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Factsheets for the (eco)toxicological risk assessment strategy of the National Institute for Public Health and the Environment (RIVM), Part III

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Abstract

Four factsheets describing risk assessment methods used at the Expert Centre for Substances (SEC) and the Centre for Substances and Integral Risk Assessment (SIR) of the National Institute for Public Health and the Environment (RIVM) are presented here with the main aim of promoting greater transparency in the risk assessment methods used at the Institute in general and within the two Centres in particular. The factsheets, listed below, reflect a state-of-the-art approach; they are also meant to function as a platform for discussion.

1. Hazard assessment for direct immunotoxicity
2. The interpretation of hepatic peroxisome proliferation and associated hepatocellular carcinogenesis
3. Environmental risk assessment scheme for plant protection products: birds and mammals
4. Environmental risk assessment scheme for plant protection products: non-target terrestrial higher plants

The first 2 factsheets are related to human risk assessment and the last 2 to environmental issues. Remarks, omissions or additional information sent to the editors (first name) will be appreciated.

Preface

This report was written within the framework of the project 'Risk Assessment of Substances: Science and Market'. The first two factsheets as presented in this report have been discussed by members of the human peer review group of the Centre of Substances and Integral Risk Assessment (SIR), and in some cases experts were consulted. All are acknowledged for their contribution. These members and experts are: M.E. van Apeldoorn, R.B. Beems, J. van Benthem, S. de Boer, A.G.A.C. Knaap, F.X.R. van Leeuwen, H. van Loveren, and M.T.M. van Raaij.
The two environmental risk assessment schemes have been peer reviewed by the members of the Panel on Environmental Risk Assessment of the European and Mediterranean Plant Protection Organization (EPPO) and the Members States of the EPPO.

Contents

Samenvatting

In dit rapport worden 4 factsheets gepresenteerd, die voor de risicoschatting van stoffen binnen het Stoffen Expertise Centrum (SEC) en het Centrum voor Stoffen en Integrale Risicoschatting (SIR) gehanteerd worden. De eerste 2 factsheets hebben betrekking op de humane risicoschatting en de overige 2 factsheets op risicoschatting voor het milieu.

In de factsheet **'Hazard assessment for direct immunotoxicity'** wordt een procedure beschreven waarmee de mogelijke immunotoxicologische eigenschappen van een stof kunnen worden bepaald tijdens de gevaarsevaluatie (hazard analysis). De factsheet beoogt beoordelaars meer houvast te geven bij het beoordelen van immunotoxische effecten en zal leiden tot meer consistentie. In de eerste plaats wordt in gangbare toxiciteitstesten gekeken of effecten optreden die duiden op potentiële immunotoxiciteit. Als tweede stap kan gebruikt gemaakt worden van functionele immunotoxiciteitstesten. Als een derde stap kunnen infectie-modellen worden gebruikt. Afhankelijk van de uitslagen in deze testen kan worden geconcludeerd of de stof wel of geen potentie heeft om directe immunotoxische effecten te veroorzaken. Bij het vaststellen van humane grenswaarden voor immunotoxische stoffen kunnen de gebruikelijke 'default' extrapolatiefactoren worden gebruikt.
De factsheet '**The interpretation of hepatic peroxisome proliferation and associated hepatocellular carcinogenesis'** wordt een procedure beschreven hoe om te gaan met stoffen die in knaagdieren (rat, muis) proliferatie van peroxisomen induceren. In de factsheet wordt een beschrijving gegeven van de mechanistische aspecten van dit fenomeen in de lever en de relatie hiervan met carcinogenese. Daarnaast wordt vastgesteld onder welke condities peroxisoomproliferatie in de rat of muis als niet-relevant kan worden beschouwd voor de mens.
In de factsheet '**Environmental risk assessment scheme for plant protection products: birds and mammals**' wordt een risicoschattingsschema beschreven waarmee de effecten van gewasbeschermingsmiddelen op vogels en zoogdieren kan worden gekwantificeerd. In tegenstelling tot vroegere risicoschattingsschema's voor vogels en zoogdieren wordt het risico gebaseerd op de dagelijkse ingenomen hoeveelheid van een stof. Hierdoor wordt voorkomen dat er niet gecorrigeerd kan worden voor verschillen in voedsel opname tussen laboratorium dieren en vrijlevende dieren in de natuur. Een andere belangrijke verbetering is dat aannames, extrapolatiefactoren en onzekerheidsfactoren expliciet vermeld worden daar waar ze thuishoren in de berekeningen. Dit maakt de aannames/veronderstellingen transparanter en geeft de gelegenheid aan de gebruiker indien meer aanvullende data beschikbaar komen, de berekening hieraan aan te passen.
De factsheet '**Environmental risk assessment scheme for plant protection products: non-target higher plants'** beschrijft een risicoschattingsschema waarmee de effecten van gewasbeschermingsmiddelen op niet doelwit hogere planten kunnen worden gekwantificeerd. Niet doelwit hogere planten zijn gedefinieerd als niet gewas planten die groeien buiten de behandelde akker. Voor de eerste stap in het risicoschattingsschema worden gegevens gebruikt die bepaald zijn gedurende screeningsfase tijdens de ontwikkeling van een bepaald gewasbeschermingsproduct (dit zijn meestal toxiciteitstesten met een enkele dosering gelijk of hoger dan de velddosering, bij voorkeur met

meer dan 6 soorten). Indien phytotoxiciteit wordt waargenomen, moeten voor minstens 6 soorten uit verschillende families waarvoor het is vastgesteld dat de stof significante effecten veroorzaakt, dose-response relaties worden vastgesteld. Gezien dit aantal toxiciteitstesten wordt aangeraden bij voorkeur een statistische methode te gebruiken om the toxiciteitswaarde uit te rekenen waarmee de risicoschatting zal worden uitgevoerd, dan een vaste extrapolatie factor van 10 te gebruiken en deze toe te passen op laagste beschikbare waarde.

Summary

This report presents 4 factsheets for the risk assessment methods used in the Expert Centre for Substances (ECS) and the Centre for Substances and Integral Risk Assessment (SIR) of the National Institute of Public Health and the Environment (RIVM). The first 2 of these factsheets are dealing with issues related to human risk assessment and the other 2 with environmental risk assessment.

The factsheet '**Hazard assessment for direct immunotoxicity'** describes a procedure to establish the direct immunotoxicity potential of substances. The factsheet is considered to provide further guidance for the evaluation of immunotoxicity effects and will lead to improved consistency. In the first tier, regular toxicity studies are evaluated for flags of direct immunotoxicity. In the second tier, functional immunotoxicity test can be used. In the third tier, disease models can be used. Depending on the results of those studies it can be concluded whether a substance has a potency for direct immunotoxicity. For the establishment of human limit values regular 'default' assessment factors can be used.
The factsheet **'The Interpretation of hepatic peroxisome proliferation and associated hepatocellular carcinogenesis'** describes a procedure to evaluate substances that induce peroxisome proliferation in rodents (rat, mouse). In this factsheet the mechanistic aspects of this phenomenon in the liver and its relation with hepatic carcinogenesis is described. In addition, it is defined under which conditions hepatic peroxisome proliferation in rat or mouse can be considered not relevant for human risk assessment.
In the factsheet **'Environmental risk assessment scheme for plant protection products: birds and mammals'** a risk assessment method is described for estimating the risk of plant protection products to birds and mammals. In contrast to the former risk assessment schemes for birds and mammals, the risk assessment is based on daily dose in order to avoid bias to different food intake rates between laboratory results and the field. An other important feature of the scheme is that all assumptions, extrapolation factors and uncertainty factors are explicitly stated in defining the calculations for the realistic worst case and most likely case scenarios. This ensures that the assumptions are transparent, and enables the user to adjust them appropriately if required (e.g. if specific data is available for part of the calculation).
The factsheet **'Environmental risk assessment scheme for plant protection products: non-target higher plants'** deals with the assessment of plant protection products on non-target terrestrial higher plants. Non-target higher plants are defined as non-crop plants outside the treatment area. For the first tier single dose phytotoxicity screening data at or above the maximum application rate on a wide range of species (e.g. at least 6 but preferably more) are used. If phytotoxicity is observed, dose-response relationships for at least 6 species representing families for which significant herbicidal action has been found are recommended to quantify the level of effect. Because of this great number of species, which is beyond that what is currently required in other areas of environmental risk assessment, it is recommended to use a statistical method to calculate the toxicity value for the risk assessment, instead of applying a fixed uncertainty value of 10 on the lowest available toxicity value.

Introduction

One of the main tasks of the Expert Centre for Substances (SEC) and the Centre of Substances and Risk Assessment (SIR) of the National Institute for Public Health and the Environment (RIVM) is to assess the risk of compounds on public health and the environment. To carry out risk assessments it is of the highest importance that adequate and up-to-date risk assessment methods are available. Some of these methods are taken over (adopted) from other organisations, but many are, for a large part, developed within the RIVM. These risk assessment methods are not rigid procedures but can be adapted based on new/developing scientific information, possible triggered by questions from policy makers or by developments in national or international organisations.
For specific problems or gaps in the assessment of (eco)toxicological effects, 'factsheets' are written by employees of SEC and SIR in co-operation with experts. In these factsheets the assessment strategies of RIVM/SEC and RIVM/SIR are described. After adoption of the factsheet by the advisory board and the head of the laboratories SEC or SIR all employees of SEC and SIR have to follow the risk assessment method described in the factsheet.

In 2001 the first eight factsheets were published in the RIVM report 601516007 (Factsheets for the (eco)toxicological risk assessment strategy of the National Institute for Public Health and the Environment, edited by Luttik and Van Raaij). In 2002 five additional factsheets were presented (edited by Luttik and Pelgrom)

In the report of 2003 four new factsheets are presented (2 factsheets related to public health issues and 2 factsheets related to environmental issues):

Factsheets concerning public health
1. Hazard assessment for direct immunotoxicity
2. The interpretation of hepatic peroxisome proliferation and associated hepatocellular carcinogenesis

Factsheets concerning the environment
3. Environmental risk assessment scheme for plant protection products: birds and mammals
4. Environmental risk assessment scheme for plant protection products: non-target terrestrial higher plants

We hope that by publishing these factsheets, the risk assessment methods followed by RIVM/SEC and RIVM/SIR will become more transparent. The authors of each factsheet have tried to describe the state of the art of their subject.
Remarks, omissions or supplementary information will be appreciated and can be send to Robert.Luttik@RIVM.NL and will be passed on to the responsible authors.

1 Hazard assessment for direct immunotoxicity

Factsheet FSV-009/00 date 22-06-2002

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1.1 Introduction and aim

For the evaluation of toxic substances, the potential for inducing immunotoxic effects received a lot of attention in last years, but the request for and use of toxicological data in hazard assessment for this endpoint is not strictly regulated in most regulatory frameworks.

There are several publications dealing with the predictivity of tests on immune parameters. This resulted amongst others in an update of OECD guideline 407 on repeated dose toxicity extended with more parameters to flag substances for immunotoxic potential, in tiered approaches for testing for immunotoxicity and in updating of regulatory guidelines.

For drugs, the EMEA has released guidelines focussing on direct immunotoxicity (CPMP/SWP, 2001b) and on hypersensitivity (CPMP/SWP, 2001c; CPMP/SWP, 2001a). The FDA is in the process of defining an immunotoxicity guideline for pharmaceuticals. The EPA established a guideline on immunotoxicity in 1998 to be applied by the Offices of Pesticides Program (OPP) and the Office of Pollution Prevention and Toxic Substances (OPPT) (EPA, 1998).
In this factsheet a strategy is established for determining the relevance of test results for hazard assessment of direct immunotoxicity and the request for additional investigations. Additionally, considerations to derive human limit values for direct immunotoxicity are given.

1.2 General background information

1.2.1 The immune system

In mammals, the immune system and its reactions consist of a fine-tuned, complex interplay between various cell types and soluble mediators secreted by those cells. Components of the immune system are present throughout the body. The major cell type is the lymphocyte (B- and T-cells). Assessory cells include macrophages, natural killer (NK) cells, eosinophils, basophils, and dendritic cells. Lymphocytes circulate among lymphoid organs throughout the body.

There are two systems to classify the lymphoid organs. The first is based on function: primary or central (bone marrow, thymus) and secondary or peripheral (spleen, lymph nodes, and lymphoid tissue along secretory surfaces like the gastrointestinal and respiratory tract). In the primary organs lymphocyte proliferation and maturation takes place independent of exogenous antigen exposure. Antigen-dependent development and maturation takes place in the secondary lymphoid organs, as well as in the bone marrow and the thymic medulla.

A second classification is based on the location of lymphoid organs, divided in internal organs (some lymph nodes and the spleen, in addition to the thymus and bone marrow)

and external organs (lymphoid tissue along secretory surfaces and the lymph nodes that drain the mucosa, named MALT[1]).
The main function of the external immune system is to produce (secretory) IgA antibody, whereas the internal immune system (mainly bone marrow) produces IgG or IgM antibody. Immune cells and cellular products are transported between lymphoid organs by blood and lymph vessels. The blood circulation contains only a minor part of the body's total pool of lymphocytes (estimated about 1%) and only a select population.

A short description of the physiology of lymphoid organs is given by IPCS (1996) and by Kuper *et al.* (2000). In appendix 1 an overview is given of the compartments, cells and functions of lymphoid tissues.

An extended description of the immune system is beyond the scope of this report and reference is made to IPCS (1996) and to the textbook of Roitt *et al*. (2000).

1.2.2 Types of immunotoxicity

The interference of substance with the immune system may exert different manifestations of immunotoxicity:

1. Direct (non-specific) immunotoxicity: an effect of the substance (and/or its metabolites) on the immune system. Immunosuppression and immunostimulation.
2. Indirect (specific) immunotoxicity: an immunologically based host response to the compound and/or its metabolites, or host antigens modified by the compound and/or its metabolites. Induction of allergy and autoimmunity.

These effects are characteristically different in dose-response and occurrence over time (Harleman, 2000). Direct effects are classical toxicological responses becoming manifest immediately or shortly after dosing and having a clear dose-response relationship. The indirect responses need an induction phase of several weeks and show marked differences in individual sensitivity and not always a clear dose response.

This factsheet deals only with direct immunotoxicity, the immunosuppression and immunostimulation. Indirect immunotoxicity requires other strategies and will not included in this document.

1.2.3 Direct immunotoxicity

Immunosuppression may become manifest as an impaired resistance to infectious pathogens (resulting in recurring, more severe or prolonged infections), exacerbation of latent infectious disease or the occurrence of tumours that escape immune surveillance. Immunostimulation may result in exacerbation of pre-existing allergy or autoimmunity.
It should be mentioned that strict immunosuppresion or -stimulation may not always occur as the immune system is a very balanced system, some responses may be suppressed, while others are stimulated simultaneously**.**

[1] i.e. bronchus associated lymphoid tissue (BALT), nose associated lymphoid tissue (NALT), and gut associated lymphoid tissue (GALT). In addition, according to this systematics, also the skin immune system (SIS) has been recognised as an immunological entity surveilling an external surface.

1.2.4 Immune reserve and immune redundancy

It has been argued that small changes in immune function should not be a significant concern, due to reserve capacity and redundancy as they exist within the immune system. Studies with TBTO have shown no effects on basal immunotoxicological parameters, while a decreased resistance to Trichinella spiralis was observed (Vos *et al.*, 1990), indicating that notwithstanding reserve and redundancy, small changes can have clinically relevant effects. Moreover, it should be mentioned that immune reserve and redundancy are relative terms, depending on the dose of the infectious agent. Additionally, immune reserve and redundancy may be applicable to individuals, but in populations where immunocompetence may be compromised there is no or only minor reserve capacity and redundancy (Karol, 1998). Generally, it is unclear how these aspects should be applied on the scale of populations (IPCS, 1996). This can be illustrated by the relationship between drop in CD4+ levels and developing AIDS. The normal human range of CD4+ cells is 800-1200 cells/µl, but this level generally declines to less than 500 cells/µl within 3 to 4 years after HIV-1 infection and to 200 cells/µl before overt infections are seen. So, for individuals a decrease of 90% is required to result in infections. However, it has been shown in seropositive humans that a drop in CD4+ cells by 7% or more in one year increases the relative risk (i.e. population level) to develop AIDS.

An ILSI panel did not support the reserve and redundancy concept, stating that any effect on the immune reserve could be important to the health of an individual (ILSI, 1995): a change in any immune function can be considered potentially deleterious in that it may increase the risk of developing clinical diseases, although a change in immune function does not necessarily precipitate as a disease or clinical health effect.

In conclusion: although immune reserve and redundancy will play a role in the ultimate effect on the development of diseases via immune suppression, a change in any immune parameter should be considered as hazardous.

1.3 Tests for direct immunotoxicity

In the last two decades, many methods have become available in animal models to assess aspects of immune disturbance after exposure to chemicals, both *in vitro* and *in vivo* (Dean *et al.*, 1982; Norbury, 1982; Basketter *et al.*, 1994).

Methods for assessing direct immunotoxicity in experimental animals may be classified into two principal categories (IPCS, 1996; Thomas, 1998).

1.3.1 Non-functional tests or immunopathology

- body and lymphoid organ (spleen, thymus, kidney, liver) weights
- complete blood and differential counts (cellularity of spleen and bone marrow)
- histopathology
- lymphocyte subpopulation assessment (FACS).

1.3.2 Functional tests: several techniques to evaluate functional competence of immune cells

1.3.2.1 *Ex vivo/in vitro* cell immune function assays

- Macrophage activity
- Natural killer cell activity
- Antigen specific antibody responses
- Primary antibody response to T-cell dependent antigen
  - Plaque-forming cell assay, measures the ability of isolated spleen (B-cells = PFCs) to generate IgM and IgG to T cell dependent antigen, for example sheep red blood cells (SRBC) or tetanus toxoid
  - Enzyme-linked immunosorbent assay of anti-sheep red blood cell or other T-cell dependent antigen antibodies of classes M, G and A in rats
- Responsiveness to B-cell mitogens (LPS)
- Responsiveness to T-cell mitogens (PHA, ConA)
- Mixed lymphocyte reaction (MRL)
- Cytotoxic T lymphocyte cytolysis

1.3.2.2 *In vivo* disease models

- Syngeneic tumour cells (PYB6 sarcoma, B16F10 melanoma)
- Bacterial models (Listerial monocytogenes, Streptococcus species)
- Viral models (influenza)
- Parasite models (Trichinella spiralis, Plasmodium yoelii)
- B16F10 Melanoma model
- PYB6 Carcinoma model
- MADB106 Adenocarcinoma model
- Experimental Allergic Encephalomyelitis (EAE)
- Adjuvant Arthritis Model
- Ovalbumin- induced Respiratory Allergy
- Delayed type hypersensitivity to a model allergen

The functional tests comprise several *in vivo* disease models, in addition to *ex vivo/in vitro* assays and are meant to study the functional consequences of immunotoxic effects. Different aspects of the immune system should be addressed to obtain a complete picture of the effects of a substance on the immune system, i.e. non-specific responses (macrophage and natural killer activity), humoral responses (antibody responses to antigens, mitogen responsiveness) and cellular responses (mitogen responsiveness, mixed lymphocyte reactions, cytotoxic T cell responses, delayed hypersensitivity). The most relevant endpoint for immune dysfunction is altered host resistance, because host resistance models assess the function of the immune system *in toto* (Van Loveren, 1995). Since defence mechanisms that are put into action vary for different pathogens, a number of host resistance models need to be employed. Some of these models can be employed in both rats and mice, while some of them showed limited success in one of these species (Van Loveren, 1995). Nevertheless, all types of pathogens/virus/bacteria etc. can be

studied in both mice and rats, although the specific pathogen used may vary between these species.

1.4 Hazard assessment and predictive value of tests/parameters

1.4.1 Introduction

Changes in several different parameters can be regarded as flags for the assessor pointing to direct immunotoxicity. A flag is an effect on any immune parameter without overt other toxicity. Because complex interactions play a role in immune reactions, generally, a combination of changes in a number of parameters will be more important than a change in one single parameter. Therefore, the whole toxicological database should be taken into account. The total picture of changes will give weight of evidence to conclude on direct immunotoxic potential or not. A complicating factor in hazard assessment is that for many substances the data on immunotoxicity will be limited.

1.4.2 Predictive value of non-functional tests or immunopathology

In recent years several studies were conducted in order to examine the predictive value for direct immunotoxicity of parameters in routine toxicity studies, like the 28-day toxicity test, and the value of additional pathology in these tests.

Body and organ weights

Changes in body weight may give an indication for effects on general health status. Changes in weights of lymphoid organs (spleen, thymus, kidney, liver) may serve as flag for immunotoxicity, but they should be evaluated in conjunction with histological examination of these organs (see below).

Blood parameters

Like with the changes in organ weigths, changes in complete blood cell counts, including differential WBC, may serve as flags for immunotoxicity. The assessment of lymphocyte subpopulation via FACS will give important additional information. Because of the complexity of the immune reactions, it is not possible to give a quantitative guide on the extent of changes before these parameters become relevant for induction of immunotoxicity (for relevance of changes in blood parameters a separate factsheet will be prepared). Usually, any statistically significant effect should be considered relevant, however, an expert judgement needs to be made. For instance, minor but statistically significant changes in granulocyte counts may only be considered relevant when other indicators of immunotoxicity are observed.

Histopathology

The thymus is the first lymphoid organ that shows morphologic alterations after exposure to many known immunotoxic agents. A decrease in size is often the first manifestation of immune toxicity (Kuper *et al.*, 2000). Atrophy of this organ is mostly the result of lymphocyte depletion of the cortex. A few compounds cause an expansion of a distinct thymic compartment without a major change in thymic weight. For example, in rats the

food colour additive TDI (2-acetyl-4)5)-tetrahydroxybutyl-imidazole) causes a reduction in number of peripheral B- and T-lymphocytes and in lymphocyte numbers in thymus, spleen and lymph nodes. TDI-induced immunosuppression is thought to be due to inhibited migration of mature T-cells into the periphery (Houben *et al.*, 1993). Most of the known thymotoxic and immunosuppressive substances also affect secondary lymphoid organs and tissues. Limited data on the effects of substances with immunostimulation or immunoadjuvant properties have shown several different effects, like an increased development of germinal centers, an increased marginal zone in the spleen, lymph node enlargement with germinal center development and increased size of paracortex, the appearance of aggregated macrophages or increased number of macrophages in lymph nodes.

Because histological changes in lymphoid organs play an important role in flagging for immunotoxicity, a revision of the OECD guideline 407 (28-day repeated dose toxicity test) was adopted in July 1995. According to the former guideline agreed upon in 1981, the only immunologically related parameters assayed are histopathology of the spleen, in addition to total and differential white blood cell counting in the circulation. In 1995 the protocol was extended and now includes weight and histopathology of the spleen and thymus, and histopathology of draining and distant lymph nodes and Peyer's patches were added.

Only six of the 18 substances were claimed not to be flagged in 28-day studies performed according the updated OECD protocol 407 (Van Loveren *et al.*, 1996). However, these could be detected by the inclusion of measurements of basal immunoglobulin levels (total serum IgG, IgM and IgA levels) as indicators of altered B-cell function, avoiding functional tests.

The ICICIS (1998) validation study was aimed to investigate whether this more detailed examination of lymphoid organs in the updated 28-day toxicity study from July 1995 could serve as 'flagging' system. It was indicated that the updated guideline is able to detect most of the non-specific immunotoxic substances.
Although only two compounds were tested in the ICICIS study, the results indicate also that adequate pathological examination has a crucial role in the identification of effects on the immune system (ICICIS, 1998; Harleman, 2000). Standardisation of the morphological examinations was an important factor determining the adequacy of pathology (Hastings, 1998; Harleman, 2000; Kuper *et al.*, 2000). They recommend to examine the lymphoid organs per compartment and to use a terminology to describe morphological reflections of functional disturbances quantitative and descriptive rather than interpretative. Cellularity and development of germinal centers are considered important aspects of histopathological examinations. It is recommended to restrict the use of the term 'altered cellularity' to changes in numbers of passenger cells that are normal constituents of a given compartment. Standardised nomenclature for the evaluation of the immune system was proposed by Kuper *et al*. (2000) and are given in Table 1.
In evaluation of toxicity studies one should be aware that this terminology is not generally used, especially in studies of older date, but it is recommended to use these terms in the assessment reports.

Table 1 Proposed diagnostic terms for identifying histologic changes in lymphoid organs and tissues (cited from (Kuper et al., 2000).

Organs and compartments	Changes (semiquantitative)
All organs, all compartments	- Increased or decreased size - Increased or decreased cellularity (lymphocytes, plasma cells, blast cells) - Increased number of cells otherwise not present or in low numbers: tingible body macrophages ('starry-sky appearance'), phagocytizing or pigmented macrophages, mast cells and granulocytes, apoptotic cells - (Micro)granulomata or macrophage aggregates
Thymus	- Cortex: medulla ratio - Effects on number and size of epithelium-free area - Increased epithelial cords and tubules
Lymph nodes, spleen and MALT	- Increased or decreased germinal center development - Erythrocyte rosette formation
Lymph nodes and MALT	- Prominent high endothelial venules
Lymphatics and lymph nodes	- Lymphatic ectasia

The ICICIS validation study not only demonstrated that histopathology is able to flag many compounds as non-specific immunotoxic, but that careful examination of the lymphoid system can also give important clues on the mechanism of action of a test material (Harleman, 2000). Additional techniques, such as morphometric analysis and the use of specific cell (surface) markers for different cell populations (T cells and subsets, B cells, macrophages) may be used if the initial examination of the H&E stained tissues gives an indication of a possible immunotoxic effect. Although these immuno-histochemical techniques and quantitative methods are still not being used in regulatory immunotoxicity testing, the results of these examinations can be important in the design of subsequent tests (Kuper, Harleman, Richter-Reichelm, and Vos, 2000).

1.4.3 Predictive value of *ex vivo/in vitro* immune function assays

Ex vivo/in vitro immune function assays are defined as assays in which constituents of the immune system are evaluated for their ability to perform a specific function. In *ex vivo* assays, experimental animals are exposed to the test compound and a challenge with a specific antigen is given. The immune function is assayed by measuring the specific immune response using serum or isolated cells (ILSI, 1995).

Luster *et al*. (1993) determined the concordance between the results of a battery of *ex vivo/in vitro* functional immune assays and a variety of host resistance (HR) models (Luster *et al.*, 1993). No single test was fully predictive for HR. A number of individual tests were good predictors (> 70% concordance) for the outcome of host resistance. Combination of several *ex vivo/in vitro* immune assays improves the accuracy of prediction. They reported, that there were no instances where HR was altered without effects seen in at least one of the *ex vivo/in vitro* immune function tests.
However, there were cases of *ex vivo/in vitro* immune test changes without effects on HR. The results of studies with TBTO from Vos *et al*. (1990) are contradictory with the conclusions of Luster *et al.* (1993). For example, in rats exposed to TBTO no effects on general toxicity or basal immunotoxicologic parameters were observed, whereas decreased resistance to Trichinella spiralis, as evidenced by increased number of muscle larvae, could already be detected (Vos *et al.*, 1990). This indicates that insignificant

changes in a number of immune parameters may together result in direct immunotoxicity that lowers HR and as such stresses the value of HR-models.

The primary antibody response to T-cell dependent antigen (e.g. sheep red blood cells, SRBC) is one of the most sensitive immunotoxicological assays. This response can be measured via an ELISA serum titers of IgG, IgM and IgA) or via the more time consuming plaque forming assay (IgG and IgM) (Van Loveren *et al.*, 1991). Luster *et al*. (1992) reported that the concordance of this assay for predicting immunotoxicity was the highest of all the functional assays (78%). Furthermore, use of this assay in combination with either the NK cell activity or surface marker analysis resulted in pairwise concordances for predictability of more than 90%.

