



Deliverable 11:

List of defining knowledge gaps for microbial BCAs

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During the REBECA project 5 workshops on the risk assessment and regulation of microbial BCAs were carried out in Innsbruck (April 2006), Salzau (September 2006), Alés (June 2007), Kiel (September 2007), Brussels (September 2007). The identification of knowledge gaps for microbial BCAs based on the discussions between scientists and representatives from regulation authorities and the BCA industry. Project partner CAU carried out a literature research on risk assessment methods for micro-organisms in order to identify starting points for research projects.

Document Abstract

REBECA carried out 5 workshops on microbial risk assessment and regulation, primarily in order to develop proposals on improvement of the current system. However, it becomes very clear in an early stage of this action that the development of such proposals is hindered so far by: significant lack on validated risk assessment methods for microbials, knowledge gaps on the natural distribution of the biocontrol micro-organisms, knowledge gaps on natural exposition of humans and other non-target organisms, missing definitions allowing the identification of low risk products. This is hampering an adequate risk assessment of microbial plant protection products. The problems with the current risk assessment methodology are summarised, potential alternatives are identified and research tasks are proposed.

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Introduction

Micro-organisms used as active substances in plant protection products in the EU are regulated according to the EU Council Directive 91/414/EEC. This Directive was amended by the Commission Directive 2001/36/EC regarding the data requirements for the Annex I inclusion of micro-organisms as active substances and national authorisation of products (Annex II and III in the directive respectively). The Uniform Principles for evaluation and authorisation of plant protection products containing micro-organisms are laid down in the Council Directive 2005/25/EC.

REBECA carried out 5 workshops on microbial risk assessment and regulation, primarily in order to develop proposals on improvement of the current system. However, it becomes very clear in an early stage of this action that the development or implementation of such is hindered so far by:

- significant lack on validated risk assessment methods for microbials
- knowledge gaps on the natural distribution of the biocontrol micro-organisms
- knowledge gaps on natural exposition of humans and other non-target organisms
- missing definitions allowing the identification of low risk products.

This is hampering an adequate and balanced risk assessment of microbial plant protection products.

Risk assessment methodology

The current risk assessment for microbial biological control agents (MBCAs) is mainly based on methods developed for chemical pesticides according to Directive 91/414 and the problems regarding the applicability of these methods for microbials are well known. Nevertheless, validated alternative methods for MBCAs are not available.

The risk assessment methodology for pesticides is mainly based on whole-animal testing. Whole-animal tests represent true physiological and metabolic relationships of macromolecules, cells, tissues, and organs. However, these tests are costly, time consuming, insensitive, and difficult to standardize and are sometimes poorly predictive of human *in vivo* response. New *in vitro* test methods target the behaviour of macromolecules, cells, tissues, organs and invertebrates in well-defined methods that control experimental conditions and standardize experimentation. Animal testing using higher vertebrates should be minimized due to animal welfare and the related high costs. This counts not only for pesticides, but as well for the assessment of chemicals in general, pharmaceuticals, cosmetics and food additives. The availability of high throughput and low cost alternatives to animal testing systems will encourage the generation of innovative new products. Better adaptation of the test systems to microbials will improve the obtainable safety by the risk assessment of microbial pesticides, biocides and food additives.

Setting up a call in the 7th Framework programme for the development of better adapted risk assessment methods and the reduction of animal testing would be in line with REACH (Regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals) and the 'Community Action Plan on the Protection and Welfare of Animals 2006-2010',

COM(2006)13. The envisaged research programme should focus on studies with the potential to refine or replace the animal tests commonly required by regulatory authorities. The validation of alternative test systems for microbials and the development of standard protocols and guidelines should be considered as important tasks. For that purpose industry, regulatory authorities and international acting institutions (European Centre for the Validation of Alternative Testing Methods (ECVAM), the European Chemicals Bureau (ECB), which is part of the Institute for Health and Consumer Protection (IHCP) i.e. ECVAM, ESTIV) must be involved into the research programme. It can be learned from earlier studies on alternative test systems (e. g. RAFBCA and 'The Biotechnology Programme' (BIOTECH 2) 1994-1998; area 7.1: prenormative research: *in vitro* alternatives to animal experiments in pharmacotoxicology), that without the involvement of the industry, ECVAM and the regulation authorities, the research results will not get implemented into the risk assessment procedures for microbials or chemicals. The involvement of the OECD biopesticide steering group should be envisaged for the same reason.

In the following the problems with the risk assessment methods are summarised and research tasks are proposed to overcome the corresponding knowledge gaps.

