment resulting from the release of biocidal product residues into the environment. This topic is being addressed by a formal request on antimicrobial resistance in the environment, managed by the Water Risk Assessment Unit within ANSES's Risk Assessment Department.

2. ORGANISATION OF THE EXPERT APPRAISAL

The expert appraisal was carried out in accordance with French standard NF X 50-110 "Quality in Expert Appraisals – General requirements of Competence for Expert Appraisals (May 2003)".

ANSES entrusted examination of this request to the "Resistance to antimicrobial biocides" Working Group (WG), belonging to the Expert Committee (CES) on "Biocidal Substances and Products". This work was therefore conducted by a group of experts with complementary skills. The Working Group met nine times between 1 March 2017 and 24 May 2019. The work was described in a report whose main conclusions are set out in this opinion.

The methodological and scientific aspects of the work were presented to the CES for discussion on 21 February 2019 and 28 March 2019. The report produced by the Working Group takes account of the observations and additional information provided by the CES members.
The WG's work was adopted by the CES at its meeting of 20 June 2019.

ANSES analyses interests declared by experts before they are appointed and throughout their work in order to prevent the risk of conflicts of interest in relation to the points addressed in expert appraisals.

The experts' declarations of interests are made public via the ANSES website (www.anses.fr).

3. ANALYSIS AND CONCLUSIONS OF THE CES AND THE WG

As a preamble to the development of an approach for assessing a resistance phenomenon, ANSES produced a literature summary on:

- The different definitions related to these resistance phenomena;
- Biocides' main modes of action, and bacterial resistance mechanisms;
- Current methodological approaches for assessing the ability of bacteria to develop resistance.

An approach for assessing a resistance phenomenon applicable to biocidal products or active substances was then proposed. An experimental protocol was adopted to make various methodological choices consistent with the uses and application conditions of antibacterial biocides. These choices were integrated into a decision tree that will ultimately be used to assess the risk of development of resistance, or even cross-resistance to other antibacterial biocides or antibiotics. Where appropriate, strategies to manage the emergence of resistance were then proposed.

3.1. Literature summary

3.1.1. Definitions

The literature review on the topic of resistance of micro-organisms to antibacterial biocides led to the finding that there are considerable differences between authors in the terms used (resistance, tolerance, reduced susceptibility, cross-resistance, co-resistance, acquired resistance, resistance and biofilm, etc.).

In the context of this work, the WG proposes using only the term "resistance", which seems the most appropriate since it is already used in the Biocides Regulation. This choice can also be justified by the frequent link with antibiotics in the case of cross-resistance or co-resistance.

A series of definitions was proposed by the WG and is presented in the report. These include the following:

"Resistance":

"*Resistance is the reduction in susceptibility of a micro-organism to an antibacterial biocide because of its ability to withstand the dose(s) of use.*"

"Cross-resistance":

"*Cross-resistance is a process in which a micro-organism resistant to an antibacterial biocidal active substance or product to which it has been exposed, is also resistant to one or more other antibacterial substance(s) to which it has not been exposed.*"

"**Adaptation":**

"Adaptation is an evolution in the behaviour of bacterial strains, which acquire new transient or stable properties concerning resistance, increased susceptibility, increased or decreased virulence, or other properties. Going well beyond the development of resistance, this term covers all possible types of evolution in bacteria's behaviour as a result of a change in their environment."

3.1.2. Biocides' modes of action and bacterial resistance mechanisms

3.1.2.1 Modes of action of the main groups of biocidal substances

The modes of action of antibacterial biocides can be defined according to the targeted bacterial structures. Four sites of action are described in the table below:

Table 1: The different groups of antibacterial biocides[1] and the targeted bacterial structures

Action on the cell wall	Action on the membrane	Action on proteins	Action on nucleic acids
Alcohols Aldehydes Bases Phenols	Acids Alcohols Quaternary ammoniums Bases Biguanides Isothiazolinones Metals Oxidants Phenols	Acids Alcohols Aldehydes Bases Biguanides Isothiazolinones Metals Oxidants Phenols	Acids Alcohols Aldehydes Biguanides Halogens and derivatives Metals Oxidants

The vast majority of antibacterial biocides act on several targets, due to the chemical reactivity of most of them.

