PROCEEDINGS

”Microbiological Plant Protection Products - Workshop on the Scientific Basis for Risk Assessment”

Stockholm, Sweden 26-28 October, 1998

KEMI
NATIONAL CHEMICALS INSPECTORATE

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Preface

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Activities within the area of microbial plant protection products have increased in recent years. Although they represent a small market share of the total plant protection market in Europe, the share is growing. Until now most microbial plant protection products have been assessed nationally and on a case-by-case basis depending on the lack of relevant knowledge. But with more products of this kind and increased knowledge, a more systematic and harmonised approach is needed with regard to data requirements and risk assessment.

Within the EU, microbial plant protection products are regulated by the same directive as chemical plant protection products, namely Directive 91/414/EEC on the placing of plant protection products on the market. According to that directive there are today 7 new applications in EU member states for plant protection products containing microorganisms as active substances.

There is a growing awareness that microbial pesticides are inherently different from chemical pesticides, with fundamentally different modes of action and that they should therefore be assessed on their own merits and problems and data requirements should be set accordingly. However, the basis for the proposed data requirements needs improved scientific justification. It was the purpose of the workshop to penetrate this topic between scientists with knowledge of the microbial area and regulators of plant protection products in order to find consensus on the nature of the risks we are regulating and what information is needed in order to assess these risks.

The National Chemicals Inspectorate in Sweden was contracted by the European Commission DG VI to hold the workshop for EU member states with the above purpose. However, it is not only in Europe that increasing attention has been drawn to microbial plant protection products. Also the United States of America and Canada have experienced increased activity. Within the OECD framework several countries have expressed their interest in harmonising data requirements. Canada and the United States have had longer experience in regulating microbial pesticides than most European countries. In order to benefit from their knowledge and experience and at the same time take the opportunity to find ways to harmonise, representatives from Canada, the United States and OECD were invited to the workshop. Researchers and industry showed a great interest in participating in the meeting which can be seen from the participants list.

The results of the workshop will be used to elaborate a new proposal to the Commission, using the existing EU working document on documentation requirements for microbial plant protection products. Our hope is also that it will show that the workshop became a catalyst for increased harmonisation and co-operation in the field of microbial plant protection products between EU member states and OECD member countries.

Authors

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Program for ”Microbiological Plant Protection Products - Workshop on the Scientific Basis for Risk Assessment”

October 26

09.00	Registration
12.00	Lunch
13.00	Introduction
13.15	DG VI information
13.30	Risk analysis and the evaluation procedure
13.45	Experiences so far within EU: DG VI
14.00	U.S. Guidelines Rationales and Risk Assessment Experiences
14.10	Assessing the Risks of Microbial Pesticides - A Canadian Approach
14.20	Harmonisation efforts within OECD
14.30	Discussion Summary of discussion
15.00	Coffee
15.30	Lecture 1:Risk analysis of beneficial microorganisms - wild types and genetically modified
16.15	Discussion Summary of discussion
18.00	Guided bus tour and dinner

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8.30	Lecture 2: Identification and characterisation of bacteria.
9.00	Discussion Summary of discussion
9.45	Coffee

10.15 Lecture 3: Pathogenic properties of microorganisms - human hazard identification

10.45 2 parallel group discussions - Summary of the discussions

12.00 Lunch

13.00 Lecture 4: Fate of micro-organisms introduced in soil, effects on autochtonous populations and activities.

13.30 2 parallel group discussions - Summary of the discussions

14.30 Comments from EU and OECD member countries on EU data requirements - Discussion
Summary of discussion

15.00 Coffee

15.30 Biological control OECD and ERBIC

15.45 Release of wild-type and genetically modified biocontrol inoculants as plant protection products.

16.00 Experiences so far within EU: DGXII-COST Action 830

16.10 Considerations and a Scientific Opinion prepared by DG XXIV Plant Protection Products Committee on the EU
Draft Data Requirements for Microbiological Plant Protection Products.

16.30 Discussion - Summary of discussion

17.30 Preparation of document

19.30 Dinner at the hotel

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8.30 Common Position on data requirements and scientific justifications

10.00 Coffee

10.30 Common Position on data requirements and scientific justifications

11.30 Conclusion and closing remarks

12.00 Lunch

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Risk analysis of beneficial microorganisms - wild types and genetically modified

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Contents

1. Subject matter

The European Union has issued several Directives considering risk. One is the well-known Directive 91/414 on plant protection products, another is Directive 90/220 on genetically modified organisms. The link between the two becomes obvious when we discuss Microbiological Plant Protection Products, briefly 'microbials'. Such products may be genetically modified or not. In either case, they have to undergo the screening prescribed by Directive 91/414. If genetically modified, another screening is prescribed by Directive 90/220 for the added risk due to the genetic modification. Directive 90/220 considers release of the genetically modified organisms into the environment and their admission to the market. For microbials, the two screenings could be performed simultaneously, and even in concert by cross references between individual regulations (Figure 1). We are still far from that situation.

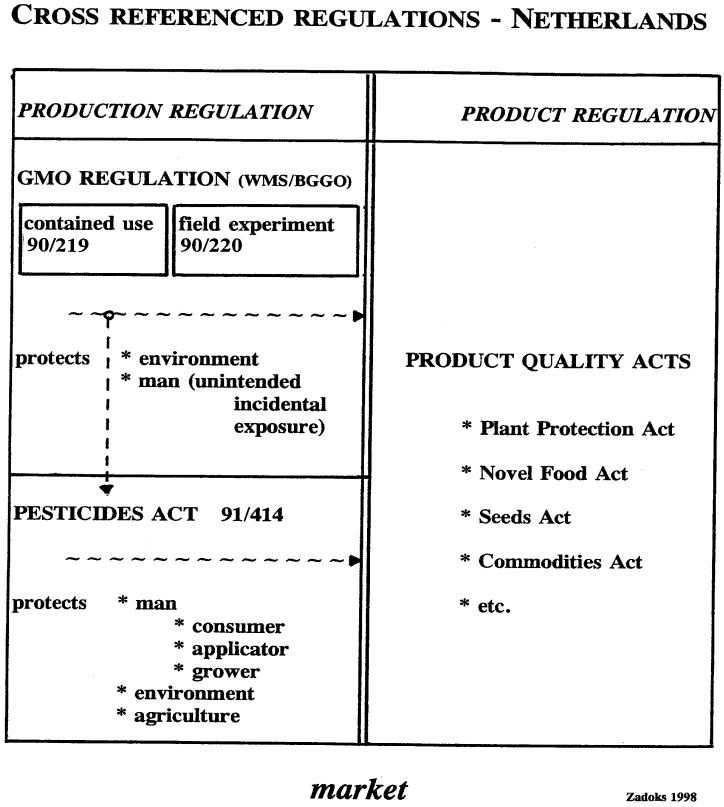

Figure 1. Cross references between various regulations at EU al level. Left column process-oriented regulations, right column product-oriented regulations. Field experiments with genetically modified microbials need consent under both 91/414 and 90/220. The original figure was proposed by Mr. P. van der Meer.

At the national level, I have been exposed to both Directives. As a member of the Pesticides Registration Board of The Netherlands[1] I was involved in the implementation of Directive 91/414 As a member of the Committee on Genetic Modification[2]. I am permanently exposed to Directive 90/220. The procedures following the respective Directives differ greatly, possibly because of differences in the body of knowledge available for either purpose, and certainly because the two Directives reflect different political concerns.

My present work makes me strongly biased toward the procedures in Directive 90/220. Here, lessons are taken from work done with plants and industrial fungi. The EU Directive prescribes an analysis of the risk which genetic modification adds to a current microbial, the 'added risk' of transgenes[3]. A merger of the procedures following the two Directives, or at least an overlay, could save valuable time and money for both the Competent Authorities and the industry. A minimum requirement is that the Competent Authorities responsible for 91/414 and 90/220 accept each others' results.

2 Risk

Considerations about risk attributed to microbials without or with transgenes are numerous, be they philosophical [75], political [1,2,11,74] or technical [9,11,12,38,40,44,46,74].

1 In the Netherlands, registration of pesticides is done by a legal semi-autonomous body, the Pesticides Registration Board (CTB = College Toelating Bestrijdingsmiddelen), under the political responsibility of the Minister of Agriculture (in cooperation with several other ministries).

2. The Committee on Genetic Modification (COGEM) is a legal body which advises the Minister of the Environment (cooperating with several other ministries) in all matters of genetic modification.

3. Whereas 91/414 considers health of humans, animals, crop plants and the environment, 90/220 tends to consider only the environment, including unintended and incidental exposure of humans and animals. To the intended exposure (genetically modified food and feed) other Directives apply.

2.1 Definition

The problem is to assess the risk of possible adverse effects of a technological development thought to be advantageous. We enter the area of decision making under uncertainty, where risk assessment and risk management are among the tools to be applied.

The usual definition of risk is hazard times probability. The procedure is simple. We identify the hazard and determine the probability that the hazard will materialize. Of course, we might envisage more than one hazard each with its own probability. Then, we have the option to accumulate risks or to concentrate on the 'worst case' scenario.

The case is relatively simple with the Dutch dikes that protect The Netherlands from the sea. If a dike breaks, a certain area is inundated, with so many persons living there, so much real estate and so much economic activity. Some people will die, many will be displaced. Real estate will be destroyed. The economic activity will be suspended for a year or more. There is sufficient experience with early dike breaks to make the necessary estimates. The hazard is known for every kilometer of dike. Past experience allows to calculate the probability of dike breaks, which depends on their height, strength and exposure. In principle, dikes are designed to break once in a thousand years at most.

2.2 Hazard identification

A hazard is any imaginable adverse effect which can be named and measured. A hazard is not an existential fear which remains unidentified, which cannot be expressed in the current kilogram-meter-second system, and to which no dollar or ECU value can be attached. In the area of chemical Plant Protection Products there is rather too much experience with hazards, but in the area of genetic modification hazard identification it is notoriously difficult. We may learn from studies on plant pathogens [15,71,72,84]. Hazards due to pesticides have been expressed in monetary value [63,67,80].

Hazards due to genetic modification are not yet so explicit. A genetically modified fungus for weed control might run out of hand and become a pathogen of a cultivated crop or, probably worse, of native wild plants. Now, the hazard is named but its extent remains vague and its dimensions are hazy. The time dimension should be considered since a threatened species might be damaged temporarily and eventually recover.

I have not come across any method of systematic hazard identification, and certainly not any science based method. Some checklists are available[4], but these contain only hazards already identified which are rarely relevant to our specific problem. Of course, the lists do not contain the unexpected hazards.

I conclude that hazard identification still is like gazing in a crystal ball. What you see is at best an extremely distorted view of your own misgivings.

2.3 Probability estimation

When the hazard is identified, a likelihood or probability has to be attached to it. I do not know of any scientific method to determine a probability of a hazard posed by microbials, and *a fortiori* by genetically modified microbials.