The introduction of an immune antigen stimulatory event in routine toxicity tests, like immunisation of the animals with sheep red blood cells (SRBC), increases the predictivity of the immunopathology (see 4.1). It has been reported that this will cause limited effects on the hematological and clinical chemistry parameters, although a slight increase in number and size of the germinal centres was observed (Harleman, 2000). However, Ladics *et al.* (1995) concluded that administration of SRBC did not significantly alter the weights and morphology of routine protocol tissues in a rat study with cyclophosphamide.

1.4.4 Predictive value of *in vivo* disease models

In vivo disease models, like host resistance models, assess the function of the immune system. The selection of the most appropriate host resistance (HR) model is dependent upon several factors including the particular exposure route of the compound, the immunologic profiles already known, or both (Van Loveren, 1995; Thomas, 1998).
The sensitivity of host resistance assays depend on the end-point measured. For example, tests involving tumour load or survival are by nature less sensitive than those with end-points that provide continuous data, such as enumeration of tumours, bacteria or soluble immune activation markers (Van Loveren, 1995). Mortality was the main endpoint used in the host resistance assays used by Luster *et al.* (1993). The observation of immune test changes without effects on host resistance might be a reflection of the sensitivity of the endpoint chosen and of functional reserve of the immune system (see also chapter 1.2.4) (Luster *et al.*, 1996). Host resistance models are considered to be very relevant tests for immunotoxicity, especially as they indicate clinically relevant changes (Van Loveren, 1995; Karol, 1998).

1.4.5 Predictive value of immune parameters, in general

Validation studies have been used to propose tiered approaches for testing on immunotoxicity (see Appendix 2), but the results can also be used to establish a strategy for hazard assessment with respect to this endpoint (see Chapter 1.7).

The certainty of conclusions on immunotoxicity is dependent on the data available, which will be dependent on regulatory framework. For hazard assessment it is important to know the predictivity of immune parameters in order to use the data available as good as

possible and to give adequate recommendations for additional studies to get more information on this endpoint.

The general conclusion of the various concordance studies was that only few (two or three) of the immune parameters are sufficient for immunotoxic hazard identification. It can be concluded that of particular value was the T-cell dependent antibody response linked with phenotypic evaluation of lymphocyte subpopulation (Luster and Kimber, 1996).
This conclusion was used in the strategy proposed in Chapter 1.7 to recommend on most appropriate studies needed to verify whether substances with flags for direct immunotoxicity based on results from repeated dose studies may indeed cause these effects.

1.4.6 Some pitfalls in hazard assessment for direct immunotoxicity

The available toxicity data should be evaluated carefully, in order to avoid false conclusions. Several pitfalls in evaluation of the results can be mentioned.

Toxicity studies are commonly designed in such way that the high doses lead to overt toxicity. This may cause severe stress and lead to slight non-specific inhibitory effects on the immune system. These findings are not indicative of a potential immunotoxic hazard.

When effects on thymus are evaluated, it should be taken into account that thymus weight, volume and function decrease with age (age-related thymic involution) (Kuper *et al.*, 2000).

Interferences between the immune system and biological systems have been established, e.g. the nervous system can interact with the immune system by a number of specific mechanisms, such as glucocorticosteroid hormones, catecholamines, additional hormones (prolactin, growth hormones) and neuropeptides (Descotes, 2000). In turn, a variety of immune system products like cytokines, provide important signals for the nervous system. These interactions result in physiological and pathological consequences. The first lymphoid organ affected by stress is the thymus. It would be valuable to distinguish immunosuppression by chemically induced stress from real, direct immunotoxicity. However, in practice, it will be very difficult to make this distinction.

1.5 Regulatory guidelines on direct immunotoxicity

At this time predictive immunotoxicity testing is mainly done in the context of general toxicity, according OECD guideline 407. The assessor should be aware of the fact, that current studies are performed according to the updated guideline published in 1995, i.e. extended with some parameters relevant to predict immunotoxicity (incorporation of thymus weight, and histopathology of lymphoid tissues), but in a number of dossiers, studies will be available which are performed before 1995 and consequently according to the former guideline of 1981.

At this moment within OECD the necessity to extend guideline OECD 407 with functional testing is discussed, i.e. measurement of antibody responses to SRBC, in addition to discussions as to the inclusion of measurement of NK activity and inclusion of FACS analysis of lymphocyte subpopulations.

The requirements on testing for direct immunotoxicity set by several organisations are different (see table 2), and currently in several fields (e.g. ICH) there are ongoing discussions to harmonise the approaches.

For drugs, the EMEA has released guidelines focussing on direct immunotoxicity (CPMP/SWP, 2001b). The FDA is in the process of defining an immunotoxicity guideline. Both EMEA and the draft FDA guidelines follow similar lines for immunotoxicity testing as the OECD. The major difference between the EMEA and the FDA guideline is that the EMEA has included functional testing in routine screening, i.e. primary antibody response to T-cell dependent antigen or NK activity and FACS analysis of lymphocyte subset populations.

According to the guideline of the EPA, histopathology and primary antibody response to SRBC are required to screen for immunotoxicity in repeated dose studies (EPA, 1998) This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) and the Toxic Substances Act (TSCA).

An overview of requirements of several organisations is given in Table 2.

Table 2 Requirements for testing on direct immunotoxicity

Organisation/field	Test requirements for direct immunotoxicity
Europe-EMEA / drugs	- OECD 407 - functional testing in routine screening (primary antibody response to T-cell dependent antigen, or NK activity and FACS analysis of lymphocyte subset population)
US-FDA / drugs	- OECD 407
Japan / drugs	Tiered approach[1] with - Tier 1: repeated dose toxicity study (including hematological tests, weights and histopathology of lymphoid organs; lymphocyte subset test for peripheral blood or immunohistochemical test of the spleen is recommended) with functional screening (primary antibody response to T-cell dependent antigen). NK cell assay is optional. - Tier 2: if an abnormal finding is observed in Tier 1. Most suitable test based on results from Tier 1 assays.
US / EPA (FIFRA and TSCA)	- Histopathology - Functional testing in routine screening (primary antibody response to SRBC)

1 = tier 1 is started after an initial study, and therefore could be considered as tier ii of the approaches described in appendix 2.

1.6 Human Limit Values and assessment factors

1.6.1 Threshold approach

Direct immunotoxicity is a classical toxicological response, present immediately after dosing and having a clear dose-response relationship (Karol, 1998; Harleman, 2000). Therefore, the threshold-approach is applicable to derive human limit values.

1.6.2 Assessment factors

Interspecies differences

The occurrence of interspecies differences for direct immunotoxicity is limitedly studied. Fundamentally, there are similarities between the immune systems of animals from different species. *In vitro* functional assays involving both non-immunosuppressive and immunosuppressive pharmaceuticals using mouse or rat spleen cells and human peripheral blood mononuclear cells point also to similarities (Lebrec *et al.*, 1995).

Parallelogram approaches have been used to compare effects in animals and humans (Selgrade, 1999). According to this approach, the human effects in the *in vivo* situation are predicted using *in vitro* data with animal and human cells and using results from *in vivo* animal experiments. This approach was applied by Selgrade *et al*. (1995) on immunotoxicity data from human clinical and rodent studies of ozone and ultraviolet radiation. However, an assessment factor for interspecies differences has not been derived.

This approach has also been applied to data on TBTO. A factor of 3.14 for interspecies variability was derived from ED_{50} values based on *in vitro* ConA responsiveness of rat peripheral (spleen) lymphocytes and human peripheral lymphocytes (van Loveren *et al.,* 1998). This factor could then be applied to predict effect levels in humans from *in vivo* data on host resistance in rats.

In conclusion, for substances inducing direct immunotoxicity, there is no indication to deviate from the default approaches as mentioned in Vermeire *et al.* (2001).

Intraspecies differences

Apart from the limited data on susceptibility of children, there are no data on differences in susceptibility of the human population.

A number of factors influence the response of the immune system to toxicant exposure. In addition to genetics, gender and age, factors such as stress, infections, medications, UV exposure, nutrition, and in women the timing of the menstrual cycle affect the immune response (Selgrade, 1999). It is mentioned that the variation in human response is great, frequently exceeding a coefficient of variation greater than 20 to 30% (Karol, 1998).

Because young adult sexually mature animals are generally used, the impact on other, possibly more susceptible populations (e.g. the fetus) may be underestimated (Thomas, 1998).

Studies with mice of different strains and/or ages revealed a difference in susceptibility of a factor > 5 (TCDD), factor 9 (ozone) to 18 (UV) (Selgrade *et al.*, 1995).
Van Loveren *et al*. (1998) estimated the intraspecies variability for TBTO, using the ED_{50} for ConA responsiveness of the most sensitive and least sensitive samples of human peripheral lymphocytes divided by the mean ED_{50} of all human samples (factor 0.64 and 1.7, respectively).

Selgrade (1999) concluded that, assuming that variability in the human population is similar to differences among mouse strains, an assessment factor of 10 for intraspecies differences as generally used assessment factor may also be reasonable for direct immunotoxic effects.

In conclusion: Taking all data together, for substances inducing direct immunotoxicity there are no indications to deviate from the default approaches (Vermeire *et al.*, 2001) for intraspecies differences.

1.6.3 Assessment factor for duration of exposure

Generally, an assessment factor is used in hazard assessment when there is only information of effects after subacute/subchronic exposure and data for chronic exposure are lacking. Very few chronic studies have been done to address immunotoxicity. These studies are complicated by the fact that immuno-responsiveness decreases in ageing animals (Selgrade, 1999). Since antigen-sensitive immunocompetent cells turn over fairly rapidly, compounds that target these cells should provide effects relatively shortly after exposure, and therefore exposure to a substance for 2-4 weeks may be representative of longer exposures.
However, if certain cell types (progenitor or stem cells) are targeted by a chemical, acute exposure may have long lasting effects.

In conclusion: The use of a default assessment factor for extrapolation from short-term exposure in the experiment to long-term exposure in the human situation is not indicated, unless there are data that point to another approach (e.g. effects on stem cells).

1.7 Assessment and RIVM Strategy

Based on the analysis in previous chapters, we have established a strategy for determining the relevance of test results for hazard assessment of direct immunotoxicity and the potential request for additional investigations. Considerations are given for the derivation of human limit values for direct immunotoxicity.

Hazard assessment
In the context of hazard assessment for direct immunotoxicity, a flag is an effect on an immune parameter without overt other toxicity. If a flag is observed, direct immuno-toxicity is considered the critical endpoint determining the LOAEL.

The amount of toxicity data on a substance is largely dependent on the regulatory framework and the possibility to ask for additional studies. This last aspect is especially important, because, generally, data on immunotoxicity will be limited.
For most substances there are data on repeated dose toxicity, and therefore these data are used as starting point in the strategy as given in figure 1.

Results from a number of studies indicate that adequate pathological examination has a crucial role in the identification of effects on the immune system.
The strategy is designed in such a way that substances with flags indicative for direct immunotoxicity should be considered as immunotoxic agents, unless results from additional tests do not confirm an effect on the functionality of the immune system (false positives). False negatives are avoided as much as possible, but it has to be noted that they may occur, especially if there are only limited data on the substance and the request for additional studies is not possible in the regulatory framework.

Principal steps in hazard assessment

The principal steps in hazard assessment for direct immunotoxicity are depicted in a flow diagram given in figure 1. The steps are described below. The numbers in the text refer to the numbers in the boxes of the figure.

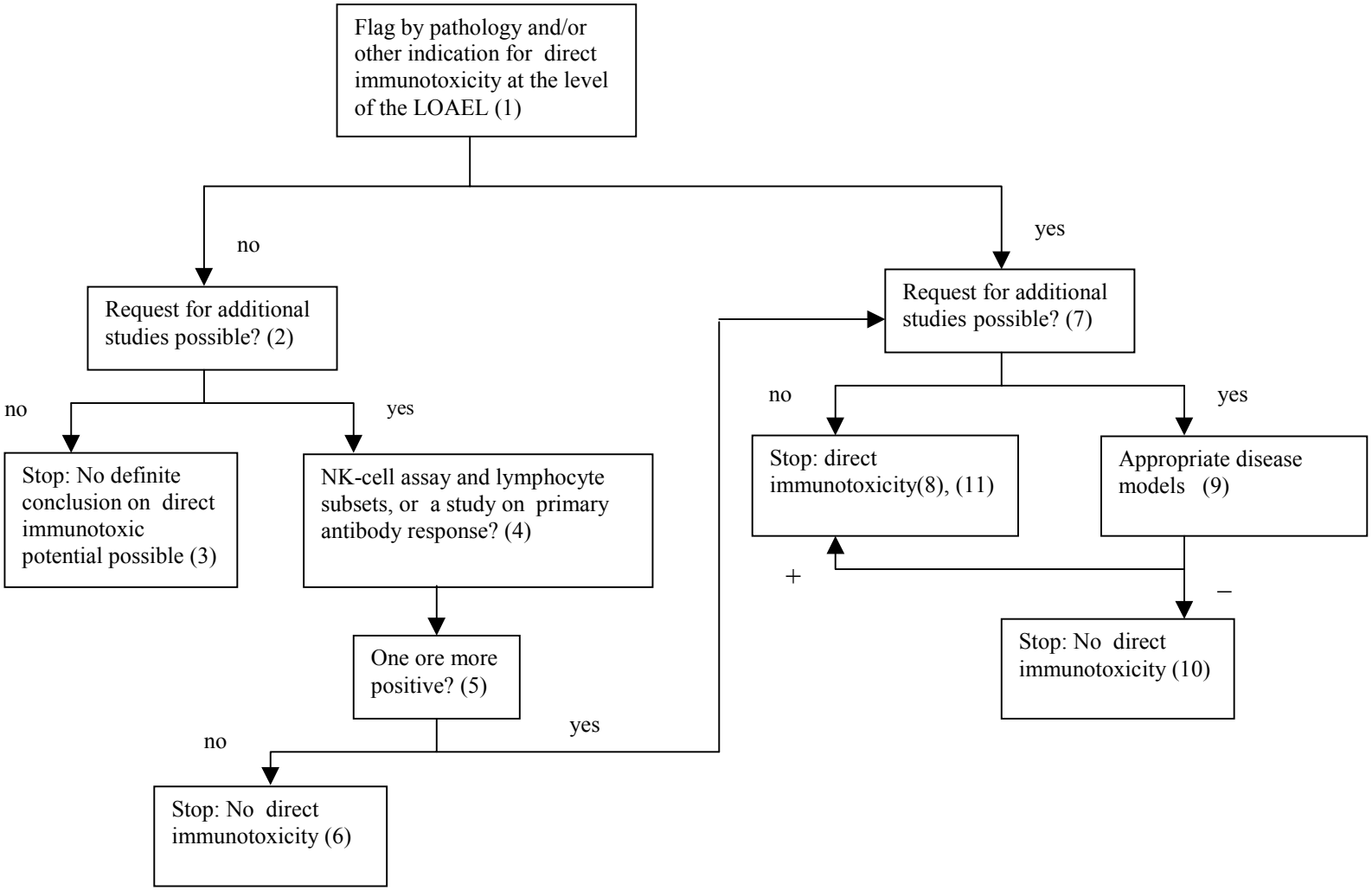

Figure 1 Decision scheme for hazard assessment of direct immunotoxic effects

1. Evaluation of flags for direct immunotoxicity
   a. Pathological changes in repeated dose toxicity studies:
      - body and lymphoid organ (spleen, thymus, kidney, liver) weights
      - complete blood and differential counts (cellularity of spleen and bone marrow)
      - histopathology
   b. Other indications, e.g. structure activity relationships.

   In chapter 4.2 some important aspects on evaluation of repeated dose toxicity studies with respect to this endpoint are given. The flags should not be evaluated on their own, but the total picture of changes will give weight of evidence to conclude on direct immunotoxic effects.
   It is important to assess whether the study is performed according to the revised version of the OECD-guideline (July 1995) or not (i.e., with or without extension of observations relevant for immune parameters). Results from repeated dose toxicity studies performed according to the OECD-protocol before July 1995 will give insufficient information on flags for direct immunotoxicity, and therefore did not allow conclusions on this endpoint.
   Additionally, it is recommended to assess whether the lymphoid organs per compartment were examined and whether the morphological reflections of functional disturbances are described quantitative and descriptive. Standardised nomenclature for the evaluation of the immune system was proposed by Kuper *et al*. (2000) and are given in table 1 and it is recommended to use these in the assessment report.

   Because of the complexity of the changes used as flags for immunotoxicity it is recommended to consult the expert in this field if flags are identified.

   It has to be noted that results from sensitization studies with guinea pigs (either positive or negative) cannot be used to conclude on direct immunotoxicity.

2. If there are no indications for direct immunotoxicity obtained in box 1 and the toxicity study (ies) is (are) performed according to the revised OECD guideline (July 1995), a definitive conclusion on direct immunotoxicity cannot be drawn without additional functional immune testing. This is always required to avoid false negatives.

3. If there were no flags for direct immunotoxicity (box 1, study performed according to OECD guideline of July 1995)) and it is not possible to ask for additional testing (box 2), there are insufficient data to draw definite conclusions on the potential of the substance to induce direct immunotoxic effects.

4. If there are no indications for direct immunotoxicity obtained in box 1 and additional testing is available and/or can be requested, the most relevant functional tests are the primary antibody response to T-cell dependent antigen (e.g. SRBC test), or the NK-cell assay in combination with determination of lymphocyte subsets.

5. The results of the studies mentioned in box 4 should be evaluated.

6. If negative results are obtained in the primary antibody response to T-cell dependent antigen or in both the NK-cell assay and in determination of lymphocyte subsets, the substance is considered to have no direct immunotoxic potential.

7. If one or more of the tests mentioned in box 4 are positive or there is a flag for immunotoxic potential obtained in box 1, additional testing is recommended to determine the relevance of these positive findings.

8. If additional testing in the situation mentioned in box 7 cannot be requested, the substance is considered as a direct immunotoxic agent, although it cannot be excluded that it concerns a false positive.

9. If additional testing in the situation mentioned in box 7 can be requested, the indications for direct immunotoxicity effects should be verified in disease models (suppression (allergy or autoimmunity) or stimulation). Attention should be paid to relevant and susceptible endpoints in these assays and it is recommended to assess the most appropriate study in consultation with the expert in this field.

10. If the results of adequate disease models are negative, the substance should be considered to cause no direct immunotoxicity, provided that appropriate *in vivo* disease models have been used. Conclusions should always be drawn in agreement with the expert.

11. If the results of adequate disease models are positive, the substance is a direct immunotoxic agent.

Principal steps in derivation of human limit values

Direct immunotoxicity is a toxicological endpoint with usually a clear dose-response relationship, and therefore, the threshold-approach can be used to derive human limit values. These values can then be calculated with default factors for inter- and intraspecies differences (Vermeire *et al.*, 2001).

A default assessment factor to account for differences in exposure time in the experiment and the human situation cannot be given. Generally, such a factor is not required because of the relatively rapid turn-over of immunocompetent cells, unless there are indications to deviate from this approach, e.g., if a substance targetted progenitor or stem cells, if exposure occurs during critical periods of development of the immune system, or if the substance is slowly eliminated.

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Appendix 1 Compartments, cells and function of lymphoid tissue (cited from Kuper, Harleman, Richter-Reichelm, and Vos, 2000)

Organs and compartments	Cells	Functions
Bone marrow	Hematopoietic cells organised as islands within fatty tissue, mature leukocytes, plasma cells	Differentiation of stem cells into cells of the erythroid, myeloid-monocytoid, platelet, and lymphoid lineage; antibody synthesis, memory cells
Thymus		
Cortex	Fine reticular epithelium, macrophages, immature T cells	Generation of T-cell competence: T-cell receptor rearrangement, positive selection (major histocompatibility complex restriction), negative selection, autoreactive cells, phenotypic changes
Medulla	Plump reticular epithelium, macrophages, dendritic cells, T lymphocytes	T-cell competence generation (negative selection), thymic hormone synthesis, antigen presentation
Cortico medullary zone	Immature and mature lymphocytes	Entrance of bone marrow-derived stem cells, exit of T cells having undergone intrathymic maturation
Epithelium–free areas	Immature T-cells, macrophages	T cell proliferation, function unknown
Lymph node and spleen		
Paracortex (lymph node) and PALS (spleen)	Interdigitating cells, T-helper and T-suppressor cells	Lymphocyte entry through high endothelial venules (lymph node) or central arteriole (spleen), antigen presentation to T-helper cells, T-cell proliferation-differentiation-regulation (T-suppressor cells)
Primary follicles, follicle mantle of secondary follicles	Dendritic cells (subtype of follicular dendritic cells), dendritic macrophages, B cells, small number of T cells	Storage of (virgin/memory) B cells, recirculating B cells (surface IgM^+ IgD^-)
Germinal center	Follicular dendritic cells, dendritic macrophages (starry sky macrophages) B cells (centrocytes, centroblasts), T-helper cells	T-cell dependent B lymphocytes, differentiation, antigen presentation in the form of immune complexes (with/without complement C3)
Medulla (lymph node), red pulp (spleen)	Plasma cells, T-effector cells, reticular cells, polymorphonuclear granulocytes	Termination of antigen-specific reaction: antibody synthesis and immune mediated clearance, reactions of T-delayed type hypersensitivity and cytotoxic cells
Marginal zone (spleen)	Marginal zone macrophages, marginal metallophilic cells	T-cell independent B lymphocyte proliferation-differentiation, eg, to bacterial polysaccharides; B-cell memory (surface IgM^+ IgD^-)

Organs and compartments	Cells	Functions
Mucosa-associated lymphoid tissue *Organised lymphoid tissue* *(Peyer's patches, NALT, BALT)*		
Epithelium covering lymphoid tissue	M (microfold) cells, lymphocytes	Transport (uptake of exogenous substances, mainly particles), initiation of immune responses including IgA response
Follicles and interfollicular areas	See lymph node and spleen	
Single cells		
Epithelium	Epithelial cells, T-cytotoxic cells, natural killer cels, T-γδ cells	First line defense, synthesis of cells, extrathymic maturation of lymphocytes, secretory component, transport of IgA (IgM)
Lamina propria	Plasma cells, macrophages, lymphocytes	Synthesis of IgA antibody, phagocytosis and killing

Appendix 2 Tiered approaches

Due to the complexity of the immune system, no single assay will encompass all the possible dearrangements, and therefore tiered approaches have been developed to assess immunotoxicity by incorporating immune parameters into the routine protocol of a repeated dose toxicity study. These approaches primarily aim at the detection of direct immunotoxicity. The animal species most frequently used are rat and mouse.
The basic philosophy is that detailed testing may be indicated if the results of repeated dose toxicity testing pose a concern. The choice for the type of tier 2 testing is based on the results obtained in tier 1.

Tiered testing in rats

At the Dutch National Institute for Public Health and the Environment (RIVM) a tiered strategy of immunotoxicity testing has been developed in rats (Van Loveren *et al.*, 1989; Vos, 1983). This approach is depicted in figure 1.

Parameter	Procedure
Tier I	
Immunopathology	Hematology, including differential cell counting Lymphoid organ weights (thymus, spleen, lymph nodes) Histology (thymus, spleen, lymph nodes, Peyer's patches) Bone marrow cellularity Splenic B- and T-cell numbers (flow cytometry) Serum IgM, IgG and IgA levels
Tier II	
Cellular immunity	Sensitisation to T-cell dependent antigens (e.g. ovalbumin, tuberculin) and skin test challenge Lymphoproliferative response to specific antigens T-cell mitogen response (ConA, PHA)
Humoral immunity	IgM, IgG, IgA, IgE responses to T-cell dependent antigens IgM response to T-cell independent antigen (LPS) B-cell mitogen response (LPS)
Non-specific immunity	Cytolysis of YAC-1 lymphoma cells by macrophages and NK cells
Host resistance	Bacterial model: Listeria monocytogenes Parasite model: Trichinella spiralis Viral model: rat cytomegalovirus Autoimmune models: adjuvant arthritis, experimental allergic encephalomyelitis

Figure 1 Tiered immunotoxicity screening in rats

In Tier I and Tier II assays rats are used, because this species is the most commonly used test species in routine toxicity testing. Tier I parameters are incorporated into the conventional 28-day test protocol. It has been suggested to include some functional parameters (e.g. NK-cell assay or antibody response to sheep red blood cells (SRBC)) in tier I (Ladics *et al.*, 1995; Van Loveren *et al.*, 1992).

Tiered testing in mice

US NTP has developed a similar tiered system in mice (Luster *et al.*, 1988). The aim of this approach is to study compounds that are already under suspicion of immunotoxic potential because of findings in routine toxicity testing. The tests from the tiers are being performed in a late phase of toxicity testing. The approach is depicted in Figure 2.

Parameter	Procedure
Pretest	General toxicity screening yielding reasons for concern regarding immunotoxicity
Tier I	
Immunopathology	Hematology, including differential cell counting Organ weights (thymus, spleen, kidney, liver) Spleen cellularity Histology (thymus, spleen, lymph nodes)
Cellular immunity	T-cell mitogen response (ConA) B-cell mitogen response (LPS)
Humoral immunity	IgM antibody plaque-forming cells to T-cell dependent antigen (SRBC) B-cell mitogen response (LPS)
Non-specific immunity	NK cell activity
Tier II	
Immunopathology	Splenic B- and T-cell numbers (flow cytometry)
Cellular immunity	Cytotoxic T-cell (CTL) cytolysis Delayed hypersensitivity
Humoral immunity	IgG antibody response to SRBC
Non-specific immunity	Macrophage function
Host resistance	Syngeneic tumor cells: PYB6 sarcoma, B16F10 melanoma Bacterial model: Listeria monocytogenes, Streptococcus species Parasite model: Plasmodium yoelii Viral model: influenza virus

Figure 2 Tiered immunotoxicity screening in mice

In the NTP studies with mice the duration of treatment is generally shorter than in the rat study (14 versus 28 days of exposure). The highest dose level tested is set lower than the MTD, because dose selection in the immunotoxicity test is based upon already existing dose-range finding data underlying the mouse carcinogenicity study. Consequently, overt toxicity is avoided. Therefore, functional assays need to be present already in Tier I in order to raise the sensitivity of the panel precluding false negatives. In the NTP-studies with mice, it was shown that gross changes as leukocyte counts, thymic weight and spleen cellularity or histopathology seem to be unreliable indicators of immunotoxicity as compared to Tier I testing in rats. This could probably be attributed to the relatively low dose levels employed in these mice studies (Luster *et al.*, 1992; Schuurman *et al.*, 1994).

Evaluation of tiered immunotoxicity testing in rodents

The interpretation of Tier I immunotoxicity panels should be based on an integrative view of changes in lymphoid tissues and immune cell populations in the context of other types of toxicity and the health status of the animals.

In both approaches Tier II is intended to confirm and further explore Tier I findings, which is especially important for compounds which slightly affect the immune system exerting subtle changes in the animal's immune system. In those cases, Tier II functional assays including host resistance models are particularly important for hazard assessment as they are tools to elucidate the actual consequences of disturbed immune system.

Caution is needed in interpreting Tier I endpoints, because of the redundancy and functional reserve in the immune system (see also chapter 9.2) (ILSI, 1995).