3.1.2.2 Resistance mechanisms

Bacterial resistance to biocides can be classified into two categories: intrinsic and acquired. Intrinsic resistance is defined as an already existing property inherent to a given species, resulting in reduced susceptibility or insusceptibility to the biocide.

Only phenomena related to acquired resistance, which may develop during the use of antibacterial biocides, were analysed when responding to this formal request.

Resistance is acquired either by transfer of resistance genes during contact between two bacteria according to a primary mechanism known as conjugation, in addition to mechanisms of expression, transduction and transformation, or by mutation in the bacterial chromosome of regulatory genes.

Concerning acquired resistance, numerous studies have shown that this is based on several major mechanisms: modulation of efflux pump activity, inactivation of the biocide, target modification, horizontal gene transfer, or modification of membrane properties. These resistance mechanisms seek to counter the biocide's mode of action, i.e. to prevent it from binding to its action site.

In addition, there are special cases such as biofilms and the protective effect of protozoa. For biofilms, in addition to the mechanisms mentioned above, there are certain effects inherent to their structures, such as the limited diffusion of biocidal substances through the matrix, the diversity of physiological states of the bacteria related to nutrient and oxygen gradients, communication phenomena, interactions with a wide variety of biofilm constituents, and a greater ability to transfer genetic material carrying resistance genes.

3.1.3. Method for assessing resistance

A common objective of studies in the literature on assessing bacterial resistance to biocidal substances is to determine whether certain bacterial species are inherently adaptable to biocide exposure by becoming more resistant to that biocide. In cases where such adaptive capability is

[1] Certain biocidal active substances mentioned in the report annexed to the opinion are still under assessment within the framework of the European Biocides Regulation (EU) No 528/2012. Their approval for a given PT was not confirmed at the time of publication of this opinion. Other active substances have already been approved while others have not yet been submitted for approval.

observed, it is then necessary to assess its extent, understand its stability, and investigate whether cross-resistance to other antibacterials (other biocides or antibiotics) is also developing.

Laboratory experiments typically involve three phases:

- The first assesses the capacity of the tested bacterial strains to react and adapt to an environment consisting of biocides.
- The second characterises the stability of this adaptation: transient (i.e. disappearing after cessation of exposure to the biocide), or stable/irreversible (remaining after cessation of exposure to the biocide).
- The third is used to determine the level of resistance.

On the basis of five examples of biocide use (surface treatment, preservatives, human hygiene products, water treatment products and biofilm treatment products), a literature review was carried out to list the different methods for assessing the ability of a biocidal product to induce resistance in target bacteria. This analysis is presented in detail in the WG's report.

The ability of bacteria to adapt is assessed following contact of the bacterial population with the tested biocide under various operating conditions (single or repeated contact, with low or high concentrations), then the stability of this resistance phenomenon in bacteria is determined (after exposure in a medium without the tested biocide). Where appropriate, cross-resistance to other biocides and/or antibiotics is also assessed. These studies may in addition be supplemented by a search for mechanisms (resistance genes, mutation, etc.).

The level of bacterial resistance to a biocide or antibiotic is generally assessed via changes in the minimum inhibitory concentration (MIC), the minimum bactericidal concentration (MBC), the destruction kinetics and the growth kinetics. Other available methods are used to refine the analysis of the observed level of resistance (flow cytometry, detection of resistance genes).

For the special cases of biofilms, there are few standard methods for assessing the susceptibility of bacterial cells within biofilms to disinfectant biocides. The most commonly used methods are as follows: determining the minimum biofilm eradication concentration (MBEC) and the ratio of concentrations (Rc) or times (Rt) required to achieve the same reduction in the planktonic or biofilm population, or comparing reductions achieved after exposure to the same concentration over the same time period.

3.2. Proposed methodological approach for assessing the risk of emergence of resistance for a biocidal product or active substance

A general methodological approach was proposed by the WG to assess the ability of a biocidal product or active substance to induce bacterial resistance. This approach, applicable in the laboratory, should be adapted for each biocidal product studied, taking parameters related to the claims into account (target micro-organisms, uses, treated media and application conditions).