The problem is not an easy one because we have in fact two probabilities, called 'if' and 'when'. One answer could be that we express the probability on a per year basis. The probability that a genetically modified fungus (GMF) intended for weed control turns into a pathogen of native wild plants then becomes, for example, one per mil per year, once in a thousand years. We still have no answer about the year, which year, when?

One could imagine that the probability is proportional to the amount of GMF applied or to the hectareage covered. Though this sounds logical, caution is needed. The fungal pathogen of wheat called yellow rust, *Puccinia striiformis*, has the ability to overcome specific monogenic resistance in a variety by mutation toward equally specific virulence. Such a specific virulence may develop at any time reckoned from the introduction of that variety. Any time means between zero and 25 years, irrespective of the hectareage under the new wheat variety [83].

The crystal ball does not answer the questions 'if' and 'when'. With microbials we do not yet have the unfortunate experience which we have with pesticides or Dutch dikes. For the time being, we cannot answer the probability question.

2.4 First set of conclusions

4. Gressel, J., Rotteveel, T. - 1998/9. Evaluation of the genetic and agro-ecological risks from biotechnologically-derived herbicide resistant crops (BD-HRC), with decision trees for less biased, regional, risk assessment. Plant Breeding Reviews (submitted).
See also [48,49,56] and figure 4.

Some preliminary conclusions may be drawn.

1. Classical, technology based risk assessment seems to be impossible (yet) in the area of biological control.

2. Nevertheless, risks are perceived and their management is imperative.

3. Another approach is needed.

3 General principles

3.1 The precautionary principle

Where science cannot give a definite answer to reasonable questions on risk, politics step in. The 'precautionary principle' was formulated at the 1992 UNCED meeting in Rio de Janeiro [1]. The precautionary principle [17] implies that politicians, facing a putatively undesirable event, need not wait for remedial action until full scientific proof of the event has been given. An example in case is the political action taken to counter the greenhouse effect which, though probable, is still not undisputable.

The precautionary principle can be implemented in a way akin to risk assessment and risk management, but we start on a different footing. It is my intention to outline a feasible approach borrowing heavily from my experience with genetically modified plants. The approach is coalled the 'familiarity based, transgene-centered approach' [26,50].

3.2 Familiarity

The OECD developed the concept of 'familiarity' [57,58], which is the combination of knowledge and experience needed for *1*) risk analysis, *2*) risk management, and *3*) continued quest for information. Familiarity implies that the scientists and the public (including the regulatory authorities) have a basic knowledge of the organism involved, its environment, its life cycle and behaviour, its advantages and disadvatages. If we talk about maize, we know what it is, what it can do for us and what it does not. Thus, familiarity is very much a household-type of concept. In industrial applications of genetically modified micro-organisms (GMMs) familiarity

is expressed in terms of an 'extended history of safe industrial use'[5] [56], and in agriculture we could speak of an 'extended history of safe agricultural use'.

Of course, the specialist will go much further in detailing familiarity than the average housewife, but that does not change the principle. Familiarity is, or should be, based on a wide consensus. The OECD systematizes consensus on familiarity in various 'consensus documents' [59,61,62]. Familiarity as a concept is not unambiguous but it is useful.

Familiarity with microbials is abundantly available. Examples are ice nucleating bacteria to control night frost damage [45], root inhabiting Pseudomonads producing antibiotics wich keep root-invading pathogenic fungi in check [12,53,81], entomopathogenic fungi to control insects such as *Beauveria bassiana* [20,29,30], fungi to control fungi [24,47,78,81] or weeds [14,22,41,70] and baculoviruses against insect pests [6,7,21,51,52].

Consensus documents for regulatory purposes, demonstrating familiarity with a microbial, could be made fast and cheap. For risk assessment under 91/414 and the necessary corollary discussions such consensus documents could form a solid foundation. For example, the OECD [62] prepares a consensus document on environmental applications involving *Pseudomonas*. These organisms could be brought under the jurisdiction of 91/414 since some have a plant protection effect.

3.3 Effects of microbials

The effects of microbials can be grouped in four major classes, competitive displacement, pathogenicity, toxicity and allergenicity (Figure 2). As to the targeting of the microbials we distinguish effects on target and non-target organisms [12].

5. The mention of 'extended history of safe industrial use' is found on page 34 of the 'Blue Book' [56]. Though the term is vague, it has been used extensively. A good example is the use of yeast (*Saccharomyces cerevisiae*) in bread making, another one the various microbes used in cheese and yoghurt making.
An 'extended history of safe agricultural use' could be proclaimed for many strains of soil-borne and rhizosphere bacteria and fungi, which can be used in biological control of plant pathogens.

ECOLOGICAL EFFECTS OF MICROBIALS

Effect	Target	Non-target
Competitive displacement	+	-
Pathogenicity	+	-
Toxicity	+	-
Allergenicity	NR	-

Figure 2. Four major classes of ecological effects due to the application of microbials. + refers to desired, - to undesired effects. NR = not relevant.

Competitive displacement is desirable when ice nucleating *Pseudomonas syringae* are temporarily displaced by genetically modified ice minus strains to protect crops from night frost damage [45], but should displacement be permanent? We touch upon the problem of 'persistence'.

Pathogenicity to humans and warm-blooded animals is undesirable, but pathogenicity to target organisms such as weeds, to be killed by plant pathogens, or insects, to be killed by insect pathogens, is desirable. However, the host range of the pathogen should be so narrow that only the target organisms is killed but not any related organism. In my view, host range is a critical item.

Toxicity to humans is undesirable but toxicity to target organisms is wanted, as e.g. for *Bacillus thuringiensis* to kill caterpillars and fluorescent pseudomonads which produce antibiotics against the soil-borne wheat pathogen *Gäumannomyces graminis*. Note that *B. thuringiensis* forms many toxins, of which the endotoxins are wanted but the exotoxin is unwanted[6].

Allergenicity to humans, cattle and companion animals is unwanted, but allergenicity to target organisms is not relevant. Several allergenicity tests are available [27].

Tests for toxicity and allergenicity to humans and higher animals need not differ much from current tests of synthetic pesticides, but tests for ecological effects

6. For registration purposes, *Bt* strains produced for crop protection and medical purposes should be free of exotoxins.

require special attention. Target specificity of microbials is imperative, but in some cases it is not available.

As a special case is the control of the forest weed *Prunus serotina* by a common fungus, *Chondrostereum purpureum*, a generally occurring wound colonizer but also a dreaded pathogen of cultivated plum trees. Treatment of a forest plot should not threaten nearby plum orchards. As the fungus is ubiquitous, a bottom-line threat to plum trees is always present. What is the acceptable level of added risk due to weed control? It was decided, somewhat arbitrarily, that the added threat should not exceed the ever present bottom-line threat. Using detailed measurements and sophisticated models, a minimum safety distance was calculated between plum orchards and plots to be treated. This distance was accepted by the Dutch Plant Protection Service [14,15; compare 71,72,82].

3.4 Applications of microbials

For microbials, three essentially different applications methods exist, called inoculative, augmentative and inundative [76](Figure 3). The consequences may differ for native and introduced microbials and the question arises whether Directive 91/414 applies to all three methods.

STRATEGIES FOR MICROBIALS

Method / Origin	Native	Introduced
Inoculative	NR	???
Augmentative	NR	???
Inundative	91/414	91/414

Figure 3. Three strategies for the application of microbials. Legal aspects may very according to their origin, native or introduced. NR = not relevant.

Inoculative control implies a local inoculation of the organism to be controlled in the expectation that the microbial will spread autonomously. Introduction of the rust fungus *Puccinia chondrillina* into Australia to control the imported skeletonweed, *Chondrilla juncea*, is a good example [33]. In short-season crops, as in glasshouses, a seasonal inoculative control may be needed. Seed bacterization (see below) may be seen as a seasonal inoculative control since the bacteria used should multiply during the season but then disappear.

Augmentative control, also called conservation, manipulates the environment to improve the effectiveness of already established natural enemies, and eliminates constraints to their survival or effectiveness.

Inundative control is inspired by and not essentially different from classical chemical control, just spraying or dusting. Application of *B. thuringiensis* to an outbreak of caterpillars, euphemistically called biological control, is nothing else than the application of insecticidal molecules produced by and packed in a bacterium. The advantage is the high specificity of the chemical involved.

3.5 Matters of scale

Scale effects are important. We consider scales of distance, time and complexity [4,84,85]. We learned from synthetic pesticides that few undesirable effects are found in the early stages of field application. The real nuisance comes later and can be predicted only in hindsight. Should the microbial spread, possibly continent-wide, and stay forever, as with inundative control? If it should not stay, can we prevent transmission to other places by wind or water, by insects or implements?

And if the microbial stays, should its population increase, remain constant, or decrease and disappear? Seasonal increases are common and desirable, as with bacterisation of seeds with *Pseudomonas putida* to control root pathogens. The bacterium should multiply and spread over the roots into the soil [28]. Similarly, the population of the fungus *Coniothyrium minitans*, an effective control agent of sclerotial plant diseases, will grow during the season as long as fresh sclerotia are available [24,25,47]. In contrast, the population of the fungus *C. purpureum* to control *P. serotina* should disappear after application [14,15].

Genetic modification of microbials helps to attain the objective of inundative application and to mimick synthetic pesticides [23]. Microbials often are slow to produce the desired effect. Genetic modification may speed up that effect and, by the same token, make the microbial suicidal, so that it rapidly disappears from the environment after application. Such is the objective of the scorpion poison engineered into baculovirus to control lepidopteran larvae.

3.6 Transgene-centred approach

The OECD introduced two ways to handle risk assessment of genetically modified organisms (GMOs), the 'case-by-case' and the 'step-by-step' approaches[7]. The case-by-case approach implies that the contribution of each transgene is considered separately. The step-by-step approach refers primarily to risk management under EU 90/219 and will not be discussed here.

In GMOs, it is the product of the transgene which makes the difference between the modified and parent strains. Thus, in The Netherlands we explicitly follow a 'transgene-centred approach'[8] [26,50]. This implies that the consequences of each transgene added to the genome will be considered separately. In addition, the combined effect of the transgenes will be considered. For each transgene, the intended gene product and its possible effects on the modified organism, on its ecology [18] and on ecologically related organisms is taken into consideration.

We first discuss the molecular aspects such as the unambiguous characterization of the transgene, observance of the borders of the construct, orientation of the transgene involved (sense or anti-sense) and numbers of transgenes, all items which can be determined using appropriate molecular techniques. Such data are needed for commercial purposes (intellectual property), for legal disputes (liability) and for risk management (monitoring, retrieval, eradication). Posttranscriptional and posttranslational effects should be considered, with due attention for desirable effects such as enhancement or silencing of target genes. The gene products should have been identified.

Then we discuss the possible ecological effects of individual transgenes and their combinations, since a construct rarely contains one transgene only. Among the imaginable ecological effects are toxicity and allergenicity to man and animals, and favourable or adverse effects on other organisms in agro-ecosystems, managed ecosystems or natural ecosystems. We have to keep in mind that our duty is limited to the added risk due to the genetic modification.