Methodology to assess infectivity

Infectivity, the capability of entering, surviving and multiplying in a susceptible host, is a unique character of microbials. The related potential risks of microbial biocontrol products for humans and animals can not be assessed by methods used for chemical products. Human pathogens are well known and for common micro-organisms the screening of the medical literature can be sufficient to assess the risks for infections (Möllby 1998). However, in case the micro-organism is not well described so far and the natural exposure of humans and animals can not be evaluated, the absence of clinical reports might be not sufficient for a presumption of safety. In such cases an assessment of the potential for infections is indispensable. Currently the infectivity is assessed by so called clearance investigations. In this investigation the clearance of the micro-organisms from the inner organs of rats or mice is assessed after an intratracheal instillation. This method has a number of disadvantages.

For several reasons intratracheal instillation can cause high control mortalities (e.g. Biopesticides registration action document: *Chondrostereum purpureum* strain PFC 2139, US EPA; Product monograph *Pseudozyma flocculosa* strain PF-A22 UL, US EPA). These reasons are the high stress for the laboratory animals, anesthetization and intoxication or irritation/sensitization of the lung by the control substances. Control substances are the 'inert' additives, which are applied together with the micro-organisms in the treated group or killed micro-organisms. Further complications can be caused by plugging of the trachea and bronchi by the test substances. This happens regularly in case microbial products are applied with low cell densities. In order to instil sufficient amounts of micro-organisms these products needs to be applied in high volumes. Sometimes an intratracheal instillation is not feasible for that reason.

In several cases a slow clearance process of the lung and other organs could be observed, even no infections and no clinical manifestations were recorded. Any microscopic particles like micro-organisms can be transported from the application site to other organs by tissue fluids and circulate in the reticular connective tissue and the reticuloendothelial system (Adlersberg et al., 1969). Therefore, relocation of micro-organisms from the injection site to other organs does not necessarily indicate an infection process. Regardless a slow clearance process, MBCAs could be proven to be not infective (e. g. *Bacillus sphaericus*:

Regulatory note *Bacillus sphaericus* Strain 2362 REG2006-02, PMRA Canada, *Trichoderma harzianum*: Technical Document *Trichoderma harzianum* Rifai Strain T-39, US EPA). Also for *B. thuringiensis* (Bt) it is known that clearance occurs not instantaneous (Siegel 2001). Bt persisted up to 49 days after intraperitoneal injection into mice (Siegel et al. 1987) and 21 days after intratracheal instillation into rats (Tsai et al., 1997) without evidence for infections. Therefore, in case no short term clearance could be observed, clinical manifestations of the laboratory animals are more relevant for the risk assessment than the clearance process. Focussing from the beginning on clinical manifestations instead on clearance might be more reliable, while reducing costs for long term clearance investigations and the number of tested animals over time. The applicability of blood test systems might be reviewed for that purpose. A highly sensitive and cost efficient candidate test system is the quantification of C-reactive protein (CRP) (Pepys 1981, Volanakis 2001, Das et al. 2003). CRP is a member of the class of acute phase reactants as its levels rise dramatically during inflammatory processes occurring in the body. CRP binds to phosphorylcholine on microbes. It is thought to assist in complement binding to foreign and damaged cells and enhances phagocytosis by macrophages, which express a receptor for CRP. It is also believed to play an important role in innate immunity, as an early defence system against infections. Highly sensitive CRP test systems (e. g. ELISA) are standard methods in clinical diagnostics and can be used with mice, rats and rabbits. In contrast to the clearance investigations CRP assessment allows to monitor the development of infections on single individuals over time. However, all potential alternatives to clearance investigations need to be checked regarding the probability for wrong negative and wrong positive results. Nevertheless, it should be taken into account that also the current methodology can not avoid wrong results in regard to human risks.

Since the investigations based on whole animal testing are very expensive, the methodology can cause many complications, and in view of animal welfare alternatives for the infectivity assessment methods should be investigated. Chicken embryo tissue assays are regularly used in medical investigations to compare the infectivity of micro-organisms. These assays are proved for several pathogens to be equivalent to or even more reliable than animal testing (Ormsbee et al. 1978, Wooley et al. 2000, Olier et al. 2002, Gibb & Wooley 2003). Further candidates for vertebrate testing alternatives are tests developed for the investigation of opportunistic microbes. For such test systems the nematodes *Caenorhabditis elegans* or *Panagrellus redivivus* can be used (Kurz & Ewbank 2000, Cardona et al. 2005, Laws et al. 2005, Sifri et al. 2005). These test systems are highly sensitive and can distinguish between pathogenic and non-pathogenic strains of opportunistic micro-organisms. However, the development of standard protocols and a critical validation for risk assessment purposes of these methods is still needed.

