Confronting the clinical relevance of biocide induced antibiotic resistance

Reporting

Project Information

BIOHYPO

Grant agreement ID: 227258

Project website

Status
Closed project

Start date
1 June 2009

End date
30 November 2012

Funded under
FP7-KBBE

Overall budget
€ 4 396 589

EU contribution
€ 2 999 705

Coordinated by
UNIVERSITA DEGLI STUDI DI SIENA
Italy

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Final Report Summary - BIOHYPO (Confronting the clinical relevance of biocide induced antibiotic resistance)

Executive summary:

The BIOHYPO project was launched in 2009 with the specific aim to investigate if biocide use in the food chain would result in clinically relevant increase of antibiotic resistance in human pathogens. The hypothesis investigated included
(i) if biocides select biocide resistance,
(ii) if biocides select antibiotic resistance and
(iii) if this antibiotic resistance would be of clinical relevance.

Four biocides were investigated and included triclosan, benzalkonium chloride, chlorhexidine and sodium hypochlorite.

Project Context and Objectives:
Main aim of the BIOHYPO project was to investigate if biocide use in the food chain would result in clinically relevant increase of antibiotic resistance in human pathogens. The project was built to investigate a series of hypothesis which included
(i) if biocides select biocide resistance,
(ii) if biocides select antibiotic resistance and
(iii) if this resistance would be of clinical relevance.

Four biocides were investigated and included triclosan, benzalkonium chloride, chlorhexidine and sodium hypochlorite.

There are four major goals met by BIOHYPO include:

- Biocide ECOFFs (Ecological cut offs): Phenotypic screening for the minimal bacteriostatic concentration (MIC) and minimal bactericidal concentration (MBC) to benzalkonium chloride, chlorhexidine, triclosan and sodium hypochlorite allowed the determination of the epidemiological cut off ECOFF values of biocide

susceptibility in a number of human pathogens including S. aureus, S. enterica, E. coli, K. pneumoniae, and E. faecalis. This effort is of great importance as it does allow the scientific community to identify phenotypes of reduced susceptibility to biocides by comparison of MICs and MBCs against a series of reference values. The relative data have been made available through a public web page (see http://kdbio.inesc-id.pt/sdlink/biohypo/Ecoff online).

- Novel biocide resistance mechanisms: Two novel biocide resistance mechanism against triclosan were identified in S. aureus. Heterodiploidy for two susceptible fabI alleles, one acquired from S. haemolyticus, represents a completely novel type of mechanism of antimicrobial drug resistance. The added sh-fabI allele was detected in about half of triclosan resistant strains. A second novel resistance mechanism was found in a strain where insertion of IS256 upstream fabI determined strong upregulation of fabI expression and phenotypic resistance to triclosan. In Gram negatives two novel types of efflux systems PFLU1632-1636 and ybhGFS have been identified to confer biocide resistance respectively to triclosan and hexachlorophene. In Candida biocide resistance was associated to over-expression of the CDR1 and CDR2 efflux systems.

- A novel fast method for biocide activity determination and resistance measurement by comparison of the inactivation kinetics curves of target microorganisms was developed. The new test is based on a semi-automated bioluminescent adenosine triphosphate (ATP) assay and linearity of the ATP detection was demonstrated over a wide range of concentrations against the standard suspension tests EN 1276 and EN 13727.

- Relationships between phenotypes of reduced susceptibility to biocides and antibiotic resistance where evaluated for the large dataset of S. aureus and Salmonella. Except for chlorhexidine and benzalkonium chloride exported by the same multi drug (MDR) efflux pumps, no correlation of reduced susceptibility could be seen between the different biocides analysed. We predict that this may change in future as plasmids carrying both qacA and sh-fabI were detected in S. aureus. Regarding the efflux resistance mechanisms for chlorhexidine and benzalkonium chloride it is important to underline that upon the qac genes in S. aureus all qac genes increased MIC, but not MBC, to benzalkonium chloride and only qacA increased MIC to chlorhexidine. Furthermore in E. coli cloning of the enterobacterial qac genes did not result in changes in biocide susceptibility of recombinants, indicating that most reports on biocide susceptibility in enterobacteria should be revisited.

