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

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Abstract

Factsheets for the (eco)toxicological risk assessment strategy of the National Institute for Public Health and the Environment - Part V

This report contains five factsheets describing risk assessment methods used at the Centre for Substances and Integral Risk Assessment (SIR) and the Expert Centre for Substances (SEC) of the National Institute for Public Health and the Environment (RIVM). The main aim is to enhance transparency and consistency in the risk assessment methods used at RIVM-SIR and RIVM-SEC. The factsheet on hepatic tumours in mice describes whether or not mouse liver tumours are considered relevant for human risk assessment. The factsheet on historical control data for tumour incidence provides the RIVM strategy on how to use historical control data in the evaluation of carcinogenicity data. The factsheet on Mononuclear Cell Leukaemia in the F344 rat strain discusses the relevance of an increased incidence of mononuclear cell leukaemia (MNCL) in F344 rats for humans and provides an approach to human hazard and risk assessment. In the factsheet on energy and moisture content and assimilation efficiency of bird and mammal food, two large datasets on the energy and water content and assimilation efficiency of various types of bird and mammal food, are merged and analysed. The last factsheet on sorption of dissociating compounds describes the main principles and limitations of QSAR models and the HPLC method for determining the adsorption coefficients of organic ionisable substances. The five factsheets reflect a state-of-the-art approach and are meant to facilitate discussion with other national and international parties involved in risk assessment.

Keywords: risk assessment; hepatic tumours; mononuclear cell leukaemia (MNCL); assimilation efficiency of food; QSAR models, HPLC method; organic ionisable substances

Rapport in het kort

Factsheets voor de (eco)toxicologische risicobeoordelingsstrategie van het Rijksinstituut voor Volksgezondheid en Milieu - Deel V

Dit rapport bundelt vijf factsheets waarin methodieken worden beschreven die worden gebruikt voor de risicobeoordeling van stoffen bij het Centrum voor Stoffen en Integrale Risicobeoordeling (SIR) en het Stoffen Expertise Centrum (SEC) van het Rijksinstituut voor Volksgezondheid en Milieu (RIVM). Het voornaamste doel is om de inzichtelijkheid en eenduidigheid van de bij RIVM-SIR en -SEC gevolgde methodieken te vergroten.
De factsheet over levertumoren in de muis beschrijft onder welke voorwaarden levertumoren in de muis als relevant voor risicobeoordeling in de mens worden beschouwd. In de factsheet over historische controlegegevens van het voorkomen van tumor wordt aangegeven hoe het RIVM bij de evaluatie van carcinogeniteit omgaat met historische controlegegevens. De factsheet over Mononucleaire Cel Leukemie in de F344 rat bespreekt de relevantie voor de mens van een toename in het voorkomen van mononucleaire cel leukemie (MNCL) in F344 ratten en levert een strategie voor de gevaar- en risicobeoordeling. In de factsheet 'Energie- en vochtgehalte en assimilatie-efficiëntie van voedsel voor vogel- en zoogdieren' zijn twee grote datasets met gegevens over het energie- en watergehalte van verschillende voedselbronnen voor vogels en zoogdieren samengevoegd en geanalyseerd. De laatste factsheet over sorptie van dissociërende stoffen geeft een uiteenzetting van de belangrijkste principes en beperkingen van de QSAR-modellen en HPLC-methoden voor het bepalen van het adsorptiegedrag van een stof. De vijf factsheets vormen de weerslag van de huidige stand van wetenschap. Ze zijn bedoeld om de discussie met andere (inter)nationale partijen op het gebied van risicobeoordeling te bevorderen.

Trefwoorden: risicobeoordeling; levertumoren; Mononucleaire Cel Leukemie; assimilatie-efficiëntie van voedsel; QSAR-modellen; HPLC-methoden

Preface

This report was written within the framework of the project 'Kennislacunes Risicobeoordeling' (*'Knowledge gaps in risk assessment'*). The factsheets presented in this report have been reviewed by members of the peer review groups of the Centre for Substances and Integral Risk Assessment (SIR) and the Expert Centre for Substances (SEC), and in some cases experts were consulted. The following persons are acknowledged for their contribution: M.E. van Apeldoorn, R. Beems, J. van Benthem, S. de Boer, A.G.A.C. Knaap, J.B.H.J. Linders, R. Luttik, M.T.M. van Raaij, T.P. Traas, A.P. Verschoor, and P. Wester.

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Samenvatting

In dit rapport worden vijf factsheets gepresenteerd die worden gebruikt voor de beoordeling van stoffen bij het Centrum voor Stoffen en Integrale risicobeoordeling (SIR) en het Stoffen Expertise Centrum (SEC) van het Rijksinstituut voor Volksgezondheid en Milieu (RIVM).

Bij blootstelling van de muis aan chemische stoffen wordt vaak een verhoogde incidentie van levertumoren waargenomen. De relevantie van levertumoren in de muis voor de humane risicobeoordeling is vaak een onderwerp van discussie geweest. In de factsheet **'Levertumoren in de muis'** wordt de cellulaire en moleculaire pathologie van de hepatocarcinogenese beschreven en er wordt aangegeven wat overeenkomsten en verschillen in levertumorvorming tussen de muis en de mens zijn. In de RIVM-strategie wordt beschreven onder welke voorwaarden levertumoren in de muis als relevant voor risicobeoordeling in de mens worden beschouwd.

In de factsheet **'Historische controle gegevens van tumorincidentie; praktische toepasbaarheid**' wordt aangegeven hoe het RIVM bij de evaluatie van carcinogeniteit omgaat met historische controlegegevens. In het algemeen worden in een chronische studie met ratten de mogelijke effecten na langdurige blootstelling onderzocht, waaronder ook de mogelijk carcinogene potentie van de stof. Vaak echter, wanneer een toegenomen tumorincidentie wordt waargenomen, ligt de verhoogde tumorincidentie op de grens van biologische en/of statistische significantie. In dat geval kunnen historische controlegegevens gebruikt worden om in te schatten of de verandering biologisch significant is.

In de factsheet **'Mononucleaire Cel Leukemie in de F344 rat'** wordt de beoordeling van de carcinogeniteit van stoffen gebaseerd op epidemiologie en/of chronische dierstudies. Echter, toename van bepaalde type tumoren in dierstudies worden soms als niet relevant voor de mens beschouwd. De relevantie van andere type tumoren is twijfelachtig. Een van de tumor typen met een twijfelachtige relevantie is mononucleaire cel leukemie (MNCL) in de F344 rat. In deze factsheet wordt de relevantie voor de mens van een toename in de incidentie van MNCL in F344 ratten besproken en een strategie voor de gevaar- en risicobeoordeling geleverd.

In de factsheet '**Energie- en vochtgehalte en assimilatie-efficiëntie van voedsel voor vogel- en zoogdieren'** zijn twee grote datasets met gegevens over het energie- en watergehalte van verschillende voedselbronnen voor vogels en zoogdieren samengevoegd en geanalyseerd. De gemiddelde calorische waarde en het vochtgehalte zijn berekend voor 16 verschillende categorieën voedsel en de assimilatie-efficiëntie wordt gepresenteerd voor 22 vogeltaxa en zeven groepen zoogdieren. De gegevens vormen de basis van de risicobeoordeling voor vogels en zoogdieren in het kader van de toelating van bestrijdingsmiddelen binnen de Europese Unie.

In de factsheet '**Sorptie van dissociërende stoffen**' wordt een uiteenzetting van de belangrijkste principes van de QSAR-modellen en HPLC-methoden voor het bepalen van het adsorptiegedrag van dissociërende stoffen beschreven. Bovendien beschrijft de factsheet de beperkingen die deze modellen en methoden hebben met betrekking tot geïoniseerde stoffen. Als aanvulling worden voor deze stoffen pragmatische benaderingen gegeven voor de risicobeoordeling. In dit document komen ook aspecten aan bod die niet in de EU TGD beschreven staan.

Summary

This report presents five factsheets for the risk assessment methods used in the Centre for Substances and Integral Risk Assessment (SIR) and the Expert Centre for Substances (SEC) of the National Institute for Public Health and the Environment (RIVM).

Upon exposure to chemicals, the liver of mice is a common and susceptible site of an increased tumour incidence. The relevance for human risk assessment of liver tumours in mice has been the subject of extensive debate. In the factsheet **'Hepatic tumours in mice'** the cellular and molecular pathology of hepatocarcinogenesis is described and similarities and differences in liver tumorigenesis between mice and humans are indicated. The question whether or not mouse liver tumours are considered relevant for human risk assessment is addressed in the RIVM strategy.

In the factsheet **'Historical control tumour incidence; its practical use'** the RIVM strategy is given on how to use historical control data in the evaluation of carcinogenicity data.
The potential long-term effects of toxic substances are usually determined in chronic toxicity studies with rodents. The outcome of a chronic test should enable a conclusion about the carcinogenicity of a given compound. However, the results often involve an increased incidence of tumours in treated animals which lies at the borderline of biological and/or statistical significance. In this case historical control values can be helpful in the interpretation of the biological significance of the change observed.