3.7 Evaluation of alternatives

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7. 'Gene-for-gene' implies that each transgene is considered separately for biosafety. In addition, transgene combinations are considered. 'Step-by-step' implies that each step from greenhouse over field experiment to market release is checked, and a next step is only allowed if the last step was satisfactory from a biosafety point of view.

8. A number of reports were prepared by the CPRO-DLO institute in the Netherlands [a.o. 26,50].

Evaluation of alternatives is not mandatory, unfortunately. No intervention whatsoever exists without undesirable side effects, the pros and cons of different possible interventions should be considered.

Take as an example the use of genes from *B. thuringiensis* used to make crops resistant to specified insects. The alternatives are:

1 - No intervention (let things go).
2 - Chemical intervention (spray insecticides).
3 - Prevention by classical resistance breeding (if available).
4 - Prevention by modern breeding (applying biotechnology).

Each alternative has its risks and benefits (Figure 4).

FOOD CHAIN EFFECTS OF MICROBIALS - Example *Bt*

Trophic level	Control	Chemical	Biological	Genetic modification	None
	RISK				
1	Host plants	Minimal	None	None	High
	KILL	*of target*			
2	Miners	Yes	No	Yes	No
	Chewers	Yes	Yes	Yes	No
	Suckers	Yes	No	No	No
	KILL	*of predators*			
3	Miners	Often	No	No	No
	Chewers	Often	???	Sometimes?	No
	Suckers	Often	Partly?	Sometimes?	No
	KILL	*of parasitoids*			
3	Miners	Mostly	No	Sometimes?	No
	Chewers	Mostly	No	Sometimes?	No
	Suckers	Mostly	No	Sometimes?	No

Figure 4. Tentative checklist for food chain effects of microbials, illustrated by the example of *Bacillus thuringienses*, applied either as a suspension of bacteria (spray) or as transgenes in the host crop.

Application of *Bt* genes in maize implies a precisely targeted killing of the European Corn Borer (*Ostrinia nubilalis*). Orgamisms of the third trophic level, predators and parasitoids, might get killed too [5,34], though possibly far less than after spraying pesticides. Transgenic resistance may suffer the same fate as most monogenic resistances obtained by classical breeding, it may not last[9] [32,39,68,83] but they buy

9. The literature on so-called breaking of vertical resistance is incredibly large, though modern writers tend to ignore it. Vertical resistance is usually due to one, mostly dominant, gene and it is effective against a

time, valuable time[10]. We have little experience in the evaluation of alternatives at field or farm level and at the large scale socio-economic level[11] [63,80].

Counterintuitively, we must concede that the use of so-called beneficials in biological control is not without its own risks[12] [3,35,36,37,40,48,49,84]. The international trade and exchange of beneficials is being regulated by FAO, to avoid unnecessary risks[13] [19]. The European Union commissioned a study on the risks involved with beneficials[14]. An introduced beneficial insect may become a pest to the local flora or fauna, e.g. by 'host shift', a rare phenomenon but not without evolutionary value. Alternatively, the introduced benefical insect may carry a new insect disease dangerous to the local insect fauna. Many beneficial bacteria and fungi operate by means of poisons, such as the *Bt*-proteins and various antibiotics. Some

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limited number of genotypes of a single pest species, be it fungus, bacterium, virus (sometimes), insect or nematode. Mutations in pest genotypes create new virulences which overcome the 'broken' host resistance gene, which remains completely unchanged but looses its effectiveness.

10. Breeding for vertical resistance has been in vogue ever since about 1900 and it still is the first line of defense against many pests and diseases, especially fungal diseases, in many crops, primarily annual field crops. Such breeding is simple, cheep, and - at least temporarily - highly effective. Some transgenes for resistance, such as the *Bt* transgene, behave as simple vertical resistance genes and thus are easy to transfer to other cultivars by classical breeding methods. See reference journals.

11. Benefit-risk analysis is mandatory for the registration of pesticides by US-EPA but excluded from Directive 91/414. Similarly, benefit-risk analysis is not mentioned in Directive 90/220 and it is definitely beyond the scope of the Netherlands Dangerous Substances Act. In my view, these are serious omissions which impede a healthy discussion with authorities, industries, clients, consumers and the general public.

12. *Some examples of EU research programs are:*
COST-816 - Biological control of weeds in crops, of which the 4th Workshop on 'Risk assessment in biological control of weeds' was held in Southampton, 9 April 1998. Publication foreseen.
Hokkanen, H.M.T. (co-ordinator) - 1997. Evaluating environmental risks of biological control introductions into Europe (ERBIC). Technical annex of project FAIR5-PL97-3489. 27 pp.
BIOT-CT91-0291, financing a.o. work on baculovirus.

13. The advance of biological control and the international trade in beneficial organisms was and is much hampered by lack of adequate international regulation. The practical effect was that several countries did not allow importation of predators and parasitoids, which are usually not regulated by Pesticides Acts (nor by 91/414), though the desired benefical effects had been well established.

14. Hokkanen, this workshop.

beneficials might even carry dangerous allergens[15] [27]. By and large, however, the odds are not against beneficials, without or with transgenes.

3.8 Second set of conclusions

We arrive at a second set of conclusions.

1. Political and technical decisions must be made under uncertainty,

2. The precautionary principle may give some guidance.

3. The OECD concept of familiarity with non-modified wildtype or domesticated organisms may provide the necessary perspective for decisions.

4. The concept of 'extended history of safe use in agriculture' may be developed.

5. In the case of transgenic microbials a transgene-centred approach may be an appropriate tool.

4. A 'familiarity-based and transgene-centred' approach

4.1 Familiarity with microbials

To proceed, it is useful to detail the concept of familiarity in the case of microbials under 90/220. The comments also apply to 91/414.

4.1.1. Wild parent species and strain

Familiarity with micro-organisms, say with the fungus *Beauveria bassiana*, to control caterpillars, is not essentially different from familiarity with a crop plant such as maize. The following items apply to microbials.

Identification

15. By and large, the number of people suffering from allergies of various nature is increasing in the western world. Regulary authorities have to consider the problem of allergies, as currently done under Directives 90/220 and 91/414. The methodology to test for allergenicity without testing humans is advancing [27] but ultimately human subjects may be needed for the tests.

a. The wild parent species must be known. Its taxonomy must be clear so that the species can be identified without doubt, also after genetic modification.

b. Similarly, the wild (or domesticated) parent strain[16] selected to be modified must be identifiable with classical taxonomic means and with molecular techniques, so that the strain can be identified without doubt, also after genetic modification.

Environmental behaviour, fate and effect

c. The life cycle of the parent microbial and strain should be known. Especially its mating behaviour is relevant[17].

d. The multiplication system(s) of the microbial should be known. In some cases it should not multiply after having done its work (e.g. insect control by baculovirus), but in other cases multiplication steps are necessary (e.g. seed bacterisation of wheat)[18].

e. The survival structures helping the micro-organism to overcome an adverse season should be known. If the microbial is to be used as a replacement of a chemical, that is by spraying it over a crop, lack of survival structures may be an asset.

f. The dispersal structures of the microbial should be known. If it is to be used as a replacement of a chemical, that is by spraying it over a crop, its dispersal should be minimized[19].

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16. Micro-organisms in general, are also beneficial micro-organisms, exist in a countless variation of types and strains, to be differentiated by cultural, biochemical, and molecular means. Modes of action, the range of affected species, the degree of pathogenicity, and the environmental requirements for effectiveness may vary. The literature on these points is voluminous.

17. The common taxonomic requirement that different species should not mate does not fully apply to fungi and bacteria. Nevertheless, lack of mating ability is an asset.

18. *Pseudomonas putida* strains used for seed bacterisation of wheat have been observed to multiply a millionfold or more per seed(ling) (JM Raaijmakers, pers. comm.) within three weeks. During a severe epidemic of the cereal rust *Puccinia striiformis* this multiplication make take over three months (per hectare) [83].
Coniothyrium minitans controls sclerotial plant diseases and multiplies during the vegetation season as long as sufficient fresh sclerotia are available.

19. If the classical adage about micro-organisms 'everything is everywhere but the environment selects' applies the micro-organism might be allowed to spread far and wide. The dictum ascribed to the Dutch microbiologist Baas Becking 1895-1963).

g. The specific ecological niche of the microbial (species and strain) to be used as a parent should be known. If it is to be used as a replacement of a chemical, it should not be able to hide away.

h. The persistence of the microbial should be known.

When the microbial is applied in the inoculative mode, good persistence is an asset. When the microbial is applied in the inundative mode, like a synthetic pesticide, low persistence is desirable and suicidal genes could be added. In rare cases, the population of the microbial is expected to grow, at least temporarily.

i. The ecological effects, desired and undesired, of adding the wild type of the microbial to the environment should be known.

Genetics

j. The genetic stability, or rather instability, of the wild or domesticated parent strain should be known.

k. The genetics of the microbial should be known, not only its mating behaviour but also its ability for parasexual exchange of genes.

l. Information on the presence of extra-chromosomal and exchangeable DNA is desirable. New inserts should be chromosomal and non-exchangeable.

Special characteristics

m. The competitive ability of the parent strain at the desired site of action should be known.

n. The specificity of the parent strain for the target organism should be known, since the microbial should not unexpectedly attack non-target organisms.

o. The pathogenic ability of the parent strain to the target organism and its relatives should be known.

p. The toxicity of the wild strain to non-target organisms, specifically to man[20] and higher animals, should be known. In principle, it should be zero.

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20. Two effects on humans must be considered. *1*. Exposure of consumers to residues on food. *2*. Exposure of labour *a*. primary exposure during handling and application of microbials and *b*. secondary exposure during handling of the crop (weeding, pruning, harvesting).
A Dutch court ruled that, in the case of genetically modified feed given to animals, the food products made from those animals should be considered in the risk analysis. Such a decision could *mutatis mutandis* be extended to feed treated with biologicals if bioaccumulation is feered.

q. The allergenicity of the wild strain should be known[21].

4.1.2 Examples of familiarity

The list of beneficials amenable to genetic modification is endless [e.g. 44]. I will mention a few.

Ice nucleating bacteria[22] are instrumental in the pathogenesis of night frost damage. Ice^+ strains are usually of the *Pseudomonas syringae* type. By genetic modification ice^- strains can be made to be sprayed for damage prevention. In California, a terrific fight exploded between pros and cons. In the end, genetically modified ice^- strains were registered. They are safely used.

Baculoviruses to control insect pests typically have an 'extended history of safe agricultural use' [6,7,21]. They are applied over a million of hectares annually [51,52]. By genetic modification the slow kill characteristic, often undesirable, can be changed into fast kill, which cripples the modified virus and makes it suicidal[79]. Several field tests have been performed already and a dynamic simulation model to test ecological effects is being developed[23].