Methodology to assess toxicity and toxigenicity

Chemical pesticides usually are based on one active ingredient in a defined concentration. Microbials in contrast can produce a broad range of metabolites. Such metabolites can be the active ingredient as in *Bacillus thuringiensis* products; some are usually produced in contact with the target organism and involved in the mode of action and some might be toxic to non-target organisms. Furthermore, different metabolites and different amounts can be produced under different environmental conditions (Baker & Griffiths 1993, Kershaw et al. 1999; Amiri-Besheli et al. 2000, Quesada-Moraga and Vey 2003, Strasser et al. 2000, Vey et al. 2001, Wang et al. 2004, Dabrowski & Sikorski 2005). Therefore, beside the toxicity of the active ingredient in the product the potential to produce toxins (toxigenicity) of the microorganism might be of interest in the risk assessment of microbials.

In the Directive 91/414 toxicity assessments with purified relevant metabolites are required. However, it is not feasible to identify and quantify all metabolites produced or potentially produced by a micro-organism. Methods are needed to detect or exclude the occurrence of relevant metabolites in relevant amounts in a first instance. Most of these metabolites are produced in very little amounts. A purification of sufficient quantities allowing whole-animal testing can be impossible or respectively extremely expensive, discouraging biocontrol companies from developing microbial BCAs (Blum et al., 2003; Zimmermann et al., 2004). Clearly, sensible, simple, better adapted and cost-effective strategies are needed for the risk assessment of metabolites from microbial BCAs.

A way out from that dilemma can be the assessment of extracts from microbial cell cultures, produced under different conditions. REBECA proposed a tiered scheme for bacterial and fungal metabolites (see deliverable 10), based on the assessment of supernatants and crude extracts from cultures of the micro-organisms in question. Microbial metabolites may have additive or synergistic toxic effects. It is conceivable that the toxicological risk associated to a particular MBCA would be better foreseen by assaying mixtures of metabolites, like those in crude culture extracts, on test organisms characterized by sensitivity to a large spectrum of different molecules, instead of assessing the toxicity of single metabolites. As a replacement of whole-animal (vertebrate) testing, it is proposed to use assays with cell lines, protozoa, arthropods or nematodes. There has been success in relating the toxicity data for certain invertebrates to toxicity in vertebrates (Walker et al. 1991, Guilhermino et al. 2000, Lagarto Parra et al. 2001, Sifri et al. 2005, Favilla et al. 2006, Skrobek et al. 2006) or human cell lines (McLaughlin et al. 1993, Solis et al. 1993, Logrieco et al. 1996). A further well known alternative to animal testing is the chicken embryo assay system, used already for the assessment of microbial toxin production (Griffin & Chu 1983, Veselý et al. 1984, Prelusky et al. 1987, Bacon et al. 1995, Sayers et al. 1995).

Different bioassay systems may differ in the susceptibility to different chemicals and may represent different groups of non-target organisms. Therefore, different combinations of invertebrate cell and/or tissue culture bioassays may needed to be evaluated for human, animal and ecotoxicological risk assessments, avoiding wrong negative and wrong positive results. Furthermore, standard protocols for sample (crude extract) preparations are needed, adapted to different chemical families.

Higher throughput and low cost test systems compared to animal testing would allow the investigation of toxic metabolite production over a broader range of environmental conditions. This might improve the risk assessment for microbials compared to the current methodology. However, research is needed to develop guidance and protocols for the assessment of toxigenicity of the microorganism considering the growth under different environmental conditions.

Genotoxicity assessment is a special part of the toxicity assessment, requiring a particular set of methodologies. Genotoxic effects are often of cumulative nature or can cause germline damage. Therefore, an acute toxicity assessment might be not sufficient to detect hidden genotoxic effects. Genotoxicity assessment is based on *in vitro* assays, because whole animal testing is acknowledged as inadequate. With conventional genotoxicity tests applied to small-molecule chemicals careful consideration needs to be given to appropriate protocols that avoid uninterpretable or misleading results when used with micro-organisms (McGregor 2005). Mutagenic and carcinogenic secondary metabolites have been identified in micro-organisms, particularly fungal species using various methods of isolation and bioassay (e.g. Enomoto & Saito 1972, Steyn 1977, Tazima 1982, Rodericks et al. 1977).

However, although specific products under consideration as microbial pesticides have been tested (e. g. Genthner et al., 1998), a general method of screening fungi or other micro-organisms for mutagenic activity has not been developed so far. The data requirements and assessment methods foreseen according Directive 91/414 has been reviewed by McGregor (2005) and judged as not most appropriate. Better adapted sample preparation protocols, guidelines on test choice and test hierarchy in a tiered system need to be developed in relation to exposure scenarios. The development of assays with crude extracts and culture supernatants should be envisaged detecting or excluding the production of genotoxic metabolites in relevant quantities in a first instance.