A public interdisciplinary workshop has been organised by BIOHYPO in March 2011 in Barcelona with over hundred participants from industry and public authorities. Objective of the meeting was to interface recent EC-funded research efforts aimed to elucidate the claimed correlation between biocide utilization and resistance to antibiotics with current efforts in redefining guidelines for efficacy evaluation and biocide related European legislation.

Importantly the results obtained by BIOHYPO were presented regularly to the Technical Committee for Biocides (TC 216) of the European Committee for Standardization (CEN). CEN has shown a strong interest and has invited the BIOHPYO participants that are also members of the TC, to provide updating on the matter at the next meetings.

Project Results:
Workpackage 1 –High throughput screening

Workpackage Objectives
- Phenotypic identification of strains, including pathogenic and non pathogenic bacteria and fungi, showing reduced susceptibility or resistance to the selected disinfectants among clinical isolates
- Correlation of biocide resistance pattern to known antibiotic resistance pattern and genetic background of strains
- High throughput molecular screening for distribution of genes and markers associated to biocide resistance

Progress Towards Objectives
The BIOHYPO had decided to focalise screening of clinical isolates on few species. The phenotypic screening for biocide susceptibility for chlorhexidine, benzalkonium chloride, sodium hypochlorite and triclosan was performed utilising CLSI MIC and MBC assays. During the first years of the project we have

screened:
- 1635 Staphylococcus aureus strains collected in 2002-3 from different geographical origins, representing both hospital and community acquired infections.
- 901 Salmonella spp. collected between 1999 and 2003 from European veterinary sources.
- 371 Escherichia coli collected between 1998 and 2011 from Spain.
- 200 Candida albicans collected in 2010 and 2011 from hospital acquired infections and vulvovaginal candidiasis in Turkey.
- 60 Klebsiella pneumoniae collected between 1991 and 2011 from Spain.
- 52 Enterococcus faecium collected between 1986 and 2009 from world-wide locations.
- 43 Enterococcus faecalis collected between 2001 and 2009 from Spain.
These numbers refer to strains screened for both MIC and MBC to biocides. In addition to this, we have screened using the MIC assay only, further Candida strains, filamentous fungi and a series of food grade bacteria.

Phenotypic screening: MIC and MBC/MFC (minimal bactericidal/fungicidal concentration) data for benzalkonium chloride, chlorhexidine, triclosan and sodium hypochlorite are shown in. In most cases it can be seen that MIC and MBC/MFC distributions for the biocides were quite similar (although MBC always higher) confirming the bactericidal nature of biocides. Exceptions to this were observed for benzalkonium chloride against C. albicans where MIC mode was 2/4 mg/L but MBC mode was 8/16 mg/L, triclosan against the enterobacteria Salmonella spp., E. coli and K. pneumoniae where MBC values were much higher than MICs.

ECOFF determination: In order to provide the community with reference values of susceptibility, we have determined the epidemiological cut off values (ECOFF) for biocide for those species screened. Generally ECOFFs produced from MIC or MBC/MFC measurement were within 1 dilution or so of each other. Triclosan or chlorhexidine, on the other hand, often showed different MIC and MBC ECOFFs, especially against S. aureus, Salmonella spp. or E. coli. Triclosan was also quite interesting because bimodal MBC distributions were observed against S. aureus and Salmonella spp. that could be used to determine an alternative ECOFF to that of the MBC99.9. Similarly, the distribution of triclosan MIC against E. coli was

bimodal. In most cases a relatively small number of isolates would be considered biocide non-wild type. The major exception to this was triclosan against S. aureus where 70 isolates had an MBC between 8 and 64 mg/L. To the contrary no bacteria with reduced susceptibility to sodium hypochlorite were detected. The ECOFF data have been made publically available through the following web interface: http://kdbio.inesc-id.pt/sdlink/biohypo/Ecoff