Bacteria, primarily pseudomonads, are known to protect plant root systems from pathogenic fungi [8,12,13,42,43,65,66,73,77,81]. Such pseudomonads are functional parts of nature's own regulatory system. The literature is comprehensive and convincing. Experiments are ongoing to enhance the effect of operational genes and to make new gene combinations by genetic modification [10,16,23,31,40,46,55,69]. Experimental risk analyses are being made [28]. The first field experiments indicate that such modified bacteria, added to the seed, spread over the root system and protect the root system, and disappear from the soil within one year [28].

Several entomopathogenic fungi are applied to control noxious insects and a wealth of literature exists on each of these fungi providing adequate familiarity. Genetic modification, enhancing the gene expression of natural killer genes, is

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21. Allergenicity to rare individuals can never be excluded but some micro-organisms are known to be more allergenic than others.

22. Ice nucleating bacteria (ice^+ types) on the leaf surface can be instrumental in the causation of night frost damage to plants. If replaced by ice^- types, night frost damage may be negligible.

23. J. Vlak and collaborators, Department of Virology, Wageningen Agricultural University, The Netherlands.

making progress [69]. Mycopathogenic fungi function in the natural homeostasis and they can be used in the control of fungal diseases [54,64,78]. Similarly, adequate familiarity exists with phytopathogenic fungi [14,15,22,41] thought to be useful in weed control.

4.1.3 Familiarity reassessed

Familiarity thus obtained with the wild or domesticated strain does not protect us from all undesirable events and such protection is not the objective of gaining familiarity. Familiarity serves as a common starting point for risk assessment, as an anchor to avoid discussions going adrift, as an assurance to the general public and the authorities that scientists know what they are talking about[24]. The familarity provides a background for the next phase in the process of risk assessment.

The hunger for knowledge is without end. One fool can ask more questions than ten wise men can answer. Moderation in the amount of knowledge required is recommended. At all times, we must try to respect the border line between 'need to know' and 'nice to know', accepting two nasty facts:

1. The border line between 'need' and 'nice' may shift in time, and

2. Much of the available knowledge does not really help us in decision making under uncertainty.

4.2 Transgene-centred approach

In The Netherlands, under 90/220, we explicitly follow a transgene-centred approach. This implies that the consequences of each transgene added to the genome will be considered separately. In addition, the combined effect of the genes added will be considered. For each transgene, the intended gene product and its effect are taken into consideration.

4.2.1 Intended genetic effects

[24] Familiarity is an asset in risk communication with the general public. If the responsible authorities and their supporting scientists can explain what is going on, it may relieve some of the pressure even though it cannot take away all concerns. Familiarity may protect the responsible authorities in court during a legal dispute, as I have experienced myself.

Since we talk about release into the environment, the primary gene effects are already known. The transgene product is relevant. The number and position(s) of the transgenes may be nice to know but not necessarily relevant. Several points must be considered.

a. The microbial should be crippled, that is, it should not be able to persist in nature[25]. When multiplication steps of the microbial are required (e.g. seed bacterisation) it should nevertheless disappear from the site of action within a reasonable time.
b. The microbial should be sharply targeted to the organism to be controlled[26].
c. The microbial should produce a fast kill, for two reasons.
   1. The commercial reason is that a product is commercially competitive only when it kills the target organisms fast, nearly as fast a a chemical control agent.
   2. The safety reason is that the probability of GMM reproduction in the target organism is inversely proportional to the killing rate.
d. For safety reasons, it is preferable to use nature's own killing technology. Thus, an enhancement of natural killing factors is preferable above new killing factors[27].
e. Generally speaking, a molecular device must be present for post-release monitoring (the 'molecular signature', and the applicant must provide a reasonably reliable and cheap method for selection and identification of the microbial in the environment.

4.2.2 Unintended genetic effects

25. The first requirement in the industry is that a fungus to be fermented at an industrial scale should be asporogenic.

26. This requirement is difficult to implement because the molecular base for host specificity is inadequately known. Fortunately, specificity tests are easy to perform. Note that they never preclude the possibility of a host shift.

27. Technically, this is feasible, in some instances at least, by enhancing gene expression. The point would be an asset in risk communication, if we consider the understandable fuss about the scorpion poison gene in baculovirus.

Unintended genetic effects of a transgene, as far as not weeded out during the development of the microbial, cannot be predicted. Field experiments without incidents add to familiarity, but they belong to the past and they cannot predict the future. Some points may be raised here, in addition to the ecological concerns of mentioned above.

f. Genetic instability. The transgene should be chromosomal. The genetic stability of the microbial should be demonstrated[28].

g. Genetic instability. The transgene should be stably inserted into the chromosome(s) of the microbial and should not be transferable to micro-chromosomes or plasmids, in order to avoid easy transfer to other micro-organisms.

h. The number of inserts must be known, not so much for safety reasons as for post-release monitoring. High numbers of inserts should be avoided.

i. Pleiotropic effects should be avoided. The product of the transgene should not change the genetic regulation of emzymatic processes other than intended.

j. Pleiotropic effects should be avoided. The regulating elements (promotor, enhancers, introns) should not change the genetic regulation of enzymatic processes other than intended.

5. Concluding remarks

5.1. Technical and political judgements

This paper is inspired by the discussion on genetically modified micro-organisms for biological control of noxious organisms, primarily in agriculture, but most ideas are applicable to non-modified beneficial micro-organisms or 'microbials'. I rejected the classical approach to risk analysis, where risk equals hazard times probability, because science-based hazard indentification is hardly possible and probability estimation has no empirical support.

28. During mass production of GMM (as with the wild strains) undesirable mutations might occur which are not foreseen in the risk analysis.

To implement the precautionary principle I propose to summarize the ecological base-line knowledge for expert judgement, thus gaining familiarity with the wild-type and/or domesticated micro-organism and strain, by means of 'consensus reports' *sensu* OECD. I propose to introduce the notion of 'extended history of safe use in agriculture'.

The consensus reports must be fair, with which I mean that the assessment is acceptable to the industry as well as to the regulatory authority, and finds wide acceptation in the scientific community.

I stress the importance of risk communication before, during and after the administrative procedures. Risk communication is facilitated by familiarity. For risk management, e.g. by post-release monitoring, consensus reports are indispensable base-line documents.

5.2. Final conclusions

I arrive at my final conclusions.

1. Registration of beneficial microbials should be preceeded by a risk analysis.

2. The procedures used under EU Directive 90/220 may help to prepare for the application of EU Directive 91/414.

3. An overlay of procedures under these two Directives is useful, since applications under 90/220 for genetically modified microbials will also be subjected to 91/414.

4. To facilitate communication between regulators and scientists appropriate consensus documents should be written on 'microbials'.

5. An 'extended history of safe use in agriculture' should be a base-line concept.

6. Consensus documents are invaluable in the areas of risk assessment, risk management (including post-release monitoring) and risk communication.

I conclude with the words which are also applicable to risk assessment: *errare humanum est.*

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Characterisation and Identification of bacteria

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The functions of the German Culture Collection, DSMZ

The German Collection of Micro-organisms and Cell Cultures (DSMZ) is the national Service Collection.

The DSMZ is an independent, non-profit organisation dedicated to the acquisition, characterisation and identification, preservation and distribution of bacteria, Archaea, fungi, plasmids, phages, human and animal cell lines, plant cell cultures and plant viruses.

The DSMZ has, at present, more than 8200 Bacteria and Archaea,
100 bacteriophages, 2300 filamentous fungi, 500 yeasts, 1200 plant cell cultures, 700 plant viruses, and 450 human and animal cell lines. Only biological material of risk group 1 and 2 (see Guidelines "Safe Biotechnology" B 004, B 006, B 007, and B 008 of the Berufsgenossenschaft der chemischen Industrie) can be accepted.

Research at the DSMZ

The research focuses on collection-related fields including microbial taxonomy, molecular biodiversity, development of preservation methods for biological material (micro-organisms, cell lines, plant viruses), cell line characterisation and identification, as well as detection and elimination of mycoplasms and viruses from human and animal cell lines. The DSMZ co-operates closely with other national or international research groups in the fields of characterisation and identification of biological material. The DSMZ is recognised as a MIRCEN (Microbiological Resources centre).

There are co-operations with other culture collections world-wide and with other relevant national or international bodies such as ECCO, WFCC, WDC, MINE1.

1 ECCO = European Culture Collection Organization; WFCC = World Federation of Culture Collections; WDCM = World Data Center on Micro-organisms; MINE = Microbial Information Network Europe

Characterisation and Identification of Micro-organisms

Identification depends upon the quality of classification and taxonomy, which includes scientific studies on the diversity of organisms and their relationships. Taxonomy consists of:

- Characterisation, the achievement of the properties of an organism
- Classification, the arranging of organisms into groups
- Nomenclature, the assignment of correct international taxonomic names to organisms (International Code of Nomenclature of Bacteria).

Identification, the assignment of an unknown organism to a particular group of organisms makes use of characterisation methods, classification and characterisation to associate a strain to a certain species.

The DSMZ Identification Service identifies more than 1000 bacterial and fungal isolates with a variety of identification methods per year.

The main reasons to work and produce only with well defined and well characterised cultures with a known species name are obvious:

- Knowledge about hazardous groups of micro-organisms are very important for safety reasons in industrial production processes, for persons who work with the cultures and for laboratory regulations.
- Knowledge of the species name (and properties) facilities research.
- Processes in biotechnology need defined strains for process controlling, safety reasons and strain improvement.
- With the systematic name, scientists get access to ecological, medical, genetical and biochemical data in the literature including electronic authorities.

When a bacterial isolate reaches the DSMZ Identification Service laboratory the unknown strain is inoculated on complex media or on media advised by the customer. In other cases the use of special media with specific carbon sources, a special pH, vitamins or minerals is essential. If contamination of bacterial isolates is detected the customer is asked for subsequent strategies. When growth is obtained a purity check is necessary to assure the existence of a pure culture. This is an important prerequisite before the proper identification procedure can be started. If the customer agrees, different colony types are separated, and - if colony types keep on showing a different morphological and microscopical appearance - are screened by fatty acid analysis or in future ribotyping analysis. If dissimilar strains exist or in case of doubt, they should be identified parallel.

In case of a pure culture, working and stock cultures as well as a glycerol stock

should be prepared. The glycerol stock prevents loosing properties, eventually located on plasmids, during the identification procedure with complex media. In order to save time, all following methods are carried out simultaneous:

- morphological and microscopical studies (i.e. detection of spores, flagella, etc.), including a microphotograph
- partial sequencing of the 16SrDNA and phylogenetic analysis
- analysis of the cellular fatty acid composition
- primary physiological/biochemical tests
  (i.e. Gram behaviour, oxidase and catalase activity, etc.)

For practical reasons the morphological and microscopical investigations are carried out as first steps in an identification procedure to get a first impression of the unknown micro-organism.