This approach proposes assessing the development of resistance in bacteria exposed to both use concentrations and lower concentrations, in order to take account of the exposure of target bacteria to possible biocide residues after the product has been removed (e.g. through rinsing). It also proposes assessing the development of cross-resistance to antibiotics or other biocidal products.

3.2.1. Development of the methodological process

The WG proposes establishing such a methodological approach in several steps:

1- Developing the experimental protocol adapted to the biocidal product:
   a. Identify as fully as possible the parameters necessary for implementing the tests:
      - ✓ Parameters related to the products (water quality and pH, use concentrations, type of treatments, contact time, application process, temperature);
      - ✓ Parameters related to the micro-organisms (choice of relevant species, inoculum size and physiological state);
      - ✓ Parameters related to the treated environments (interfering substances, type of surfaces, etc.).
   b. Determine the method for assessing resistance of the target bacterial species to the studied biocide, given its uses and application conditions.

2- Conducting tests

The experimental protocol should be developed in two steps:

- ✓ Step 1: exposure of bacterial populations to the biocide, in order to determine the bacteria's ability to adapt, according to the most appropriate methods;
- ✓ Step 2: if resistance is observed in step 1, confirm or rule out the stability of this resistance using a suitable method;
  Where appropriate, cross-resistance to other biocides and antibiotics should be investigated through a literature search.

In order to provide a practical illustration of the development of these experimental protocols, representative examples of products found in the main areas of use of antibacterial biocides are presented in the WG report.

3- Analysing the results and concluding on the appearance or not of a resistance phenomenon using a decision tree.

3.2.2. Example of a decision tree

Using the data generated from the above protocol, the potential risk of resistance development can then be assessed. An example of a decision tree is provided for this purpose (Figure 1). This decision tree is considered a "simplified" decision tree, not intended to exhaustively describe all possible cases.

It should be noted that:

- The entire methodological approach described below applies to the assessment of a resistance phenomenon in the context of a "Biocidal Product" dossier. In the case of an "Active Substance" dossier, this approach may also apply insofar as the assessment should be carried out on the representative product described in the dossier.
- The same approach should be followed in the case of a biocidal product with several active substances.

The decision tree proposed below describes the case of a disinfectant product X, intended for treating hard surfaces, without rinsing, applied at its use concentration (UC). This example assumes a single application. Exposure is achieved at the use concentration "UC" and at two other concentrations, UC/2 and UC/4.

The experimental protocol consists of two steps:

Step 1: Determination of the bacteria's ability to adapt to the antibacterial biocide being tested.

Step 2: Determination of the stability of the resistance phenomenon and search for possible cross-resistance to other biocides and antibiotics via a literature search. This step is only performed if adaptation is observed in step 1.

Here, the MIC has been chosen as the method of assessing resistance. Nevertheless, in the report, a range of methods is proposed. The applicant is free to use the most appropriate method to determine the occurrence of a resistance phenomenon.

Prior to step 1, the "initial" MIC of the strains collected in the field (and, if applicable, of the collection strains of the applicable standards of the CEN/TC 216) should be determined according to the methods described above.

Following application of product X, the bacteria's ability to adapt is assessed (step 1), and then the resistance level is determined according to the MIC method:

- If the MIC after the field strain adaptation step is the same as the initial MIC, it is considered that there is no risk of development of resistance under the application conditions of product X. No testing is required to check for cross-resistance to other biocides and/or antibiotics.
- If the MIC after the adaptation step is higher than the initial MIC of the field strains, then adaptation to the biocidal product X under study is observed. In this case, the experimental protocol will proceed with step 2 and will then be checked in the same way:
    - Whether or not this adaptation is stable in the absence of biocidal product X, and;
    - Where appropriate, on the basis of literature data, whether there is a risk of development of cross-resistance to other biocides (range of biocides from different chemical groups that are representative of the area of use) and/or antibiotics (typically used in humans and/or animals).