Molecular screening: Work in WP3 investigated correlation of phenotypes with reduced susceptibility to molecular markers of resistance mainly focusing on S. aureus and enterobacteria. In S. aureus a perfect overlap was found between reduced susceptibility to triclosan and presence of either mutations in fabI or an added allele of sh-fabI, while in the case of reduces susceptibility to chlorhexidine and benzalkonium chloride a high frequency of qac efflux genes were detected (see WP3). To provide an overall picture the whole population of 1602 S. aureus strains was analysed for presence of qacA, qacB, qacC, qacG and qacH. This screening indicated that all qac genes reduce the mode MIC of clinical isolates by at least one dilution for benzalkonium chloride, but only qacA and qacB reduce the mode MIC of chlorhexidine in clinical isolates. Importantly none of the qac efflux genes was associated to any decrease in MBC,

indicating that Qac efflux pumps do not protect from the ‘cidal’ effect of biocides.

Ranking of relationships between biocide and antibiotic susceptibility.

1) Evaluations of the relationship between any non-antibiotic parameter available (including biocides, but excluding antibiotics) for prediction of antibiotic resistance
The rise of antibiotic resistance in pathogenic bacteria is a significant problem for the treatment of infectious diseases. Resistance is usually selected by the antibiotic itself, however, biocides might also co-select for resistance to antibiotics. Although resistance to biocides is poorly defined, different in vitro studies have shown that mutants presenting low susceptibility to biocides also have reduced susceptibility to antibiotics. However, studies with natural bacterial isolates are more limited and there are no clear conclusions as to whether the use of biocides results in the development of multidrug resistant bacteria.

2) Evaluations of the relationship between any parameter available, including biocides and antibiotics, for prediction of antibiotic resistance were computed both using non-linear correlations and binary classification tests.

2a) A bivariate correlation analysis was performed between biocides and antibiotics' variables. Spearman's correlation coefficient was computed for each bivariate combination of these variables in order to find non-linear associations between the biological variables. Bivariate correlations were calculated using Matlab. For each computed Spearman's correlation coefficient, a hypothesis test was performed in order to test for statistical association between each pair of variables and a p-value calculated, assuming statistical significance at a level of 5%. When performing a set of multiple statistical inferences it is likely that a multiple testing problem of incorrectly rejecting the null hypothesis will occur. To control the familywise error rate, the classical conservative approach of Bonferroni correction was applied leading to a critical value at level.

By using this methodology there was no clear correlation between the biocide and antibiotic phenotype variables.

Indeed, the data analysed showed weak bivariate correlations. This is a result that matches with previous studies of smaller data sets. After performing the correction for family-wise error rate, we found some pairs of variables with a statistically significant Spearman’s correlation coefficient. In spite of having a statistically significant correlation coefficient, the pair of variables in bold have a considerably low absolute value for the coefficient so one cannot assume a strong correlation between those variables. The ratio between MBC and MIC (Fold Change) of each biocide was also analysed as this may indicate a difference in biocide reduced susceptibility. Kendall's correlation coefficient was also computed for the same pairwise test variables and the results were similar. When comparing statistically our complete dataset we found (1) no statistical correlation between reduced susceptibility to triclosan or sodium hypochlorite and any antibiotic resistance and (2) significant correlation with a low correlation coefficient between decreased susceptibility to chlorhexidine and benzalkonium chloride and resistance towards most clinically relevant antibiotics.