2 The interpretation of hepatic peroxisome proliferation and associated hepatocellular carcinogenesis

Factsheet FSV-010/00 date 24-04-2003

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2.1 Introduction

This factsheet has been developed to assist in the interpretation of peroxisome proliferation in hepatocytes ('parenchymal liver cells') in rodents. Many substances may cause this phenomenon together with e.g. liver enlargement and hepatocarcinogenesis. The relevance of peroxisome proliferation for human hazard assessment has been under discussion for a long period and it has been stated that humans are refractory to peroxisome proliferation, suggesting that the related carcinogenicity in rodents does not pose a hazard to humans (e.g. Ashby *et al.*, 1994; Cattley *et al.*, 1998; IARC, 1995, 2000).

In this factsheet a strategy is described to assess the relevance of animal data on peroxisome proliferation in connection with other effects that can be observed in the liver for human hazard and risk assessment. The text below is intended to provide insight in the background of peroxisome proliferation and its relationship to rodent carcinogenicity. Data requirements are defined that are necessary to evaluate the relevance of the animal hepatocarcinogenicity to humans.

In the context of this factsheet only peroxisome proliferation in hepatocytes (i.e. the parenchymal liver cells) and the relationship with hepatocarcinogenicity is taken into consideration, because peroxisome proliferation in these cells is known to be associated with hepatocellular carcinogenesis in some rodent species. Peroxisome proliferation can also occur in non-parenchymal liver cells and extra-hepatic cell types, but in these cells the relationship between peroxisome proliferation and possible carcinogenicity is unknown and are therefore beyond the scope of this factsheet.

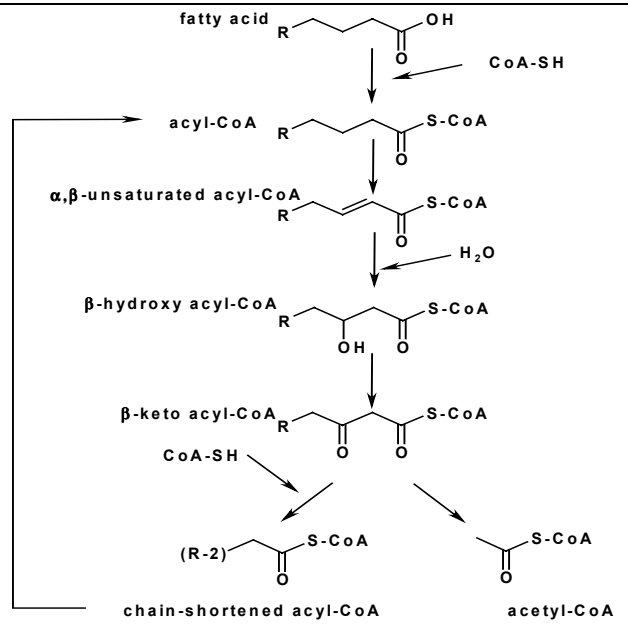
2.2 Mechanism of action and background information

2.2.1 Peroxisomes: histological aspects

Peroxisomes, in older literature also known as microbodies, are small cytosolic organelles (see Appendix I). They contain high amounts of catalase. This enzyme is used to identify peroxisomes in electron micrographs by means of specific staining techniques. In electron micrographs of rat and hamster peroxisomes, a polytubular structure, the so called 'crystalloid core' can be seen, in which the enzyme urate oxidase is located. This crystalloid core does not occur in the peroxisomes of primates and these species lack urate oxidase. Peroxisomes are present in virtually all eukaryotic cells, notably in the kidney and the liver. (Handler and Thurman, 1998a; Bloom and Fawcett, 1975; Alberts *et al.*, 1983; Usuda *et al.*, 1998).

2.2.2 Peroxisomes: biochemical and physiological function

It has been demonstrated that peroxisome β-oxidation may contribute to as much as 30% of the total fatty acid oxidation in the liver. As a result of this oxidation, peroxisomes generate hydrogen peroxide (see below). It has been suggested that hepatic peroxisomes are also involved in various other metabolic processes such as bile acid synthesis, dicarboxylic acid metabolism, and oxidation of xenobiotics. (Handler and Thurman, 1998a,b; Bloom and Fawcett, 1975; Alberts *et al.*, 1983).

Mitochondrial β-oxidation		**Peroxisomal β-oxidation**
Thiokinase		thiokinase
acyl dehydrogenase[i] yielding $FADH_2$		acyl oxydase[i] yielding H_2O_2
Enoyl CoA hydratase[ii]		combined activity of a bifunctional enzyme[ii] producing $NADH + H^+$
β-hydroxyacyl dehydrogenase[ii] producing $NADH + H^+$		
thiolase[iii]		thiolase[iii]

i. In mitochondria the conversion of acyl CoA compounds to enoyl CoA compounds is carried out by an acyl CoA oxidase, which concomitantly reduces FAD to $FADH_2$. In contrast, the peroxisomal system contains an acyl CoA oxidase which metabolises acyl CoA compounds to enoyl CoA compounds with the concomitant reduction of O_2 to H_2O_2.

ii. In the mitochondria the enoyl-CoA hydratase and β -hydroxyacyl-CoA dehydrogenase activities are two separate enzymes. In peroxisomes the corresponding steps in the fatty acid β-oxidation are carried out by a single bifunctional enzyme

iii. The peroxisomal thiolase is a protein, distinctly different from the mitochondrial enzyme

Figure 1. Mitochondrial and Peroxisomal β-oxidation, similarities and differences

In humans, the importance of peroxisomes for normal physiological processes is illustrated by the existence of at least 16 genetic diseases in which there is an impairment in one or more peroxisomal functions, notably in fatty acid α- and β-oxidation, ether phospholipid synthesis, isoprenoid biosynthesis or plasmologen biosynthesis. The most severe form of such a disease is the Zellweger syndrome, in which the incorporation of enzyme systems in the peroxisome is impaired. Patients suffering from this disease do not survive for more than one or two years (Wanders, 1995, Wanders *et al.*, 1995; Gärtner, 2000).

2.2.3 Comparison of peroxisomal vs. mitochondrial β-oxidation

Mitochondrial and peroxisomal β-oxidation are both cyclic processes in which fatty acids are broken down by sequential splitting off of di-carbon units. Schematically, this process is presented in figure 1 (Bell *et al.*, 1976; Handler and Thurman, 1998a), in which also the various enzymes have been indicated.

Several important differences between the enzymes involved in mitochondrial and peroxisomal β-oxidation exist. In mitochondria as well as in peroxisomes, for each molecule of activated fatty acid (acyl-CoA) which completes one loop in the β-oxidation cycle, one molecule NADH (+ H^+) is generated, but in contrast to mitochondria, peroxisomes produce simultaneously one molecule of hydrogen peroxide per cycle. In contrast to the mitochondrial system, the peroxisomal β-oxidation system is not linked to the electron transport chain. Therefore, peroxisomes are able to catalyse fatty acid β-oxidation in the presence of the cytochrome-c-oxidase inhibitor CN^-, provided a sufficient supply of NAD^+ is present, whereas mitochondrial β-oxidation is completely blocked by CN^-. Consequently, cyanide-insensitive reduction of NAD^+ to NADH has been used widely to assay peroxisomal β-oxidation activity.

2.2.4 Biochemical and histological changes during peroxisome proliferation

Peroxisome proliferation can be defined as a cellular process characterised by an increase in both volume density of peroxisomes (that part of the hepatocellular volume that is occupied by the total peroxisomal compartment; see Appendix I, figure A1) and peroxisomal fatty acid β-oxidation activity (IARC, 1995). After exposure to a peroxisome-proliferating stimulus, in rodent hepatocytes the increase of the peroxisomal compartment may be about ca. 10-fold: from 2% of the total cellular volume to up to 25%. Proliferation of the peroxisomal compartment is accompanied by hepatocyte hypertrophy and hepatocyte hyperplasia. Acute hepatocellular proliferation may affect as much as 50% of the hepatocyte population and can be seen from 48 hr after the first administration of a peroxisome proliferating stimulus. In chronic experiments the increase in the replication rate (i.e. the speed with which the cells divide) is less than after acute exposure, however, the total hyperplastic response (i.e. the number of cells involved in the response) is much greater (IARC, 1995).

The morphological and biochemical changes persist as long as the animal is exposed to the 'peroxisome proliferator'. The reversal depends both on the elimination half-life of the peroxisome proliferator, and the biological half-lives of induced enzymes (which is not substance specific). Several other, non-peroxisomal enzymes may also be affected by peroxisome proliferators among which UDP-glucuronyl transferase, glutathione peroxidase, glutathione transferase, superoxide dismutase and cytochrome P450 4A-subfamily members.

2.2.5 Substances which may cause peroxisome proliferation

The group of peroxisome proliferators consists of a variety of compounds, among which well-known hypolipidaemic drugs, such as the fibrates (see appendix II). Clofibrate can be considered as the archetype of the peroxisome proliferators. Many other chemicals, such as phthalate plasticizers and substances like tri- and tetrachloroethylene and trichloroacetic acid have the ability to induce peroxisome proliferation, too.

The potency of these substances varies greatly and according to Barber *et al.* (1987), the longer chain dialkylphthalates are more potent for the induction of peroxisome proliferation than the shorter chain ones and branched chain phthalates seemed more potent than linear ones. Cyprofibrate is orders of magnitude more potent to induce peroxisome proliferation than the plasticizer DEHP (di-ethylhexyl phthalate). Although general structure activity relationships have not been established, a common feature of these chemicals is that they contain carboxylic acid groups, or that they are metabolised to carboxylic acids.

2.2.6 Determination of peroxisome proliferation

Based on the definition of peroxisome proliferation given above, ideally two characteristics should be studied, namely volume density and peroxisomal β-oxidation activity. There are also other parameters that can be studied, but some of these are less specific. In the paragraphs below these parameters are discussed.

- Peroxisomes can be made visible under the light microscope (‘microbodies’) but studied in this way it is not possible to quantify changes in volume density.
- Peroxisomes can be made visible under the electron microscope by staining with reagents, which react specifically with catalase. By morphometric analysis changes in the total number and size of the peroxisomes can be monitored, and changes in the volume density of the total peroxisomal compartment can be quantified.
- The functionality of peroxisomes can be studied by measuring biochemical activities located specifically in these organelles, notably CN^--insensitive palmitoyl CoA oxidation (usually in cell homogenates). Other enzymes that can be studied are carnitine acetyltransferase or catalase but these enzymes are not very suitable to monitor peroxisome proliferation. Carnitine acetyltransferase is less specific because it is located in both mitochondria and peroxisomes and effects on catalase activity are often of a rather limited extent. Urate oxidase, although specifically located in the peroxisome, is not affected by treatment with peroxisome proliferators (Usuda *et al.*, 1988).
- Substances that induce peroxisomal enzymes also increase lauric acid ω- and ω-1 fatty acid oxidation by induction of enzymes of the cytochrome P450 4A subfamily in the liver. However, these enzymes are located on the smooth endoplasmic reticulum and are therefore not considered as direct markers for peroxisome proliferation, but the induction of these enzymes indicates that the inducing substance might be a peroxisome proliferator.

2.2.7 Regulation of peroxisome proliferation

The genes encoding for the endoplasmic, mitochondrial and peroxisome proteins that are induced after exposure to peroxisome proliferators are under control of a *peroxisome proliferator*

activated receptor (PPARα). It has been demonstrated that most peroxisome proliferators bind to PPARα with moderate affinity. This receptor is most abundant in liver and kidney and is associated with the events occurring in peroxisome proliferation. Furthermore, this receptor controls the serum cholesterol level, in particular high-density lipoprotein cholesterol. In PPARα-null mice an increased level of serum cholesterol can be seen as compared to wild-type mice, which cannot be influenced by treatment with fibrates. However, it has also been demonstrated that in liver cells of these PPARα-null mice peroxisomes do occur and that mRNA levels of several peroxisome-related proteins are expressed to a similar level as in hepatocytes from wild-type mice. These observations suggests that peroxisome proliferation but not the constitutive expression of the respective genes is under control of PPARα (Lee *et al.*, 1995; Gonzalez *et al.*, 1998; Vanden Heuvel, 1999).

2.2.8 The link between peroxisome proliferation and liver carcinogenicity

After chronic exposure to peroxisome proliferators, increases in the frequency of hepatocellular cancer have been observed in mice and rats in conjunction with significant peroxisome proliferation. Generally, there is a strong correlation between the dose-response curves of peroxisome proliferation and hepatocarcinogenicity (Dybing *et al.*, 1995). Ashby *et al.* (1994) found that in approximately 80% of the cases in which peroxisome proliferation is seen, hepatocellular carcinogenicity is observed as well. This also shows that there are a number of cases in which animals exhibit peroxisome proliferation, in absence of developing hepatocellular carcinomas. It is thought that in these cases the compounds involved generally have a weaker potency to cause peroxisome proliferation and it seems plausible that a certain amount of peroxisomal increase is necessary to trigger the development of hepatocellular tumours (Dybing *et al.*, 1995).

The mechanisms by which treatment with peroxisome proliferators may elicit carcinogenicity are complex. Many peroxisome proliferators are known to be non-mutagenic and they do give DNA adducts (Gonzalez *et al.*, 1998). One of the oldest explanations for the carcinogenic activity of peroxisome proliferating compounds is the hypothesis that following peroxisome proliferation, increased amounts of hydrogen peroxide, produced by peroxisomal fatty acid oxidation, would diffuse through the liver cell into the nucleus causing DNA damage. Other peroxisomal enzymes such as glycollate oxidase may also contribute to an increased production of hydrogen peroxide (Handler and Thurman, 1998a). Thus, this theory assumes a direct link between peroxisome proliferation and hepatocarcinogenesis.

Currently it is thought that a multitude of processes may lead to tumour formation, which are not mutually exclusive but could co-operate with one another in a way which has been depicted in Figure 2. This diagram shows that the proliferation of the peroxisomal compartment (fig 2; oval 3) as such is not at the basis of the carcinogenicity (fig 2; oval 2), but that both peroxisome proliferation and hepatocarcinogenicity are different manifestations of one event being the interaction of a putative peroxisome proliferator with the PPARα (fig 2; oval 1).

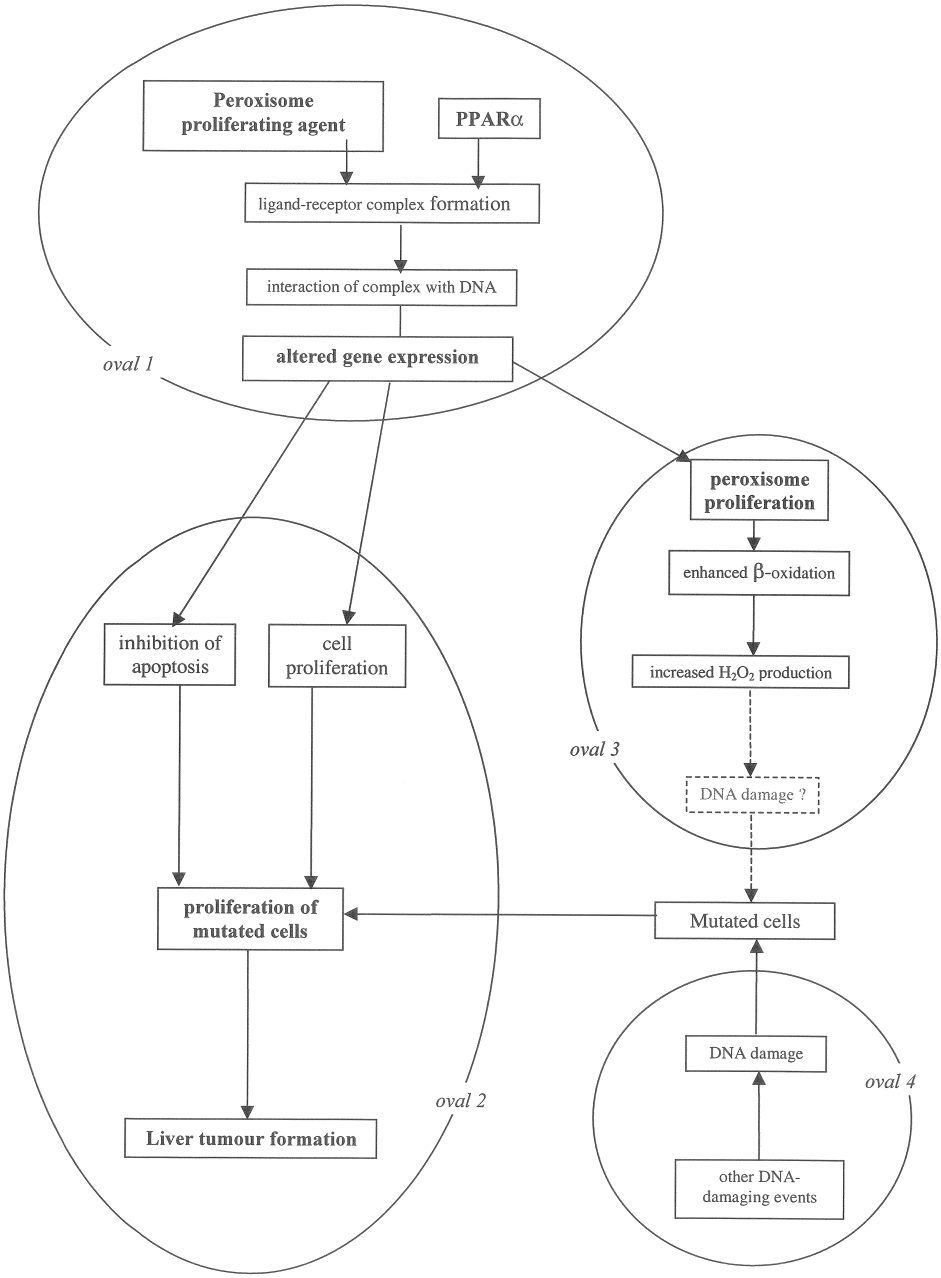


Figure 2. The relationship between peroxisome proliferation and carcinogenicity.
In oval 1 processes are described which control the expression of genes, responsive to stimulation with peroxisome proliferating agents. Oval 2 describes the responses which affect cell cycle (see text under sections) which may result in the promotion of tumour growth, initiated via processes described in oval 3 (possible initiating events related to peroxisome proliferation) and oval 4 (initiating events resulting from genotoxic stimuli unrelated to peroxisome proliferation. e.g. from exposure to environmental genotoxicants).
Note that the possible genetic damage in oval 3 results secondary to increased peroxisomal activity, which is a threshold phenomenon. Hence, if peroxisome proliferation does not occur, there is no genotoxicity hazard via this route.

Directly relevant processes mentioned in figure 2 are discussed below:

- Suppression of apoptosis (figure 2; oval 2).
  Apoptosis is an important mechanism by which living organisms can get rid of cells with a compromised genetic constitution (King, 1996). Peroxisome proliferators have been demonstrated to reduce apoptosis in liver cells, an effect that may lead to propagation of erroneous genetic information to daughter liver cells. In rat hepatocytes cultures, nafenopin-induced suppression of apoptosis could be prohibited when these cells were transfected with a clone of a normal variant of human PPARα gene, which is transcriptionally inactive (Roberts *et al.*, 1998).
- Cell proliferation (figure 2; oval 2).
  A strong correlation does exist between the ability of peroxisome proliferators to induce an increase in replicative DNA synthesis and their tumour-promoting activity (Vanden Heuvel, 1999). In PPARα-knock-out mice, treatment with peroxisome proliferators does not lead to transcriptional-activation of target genes, nor to hepatic cell proliferation or hepatomegaly (Lee *et al.*, 1995).
- Oxidative stress-induced DNA damage (figure 2; oval 3).
  Indeed, an increase in peroxisomal hydrogen peroxide production is not fully counterbalanced by an increase in hydrogen-peroxide scavenging enzymes (e.g. catalase) leading, in theory, to increased intra-cellular levels of reactive oxygen species (Rao and Reddy, 1991). However, convincing evidence for increased levels of reactive oxygen species in vivo is lacking and treatment with peroxisome proliferators is poorly related to lipid peroxidation, an indicator of oxidative stress. Moreover, oxidative DNA damage is a common event in cells as a result of normal physiology, and it is questionable whether the contribution of hydrogen peroxide from peroxisomal β-oxidation contributes significantly to the 'normal' background of oxidative stress.
  It has been claimed that in rats, treatment with the peroxisome proliferator ciprofibrate for 16 or 28 weeks resulted in increased average 8-OH-deoxyguanosine (8-OH-dG) levels in the liver of 3.4 ± 0.3 and 4.3 ± 0.5 / 10^5 dG bases, respectively, as compared to 2.4 ± 0.2 / 10^5 dG bases in the liver from 40 week control animals (statistically significant). No statistically significant increase was observed after 36 weeks of treatment (4.0 ± 1.4 / 10^5 dG bases). In livers from animals treated for 40 weeks, 8-OH-dG levels of 8.3 ± 2.4 / 10^5 dG bases were observed which were statistically significantly higher than the levels observed in other 40 week control animals (4.0 ± 1.3 / 10^5 dG bases). No increase was seen at 3, 16 or 24 hrs post dosing in livers from animals that received a single dose of ciprofibrate (1.9 ± 0.5; 1.4 ± 0.3 and 1.4 ± 0.3 / 10^5 dG bases, respectively) as compared to 1.8 ± 0.6 found in control animals killed at 16 hrs post dosing with vehiculum (Kasai *et al*., 1989).
  However, the variability of the various control groups from the study by Kasai *et al*. (1989) casts some doubts on the stability of the analytical method used. It has also been argued that the observed increase in 8-OH-dG was observed in DNA that may have originated from both mitochondria and nuclei, where the former is more susceptible to oxidative stress than the latter. In addition, no increase in hydroxy-DNA adducts was found in purified nucleic DNA isolated from livers treated with peroxisome proliferators (Ashby *et al.*, 1994; Gonzalez *et al.*, 1998), suggesting that DNA adducts are of mitochondrial origin. Thus, the relevance of the reported increase in 8-OH-dG is not clear.

2.3 Biochemical and morphological reference values

The normal physiological activity of peroxisomal enzymes is strongly dependent on animal strain and dietary composition. Reference values for the parameters reflecting peroxisome proliferation are not available. However, from open literature, some indication of the activity of several enzymes and other peroxisomal parameters under control conditions and after treatment with peroxisome proliferating agents can be obtained. These data are summarised in Appendix III for *in vivo studies* and in Appendix IV for *in vitro* studies. The tabulated data are not intended to be used as reference values. Decision whether a substance is a peroxisome proliferator or not should be made by comparison with an appropriate (concurrent) control group.

2.4 Species differences in sensitivity and possible explanations

It is well known that major species differences do exist in the responsiveness to peroxisome proliferators (see also Appendix III and IV). From both *in vivo* and *in vitro* studies a sequence of species sensitivity can be derived showing that rats and mice are clearly responsive, that guinea pigs, dogs and humans are refractory or non-responsive, and that hamsters are somewhat in between. There is no evidence for a significant response in marmosets, rhesus or Cynomolgus monkeys.

In vitro studies with human hepatocytes have shown that human liver cells do not react to several peroxisome proliferators. In humans treated with several hypolipidaemic drugs no significant changes were observed in the liver. However, in one study with clofibrate a 50% increase in the number of peroxisomes in the liver was seen, but the volume density of the peroxisomal compartment was not changed, indicating an essential negative response in this study, as well. The limited number of reliable epidemiological studies with hypolipidaemic drugs, notably clofibrate, does not provide any indication for an increased tumour incidence in humans (IARC, 1995; Ashby *et al.*, 1994).

It is not clear why some species do respond with proliferation of the peroxisomal compartment while others do not, given the fact that PPARα has been detected in liver cells of many species. Ashby *et al.* (1994) have presented a number of theoretical explanations for the differences in species responsiveness:

- *absence of functional PPARα in non-responsive species:*
  Not likely because PPARα receptors are highly homologous. Moreover, in *in vitro* transfection studies in a mouse hepatoma cell line, human PPARα can be activated by several peroxisome proliferators, resulting in the expression of genes known to be responsive to peroxisome proliferators *in vivo* (Sher *et al.*, 1993).
- *non-responsive species have low levels of PPARα:*
  In general, low levels of functional receptor may be insufficient to generate a peroxisome-proliferative response. Indeed rat and mouse livers have much higher concentrations of PPARα mRNA than guinea pig liver. Data also indicate low mRNA levels of the receptor in human liver. In addition, PPARα's of different species may have different thresholds for activation.
  According to Gonzalez *et al.* (1998) the concentration of PPARα in human liver is at least 10 times less than in the liver of mice. However, in humans treatment with hypolipidaemic drugs

is effective to reduce serum cholesterol. Thus, hepatic PPARα concentrations in humans might be sufficiently high to stimulate (mitochondrial) fatty acid metabolism to a degree to allow hypolipidaemic drugs to control serum cholesterol and serum lipids in hyperlipidaemic patients. In contrast, PPARα concentrations in humans might be insufficient to elicit proliferation of the peroxisomal compartment (Gonzalez *et al.*, 1998).

- *Hampered binding of PPARα to DNA:*
  Binding of activated PPARα to DNA occurs as heterodimers with retinoid X receptor (RXR). Thus, binding may be hampered in refractory species because of an insufficient level of RXR-co-factor or because of little reactivity between PPARα and RXR. This hypothesis is not likely, because other ligand receptor-systems that rely on the RXR co-factor do not show marked species differences. It has been demonstrated that human PPARα is quite capable to bind to RXR.
- *Lack of responsive elements in non-responsive species:*
  Genomic responsive elements, which are responsible for the gene-regulation of peroxisome proliferator-elicited events have only been detected in rats and rabbits. Such responsive elements might be lacking in refractory species, but these species have not been studied well. It is hard to imagine that all such elements are lost from the genome during evolution. Loss of one responsive element is more likely.
- *Absence of an as yet unknown PPARα activating factor which is absent in refractory species*:
  It cannot be ruled out that a still unknown factor is required for the activation of PPARα, which is present in responsive species but absent in refractory ones. It is also possible that peroxisome proliferators perturb intermediary metabolism increasing activities of an endogenous PPARα activating ligand in rats and mice but not in e.g. monkeys and humans.
- *Presence of an as yet unknown factor in refractory species which prohibits PPARα activation in vivo*:
  It cannot be ruled out that although PPARα from refractory species can be activated *in vitro*, activation *in vivo* is impossible e.g. because of an intracellular factor which interferes with ligand-receptor binding, receptor DNA binding or transcriptional activation.

The above possible explanations for differences in species susceptibility focus on differences in functional aspects of PPARα in various species. Although no conclusive explanation for the species differences can be given, it is nevertheless clear that species differences in the susceptibility to peroxisome proliferators exist and that these differences should be taken into account in hazard or risk assessment procedures.

2.5 Assessment and RIVM strategy

The biological phenomena which can be observed in responsive species after exposure to any peroxisome proliferator show great inter-species consistency. Based on this consistency and on the absence of such a response in non-responsive species, among which humans, the relevance of the peroxisome proliferator-induced hepatotoxicity and hepatocarcinogenicity in rodents for human hazard assessment is scientifically questioned (IARC, 1995).

An RIVM strategy has been developed by which the relevance of peroxisome proliferation and other associated effects in the liver (e.g. tumours) in rodents can be determined. This strategy is focussed on the following:

- Peroxisome proliferation and hepatocarcinogenicity are both phenomena that ultimately reflect one and the same event namely interaction of a substance with the PPARα (figure 2; oval 1).
- This interaction is followed by changes in gene expression which in responsive species leads to induction of peroxisomes (figure 2; oval 3), increased hepatocellular mitogenesis and decreased hepatocellular apoptosis (figure 2; oval 2).
- None of these responses is considered relevant for species refractive to peroxisome proliferation including humans, because these species do not respond to PPARα stimulation in the same way as rodents.