In the factsheet '**Mononuclear Cell Leukaemia in the F344 rat strain'** the assessment of the carcinogenicity of substances is based on the results of epidemiology and/or chronic animal studies. However, increases of some types of tumours in animal studies are sometimes claimed not to be relevant to humans. The relevancy of other tumour types is questionable. One of the questionable tumour types is mononuclear cell leukaemia (MNCL) in the F344 rat. In this factsheet, the relevance of an increased incidence of MNCL in F344 rats for humans is discussed, and an approach for human hazard and risk assessment is provided.

In the factsheet '**Energy and moisture content and assimilation efficiency of bird and mammal food'**, two large datasets on the energy and water content and assimilation efficiency of various types of bird and mammal food are merged and analysed. Average caloric and moisture values are determined for 16 different food types, and assimilation efficiencies are presented for 22 bird orders and seven groups of mammals. The data are used as basic input values for the risk assessment for birds and mammals within the framework of pesticide authorisation in the European Union.

In the factsheet '**Sorption of dissociating compounds'** some of the main principles of the QSAR models and the HPLC method are presented. Furthermore, the limitations they have in the treatment of organic ionisable substances with respect to adsorption coefficients are described. In addition, pragmatic approaches will be given for the risk assessment of organic ionisable substances. This factsheet specifically gives guidance to some issues that fall outside the current EU Technical Guidance Document.

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 for public health and the environment. The availability of adequate and up-to-date risk assessment methods is of the highest importance to fulfil this task. Some of these methods follow international guidance, but many have been developed within the RIVM during the process of evaluation. These risk assessment methods are not rigid procedures but can be adapted based on new/developing scientific information, possibly triggered by questions from policy makers or by developments in (inter)national 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. These factsheets describe the current assessment strategies of SEC and SIR, and their main aim is to provide a transparent and accessible guidance for issues that are not covered by regular guidance documents. 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 an RIVM report[1], followed by similar reports in 2002, 2003 and 2004[2,3,4]. The present report contains five factsheets that were produced in 2004 and 2005 by SIR and SEC:

1. Hepatic tumours in mice
2. Historical tumour incidence; its practical use
3. Mononuclear Cell Leukaemia in the F344 rat strain
4. Energy and moisture content and assimilation efficiency of bird and mammal food
5. Sorption of dissociating compounds

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 Jacqueline.Scheepmaker@rivm.nl and will be passed on to the responsible authors.

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1. Hepatic tumours in mice

Factsheet FSV-014/00, date 24-08-2005

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1.1 Introduction and aim

For the evaluation of toxic substances, effects of chronic exposure are usually determined in chronic toxicity/carcinogenicity studies with rodents. These effects include the potency of a substance to induce carcinogenic effects. The relevance for human risk assessment of some types of tumours in rodent bioassays has been the subject of extensive debate [e.g. 1, 2, 3].
In rodents, in particular in mice, the liver is a common site of an increased tumour incidence associated with chemical exposure; one-third to one-half of all chemicals that are carcinogenic in rodents are hepatocarcinogens [4, 5]. Worldwide, hepatocellular carcinoma (HCC) is also one of the most frequent visceral tumours in humans [6, 7, 8].
Many experts have addressed the susceptibility of the mouse liver to tumour development. It is now recognised that there is a variety of mechanisms by which chemicals can give rise to tumour development in the liver, and that their mechanisms may have different implications for human risk assessment.
In this factsheet the cellular and molecular pathology of hepatocarcinogenesis is described and similarities and differences in liver tumorigenesis between mice and humans are indicated. Whether or not mouse liver tumours are considered relevant for human risk assessment is addressed in the RIVM evaluation strategy in paragraph 5.
Apart from hepatocellular adenoma (HCA) and HCC, a chemical may induce other types of liver tumours in mice, (e.g. haemangiomas, haemangiosarcomas, isolated hepatoblastomas) which are considered relevant for humans. It must be noted that the RIVM evaluation strategy only applies to the HCA and HCC types of liver tumours in mice.

1.2 Mechanism for the development of the effect, and background

1.2.1 Pathogenesis of liver cancer

The differences and similarities in the pathogenesis of liver cancer in different species have been reviewed by Grisham [9]. Hepatocarcinogenesis has been analysed both at the cellular and molecular level. For many hepatocarcinogens the mechanism of action has been clarified. On the basis of the mechanism of action carcinogens can be classified as DNA-reactive or epigenetic hepatocarcinogens, but for a number of chemicals which induce liver tumours, sufficient information is not yet available for mechanistic classification (see ref. 20).

Cellular pathogenesis
Mice and rats
In rodents, hepatocarcinogenesis is a sequential, multi-step process, with stages termed initiation, promotion, and progression which can be delineated experimentally [9]. The cellular pathway is thought to lead from foci of cellular alteration (FCA), which are presumed to contain initiated cells, through hepatocellular adenoma (HCA) to hepatocellular carcinoma. Hepatocytes, liver stem cells, or both may be initially affected by genetic damage that initiates the carcinogenic process. Some proliferative lesions may be less well-defined in mice; e.g. hyperplastic nodules in combination with hepatocellular damage are generally associated with regeneration rather than (pre)neoplasia. It is often difficult to distinguish between HCA and HCC in mice. Mouse liver tumours generally do not invade surrounding extrahepatic tissues and they rarely metastasise [10].
Humans
A similar, rather simple pathway is not applicable to all HCC in humans. In humans three pathways are known. Pathway 1 is the most frequent, occurring in about 75% of all cases. In this pathway chronic hepatitis and cirrhosis precedes the development of HCC, which appears normally to develop in rapidly expanding cirrhotic nodules. In a second pathway, occurring in about 25% of all cases, chronic hepatitis and focal parenchymal hyperplasia are prominent features preceding the development of HCC. In the third pathway, which represents less than 1% of HCC, the lesion that precedes HCA is unknown. The risk of progression from HCA to HCC is very small and HCA may regress by unknown mechanisms [9].
The prognosis of HCC in humans is poor. HCC often metastasises and the 5-year survival rate is 6% [11].

Molecular alterations in pathogenesis of liver tumours
With respect to the molecular pathogenesis of HCC in rodents and in humans many alterations involving expression of growth factors/receptors, proto-oncogens, and tumour suppressor genes have been identified at various stages of hepatocarcinogenesis. The most important alterations are [9]:

- Increased expression of the growth factors TGFα or IGF-II, in mice and rats as well as humans.
- Increased expression of the H-*ras* and *myc* proto-oncogenes.
- The level of methylation of a gene is one of the mechanisms involved in the control of gene expression. A hypomethylated gene can be considered to possess an increased potential for expression as compared to a hypermethylated gene [12]. Hypomethylation of proto-oncogenes has been demonstrated in HCC of rats and mice [e.g. 13, 14]. Elevated c-myc expression is also a common feature of HCC and preneoplastic lesions in humans.
- Mutated *ras* genes.

Frequently *ras* genes are mutated in HCC of mice. The H-*ras* proto-oncogene is mutated in 30-60% of the spontaneous HCC in $B_6C_3F_1$ or C_3H mice. The mutation is less frequent in HCC of strains of mice that are less prone to development of HCC [15]. Even in HCC-susceptible mouse strains other molecular pathways than the *ras* mutations exist. In contrast to mice, mutations of *ras* genes are infrequent in HCC of rats and humans.

- Increased expression or mutations of p53 tumour suppressor gene.
  Changes in expression or mutations of the p53 tumour suppressor gene have been extensively studied in human cancer, including HCC [16]. It has been suggested that the mutation of the p53 gene may have a role in the development of HCC in humans. Mutations of the p53 gene have not been identified in HCC in mice [17, 18, 19].
- Changes in additional tumour suppressor genes.
  Other known and putative tumour suppressor genes are also assumed to be involved in the pathogenesis of HCC in humans [9].

1.2.2 Mechanisms for liver tumour induction

In the liver, several mechanisms of chemically induced carcinogenesis have been identified. These mechanisms can be categorised as follows (according to ref. 20).

A. DNA-reactive hepatocarcinogens

Many DNA-reactive agents, such as aromatic amines [16], can induce cancer in the liver of rodents in addition to producing cancer at other sites. For hepatocarcinogens, there is a relationship between DNA binding and carcinogenic potency. Most DNA reactive carcinogens are more hepatocarcinogenic in male rats and mice than in females rats and mice [20].

B. Epigenetic hepatocarcinogens

Some agents produce increases in rodent liver tumours by epigenetic mechanisms that do not involve reactivity of the chemical with DNA. These agents can be assigned to several classes based upon the mechanism of action through which they produce an increase in the occurrence of neoplasms.