Molecular genetic methods like 16S rDNA sequencing facilitate subsequent selection of tests to be implied in species identification. The partial sequence of the first 500 bp of the 16S rDNA bacterial gene is used by this method to determine the phylogenetic position of the unknown isolate next to it's neighbour included in the extensive DSMZ sequence databases. Sequence data from the European Molecular Biology Laboratory (EMBL)2 and the Ribosomal Database Project (RDP)3 are part of the DSMZ databases.

The Microbial Identification System (MIS) Whole-Cell Fatty Acid Analysis by gas chromatography (MIDI, Newark, USA) compares the resulting fatty acid pattern with a database provided by the manufacturer, which is completed by several DSMZ databases for special groups or new species.

In identification of micro-organisms certain general characteristics are of primary importance for determining to which phenetic group the new isolate most likely belongs (primary tests). For the 16SrDNA sequence analysis these tests are not essential but simplify the database research.

While the above mentioned identification methods are in progress, additional commercially available biochemical tests systems, like the API test strip or the BIOLOG-system can be performed to determine some of the most important physiological characters (secondary physiological tests). They show the advantages of miniaturisation, standardisation and speed determination of the result with a computer aided evaluation.

In the physiological identification of micro-organisms, however, the number of tests applied should, for practical reasons, be kept to a minimum. Usually, only characters known to have distinguishing value are studied. In addition, with respect to time and effort one should concentrate on simple methods as far as

2 http://www.ebi.ac.uk/ebi_docs/embl_db/ebi/topembl.html
3 http://www.cme.msu.edu/RDP

possible.

With the results of the sequencing and phylogenetic analysis, the fatty acid pattern and the partly automated biochemical identification systems, individual tertiary physiological tests to confirm the species or even subspecies identification are selected. The kind of properties to be tested to obtain a useful identification partly depends on the type of organism under study. Many characters used in microbial characterisation may be affected by cultural or other conditions. For these reasons such test methods must be standardised. These tertiary physiological and biochemical tests are in most cases carried out according to the 'Bergey's Manual of Determinative Bacteriology' (1994), the 'Bergey's Manual of Systematic Bacteriology' (1984-1989) and other relevant identification literature. For a number of isolates a more precise literature research, i.e. in the publications of the International Journal of Systematic Bacteriology (IJSB), is necessary to choose a test set for a reliable identification. A list of selected literature for identification purposes can be found in appendix 1.

For some groups of bacteria additional chemotaxonomical analyses are useful but not always essential for identification purposes. These methods are i.e. the HPLC-analysis of quinones or mycolic acids or determination of the G+C mol% composition of the DNA, thin layer chromatographic analysis of the cell wall peptidoglycan, DNA-DNA hybridisation, etc. Nevertheless, for some Gram-positive organisms, the analysis of the cell wall composition is a useful tool for identification.

The identification scheme of the DSMZ identification service can be seen in the appendix 2.

For some more 'exotic' isolates one has to deviate from this rigid scheme to receive satisfactory identification results. Due to the fact that different organisms or different taxonomic groups have been studied and classified by such a variety of methods, standardisation of identifications cannot be achieved (at the moment). Furthermore numerous genera and species were given new names due to their new discovered phylogenetic relationships and many new species and genera are described every year. Another fact to consider is that the molecular weight of the DNA in most micro-organisms is between 1×10^9 and 8×10^9, enough to specify some 1500 - 6000 average-sized genes. Therefore, even a battery of 300 biochemical tests would, at most, assay only between 5 and 20 % of the genetic potential of a given organism.

When failing to identify a culture one should check: (1) whether the appropriate tests had been carried out; (2) whether the methods are satisfactory (easily proved when a known control strain is included in the tests); and (3) whether the various methods and databases had been used correctly.
For identification at strain level the species or at less the genus membership of the isolate should be known before starting a more precise investigation.

Comparison of different isolates with each other can be done without a database. For identification purposes a nearly complete database should be available.

Suitable methods for this aim are the Ribotyping Analysis, DNA fingerprinting methods like Macrorestriction Analysis (with Pulse Field Gel Electrophoresis), Restriction Fragment Length Polymorphism, Fourier -Transformation Infra-Red spectroscopy and others. These methods need to be standardised strictly. If they are standardised (like Riboprinting), they share the advantage of high discriminative power.

For a reliable identification result most bacteria need the above mentioned simultaneous performed identification methods combined with secondary and in most cases some tertiary biochemical tests. By experience of the DSMZ Identification Service, a combination of primary, secondary and tertiary biochemical test, one or two partly automated commercially available test systems (API, BIOLOG) and the sequencing of the 16SrDNA bacterial gene usually leads to the affiliation of an isolate to a certain species.

For most of the identification systems the DSMZ has excellent databases. Commercially available databases are improved by numerous strains from the collection, because each identification method can only be as good as the included database allows.

A table with selected tests used for identification purposes can been seen in the appendix 3.

Identification - Literature

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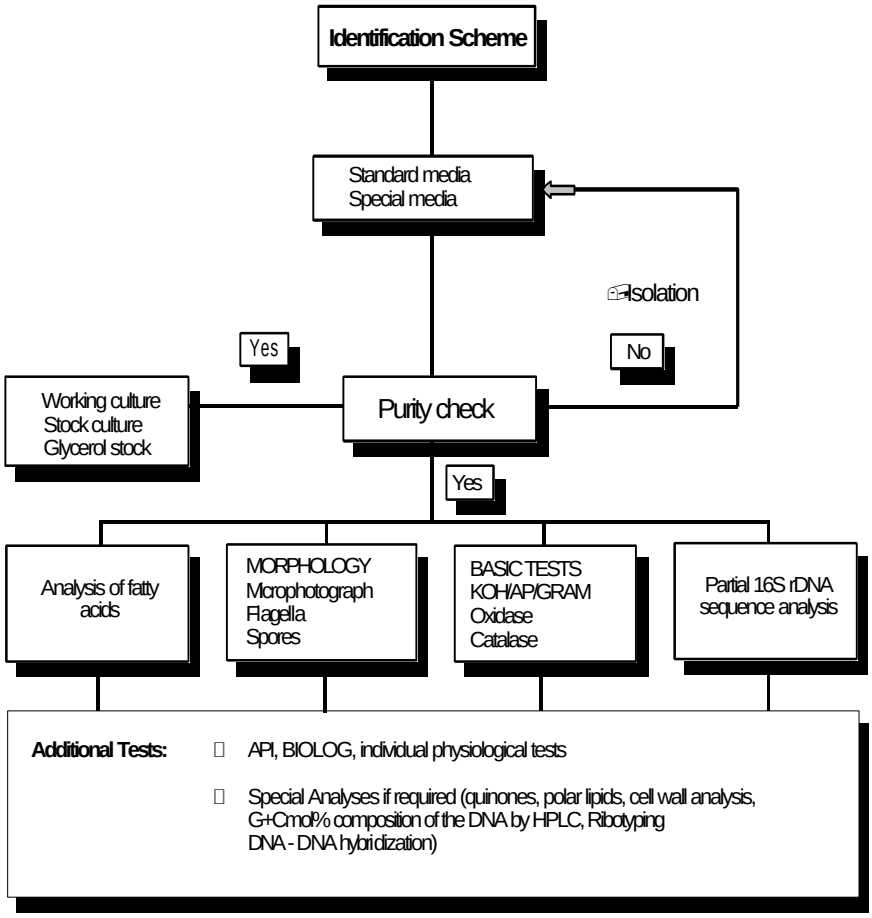
Steffen
Appendix 2



Selected Tests used in the Characterization of bacteria for Identification purposes

	Cell morphology and Gram behaviour	**Cultural characters**	**Molecular biological/ chemotaxonomical characters**	**Physiological characters**	**Biochemical characters**
Primary tests	Shape Arrangement Irregular forms Spores Sheats Buds, Appendages, Prosthecae, Stalks, Holdfasts Motility: > by swimming > by gliding GRAM reaction	Pigmentaion Colony morphology: > shape > size > elevation > edge > surface > opacity > consistency > emulsifiability	16SrDNA sequencing (complete or partial) Fatty acid analysis	Phototrophic Chemoautotrophic Chemoheterotrophic Aerobic growth Anaerobic growth Optimum temperature	Catalase activity Oxidase activity Dissimilation of sugars: > oxidative > fermentative > inactive
Secondary and tertiary tests		Growth in broth: > surface > turbidity > deposit	Ribotyping DNA/DNA homology G+C value DNA fingerprinting Cell wall composition Polar lipid analysis Menaquinones Ubiquinones Protein pattern Serology Phage susceptibility Pigment analysis And others	Temperature range pH range Nutrition Need for growth factors Gaseous needs Heat resistance Inhibition by: > antibiotics > selective media > NaCl > chemicals	Gas from glucose Acid from sugars ONPG Nitrite from nitrate Denitrification Voges-Proskauer Methyl red ADH ODC LDC Indol reaction Hydrogen sulfide Urease activity Lecithinase

					Phenylalanindeaminase Hydrolysis of: > starch > casein > DNA > esculin > gelatine > tween 80 Utilization of: > citrate > propionate > malonate > acetate etc Hemolysis Fermentation products And many others

Lecture at
Microbial plant protection products - Workshop on the scientific basis for risk assessment

Pathogenic properties of microorganisms - human hazard identification

Roland Möllby
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Ecology and pathogenesis

Pathogenic microorganisms consist of bacteria, virus, fungi and protozoa. I will mostly deal with bacteria, but most of the issues are the same for fungi and protozoa. For viruses, there are some different issues, that will be adressed separately.

Bacteria were the first living organisms on this globe, out of which fungi, algae and protozoa developed. Only ”lately” did mammals including humans evolve. Man has thus evolved together with all existing microorganisms and lives in a sometimes delicate balance with these. A number of different organisms, hundreds of species in fact, are constitutents of man´s ”normal bacterial flora”, but a limited number of species are known to be able to disturb this balance and under more or less normal circumstances cause harm or disease to humans. The ability to cause disease is named pathogenic ability. This property is for bacteria only interesting as long as it does give them a possibility to survive and grow better. However, the death of a host only diminishes the number of available hosts to grow upon and is not wanted by the bacteria.

For viruses this is another issue, since viruses are truly dependent upon specific host cells, that in many cases only human beings can provide, and the virus sometimes grows as much as possible without limitations.

Colonization and infection

It is thus apparent that many bacteria, useful, symbiotic and pathogenic may grow on man as ”substrate”, a condition called colonization. Some of the pathogenic bacteria may cause a disease, i.e. harmful effects to the host, which we may call an infection. Infectious diseases constitute a well studied concept in

medicine and are in numbers the most prevalent diseases in our society together with psychosomatic diseases.

There are two special properties of an infectious disease compared to other diseases: 1) it may spread to another person 2) you may treat it with drugs that affect other living organisms besides the cells of the host. The first property has always made the public very conscious and alert to these diseases, not to say scared in many cultures. In the European cultures, you might say that most dangerous infectious diseases are under control, due to measures taken by the society. These facts of course make it even more important not to allow actions that may increase the number of infectious diseases.