Hence, the fact that in human liver a peroxisome proliferating response (figure 2; oval 3) does not occur can be considered as proof that events leading to expression of peroxisome-regulating genes do not occur (figure 2, oval 1) and that therefore responses leading to liver tumour promotion (figure 2; oval 2) do not occur, either. For substances which are hepatotoxic and /or hepatocarcinogenic in rodents, these effect can be dismissed if it is sufficiently demonstrated that these effects are solely the result of a PPARα-mediated response. This also means that for genotoxic peroxisome proliferating substances, or for peroxisome proliferating substances for which absence of genotoxicity has not been sufficiently demonstrated, this carcinogenicity cannot be dismissed without further evaluation of the genotoxicty data.

In order to decide whether hepatic effects (including carcinogenicity) in rodents can be dismissed for human hazard assessment on the basis of peroxisome proliferative activity, two questions have to be answered in a tiered approach, starting with the question in section 2.5.1:

2.5.1 Is it plausible that the effects observed in the livers of rodents can be attributed to interaction of the substance with PPARα?

Implicitly, this question falls apart in two sub-questions, a) is interaction with the PPARα receptor demonstrated and b) do peroxisome proliferation and other events in the liver occur at about the same exposure levels?

a) In order to decide whether the substance elicits its effects by interaction with the PPARα receptor it is necessary to monitor parameters which are specific for the biological response resulting from this interaction, or in other words whether the substance is a peroxisome proliferator in hepatocytes. For this, an increase in peroxisome volume density must have been demonstrated by morphometric analysis. In addition, peroxisome proliferation must also have been demonstrated by determination of at least one specific biochemical parameter. The most common biochemical parameters are CN^--insensitive palmitoyl CoA oxidation. Less commonly used parameters, but equally acceptable, are the activities of peroxisome-specific bifunctional enzyme or thiolase (see figure 1). Strongly indicative for peroxisome proliferation is induction of cytochrome P450 4A, the latter either at mRNA, protein level or by biochemical activity (lauric acid ω- or ω-1 oxidation).

Determination of catalase and carnitine acetyl transferase can only provide supportive information. Determination of changes in total cytochrome P450 levels, hyperplasia and/or hypertrophy of hepatocytes are not specific enough and cannot be used to prove peroxisome proliferation. Determination of the formation of a complex between PPARα and the substance is also not sufficient, because it is not certain that formation of such a complex will always lead to the biological response.

b) The exposure levels at which peroxisome proliferation-related liver effects (hepatocellular hypertrophy and hyperplasia, increased liver weight and hepatocarcinogenicity) occur, should be similar or higher than those at which induction of peroxisomes can be demonstrated. If only *in vitro* data on peroxisome proliferation are available, these data can still be used, provided that adequate kinetic data show that the concentrations tested in the *in vitro* studies are relevant with respect to the i*n vivo* observations.

If the answer to this question is 'no' or if the data are equivocal, there is insufficient ground to dismiss hepatotoxicity on the basis of the argument that the substance is a peroxisome proliferator. If the answer is yes, the question in section 5.2 should be answered as well:

2.5.2 Are there indications that the substance causes hepatotoxicity via a mechanism independent of interaction with PPARα?

Several possibilities can be distinguished indicating the presence of a mechanism of toxicity which is not related to PPARα-mediated events:

1. hepatotoxic effects are observed at exposure levels far below those at which peroxisome proliferation is seen.
2. clinical chemical or morphological evidence is available, indicating effects on the hepatocytes, which are qualitatively different from the effects that are described in the commentary to the question in heading 5.1.
3. effects are observed in other (i.e. non-hepatocellular) cell types in the liver.
4. hepatotoxic effects have been observed in an animal species known to be refractory to peroxisome proliferation.

If the answer is 'yes' or 'questionable', a direct link between peroxisome proliferation and the (other) hepatotoxic events is not plausible (or is even not existing) and the hepatotoxic effects, in particular those which cannot be attributed to the peroxisome proliferating response, should be taken into account. If the answer to this question is 'no', all hepatotoxic effects including carcinogenicity can be dismissed.

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Appendix I Morphological appearance of peroxisomes in electron micrographs of hepatocytes

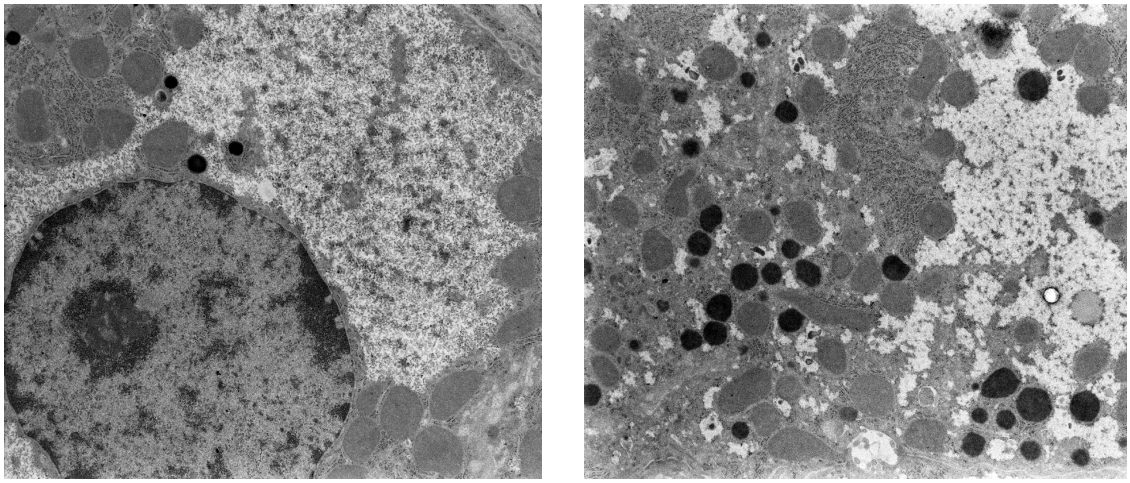

Figure A1. Peroxisome proliferation in rat liver cells.
The left image is from a control animal; the right image is from an animal exposed to 200 mg DEHP/kg diet for 14 days. Peroxisomes, stained for catalase show as deep-dark spots. Because of the dark staining, the 'crystalloid core' is invisible. 1 cm in the images corresponds to about 6 µm in the tissue slides. (Photographs by courtesy of J. Dormans, LPI, RIVM, Bilthoven, The Netherlands)

Appendix II Examples of agents causing peroxisome proliferation in rodent liver

Pharmaceuticals	Acetyl salicylic acid, bezafibrate, beclobric acid, bifonazole, ciprofibrate, clobuzarit, clofibrate, DL-040, fenofibrate methylclofenapate, LY 171883, nafenopin, tiadenol, Wy-14643
Steroids	Dehydroepiandrosterone
Plasticizers	DEHA, DEHP, DIDP, DINP, di-(2-ethylhexyl)terephthalate, tri-(2-ethylhexyl)trimellitate
Solvents and industrial chemicals	Chlorinated paraffins, tetrachloroethylene, trichloroethylene, perfluoro-n-octanoic acid, 2,4-D
Food flavourings and natural products	Cinnamyl anthanilate, citral, linalool

(modified after Lake , 1995)

Many other examples of substances which cause peroxisome proliferation can be found in ECETOC monograph 17 (ECETOC, 1992). In this report also some Structure-Activity aspects have been discussed.

Appendix III Parameters describing the peroxisomal compartment and peroxisome proliferation: *in vivo* data

Parameter values are presented for several animal species before and after treatment *in vivo* treatment with peroxisome proliferating substances. Note that the data are not representative for the species, but are only representative for the particular experiment. These data should not be considered as reference values.

Orton et al., 1984; peroxisome proliferator used : clobuzarit

species	dose	period	CN-insens. Palm.-CoA oxid.	activity P450 4A	morphology	Peroxisome number	Peroxisome surface
	mg/kg bw/d		µmol/min/g liver	nmol/min/mg prot		per 100 µm2	% cytosol surf *
rat	0	14 d	0.24	2.15	nomal	15.2	1.9
male	3		0.66	11.97	marked hypertrophy	19.2	1.8
	10		1.12	12.94	marked hypertrophy	35.7	4
mouse	0	15 d	0.34	2.1	normal	15.5	
male	20		1.29	9.91	normal	35.8	
	60		3.25	19.32	minimal hypertrophy	53.9	
Syrian hamster	0	15 d	1.15	1.31	normal	16.6	
male	10		1.86	-	normal	21.8	
	25		1.51	14.13	normal	26.8	
dog	0	15 d	0.88	1.51	normal	23.4	
male	15		1.02	-	normal	26.5	
	45		0.78	1.15	mild inflammation, pigment. deposition/bile duct proliferation.	24.3	
marmoset	0	15 d	0.27	1.49	normal	6.6	
male	15		0.32	-	normal	4.7	
	45		0.26	1.39	normal	7.3	

* % cytosolic surface: the percentage of the electron micrographic cell image which is covered by peroxisomes; an indicator of the change in volume density.

Gray & Iglesia, 1984; peroxisome proliferator used : gemfibrosil

species	dose	period	morphology	Number of peroxisomes	Peroxisomal volume density
	mg/kg bw/d			per cell	% cell vol
rat					
male	0			718	1.56
	300	1 Y	increased hepatocyte volume	4959	29.68
female	0			715	1.23
	300	1 Y	decreased hepatocyte volume	2226	4.51
Syrian hamster					
male	0			462	2.77
	400	14 d	increased hepatocyte volume	1562	2.96
female	0			354	2.96
	400	14 d	increased hepatocyte volume	1085	3.08
dog					
male	0			791	2.6
	400	1 Y	normal hepatocyte volume	1039	2.76
female	0			423	1.76
	400	1 Y	increased hepatocyte volume	808	2.33
Rhesus monkey					
Young					
male	0			425	2.48
(data extracted from graph)					
	400	3 m	normal hepatocyte vol	700	3.22
female	0			400	1.59
	400	3 m	normal hepatocyte vol	450	3.1
Aged					
male	0			326	0.99
	400	14 d	incr. hepatocyte vol	985	2.05
female	0			373	1.15
	400	14 d	incr. hepatocyte vol	1267	1.39

Appendix IV Parameters describing the peroxisomal compartment and peroxisome proliferation: *in vitro* data

Parameter values are presented for several animal species before and after treatment *in vitro* treatment of hepatocyte cultures with peroxisome proliferating substances. Note that the data are not representative for the species, but are only representative for the particular experiment. These data should not be considered as reference values.

Blaauboer et al., 1990; cells exposed for 72 h

SPECIES	**medium concentration** µM	**CN-ins. Palm.-CoA-oxidation** nmol/min/mg prot	**peroxisome number** per arbitrary surface unit	**peroxisome volume** arbitrary units
clofibric acid				
rat				
male	0	0.34	55.5	24.3
	300	3.12	152	35.5
	1000	n.d.	123	27.8
Cynomolgus monkey				
male /female	0	0.71	24.1	20.9
	300	0.82	n.d.	n.d
	1000	0.76	24.1	21.7
human				
male /female	0	0.43	59	17.1
	300	0.49	n.d.	n.d
	1000	0.41	70.5	15
beclobric acid				
rat				
male	0	0.34	55.5	24.3
	300	2.8	167	41
Cynomolgus monkey				
male /female	0	0.71	24.1	20.9
	300	0.78	22.7	19.7
human				
male /female	0	0.43	59	17.1
	300	0.62	50	18.3

Foxworthy et al., 1990; cells exposed for 72 h; data extracted from graph

		CN-ins. Palm.-CoA oxidation (nmol/mg prot/min)		
	medium concentration	**male rat**	**dog**	**male/female Rhesus monkey**
	µM			
control	0	3	3	2
ciprofibrate	100	25	4	2
bezafibrate	100	18	4	2
LY171883	100	15	2	1.5

3 Environmental risk assessment scheme for plant protection products: birds and mammals

Factsheet FSM-005/00 date 05-06-2003

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3.1 Introduction

Adverse effects on birds and mammals are among the factors which most strongly influence the acceptability of a plant protection product. The greatest concern is effects at the population level. However, there is also strong public concern regarding the deaths of individual birds and mammals from the use of plant protection products, although they may not have any significant effect on the population. Partly for that reason, and because of the lack of agreed criteria for the acceptability of effects at the population level, this scheme is concerned with the risk to individual animals. The result can also be used to provide an indication of the potential impact at the population level.

Scope
The scope of this risk assessment scheme is the determination of direct risk of a plant protection product to non-target birds and mammals. The direct risk is defined as the risk from the product itself including exposure via treated or contaminated food. The scheme determines the risk from primary and secondary exposure, where primary exposure is defined as direct consumption of product or of treated food, and secondary exposure is defined as consumption of food that, although not directly treated, contains the active substance (e.g. bioaccumulation in fish).

Exposure by non-dietary routes is not considered in this scheme. There is some evidence that non-dietary routes can contribute significantly to exposure in some situations (Driver *et al.*, 1991; Mineau et al., 2001), but there is no generally accepted approach to assessing them and in practice they are frequently omitted from routine assessment. It would be advisable to consider assessing non-dietary routes, especially in those cases where the level of risk from dietary exposure alone is approaching an unacceptable level. Approaches to assessing non-dietary routes have not developed significantly since the previous edition of this scheme (OEPP/EPPO, 1994) and the user is referred to that source for guidance.

For some specialised products e.g. slug pellets or rodenticides, the assessment requires some extra considerations. However, the basic concept of risk assessment is the same (processing of toxicity data, definitions of exposure-toxicity ratio (ETR)). Such cases can be assessed by taking one of the modules of the scheme (for sprayed products and seed treatments or for granular formulations) as a model and deciding on:

- suitable indicator species
- relevance of the three time-scales (short-, medium- or long-term exposure)
- exposure model for reasonable-worst-case and most-likely-case scenarios.

With regard to rodenticides some background information is provided in Note 26.

The risk assessment is done at field scale and not at landscape scale, i.e. the risk to non-target birds and mammals frequenting the treated field. No consideration is made of the risk from applications of the same plant protection product to neighbouring fields. It should also be noted that there is no consideration of the risk from mixtures, i.e. the risk from two different active substances or plant protection products to the same crop at the same time. Although these risks may be important, it is considered that the associated assessment is extremely complex and not part of current regulatory practice.

The scheme does not include the risk to non-target birds and mammals from indirect effects, i.e. the removal of food sources due to the action of the plant protection products or alteration of habitat structure.

Time scales of assessment
In determining the level of risk, the following time-scales are considered:

- *short-term*: minutes to hours, representing gorging behaviour, diurnal peaks in feeding (e.g. dawn and dusk) and products which depurate or dissipate very rapidly.
- *medium-term*: hours to days, representing scenarios with relatively high exposures over several days. Also appropriate for acutely toxic compounds with delayed effects (e.g. rodenticides).
- *long-term*: days to weeks, representing long-term, low level exposures. Especially relevant to pesticides with bioaccumulative effects. The assessment determines the risk of both chronic lethal and reproductive effects on birds and mammals.

It should be noted that, in the short-term exposures, repeated applications of particular products can lead to exposure concentrations higher than the initial values. However, in the majority of cases, this is considered to be unlikely for the following reasons:

- decline in residue on plant material due to degradation and plant metabolism
- dilution of residue due to the growth of the plant
- dislodgement of dead insects.

Therefore, for the short-term risk assessment, the issue of multiple applications is not considered. However, for the medium and long-term assessment, possible exposure due to multiple applications should be considered.

Exposure-toxicity ratio (ETR)
The measure of risk used in the scheme is an 'exposure-toxicity ratio' (ETR). This is preferred because it increases with increasing risk, which is more intuitive than a toxicity exposure ratio (TER, which increases as risk decreases). ETRs are calculated for two types of scenarios for each time scale: the 'reasonable worst case' (RWC) and 'most likely case' (MLC) scenarios. The assumptions used for the RWC are more conservative (i.e. lead to higher estimates of risk) than the assumptions for the MLC. The purpose of using these two scenarios is to enable the risk to be classified into three categories consistent with the general approach of EPPO risk assessment schemes: high risk, low risk, and uncertain (or intermediate) risk. If little impact is expected in the RWC scenario, then the risk is low. If substantial impacts are expected even in the MLC (i.e. under relatively favourable assumptions), then the risk is high. If impacts are expected in the RWC but not in the MLC, this implies that further refinement of the assessment is necessary to evaluate how frequently impacts can really be expected in the range of conditions that occur in the field.

Reasonable worst-case scenario
The assumptions used for the 'reasonable worst case' (RWC) are intended to represent a scenario that is tending to worst case but is not beyond the bounds of possibility. The RWC for the spray and granule assessment uses the following assumptions (see Notes and Tables for details):

- the toxicity is based on a sensitive species (e.g. based on the 5th percentile of the sensitivity distribution or a fixed extrapolation factor)
- the active substance in/on the food or in the granules does not decay over time (except in the long-term time scale)

- the animal obtains all its food and its grit requirement from the treated field
- the animal shows no avoidance response to the treated food or to the granules
- the animal feeds entirely on the food type with highest residues
- the reasonable worst case for the short-term exposure concentration of plant protection product in/on food is based on the 90th percentile of the residue data (i.e. 90% of the time, residues will be lower)
- the percentages of soil/grit in the diet are based on the 90th percentile estimates
- the granule does not decay over time (except in the long-term time scale).

A short- or medium-term ETR less than 1 in the RWC implies that less than 50% mortality is expected under these conditions. A long-term ETR less than 1 in the RWC implies that no adverse effects (sub-lethal or lethal) are expected under these conditions. Because conditions worse than the RWC will occur only for a small proportion of species (5%) and only a small proportion of the time (no more than 10%, and probably much less), the risk is classified as low.

If a substance is classified as low-risk on the basis of RWC conditions, then the assessment process for that time period is complete and the next time period is considered. If, however, a compound is not classified as 'low risk', then further assessment is required to consider MLC conditions.

'Most likely case' scenario
The assumptions used for the 'most likely case' (MLC) for products applied as sprays or granules are intended to represent a scenario that is 'average', or about midway between 'worst case' and 'best case'. The MLC uses the following assumptions (see Notes and Tables for detail):

- the toxicity is based on the geometric mean of the available endpoints (average sensitivity)
- the active substance in/on the food or in the granules decays over time (but in the short-term time scale the decay is small)
- the animal obtains only part of its food or its grit requirements from the treated area
- the animal may show an avoidance response to the treated food or the granules, if there is evidence of this from relevant laboratory or (semi)field studies
- the animal may consume a mixed diet of several food types with differing levels of residues
- the concentration of plant protection product in/on food is based on the 50th percentile (median) of the relevant residue data (i.e. 50% of the time, residues will be higher)
- the percentages of soil/grit in the diet are based on the geometric mean estimates
- the granule may decay over time.

A short- or medium-term ETR greater than 1 in the MLC implies that a species of average sensitivity will suffer 50% mortality under average exposure conditions, and for 50% of the species the effects will be greater still. A long-term ETR greater than 1 in the MLC implies that adverse effects (sub-lethal or lethal) are expected for species of average sensitivity under average exposure conditions. Products of this type are classified as 'high risk'.

The 'most likely case' (MLC) is not a refined risk assessment step and should not be used as such. The MLC merely gives an indication as to the possibility of impacts under more average conditions (as defined above) and hence a step in the process for deciding whether a compound should be classified as 'high risk' or 'uncertain risk'.

Analysis of uncertainty
An important feature of this approach is that all assumptions, extrapolation factors and uncertainty factors are explicitly stated in defining the calculations for the RWC and MLC scenarios. This ensures that the assumptions are transparent, and enables the user to adjust them appropriately if required (e.g. if specific data is available for part of the calculation). In certain stages in the risk assessment, the most appropriate endpoints are not available (e.g. mammalian LC50) and extrapolation factors have therefore been used to estimate the endpoint which should be used in the assessment. In such cases, the rationale behind the extrapolation factor is fully explained in the notes. Because these factors are allowed for in the calculation of exposure and toxicity, the ETR is compared to a 'trigger value' of 1, rather than incorporating extrapolation factors into the trigger value as was done in the previous version of this scheme (OEPP/EPPO, 1994).

Interpretation of results
As stated above, each of the modules results in classification of the plant protection product as either 'low risk', 'uncertain risk' or 'high risk'. These terms do not indicate any degree of acceptability, they simply indicate the potential level of impact as defined by the 'reasonable worst case' and 'most likely case' scenarios. Acceptability is beyond the remit of the scheme, since various factors (e.g. social and political) may be considered before the use of a plant protection product is considered to be acceptable.

- *Low risk*: Effects are expected only for very sensitive species and under infrequent, worst-case exposure conditions.
- *High risk*: Substantial casualties are expected for a significant proportion of species. In this situation refinement on its own is unlikely to change the assessment outcome to 'low risk', so both mitigation and refinement should be considered. It should be noted that in certain cases mitigation followed by refinement may still result in a high risk.
- *Uncertain risk*: The risk assessment has indicated that the risk is uncertain, i.e. intermediate between the 'high' and 'low' categories, so refinement of the assessment is necessary to try and determine how frequently impacts on non-target birds and mammals will occur. This could result in the risk being assessed as either high or low.

Limitations of the current scheme
Any scheme of this type is necessarily limited by the current state of scientific knowledge. Many, if not all, elements of the scheme are affected by uncertainties and attention is drawn to many of these in the accompanying Notes. Examples of significant limitations include:

- lack of a firm basis for quantifying between-species variation in medium- and long-term toxicity (see Note 13)
- lack of a satisfactory method for assessing reproductive effects of short- and medium-term exposures (see Note 16)
- lack of established methods for assessing non-dietary routes of exposure (see discussion of scope, above, and Note 18).
- limitations of current knowledge regarding the factors influencing ingestion of granules (see Steps 28 – 71).
- limitations of current knowledge regarding factors influencing the avoidance response and its variation between species (see Note 19).
- limitations of current data for estimating residues on invertebrates (see Note 8).

Further research is required to establish scientifically robust ways of addressing these and other issues in risk assessment. Users of this scheme are advised to incorporate improved approaches as and when they become available. The user is advised to keep a note of the

outcome for each scenario until the second phase is reached (steps R1 to R6) where refinement/mitigation is carried out for those scenarios which need it.

3.2 Risk assessment scheme

Details of the product and its pattern of use

1. Obtain the basic information on the product (active substances) and its pattern of use including crops, application rates and timing, and formulation type.

Go to 2

Possibility of exposure

2. Is the exposure of terrestrial vertebrates possible (see Note 1)?

If no **Classify as negligible risk for vertebrates (i.e. birds and mammals) and exit scheme**

If yes **Go to 3**

Toxicity data

3. Obtain existing information on the toxicity of the active substance to mammals and birds, including data from human health assessments (see Note 2).

For seed treatment and granular applications **Go to 4**

For spray applications **Go to 7**

One seed / one granule criterion

This part of the scheme can be considered as a precautionary warning system for potential high risk applications for birds and mammals. It enables the user to calculate whether a generic species of either bird or mammal has a 50% chance of dying following the consumption of only one treated seed or one granule. The following assumptions are made in the calculation of the risk associated with the consumption of one seed or granule: an average concentration [amount] of the compound on/in 1 seed/granule is used, the 5th percentile of the toxicity distribution ($LD50^{5th\ percentile}$), and a body weight of 25 grams (Note that this is not worst case, the smallest birds and mammals in Europe have a body weight of several grams).

4. Calculate a 5th percentile of the toxicity distribution ($LD50^{5th\ percentile}$) according to Notes 10-12.
Calculate for the appropriate generic bird and mammal the exposure level as a result of the consumption of 1 seed or 1 granule (use shortcut values of Table 1).

One seed dose (OSD) = shortcut value * $S_{loading}$ or
one granule dose (OGD) = shortcut value * $G_{loading}$ (see Note 3)

Go to 5

5. Calculate $ETR_{1\ seed\ or\ 1\ granule}$ (= OSD or OGD / $LD50^{5th\ percentile}$) for the consumption of 1 seed or granule.

$ETR_{1\ seed\ or\ granule} > 1$ **Go to 6**

$ETR_{1\ seed\ or\ granule} \leq 1$ **Go to 7 for seed treatments or 28 for granular applications**

6. The seed treatment or granular application should be classified as a potential high risk application for birds and mammals. Proceeding with the assessment should give a better insight of the potential risk under normal agricultural circumstances (mid field and end row). As the potential risk is high it may be worthwhile to stress caution when applying this product/formulation for seed treatment or granular applications under conditions of good agricultural practice (e.g. preventing spills and ensuring full incorporation of treated seed or granules).

Consider adding safety phrases on the label and go to 7 for seed treatments or to 28 for granular applications

3.2.1 Risk assessment module for sprayed products and seed treatments

Risk characterisation

In almost all cases, the initial risk characterisation is done by means of exposure-to-toxicity ratios (ETR) or the inverse relationship (toxicity-to-exposure, TER). In this module, the risk is assessed for 3 time-scales: short (acute), medium, and long-term. The distinction between medium and long-term for mammals is poorly defined according to current toxicity input data. When the same type of medium-term toxicity data is available for mammals as for birds, the normal procedure for birds should be followed. In cases where relevant toxicity data is lacking the medium-term assessment may be omitted for mammals.

When exposure estimates and toxicity data are put into an ETR both figures have to match with regard to time scale and should be based on the same unit, either daily dose or concentration. However, for scientific reasons, ETRs are better based on daily dose in order to avoid bias to different food intake rates between laboratory results and the field. Therefore, this approach is followed in this document (see Note 4)

Short-term exposure (minutes to hours) - reasonable worst case assessment

7. Identify which of the generic species listed in Table 2 (see also Note 5 and 6) are relevant to the crop and use pattern under assessment (generally this will be more than one mammal and more than one bird).
   Calculate the reasonable worst case Daily Dietary Dose (DDD_{rwc}) for each of the relevant species using the shortcut value(s) presented in Table 2. DDD_{rwc} = Shortcut value * application rate in kg a.s./ha [mg per kg body weight per day] or application rate in g/kg seed.

   Go to 8

8. Either calculate a 5th percentile of the toxicity distribution ($LD50^{5th\ percentile}$) according to Notes 10-12 or use a fixed extrapolation factor on the lowest available toxicity value (Note 12).

   Go to 9

9. Calculate ETR_{rwc} (= $DDD_{rwc}/LD50^{5th\ percentile}$) for short-term exposure.
   $ETR_{rwc} \leq 1$ **Classify as <u>low risk for short-term exposure</u> and go to 13**
   $ETR_{rwc} > 1$ **Go to 10**

Short-term exposure (minutes to hours) - most likely case assessment

10. Identify which of the generic species listed in Table 3 are relevant to the crop and use pattern under assessment (generally this will be more than one mammal and more than one bird).
Calculate the most likely case Daily Dietary Dose (DDD_{mlc}) for each of the relevant species using the shortcut values presented in Table 3.

Go to 11

11. Calculate the geometric mean of the LD50s for the species tested. If more than one LD50 is available for any one species (see Note 12.)

Go to 12

12. Calculate ETR_{mlc} (= DDD_{mlc}/mean LD50) for short-term exposure for each of the relevant generic species.
$ETR_{mlc} \geq 1$: **Classify as <u>high risk for short-term exposure</u> and go to 13**
$ETR_{mlc} < 1$: **Classify as <u>uncertain risk for short-term exposure</u> and go to 13**

Medium-term exposure (days to weeks) - reasonable worst case assessment

13. Use the same generic species as were identified for the short-term assessment, and calculate DDD_{rwc} by using the shortcut values presented in Table 4. In addition, include when appropriate and when the Log $K_{ow} \geq 3$ earthworm-eating and fish-eating mammals and birds in the assessment to account for bioaccumulation (see Note 20) and estimate their exposure according to the shortcut values shown in Table 4.