Promoters

Tumour promoters act by facilitating the clonal expansion of initiated cells. FCAs are presumed to contain initiated cells and FCAs are considered to be precursors of liver neoplasms. Promoter-mediated development of FCA could be a consequence of enhanced cell proliferation under the influence of the promoter (e.g. phenobarbital) [21, 22].
Phenobarbital was found to increase the occurrence of liver tumours in rodents when administered chronically at high levels [23]. Phenobarbital was the first compound of a group of liver enzyme inducers found to be promoters of liver tumours. Thus, the concept arose that phenobarbital-type promoters acted through enzyme induction. Indeed, there is a good correlation between enzyme induction and promoting activities [24]. However, no clear mechanistic explanation has been provided for the role of enzyme induction in development of liver tumours.
In addition to phenobarbital and other barbiturates, a number of organochlorine compounds have been found to increase the occurrence of neoplasms in the livers of mice and rats; these include some pesticides, PCBs, and 2,3,7,8-TCDD [25].

Peroxisome proliferating agents

After chronic exposure to peroxisome proliferators, increases in the incidence of HCC have been observed in several rodent studies in conjunction with significant peroxisome proliferation

(see for a review ref. 26), especially in mice and rats. In a number of these studies also tumours in other tissues have been found. A strategy regarding peroxisome proliferating agents is presented in a separate factsheet [26].

Cytotoxins
Cytotoxicity gives rise to regenerative cell proliferation and this may be involved in the pathogenesis of the neoplasms [27]. In addition, selective growth of pre-neoplastic lesions, which are resistant to the cytotoxic agents, may also be involved [28]. Carbon tetrachloride and chloroform are two hepatotoxic agents that also increase liver tumour incidence in mice. However, these agents are not known to increase cancer in humans, probably because exposures are not sufficiently high enough for chronic hepatotoxicity. Ethanol is hepatotoxic in humans, and repeated episodes of hepatotoxicity are associated with increases in liver cancer. Interestingly, it has not been possible to replicate this mechanism in rodents [29].

Hormones
In humans, oral contraceptive use is associated with increased risk of liver tumours [30]. In rats, liver tumours have been induced by synthetic estrogenic agents such as ethinylestradiol [31, 32] and diethylstilbestrol [33]. Estrogens have been shown to have a promoting effect in rat liver hepatocarcinogenesis and enhancement of cell proliferation by estrogenic hormones has been documented in rat liver [31]. No effect of diethylstilbestrol [33, 34] or estrogens or progestagens on mouse liver tumour incidence has been reported [35].

Viral infections
In humans, viral hepatitis is a major factor for liver cancer, possibly through development of cirrhosis and regeneration [36]. In woodchucks, hepatitis virus also leads to development of liver cancer [37]. In mice, however, hepatitis does not appear to be associated with an increase in liver tumour incidence, but an increase is seen in mice with *Helicobacter hepaticus* infection [38] and in transgenic mice expressing the RNA for the hepatitis B virus [39]. Increased cell turnover is a common factor in all these conditions.

1.3 Normal values and natural variation

Experimental animals

Table 1-1: Incidences of spontaneous hepatic tumours in rodent species in long-term studies

Species	Strain	Spontaneous incidence (%)				References
		Male		Female		
		HCA	HCC	HCA	HCC	
Mice	C_3H	18-100*		0*		[40]
	C_3He	78*		0*		[40]
	BalB/c	0*		0-1*		[40]
	Charles River	2*		1.5*		[40]
	CF-1	15-20*		0-13*		[40]
	CBA	41*		27*		[40]
	TF-1	13*		5*		[40]
	$B_6C_3F_1$	18-74	8-70	6-80	0-42	[41]
Rats	Charles River CD	1.4*		2.7*		[42]
	Osborne-Mendel	0.5*		2.4*		[42]
	AES	2.6*		1.3*		[42]
	Sprague-Dawley	1.1*		0.7*		[42]
	Wistar	1.8*		1.1*		[42]
	Fischer 344	0-10	0-6	0-2	0-1	[41]

HCA= hepatocellular adenoma, HCC=hepatocellular carcinoma
*: no distinction was made between HCA and HCC.

Table 1-1 indicates that the spontaneous occurrence of HCA/HCC is a very common finding in mice, and that there is a high variability in the incidences of HCA/HCC between mice of different strains. In rats the spontaneous incidence of liver tumours is lower than in mice.

Three factors seem to influence the occurrence of spontaneous hepatocellular neoplasms in mice, namely strain, sex and food intake (ref. 43; see also paragraph 2).

Strain
As shown in Table 1-1 the incidence of spontaneous liver tumours in mice varies greatly among strains. Mice strains with a high incidence in spontaneous liver tumours also show an increased incidence in mutated H-*ras* genes (see paragraph 2, molecular pathogenesis). The relative susceptibilities of various strains to chemically induced hepatocarcinogenesis closely follows the incidence of spontaneous liver tumours in mice [20].

Sex
Male mice generally display higher incidences of spontaneous liver tumours and are more susceptible to hepatocarcinogenesis than females. The mode of action for this difference between males and females is unknown. However, since androgenic hormones have not been reported to induce rodent liver cancer, it is unlikely that it is a direct effect of male sex hormones. Castration or treatment of males with diethylstilbestrol reduced the occurrence of hepatocellular neoplasm in males [43, 44, 45]. In contrast, treatment of females with synthetic estrogenic agents increased liver tumour incidences [25].

Data from NCI/NTP studies indicate that there is a strong correlation between chemically induced male and female (liver) tumours in $B_6C_3F_1$ mice, i.e. when liver tumours are observed in one sex they will also appear in the opposite sex [5, 46].

Food intake
Chronic dietary restriction induces an increase in apoptosis rate and a decrease in cell proliferation rate in hepatocytes of 12-month-old $B_6C_3F_1$ mice compared to ad libitum feeding [47]. This diet-induced shift in cell death/proliferation rates was associated with a marked reduction in spontaneous hepatoma and a marked increase in disease-free life span in dietary restricted mice compared to ad libitum fed mice. These results suggest that total caloric intake may modulate the rates of cell death and proliferation, showing a cancer-protective effect with dietary restriction and a cancer-promoting effect in ad libitum fed mice [47]. High-protein or high–fat diets also led to higher incidences of hepatic tumours in rodents [48]. It was shown that the inhibiting effect of dietary restriction on growth of diethylnitrosamine (DEN) induced glucose-6-phosphokinase-deficient (G6Pd) preneoplastic hepatic foci in Swiss OF1 mice was associated with reduced levels of insulin and IGF-I, two growth stimulating factors [49, 50]. Caloric restriction in SV129 mice was shown to modify nuclear receptor transcription (involved in regulation of growth factors and oxidizing enzymes) patterns which may play a role in liver tumour formation induced by hepatotoxic compounds [51].

Humans
In the age-adjusted standard population of humans, the incidence rate in hepatocellular carcinoma in Europe and North America is 0.003-0.005%. In Africa (0.030%) and in Asia (0.035%) incidence rates are relatively much higher [11]. The differences in incidence rates in humans from different parts of the world are mainly due to high incidences of chronic infection with the hepatitis B and C virus in Africa and Asia.

1.4 Susceptible species / Subpopulations

Interspecies differences
There are several differences between mice and humans with respect to the development of spontaneous or chemical-induced hepatic tumours.

- Several strains of mice, e.g. B6C3F1, have very high and variable spontaneous tumour incidences, which indicates that their liver contains a significant population of ‘initiated’ or latent tumour cells [9]. These cells would be expected to be susceptible to the promoting effects of cellular proliferation. According to a report by the ‘international expert advisory committee to the Nutrition Foundation’, a similar susceptible population does not appear to exist in human livers [40].
- Chemically induced liver tumours are often observed in mice. Analysis of the long-term carcinogenicity studies in rodents from the NCI/NTP database [46] revealed that from the 313 chemicals tested for liver tumours, 78 (25%) were positive in mice and 33 (11%) in rats. In comparison, as of July 1996 IARC had identified only a few chemicals or therapeutic drugs that were considered to be associated with HCC in humans [30]. Of 183 pharmaceutical agents analysed by IARC, 3 substances (contraceptive steroids, azathiopine and anabolic steroids) were considered to be associated with risk of HCC in humans [30, 33]. Furthermore exposure to arsenic, aflatoxin B1, ethanol and vinyl chloride monomer are considered risk factors for development of HCC in humans. It must be noted that the exposure level to a chemical in experimental mice in long-term studies is much higher than in humans, which may be an important factor for the observed differences between mice and humans.

- In contrast to human liver tumours, mouse liver neoplasms generally do not invade surrounding extrahepatic tissues and they rarely metastasise [10].
- Hepatitis B and C viral infections are major factors for HCC in humans, whereas mice and rats are not known to be susceptible to a species-specific hepatitis virus.
- There is a lack of concordance in the occurrence of chemically induced HCC in humans and mice. For instance, ethanol is hepatotoxic in humans and is associated with increased risk of liver tumours. These effects of ethanol are not observed in rodents. Similarly, aflatoxin B1 is a known hepatocarcinogen in rats and humans whereas mice appear to be resistant, probably due to a difference in metabolism of aflatoxine B1 [4, 5, 36].
- In mice and rats the major hepatocarcinogens identified are chemicals. In human epidemiological studies, the effect of a chemical on liver tumour induction is less clear and cannot be easily distinguished from other factors. An exception may be aflatoxin B1, for which most epidemiological studies show a correlation between exposure and increased incidence of liver cancer [52].