2b) The classical binary classification test for prediction of utility of a test (sensitivity, specificity, PPV,

NPV) were computed for each variable against methicillin resistance which is the ‘gold standard’ identifying multi-drug-resistance in S. aureus. Similar to the results obtained by multivariate correlation we could clearly define that two of the biocides, triclosan and sodium hypochlorite, did not show for any of the four parameters analysed (negative predictive values, positive predictive values, sensitivity and specificity) any indication of utility to predict methicillin resistance in S. aureus. In contrast, both chlorhexidine and benzalkonium chloride did show intermediate values of specificity, PPV and likelihood ratio. There is a clear cut off between capacity of macrolides and quinolones to predict methicillin resistance and hence multi drug resistance in staphylococci, and the likelihood ratio calculated using biocide susceptibility data or qac gene distribution. The values of benzalkonium chloride and chlorhexidine are more similar to those of teicoplanin or tetracycline, two antibiotic resistances well known not to cluster with MDR resistance in staphylococci.

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Workpackage 2 – Integrated bioinformatics and data management

Workpackage Objectives
- Development of a web-based S3DB software
- Development of dedicated data processing services
- Development and maintenance of an Application Programming Interface
- Analysis of data form high throughput screening

Progress Towards Objectives

The integrative bioinformatics workpackage consisted of three parallel efforts by the team members of B4. Firstly, a semantic web infrastructure was developed to provide a flexible web-based data management resource for all participants, where both the raw data and the results of its processing can be hosted, shared and selectively disseminated. Secondly, the web based repository have its application programming interface (API) exposed through REST web-services. This feature enables the different participants to pursue their own development or adoption of data analysis tools without requiring the use of a specific programming language. Thirdly, data integration and analysis was performed in order to characterize biocide induced antibiotic resistance hypothesis. Data on phenotypic and molecular biocide resistance matched to the data of the strain database was used to validate statistically the hypothesis outlined in the first pages of this project. Since the data needed to test most hypothesis has been generated by multiple participants and the results of the data analysis services triggered by the upload to the web-based repository are ultimately validated by those very participants that contribute the data, WP2 was in fact a package contributed by all participants. The main strain collection, subjected to high throughput screening, was provided by partner B2 and it included strains from all over the world that are characterized for their geographical distribution, year of isolation clinical origin, species, subspecies or type, and drug resistance profile.

Despite the deep knowledge of the domain by all the team members of this project, the complexity of all data and parameters was not totally predicted (or known) at the first stage, and thus the last stages of

integrated data analysis presented some difficulties. However, it was possible to develop a machine learning approach for extracting biological information from large epidemiological data sets and at the end, all data was analysed according to the original plan.

One of the main goals of this workpackage was to develop a semantic web data management system. The final system should include tools for data processing, analysis and visualization. Particular attention should be given to the user interfaces. Different interfaces for data visualization, retrieval and querying should be developed. Interfaces to add data to the database should be designed having in mind the target users, in this context biologist are used to Excel spread sheets for data collection.

Due to the difficulties experienced by the biologists when using the S3DB framework, the INESC-ID team (B4 partner) decided to develop a new semantic web based data management system. The new system, named sdlink, implements the concept of linked data, a recommended best practice for exposing, sharing, and connecting pieces of data, information, and knowledge on the Semantic Web using URIs and RDF. The new system can be accessed through the following URL: http://kdbio.inesc-id.pt/sdlink/biohypo

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5. Coelho, J. R., Carrico, J., Knight, D., Martinez, J.-L. Morrissey, I., Oggioni, M. R., Freitas, A. T., Computational approaches for antimicrobials resistance analysis, Workshop on Mathematical modeling of antibiotic resistance, Paris, France, May 2012

Workpackage 3 – Detection and characterisation of biocide resistance markers

Workpackage Objectives
- Identification of genes conferring reduced susceptibility to biocides
- Evaluation of mobile genetic elements involved in mobilisation of these genes
- Evaluation of resistance mechanisms based on mutated or over-expressed genes
- Evaluation of extent of co- and cross-resistance to antibiotics

Progress Towards Objectives

WP3 focussed on the analysis of biocide resistance phenotypes and genes in Staphylococcus aureus,

Enterobacteria, and fungi.