Knowledge of infectious diseases and their causes

Since the discoveries of the relations between infectious disease and microorganisms during the end of the former century, scientists have learnt more and more about the causes of infectious diseases and the possible agents. Interestingly, although more than 100 years have elapsed, we still (re)discover old microorganisms as well as completely new microorganisms as causes of disease. In rare cases, we even discover new diseases casued by ”old” or ”new” organisms. However, to almost all infectious diseases known, the causative microorganisms are well documented.

The discovery of a new infectious disease always appears from the collections of symtoms or syndromes observed in patients. These findings alert the medical society and microbiologists start looking for causative organisms. Eventually such organisms are found through epidemiological connections and laboratory work, sometimes on experimental animals if available models exist, but the latter is often not the case. Thus, we do not discover new infectious diseases through investigating microorganisms, nor in the laboratory nor in the animal house. This fact of course makes predictions from laboratory investigations on hitherto non-recognized pathogens difficult to interpret.

In certain cases, with very sensitive patients, like very young and very old, immunosuppressed, otherwise very ill patients, etc. almost any bacterium or fungus may be pathogenic. E.g. the well known health promoting species *Lactobacillus* has been isolated from the bloodstream of diseased patients, i.e. probably causing a septicaemia. Also environmental bacteria or fungi may appear as pathogens in such patients. This fact makes the distinctions between pathogenic and non-pathogenic somewhat arbitrary, but we usually consider possible effects on normal, healthy children or adults.

In almost all infectious diseases we know the cause of the infection. This means that we also know which bacteria that may cause a disease. If a certain species never has been incriminated as a cause of disease, it is highly unlikely that it would cause a disease only because it is used e.g. as a pesticide. In fact, bacteria that may act as pesticides are biologically very unlikey to be able to grow

favourably in human bodies and there compete with the natural flora. Furthermore, such a finding would be a scientifical rarity and would cause much alarm and interest from medical and microbiological societies.

Pathogenic microorganisms

The above statements mean that:

on the one hand

- we know what microorganims that are pathogenic and which are not

but on the other hand

- there is no clear difference between pathogenic and non-pathogenic microorganisms.

Human hazards from microorganisms

1. ”Normal” infectivity and pathogenicity
2. Infectivity from high concentrations (e.g. in the production process)
3. Toxin production
4. Sensitization
5. Infectivity to very sensitive individuals

1. ”Normal” infectivity and pathogenicity

In reality, it is relatively simple for a medical microbiologist to tell whether a certain species may cause a disease or not during normal circumstances, since the pathogenic microorganisms are known. However, in a limited number of cases there may be doubts as to the infectivity and pathogenicity of a species or a specific strain thereof. In such cases certain test procedures may be needed to evaluate the pathogenic properties of the microorganism.

2. Infectivity from high concentrations (e.g. in the production process)

The infectivity of high concentrations of certain bacteria may be unknown. To a limited extent it can be estimated from animal experiments, appropiate experimental models for aerosol inoculations do not exist. The production process is supposedly designed so as not to expose the workers to aerosols of high bacterial concentrations, but accidents may occur and a preknowledge of existing hazards is good to have.

3. Toxin production

Microorganisms may produce toxins that stay in the product or possibly are formed in the environment after spreading. Whether such toxins are formed may be possible to assay in advance, although the circumstances during which they may be formed are often difficult to reproduce. When the fact that toxins are present is established, this might be considered as a question of a contamination by chemical compounds, but need to be handled by specialised toxinologists.

4. Sensitization

Finally, microorganisms are made up by biological, i.e. organic compounds, and they always have an allergenic potential. Some compounds are probably more sensitizing than others, but again, there are few relevant animal models to test for this capacity. The sensitizing problem may be related to the microorganisms as such and/or extracellular products, like toxins or enzymes. Again, this problem is most relevant during production and handling of the products.

5. Infectivity to very sensitive individuals

This constitutes a problem, both with regard to definition of pathogenic microorganisms and as to testing for such infectivity. It has to be assumed in the risk evaluation, that individuals to become exposed to the product are of reasonably normal health, i.e. they do not belong to the cathegory of very sensitive individuals. It can be reasoned that very sensitive individuals are not in the production or handling of the product line because of their handicap/disease. When they are out in the environment where they accidentally may become exposed to the product, the risks from all other microorganisms in the environment, especially from humans around, is of a much greater magnitude.

Pathogenicity

Colonisation requisites	**Pathogenic (damaging) mechanisms**
Ability to:	Ability to:
•spread and infect	•degrade (enzymes)
•metabolise/grow/divide	•kill local cells (local toxins, attachment)
•survive defense mechanisms	•invade cells
•stay on the site (adhesins)	•be toxic (periferic toxins)

Tests for pathogenicity

Unfortunately there are very few good tests available for *in vitro* pathogenicity evaluations. Cultured cells may be used to show the ability of bacteria to specifically attach to or to invade human cells, but that is only valid during very limited circumstances. When certain virulence factors of a microorganism are known and anticipated, like enzymes, toxins or adhesins, the production of them can be measured by normal protein (immuno)techniques or the existence of their genes by standard DNA techniques (probing, PCR), but this can only be used to rule out known risk factors.

Furthermore, it is evident that the colonisation factors (above square 1) are very difficult to assay for *in vitro* , i.e. outside a living body. This leaves us with animal experiments.

Animal experimental models

On animals we can assay the infectivity of a microorganism, i.e. whether it may cause an infection or not, taken into consideration all of the above virulence criteria. Different routes of administration may be used and the infectious dose may be determined through titration. However, besides the ethical problems with this there are a number of biological uncertainties, which are more or less self-evident if one starts to scrutinize these methods. In short, we do not have the experience to predict a disease by microorganisms from animal experiments, since this has not been done earlier.

Some problems with animal models

Correct animal species.
How can the results be transferred/translated to humans? All experiments can not be performed in monkeys, which are the only reasonably reliable species to be used, but even that is not perfect. There are several common human infections, for which we do not have any animal experimental model at all, including monkeys. The popular rodents are not selected because of their biological similarities to humans, but because of their ease at handling.

Route of administration.
How do we expect the pesticide product to get in contact with humans? Consumption, breathing of dust, bathing water, aerosol inhalation, animal contacts, etc. How can that be mimicked in the animal experiment ? Definitely, in almost no case do we expect the product to be injected intraperitoneally...

Infective dose.
What doses of microorganisms do we expect humans to be submitted to and how can that be translated to/from the animal experiment? Different species are susceptible to varying degrees, why this is related to the first problem.

Very sensitive individuals.
We would like to test the hazards of the specific microorganism also for more sensitive individuals, and at least in mice and rats there are a number of breeds with various decreases in their immune response. However, again this is difficult to translate to the human situation, but at best it can give some hints as to which kinds of immune deficient patients that may be at a higher risk.

Testing of microorganisms versus chemical compunds

The tests for microorganisms are always compared to those for chemicals to be used in nature. There are, however, some points of considerable difference between the two types of testings.

Natural versus synthetic (new) substances

Microorganisms are naturally living cells, as stated above existing long before mankind, and now living in a stable equilibrium with other living creatures. The existing pathogenic micoorganisms are relatively well known. New synthetic chemicals, on the other hand, did not exist earlier and thus we have no biological knowledge about them whatsoever, and their are a number of examples on how they have unexpectedly damaged the environment and human health.

As for genetically manipulated organisms (GMO:s), the difference is made up by the added/deleted gene. It is comparatively easy to anticipate or test for whether this genetic change has increased or possbily decreased the pathogenic potential of the actual microorganism, since we here deal with known organisms and genes.

Infection versus toxicity

Chemicals do not cause an infection. However, microorganisms may be toxic, i.e. some of their exo-products or endogenous parts, like the cell wall and the membrane lipopolysaccharide (endotoxin), may cause a toxic response in the host upon contact. This problem is a matter for (microbial) toxinologists, and poses normally different questions than those handled by traditional toxicologists.

Acute versus long-term toxicity

Microorganisms produce among other compounds nucleic acids, proteins and carbohydrates, with and without lipid parts, i.e. normally big polymeric molecules that affect the immune system of the individual. This means that the hosts immune system is activated upon the first contact, and the effects of this activation increases for every new contact. Thus, upon repeated exposure to the same microorganism or its products, the host is normally immunized and long

term toxicity does not exist for microorganisms. There are a few exceptions to this statement, e.g. certain fungal mycotoxins are small molecules and may cause a long term toxicity. The production of these may be assayed separately.

Sensitization

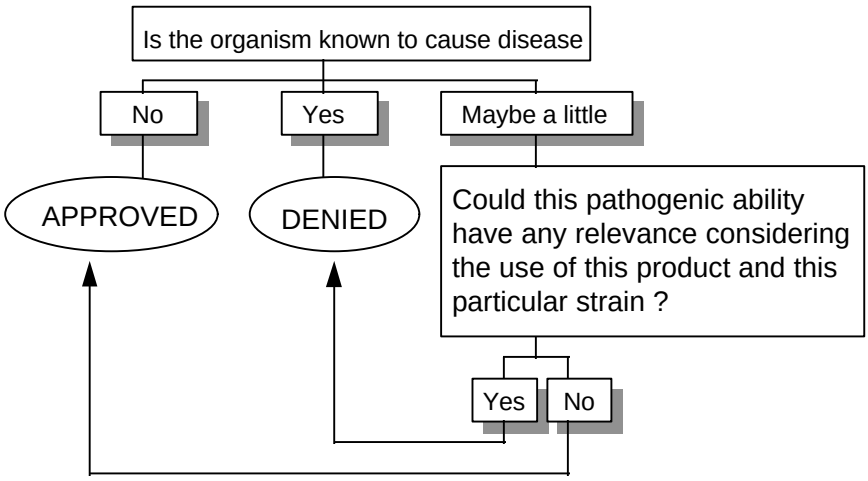
This may pose a problem by certain microorganisms, as may repeated impropriate administration to the host of any big molecules (like polycarbohydrates and proteins) do. Again, this is difficult to test for, both for chemicals and microorganisms, but some standardized animal test systems exist, the translatable value of which is hitherto unrecognized. For chemicals, the problem is often skin allergy, while microorganisms may cause a kind of allergic response in the bronchi upon repeated inhalation of larger doses. It is difficult to test for but should be kept in mind.

Should we have lower demands on scientific biological knowledge for microorganisms as compared to chemical compounds ?

It is of course important to make clear that the demands on sound scientific facts for the safety judgements of microorganisms should be no less than for other agents. They may, however, be different. They may, also, in many cases come from existing knowledge on the microorganism to be judged, and less from tests performed by the producer. Lastly, since the infectious process may be considered as a disturbance of the ecological balance between micro- and macroorganisms (e.g. bacteria and man), and in spite of all biomedical research for more than a century, the mechanisms behind this balance are so poorly understood, uncritical demands for tests to be performed on microorganisms may give us information that is of little value to the important final judgements to be made. Instead, there is a need for more toxinological and microbiological expertise in this field, in order to avoid stereotypical forms and animal experiments, and who are instead able to ask for the exact relevant information in each case.