Human variability
The risk of developing HCC in humans is associated with several inheritable metabolic abnormalities, e.g. primary hemachromatosis, tyrosinemia, glycogen storage disease, porphyria cutanea tarda and acute intermittent porphyria [9]. Most of these are characterised by inborn errors of metabolism that lead to accumulation of metabolic products in hepatocytes.
In humans chronic infection with either hepatitis B or C greatly increases the risk of hepatic cell carcinoma (100-200-fold increased risks are found in epidemiological studies) [53]. Epidemiological evidence suggests that concurrent chronic infections with hepatitis B or C and exposure to aflatoxin [54], ethanol abuse [55], and possibly tobacco smoking [56], may amplify the risk of development of HCC.

1.5 Assessment and RIVM strategy

The biological relevance of chemically induced mouse liver tumours for human hazard identification has been the subject of considerable debate.
In contrast to some other rodent tumours where a specific mechanism has been identified (e.g. α2μ-globulin associated renal tumours in male rats [57]), the evidence regarding the relevance of hepatic tumours in mice for human risk assessment is less straightforward. The scientific evidence to date indicates that in many ways mouse liver tumours have different characteristics compared to HCC in humans [10].

The relevance of hepatic tumours in mice has been discussed by the 'International Expert Advisory Committee to the Nutrition Foundation' (1983 [40]), 'ECETOC' (1982 [58]), and in a 'Society of Toxicology'-sponsored meeting (1997 [48]). In all discussions it was stated that for non-genotoxic hepatic tumour promoters, the weight of evidence indicates that a mouse liver tumour response is of questionable relevance to humans.
'The International Expert Advisory Committee to the Nutrition Foundation' stated that it is necessary that the total weight of evidence on carcinogenicity be considered in making regulatory decisions. More concern regarding the relevance of hepatic tumours in mice is warranted when there is:
1. unequivocal evidence of genotoxicity,
2. specific metabolic data demonstrating that the substance interacts with cellular genetic material,
3. evidence to demonstrate that the pharmacokinetic and metabolic data on the substance in the mouse more closely resembles man than other species tested,

4. evidence from epidemiological investigations indicating the substance has the potential to produce an increased risk to humans.

According to the committee a case-by-case approach should be applied to evaluate all available evidence.

With respect to risk assessment of pesticides, the use of hepatic tumour induction in mice has been addressed by JMPR on several occasions, as reviewed by the World Health Organization in the 'Principles for the Toxicological Assessment of Pesticide Residues in Food' [59]. In their review it is stated that 'JMPR has generally considered it unwise to classify a compound as a carcinogen solely on the basis of an increased incidence of tumours of a kind that commonly occur spontaneously in the species and strain under study'. With respect to liver tumours it is stated that 'the position of JMPR is that mouse liver tumours are of little relevance in predicting human cancer risk' [60].

For the classification and labelling requirements for dangerous substances and preparations in the European Union (Annex VI of Directive 67/548/EEC, 2001[61]), compounds may not be classified as carcinogenic if the only available tumour data are liver tumours in certain sensitive strains of mice, without other supplementary evidence. No further reference was supplied in this guideline and the sensitive strains are not specified.

RIVM strategy
Apart from hepatocellular adenoma (HCA) and HCC, a chemical may induce other types of liver tumours in mice, (e.g. haemangiomas, haemangiosarcomas, isolated hepatoblastomas) which are considered relevant for humans. It must be noted that this RIVM evaluation strategy only applies to the HCA and HCC types of liver tumours in mice.
Overall, the data presented in paragraph 4 indicate that there are considerable differences in liver tumours between mice and humans. In order to establish the relevance of substance-related mouse liver tumours for human risk assessment the following points must be considered:

1. The substance is genotoxic.

When the substance is genotoxic, it will be evaluated as if it presents a carcinogenic hazard to humans. Since genotoxic substances may induce irreversible damage to the DNA, it is generally assumed that there is no exposure level below which damage cannot occur. If a substance is genotoxic, all tumours, including mouse liver tumours, are considered relevant. In the risk assessment a non-threshold approach is applied to the mouse liver tumours, or if multiple sites or more than one species are affected, to the most critical tumour observed.
Note: The available database for a given substance may indicate that cytotoxicity rather than genotoxicity is the determining factor for mouse liver tumour induction. In that case, the available data should be subject to expert consultation, in order to decide on the primary mode of action and the way forward in risk assessment.

2. The substance is not genotoxic

If the substance is not genotoxic the following situations may apply:

A. The substance is a peroxisome proliferator.
If liver tumours in the mouse are induced by a peroxisome proliferator, at doses at which peroxisome proliferation occurs, in most cases they will be considered not relevant for human risk assessment. Details of the RIVM strategy on interpretation of hepatocarcinogenesis induced by peroxisome proliferators are described in RIVM/SIR factsheet [26].

B. The substance induces only liver tumours in the mouse.
If the non-genotoxic substance induces only hepatic tumours in the mouse these tumours are considered not relevant for human risk assessment.

C. The substance induces other tumours.
Apart from liver tumours in the mouse, a chemical may induce other types of tumours in the mouse or tumours in another species. In this case, expert judgement is needed to establish the relevance of the liver tumours for humans.

The reasons for accepting or discarding mouse liver tumours as being relevant for human risk assessment should always be justified.

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2. Historical control tumour incidence; its practical use

Factsheet FSV-015/00, date 11-01-2005

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Contents

2.1 Introduction

For the evaluation of toxic substances, the potential long-term effects are usually determined in chronic toxicity studies with rodents. Potential effects after chronic exposure also include possible carcinogenic effects.
Under ideal conditions, the outcome of a chronic test should enable a conclusion about the carcinogenicity of a given compound. However, the actual test results do not always allow such a clear distinction. Experience has shown that the results often involve an increased incidence of tumours in treated animals which lies at the borderline of biological and/or statistical significance. Evaluations about the significance of such findings in animals (and man) are complex [1]. In this case 'historical control values', i.e. data on the normal variation of a parameter in the test species, can be helpful in the interpretation of the biological significance of the change observed. However, the incidence of spontaneous changes is often highly variable among control groups of the same species and strain in different studies conducted [2]. This factsheet will describe when and how historical control data are helpful in the evaluation of carcinogenicity studies.

2.2 Background information

The primary goal of the chronic rodent carcinogenicity study is to assess the development of tumours in animals exposed to a chemical of concern as compared with controls. Four types of neoplastic responses are considered to be evidence of chemically induced carcinogenesis:
1. An increase in the frequency of one or several types of tumours that also occur in the controls;
2. The development of tumours not seen in controls;

3. The occurrence of tumours at an earlier time point than in controls;
4. An increase in the number of tumours per individual animal (multiplicity), compared to the controls.

Further a dose response relationship should be apparent. However, one should be aware that there may be a lower tumour incidence in the high(er) dose group(s) resulting from poor survival in these animals [3,4,5].
The tumours in the experimental animals may not be at the same stage of development. The stages include, for example, atypical hyperplasia (putative preneoplastic), benign tumours, carcinomas in situ, invasion of adjacent tissues and metastasis to other parts of the body. Although tumours of the same type but at different neoplastic stages could be separately tabulated, they should be combined for statistical purposes [3,5].
Factors to be weighed in the analysis of tumorigenesis include: (i) the occurrence of (cyto)toxic effects in the target organ(s), (ii) the occurrence of toxic effects in non-target organs (iii) the tumour incidence in concurrent controls, (iv) the presence and nature of preneoplastic lesions, (v) the species- and organ- specific sensitivity of tumourigenic response (e.g. liver tumours in B6C3F1 mouse), and (vi) the presence of a shift from 'benign' towards 'malignant' tumours with increasing dose.

In table 2-1 and 2-2, a top ten rank order of chemically induced site specific tumours in mice and rats based on the NTP database (B6C3F1 mice and F344 rats) is given [4].