UC = use concentration
UC/2, UC/4 are the concentrations encountered when under-dosing or improperly disposing of the product
(*) Disinfectant
(**) Initial MIC of strains taken from the field
(***) via a literature search

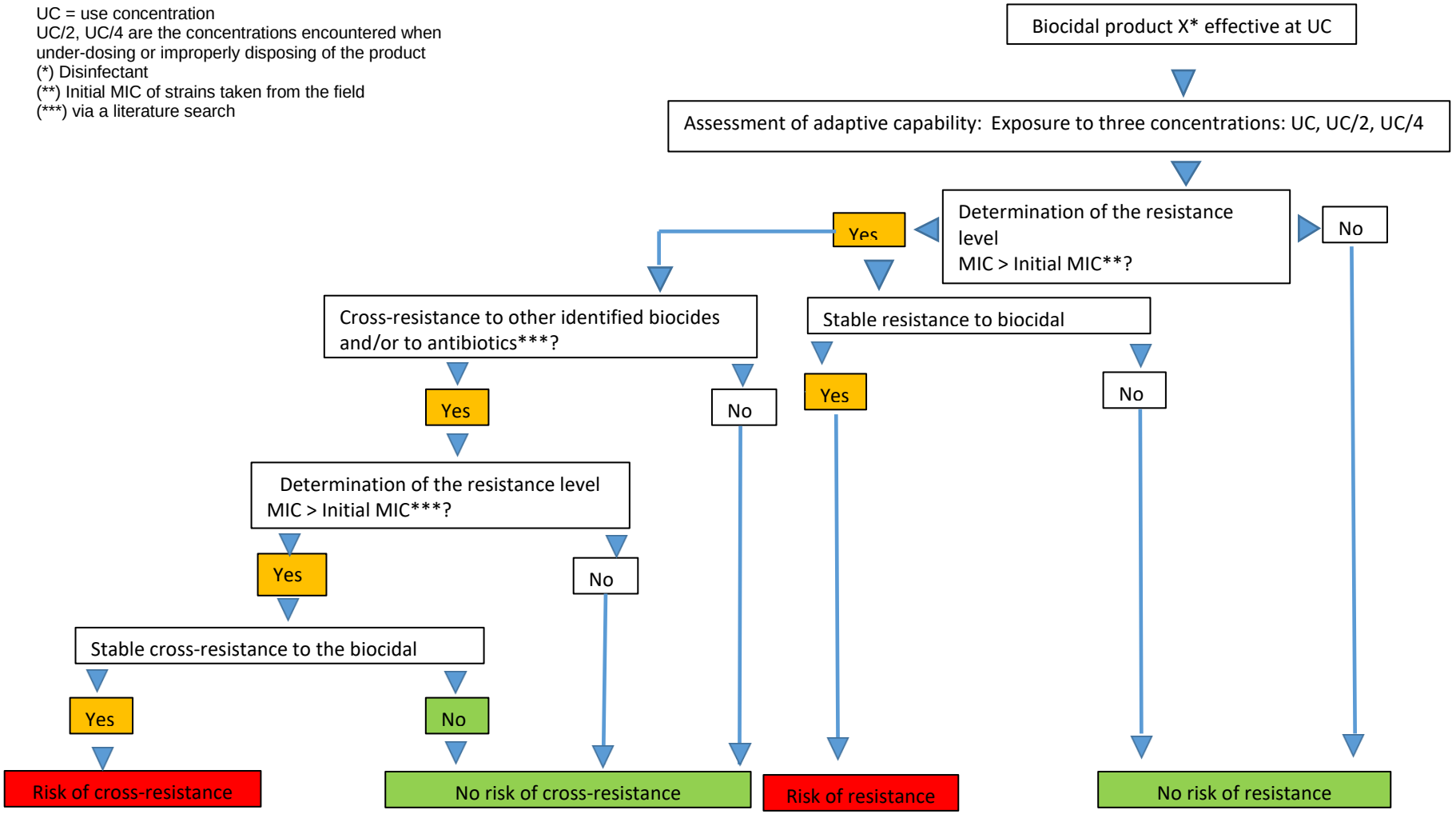

Figure 1: Example of a decision tree illustrating the resistance assessment protocol for a surface disinfectant product

3.3. Strategy for managing the emergence of resistance

Under the Biocides Regulation (point 75 of Annex VI), if resistance or cross-resistance to the active substance in the biocidal product is likely to develop, the evaluating body must consider actions to minimise the consequences of this resistance.