An example of a suggested simplified questionnaire, or at least a way of reasoning is shown below.

Microbial products to be released in the environment



Follow up demands

In summary, we do not have enough knowledge on how to extract information from animal experiments to the human situation, and all infectious diseases have primarily been discovered as diseases and not as organisms. It is thus of outmost importance to follow up the use of the microbiological pesticides and carefully monitor for diseases in workers producing and using them and in the possibly exposed population. For this purpose relevant authorities dealing with occupational and with community health have to be alerted to possible unexpected health problems and cooperative monitoring groups should be formed for each new microbiological product.

Biological products and humans are **biological**, i.e they constitute a part of the great mystery of life, and are therefore less predictable than we would wish.

Fate of micro-organisms introduced into soil, effects on autochthonous communities and activities

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1 - Introduction

In agriculture and forestry, different types of beneficial organisms can be applied to either improve the growth of the plant (PGPR : Plant Growth Promoting Rhizobacteria), increase the yield (Y.I.B. : Yield Increasing Bacteria) or control diseases and pests (BCA : Biological Control Agents). These beneficial organisms include viruses, bacteria and fungi, but also nematodes, mites and insects belonging to several families. The European Directive 91/414 only applies to micro-organisms used to control diseases or pests, which are considered as pesticides! The micro-organisms applied to improve plant growth and often considered as biofertilizers escape to this directive. To our opinion, whatever the intended use, all micro-organisms released in the environment should be considered similarly. Risk assessment has to be based on the biology of the micro-organisms including their modes of action, ability to survive in the environment and innocuousness for non-target organisms.

This paper will briefly summarise the main modes of action of beneficial organisms used in agriculture, and then consider their fate in soil and effects on non-target communities. Based on the already available knowledge it seems possible to conclude that microbial inoculation of soil has only a transient effect although some traditional cultural practices affect more drastically the microbial balance of the soils.

2 - Modes of action of beneficial micro-organisms

In this short presentation, we will have no time to review in details the mechanisms by which micro-organisms improve plant growth or control diseases and pests. However it is absolutely needed to summarise briefly our knowledge in this domaine. Indeed, if we believe that growth promoters and biocontrol agents have to be considered similarly, it is mainly because their modes of action and their fate in the environment are similar. Knowledge of the modes of action is important to determine hazard and evaluate the risks linked to application of these micro-organisms for agricultural purposes.

Growth promoter micro-organisms may:

(i) produce plant hormones or metabolites similar to plant hormones, having a direct effect on plant growth,

(ii) help the plant to assimilate nutrients such as nitrogen, phosphorus or micro-nutrients,
(iii) modify the microbial balance in the rhizosphere and control deleterious micro-organisms as BCAs,
(iv) induce systemic resistance into the host plant by stimulating the natural defence reactions of the plant.

Biocontrol agents limit plant disease incidence or severity through four main mechanisms :
(i) hyperparasitism : it is by recognition of the microbial host, penetration into the host cell and enzymatic digestion of the cell content,
(ii) antibiosis : that is by production of different types of secondary metabolites (antibiotics, enzymes, antifungal compounds, ..) inhibiting the growth or the activity of the target pathogen,
(iii) competition for nutrients, oxygen or space ; it must be recalled that the soil is an oligotrophic environment where micro-organisms are suffering from starvation,
(iv) Induction of local or systemic resistance into the host plant by stimulating the natural plant defence reactions.

It must be stressed that most of the micro-organisms have several modes of action acting independently, successively or simultaneously. It is therefore always difficult to fully understand the modes of action responsible for the beneficial effect of the micro-organism on plant growth or disease control.

Obviously, knowledge of the modes of action is useful when addressing the question of fate of the introduced micro-organisms in the environment. The modes of action described above are not strictly directed against the plant pathogen, they may have effects on other saprophytic micro-organisms and help the biological control agent to survive in the soil or to colonise specific niches.

3 - The soil: an heterogeneous environment harbouring a large diversity of microbial communities

The soil is made of a solid phase representing 93 to 95% of the total weight of the soil, a liquid phase and gazes. The minerals come from the degradation of the parent rocks and the individual particles are usually bound together into aggregates of different sizes. These aggregates are made of grains of sand and silt bound together with clay and organic matter particles by organic cements such as polysaccharides and mucigels produced by micro-organisms and plant roots. The space between aggregates forms a network of pores of different sizes, filled with either an aqueous solution or gazes.

This abiotic matrix harbours a large diversity of organisms of different sizes. The soil microbiologists focus on bacteria and fungi, which represent the largest biomass in the soil. But the microbial community also includes protozoa, algae, nematodes and micro-arthropods, and the total biomass of a soil includes larger organisms such as earthworms, springtails and mites. These organisms

are not evenly distributed in the soil. And just to focus on micro-organisms, it is

surface of the soil aggregates, but some of them can be included with organic matter inside the aggregates. The hyphae of fungi are mostly located at the

also run from one aggregate to another through the pores.

Interactions between BCAs and target populations will depend on the

important to consider since, depending on their location, the population density of given micro-organisms will be more or less difficult to assess.

of sampling when tracking an introduced population into soil.

To finish with this brief presentation of the complexity of the soil

fact that probably no more than 10 per cent of the existing microbial populations are actually known. Using molecular techniques, it is possible to

species of micro-organisms. Whether these molecules of DNA correspond to living organisms, not yet cultivable, or to DNA of dead organisms, attached to

physiological state, a given species of micro-organisms may change of shape in the soil and become uncultivable although still alive and able to exchange

gene dissemination in the environment, one must remember that there are plenty of DNA sequences of unknown origin in the soil.

4.1. – Direct methods

The soil being an heterogeneous and opaque milieu, direct observation

When or where very high population densities are present it may be possible to prepare soil smears and to directly observe bacterial cells or fungal propagules

used, to locate microbial populations in relation to the abiotic soil constituents. The new environmental scanning microscopy should be very useful to observe

When antibodies can be raised to specifically bind with the epitopes of the bacteria at the strain level, quantification of these bacteria can be realised by

detection threshold, which is generally high with regard to the requirement for risk assessment.

With the exception of the latter technique, other direct observation techniques are not quantitative and do not allow following the population dynamics of a population of micro-organisms introduced in the soil.

4.2. – Indirect methods

All the other techniques are indirect techniques; they imply either the growth of the microorganism on a specific medium or the extraction of the microorganism, or of the nucleic acids from the soil.

The isolation based methods are suitable only for cultivable organisms. The main problem is that one can not differentiate the introduced strain from the autochthonous strain belonging to the same species, unless specific labelled antibodies can be used to discriminate the strain.

More sensitive are the molecular methods targeting at specific DNA or RNA sequences. Although different genes can be targeted, the most utilised methods are based on the analysis of ribosomal DNA sequences. Indeed these genes are universal and contain both conserved and variable regions, enabling discrimination of the micro-organisms at the genus, the species and the strain level.

Two main types of methods can be used: either PCR amplification of specific DNA sequences using primers allowing detection of the target organisms, or hybridisation with fluorescently labelled rRNA targeted probes. This second approach targeting to the ribosomal RNA has the advantage to give information on the general metabolic activity of the micro-organism since there is a direct correlation between ribosomal RNA and the activity of the cell. Hybridisation can be applied on microbial colonies allowing a rapid identification of the target organism. Both methods can also be used *in situ* not only to detect but also to quantify the population density of a micro-organism.

Fluorescent oligonucleotide probing can be used not only to monitor the distribution and follow the survival of an introduced micro-organism but also to study the structure of the microbial communities. Although very sensitive these molecular techniques are not yet widely used to monitor the population kinetics of an introduced strain; there is a need for further technical development to make them routinely available.

4.3. – Use of marked strains

Today, the most common approach to track a given organism in the soil is to use marked strains that can be easily differenciated from the autochthonous population of the same species.

Different types of marked strains can be produced either by mutation or transformation of the wild-type micro-organisms. A first category of very useful marked strains are natural or induced mutants resistant to antibiotics or

fungicides. A second category are micro-organisms in which a luminescence gene g*us, lux, gfp* has been introduced.

The main problem encountered with this approach is that one follows the survival of the marked strain and not that of the wild-type strain. The mutation or the transformation might have affected some traits controlling the antagonistic capacity or the saprophytic ability of the micro-organisms. Moreover these techniques enable the detection and the quantification of viable or metabolically active micro-organisms; they do not allow detection of the uncultivable forms of the micro-organisms.

5 - Survival in soil of an introduced micro-organism

The fate of a microbial population introduced into soil either directly or through seeds or planting material, obviously depends on the type of micro-organism, and on its origin. Whether the micro-organism belongs to a genus or species naturally occurring in soil or not, it will either establish in soil for very long periods of time or disappear very quickly.

One example taken from Richaume *et al*.(1990) demonstrates that the density of a strain of *Escherichia coli*, which normal habitat is the digestive tractus of animals, declines rapidly after its introduction into soil and, as soon as 25 days after inoculation, it is no more possible to detect this bacteria in the soil. On the contrary, when inoculated at a high density close to 10^8 bacteria g^{-1} soil the telluric bacteria (that is bacteria which have soil as a normal habitat) show first a clear decline of their population densities which later establish at a level close to 10^5 cells g^{-1} soil for *Azospirillum lipoferum*, *Agrobacterium radiobacter*, or to 10^6 cells g^{-1} soil for *Pseudomonas aeruginosa*. These results obtained in microcosm experiments have been confirmed by field experiments showing that a strain of *Bradyrhizobium japonicum* was still easily detectable in 52 field soils where it has been introduced 13 years ago.

The same good ability to survive in soil has been showed for telluric fungi, able to colonise the organic matter or to live saprophytically at the root surface of several plant species. For example, Davet (1983) demonstrated that *Trichoderma harzianum* was able to survive saprophytically for at least 3 years in a field soil. The population of *Trichoderma* reached a density greater than 10^5 CFU g^{-1} soil immediately after inoculation, then decreased slowly. Three years later, at the end of the experiment, the population density of *T.harzianum* was still 10 times greater in the treated plot than in the control, where the wild population of *Trichoderma* spp. established at 3×10^3 CFU g^{-1} soil.

Many other examples from the literature show that the micro-organisms originating from soil will survive for long time after their re-introduction into soil.

Obviously, the population dynamics of introduced micro-organisms is depending on the strain and on the soil type (Alabouvette and Steinberg 1995). We do not have time to develop this aspect, but it is important to stress that

different strains of micro-organisms belonging to the same species may differ in their survival capacity in soil. And there is also many papers reporting that a given strain of bacteria or fungus has established at different levels in soils of different textures.

The main abiotic characteristics that affect population dynamics and microbial activity are the pH, the texture and the nature of the clays. Plant pathologists know very well that some diseases induced by soil-borne plant pathogens only occur in soil of low pH (*Plasmodiophora brassicae*) when others are favoured by higher values of pH (*Streptomyces scabies*).