Table 2-1: Top ten organs/systems developing tumours in carcinogenesis studies – mice [a]

ranking	males	females
1	Liver	Liver
2	Lung	Lung
3	Forestomach	Forestomach
4	Circulatory system	Haematopoietic system
5	Haematopoietic system	Circulatory system
6	Thyroid gland	Mammary gland
7	Harderian gland	Ovary
8	Adrenal gland	Thyroid gland
9	Kidney	Uterus/cervix
10	Five sites [b]	Harderian gland

a. Based on 379 long-term chemical carcinogenesis studies from the National Toxicology program (NTP) data base

b. Heart, nasal cavity, preputial gland, skin, urinary bladder

Table 2-2: Top ten organs/systems developing tumours in carcinogenesis studies – rats [a]

ranking	males	females
1	Liver	Liver
2	Kidney	Mammary gland
3	Zymbal gland	Zymbal gland
4	Forestomach	Thyroid gland
5	Thyroid gland	Forestomach
6	Skin	Urinary bladder
7	Haematopoietic system	Clitoral gland
8	Urinary bladder	Haematopoietic system
9	Intestines	Kidney
10	Nasal cavity	Uterus/cervix

a. Based on 379 long-term chemical carcinogenesis studies from the National Toxicology program (NTP) data base

2.3 Normal values and variation

Spontaneous carcinogenesis occurs in rodents as well as in humans. Thus, a degree of background tumour formation is always observable in control animals from chronic rodent carcinogenicity studies. Certain organs/tissues seem to be more susceptible to spontaneous tumour formation than others. Spontaneous tumour incidences vary by species, strain, stock or breeder and by gender [4,6,7,8]. Therefore, knowledge of the natural incidence of neoplastic lesions is essential for interpretation of experiments designed to reveal the effects of potential carcinogens [6,9]. Organs most susceptible to spontaneous tumour formation are not always the

ones most susceptible to chemically induced carcinogenesis as occurs in chronic rodent bioassays [4,10].

Examples of differences in tumour incidences between strains are: C57BL/6 mice have a much lower liver tumour rate than do C3H mice, female Sprague Dawley rats have considerably more mammary tumours than do Fisher 344 rats, and Fischer 344 rats have a higher rate of leukaemias and tumours of the testis [10].
Examples of differences in tumour incidences between genders are: the most frequently occurring neoplasms in untreated male F344 rats are testicular adenoma (89.1%) and mononuclear cell leukaemia (50.5%) and for untreated female F344 rats pituitary gland neoplasms (54.2%) and mammary gland fibroadenoma (41.2%). For B6C3F1 mice the most frequently occurring neoplasms for untreated males are liver adenoma/carcinoma (42.2%) and lung adenoma/carcinoma (20.5%) and for untreated female mice liver adenoma/carcinoma (23.6%) and malignant lymphoma (haematopoietic system) (20.9%). These examples are based on the 1997 NTP data [11].

In table 2-3 and 2-4, a ranking on incidence of spontaneous tumour formation (benign and malignant) is given for the organs of mouse and rats, based on the mean of the highest incidence found for various strains (CD1 and B6C3F1 mice and F344, Sprague Dawley and Wistar rats) [4]. These tables are only meant as illustration.

Table 2-3: Ranking of mouse organs based on the incidence of spontaneous tumour formation (benign and malignant)

	males				**females**		
	organ	**incidence** [a] (%)			**organ**	**incidence** [a] (%)	
1	Liver	28.7		1	Blood/lymphoid tissue [b]	29.1	
2	Lung/trachea	24.3		2	Lung/trachea	22.9	
3	Adrenal	14.7		3	Pituitary	11.6	
4	Blood/lymphoid tissue [b]	13.8		4	Liver	8.8	
5	Stomach	3.0		5	Uterus/vagina	7.6	
6	Circulatory system	2.9		6	Mammary gland	4.3	
7	Skin/subcutaneous	2.4		7	Ovary	2.8	
8	Pancreas	2.1		8	Circulatory system	2.4	
9	Thyroid	1.6		9	Adrenal	2.2	
10	Kidney	1.5		10	Stomach	2.1	
11	Pancreas islets	1.2		11	Skin/subcutaneous	1.8	
11	Testes	1.2		12	Thyroid	1.7	
12	Urinary bladder	1.1		13	Kidney	1.4	
13	Pituitary	0.5		14	Urinary bladder	1.2	
14	Intestines	0.4		15	Brain	1.1	
15	Brain	0.1		16	Pancreas	<1.0	
15	Heart	0.1		16	Pancreas (islets)	0.8	
15	Body cavities	0.1		17	Body cavity	0.3	
				18	Intestines	0.2	
				19	Heart	0.1	

a mean of highest reported percent incidence of spontaneous tumour formation for various mouse strains
b Leukaemia/lymphoma

Table 2-4: Ranking of rat organs based on the incidence of spontaneous tumour formation (benign and malignant)

	males				**females**		
	organ	**incidence** [a] (%)			**organ**	**incidence** [a] (%)	
1	Pituitary	42.1		1	Pituitary	61.4	
2	Testes	38.8		2	Mammary gland	43.7	
3	Adrenal	31.4		3	Adrenal	24.5	
4	Pancreas	28.9		4	Uterus/vagina	18.1	
5	Blood/lymphoid tissue [b]	20.7		5	Blood/lymphoid tissue [b]	14.1	
6	Thyroid	12.5		6	Thyroid	11.4	
7	Skin/subcutaneous	12.1		7	Liver	6.0	
8	Pancreas (islets)	10.9		8	Skin/subcutaneous	4.0	
9	Brain	6.3		9	Brain	2.7	
10	Body cavities	3.5		10	Lung/trachea	2.1	
10	Mammary gland	3.5		11	Pancreas (islets)	1.9	
11	Lung/trachea	3.4		11	Ovary	1.9	
12	Liver	3.2		11	Intestines	1.9	
13	Circulatory system	2.5		12	Preputial gland	1.8	
14	Preputial gland	2.4		12	Circulatory system	1.8	
15	Intestines	2.1		13	Pancreas	1.7	
16	Kidney	1.7		14	Body cavity	1.4	
17	Stomach	1.2		15	Urinary bladder	1.1	
18	Urinary bladder	0.9		15	Stomach	1.1	
19	Heart	0.2		16	Kidney	1.0	
				17	Heart	<0.1	

a mean of highest reported percent incidence of spontaneous tumour formation for various mouse strains
b Leukaemia/lymphoma

For up to date information on the historical control values of individual tumour types in certain organs, one can look on the internet for actual databases on historical controls like the NTP database [12], the database of Charles River Laboratories [13] and Mouse Tumor Biology database [14]. Other databases that provide a standardised and reliable source of historical control data are the North American Control Animal Database (NACAD) [15] and the European Registry of Industrial Toxicology Animal-data (RITA) system [8]. Both can be found on the world wide web, but need membership to use it.

For certain commonly-occurring rodent tumour types, which give rise to controversy in relation to their relevance for man, an overview of historical control values can be found in earlier factsheets dealing with this subject:

- Factsheet FSV 003/00 [16]: pheochromocytomas
- Factsheet FSV 006/00 [17]: kidney tumours related to alpha$_{2u}$- globulin
- Factsheet FSV 007/00 [18]: follicular thyroid tumours
- Factsheet FSV 010/00 [19]: liver tumours related to peroxisome proliferation
- Factsheet FSV 011/00 [20]: forestomach tumours
- Factsheet FSV 012/00 [21]: Leydig cell tumours

2.4 Aspects that influence spontaneous tumour incidences

Many factors can be of influence on spontaneous tumour formation and generally fall into two categories: a) study design and b) histological procedures.

a) Study design
The factors related to study design are diet (restricted diet vs. ad libitum), body weight, housing conditions (individually vs. group), duration of the study, environment (e.g. the laboratory), genetic drift, survival differences (age), time (calendar year), presence of infectious organisms such as Helicobacter (increase of liver neoplasms in B6C3F1 mice), exposure scenario (gavage exposure vs. dietary exposure) and/or type of control treatment (e.g. untreated, corn oil gavage) [4,6,7,8,11,15,22].
For example, a comparison of the historic control database from the NTP in 1997 with that of a decade earlier showed higher body weights and lower survival for rats and a similar trend for mice. It showed that many spontaneous tumour rates had increased and in some cases the higher body weight could account for these increases (e.g. liver tumours in mice (m/f) and mammary tumours in female rats) [11].
Some of the key environmental influences include lighting and numbers of animals per cage. However, one of the most important experimental variables is age, as differences in tumour rates may simply reflect the greater survival of one group over another [15]. When tumour rates found in lifespan studies were compared with 2-year historic control tumour rates in F344 rats, it was found that the variety of neoplastic lesions in animals that were allowed to live their life-span was not greater than that in animals that were killed between 110 and 116 weeks of age, thus older age was not characterised by unique neoplasms. A second finding was that the incidence of certain neoplasms increased markedly after 110-116 weeks. No tumours were found in animals of either sex that died before 59 weeks of age and most of the common types of neoplasms were already present between 85 and 97 weeks of age [9]. A similar finding that increasing age is correlated with increasing incidence of spontaneous tumours was observed for Sprague Dawley rats. The spontaneous tumour frequencies of all rats (male and female together) were only 6% at 15 months, 13% at 21 months, 66% at 26 months, and 86% at 32 months [23]. The data with F344 rats showed that hepatocellular neoplasms (neoplastic nodules) and pancreatic acinar cell adenomas occurred relatively late in lifespan. These were seen for the first time in animals that died between 98 and 100 weeks of age and between 111 and 128 weeks of age, respectively [9].

b) Histopathologic procedures
The second major category of factors include methods for tissue preparation and histopathologic evaluation like gross necropsy, tissues selected, orientation in trimming, number of sections surveyed, slide preparation procedures, staining procedures and diagnostic criteria applied by the pathologist (histopathology diagnoses and nomenclature of pre-neoplastic lesions and tumours) [6,8,15,22,24].
For example a standard dissection is necessary to ensure that all animals are examined in a similar manner for the detection of gross lesions and collection of all required organs. As number of tumours can increase with multiple sections or changes in orientation [25,26], all groups within a carcinogenicity study should have tissues prepared in the same way. Staining procedures should also be as consistent as possible with animals from each group processed concurrently such that any variations in staining will be spread across all groups [15].