The widely used biocide triclosan selectively targets FabI, the NADH-dependent trans-2-enoyl-acyl carrier protein reductase, which is an important target for narrow-spectrum antimicrobial drug development. In relation to the growing concern about biocide resistance, we compared in vitro mutants and clinical isolates of S. aureus with reduced triclosan susceptibility. Clinical isolates of S. aureus as well as laboratory-generated mutants were assayed for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) phenotypes and genotypes related to reduced triclosan susceptibility. A potential epidemiological cut-off (ECOFF) MBC of greater than 4 mg/L was observed for triclosan in clinical isolates of S. aureus. These showed significantly lower MICs and higher MBCs than laboratory mutants. These groups of strains also had few similarities in the triclosan resistance mechanism. Molecular analysis identified novel resistance mechanisms linked to the presence of an additional sh-fabI allele derived from Staphylococcus haemolyticus. The lack of predictive value of in-vitro-selected mutations for clinical isolates indicates that laboratory tests in the present form appear to be of limited value. More importantly, detection of sh-fabI as a novel resistance mechanism with high potential for

horizontal gene transfer demonstrates for the first time that a biocide could exert a selective pressure able to drive the spread of a resistance determinant in a human pathogen [1]. To investigate if the lack of predictive value of in vitro mutants was due to fitness defect we performed a killing assay in wax moth larvae, but none of the mutants showed any fitness defect [2].

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Workpackage 4 – Methodologies and quality control

Workpackage Objectives
- Definition of parameters for high through put testing;
- Meliorations rendering biocide testing efficient and cost effective;
- Definition of tests for selection of biocide resistance as model to set up of a standard test for risk assessment;
- Definition of standards for biocide evaluation, including work on biofilms.

Progress Towards Objectives

Workpackage 4 had four main tasks; of these, two regarding the setup of a fast method for biocide testing

and the definition of protocol(s) for the assessment of risk for biocide to induce resistance. For the other two, which concerned the setup of method(s) for evaluation of impact of biofilms on resistance to biocides and the development of guidelines for biocide testing, an extension until the end of the project was requested.

To summarize:
Methods to evaluate the potency of biocides to induce stable mutations have been set up and evaluated on both gram-positive and gram-negative organisms. Several condition mimicking those potentially occurring in nature were evaluated. Of the biocides tested, only Triclosan appeared to induce stable, transmissible mutation, with significant shift of the MIC values compared to the parent strains, while benzalkonium chloride and chlorexidine did not [1, 2, 3].

In all cases, data clearly indicate that, since both the phenotype and the genotype of in vitro generated mutants and of clinical isolates are completely different, any in vitro tests appear to be irrelevant to predict any risk of resistance development of strains of clinical relevance. This point is of particular relevance to

the ongoing discussion on risk evaluation of biocides, both in the case of selection of biocide resistance, as in the case of selection for co- or cross-resistance to antimicrobial compounds including antibiotics.

We would thus suggest that a single in vitro test for prediction of biocide resistance generation is not feasible, at least with the current knowledge; nevertheless the data obtained by the project in toto, allowed the presentation of a strategy to approach, at guidelines level, the problem of risk assessment. The results obtained were presented to the Technical Committee for Biocides (TC216) of the European Committee for Standardization (CEN) [4]. CEN has shown a strong interest and has invited the BIOHYPO participants that are also members of the TC, to provide updating on the matter at the next meetings.

A new method for the evaluation of the biocidal activity, comparable to the EN standard tests, but much faster and cost-efficient was developed and validated [5]. The comparability study performed using the current European Normalization standards as a reference method has demonstrated the equivalence of the new method with the EN standard suspension tests, but allowing the undertaking of up to 20-fold more assays per week than the current European Norms (EN) and it is ready to be used for testing the resistance to biocides of the antibiotic resistant selected strains.