Go to 14

14. For birds **Go to 15**
For mammals **Go to 18**

15. Is one or more avian LC50 study available?
If yes **Go to 16**
If no **Go to 17**

16. Convert each LC50 test result (mg/kg food) to an estimated LD50 (mg/kg body weight per day) (see Note 4). Calculate a $LD50^{5th\ percentile}$ according to Note 12 (or Note 11), applying the same extrapolation factors as for the short-term exposure scenarios (see Note 13). Alternatively, if it is established in legislation, then use a fixed extrapolation factor on the lowest available toxicity value.

Calculate ETR_{rwc} (= DDD_{rwc} / $LD50^{5th\ percentile}$) for medium-term exposure.
$ETR_{rwc} \leq 1$ **Classify as <u>low risk for medium-term exposure</u> and go to 22**
$ETR_{rwc} > 1$ **Go to 19**

17. Carry out the medium-term avian assessment using information from the avian reproduction study. If this indicates a low risk in the reasonable worst case, it will avoid the need to conduct an avian LC50 study (See Note 14). Derive a No Observed Effect Concentration (NOEC) based on the no-parental-mortality-level (mg/kg food) from each avian reproduction toxicity study and express each NOEC into a No Observed Effect Dose (NOED) in mg per kg body weight per day (See Note 4). Either

calculate a 5th percentile of toxicity distribution of converted NOED values ($NOED^{5th\ percentile}$) according to Note 12 (or Note 11), applying the same extrapolation factors as for the short-term exposure scenarios (see Note 13), or use a fixed extrapolation factor on the lowest available toxicity value.

Calculate ETR_{rwc} ($=DDD_{rwc} / NOED^{5th\ percentile}$) for medium-term exposure.

$ETR_{rwc} \leq 1$ **Classify as <u>low risk for medium-term exposure</u> and go to 22**

$ETR_{rwc} > 1$ **Conduct an avian 5-day dietary LC50 study and go to 16**

18. For mammals, take the NOED based on mortality from the 28-day rat study, expressed in mg per kg body weight per day (see Note 15). If no 28 day NOED is available, take the closest toxicity test available and use expert judgement to derive an appropriate endpoint. Either calculate a 5th percentile of toxicity distribution of NOED values ($NOED^{5th\ percentile}$) according to Note 12 (or Note 11), applying the same extrapolation factors as for the short-term exposure scenarios (see Note 13), or use a fixed extrapolation factor on the lowest available toxicity value.

Calculate ETR_{rwc} ($=DDD_{rwc} / NOED^{5th\ percentile}$) for medium-term exposure.

$ETR_{rwc} \leq 1$ **Classify as <u>low risk for medium-term exposure</u> and go to 22**

$ETR_{rwc} > 1$ **Go to 19**

Medium-term exposure (days to weeks) - most likely case assessment

19. Calculate the Daily Dietary Dose (DDD_{mlc}) for all generic species relevant to the use pattern, using the shortcut values presented in Table 5. In addition, include when appropriate and when the Log $K_{ow} \geq 3$ earthworm-eating and fish-eating mammals and birds in the assessment (see Note 20) and estimate their exposure according to the shortcut values shown in Table 5.

Go to 20

20. Calculate the geometric mean of the converted LD50 values (from Step 16-18, one for birds and one for mammals).

Go to 21

21. Calculate ETR_{mlc} ($= DDD_{mlc}$/mean LD50) for medium-term exposure for each of the relevant generic species.

$ETR_{mlc} \geq 1$: **Classify as <u>high risk for medium-term exposure</u> and go to 22**

$ETR_{mlc} < 1$: **Classify as <u>uncertain risk for medium-term exposure</u> and go to 22**

Assessment of long-term exposures (weeks to months) - reasonable worst case assessment

The assessment of long-term exposure (see also Note 16) should be based on the lowest available relevant endpoint either for parental effects or reproductive effects. If the outcome of the first assessment is uncertain or high risk, and the outcome of the subsequent refinement process indicates that the assessment based on this endpoint is of no concern (low risk), one should check whether a risk assessment should be carried out for the other endpoints.

22. Calculate the Daily Dietary Dose (DDD_{mlc}) for all generic species relevant to the use pattern, using the method presented in Table 6.

Go to 23

23. Derive a NOED from the relevant long-term toxicity study (the lowest one either based on parental effects or reproductive effects). Either calculate a $NOED^{5th\ percentile}$ according to Note 12 (or Note 11), applying the same extrapolation factors as for the short-term exposure scenarios (see Note 13), or use a fixed extrapolation factor on the lowest available toxicity value.

Go to 24

24. Calculate ETR_{rwc} (= DDD_{rwc} / $NOED^{5th\ percentile}$) for long-term exposure.
    $ETR_{rwc} \leq 1$ **Classify as <u>low risk for long-term exposure</u> and go to R1**
    $ETR_{rwc} > 1$ **Go to 25**

Assessment of long-term exposures (weeks to months) - most likely case assessment

25. Calculate the Daily Dietary Dose (DDD_{mlc}) for all generic species relevant to the use pattern, using the method presented in Table 7.

Go to 26

26. Calculate the geometric mean of the converted NOED values (from Step 23) (one for birds, one for mammals).

Go to 27

27. Calculate ETR_{mlc} (= DDD_{mlc}/ mean of converted NOED values) for long-term exposure for each of the relevant generic species.
    $ETR_{mlc} \geq 1$ **Classify as <u>high risk for long-term exposure</u> and go to R1**
    $ETR_{mlc} < 1$ **Classify as <u>uncertain risk for long-term exposure</u> and go to R1**

3.2.2 Risk assessment module for granular formulations

Risk characterisation
Grit consumption by farmland birds is an important constituent of dietary intake both for mineral content and mastication (Best & Gionfriddo, 1994). Significant differences exist between granivorous and non-granivorous species in respect to size of grit ingested, with non-granivores generally taking in grit indiscriminately with soil particles, while granivore species pick up grit particles selectively. Accordingly, the type of soil and its constituent composition can substantially influence the extent to which birds may be exposed to granular products. In general, detailed information on the particle-class size distribution in the top layer of soil is not available for a large number of representative soil types. Accordingly, the data presented on particle size classification should be assessed in the context of its relevance to the particular use situation.

Granules may be ingested accidentally when birds probe for or peck at food in or on treated soil, or they may be ingested intentionally by animals that mistake them for grit or food. Of the commonly used carriers, corncob granules most likely seem to be mistakenly ingested as food. Exposure assessment for granular products formulated on corncob carrier should follow the methodology presented in the decision-making module for treated seeds, starting at Step 7.

Other types of granules may possibly be mistaken by birds when searching for weed seeds but it is unknown whether this actually occurs and there is no established methodology for assessing it. It is recommended that assessors examine and compare samples of the granules

and of relevant weed seeds. If it appears possible that the granules could be mistaken for weed seeds, then consideration could be given to adopting a modified version of the approach recommended below for intentional ingestion of granules as grit (starting at Step 51). In this case, the daily grit dose (DGD) would be replaced with an estimate of the number of seeds eaten daily, and the number of soil particles (SP) would be replaced with an estimate of the number of exposed weed seeds (m^{-2}).
Assessing the exposure of birds to granules presents special difficulties, and scientific knowledge in this area has continued to develop since the presentation of the first decision-making sub-scheme for the environmental risk assessment of plant protection products for terrestrial vertebrates (OEPP/EPPO, 1994). However, significant uncertainties remain and there is currently no generally accepted approach for this issue (see also notes 21, 24 and 25).

The following routes by which birds and mammals might be exposed can be identified:
ingestion of granules by birds seeking grit
ingestion of granules by animals seeking seeds as food
ingestion of residues in other food items such as earthworms and plant seedlings
ingestion of granules with soil, unintentionally ingested with food/water
ingestion of residues in drinking water.

It is not usual to assess all these routes in detail. However, when only one or a few granules would be sufficient to achieve a lethal dose it is recommended to check whether one of these routes can be of concern. A method for assessing the potential risk for the two major routes, ingestion of granules when seeking grit and ingestion of soil when seeking food, is presented in steps 28 to 71.

28. Is the ingestion predominately accidental? **Go to 30**
Is the ingestion predominately intentional? **Go to 29**

If it is uncertain whether ingestion is predominately accidental or intentional, then both routes should be examined by proceeding from both Step 29 and (separately) Step 30.

29. Do animals mistake the granules for food (see above)? **Go to 7**
Do animals (e.g. birds) mistake the granules for grit? **Go to 51**

3.2.2.1 Ingestion of granules accidentally (as part of soil ingestion)

Short-term exposure (minutes to hours) - reasonable worst case assessment

30. Calculate the reasonable worst case Daily Dry Soil Dose ($DDSD_{rwc}$) for a 25 gram bird and mammal using the shortcut values presented in Table 8 (see also Note 22).
$DDSD_{rwc}$ = Shortcut value * application rate in kg a.s./ha [mg per kg body weight per day].

Go to 31

31. Either calculate a 5th percentile of the toxicity distribution ($LD50^{5th\ percentile}$) according to the Notes 10-12 or use a fixed extrapolation factor on the lowest available toxicity value.

Go to 32

32. Calculate ETR_{rwc} (= $DDSD_{rwc}/LD50^{5th\ percentile}$) for short-term exposure.

$ETR_{rwc} \leq 1$ **Classify as <u>low risk for short-term exposure</u> and go to 36**
$ETR_{rwc} > 1$ **Go to 33**

Short-term exposure (minutes to hours) - most likely case assessment

33. Calculate the most likely case Daily Soil Dose ($DDSD_{mlc}$) for a 25 gram bird and mammal using the shortcut values presented in Table 8.
DSD_{mlc} = Shortcut value * application rate in kg a.s./ha [mg per kg body weight per day].

Go to 34

34. Calculate the geometric mean of the LD50s for the species tested. If more than one LD50 is available for any one species (see Note 12).

Go to 35

35. Calculate ETR_{mlc} (= $DDSD_{mlc}$/mean LD50) for short-term exposure.
$ETR_{mlc} \geq 1$: **Classify as <u>high risk for short-term exposure</u> and go to 36**
$ETR_{mlc} < 1$: **Classify as <u>uncertain risk for short-term exposure</u> and go to 36**

Medium-term exposure (days to weeks) - reasonable worst case assessment

36. Calculate the reasonable worst case Daily Soil Dose ($DDSD_{rwc}$) for a 25 gram bird and mammal using the shortcut values presented in Table 8.
$DDSD_{rwc}$ = Shortcut value * application rate in kg a.s./ha [mg per kg body weight per day].

Go to 37

37. For birds **Go to 38**
For mammals **Go to 41**

38. Is one or more avian LC50 study available?
If yes **Go to 39**
If no **Go to 40**

39. Convert each LC50 test result (mg/kg food) to an estimated LD50 (mg per kg body weight per day) (see Note 4). Either calculate a $LD50^{5th\ percentile}$ according to Note 11 or 12, applying the same extrapolation factors as for the short-term exposure scenarios (see Note 13), or use a fixed extrapolation factor on the lowest available toxicity value.

Calculate ETR_{rwc} (= $DDSD_{rwc}$ / $LD50^{5th\ percentile}$) for medium-term exposure.
$ETR_{rwc} \leq 1$ **Classify as <u>low risk for medium-term exposure</u> and go to 45**
$ETR_{rwc} > 1$ **Go to 42**

40. Carry out the medium-term avian assessment using information from the avian reproduction study. If this indicates a low risk in the reasonable worst case it will avoid the need to conduct an avian LC50 study (See Note 14). Derive a NOEC based on the no-parental-mortality-level (mg/kg food) from each avian reproduction toxicity study and convert each NOEC into a NOED in mg per kg body weight per day (See Note 4). Either calculate a 5^{th} percentile of toxicity distribution of converted NOED values ($NOED^{5th\ percentile}$) according to Note 11 or 12, applying the same extrapolation

factors as for the short-term exposure scenarios (see Note 13), or use a fixed extrapolation factor on the lowest available toxicity value.

Calculate ETR_{rwc} (=$DDSD_{rwc}$ / $NOED^{5th\ percentile}$) for medium-term exposure.
$ETR_{rwc} \leq 1$ Classify as low risk for medium-term exposure and go to 45
$ETR_{rwc} > 1$ Conduct an avian 5-day dietary LC50 study and go to 39

41. For mammals, take the NOED based on mortality from the 28-day rat study, expressed in mg per kg body weight perday (see Note 15) If no 28 day NOED is available take the closest toxicity test available and use expert judgement to derive an appropriate endpoint. Either calculate a 5th percentile of toxicity distribution of NOED values ($NOED^{5th\ percentile}$) according to Note 11 or 12, applying the same extrapolation factors as for the short-term exposure scenarios (see Note 13), or use a fixed extrapolation factor on the lowest available toxicity value.

Calculate ETR_{rwc} (=$DDSD_{rwc}$ / $NOED^{5th\ percentile}$) for medium-term exposure.
$ETR_{rwc} \leq 1$ **Classify as low risk for medium-term exposure and go to 45**
$ETR_{rwc} > 1$ **Go to 42**

Medium-term exposure (days to weeks) - most likely case assessment

42. Calculate the most likely case Daily Soil Dose ($DDSD_{mlc}$) for a 25 gram bird and mammal using the shortcut values presented in Table 8.
DSD_{mlc} = Shortcut value * application rate in kg a.s./ha * time weighted average (TWA) factor [mg per kg body weight per day].

Go to 43

43. Calculate the geometric mean of the converted LD50 values (from Step 39-41), one for birds and one for mammals).

Go to 44

44. Calculate ETR_{mlc} (= $DDSD_{mlc}$/mean LD50) for medium-term exposure for each of the relevant generic species.
$ETR_{mlc} \geq 1$: **Classify as high risk for medium-term exposure and go to 45**
$ETR_{mlc} < 1$: **Classify as uncertain risk for medium-term exposure and go to 45**

Long-term exposures (weeks to months) - reasonable worst case assessment

The assessment of long-term exposure (see also Note 16) should be based on the lowest available relevant endpoint either for parental effects or reproductive effects. If the outcome of the first assessment is uncertain or high risk, and the outcome of the subsequent refinement process indicates that the assessment based on this endpoint is of no concern (low risk), one should check whether a risk assessment should be carried out for the other endpoints.

45. Calculate the reasonable worst case Daily Soil Dose ($DDSD_{rwc}$) for a 25 gram bird and mammal using the shortcut values presented in Table 8.
$DDSD_{rwc}$ = Shortcut value * application rate in kg a.s./ha * TWA factor [mg per kg body weight per day].

Go to 46

46. Derive a NOED from the relevant long-term toxicity study (the lowest one either based on parental effects or reproductive effects). Either calculate a $NOED^{5th\ percentile}$ according to Note 11 or 12, applying the same extrapolation factors as for the short-term exposure scenarios (see Note 13), or use a fixed extrapolation factor on the lowest available toxicity value.

Go to 47

47. Calculate ETR_{rwc} (= $DDSD_{rwc}$ / $NOED^{5th\ percentile}$) for long-term exposure.
$ETR_{rwc} \leq 1$ **Classify as <u>low risk for effects of long-term exposure</u> and go to R1**
$ETR_{rwc} > 1$ **Go to 48**

Long-term exposures (weeks to months) - most likely case assessment

48. Calculate the most likely case Daily Soil Dose ($DDSD_{mlc}$) for a 25 gram bird and mammal using the shortcut values presented in Table 8.
$DDSD_{mlc}$ = Shortcut value * application rate in kg a.s./ha * TWA factor [mg per kg body weight per day].

Go to 49

49. Calculate the geometric mean of the converted NOED values (from Step 46) (one for birds, one for mammals).

Go to 50

50. Calculate ETR_{mlc} (= $DDSD_{mlc}$ / mean of converted NOEL values) for long-term exposure for each of the relevant generic species.
$ETR_{mlc} \geq 1$: **Classify as <u>high risk for long-term exposure</u> and go to R1**
$ETR_{mlc} < 1$: **Classify as <u>uncertain risk for long-term exposure</u> and go to R1**

3.2.2.2 Ingestion of granules intentionally (as part of grit ingestion)

Short-term exposure (minutes to hours) - reasonable worst case assessment

51. Calculate the reasonable worst case Daily Granule Dose (DGD_{rwc}) for a representative generic species using the values presented in Table 9 and Note 24.
$DGD_{rwc} = DGI_{rwc} * (G_{surface}/(SP_{surface}+G_{surface})) * G_{loading}$ [mg per kg body weight per day].

Go to 52

52. Either calculate a 5th percentile of the toxicity distribution ($LD50^{5th\ percentile}$) according to the Notes 10-12 or use a fixed extrapolation factor on the lowest available toxicity value.

Go to 53

53. Calculate ETR_{rwc} (= $DGD_{rwc}/LD50^{5th\ percentile}$) for short-term exposure.
$ETR_{rwc} \leq 1$ **Classify as <u>low risk for short-term exposure</u> and go to 57**
$ETR_{rwc} > 1$ **Go to 54**

Short-term exposure (minutes to hours) - most likely case assessment

54. Calculate the most likely case Daily Granule Dose (DGD_{mlc}) for a representative generic species using the values presented in Table 9 and Note 25.
$DGD_{mlc} = DGI_{mlc} * (G_{surface}/(SP_{surface}+G_{surface})) * G_{loading}$ [mg per kg body weight per day].

Go to 55

55. Calculate the geometric mean of the LD50s for the species tested. If more than one LD50 is available for any one species, see Note 12.

Go to 56

56. Calculate ETR_{mlc} (= DGD_{mlc}/mean LD50) for short-term exposure.
$ETR_{mlc} \geq 1$: **Classify as <u>high risk for short-term exposure</u> and go to 57**
$ETR_{mlc} < 1$: **Classify as <u>uncertain risk for short-term exposure</u> and go to 57**

Medium-term exposure (days to weeks) - Reasonable worst case assessment

57. Calculate the reasonable worst case Daily Granule Dose (DGD_{rwc}) for a representative generic species using the values presented in Table 9 and Note 25.
$DGD_{rwc} = DGI_{rwc} * (G_{surface}/(SP_{surface}+G_{surface})) * G_{loading}$ [mg per kg body weight per day].

Go to 58

58. For birds **Go to 59**
For mammals **Go to 62**

59. Is one or more avian LC50 study available?
If yes **Go to 60**
If no **Go to 61**

60. Convert each LC50 test result (mg/kg food) to an estimated LD50 (mg per kg body weight per day) (see Note 4). Either calculate a $LD50^{5th\ percentile}$ according to Note 11 or 12, applying the same extrapolation factors as for the short-term exposure scenarios (see Note 13), or use a fixed extrapolation factor on the lowest available toxicity value.

Calculate ETR_{rwc} (= DGD_{rwc} / $LD50^{5th\ percentile}$) for medium-term exposure.
$ETR_{rwc} \leq 1$ **Classify as <u>low risk for medium-term exposure</u> and go to 66**
$ETR_{rwc} > 1$ **Go to 63**

61. Carry out the medium-term avian assessment using information from the avian reproduction study. If this indicates a low risk in the reasonable worst case it will avoid the need to conduct an avian LC50 study (See Note 14). Derive a NOEC based on the no-parental-mortality-level (mg/kg food) from each avian reproduction toxicity study and express convert each NOEC into a NOED in mg per kg body weight per day (See Note 4). Either calculate a 5^{th} percentile of toxicity distribution of converted NOED values ($NOED^{5th\ percentile}$) according to Note 11 or 12, applying the same extrapolation factors as for the short-term exposure scenarios (see Note 13), or use a fixed extrapolation factor on the lowest available toxicity value.

Calculate ETR_{rwc} (=DGD_{rwc} / $NOED^{5th\ percentile}$) for medium-term exposure.
$ETR_{rwc} \leq 1$ **Classify as <u>low risk for medium-term exposure</u> and go to 66**
$ETR_{rwc} > 1$ **Conduct an avian 5-day dietary LC50 study and go to 60**

62. For mammals, take the NOED based on mortality from the 28-day rat study, expressed in mg per kg body weight per day (see Note 15) If no 28 day NOED is available take the closest toxicity test available and use expert judgement to derive an appropriate endpoint. Either calculate a 5th percentile of toxicity distribution of NOED values ($NOED^{5th\ percentile}$) according to Note 11 or 12, applying the same extrapolation factors as for the short-term exposure scenarios (see Note 13), or use a fixed extrapolation factor on the lowest available toxicity value.

Calculate ETR_{rwc} (=DGD_{rwc} / $NOED^{5th\ percentile}$) for medium-term exposure.
$ETR_{rwc} \leq 1$ **Classify as <u>low risk for medium-term exposure</u> and go to 66**
$ETR_{rwc} > 1$ **Go to 63**

Medium-term exposure (days to weeks) - most likely case assessment

63. Calculate the most likely case Daily Granule Dose (DGD_{mlc}) for a representative generic species using the values presented in Table 9 and Note 25. If appropriate, adjust to allow for granules lost from a soil surface (through disintegration or by being covered with soil during rainfall or by wind erosion).
$DGD_{mlc} = DGI_{mlc} * (G_{surface}/(SP_{surface}+G_{surface})) * (G_{loading} * TWA_{factor\ for\ a.s.})$ [mg per kg body weight per day].

Go to 64

64. Calculate the geometric mean of the converted LD50 values (from Step 60-62), one for birds and one for mammals).

Go to 65

65. Calculate ETR_{mlc} (= DGD_{mlc}/ mean LD_{50}) for medium-term exposure for each of the relevant generic species.
$ETR_{mlc} \geq 1$: **Classify as <u>high risk for medium-term exposure</u> and go to 66**
$ETR_{mlc} < 1$: **Classify as <u>uncertain risk for medium-term exposure</u> and go to 66**

Assessment of long-term exposures (weeks to months) - reasonable worst case assessment

This part of the decision tree is very often superfluous because the granules will be disintegrated or covered by soil or the concentration of the compound in the granule is lower due to degradation. The resulting risk to birds is probably covered by the soil ingestion part of this module. If appropriate (based on the characteristics of the granule), a long-term risk assessment for granules step 66 to 71 can be followed. Otherwise, one can go immediately to the refinement loop (=R1). The assessment of long-term exposure (see also Note 16) should be based on the lowest available relevant endpoint either for parental effects or reproductive effects. If the outcome of the first assessment is uncertain or high risk, and the outcome of the subsequent refinement process indicates that the assessment based on this endpoint is of no concern (low risk), one should check whether a risk assessment should be carried out for the other endpoints.

66. Calculate the reasonable worst case Daily Granule Dose (DGD_{rwc}) for a representative generic species using the values presented in Table 9 and Note 25. If appropriate, adjust $G_{surface}$ to allow for granules lost from the soil surface (through disintegration, or by being covered with soil during rainfall, or by wind erosion).
$DGD_{rwc} = DGI_{rwc} * (G_{surface}/(SP_{surface}+G_{surface})) * (G_{loading}* TWA_{factor\ for\ a.s.})$ [mg per kg body weight per day].

Go to 67

67. Derive a NOED from the relevant long-term toxicity study (the lowest one either based on parental effects of reproductive effects). Either calculate a $NOED^{5th\ percentile}$ according to Note 11 or 12, applying the same extrapolation factors as for the short-term exposure scenarios (see Note 13), or use a fixed extrapolation factor on the lowest available toxicity value.

Go to 68

68. Calculate ETR_{rwc} (= DGD_{rwc}/ $NOED^{5th\ percentile}$) for long-term exposure.
$ETR_{rwc} \leq 1$ **Classify as <u>low risk for effects of long-term exposure</u> and go to R1**
$ETR_{rwc} > 1$ **Go to 69**

Assessment of long-term exposures (weeks to months) - most likely case assessment

69. Calculate the most likely case Daily Granule Dose (DGD_{mlc}) for a representative generic species using the values presented in Table 9 and Note 25. If appropriate, adjust $G_{surface}$ to allow for granules lost from the soil surface (through disintegration, or by being covered with soil during rainfall, or by wind erosion).
$DGD_{mlc} = DGI_{mlc} * (G_{surface}/(SP_{surface}+G_{surface})) * (G_{loading}* TWA_{factor\ for\ a.s.})$ [mg per kg body weight per day].

Go to 70

70. Calculate the geometric mean of the converted NOED values (from Step 67) (one for birds, one for mammals).

Go to 71

71. Calculate ETR_{mlc} (= DGD_{mlc}/ mean of converted NOED values) for long-term exposure for each of the relevant generic species.
$ETR_{mlc} \geq 1$: **Classify as <u>high risk for long-term exposure</u> and go to R1**
$ETR_{mlc} < 1$: **Classify as <u>uncertain risk for long-term exposure</u> and go to R1**

Table 1 Shortcut values for 1 seed and 1 granule criterion

Species	Size of seeds and granules	BW (g)	Shortcut value (number of seeds consumed per kg BW)
Mammals	All seeds and all granules	25	40
Small bird	Small seeds and small granules (<3.5 mm)	25	40
Large bird	Large seeds (e.g. maize, peas, beans) and large granules (≥ 3.5 mm)	300	3.3

Table 2 Estimation of reasonable worst case short-term exposure for generic bird and mammalian species (no degradation is assumed and RUD is based on the 90th percentile of residue values)

Select all species which are relevant to the use pattern under consideration and estimate DDD_{rwc} by multiplying the maximum application rate by the shortcut values given in the table: DDDrwc [mg/kg BW per day] = shortcut value * application rate in kg/ha or g/kg seed for seed treatments.

Crop	Crop stage	Indicator species	Body weight	Food types (PD)	DFI kg/kg BW/d	RUD Residue Unit Dose mg/kg food	Shortcut value
Grassland	-	Herbivorous mammal	25	Short grass	1.389	142	197
		Herbivorous bird	3000	Short grass	0.441	142	63
Cereals	Early	Herbivorous mammal	25	Cereal shoots	1.389	142	197
		Herbivorous bird	3000	Cereal shoots	0.441	142	63
	Late	Herbivorous mammal	25	Non-grass herbs	1.138	87	99
		Insectivorous bird	10	Small insects	1.033	52	54
Vegetables	Early	Herbivorous mammal	25	Non-grass herbs	1.138	87	99
(e.g. oilseed		Herbivorous bird	300	Non-grass herbs	0.958	87	83
rape, sugar	Late	Herbivorous mammal	25	Non-grass herbs	1.138	87	99
beet etc.)		Insectivorous bird	10	Small insects	1.033	52	54
Vine/	Whole year	Herbivorous mammal	25	Short grass	1.389	H:142, I&F: 71*	H:197, I&F:99*
orchards/		Insectivorous bird	10	Small insects	1.033	52	54
hops		Fruit-eating bird	80	Fruit	2.160	11	24
Seed	Whole year	Seed-eating mammal	25	Treated seeds	0.226	1000	226
treatment		Seed-eating bird	15	Treated seeds (small)	0.304	1000	304
		Seed-eating birds	300	Treated seeds (large)	0.138	1000	138

* For canopy applications (e.g. insecticides (I) and fungicides (F) but not herbicides (H)) an interception factor of 0.5 is assumed.

Table 3 Estimation of most likely case short-term exposure for generic bird and mammalian species (no degradation is assumed and RUD is based on 50th percentile of residue values)

Select all species which are relevant to the use pattern under consideration and estimate DDD_{mlc} by multiplying the maximum application rate by the shortcut values given in the table and when applicable by the avoidance factor (AV_{factor}): DDD_{mlc} [mg/kg BW per day] = shortcut value * AV_{factor} * application rate in kg/ha or g/kg seeds for seed treatments.