2.5 The use of historical control values

In the OECD guideline 451 for carcinogenicity study [27] it is stated: 'A good knowledge of the tumour profile of the animal strain throughout the life span is highly desirable in order to evaluate the results of experiments in a proper way' and 'The incidence of tumours and other suspect lesions normally occurring in the strain of animals used (under the same laboratory conditions – i.e. historical control) is desirable for assessing the significance of changes observed in exposed animals'.
The overall opinion of many authors [5,8,11,22,24,28,29,30] is that historical control information is useful in the interpretation of experimental results from similar studies, especially for rare neoplasms and for borderline effects, though all emphasize that the most appropriate and important comparison of an experimental group is with its concurrent control. However, there are specific instances in which historical control rates provide relevant data needed to interpret results of rodent carcinogenicity studies and as such are useful in deciding whether or not the apparent increase in neoplasm incidence (either statistically or not-statistically significant) is a biological meaningful effect:

- the evaluation of rare neoplasms (i.e. with spontaneous tumour rates of 1% or less) may require less stringent statistical evidence in a given study if the low spontaneous rate can be demonstrated from an established historical control database.
- historical control data may be helpful in the evaluation of a neoplasm showing a marginal increase in incidence relative to concurrent controls.
- historical control data may also be useful from a quality control point of view, to help determine if concurrent control neoplasm rates are consistent with past experience. Like in the case of aberrant frequencies (i.e. control frequencies outside the expected control range) of neoplastic or pre-neoplastic lesions, there are certain limitations of the concurrent control. A lower than normal tumour frequency in the concurrent control animals may lead to a statistically significant increased incidence of lesions in the dose groups (false positive). A higher than normal tumour frequency in the concurrent control, on the other hand, might mask a carcinogenic response in dosed animals (false negative). In such cases historical control data (which may provide a more appropriate demonstration of the true mean for the population and the variability of that mean) may support substantially the assessment of a potential carcinogenic risk.

One should be aware that false positive results may occur at sites with high and variable tumour rates and that these results are dependent on the rate of spontaneous tumour incidence.

When it is decided to use historic control data, it is essential that the study being evaluated is comparable to the studies in the historic control database with respect to those factors that are known to influence tumour occurrence (see paragraph 3 and 4). And, due to time-related variability of tumour incidences, historical control data should be limited to more recent studies. For that reason, windows of 3-5 years are proposed [8,11,22,24].
In the literature only few examples were found showing how historical data have been used in the evaluation process. Two examples (see below) are copied from Haseman (1992) [24] and are based on the NTP technical Reports. The other two examples are based on the RITA database and are copied from Deschl et al. (2002) [8]. One should be aware that these are only examples without giving definite rules of how to interpret the results of a study.

Example 1: rare tumour
In the kidney of female F344 rats a striking increase in transitional cell hyperplasia of the renal pelvis was observed (control 7/50; low dose 20/50; high dose 37/50). However, only two transitional cell tumours (carcinomas) were found in the high dose group, which was not

statistically significant. The historical control value was 0%. Because of the rarity of the tumour (and the supporting preneoplastic response), the two tumours were considered to be chemically related [24].
Example 2: rare tumour
In the case of transitional cell papillomas of the urinary bladder in female Wistar rats, there was an increase seen in a long-term rodent bioassay. In the high dose females only, 5 (10%) transitional cell papillomas were observed versus 0 in control, low and mid dose groups. The historical control data of the RITA database clearly showed that there are very low tumour incidences: total Wistar database 0.2% (range 0-2.2%), breeder Wistar database 0.4% (range 0-2.1%) and company Wistar database 0%. Since there was no overlap in the incidences observed in the database and in the carcinogenicity study, the company considered the tumour response to be treatment related [8].
Example 3: aberrant control value
A highly significant increase in adrenal medullary pheochromocytomas (control 2/50; low dose 9/50; high dose 12/50) was found in male rats. Medullary hyperplasia was marginally elevated: 1/50; 6/50; 3/50). However, historical control data indicated that the background rate in male F344 rats was 18% throughout the NTP program, and that in the only 2 previous studies at the contract laboratory, the control rates were 12/50 and 13/50. It seems clear that the statistical significance was a result of the concurrent control being unusually low rather than the dosed groups being unusually high. Thus the effect was only considered to be equivocal [24].
Example 4: aberrant control value
In a case of significantly increased incidences of pituitary adenoma of the pars distalis in female Wistar rats, historical control data of the RITA database have been applied. The tumour incidences were 28, 44, 40 and 48% respectively for the control, low, mid and high dose females. The incidence of the control group were compared to the total RITA data base (55 studies) on female Wistar rats, the data base of the specific commercial breeder of this Wistar strain (16 studies) and the database of the company/laboratory for that specific strain (11 studies). The mean incidence of pituitary gland adenoma and within brackets the range for these 3 databases were 55.1% (15-80%), 42.9% (31.2-62.0%), and 44.4% (32.7-62.0%), respectively. In addition, there was no increase in adenomas of the pars distalis in the pituitary gland in males and there was also no increase in hyperplasias of the pars distalis in either sex. Therefore the positive trend seen in this study was due to a distinctively low concurrent control incidence [8].

To date, many statistical procedures have been proposed for incorporating historical control data into a statistical analysis of possible chemically related carcinogenic effects. Any statistical methodology that uses historical control data should take into account study-to-study variability in tumour incidence and survival differences. Various techniques has been proposed for this purpose [11,31,32,33], but no method has been universally accepted as best. Such procedures may be most valuable for rare tumours because the relatively infrequent tumour occurrences minimize the problems associated with study-to-study variability [11]. Thus, if historical control data are to be utilized in a formal testing framework, care must be taken to ensure that studies in the database are comparable to the concurrent control group: all potentially confounding factors noted above (paragraphs 3 and 4) must be considered. In practice, evaluation of the influence of these various factors will be difficult, and thus the value of historical control data may be limited [24].
However, historical control data from the more recent databases like NACAD and RITA provide useful data since these databases make use of standardized study designs, standardized histopathological methods and standardized nomenclature. With the availability of these kind of standardized databases, it will become more easy to use them in the assessments.

2.6 Opinions of (inter)national bodies

International bodies who deal with evaluations of chronic/carcinogenicity data like IARC [34], WHO/IPCS [35,36], and USA [5,22,30] subscribe the view that the most appropriate control group in long-term bioassays is the concurrent control. This concurrent control group should be compared with the experimental groups. And, when there is increased occurrence of rare or marginally increased tumour incidences in treated groups compared to concurrent controls, historical control data may provide additional information for carcinogenic risk evaluation. Recently the OECD has published a 'Guidance notes for analysis and evaluation of chronic toxicity and carcinogenicity studies' [37], which was established as part of the OECD project to develop harmonized guidance on the preparation of data reviews for toxicity studies. In paragraphs 3.1, 5.2 and 5.7 of the OECD document attention is given to the historical control data. As such this document gives the harmonized views of all (inter)national bodies on the use of historical control data in relation to chronic toxicity/carcinogenicity studies (see Annex 1).

2.7 RIVM/SIR strategy

As mentioned by the Office of Science and Technology [5]: Rigid rules or definitions of how to interpret the results of a carcinogenicity study are not very useful. The ultimate decision must be based on the knowledge of experienced pathologists and toxicologists, the weight of corroborative evidence and careful statistical evaluation.

The RIVM strategy of how to use historical control data in the evaluation of carcinogenicity data is largely based on the Guidance notes of the OECD [37] (see chapter 6).
The main points are highlighted:

- Historic control data may be used in 3 primary ways:
  1) To identify aberrant control values;
  2) To evaluate the relevance of low-incidence findings (e.g. rare tumours);
  3) To evaluate the relevance of marginal increases in tumour incidences, especially for tumours with high background values.
- Historical control data should be from the same species, strain, age, sex, and laboratory, and should come from contemporary control animals.
- Historic control data should only include studies conducted within an appropriate time period on either side of the study under review (ideally 2-3 years at either site), with identification of study methodology (e.g. presampling conditions such as fasting or nonfasting, assay methodology for study parameters, histopathological criteria for lesion identification, time of terminal sacrifice, control treatment etc) that could have affected the results.
- If the authors of a report rely on historical control data in their interpretation of effects these should be provided together with the information necessary to assess their quality
- Literature values for normal ranges which do not specify the method by which they are obtained should not be used!