Methodology to assess sensitisation and irritation properties

Sensitisation or hypersensitivity is a delayed inflammatory reaction induced by a reaction of the immune system to a chemical compound. In contrast to irritation, which is a direct inflammatory response to a substance, sensitisation can only be routinely assessed by whole-animal testing (Chew & Maibach 2006, Simion 2006).

The available methodology is based on assays developed for pure chemicals, producing inconsistent results with microbials. Injective induction and challenge with foreign proteinaceous components into a laboratory animal regularly yields a positive response. On the other hand, topical induction and challenge with the active microbial ingredient would most probably lead to a negative response. No tests are available assessing the potential sensitisation by inhalation of micro-organisms, most probably a greater problem compared with dermal exposure. The lack of suitable methods assessing the sensitising potential of microbials is acknowledged by the European and North American regulation authorities. As a consequence of the absence of proper test methods the Directive 2001/36/EC advises that all micro-organisms should be labelled as potential sensitizers, unless the applicant wants to demonstrate the non-sensitising potential by submitting data. The producers of microbial plant protection products demand applicable test methods, since the sensitizer label might unnecessarily restrict the use of their products, especially in organic farming and the amateur market. The same counts for the irritation assessment. The used methods should be better adapted to products containing micro-organisms, considering alternatives to whole animal testing.

Natural distribution of biocontrol micro-organisms and natural exposition of humans and other non-target organisms

Knowledge on the natural distribution of MBCAs or potential MBCAs and the related natural exposure of humans and animals will support a balanced risk assessment. Most of the micro-organisms used in biocontrol are very common and therefore, a regular exposure of humans and animals can be presumed. Nearly all microbial BCAs originated or can be isolated from the soil and from plants, including food and feed (e. g. Damgaard 2000, Ramos 2004, Meyling & Eilenberg 2007). However, data on background and natural exposure levels are often not available. Such data will help to estimate the risks caused by an artificial application of MBCAs to the environment, will help to identify low risk agents and can deliver the rationale for waivers on data requirements regarding infectivity, toxicity, non-target

effects and fate in the environment. However, the biocontrol industry depends on public research in that field, since they have usually not the human and monetary resources for such investigations. Support of related research areas in the 7th Framework programme would promote the registration of low risk plant protection products in the EU. Case studies on relevant types of micro-organisms used as MBCAs (endophytes, rhizosphere colonisers, soil microbiota, insect pathogens, spore formers, conidia formers, etc.) need to be carried out. Natural background levels should be determined, with a focus on agricultural environments and food/feed. Hot spots should be identified and exposure levels for human and animals evaluated that are in regular contact to such spots. Such exposure data could be also related to data obtained for plant pathogens identified as toxic metabolite producers, causing poisoning of humans and animals.

Specification and identification of low risk products

In the Commission's proposal for a new pesticide Regulation (2006/0136 COD) the introducing of a simplified procedure for low risk substances and products was envisaged. However, the deficiencies of that proposal are that the identification of low risk products is only possible after the risk assessment and the absence of a clear definition for "low risk products". REBECA developed a proposal for an environmental 'risk indicator' published in 2008 by Laengle & Strasser, allowing a comparative assessment of biological and chemical plant protection products and the identification of low risk products (see deliverable 28: Specification of low risk products). This risk indicator is a refinement of earlier models (Environmental Impact Quotient (EIQ, Kovach et al., 1992), ERBIC (Hokkanen et al., 2003), Norwegian Indicator (NARI), Québec Pesticide Risk Indicator (IRPeQ, Onil et al., 2007), Canadian Agri-Environmental Standards (NAESI, Mineau *et al.*, 2008). The proposed model is the first indicator allowing a direct numerical comparison of relative environmental risks posed by microbials and conventional chemical pesticides. The suitability of the model was demonstrated by calculating the risk scores for seventeen selected products (chemicals and MBCAs). Data gaps regarding one product can be filled in the model with worst case assumptions. This offers the opportunity to identify low risk products using public data and during and after the risk assessment process, delivering a scientific basis for simplified registration procedures at any stage of the registration process. The general applicability of the model proposed by Laengle & Strasser needs to be checked on a broader basis of different kinds of plant protection products and may need to be refined further. The EU should support the further development of the proposed risk indicator model, also in view of a comparative risk assessment of plant protection products, demanded by the EU parliament and several member states (2006/0136 COD).

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