3.3.1. Management measures

In general, to prevent the emergence of resistance, it is important to limit any misuse that could lead to the target bacteria being exposed to sublethal concentrations that facilitate their adaptation. The instructions for use given in the summary of product characteristics (SPC) annexed to the authorisation must therefore mention absolute compliance with the conditions of authorisation:

- Always read the label or leaflet before use and follow all instructions.
- Comply with the product's conditions of use (concentration, contact time, temperature, pH, etc.).

The obligation for professional users to analyse the causes of the treatment’s ineffectiveness and to inform the authorisation holder (AH) holder if the treatment is ineffective also contributes to monitoring of the risk of development of resistance. The WG indicated that this should be reiterated in the product's SPC.

Lastly, the obligation for the AH to notify the competent authority in the event of unexpected or adverse effects, especially the emergence of resistance, is laid down in the Biocides Regulation (Article 47(b)). It is proposed that this obligation also be reiterated in the SPC.

3.3.2. Post-authorisation or post-approval monitoring (field trials)

Moreover, as part of the assessment of an MA application or active substance approval dossier, the laboratory studies provided in the dossier or a review of the scientific literature may reveal suspected resistance or cross-resistance to other antibacterials (antibiotics or biocides). Resistance management should then be considered. Applicants may then be asked to set up additional experiments, either post-approval (as part of the approval of an active substance) or post-MA (when a product is placed on the market). Laboratory experiments will initially be requested, then field monitoring may be required if a stable resistance phenomenon is confirmed following laboratory experiments.

If the resistance and/or cross-resistance is observed under conditions that do not comply with the product's conditions of use (below the use concentration, for example), the AH must make every effort to inform users of these risks and advise them on how best to comply with the product's application conditions. If these phenomena are triggered at residual levels, it should direct users towards a choice of suitable control methods.

If resistance and/or cross-resistance is observed under conditions that are in accordance with the authorised conditions of use, these results should be confirmed in the field.

It should be emphasised that unlike laboratory studies, there are few protocols targeting biocide resistance in the field. Most field studies on the subject of biocide resistance are associated with antibiotic resistance. On the basis of these studies, monitoring of bacterial populations in the field could therefore be proposed, at several field sites and lasting from several weeks to several months, in areas where the product is used strictly in accordance with its authorised conditions of use. Characterisation of the strains present and their resistance can be investigated using the most appropriate techniques.

4. AGENCY CONCLUSIONS AND RECOMMENDATIONS

The Biocides Regulation (EU) No 528/2012 states that it is necessary to ensure that each approved active substance or biocidal product placed on the market has no unacceptable effects on the target organisms, in particular resistance or cross-resistance.

In the absence of guidelines dealing with the subject of resistance, in particular for antibacterial biocides, an approach for assessing this resistance phenomenon needs to be proposed, in order to help both competent authorities and applicants meet the requirements laid down in various points of the Biocides Regulation.

The work of the WG on "Resistance to antimicrobial biocides" led to a proposed methodological approach for assessing bacterial resistance to the use of antibacterial biocides. This approach, to be adapted on a case-by-case basis, should make it possible to assess the bacteria's ability to adapt to an antibacterial biocide, confirm whether this resistance phenomenon is stable, and measure the level of this resistance to the biocide. The approach also provides an example of a decision tree for practical implementation.

In general, to prevent the development of resistance, it is important to limit any misuse that could lead to the target bacteria being exposed to sublethal concentrations that facilitate their adaptation. A set of generic instructions for use indicated in the MA is therefore proposed. Moreover, if it is found that the use could lead to the development of resistance, management of this resistance should be envisaged by considering, on a case-by-case basis, whether or not field experiments need to be developed, or even by setting up specific monitoring of the use of a given product over a long enough period.

On the basis of the expert appraisal carried out by the WG on "Resistance to antibacterial biocides", the French Agency for Food, Environment and Occupational Health & Safety recommends that this approach for assessing the ability of an antibacterial biocide to generate resistance or cross-resistance in target species be presented at European level, within the "Efficacy" Working Group of the European Chemicals Agency (ECHA), with a view to drawing up European guidelines for applicants for marketing authorisations or approval of active substances, which would also be taken into account when assessing biocide dossiers.

Dr Roger Genet

KEYWORDS

Biocides, résistance, adaptation, désinfectant, conservateur, bactéries
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