As a final remark:
The use of historical control data should be viewed as a tool for understanding better the events or apparent differences observed within a study. They should not be seen as a convenient device for discounting unwanted or difficult findings [29].

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Annex 1. 'Guidance notes for analysis and evaluation of chronic toxicity and carcinogenicity studies'5

Paragraphs 1- 4 are text blocks copied from the above mentioned document which relate to historical control data:

1 (section 3.1 in OECD document):
'Ideally, all historical control data submitted for consideration are obtained from the laboratory at which the study being assessed was carried out, and relate to animals of the same strain, age and sex, and obtained from the same supplier, as those used in the study. They should come only from studies conducted within five years, or two to three years either side, of the study under review. Any study methodology that could have affected the results should be identified. Relevant parameters include pre-sampling conditions such as fasting or non-fasting, haematology and clinical chemistry assay methods, histopathological criteria for lesion identification, and time of terminal sacrifice. European requirements for submission of historical control data are fully described in Section 5.5, Annex II, of Directive 91/414/EEC: Plant Protection Products. Where historical data are used in an assessment, they should be clearly identified (see Section 5.2)'.

2 (section 5.2 point 13 in OECD document):
'It may be necessary to compare results from the treated and control groups with historical control means or ranges. This can be particularly important in judging the biological significance of rare or unusual tumours and non-neoplastic abnormalities, and in cases where the concurrent control group displayed results that were atypical for untreated animals of the same strain, age, etc. It should be borne in mind that among the factors that influence the reported incidence of spontaneous lesions are diet, genetic background, housing conditions, age, and the techniques used to prepare and examine biological tissue. Even though adoption of harmonised GLP and test guidelines should be reducing the differences between laboratories, it remains highly desirable to use historical control data generated with the same strain of the test species as used in the study under review, at the same study laboratory, and no more than two to three years before or after the study under comparison. (The US FDA permits a five-year span within which the study under evaluation can fall anywhere.) Information should be provided on the source of the historical data and how closely or otherwise it matches the study being evaluated. The EC has formal requirements regarding the submission of historical control data, described in Annex II, V. 5.5 of Directive 91/414/EEC. The relative weight given to concurrent and historical controls will depend on the circumstances, and should be made clear to the reader'.

3 (section 5.7 in OECD document):
'The clearest indication of a positive carcinogenic response is obtained when the incidence of tumours rises above concurrent and historical control levels in both sexes and is higher at higher doses. Further significant observations in treated animals include an increase in rare types of tumour, metastases, reduced latency and the presence of tumours at multiple sites. However, rodent carcinogenicity data may not be so clear-cut. The choice of doses may prevent

[5] OECD. Guidance notes for analysis and evaluation of chronic toxicity and carcinogenicity studies. OECD Environment, Health and Safety publications. Series on testing and assessment no. 35 and series on pesticides no.14. OEDC Environment directorate. Joint Meeting of the chemical committee and the working party on chemicals, pesticides and biotechnology. Paris 2002.

a dose–response relationship or a NOEL for tumorigenicity being established, while some types of tumour are sex limited, particularly those arising from perturbation of endocrine hormone levels. In rodents, gender-related differences in metabolism of the test compound may also lead to sex-related differences in sensitivity to tumours, as in the case of thyroid tumours in rats and liver tumours in rats and mice. The incidence of some types of tumour may also increase with age, or with body weight differences between the treated groups and their concurrent controls (see the example in Subsection 1.2.4 under 'Spontaneous carcinogenesis')'.

4 (section 5.7 in OECD document):
'Although statistical comparisons are of treated animals and concurrent controls, additional insights into the significance of tumours can be obtained from examination of historical control data (Tarone, 1982). Such data can add to an analysis particularly by enabling identification of uncommon types of tumour or the high spontaneous incidence of a tumour in a given animal strain. Caution should be exercised in simply comparing the test group response with the historical range, because the range data ignore differences in the survival of animals among studies and is related to the number of studies in the database (US EPA, 1996). The more studies in the database, the wider the range.
In analysing the results for uncommon tumours in a treated group that are not statistically significant in comparison to concurrent controls, the evaluator can use the experience of historical controls to conclude that the result was in fact unlikely to be due to chance. In the analysis of results for common tumours, a different set of considerations comes into play. Generally speaking, statistically significant increases in tumours should not be discounted simply because incidence rates in treated groups are within the range of historical controls, or because incidence rates in the concurrent controls are somewhat lower than average. Random assignment of animals to groups and proper statistical analysis should have ensured that statistically significant results are unlikely to have arisen by chance alone.
However, caution should be exercised in interpreting results that are barely statistically significant, or in which incidence rates in concurrent controls are unusually low relative to historical controls (US EPA, 1996). As stated previously (Section 5.2, point 13), the most relevant historical data come from the same laboratory as, and from studies that used animals from the same supplier as, and were conducted within two to three years of, the study under review'.

3. Mononuclear Cell Leukaemia in the F344 rat strain

Factsheet FSV-016/00, date 11-04-2005

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Contents

3.1 Introduction and problem definition

he assessment of the carcinogenicity of substances is based on the results of epidemiology and/or chronic animal studies. However, increases of some types of tumours in animal studies are sometimes claimed not to be relevant to humans. The relevancy of other tumour types is questionable. One of the questionable tumour types is mononuclear cell leukaemia (MNCL) in the F344 rat. This type of tumour is also known as large granular lymphocyte leukaemia. A review by Caldwell (1999) is available questioning the relevance of MNCL induction. MNCL is found at high incidence in untreated aged F344 rats, the standard strain for studies of the National Toxicology Program (NTP). The incidence of MNCL increases significantly after chronic treatment with some substances. In this fact sheet, the relevance of an increased incidence of MNCL in F344 rats for humans is discussed, and an approach for human hazard and risk assessment is provided.

3.2 Background information

3.2.1 Pathology

Mononuclear cell leukaemia is a heterogeneous leukaemia seen in aged F344 rats. The tumour cells originate from the population of large granular lymphocytes. Large granular lymphocytes are cytotoxic T lymphocytes and natural killer cells which kill virally infected and tumorigenic cells by secreting cytotoxic proteins from the granules into their target cells. The tumour cells are always found in the spleen, often in the liver and sometimes in other organs. The tumour cells have an erythrophagocytic activity resulting in severe anaemia. The anaemia results in various secondary effects including necrosis of the liver.

An extensive description of the pathology of MNCL in Fischer 344 rats is provided amongst others by Stromberg et al. in three publications (1983a, 1983b and 1983c) and reviewed by IARC (Ward et al., 1990).

3.2.1.1 Clinical signs

The clinical signs were: depression, inactivity, weight loss and enlarged palpable spleen (mostly). Many animals showed pallor of the eyes and external ears and exhibited dyspnoea. Some animals were icteric. Duration of the illness from onset of clinical signs to death ranged from one to two weeks. The duration of illness from circulating neoplastic cells was 5 (Moloney et al., 1970) or 6 weeks. MNCL was the cause of death in a significant proportion in aged F344 rats. The first deaths due to MNCL were found after 12 months and the cumulative incidence increased strongly after approximately 20 months.

3.2.1.2 Haematology

In most F344 rats with MNCL, the total white cell count was markedly increased. Differential white cell counts showed marked neutrophilia, an increase in eosinophils and a moderate decrease in lymphocytes. Furthermore, there were increases in nucleated erythrocytes, reticulocytes and spherocytes and a strong reduction in PCV, RBC and Hb. Platelets were decreased. Erythrocytes showed a strong right shift, indicating an increase in cell volume. Prothrombin times were increased. Erythrophagocytosis by tumour cells was observed commonly in peripheral blood smears. Most tested rats were positive for anti-erythrocyte immunoglobulin G with the direct Coomb's test.

3.2.1.3 Clinical chemistry

MNCL rats showed strong increases in serum bilirubin, aspartate aminotransferase, alanine aminotransferase, serum alkaline phosphatase and lactate dehydrogenase (LDH). The changes in iso-enzyme profile for LDH were consistent with an interpretation of liver damage. Serum lysozyme activity was not elevated (Moloney et al., 1970).
The urine from rats with MNCL contained abundant mucoid proteinaceous material, elevated levels of urine urobilinogen, bilirubin, and haemoglobin but no cells and had an amber colour instead of a light yellow colour.

3.2.1.4 Gross and microscopic pathology

Depending on the stage of MNCL, the spleen was slightly to markedly enlarged (up to 25 fold in weight) and was dark red, pulpy, friable, and oozed blood. By definition, the tumour cells were always seen in the spleen usually diffuse throughout the red pulp sinusoids. A decrease or loss of splenic white pulp was a common finding. A striking and unique feature was the widespread erythrophagocytosis by tumour cells in 41% of the spleens.
The liver can be moderately to markedly enlarged and nodular with variable degenerative changes such as centrilobular hepatocellular degeneration and necrosis associated with the accumulation of neoplastic cells. Sinusoidal infiltration of tumour cells was seen in almost all rats with MNCL. Fewer rats had dense infiltrations with discrete masses of tumour cells.
The presence of tumour cells in other organs was increased in more advanced stages of the disease.