Crop	Crop stage	Indicator species	Body weight	Food types (PD)	DFI kg/kg BW/d	RUD mg/kg food	Proportion of time spent in crop (PT)	Shortcut value
Grassland	-	Herbivorous mammal	3000	Short grass	0.338	61.6	0.5	10.4
		Herbivorous bird	3000	Short grass	0.441	61.6	0.5	13.6
Cereals/ Grassland	Early	Herbivorous mammal	3000	Cereal shoots	0.338	61.6	0.5	10.4
		Herbivorous bird	3000	Cereal shoots	0.441	61.6	0.5	13.6
	Late	Omnivorous mammal	25	3 types of food[1]	0.362	17.6	0.5	3.2
		Omnivorous bird	300	3 types of food	0.240	26.3	0.5	3.2
Vegetables (eg oilseed rape, sugar beet etc.)	Early	Herbivorous mammal	3000	Non-grass herbs	0.277	25	0.5	3.5
		Omnivorous bird	300	3 types of food	0.240	26.3	0.5	3.3
	Late	Omnivorous mammal	25	3 types of food	0.362	17.6	0.5	3.2
		Omnivorous bird	300	3 types of food	0.240	26.3	0.5	3.2
Vine/ Orchards/ hops	Whole year	Insectivorous bird	10	Small insects	1.033	29	0.5	15
		Fruit-eating bird	80	Fruit	2.160	2.3	0.5	2.5
		Omnivorous mammal	25	3 types of food	0.362	17.6	0.5	3.2
		Omnivorous bird	300	3 types of food	0.240	26.3	0.5	3.2
Seed treatment	Whole year	Seed-eating mammal	25	Treated seed	0.226	1000	0.5	113
		Seed-eating bird	25	Treated seed (small)	0.261	1000	0.5	131
		Seed-eating bird	500	Treated seed (large)	0.100	1000	0.5	50

[1] = 33% non-grass herbs, 33% insects and 33% seeds.

Table 4 Estimation of reasonable worst case medium-term exposure for generic bird and mammalian species (no degradation is assumed and RUD is based on 50th percentile of residue values)

Select all species which are relevant to the use pattern under consideration and estimate DDD_{rwc} by multiplying the maximum application rate by the shortcut values given in the table: DDD_{rwc} [mg/kg BW per day] = shortcut value * application rate in kg/ha or in case of seed treatments in g/kg seed. For multiple applications see Note 6. FI is the fraction of the application rate that will reach the soil after interception by the crop (see Chapter 4 Soil). FD is the fraction of the application rate that will reach the surface water by drift (see Chapter 6 Surface water). The bioconcentration factors (BCF_{worm} and BCF_{fish} are explained in note 20).

Crop	Crop stage	Indicator species	Body weight (g)	Food types (PD)	DFI kg/kg BW/d	RUD mg/kg food	Shortcut value
Grassland	-	Herbivorous mammal	25	Short grass	1.389	61.6	86
		Herbivorous bird	3000	Short grass	0.441	61.6	27
Cereals	Early	Herbivorous mammal	25	Cereal shoots	1.389	61.6	86
		Herbivorous bird	3000	Cereal shoots	0.441	61.6	27
	Late	Herbivorous mammal	25	Non-grass herbs	1.138	25	28
		Insectivorous bird	10	Small insects	1.033	29	30
Vegetables (eg oilseed rape, sugar beet etc.)	Early	Herbivorous mammal	25	Non-grass herbs	1.138	25	28
		Herbivorous bird	300	Non-grass herbs	0.958	25	24
	Late	Herbivorous mammal	25	Non-grass herbs	1.138	25	28
		Insectivorous bird	10	Small insects	1.033	29	30
Vine/ orchards/ hops	Whole year	Herbivorous mammal	25	Short grass	1.389	H:62, I&F:31*	H:86. I&F:43*
		Insectivorous bird	10	Small insects	1.033	29	30
		Fruit-eating bird	80	Fruit	2.160	2.3	5
Seed treatment	Whole year	Seed-eating mammal	25	Treated seeds	0.226	1000	226
		Seed-eating bird	15	Treated seeds (small)	0.304	1000	304
		Seed-eating birds	300	Treated seeds (large)	0.138	1000	138
Soil	Early	Worm-eating mammal	10	Worms	1.359	1.33	1.81* FI * BCF_{worm}
		Worm-eating bird	100	Worms	1.134	1.33	1.51* FI * BCF_{worm}
water	Whole year	Fish-eating mammal	7000	Fish	0.101	0.3	0.030 * FD * BCF_{fish}
		Fish-eating bird	900	Fish	0.222	0.3	0.067 * FD * BCF_{fish}

* For canopy applications (e.g. insecticides (I) and fungicides (F) but not herbicides (H)) an interception factor of 0.5 is assumed.

Table 5 Estimation of most likely case medium-term exposure for generic bird and mammalian species (f_{twa} = 0.85 and RUD is based on 50th percentile of residue values)

Select all species which are relevant to the use pattern under consideration and estimate DDD_{mlc} by multiplying the maximum application rate by the shortcut values given in the table and when applicable by the avoidance factor: DDD_{mlc} [mg/kg BW per day] = shortcut value *AV_{factor}* application rate in kg/ha or in cases of seed treatments in g/kg seed. For multiple applications see Note 6. For explanation of FI, FD and Bioconcentration factors see Table 4. fTWA= time weighted average factor

Crop	Crop stage	Indicator species	Body weight (g)	Food types (PD)	DFI kg/kg BW/d	RUD mg/kg food	PT	f_{TWA}[2]	Shortcut factor
Grassland	-	Herbivorous mammal	3000	Cereal shoots	0.338	61.6	0.5	0.85	8.9
		Herbivorous bird	3000	Cereal shoots	0.441	61.6	0.5	0.85	11.5
Cereals	Early	Herbivorous mammal	3000	Cereal shoots	0.338	61.6	0.5	0.85	8.9
		Herbivorous bird	3000	Cereal shoots	0.441	61.6	0.5	0.85	11.5
	Late	Omnivorous mammal	25	3 types of food[1]	0.362	17.6	0.5	0.85	2.7
		Omnivorous bird	300	3 types of food	0.240	26.3	0.5	0.85	2.7
Vegetables (eg oilseed rape, sugar beet etc.)	Early	Herbivorous mammal	3000	Non-grass herbs	0.277	25	0.5	0.85	2.9
		Omnivorous bird	300	3 types of food	0.240	26.3	0.5	0.85	2.7
	Late	Omnivorous mammal	25	3 types of food	0.362	17.6	0.5	0.85	2.7
		Omnivorous bird	300	3 types of food	0.240	26.3	0.5	0.85	2.7
Vine/ orchards/ hops	-	Insectivorous bird	10	Small insects	1.033	29	0.5	0.85	12.7
		Fruit-eating bird	80	Fruit	2.160	2.3	0.5	0.85	2.1
		Omnivorous mammal	25	3 types of food	0.362	17.6	0.5	0.85	2.7
		Omnivorous bird	300	3 types of food	0.240	26.3	0.5	0.85	2.7
Seed treatment	-	Seed-eating mammal	25	Treated seed	0.226	1000	0.5	0.85	96
		Seed-eating bird	25	Treated seed (small)	0.261	1000	0.5	0.85	111
		Seed-eating bird	500	Treated seed (large)	0.100	1000	0.5	0.85	42
Soil	-	Worm-eating mammal	10	Worms	1.359	1.33	0.5	Use DT50 of soil	0.77 * FI *BCF_{worm}
		Worm-eating bird	100	Worms	1.134	1.33	0.5	Use DT50 of soil	0.64 * FI * BCF_{worm}
Water	-	Fish-eating mammal	7000	Fish	0.101	0.3	0.5	Use DT50 of water	0.013 * FD * BCF_{fish}
		Fish-eating bird	900	Fish	0.222	0.3	0.5	Use DT50 of water	0.028 * FD * BCF_{fish}

[1] = 33% non-grass herbs, 33% insects and 33% seeds; [2] = Default DT50 10 days (see EU document) and fixed time window of 5 days. In principle only applicable to residue levels on plant material, but probably the same for insects and perhaps even less because of immigrating and emigrating insects.

Table 6 Estimation of reasonable worst case long-term exposure for generic bird and mammalian species (f_{twa} = 0.53 and RUD is based on 50th percentile of residue values)

Select all species which are relevant to the use pattern under consideration and estimate DDD_{rwc} by multiplying the maximum application rate by the shortcut values given in the table: DDD_{rwc} [mg/kg BW per day] = shortcut value * application rate in kg/ha or in case of seed treatments in g/kg seed. For multiple applications see Note 6. See for explanation of FI, FD and Bioconcentration factors Table 4.

Crop	Crop stage	Indicator species	Body weight (g)	Food types (PD)	DFI kg/kg BW/d	RUD mg/kg food	f_{TWA}[2]	Shortcut value
Grassland	-	Herbivorous mammal	25	Short grass	1.389	61.6	0.53	45
		Herbivorous bird	3000	Short grass	0.441	61.6	0.53	14
Cereals	Early	Herbivorous mammal	25	Cereal shoots	1.389	61.6	0.53	45
		Herbivorous bird	3000	Cereal shoots	0.441	61.6	0.53	14
	Late	Herbivorous mammal	25	Non-grass herbs	1.138	25	0.53	15
		Insectivorous bird	10	Small insects	1.033	25	0.53	14
Vegetables (eg oilseed rape, sugar beet etc.)	Early	Herbivorous mammal	25	Non-grass herbs	1.138	25	0.53	15
		Herbivorous bird	300	Non-grass herbs	0.958	25	0.53	13
	Late	Herbivorous mammal	25	Non-grass herbs	1.138	25	0.53	15
		Insectivorous bird	10	Small insects	1.033	29	0.53	14
Vine/ orchards/ hops	Whole year	Herbivorous mammal	25	Short grass	1.389	H:61.6, I&F:31[1]	0.53	H:45, I&F:23
		Insectivorous bird	10	Small insects	1.033	25	0.53	14
		Fruit-eating bird	80	Fruit	2.160	2.3	0.53	3
Seed treatment	Whole year	Seed-eating mammal	25	Treated seeds	0.226	1000	0.53	120
		Seed-eating bird	15	Treated seeds (small)	0.304	1000	0.53	161
		Seed-eating birds	300	Treated seeds (large)	0.138	1000	0.53	73
Soil	-	Worm-eating mammal	10	Worms	1.359	1.33	Use DT50 of soil	0.96 * FI * BCF_{worm}
		Worm-eating bird	100	Worms	1.134	1.33	Use DT50 of soil	0.80 * FI * BCF_{worm}
water	-	Fish-eating mammal	7000	Fish	0.101	0.3	Use DT50 of water	0.016 * FD * BCF_{fish}
		Fish-eating bird	900	Fish	0.222	0.3	Use DT50 of water	0.035 * FD * BCF_{fish}

[1] = For canopy applications (e.g. insecticides (I) and fungicides (F) but not herbicides (H)) an interception factor of 0.5 is assumed.

[2] = Default DT50 10 days (see EU document) and fixed time window of 21 days. In principle only applicable to residue levels on plant material, but probably the same for insects and perhaps even less because of immigrating and emigrating insects.

Table 7 Estimation of most likely case long-term exposure for generic bird and mammalian species (f_{twa} = 0.53 and RUD is based on 50th percentile of residue values)

Select all species which are relevant to the use pattern under consideration and estimate DDD_{rwc} by multiplying the maximum application rate (kg/ha) by the shortcut values given in the table and when applicable by the avoidance factor: DDD_{mlc} [mg/kg BW per day] = shortcut value *AV_{factor} * application rate in kg/ha or in case of seed treatments in g/kg seed. For multiple applications see Note 6. See for explanation of FI, FD and Bioconcentration factors Table 4. PT=Fraction of diet obtained in treated area

Crop	Crop stage	Indicator species	Body weight (g)	Food types (PD)	DFI kg/kg BW/d	RUD mg/kg food	PT [2]	f_{TWA} [3]	Shortcut value	
									Breeding season	Other seasons
Grassland	-	Herbivorous mammal	3000	Cereal shoots	0.338	61.6	0.5 and 0.1	0.53	5.5	1.1
		Herbivorous bird	3000	Cereal shoots	0.441	61.6	0.5 and 0.1	0.53	7.2	1.4
Cereals	Early	Herbivorous mammal	3000	Cereal shoots	0.338	61.6	0.5 and 0.1	0.53	5.5	1.1
		Herbivorous bird	3000	Cereal shoots	0.441	61.6	0.5 and 0.1	0.53	7.2	1.4
	Late	Omnivorous mammal	25	3 types of food[1]	0.362	17.6	0.5 and 0.1	0.53	1.7	0.34
		Omnivorous bird	300	3 types of food	0.240	26.3	0.5 and 0.1	0.53	1.7	0.33
Vegetables (eg oilseed rape, sugar beet etc.)	Early	Herbivorous mammal	3000	Leaves/vegetables	0.277	25	0.5 and 0.1	0.53	1.8	0.37
		Omnivorous bird	300	3 types of food	0.240	26.3	0.5 and 0.1	0.53	1.7	0.33
	Late	Omnivorous mammal	25	3 types of food	0.362	17.6	0.5 and 0.1	0.53	1.7	0.34
		Omnivorous bird	300	3 types of food	0.240	26.3	0.5 and 0.1	0.53	1.7	0.33
Vine/ orchards/ hops	-	Insectivorous bird	10	Small insects	1.033	29	0.5 and 0.1	0.53	7.9	1.59
		Fruit-eating bird	80	Fruit	2.160	2.3	0.5 and 0.1	0.53	1.3	0.26
		Omnivorous mammal	25	3 types of food	0.362	17.6	0.5 and 0.1	0.53	1.7	0.34
		Omnivorous bird	300	3 types of food	0.240	26.3	0.5 and 0.1	0.53	1.7	0.33
Seed treatment	-	Seed-eating mammal	25	Treated seed	0.226	1000	0.5 and 0.1	0.53	60	12
		Seed-eating bird	25	Treated seed (small)	0.261	1000	0.5 and 0.1	0.53	69	14
		Seed-eating bird	500	Treated seed (large)	0.100	1000	0.5 and 0.1	0.53	26	5
Soil	-	Worm-eating mammal	10	Worms	1.359	1.33	0.5 and 0.1	Use DT50 of soil	0.48 or 0.096 * FI * BCF_{worm}	
		Worm-eating bird	100	Worms	1.134	1.33	0.5 and 0.1	Use DT50 of soil	0.40 or 0.08* FI * BCF_{worm}	
Water	-	Fish-eating mammal	7000	Fish	0.101	0.3	0.5 and 0.1	Use DT50 of water	0.02 or 0.0035 * FD * BCF_{fish}	
		Fish-eating bird	900	Fish	0.222	0.3	0.5 and 0.1	Use DT50 of water	0.01 or 0.0016 * FD * BCF_{fish}	

[1] = 33% non-grass herbs, 33% insects and 33% seeds.: [2] = It is assumed that the time spent in treated crop is 0.5 in the breeding season and 0.1 outside the breeding season.
[3] = Default DT50 10 days (see EU document) and fixed time window of 21 days. In principle only applicable to residue levels on plant material, but probably the same for insects and perhaps even less because of immigrating and emigrating insect

Table 8 Estimation of reasonable worst case (RWC) and most likely case (MLC) for short, medium and long-term exposure via contaminated soil for a generic bird and mammalian omnivorous species of 25 gram.

Estimate $DDSD_{rwc}$ or $DDSD_{mlc}$ by multiplying the maximum application rate (kg/ha) by the shortcut values given in the table DDSD [mg/kg BW per day] = shortcut value * application rate in kg/ha (* time weighted average factor (f_{TWA} should be based on DT50 soil, see Note 9)).

Exposure duration	RWC /MLC	Species	Daily Dry Food Intake (DDFI) g kg^{-1} BW d^{-1}	% of soil in diet (%soil)	Daily Dry Soil Intake (DDSI) g kg^{-1} BW d^{-1}	RUD mg/kg dry soil	PT	Shortcut Value
Short-term	RWC	Mammal	164	9.4	17.0	6.667	1	0.113
		Bird	323	18	70.9	6.667	1	0.473
	MLC	Mammal	164	3.8	6.5	6.667	0.5	0.022
		Bird	323	7.9	27.7	6.667	0.5	0.092
Medium-term	RWC	Mammal	164	9.4	17.0	1.333	1	0.023
		Bird	323	18	70.9	1.333	1	0.095
	MLC	Mammal	164	3.8	6.5	1.333	0.5	0.004 * f_{TWA}
		Bird	323	7.9	27.7	1.333	0.5	0.018 * f_{TWA}
Long-term	RWC	Mammal	164	9.4	17.0	1.333	1	0.023 * f_{TWA}
		Bird	323	18	70.9	1.333	1	0.095* f_{TWA}
	MLC	Mammal	164	3.8	6.5	1.333	0.5	0.004 * f_{TWA}
		Bird	323	7.9	27.7	1.333	0.5	0.018 * f_{TWA}
		Mammal	164	3.8	6.5	1.333	0.1	0.0009 * f_{TWA}
		Bird	323	7.9	27.7	1.333	0.1	0.004 * f_{TWA}

Table 9 Estimation of reasonable worst case and most likely case values for short, medium and long-term exposure scenarios for birds ingesting granules intentionally when seeking grit. It is assumed that small granules (0.5-1.5mm) are taken by small birds e.g. finches and large granules (2-6 mm) are taken by large birds (e.g. partridge and wood pigeon).

Exposure duration	RW C/M LC	Size of birds	Number of grit per day[1]	Number of soil particles ($SP_{surface}$)[3]	PT	f_{TWA} for number of granules[4]	f_{TWA} for the active substance[4]
Short-term	RW	Large	2453	71	1	No	No
	C	Small	651	15200	1	No	No
	ML	Large	1306	71	0.5	No	No
	C	Small	386	15200	0.5	No	No
Medium-term	RW	Large	2453	71	1	No	No
	C	Small	651	15200	1	No	No
	ML	Large	1306	71	0.5	Yes	Yes
	C	Small	386	15200	0.5	Yes	Yes
Long-term	RW	Large	2453	71	1	Yes	Yes
	C	Small	651	15200	1	Yes	Yes
	ML	Large	1306	71	0.5	Yes	Yes
	C	Small	386	15200	0.5	Yes	Yes
		Large[2]	1306	71	0.1	Yes	Yes
		Small[2]	386	15200	0.1	Yes	Yes

[1] = Grit conversion factor is 4.2 (see Note 25)
[2] = Use these scenarios only in the non-breeding season.
[3] = SP is based on 3 samples from 3 Dutch soils, 2 sands and 1 clay. If appropriate, replace these numbers with data for other soils. This is especially important for peaty soils as they have lower grit content.
[4] = In case DT50s are available for the number of granules and for the active substance in granules time weighted average values can be used see also.

3.2.3 Refinement of risk assessment

This section provides a general procedure for the mitigation and/or refinement of risk. Specific options for mitigation and refinement may vary from case to case.

R1. Have any of the assessment scenarios (short-, medium- or long-term exposure, parental or reproductive effects) led to a classification of uncertain risk or high risk?
If yes **Go to R2**
If no **Low risk for all assessment scenarios. No further assessment required**.

R2. Have any of the assessment scenarios (short-, medium- or long-term exposure, parental or reproductive effects) led to a classification of high risk?
If yes **Go to R3**
If no **Go to R4**

R3. The proposed use of this formulation or compound is classified as high risk. This means it is likely to cause frequent substantial adverse effects on many species. Refinement of the risk assessment can be carried out but, on its own, is unlikely to lead to a conclusion of low risk. It may therefore be advisable to consider mitigation options first. If mitigation is effective, a review of the initial assessment may indicate low risk. Do you wish to consider mitigation?
If yes **Select appropriate mitigation options (see Note 17) and then review the initial assessment from the beginning, Go to 1**
If no **Go to R4**

R4. For each scenario which was classified as high or uncertain risk, refine the assessment using more precise data and/or replacing conservative assumptions with appropriate data, and/or carry out laboratory, semi-field or field studies to measure effects under specific conditions. It may also be advisable to consider the potential contribution of non-dietary routes to overall exposure and risk. See Note 18 for a brief discussion of refinement options.

Go to R5

R5. Consider the risk for each reassessed scenario. Is the risk defined precisely enough to make a decision?
If low risk
Classify as low risk for the scenario under consideration and go to R6
If high risk
Classify as high risk for the scenario under consideration or go to R3
If uncertain
Classify as uncertain risk for the scenario under consideration or go to R4

R6. Are all the assessment scenarios now classified as low risk?
If no **Go to R2**
If yes **Low risk for all assessment scenarios. No further assessment required.**

3.3 Explanatory notes

Note 1 - What is negligible?
If a product is to be used indoors – e.g. glasshouse, domestic premises, factories, grain stores and other enclosed structures, then the primary risk to non-target birds and mammals is considered to be negligible. Certain plant protection products used outdoors may also pose a similar negligible risk e.g. wound healing and sealant treatments. In cases where the exposure or risk is considered negligible, an appropriate justification should be given.
In some cases, where a product is used indoors, the secondary risk (indirect exposure through the food chain) should be considered. For example, use of rodenticides indoors may result in exposure of non-target birds (barn owl) or mammals (polecat), or soil invertebrates, contaminated due to the use of soil incorporated sprays and soil sterilants, may be used as food by non-target birds and mammals. In these cases, the potential risk of secondary poisoning should be considered.

Note 2 - Toxicity tests
Toxicity tests are normally conducted with a small number of laboratory strains or species of birds and mammals. Such tests are necessary in order to evaluate the risk of active substances to humans as well as terrestrial vertebrates. These tests aim to assess the effects of potential exposure through various routes and over various time scales. The extent of the data required will be contingent on the nature of the active substance, the manner of use and the extent and scale of application.
Generally for initial screening of active substances, a core data base will be necessary which will assess acute oral toxicity, long-term toxicity and reproductive toxicity in a rodent species and acute oral toxicity, short-term dietary toxicity and reproductive toxicity in avian species.
The test species selected for these test protocols are regarded as surrogates for the ecological species considered to be potentially at risk. However, due to the inherent variability in sensitivity between species and within species in their response to chemical toxicants, a degree of uncertainty may persist in the extrapolation of test findings to particular species of ecological concern. In addition, extrapolation of toxicity from laboratory tests to wild species should be done using appropriate extrapolation factors (see Notes 10-13).
For higher level assessment, it may be necessary to conduct more specific tests on species of more ecological relevance in the context of the proposed use. However, for welfare reasons, preference should generally be given to refining the assessment without conducting additional animal studies where possible, e.g. by refining the exposure assessment.

Note 3 – One-seed dose (OSD) or one-granule dose (OGD) and use of shortcut values
The one-seed dose or one-granule dose is the amount of the active substance per kg body-weight that a bird/mammal will consume (be exposed to) when eating one seed or one granule. For mammals, a generic species of 25 g will be used to estimate the potential risk (voles will eat all types of seed). For birds, a differentiation is made between small birds (seeds like maize, peas or beans are rarely swallowed whole by small birds) and larger birds. The body weight of a small bird is assumed to be 25 g, whereas that of a larger bird is assumed to be 300 g. No differentiation between species is made for the accidental or intentional consumption of granules. Because the endpoint of the toxicity test (LD50) is expressed as mg a.s./kg body weight the one seed or one granule dose has to be adjusted to a body weight of 1 kg. This is the shortcut value in the following formula (see also Table 1):

One seed dose (OSD) = shortcut value * $S_{loading}$
One granule dose (OGD) = shortcut value * $G_{loading}$

$S_{loading}$ is the amount of the active substance on one seed
$G_{loading}$ is the amount of the active substance in one granule.

Note 4 – Conversion of mg/kg food into mg/kg body weight per day
The standard unit for avian dietary studies is mg active substance (a.s.) per kg food (mammalian studies are sometimes also expressed in the same unit). If the mean body weight and the mean food consumption per day are known this unit (mg/kg food) can be converted into the unit mg/kg body weight per day. Let us assume that the LC50 is 50 mg a.s. per kg food, the mean body weight is 200 gram and the mean food consumption is 29 gram per day, then the DFI = 29 * 1000 / 200 = 145 gram food per kg body weight per day and the LD50 = 50 * 145 / 1000 = 7.25 mg/kg BW per day. In the same way a NOEC can be converted into a NOED. It should be noted that data on body weight is normally not reported on a daily basis, but for the complete 5-day exposure period (e.g. 5 days for the standard dietary test).
The outcome of standard dietary LC50 studies may often not result from increased intake of chemical, but from decreased food consumption. This food avoidance behaviour can be induced by repellent properties of the chemicals (see Note 19). Because repellency dictates, sometimes to a high degree, the outcome of the LC50 value, these values should be used with caution in the risk characterisation (Mineau *et al.* 1994 and Luttik 1998).

Note 5 - Selection of input parameters for exposure scenarios
In Tables 2 to 7 generic bird and mammalian species (column 3) can be found for several important crop combinations (column 1). If appropriate, a differentiation is made between earlier (spring) and later applications (column 2). If the generic species are adequate for the first-tier risk assessment the daily dietary dose [DDD] can be calculated by using the shortcut value(s):

DDD [mg per kg body weight per day] = Shortcut value * application rate in kg a.s./ha

The underlying formulae for calculating the DDDs for scenarios in which only one type of food is consumed (PD=1) are as follows:

DDD = (DFI / BW) * 1000 * C * AV * PT * PD [mg a.s. per indicator species per day]

In which:
DFI = Daily food intake of the indicator species (g fresh weight per day)
BW = Body weight (g)
C = Concentration of compound in/on fresh diet (mg/kg)
AV = Avoidance factor (1 = no avoidance, 0 = complete avoidance, see Note 19)
PT = Fraction of diet obtained in treated area (number between 0 and 1)
PD = Fraction of food type in diet (1 when only one type of food)

The DFI can be calculated with the following formula:

DFI = DEE / (FE * (1-(MC/100)) * (AE/100)) [g fresh weight per day]

In which:
DEE = Daily Energy Expenditure of the indicator species [Kj per day]
FE = Food Energy [Kj per dry gram]
MC = Moisture Content [%]
AE = Assimilation Efficiency [%]

Mean estimates for the factors DEE, FE, MC and AE can be found in Crocker *et al.* (2002) or in the EU document on refined risk assessment for birds and mammals (EU, 2001).
In cases where more than one food type is consumed, the total requirement for each type is estimated as if it were the sole diet, and the appropriate proportions of each (based on PD) are then added to estimate the total amount consumed. This implies that PD is estimated in terms of dry weight. If PD is expressed in terms of wet weight, the calculations need to be modified accordingly. For multiple applications see Note 7.

Note 6 – General explanation of tables 2 to 7
Crop – choose the crop that corresponds most closely to the proposed use of the product.
Crop stage – choose the relevant crop stage. 'Early' means application to young plants, usually between late autumn and early spring. If in doubt or if both periods apply, use 'Early' for the initial assessment.
Species, Body Weight and Food Type – in general, the highest theoretical risk applies for a species of small size, feeding on short grass. This is because smaller animals consume a larger proportion of their body weight as food, and estimated residues are higher for short grass than other food types. However, this scenario (small herbivores) is not reasonable for all crop types. The species and body weights listed are therefore those which are considered to represent the reasonable worst case exposure and the most likely case for each crop type. Fish-eating mammals and birds are not included in the short-term exposure scenarios, because it is considered that, in general, residues in fish will not reach sufficient levels to cause significant exposure over short time scales. In the reasonable worst case scenarios it is assumed that all generic species will consume one type of food (the type with the highest residue levels). In the most likely case, it is very often assumed that the generic species will eat different types of food.
Daily Food Intake (DFI) – the DFIs are based on mean estimates for the factors DEE, FE, MC and AE presented by Crocker *et al.* (2002). No differentiation is made for the reasonable worst case and the most likely case.