Using this new rapid microbiological test method, several concentrations of a test product can be assayed in the same run, resulting in the capacity of the new method to generate inactivation curves for each strain that allows the calculation of the Minimal Biocidal Concentration of a biocidal product for a defined contact time, in a fast and cost-effective way. This innovation in testing, apart from being crucial for the objectives of the BIOHYPO Project, is also very important in the context of the BPD (Biocidal Product Directive 98/8/CE) implementation where a well-balanced trade-off between product efficacy and its toxicological and environmental safety is sought.

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A basic test for evaluation of the efficacy of a given biocide on cells embedded in a biofilm formed under static condition was designed and standardized. This was used with different bacterial species to evaluate its applicability. Also, for S. aureus, the results of short-time disinfection experiments were compared to those obtained in a model where biofilm was formed under flow conditions. The latter resemble more

closely what happen in nature, though being more time consuming especially for what concerns the setup of the experiment. The log reduction appreciable with the flow model was larger than the one with the static model although, given the differences in the scale range, comparable. With both systems it is possible to provide the information on the efficacy of a given biocide on the killing activity and on the capacity to remove the biofilm biomass, which is also a very important point to consider.

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6. PRODUCT TYPES 1 TO 5 - DISINFECTANTS AND GENERAL BIOCIDAL PRODUCTS - PT2 TNsG – Chapter 11 – Biofilm.

Workpackage 5 – Environmental sampling and metagenomics

Workpackage Objectives
- Collection of an environmental set of water samples
- Bacteriology and Metagenomics on samples
- Metagenomics in silico

Progress Towards Objectives

Biocides and antibiotics are released in water after their use at industries (including food-processing plants), hospitals and houses. These places, which can contain as well clinically-relevant human and animal linked microbiota together with environmental microorganisms that act as donors of resistance elements, are important ecosystems for the selection of biocide and antibiotic resistance. Also, when raw water sources containing biocides are subjected to treatment in water treatment plants, their biocide content may experience reduction and/or conversion to other by-products, depending on the physicochemical process(es) applied in the water treatment plants.

In silico analysis of genomes and metagenomes

We have analysed currently available genomes and metagenomes databases by bioinformatics tools in the aim of detecting determinants relevant for the association of antibiotic resistance and biocide resistance. Among those elements, particularly relevant are the Type I integrons, which are gene-capture units that carry qac genes (qacE, qacI, qacF, qacG), that provides resistance to quaternary amines such as the biocide benzalkonium chloride and eventually to biguanides as chlorhexidine, linked to different arrays of antibiotic resistance determinants. The search of 1014 whole genome sequences demonstrated the presence of Type 1 integrons in the chromosomes of 24 microorganisms, belonging to the following bacterial species: Salmonella enterica, Acinetobacter baumannii, Klebsiella pneumoniaae, Escherichia coli, Aeromonas salmonicida, Yersinia pestis, Vibrio cholerae, Pseudomonas aeruginosa and Corynebacterium diphtheriae. Excepting Aeromonas salmonicida, which is a relevant fish pathogen, all the other organisms are human pathogens, whereas Type I integrons were not found in environmental bacteria. This indicates that Type I integrons are enriched in human- and animal-linked microbiota, but not in the environmental one. Given that these integrons contain in their DNA backbone different antibiotic resistance determinants, it is highly possible that they have been selected as the consequence of antibiotic selective pressure more than because of the utilization of biocides. In favour of this possibility is the finding

of resistance genes, associated, but located outside the integron, and the existence integrons that carry just the sul gene, which confers resistance to sulphonamides, and lack the biocide resistance gene qac.

Functional metagenomics for searching biocide resistance elements

We have analysed the presence of genes conferring resistance to biocides in phosmids metagenomic libraries available from pristine and contaminated water ecosystems, and were screened for the presence of clones resistant to the biocides hexachlorophene, benzalkonium chloride and triclosan. Several clones were obtained and, after retransformation to assure that the phenotype was due to the presence of a resistance gene within the phosmid, and not to a mutation of the bacterial host, two phosmids were selected for further analyses. The phosmids were sequenced using the Illumina technology. The phosmids conferring resistance to triclosan harboured the putative ABD-type efflux pump from Pseudomonas fluorescens PFLU1632-1636, whereas the phosmid conferring resistance to hexachlorophene harboured the ABC-type multidrug efflux pump from E. coli ybhGFS. None of these two efflux pumps have been reported before to confer resistance to these biocides, which indicates that our metagenomic studies provide information on novel mechanisms of biocide resistance in bacteria.