Results obtained in one soil type can never be generalised to other soil types, and fate of an introduced organism used for growth promotion or biological control has to be assessed in a small collection of soils representative of the main categories of agricultural soil.

6 - Effects on the autochthonous communities and activities

As already stated, the modes of action of the biological control agents are not specifically directed against the target pathogens and most of the growth promoting micro-organisms also modify the microbial balance in the rhizosphere of the plant where they are introduced. Therefore there is a need to consider the effects of inoculum introduction on the autochthonous communities.

One must admit that there are, at the present time, no well-established methodologies to assess the modifications of the microbial balance induced by massive introduction of exogenous micro-organisms.

Two main types of approaches can be followed: assessment of changes affecting the diversity of the microbial communities or assessment of changes affecting the pattern of activities of the microbial communities in soil.

The first approach is now possible due to the development of a molecular technique called « denaturing gradient gel electrophoresis » (DGGE) which enables the detection and the characterisation of known bacterial and fungal species in soil without having to isolate them.

The second approach is based on the use of the « Biolog » system, which enables the characterisation of the metabolic activities of the global soil microflora.(Garland and Mills, 1991)

Both methods present advantages and inconveniences. The first one can be used to detect modifications affecting the microbial communities. It allows detecting new genotypes by comparing the DNA patterns before and after the introduction of the beneficial micro-organism. The second one enables to detect modifications in the ability of the soil communities to degrade given substrates, by comparing the patterns of microbial activities before and after inoculation of the soil.

Both methods have to be used at several time intervals after the introduction of the micro-organisms not only to detect changes but also to determine the speed at which the soil will return to its initial stage. Indeed to

our opinion, rather than to focus on the changes affecting the microbial balance, it would be more interesting to determine the capacity of the soil to return to its initial stage.

Recently we utilised the Biolog System to characterise the microbial activities in 3 soils showing different levels of soil suppressiveness, and to detect changes affecting the microbial communities after introduction of a biocontrol agent in these three soils.

Suppressiveness caracterises the capacity of some soils to control plant disease induced by soil-borne plant pathogens. In suppressive soils, disease incidence remains low in spite of the presence of a high inoculum density of the pathogen. Suppressiveness is an important concept stating that any soil has some capacity to control both the survival and the activity of any introduced micro-organism.

The three soils differing by their level of suppressiveness to fusarium wilts also greatly differ in their pattern of metabolic activities as revealed by the Biolog System.

After introduction of the biocontrol fungus aiming at controlling fusarium wilts, the level of soil suppressiveness of the soil has changed, even the conducive soil was suppressive. Correlatively the pattern of microbial activities has changed, reveally some effect of the biocontrol fungus on the autochtonous communities. However, this effect was transient and one month after application of the BCA, the metabolic activities seem to return to their initial level.

Conclusion

Release of micro-organisms either natural wild strains or genetically modified strains requires risk assessment studies, since safety is a major component determining public acceptance of this new technology. For micro-organisms as for chemicals, information is needed on their fate and possible undesirable effects in the soil ecosystem.

As micro-organisms totally differ from pesticides, it is not possible to have the same requirements and the guidelines for risk assessment have to be adapted for micro-organisms. It must be stressed that there are generally no toxic chemical residues, the « residues » being the micro-organisms themselves and their metabolites surviving in the environment. The population density of the introduced micro-organism will be regulated according to general laws of microbial ecology that state that, in the absence of a selection pressure, a microbial population will reach an equilibrium with the soil microbiota, corresponding to the « carrying capacity » of the soil. This carrying capacity depends both on the characteristics of the soil and on the microbial strain. That means, that the introduced strain will survive among other strains belonging to the same species without inducing significant changes of the population density of this species. Indeed, inoculation of a soil generally results only in a temporally and locally increase in the population density. It is why many studies are aiming at modifying beneficial strains to improve their survival capacity in

the soil environment. It would be meaningless to require the disappearance of a micro-organism from soil when it is a naturally soil-borne micro-organism.

Since the introduced strain will survive, one must address the question of effects on the rest of the microbiota.

Until now there is no generally adopted method to study effects of microbial inoculation of soil on the autochthonous communities Two main types of techniques are being developed, one is describing modifications affecting the genetic structure of the microbial diversity, and the other is also dealing with modifications of the diversity, assessed through measurements of the metabolic activities of the soil biota. More research is needed to compare advantages and inconveniences of these two approaches, but one may predict that a combination of both methods will be the best approach to characterise effects of microbial inoculation.

But, based on already published data, one can conclude that introduction of microbial strains, even at a high concentration, generally induces fewer effects than some traditional cultural practices. Without speaking of manure applications that correspond to the introduction of millions of micro-organisms of different species, including human pathogens, compost application, which is actually encouraged by the environmental agencies, considerably affect the microbial balance of the soil. Studying the beneficial effect of compost amendment on the level of suppressiveness of a soil to fusarium diseases we were able to show that introduction of the sterilised compost (sterile organic matter) induced dramatic changes in the microbial balance and activity of the soil.(Serra-Wittling et al.,1996).

Thus, when regulating release of micro-organisms for agricultural purposes, one must remember that the soil has an enormous buffering capacity and that the most common agricultural practices, such as tillage, modify much more the microbial balance than the introduction of a single strain of micro-organisms.

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Workshop on the Scientific Basis for Risk Assessment of Microbiological Plant Protection Products, Stockholm, Sweden 26-28 October, 1998

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Agreed document

Identification and characterisation / biology of micro-organisms

Each micro-organism should be i) deposited at a recognised culture collection and given an accession number; ii) identified and named at the species level; iii) characterised at the strain level using best available and relevant technologies. Best available technology, e.g. bioassay and molecular tests, should be used to characterise the effective strain. Identification including characterisation may become the key point for decision making for authorisation of microbials.

The principal mode of action should be indicated. A producer finding a micro-organism/strain usable for plant protection usually has knowledge about and is familiar with its phytopathological characteristics and mode of action. In connection with the mode of action it is also important to know if the micro-organism produces a toxin.

Many micro-organisms produce some antibiosis substances. Interference with the use of antibiotics in human and veterinary medicine must be avoided.

Familiarity, interpreted as the availability of substantial knowledge of the micro-organism, should be included in the submitted documentation.

It was recognised as desirable and could facilitate authorisation to have consensus documents on existing knowledge and to have a consolidated list of micro-organisms generally regarded as safe.

Production control

There is a need for continuous quality control, by the applicant, of both production and end product. In particular, the occurrence of spontaneous changing of major characteristics of micro-organisms and of the absence/presence of significant contaminants should be monitored. The applicant should submit the quality criteria for the end product.

It is important to control the manufacturing process. It is desirable to have microbial PPPs without contaminants, if possible. Level of acceptable contaminants should be judged from a ”risk acceptance” point of view.

Fate and behaviour/environmental adverse effects

Experimental data are not normally required *i.e.* where it can be justified that an assessment of fate and behaviour in the environment can be performed with available information.

The choice of appropriate non-target organisms for testing should be based on the identity of the micro-organism (including host specificity, mode of action and ecology).

Post-approval monitoring should be considered for all areas of risk assessment. This is particularly the case when exotic micro-organisms are considered for approval. Conditional approval in combination with monitoring could be considered.

Adverse effects on human health

There is a need to identify ”critical endpoints” to define the risks for human health when using micro-organisms as plant protection products. By identifying such critical endpoints, priorities can be set for development and establishment of appropriate data requirements and guidelines. This will also be useful when defining mutual acceptance criteria for inclusion of the micro-organism in Annex I of directive 91/414/EEC

The first and most important step in the evaluation of a micro-organism would be to thoroughly characterise the micro-organism concerning its:
- ability to colonise
- ability to cause damage
- ability to produce toxins (endo- and exotoxins) and other relevant metabolites

Available information based on the properties of the micro-organism and corresponding micro-organisms, including health and medical reports may be sufficient for a decision whether the micro-organism would cause adverse health effects (infectious/pathogenic/toxic) in humans or not. In this case no further testing is necessary. If, however, the information is not sufficient for such a judgement, it would be necessary to perform further studies. In order to correctly interpret the obtained results, it is of greatest importance that the suggested test methods are relevant regarding sensitivity, administration route

etc. and relevant from a biological and toxinological[1] point of view. New test guidelines have to be developed and validated preferably within an OECD context.

Sensitisation

The available methods for testing dermal sensitisation are not suitable for testing micro-organisms. Sensitisation by inhalation is most probably a greater problem compared with dermal exposure to micro-organisms but so far, there are no validated test methods. Development of these kinds of methods are therefore of great importance. Until then, all micro-organisms should be regarded as potential sensitisers. This approach also takes into consideration immuno-compromised or other sensitive individuals in the population (pregnant women, new-born children or elderly).

Residues and contaminants

When no adverse effects are identified from the proposed use, residue data are not relevant. The term residue in food is only relevant for such micro-organisms producing toxins that may be of biological significance. Maximum residue levels (MRL:s) have to be established for the toxin. The term contaminants is here taken to mean micro-organisms (from the product) remaining on the harvested plant for use as food.

Comments on suggested test in the present data requirement document in directive 91/414

Is the list of tests in the guidance document acceptable or not?

- Suggested test on immuno-suppressed animals is not relevant
- Skin and eye irritation?
  North America: skin and eye tests are preferably performed on the formulated product.
  EU: eye irritation studies do not take eye infections into consideration.
- Acute infectivity/pathogenicity/toxicity tests are necessary and all administration routes could be of relevance:
  What end points do we expect?
- Genotoxicity tests: if there is relevant exposure to toxins there is a need for testing (fungi and *Actinomycetes* should be specially characterised, and consideration be made if tests are needed)
- Short-term toxicity testing (subacute or subchronic): Justification, both for performing the tests or not, is needed.

[1] Toxinology is interpreted as the learning of toxins produced by living organisms

Summary: more questions than answers were generated by the discussion.

Conclusion

The most important and informative information is obtained by the characterisation and identification of a micro-organism. Further tests and studies, if necessary, should be performed on a case-by-case basis within the established list of a pre-set of data requirements. It is appropriate to continue with the tiered approach for assessing risks.

Use pattern and worker exposure

Is it a good way to provide precautionary measures even if there is no known danger? All micro-organisms we are speaking about may have biological effects even if they are not pathogenic. We have to base the precaution on special characteristics. If there is no risk identified, it is not necessary to have precautionary measures. However, all micro-organisms should be regarded as potential sensitisers. The operator may have to use protective equipment but in a small-scale use (gardening) it is argued that this might be problematic.

There can be a difference in exposure due to use pattern (where for instance, contained use is to be handled different compared with use in the open field).

Post-approval monitoring should be considered for all areas of risk assessment.

Formulation

Development stages of the micro-organism as well as the type of formulation have to be taken into consideration when assessing the product.

The exposure can be limited by choosing appropriate formulations or appropriate application methods.

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