Note that the DFI estimates based on energy expenditure are estimates of what DFI would need to be if the animal balanced its energy budget and fed only on the specified diet. In reality, when this results in an excessive figure for DFI, other things might happen. The animal may change to a mixed diet (if available) with some higher-quality food in it, or it may reduce its energy expenditure (e.g. by resting), or it may run a negative energy budget (burn fat). DFIs greater than 1 times the body weight are possible (Crocker *et al.,* 2002), but most DFIs will be smaller than the body weight.

Residue unit dose (RUD) – the values provided in this column are the residue levels in mg/kg fresh food which occur immediately after spraying of 1 kg a.s./ha (see Note 8). For the short-term reasonable worst case assessment (minutes to hours), it is appropriate to use the 90^{th} percentile residue levels. In all other scenarios, the 50^{th} percentile residue levels should be used, because averaging of residues are expected to occur. If appropriate, multiple application factors (MAFs) should be applied (see Note 7). In case of the long-term exposure scenarios (RWC and MLC) and in case of the other most likely case scenarios (short and medium-term exposures), it is appropriate to use time weighted average factors (see Note 9).
PT and AV – in the reasonable worst case scenarios, it is assumed that the contaminated diet will not be avoided (AV factor is 1, see Note 19) and that the animals will obtain their entire DDD from the treated area (PT =1). In the most likely scenarios, AV may take lower values (see Note 21) and PT = 0.5, except outside the breeding season where PT is 0.1 in the long-term exposure assessment.

f_{TWA} Time weighted average factors – see Note 9 for time weighted average.

In Table 10 the different assumptions for the different reasonable worst case (RWC) and most likely case (MLC) scenarios are summarized.

Table 10 Assumptions for the different RWC and MLC exposure scenarios

Exposure	Type of case	RUD	PT	PD	BW	AV	f_{TWA}	MAF
Short-term	RWC	90th perc.	1	1 type	Small	No	No	Yes
	MLC	50th perc.	0.5	3 types	Medium	See Note 21	(no or default = 0.97)	Yes
Medium-term	RWC	50th perc.	1	1 type	Small	No	No	Yes
	MLC	50th perc.	0.5	3 types	Medium	See Note 21	Default = 0.85	Yes
Long-term	RWC	50th perc.	1	1 type	Small	No	Default = 0.53	Yes
	MLC	50th perc.	0.5 or 0.1	3 types	Medium	See Note 21	Default = 0.53	Yes

Note 7 - How to deal with multiple applications
In case an active substance is applied several times in a growing season, depending on the half-life of the substance, higher concentrations can be expected in the field (e.g. residues on food items) than for a single application. For all reasonable worst case exposure assessments, this higher estimate of the initial concentration should be used. This concentration can be calculated using the following formula:

$C_{multiple\ application} = C_{t=n-1} + C_0$

$C_{t=n-1}$ = concentration at time t for n-1 applications = $(C_{t=n-2} + C_0) * e^{-kt}$
t = number of days between applications
n = number of applications
C_0 = initial concentration
k = first order rate constant = $\ln 2/DT_{50}$
DT_{50} = half-time of decline

In the first tier, a default factor of 10 days for DT50 on vegetation is used (see EU, 2001). Multiple Application Factors (MAF), which give the ratio of the initial concentration after n applications compared with the initial concentration after the first application, are presented in Table 11. MAF is a function of the number of applications, interval and DT50.

Table 11 Multiple Applications factors (MAF) for residues in vegetation based on a DT50 of 10 days (equation and example calculations)

$MAF = (1-e^{-0.069ni})/(1-e^{-0.069i})$ i = interval; n = number of applications							
Interval (d)	Number of applications						
	2	3	4	5	6	7	≥8
7	1.6	2.0	2.2	2.4	2.5	2.5	2.6
10	1.5	1.8	1.9	1.9	2.0	2.0	2.0
14	1.4	1.5	1.6	1.6	1.6	1.6	1.6

Note 8 – Residue unit dose (RUD)
Many of the first tier risk assessments in the world are based on the so-called 'Kenaga Nomogram' (Hoerger and Kenaga, 1972; Urban and Cook, 1986). Recently, several studies have been carried out; firstly to check whether, nowadays, the results of the research of 1972 are still valid now (different compounds, low volumes, etc.) and, secondly, to provide better data for small and large insects (Fletcher *et al*., 1994; Pfleeger *et al*., 1996; Fishers and Bowers, 1997; Brewer *et al.,* 1997 and Edwards *et al.,* 1998). These studies have been summarized by Luttik (2001) and 50th and 90th percentiles have been calculated according to the distributions of residue values. Residue levels for the relevant dietary items for the species and crop type are based on Fletcher *et al.* (1994). Data presented by Fischer and Bowers (1997) and Brewer *et al.* (1997) suggest that this results in substantial over-estimation of residues on insects, but more detailed analysis and a larger dataset is required to establish definitive estimates for general use (SCP, 2002). The EU Scientific Committee on Plants (SCP, 2002) recommends that, until better data is available, different approaches should be used for 'large insects' and 'small insects'. If suitable data are wanted for small insects it is recommended to draw upon the surrogate values proposed by Kenaga (1973) which have been widely used in the past. Depending on the time scale either the 'maximum value' should be used (52 mg/kg) or the 'typical value' (29 mg/kg). Small birds are assumed to prefer small insects. Insectivorous mammals always are assumed to eat large insects. The estimates resulting from application of these are presented for different food types in Table 12[1].

Table 12 Relationship between realistic worst case and most likely case residue concentrations on plant or parts of plants and insects (in mg/kg fresh weight) and the dosage (D) of plant protection products (in kg active substance per hectare) immediately after spraying (after Kenaga, 1973 and Luttik, 2001).

Food type	Realistic worst case 90th percentile	Most likely case 50th percentile
Short grass	142 * D	62 * D
Long grass	69 * D	21 * D
Leaves, leafy crops, forage crops and small seeds	87 * D	25 * D
Fruit, pods and large seeds	11 * D	2.3 * D
Small insects	52 * D	29 * D
Large insects	11 * D	2.7 * D

Note 9 – Time weighted average factors
In the long-term exposure assessments (both reasonable worst case and most likely case) and the most likely case of the medium-term exposure assessment, time weighted average residues rather than initial residues. With the length of the time window (averaging time) fixed at 5 or 21 days, the TWA (time weighted average) factor depends only on the half-life of the compound. If data show that the DT50 is shorter than 10 days (used as default value in tier one), then the f_{TWA} should be recalculated (see Table 13).

[1] The residue estimate for small insects appears unsatisfactory, and as soon as better information becomes available the surrogate values should be replaced. Research is highly desirable to develop more robust data, also with regard to the temporal pattern.

Table 13 Time weighted average factors (assuming first order kinetics), equation and example calculations for two different time windows and DT50 of 1 to 10 days

$f_{TWA} = (1-e^{-kt})/kt$ (k = ln2/DT50 and t = averaging time in days)										
Time window	DT50 in days									
	1	2	3	4	5	6	7	8	9	10
5 days	0.28	0.48	0.59	0.67	0.72	0.76	0.79	0.81	0.83	0.85
21 days	0.07	0.14	0.20	0.27	0.32	0.38	0.42	0.46	0.50	0.53

Note 10 - Fixed extrapolation factors or the 5th percentile of the species sensitivity distribution

Under current testing requirements for avian risk assessment, only one or two species are usually tested. The uncertainty associated with extrapolating from studies conducted on usually no more than two species to the universe of possible wild species is large. Very often fixed extrapolation factors (e.g. a factor of 10) are embedded (laid down) in the legislative procedures to overcome this problem. But the use of a fixed extrapolation factor does not exclude over- or under-estimation of the potential risk of a plant protection product for birds and mammals. For example, by using the historical database described in Luttik and Aldenberg (1997) it can be calculated that after applying an extrapolation factor of ten the sensitivity of the 5th percentile bird species will be underestimated in 25% of the cases (in 3% of the cases by a factor of 10 to 100).

A more statistical approach towards extrapolating laboratory toxicity is to consider and to estimate the species sensitivity distribution (SSD) for the toxicity endpoint. One way of extrapolating toxicity data to acceptable values for the risk assessment is to estimate the fifth percentile of the SSD (Kooijman, 1987; van Straalen and Denneman, 1989; Wagner and Løkke, 1991; Aldenberg and Slob, 1993; Aldenberg and Jaworska, 2000). Although this procedure formally works from n=2 onwards, in practice the method is of little use for very small samples (e.g. n=2 or 3). In these cases the extrapolation factors are large, especially for the lower confidence limit, due to the uncertainty in estimating the SSD mean and standard deviation from a small sample. When only one toxicity value is available (n=1), the method cannot be applied, since it is impossible to estimate the SSD standard deviation from the sample. In practice, this method is only used if more than 6 (sometimes 4) toxicity values are available for one compound (see note 11).

Two similar methods for estimating the 5th percentile when only small samples sizes are available (n= 1 to 5) were proposed independently (Baril *et al.*, 1994; Baril and Mineau, 1996; Luttik and Aldenberg, 1997). In this scheme it is proposed to use the method of Luttik and Aldenberg (see note 11) because it can be applied to both birds and mammals, whereas the method of Baril and Mineau is only applicable to birds. Furthermore, the method of Baril and Mineau is mainly based on insecticides, whereas the method of Luttik and Aldenberg is mainly based on pesticides (not only insecticides) and also on other compounds. The method of Luttik and Aldenberg does not allow for scaling of toxicity with body weight, whereas this is part of the method of Baril and Mineau. In practice, the two methods provide similar answers.

Note 11 - Extrapolation between species with LD50s for 6 or more species
If LD50s are available for 6 or more species, the method of Aldenberg and Slob (1993) may be used to estimate the 5th percentile from the available data, by applying an extrapolation factor (See Table 14) to the geometric mean of the species tested:

$$5^{th} \text{ percentile of SSD} = 10^{(\text{mean log toxicity value} - \text{extrapolation factor} * \text{standard deviation})}$$

Table 14 Extrapolation factors for the median and one-sided left 95% confidence limits for the log-logistic and log-normal distribution (after Aldenberg and Slob, 1993; Aldenberg and Jaworska, 2000)

Sample size	Median estimate		One-sided left confidence limits	
	Log-logistic	Log-normal	Log-logistic	Log-normal
6	1.81	1.75	3.93	3.71
7	1.78	1.73	3.59	3.40
8	1.76	1.72	3.37	3.19
9	1.75	1.71	3.19	3.03
10	1.73	1.70	3.06	2.91
11	1.72	1.70	2.96	2.82
12	1.72	1.69	2.87	2.74
13	1.71	1.69	2.80	2.67
14	1.70	1.68	2.74	2.61
15	1.70	1.68	2.68	2.57

The following is an example of the calculation: for available toxicity data of 10, 15, 50, 100, 130 and 200 mg/kg body weight (six different species) the log mean toxicity = 1.715 and the log standard deviation = 0.526. The median estimate of 5th percentile for log-logistic distribution is 5.78 mg/kg BW, and for log-normal distribution is 6.22 mg/kg BW, the one-sided left confidence limits of the 5th percentile are respectively 0.44 and 0.58 mg/kg BW.

The standard approach for the Aldenberg and Slob method is to use the median estimates. In cases where more than one toxicity value is available for 1 species calculate the geometric mean of the values and use this outcome as input for the 5th percentile calculation.

Note 12– Extrapolation between species with LD50s for 5 or less species
If LD50s are available for 5 or fewer species, the method of Luttik and Aldenberg (1997) may be used to estimate the 5th percentile from the available data, by applying the extrapolation factors especially derived for small samples (See Table 15) to the geometric mean of the species tested:

$$5^{th} \text{ percentile of SSD} = 10^{(\text{mean log toxicity value})} * \text{extrapolation factor}$$

The following is an example of the calculation: for toxicity data 100, 350, and 600 mg/kg body weight (3 different species of birds or 3 mammalian species), the log mean toxicity = 2.4407 and the geometric mean is 275.9 mg/kg BW. The median estimate of 5th percentile for birds is 48.4 mg/kg BW, and for mammals 72.6 mg/kg BW, the one-sided left confidence limits of the 5th percentile for birds is 17.7 mg/kg BW and 32.8 mg/kg BW for mammals.

Table 15 Extrapolation factors for the median and one-sided 95% left confidence limits for the log-logistic distribution for birds and mammals (after Luttik and Aldenberg, 1997)

Sample size	Median estimate		One-sided left confidence limits	
	Birds	Mammals	Birds	Mammals
1	5.7	3.8	32.9	14.9
2	5.7	3.8	19.6	10.0
3	5.7	3.8	15.6	8.4
4	5.7	3.8	13.7	7.6
5	5.7	3.8	12.4	7.0

In agreement with Luttik and Aldenberg it is recommended to use the more conservative method (95% confidence limit) for the extrapolation, because the method is based on pooled ln standard deviations and not on worst case assumptions. In case there are indications that the available LD_{50} could be derived from a test with a sensitive species one could consider using the safety factor for a median estimate. In cases where more than one toxicity value is available for 1 species calculate the geometric mean of the values and use this outcome as input for the 5^{th} percentile calculation.

Note 13- Extrapolation between species for medium- and long-term toxicity.
Between-species variation in medium- and long-term dietary toxicity should be taken into account. However, because medium- and long-term studies are rarely available for more than two or three species it is more difficult to develop statistically based extrapolation factors than for acute toxicity, and none have yet been developed. In the meantime, interspecific variation for medium- and long-term toxicity can be reasonable assumed to be at least as great as that for short-term toxicity, because the former involves processes (metabolism over longer periods), greater uncertainty in consumption of treated food, and the use of more endpoints in the long-term studies, which are likely to add extra variation between species. Therefore, the extrapolation factors in Table 15, which were derived from short-term toxicity data, can also be used here for medium- and long-term toxicity (including reproductive toxicity). Extrapolation factors based on analysis of medium- to long-term toxicity data should be used when they become available.

Note 14 - Use of avian reproduction study for medium-term assessment
The SETAC Workshop on Harmonised Approaches to Avian Effects Assessment (Hart *et al.,* 2001) considered that the 5-day avian dietary study might not be necessary for every pesticide, if an assessment using the avian reproduction study was sufficient to demonstrate acceptable risk from medium-term exposures, and this is the approach included as an option in this scheme. It is much more conservative than the standard assessment using the LC50 study because (a) a no-mortality level is used instead of a median lethal level, (b) the test involves exposure over 6-22 weeks instead of 5 days. Nevertheless, in some cases (pesticides with a high NOEL and low application rate) this approach may produce an ETR of less than one for the reasonable worst case, in which case the need to conduct an LC50 study would be avoided.

Note 15 - 28-day-mammalian study
For pesticides, a 28-day-study according to OECD 407 is usually available for rats and often also for mice. The test substance is applied by daily intubation or (more commonly) by mixing into the feed. Dose levels are given in the report as concentration in feed and additionally as achieved dose (mg/kg BW per day). Besides observation of mortality and signs of intoxication, haematological, clinico-chemical and histological evaluations should be done. If the test substance is applied via feed, information on avoidance can also be obtained. The aim of the test is to determine a NOEL. For the present purpose (medium-term effects assessment) the no-mortality-level should be used.
The 28-day-mammal-study is more conservative as a measure of medium-term toxicity than the avian LC50 study in several respects. First, the study duration is longer. Second, the test endpoint is a NOEL and not a median lethal dose (LD50). Third, the assessment considers dietary exposure but the 28-day rat study is sometimes a repeated oral dose study that is more severe. If no 28-day NOED is available, the closest toxicity test available should be taken and expert judgement should be used to derive an appropriate endpoint. The critical point is that the endpoint is mortality but the test concentrations are structured so as to avoid excessive mortality and to determine sub-clinical effects which are followed up in longer-term studies. In effect the highest dose [concentration] may be in many cases the NOED [mortality] or LD0.

Note 16 - Exposure time scales considered for reproductive effects.
The scheme only assesses the risk of reproductive effects due to long-term exposures. In theory, reproductive effects could also occur as a result of short- or medium-term exposures during sensitive periods of the animal's reproductive cycle, and ideally these scenarios should be considered in risk assessment (ECOFRAM, 1999). Currently, however, reproductive effects are only tested in studies with long exposure periods (6 weeks to 2 years). Although effects may be caused by only a short part of the exposure period, it is not possible to determine this from the results of the current standard tests. Therefore, this scheme does not attempt an explicit assessment of reproductive effects for short- or medium-term exposures. However, the potential for such effects may be indicated by the reasonable worst case assessment of reproductive effects for long-term exposures, as this compares the initial DDD with the long-term NOEL. If the assessor wishes to examine more closely the potential for reproductive effects of short- or medium-term mortality risks, one option may be to derive NOELs for sub-lethal parental effects in the LD50 and LC50 studies and compare these with short- and medium-term DDD estimates. If the behaviour of parent animals is affected, there may or may not be consequences for reproductive performance.

Note 17 - Risk mitigation
Possible options for mitigation may include: reducing application rate, reducing maximum number of repeat applications, increasing minimum interval between applications, and changing the crop stage at which applications are made. The user is advised to review the entire assessment if risk reduction is proposed, rather than just the refined assessment. This is because it is simpler to repeat the initial assessment, and it may show that refinement is unnecessary for the mitigated use. However, depending on the type of risk reduction option adopted, some parts of the initial assessment may be unchanged.

Note 18- Refining the assessment
Refinement is only necessary if the initial assessment does not enable the user to classify the risk with adequate certainty. Refinement should accordingly focus on reducing uncertainty to the point where risk can be classified. However, it may not be necessary to refine all aspects

of the assessment. It will be more efficient to identify the major sources of uncertainty, consider how those uncertainties might be reduced, and then select those refinement options which are most likely to achieve adequate certainty at reasonable cost (in terms of resources and time).

The main types of refinement are:

1. To refine the initial assessment using more precise data and/or replacing conservative assumptions with appropriate data. The refinements should aim to identify more precisely the conditions under which unacceptable effects are expected and how often these conditions will apply. It is important to remember that a wide range of conditions occurs in the field, even if only one or two specific scenarios are considered explicitly in the risk assessment. Probabilistic methods provide a means of taking account of the range of conditions and their relative frequency, but are not yet fully developed at the time of writing (Hart, 2001). Until probabilistic methods are established, it may be best to start with the reasonable worst case assessment and gradually replace the conservative assumptions with measured data from a representative range of real field scenarios.
2. To carry out laboratory, semi-field or field studies to measure effects under specific conditions. These studies provide a more certain measure of risk for the conditions they examine. However, it is unlikely to be practical to repeat the studies for the wide range of conditions which occur in the field. Expert judgement is therefore required to extrapolate the results of such studies to other conditions. This will be easier if (a) the conditions for the studies are carefully chosen in advance (e.g. to represent a reasonable worst case), and/or (b) the conditions are quantified in the studies, so that the outcome can be compared directly with the ETR assessment.
3. To assess the potential contribution of non-dietary routes of exposure. If these are substantial, they may raise the level of risk to the 'high' category. Possible approaches to assessing non-dietary routes are described in the previous edition of this sub-scheme (OEPP/EPPO 1994). Care is required when combining exposures by different routes, and in selecting appropriate measures of toxicity.

Examples of refinement options include:

- Field measurement of the actual distribution of residue levels on bird/mammal food items, especially for residues on invertebrates as these may be substantially over-estimated in the reasonable worst case assessment.
- Obtaining more sophisticated information on the diets or on the soil or grit consumption of species at risk or the proportion obtained from treated areas - though reliable quantitative data on these variables may be difficult and expensive to obtain.
- Toxicity studies for additional species may produce lower or higher results than the initial studies, but the increased number of species tested will increase the precision of extrapolation to other species and therefore decrease the reasonable worst case ETR.
- Special laboratory or semi-field tests of effects, e.g. to quantify avoidance under more realistic conditions than the LC50 test. The degree of avoidance depends strongly on test conditions, so these must be chosen with care (see Note 19).
- Field studies (Somerville and Walker, 1990).
- Loading of the granules. In principle the nominal values as applied by the manufacturer is used in the risk assessment. In practice lower concentrations can be found.
- Incorporation efficiency of the granules. In principle default values are used in the risk assessment. Especially when new types of machinery are used other values can be found in practice.

Detailed guidance on refining avian effects assessment is provided by Hart *et al.* (2001).

For welfare reasons, additional studies involving animals should only be conducted if fully justified. Generally, elements of the assessment that do not require animal testing should be refined first. Full dose-response tests should not be carried out if approximate tests are sufficient (e.g. if an estimate of slope is not required).

Note 19– Avoidance
Avoidance may be a significant factor that reduces the exposure. It may be a chemically-mediated response to the active substance being a primary repellent, a secondary repellent or inducing anorexia. Indications may be seen in the dietary toxicity test (Luttik, 1998). However, in the case of granules and treated seeds avoidance is often observed even if the active substance is not repellent, which then is due to co-formulants, colour, shape, texture and/or other features of the material. To characterise avoidance, it is usually necessary to carry out palatability tests. There is no consensus yet on which approaches should be recommended. The key issue is that the extent of avoidance (and hence its effectiveness in reducing risk) is dependent on many factors which may differ between lab and field. Assessors should therefore select a study design which manipulates the most important factors in a realistic way for the case in hand. General guidance on the factors to consider may be found in the report of the Pensacola workshop (OECD, 1996) and the Woudschoten workshop (Hart *et al.*, 2001).

Studies in which food consumption is measured (including some LC50 studies) can be used to provide estimates of avoidance factor (AV) for use in the MLC calculations for exposure to sprays and treated seeds (AV should be set to 1 in RWC calculations). For example, if consumption of treated material is 10 % of normal consumption, then AV can be estimated as 0.1 and used to calculate a revised TER. Note that avoidance is normally dependent on concentration, so the value for AV should be taken for treatment concentrations close to the concentration assumed in the MLC calculation. Other test designs are to be regarded as simulated field tests which quantify effects rather than consumption. The results from these tests cannot be interpreted in terms of a revised TER but immediately give an indication of the likelihood of a certain effect. For all these types of studies, however, it is essential to take careful account of the test conditions when interpreting the results.

In assessing the effect of avoidance on acute avian risks from contaminated food, the key factor is the rate at which test birds feed during their first few hours of exposure. This depends on the test conditions and on the way birds are prepared for the test (Fryday *et al.,* 1998; Hart *et al*., 1999,; Pascual *et al.,* 1999). If the feeding rate in the test is close to the maximum in the field, then the result of the test can be regarded as close to worst-case. If the feeding rate is lower, then the test is likely to under-estimate the potential for risk in the field. Such a test is useful in indicating the potential for avoidance but should not be used for a definitive assessment of risk. If the feeding rate in the field is unknown, it will be difficult to interpret the result of a test unless the feeding rate in the test is close to the maximum the species can achieve (in which case the test is worst-case). In principle the same considerations apply to acute mammalian risks, although mammals may show less variation in feeding rate than birds.

For granules, the assessment of avoidance may require different approaches. Food consumption in the avian dietary study is less relevant to granules, but it may still be used for estimating AV in the MLC scenario for granules in order to indicate whether avoidance is

potentially important. As mentioned above, it should not be used in the RWC scenario. If it is decided to quantify avoidance of granules in a refined assessment, specialised studies will be required. Factors to be considered in designing such studies for granules are discussed in OECD (1996).

When interpreting the results of avoidance studies, whether for granules or any other pesticides, it is important to consider the potential for variation between species. Other species may be more or less sensitive to the pesticide, and may show stronger or weaker avoidance responses. If avoidance is a critical factor in reducing the assessment of risk, then it may be necessary to conduct avoidance tests with several species in order to be confident that the response is sufficiently consistent between species.

Note 20 - bioaccumulation (fish and earthworms)
For organic chemicals a trigger value of logKow of > 3 is used for carrying out a risk assessment for fish-eating or worm-eating birds and mammals. As bioaccumulation processes are often slow it is more appropriate to carry out a medium and long-term assessment rather than a short-term assessment.

Preferably, real measured BCF values (based on whole body residues and on wet weight base) should be used for the risk characterisation. This information can be found in the EPPO Chapters on Aquatic organisms and Soil organisms and functions (OEPP/EPPO, in press). In case no real measured BCFs are available the following QSARs can be used:

- QSAR for earthworms

$BCF = (0.84 + 0.01\ K_{ow}) / f_{oc}\ K_{oc}$

In which: K_{ow} = Octanol water partition coefficient
K_{oc} = Organic carbon adsorption coefficient (available from physical/chemical data)
f_{oc} = Organic carbon content of soil (take 0.02 as a default value)

The equation originates from works of Jager (1998). The BCF is defined as earthworm fresh weight to soil dry weight. The model is empirically based on non-ionised, organic chemicals in the $\log K_{ow}$-range from 1 to 8, and it should not be applied to other types of substances or highly reactive substances.

- QSAR for fish

$BCF = 0.048 * K_{ow}$ (MacKay, 1982)

Note 21 – Estimation of reasonable and most likely case for soil ingestion
The assessment is based on a relatively small species, because food consumption is related to the body weight. Smaller animals consume a larger proportion of their body weight as food than larger animals. The default body weight is 25 gram (comparable with the body weight of voles and finches). The DFI (Note 22) intake is based on the diet of an omnivorous animal and is defined as 1/3 leaves 1/3 seeds and 1/3 insects.

The reasonable worst case (90^{th} percentile) and most likely case (geometric mean) estimates of the percentages of soil in the daily diet are based on data collected by Beyer *et al.* (1994).

For mammals the following data are available: <2, <2, <2, <2, <2, <2, 2.3, 2.4, 2.7, 2.8, 5.4, 6.3, 6.8, 7.7, 9.4, 9.4 and 17% (geometric mean 3.8% and 90th percentile 9.4% (17 different species)). For birds data on 11 species are available (no data on passerines): <2, <2, 3.3, 7.3, 8.2, 9.3, 10.4, 11, 17, 18 and 30% (geometric mean 7.9% and 90th percentile 18%)). It is important to note that Beyer *et al.* 's estimates are expressed as dry weight/dry weight.

In the reasonable worst case scenarios, it is assumed that the contaminated soil will not be avoided (AV factor is 1), but in the most likely case scenarios it is appropriate to take avoidance into consideration (see Note 19). In the reasonable worst case scenarios, it is assumed that the animals will obtain their entire daily soil dose from the treated area (PT =1, see also Note 5). In the most likely scenarios, PT = 0.5, except outside the breeding season where PT is 0.1 in the long-term exposure assessment.

Note 22 – Selection of input parameters for exposure scenarios
Table 8 gives estimations of reasonable worst case (RWC) and most likely case (MLC) for short, medium and long-term exposure scenarios for a generic bird and mammalian omnivorous species of 25 g. It is assumed that the animals will eat equal parts on dry weight base of non-grass herbs, insects and seeds with an average caloric content of 20.3 Kj/g dry weight and an average assimilation efficiency of 81.7% for mammals and 59% for birds.

If the generic species are adequate for carrying out the first-tier risk assessment, the daily dry soil dose (DDSD) can be calculated by using the shortcut value(s) for soil ingestion:

DDSD = Shortcut value * application rate in kg a.s./ha [mg a.s. per kg body weight per day].