Workpackage 6 – Management and Dissemination

Workpackage Objectives

- To coordinate the project within the consortium
- To coordinate contact between the consortium and the EC
- To coordinate contact between the consortium and the advisory board
- To disseminate, explain and exploit the objectives and results of the project with stakeholders such as the scientific community, public authorities and industry

Progress Towards Objectives

During the lifetime of the project nine project consortium meetings were held. All partners participated in, and contributed to, all of the consortium meetings. In addition, meetings between partners took place alongside other conferences and meetings where opportunity arose. On-going written and verbal communication, facilitated by Partner 1, took place between the project partners in order to ensure the effective management of the project.

Potential Impact:
BIOCIDE REGULATIONS: According to Directive 98/8/EC concerning the placing of biocidal products on the market (from 1 September 2013, repealed and replaced by Regulation (EU) Nº 528/2012 concerning the making available on the market and use of biocidal products), information on the occurrence or possible occurrence of development of resistance and appropriate management strategies is required to support the approval of an active substance as biocidal product. For this reason, the problem of the occurrence of resistance to biocides and possibly the co- or cross resistance with other antimicrobials is dealt with in the 2009 revision of chapter 6.2 (Common Principles and Practical procedures for the Authorisation and Registration of Products) of the Technical Notes for Guidance (TNsG) on Product

Evaluation, and on the revision of chapter 10 (Assessment for the potential for resistance to the Active Substance) of the TNsG on Annex I inclusion. Up to now, a standard test(s) to determine such feature of a biocidal product/active substance is not available and the use of a strategy, possibly involving a tiered approach, is suggested, but not specified, in the same TNsG.

BIOHYPO has investigated, with the available technological knowledge, several aspects that have the potential for being very important in the field of evaluation and commercialization of biocidal products.

Currently, the activity of biocidal products/substance is tested according to the EN standards. These tests are time consuming and generally allow only limited parallel runs of different test conditions. Such approach has also been criticized by some authors for being single endpoint tests with many parameters that may influence the test outcome, especially for biocides with non-linear kinetics. It has been suggested that carrying out a complete kinetic examination would be more reliable to obtain true-rate data.

Part of BIOHYPO's efforts has been devoted to set up a fast method for biocide testing which successfully resulted in the definition of an ATP-based method. Such method, developed and validated against the EN standards, maintain the same approach but seeks for the ability of the method to generate enough data to obtain the inactivation curves for each microorganism, in a fast, easy and cost effective way. The main innovations of the method are its semi-automated nature and the speed of cell viability analysis through the use of an ATP quantification system. In devising the method, 4 internal controls were introduced to ensure that the quantified reduction is solely due to the test product during the contact time assayed. Furthermore, performing the verification tests in replicate allowed intra-experiment variance to be assessed and the coefficient of variance for considering a test valid to be established.

Another important issue related to the evaluation of biocidal products/active substances according to the Directive 98/8/EC is the unavailability of standard tests to determine the efficacy of a given product on biofilm-embedded organism. Biofilms are increasingly cited as sources of infection and disease in humans: it is estimated that most human bacterial diseases treated in the past few years were actually biofilm-related and that treatment of these biofilm-based infections in the United States costs more than $1 billion annually.

Besides the lack of specific standard procedures, the current edition of the TNsG do not contain a section specifically focused on biofilm. While setting up and comparing standardizable procedures for biocide activity evaluation on biofilms (included among the purposes of BIOHYPO), a chapter on biofilm was drafted to be included in the revision of the currently available Technical Notes for Guidance (TNGs) on Product evaluation (Chapter 7 – Efficacy Assessment). The revised draft is, at the moment, under scrutiny of the Competent Authorities (CAs), whose endorsement will possibly be available by the end of February 2013.

The different procedures and documents devised or produced by BIOHYPO has the potential for presenting a very high impact on the future of biocidal regulation. On one side, the availability of a fast, reliable assay for biocide efficacy evaluation, which has also the possibility to verify the activity of biocides with non linear kinetics, would allow a finer tuning of the in-use concentration of biocides, leading to a lower impact on the environment. Moreover, the information provided on the changes that may occur in

microbes following biocide exposure – that not always are so straightforward as significant changes in the minimal microbicidal concentrations could be – would led the way to the definition of a strategy to risk assessment evaluation of substances/products before the introduction on the market.

Finally, the introduction of a specific biofilm chapter within the Technical Notes for Guidance for Product Evaluation has filled a strongly felt gap on the necessity to test substances/products on a mode of growths of microbes which is widespread and more likely to affect the microbicidal action of a biocide.

BIOCIDE RESISTANCE: BIOHYPO has screened over four thousand strains of multiple species including Staphylococcus aureus (1600 strains), Salmonella enteritidis (900 strains), Escherichia coli (500 strains), and the yeast Candida albicans (600), as also species of dairy interest including streptococci, lactococci and lactobacilli. This large effort of susceptibility testing for biocides allows for the first time to define epidemiological cut off values (ECOFFs) allowing to clearly identify the 'normal' susceptibility profile of many bacterial species for biocides. The ECOFF values are under publication and the graphs are available on the project website (see http://kdbio.inesc-id.pt/biohypo/ online). BIOHYPO is in contact with EUCAST regarding the online positing of biocide ECOFF data on the EUCAST web page, which is the reference web page for ECOFFs to antibiotics.

The availability of biocide ECOFF values will allow in the future to identify immediately strains with reduced susceptibility to biocides, by simply comparing actual susceptibility data to those posted on the web site and indicating the 'normal population' and the cut off above which a strains can be considered non-wild type.

DISSEMINATION: One public conference and one workshop was held to disseminate the key results of the project. The public meeting (Workshop on the relationship between biocide susceptibility and antibiotic resistance. 4 March 2011, Parc Científic de Barcelona, Barcelona, Spain) was a first very significant event of publication of the research results of the project and had wide resonance and significant impact as confirmed by representatives of SMEs, industry and legislative bodies. The project and its possible outcomes were highly appreciated by representatives from public institution and legislative authorities and

from many industries. This very significant interaction with stakeholders was one of the highlights of dissemination of the BIOHYPO project during the second reporting period. Attendees to the BIOHYPO meeting included, in addition to academic attendees, importantly many attendees from legislative and regulatory authorities and many participants from industry. A workshop was organised in the last weeks of BIOHYPO to disseminate results of the project to a larger audience. The organisers of the II ICAR conference in Lisbon Portugal on November 2012 gave BIOHYPO the opportunity to organise a workshop as the opening event of the conference. A meeting report for the workshop is being published in the April 2013 issue of Expert Review of Anti-infective Therapy.

The project website, http://kdbio.inesc-id.pt/biohypo/ was developed in the first year and maintained throughout the lifetime of the project to disseminate information about the project's activities. The web page allows importantly the free online access to ECOFF data obtained in BIOHYPO.

BIOHYPO was designed to provide, not only the scientific community with a more detailed knowledge, but most importantly to allow non governmental agencies to take decisions and draft guidelines relative to

biocides. In this context the results obtained in BIOHYPO were presented at different occasions to the Technical Committee for Biocides (TC216) of the European Committee for Standardization (CEN). CEN has shown a strong interest and has invited the BIOHYPO participants that are also members of the TC, to provide updating on the matter at the next meetings.

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List of Websites:

http://kdbio.inesc-id.pt/biohypo/

Last update: 12 June 2013
Record number: 56830