The underlying formulae for calculating the shortcut value are:

Shortcut value = DDSI * RUD/1000 * PT * AV

In which: DDSI = Daily Dry Soil intake of the indicator species [gram per day]
RUD = Residue unit dose (concentration in soil as a result of an application rate of 1 kg a.s./ha, see also Note 23)
PT = Fraction of diet obtained in treated area (number between 0 and 1)
AV = Avoidance factor (1 = no avoidance, 0 = complete avoidance, see Note 7)

DDSI = DDFI / (100-%soil) * %soil

In which: DDFI = Daily dry food intake of the indicator species (gram dry weight per day)
%soil = Percentage of dry soil in dry diet of indicator species.

DDFI = DEE / (FE * (AE/100)) [g dry weight per day]

In which: DEE = Daily Energy Expenditure of the indicator species [Kj per day]
FE = Food Energy [Kj per dry g]
AE = Assimilation Efficiency [%]

Mean estimates for factors DEE, FE and AE can be found in Crocker *et al.* (2002) or in the EU document on refined risk assessment for birds and mammals (EU, 2001). If multiple applications are possible see Note 7.

Note 23 - Residue per unit dose (RUD) for soil-applied pesticides
The values for the Residue Unit Dose in Table 8 are based on an application rate of 1 kg a.s./ha and on the assumption that the formulation is broadcasted (no incorporation). For the short-term exposure assessment, it is assumed that the compound is equally mixed in a layer of 1 cm soil, for the medium and long-term exposure it is assumed that the compound is mixed over a layer of 5 cm. If other incorporation depths are preferred, the RUD value and shortcut values for a number of depths are presented in Table 16. The calculations are based on a dry bulk density of 1500 kg/m^3.

Table 16 Shortcut values for different incorporation depths (e.g. 10, 15, 20 and 25 cm)

Duration of exposure	RWC/ MLC	Species	RUD mg/kg soil (in layer of x cm)				Shortcut Value			
			10 cm	15 cm	20 cm	25 cm	10 cm	15 cm	20 cm	25 cm
Short-term	RWC	Mammal	0.667	0.444	0.333	0.267	0.011	0.008	0.006	0.005
		Bird	0.667	0.444	0.333	0.267	0.047	0.031	0.024	0.019
	MLC	Mammal	0.667	0.444	0.333	0.267	0.002	0.001	0.001	0.001
		Bird	0.667	0.444	0.333	0.267	0.009	0.006	0.005	0.004
Medium-term	RWC	Mammal	0.667	0.444	0.333	0.267	0.011	0.008	0.006	0.005
		Bird	0.667	0.444	0.333	0.267	0.047	0.031	0.024	0.019
	MLC	Mammal	0.667	0.444	0.333	0.267	0.002 * twa	0.001 * twa	0.001 * twa	0.001 * twa
		Bird	0.667	0.444	0.333	0.267	0.009 * twa	0.006 * twa	0.005 * twa	0.004 * twa
Long-term	RWC	Mammal	0.667	0.444	0.333	0.267	0.011 * twa	0.008 * twa	0.006 * twa	0.005 * twa
		Bird	0.667	0.444	0.333	0.267	0.047 * twa	0.031 * twa	0.024 * twa	0.019 * twa
	MLC	Mammal[1]	0.667	0.444	0.333	0.267	0.002 * twa	0.001 * twa	0.001 * twa	0.001 * twa
		Bird[1]	0.667	0.444	0.333	0.267	0.009 * twa	0.006 * twa	0.005 * twa	0.004 * twa
		Mammal[2]	0.667	0.444	0.333	0.267	0.0004 * twa	0.0003 * twa	0.0002 * twa	0.0002 * twa
		Bird[2]	0.667	0.444	0.333	0.267	0.002 * twa	0.001 * twa	0.001 * twa	0.0007 * twa

[1] = PT of 0.5 and [2] = PT of 0.1

Note 24 - Estimation of reasonable and most likely case for granule/grit ingestion
Table 9 gives estimations of reasonable worst case (RWC) and most likely case (MLC) for short-, medium- and long-term exposure scenarios for a small generic bird (e.g. finches) and a large bird (e.g. partridge or woodpigeon). It is assumed in the assessment that small granules (size between 0.75 and 2 mm) are taken by small birds and that large granules (size between 2 and 6 mm) are taken by large birds.

The reasonable worst case (90th percentile) and most likely case (geometric mean) estimates of the numbers of grit particles in the gizzards of a small and a large bird are based on research carried out by de Leeuw *et al.* (1995) (see also Luttik and de Snoo, 2003). For small birds data on 6 European predominantly granivorous species (Greenfinch, Chaffinch, Linnet, Twite, Brambling and Goldfinch) were available: 95, 65, 100, 122, 188 and 43 grit particles in the gizzard (mean values). The geometric mean is 92 and the 90th percentile is 155 grit particles. For larger birds data on three species were available (Grey partridge, Woodpigeon and Pheasant: 676, 208 ad 214 particles (geometric mean 311 and 90th percentile 584). To convert these gizzard counts into a daily intake a conversion factor of 4.2 is used (see Note 25).

For the number of soil particles in the same size classes as the granules (i.e. 0.75 to 1.5 mm and 2 to 6 mm) the geometric mean of three Dutch soils have been used as default (Luttik and de Snoo, 2003). On average (geometric) 15200 soil particles of the size 0.75 to 1.5 mm can be found per m^2 and 71 soil particles of the size 2 to 6 mm.

The Daily Grit Dose (DGD) can be calculated with the following formula:

$$DGD = DGI * (G_{surface}/(SP_{surface}+G_{surface})) * G_{loading} \text{ [mg per kg BW per day]}.$$

In which:
- DGI = daily grit intake of birds,
- $G_{surface}$ = number of granules at soil surface,
- $SP_{surface}$ = number of soil particles at soil surface in the same size classes as granules, and
- $G_{loading}$ = the amount of the active substance in one granule.

In the reasonable worst case scenarios, it is assumed that the granules will not be avoided (AV factor is 1, see Note 7) and that the birds will obtain their entire daily granule dose (DGD) from the treated area (PT =1). In the most likely scenarios, PT = 0.5, except outside the breeding season where PT is 0.1 in the long-term exposure assessment. Avoidance may be taken into consideration when appropriate, but requires a different approach for granules than for other types of pesticide (see Note 19). Sometimes it is appropriate to include TWA factors (see also Note 9): one for the decline in numbers of granules over time and one for the degradation of the compound.

The estimate of soil particle density is based on just one sample from each of three Dutch soils, one clay and two sands, which would be expected to have relatively high grit contents. Peaty soils contain much less grit and would therefore lead to a higher estimate of daily granule dose. Therefore, if granules may be used on peaty soils, data on grit densities on relevant soils should be obtained and used to modify the assessment calculations. Even for clay and sandy soils, it would be desirable to base the assessment on larger numbers of samples but these are not currently available.

Note 25 - Grit turnover rate
On basis of Fischer and Best (1995), a 4.2 conversion factor will be used to take account for the turnover rate of grit. This factor should be used with caution for two reasons: 1) this value is only based on one experimental design using only one species 2) the blank silica granules were intermixed with dog food and there was a great deal of scatter in the data depicting the relationship between granule consumption and gizzard granule counts.
Additional research is needed to validate the general applicability of using a conversion factor and to determine the degree to which such a factor may vary among species and under different environmental conditions.

Note 26 - Rodenticidal baits
Basically two ways of exposure are considered, direct and indirect: rodenticide baits consist of cereals, grease or wax; so direct exposure is relevant mainly for rodents and seed eating birds. Accessibility depends on where baits are placed (indoors, outdoors, field), and how (bait stations, burrow baiting, surface spreading). Scavengers and predators are indirectly exposed by secondary poisoning.

With respect to toxicity, it should be noted that anticoagulants do not cause death until a number of days after ingestion of a lethal dose. Furthermore they are far more toxic if consumed repeatedly over several days than in a single dose. Thus dietary studies or mammalian repeat-dose studies may be more relevant than the single dose LD50.

To determine the relationship between exposure and residues in rodents, a model calculation can be based on nominal concentration of the active substance in/on bait, bait uptake rate of target rodent and estimated time to death of target rodent. However, as no elimination is assumed, such an estimate usually is unrealistically high. Fortunately, in nearly all cases, measured residues in rodents are available from different sources (special laboratory studies, secondary poisoning studies, monitoring of rodent control operation). For the purpose of exposure assessment, whole body residues are relevant (not liver residues).

Generally, risk management with rodenticidal baits aims at reducing the accessibility to non-target species. According to the intended use (target organism, area of application), the following use instructions could be envisaged: do not spread on bare soil, apply deep into rodent burrows, lay bait in covered stations only, remove dead and moribund rodents, and remove bait remains after completion of the control operation.

Various aspects of the risk assessment of rodenticides are presented by Luttik *et al.* (1999). For data on secondary poisoning and design of such studies see Joermann (1998) and OEPP/EPPO (1995). A case study is described by Joermann *et al.* (2001).

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4 Environmental risk assessment scheme for plant protection products: non-target higher plants

Factsheet FSM-007/00 date 05-06-2003

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4.1 Introduction

This scheme deals with the assessment of plant protection products on non-target terrestrial higher plants. Non-target plants are defined as non-crop plants outside the treatment area. The assessment of the risk to crop plants is outside the scope of this scheme, guidance on how to assess the phytotoxicity of plant protection products to crop species can be found in EPPO Guidelines PP/1/135(2) and PP/1/207(1)). Risk assessment scheme is required to help protect biodiversity both within the plant kingdom as a whole and in agro-ecosystems. Ideally, this scheme should also provide an accurate categorisation of the risk to non-crop plant species within the cropped area. However, for numerous reasons (which include lack of knowledge of weed threshold values, variation in composition and sensitivity of weed communities, the role of the seed bank, the impact of fertilisers and crop rotations), it is not currently possible to develop a readily useable scheme for the 'in crop' scenarios.
Since it is known that phytotoxic effects may occur for products other than herbicides, some level of testing of all plant protection products is necessary, unless it can be shown that exposure will not occur.
The scheme follows a sequential or tiered approach. Toxicity values are compared with predicted environmental concentrations to develop an exposure-to-toxicity ratio (ETR). The ETR is then compared with safety factors based on expert judgement or empirical derivation. Progression to the next tier is warranted if the safety margin is not met, or testing is stopped if the safety margin is met or exceeded. Notes 8 and 10 discuss safety margins, but expert judgement may be needed to decide suitable values for a given region.

Research results (Fletcher, 1991) indicated that significant differences may exist among species in their response to plant protection products. While it would be ideal to develop dose-response data for a broad range of species of different genera and families, the cost of doing such studies under Good Laboratory Practice has to be considered. Furthermore, such extensive testing would be beyond that currently required in other areas of environmental risk assessment. Therefore, an alternative approach, using single-dose phytotoxicity screening data for terrestrial non-target plants (Boutin *et al*., 1993; Aldridge *et al*., 1993) at or above the maximum application rate on a wide range of species (e.g. at least 6 but preferably more), is recommended as a first tier to determine whether the plant protection product has phytotoxic properties. If phytotoxicity is observed, dose-response relationships for at least 6 species representing families for which significant herbicidal action has been found are recommended to quantify the level of effect using both soil and foliar exposure scenarios under tier 2.

The scheme contains decision points where expert judgement is specifically requested. This is important because, if the scheme is to be useful throughout the EPPO region, it must incorporate sufficient flexibility to take into account regional differences in farming practices, climate, etc. It should therefore not be operated without the input of an ecotoxicologist and a plant protection expert. It is primarily designed to ensure consistency in the overall approach among different regulatory authorities.

4.2 Risk assessment scheme

Details of the products and its pattern of use

1. Obtain the basic information on the product and its pattern of use:
   Mode of action (if known /understood)
   Uses (crop on which it is used, including, when relevant growth stage)
   Formulation type
   Dosage and frequency
   Time of the year
   Vapour pressure
   Water solubility

   Go to 2

Possibility of exposure

Higher plants in non-target areas can be exposed to plant protection products via the air or by run-off events. The major route of potential exposure is through drift (particulates that become airborne during application). The other route through the air is the release of vapour from the applied product followed by gaseous transport and exposure, predominant after application.

2. Is the exposure of non-target, terrestrial, higher plants possible (see Note 1)?
   If no **Classify as negligible risk, go to 17**
   If yes **Go to 3**

3. To which group does the active substance belong (see Note 2)?
   Herbicide or plant growth regulator **Go to 7**
   Other **Go to 4**

4. Obtain screening data (efficacy data can also be used) from at least 6 plant species from 6 different families including both mono- and dicotyledons (see Note 3).

   Go to 5

5. Do the results in one or more of the screening or efficacy tests with vascular plant species show ≥ 50% phytotoxic effects at or above the maximum recommended application rate (MRR) (see Notes 4 and 5)?
   If no **Classify as low risk, go to 16**
   If yes **Go to 6**

6. Obtain concentration/response tests on the affected species (see Note 6).
   If MRR/EC50 is < 1 for all affected species **Classify as low risk, go to 16**
   If MRR/EC50 is ≥ 1 for one or more affected species **Go to 7**

7. Obtain concentration/response tests on at least 6 species representing families for which significant herbicidal action has been found (see Note 7) and determine the EC50 value for each of the tests.

   Go to 8

8. Using the EC50 values for 6 or more different species, generate the 5th percentile of the log-logistic or the log-normal distribution according to Aldenberg and Slob (1993) or Wagner and Løkke (1991) or the final acute value (triangular distribution) according to Stephan *et al.* (1985) depending on the most likely shape of the toxicity distribution curve (see Note 8) and use this calculated toxicity value in the risk assessment.

Go to 9

Drift events

9. Obtain information about the drift concentration of the active substance (a.s.) that can be expected at distances of 1 and 5 metres from the treated area or 3 and 7 metres for orchards (see Note 9).

Go to 10

10. Calculate the exposure over toxicity ratio.

If PEC(1 m) /calculated toxicity value < 1

Classify as low risk for drift events, go to 11

If PEC(5 m) /calculated toxicity value < 1

Classify as medium risk for drift events, go to 11

If PEC(5 m) /calculated toxicity value > 1

Classify as high risk for drift events, go to 11

Gaseous transport events

For most products, gaseous transport events are not likely to be significant, compared with drift events. However, under certain conditions, it may be the major route of contamination (for instance, compounds with a high volatility). In some cases, indications for possible effects of gaseous transport events can be found on the application label (e.g. ‘do not use this product near a certain type of crop’).
Atmospheric deposition consists of dry and wet deposition. The dry deposition flux of gaseous substances is proportional to the dry deposition velocity. At a low dry deposition velocity, the total deposition flux is mainly determined by wet deposition. The wet deposition flux of a substance depends on its solubility in rain and the precipitation intensity. For a substance that is highly soluble, wet deposition is more important than for a substance that is poorly soluble. Guidance can be found in the Chapter on Air published in 2003 by the EPPO.

11. Obtain information about the concentration of the a.s. that can be expected at a certain location.

If PEC(gaseous transport) ≤ PEC(drift) at 1 m distance **Go to 12**

If PEC(gaseous transport) > PEC(drift) at 1 m distance **Go to 13**

12. No further risk assessment for gaseous transport events is necessary, because the outcome of this risk assessment will always be lower than that based on drift events.

Go to 14

13. Calculate the exposure over toxicity ratio (see note 10).
    If PEC(gaseous transport)/calculated toxicity value is < 1
    Classify as low risk for gaseous transport, go to 14
    If PEC(gaseous transport)/calculated toxicity value is >1 and <10
    Classify as medium risk for gaseous transport, go to 14
    If PEC(gaseous transport)/calculated toxicity value is >10
    Classify as high risk for gaseous transport, go to 14

Run-off events

For most products run-off events are not likely to be significant compared with drift events. However, under certain conditions, it may be the major route of contamination (for instance, compounds used on paved areas and/or in areas with steep slopes). Whether a run-off event will occur depends on the location (shape of the landscape) and the weather conditions. Guidance can be found in the recommendations of the FOCUS working group on surface water (standard scenarios for certain region of Europe) (FOCUS, 2001).

14. Is the uptake of the active substance mainly via the roots (in general soil-applied compounds/formulations)?
    If yes **Go to 15**
    If no **Classify as low risk for run-off events, go to 16**

15. Obtain information about the concentration of the active substance that can be expected at the scenario location (see note 10).
    If PEC(run-off scenario)/calculated toxicity value is < 1
    Classify as low risk for run-off events, go to 16
    If PEC(run-off scenario)/calculated toxicity value is >1 and <10
    Classify as medium risk for run-off events, go to 16
    If PEC(run-off scenario)/calculated toxicity value is >10
    Classify as high risk for run-off events, go to 16

Analysis of uncertainty

After completing the risk assessment based on data reflecting normal use of the product (e.g. average data for drift), it is necessary to consider whether errors in measurement, or variations in conditions of use, or the outcome of field studies, might alter the conclusions. This is appropriate for products initially categorised as medium or low risk to higher plants, to detect cases in which risk might be higher in practice. It is also appropriate for products initially categorised as high risk to consider the possibility that several of the assumptions may have been over-conservative (see also note 11).

16. Review the data that led to high, medium or low risk category and check whether the conclusion is correct
    If yes, confirm assessment **Go to 17**
    If no, obtain more information as needed **Go to 2**

Risk management

17. In most cases, products classified as negligible risk do not require any particular risk management measures.
    Products in the low risk category are not likely to affect non-target plants when used according to good plant protection practice up to the highest recommended application rate.
    Products in the medium risk category may have some effect on some non-target plants adjacent to treated areas. They should be labelled accordingly. The range of plant species at risk should be considered before a risk phrase (e.g. 'avoid use within a distance of x metres from sensitive areas') appears warranted.
    Products in the high risk category are likely to have a negative impact on non-target plants. They should be labelled accordingly and, where necessary, the range of plant species at risk should be considered when the appropriate risk mitigation measures (e.g. 'do not use within a distance of x metres from sensitive areas') are added to the label. Sensitive areas can be defined as areas containing the species at risk and/or generally particular non-target areas such as hedge rows, forest margins or wetlands.
    Measures to prevent drift or run-off may lead to lower exposure of non-target higher plants outside the field of application. This can be achieved by using low-emission application techniques such as low-drift nozzles or directed applications, or by creating buffer zones. Alternative formulation types and/or application techniques (i.e. as granules than to foliage) should be considered. Formulation characteristics and mode of application will determine the size of the buffer zones. Another way of preventing exposure of non-target higher plants to plant protection products is to insert barriers, e.g. artificial or natural wind shields. However, where windbreaks have been inserted for drift interception purposes, they should not be regarded as sensitive areas of particular concern, as discussed above). A boom sprayer application may, in some cases, be replaced by a row sprayer application (which is possible within the seed or plant furrow), or a seed treatment might be substituted in other cases. A change in the type of formulation might also be considered; e.g. a coarse granulate instead of a spray. Lower application rates or less frequent use represent other possible risk management tools.

4.3 Explanatory notes

Note 1 Negligible risk
If the nature of the product and its use are such that exposure of higher plants in the non-target area will most likely not occur (e.g. substances used for wound protection, for stored products, substances used in glasshouses, rodenticides, substances used for seed treatment or by stem injection) no further consideration of the effects on plants is necessary.

Note 2 Type of plant protection products
Herbicides and plant growth regulators are designed and/or selected to affect higher plants in one way or another. Although screening data are available for these compounds, steps 4 to 6 are omitted and concentration/response data are necessary to evaluate the potential risk of these compounds on non-target higher plants.
In most countries, products are classified according to their major use. When a product is also used as a herbicide or plant growth regulator, it should be treated as such even if the main use is different (e.g. insecticide/fungicide).
For active substances with a specific mode of action or a specific type of application, which would not be detected in the two standard tests (vegetative vigour test and seedling

emergence test conducted according to OECD 208 or other appropriate methods, see note 12), specifically designed methods using expert knowledge should be employed (this may be needed, for example, for soil sterilants working mainly via the gaseous phase).

Note 3 Screening tests for phytotoxicity
Data should be provided on all vascular plant species that have been tested during the screening process. The tested concentration should be equal to or higher than the maximum recommended application rate (MRR). Screening data and efficacy data allow one to assess whether phytotoxic properties of the test substance can be expected. They are usually conducted prior to the registration process and not according to GLP. For assessments of these data, it should include a certain minimum set of information (for details, see OECD guideline 208).

Screening data will usually be obtained with some kind of standard formulation. This is regarded to be acceptable, as such tests are generally conducted so as not to miss any significant activity (e.g. by adding significant amounts of effective additives). They may, therefore, be regarded to be at least as sensitive or to represent a worst case.

Note 4 Why 50% effect is enough?
In higher-plant testing, quantitative endpoints like biomass, plant height, number of leafs, percent chlorosis, percent ground cover etc. are nearly always measured. In such tests, the reaction of a plant population or any sub-population is of interest and testing is therefore usually done with more than one plant so that even a qualitative endpoint like germination becomes a quantitative endpoint and is expressed as percent germination.
In concentration/response tests studying the effect of plant protection products on plants, an EC_{50} value should be determined for each species by non-linear regression, and the 95% confidence interval should be calculated. As non-lethal endpoints are determined for the calculation of the EC_{50} value, the EC_{50} value is proposed as the basis for further assessment steps and not a NOEC value. Furthermore, a 50% reduction of plant biomass production in an early stage or of seedling emergence can be compensated largely by a plant population. The EC_{50} value is also proposed for technical reasons. To estimate a NOEC value on the basis of a concentration/response curve means to determine an EC_5 or EC_{10} value. This is extremely difficult due to the high variability within the plant populations, particularly if non-crop species are used.

Note 5 False positives
To exclude false positive outcomes, additional tests may be carried out if 10%, or at most 20%, of the species in the screening tests show phytotoxic responses (e.g. when the tested concentrations are above the maximum recommended application rate values).

Note 6 Definitive tests
A definitive test is a test conducted with a range of concentrations of the product, preferably in a geometric progression, but covering the EC_{50} values for the test species selected (e.g. OECD 208). For post-emergence herbicides and other plant protection products, the vegetative vigour test should be used (unless the mode of action otherwise indicates a specific test, for example, growth regulators influencing flowering, or some inhibitors of cell division causing flower sterility). For pre-emergence herbicides, the seedling emergence test is more applicable. The definitive tests should be conducted using an appropriate lead formulation.

Note 7 Number of species.
A minimum number of 6 species, representing families for which significant herbicidal activity has been claimed, should be tested. Statistical methods should be applied to calculate the 5th percentile of the available toxicity data (see Note 8). These methods apply uncertainty factors that depend on the sample size; the safety factors decrease with increasing sample size.

Note 8 Species sensitivity distributions
In contrast to the situation with many of the other species of concern in the risk assessment of plant protection products (e.g. fish, daphnids, birds), the risk assessment for higher plants exposed to phytotoxic compounds can be based on toxicity data for 6 or more species. This starting point makes it possible to use a statistical method to calculate the toxicity value for the risk assessment, instead of applying a fixed uncertainty factor of 10 to the lowest available toxicity value. The statistical methods available assume that the toxicity data follow a certain type of distribution:

Stephan *et al*. (1985)	Triangular distribution
Wagner and Løkke (1991)	Log-normal distribution
Aldenberg and Slob (1993)	Log-logistic distribution

For the method of Stephan *et al*. (1985), data for at least eight species are required. The acute final acute value calculated in this method is based on only the three lowest available toxicity data. The other two methods calculate the 5th percentile of the log-normal or log-logistic distribution for all available toxicity data. The methods of Wagner and Løkke (1991), Van Straalen and Denneman (1989) and Aldenberg and Slob (1993) estimate, on basis of the NOEC data, the hazardous concentration for 5% of the species (the 5th percentile of the log-normal or log-logistic distribution of all NOECs). This method can also be used for calculating the 5th percentile of the log-logistic distribution of all EC_{50} values:

$$5^{th} \text{ percentile} = 10^{(AVG - E * STD)}$$

In which:

AVG = the mean of the $\log_{10}$ transformed EC_{50} values
STD = the standard deviation of the $\log_{10}$ transformed EC_{50} values
E = Extrapolation factor dependent on sample size (see Table 1).

All three methods are included in the E_TX program of Aldenberg (1993) and, in addition, a goodness-of-fit test, based on the Kolmogorov-Smirnov test statistics according to D'Agostino and Stephens (1986), is provided for testing the log-normal or log-logistic distribution.

Table 1 Extrapolation factors for the median and one-sided left confidence limits for the log-logistic and log-normal distribution (after Aldenberg and Slob, 1993; Aldenberg and Jaworska, 2000)

Sample size	---- Median estimate ----	
	Log-logistic	Log-normal
6	1.81	1.75
7	1.78	1.73
8	1.76	1.72
9	1.75	1.71
10	1.73	1.70
11	1.72	1.70
12	1.72	1.69
13	1.71	1.69
14	1.70	1.68
15	1.70	1.68

Note 9 Non-target area

The aim of this risk assessment scheme is to protect higher plants in the non-target area from unacceptable phytotoxic effects of plant protection products under realistic conditions. The non-target area is defined as the area outside the treated area. The type of vegetation and plant species composition in these areas is extremely diverse and cannot be easily classified. Such areas include hedgerows, shelter belts, windbreaks, wetland, wood lots, and grassland, which are widespread terrestrial plant habitats in a landscape. Hardly any of these structures borders on a treated area directly. Usually, there is a more or less wide strip between the treated area and these structures. The flora of these transitional zones (field margins) is often a typical permanent grassland vegetation, strongly influenced by the cropping system of the adjacent fields. This influence is not restricted to the entry of plant protection products but also to the entry of fertiliser, to soil disturbance by machinery, etc. Therefore, the impact of the cropping system is more significant right next to the treated area than further away. As the risk assessment should be done under realistic conditions, a distinction for the classification of plant protection products to risk categories is made according to the distance to the treated area. Up to a distance of 1 m to the treated area, a greater impact can be tolerated than at a greater distance.

For orchards, a field margin of 3 m is assumed and for all other crops one of 1 m. Risk assessment is carried out at 3 m and at 7 m for orchards and at 1 m and 5 m for the other crop types.

Note 10 Safety factors

No scientific reason can be given for the safety factor of 10. This value is chosen because it offers roughly the same level of protection as provided in the drift scenario (differences in percentage of drift at 1 m and 5 m).

Note 11 Analysis of uncertainty

Where, on the basis of laboratory toxicity tests and reasonable worst-case exposure assumptions, a high risk is identified, the following risk refinement options should be considered. It may be possible to consider more realistic exposure scenarios i.e. the use of PECs which are not based on 95th percentile estimates of exposure. This area is currently the

subject of international discussion. Dose justification should be critically reviewed. Toxicity values may be refined by further testing, which will be conducted under more environmentally relevant conditions. It may be possible to perform these tests under glasshouse or semi-field conditions. The use of techniques which better simulate drift, (where this is the major route of exposure) should be considered. Application at less sensitive growth stages should be considered, if appropriate to the plant protection product and its intended use pattern. The use of test plants which have been grown outside should be considered. The importance of the seed bank for the species of concern should be assessed in relation to the proposed use. It is often difficult to measure the effects of pesticides on natural communities in the field because of the inherently high variability in natural populations (Marrs and Frost, 1997). These authors propose a semi-field (microcosm) approach, which may provide a suitable method for studying the impact of repeated applications. It is recommended that field studies should only be used after consideration of other refinement options such as studies in a glasshouse or on small field plots.

Note 12 Test guidelines

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