3.2.2 Description of tumour cells

The neoplastic cells were small but pleomorphic and the nucleus was often located eccentrically in the cell. Nuclei were round with either uniform or irregular nuclear membranes, coarsely clumped and marginated chromatin, and a single small nucleolus. Tumour cells

contained a small amount of cytoplasm. Most leukaemic cells but not all contained various numbers of prominent azurophilic cytoplasmatic granules (Ward and Reynolds, 1983). The cytoplasmatic granules stained pink to red or red-purple with Romanovski-staining and red with Wright-Giemsa staining. No granules were visible in haematoxylin and eosin stained fixed tissue sections. The granules also reacted to granule antigens like serine esterase. Ultrastructurally, the cytoplasmatic granules appeared as lysosomes in association with Golgi vesicles.

3.2.2.1 Enzymatic activity of MNCL cells

Histochemically, leukaemic cells were positive for beta-glucuronidase and acid phosphatase. Dieter et al. (1985) found increased enzymatic activity of mononuclear cells isolated from spleen and blood for glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, pyruvate kinase, lactate dehydrogenase, isocitric dehydrogenase and malate dehydrogenase.

3.2.2.2 Other features of MNCL cells

The antigenicity of the MNCL cells was tested for a broad range of markers and compared to other white blood cells (Ward et al., 1990; Stromberg et al., 1983). The results indicate that MNCL originates from large granular lymphocytes (LGL). Aneuploidy and morphological abnormal chromosomes were seen in some studies but only a limited number of MNCL were tested (Losco and Ward, 1984).

3.2.3 Development of tumours

No mechanism is known for the formation of spontaneous and chemically induced MNCL. However, some factors are known to influence the formation of MNCL.

Dietary restriction delayed the onset of MNCL and prolonged the lifetime in F344 rats. However, the proportion of rats developing leukaemia over lifetime was increased in rats with dietary restriction. The increase in MNCL in diet restricted rats was probably caused by the extended mean life span (Shimokawa et al., 1993; Thurman et al., 1994). Dietary restriction also reduced the growth rate of transplanted MNCL cells (Hursting et al., 1993). This seems to be mediated through reductions in the levels of growth hormone (GH) and insulin-like growth factor (IGF).
Comparison of the control groups in the NTP carcinogenicity studies showed that gavage treatment with corn oil significantly reduced the incidence of MNCL in male but not in female F344 rats (Haseman et al., 1985 and 1992) (Table 3-1). Corn oil gavage also inhibited the growth of transplanted MNCL in male F344 rats (Hursting et al., 1994). The corn oil treated rats compensated for the increased energy intake by gavage by a reduced uptake of feed. Serum growth hormone levels were reduced. No effect was seen in females. A dose dependent decrease in MNCL and food uptake was also seen in chronic studies in male F344 rats with three dose levels of corn oil, safflower oil and tricaprylin (NTP, 1994). These results confirm the delay in onset of MNCL by dietary restriction and/or dietary composition.

Moloney et al (1969) studied the effect of gavage exposure to 3-methylcholanthrene (MCA) on female Wistar-Furth rats. Rats were either treated with 80 mg (5-10 mg/day) at 2 months of age or with 180 mg at 4 or 12 months of age. Treatment at 4 or 12 months did not affect the formation of MNCL over lifetime. Treatment at 2 months increased the incidence from 15.8% to 38%. Treatment of Wistar-Furth rats of unspecified sex at 1 month with 40 mg MCA

increased the incidence from 20% to 40% (Moloney et al., 1971). Treatment of F344 with 80 mg at 2 months did not affect the MNCL incidence.
Irradiation of F344 or Wistar-Furth rats with 450 R of X-rays at 2 months of age significantly reduced the life-time incidence of MNCL. Treatment at 12 months did not affect the incidence of MNCL (Moloney et al., 1969 and 1971; Hellman et al., 1982).
Splenectomy at 1 or 2 months of age reduced the incidence of MNCL over lifetime in F344 and Wistar-Furth rats (Moloney and King 1974; Moloney et al., 1971). Splenectomy in Wistar-Furth rats at 2.5 months resulted in only a minor reduction of the development MNCL.
These results indicate that MNCL originates from the spleen and that the process resulting in MNCL starts at an early age.

MNCL can be transplanted into isogenic rats resulting in the development of MNCL (Losco and Ward 1984). Dieter et al. (1989) compared the effect of 5 substances on MNCL rates in the NTP chronic assay with the effect on the rates in a short term assay with transplanted MNCL cell line in male F344 rats. The three chemicals which increased the MNCL rate in the chronic study (pyridine, 2,4,6-trichlorophenol and dichlorovos) also showed an acceleration of the growth rate of the leukaemia transplants. The two chemicals which reduced the MNCL rate in the chronic study (2-ethoxyethanol and 4-hexylresorcinol) also showed a reduction of the growth rate of the leukaemia transplants. This indicates that at least part of the effects of substances on the formation of MNCL is due to promotion of the outgrowth of existing MNCL.

Lijinsky et al. (1993) studied the effect of treatment with alkylating substances on the formation of MNCL in F344 rats. Treatment with these substances increased the incidence of MNCL in female rats compared to control rats dying at the same age but not overall and not in male rats. Treatment with direct acting alkylating agents did not increase the incidence of MNCL over lifetime, probably because most treated rats died from other tumours.

Elwell et al. (1996) identified 20 chemicals with a significant decrease in MNCL in at least one but generally both sexes of F344 rats in the NTP database. Sixteen of these substances contained an amine and showed effects in the spleen including increased haematopoiesis, haemosiderosis and/or fibrosis of the capsule through methaemoglobin formation. The decrease in MNCL for these substances was not associated with decreased body weight or reduced survival. This indicates the importance of the spleen in the formation of MNCL.

Cell free passage of the leukaemia into young Wistar-Furth rats was tested by Moloney et al. (1971), as an indication of the involvement of a virus in the formation of MNCL. No increase in MNCL was found. No oncogenes, detectable by transfection analysis, were found in 3 primary MNCLs (Reynolds et al., 1986).

Infection of F344 with rat parvo virus reduced the severity of the MNCL and delayed the onset in a MNCL transplantation model (Ball-Goodrich et al., 1998).

Conclusion
MNCL originates in the spleen at a young age and requires a long period before clinical effects are seen. The progression of the initial lesion to MNCL is affected by substances, dietary restriction and/or composition, radiation and viruses at least partially through promotion or suppression of the growth of the tumour cells.

3.3 Spontaneous tumour incidence

3.3.1 Fischer 344

The rate of MNCL in control F344 rats was variable and dependent upon several factors including: sex, corn oil treatment, type of diet, duration of the study, diagnostic criteria and period of the study as shown in table 3-1 and by others (Haseman et al., 1989, Rao et al., 1990). Seen the importance of these factors on the MNCL incidence in untreated rats, the incidences shown below should not be used as historic control data. Data from the same laboratory and period using comparable conditions should be used.

Table 3-1: Incidence of MNCL in untreated F344 rats (%)

	Period	Source	Duration	Males	Females	Reference
F344/N	±2000	Japan	lifetime	83.6	57.5	Miyaishi et al., 2000
F344/N	±1984	NTP	33 months	36.1	38.2	Solleveld et al., 1984
F344/N	1974-1981	NTP	26 months	29.7	19.0	Solleveld et al., 1984
F344/N diet	1980-1984	NTP	26 months	48.9±10.3	25.2± 8.1	Haseman and Rao, 1992
F344/N corn oil gavage	1980-1984	NTP	26 months	21.4± 9.0	22.9± 7.7	Haseman and Rao, 1992
F344/N water gavage	1980-1984	NTP	26 months	43.8±12.6	26.2±10.6	Haseman and Rao, 1992
F344/DuCrj	1978-1983	Japan	26 months	7.1 (3.8-12.5)	9.5 (3.8 – 15)	Maita et al., 1987
F344/DuCrj	1975-1981	Japan	26 months	23.6 (13.2-40.4)	21.9 (9.8-29.8)	Maekawa et al., 1983

3.3.2 Wistar-Furth rats

Incidences of MNCL in untreated Wistar-Furth rats over lifetime were reported to be 22% in males and 16% in females (Moloney et al., 1969).

3.3.3 Other rat strains

Overviews of incidence of spontaneous tumours in other rat strains do not always provide information on the incidence of MNCL but mostly on haematopoietic tumours or lymphomas and leukaemia without specification of subtypes. Therefore, these data are used as an upper level for MNCL in these rat strains (Table 3-2). The incidence of granular cell leukaemia in Wistar rats was 1.1% (range 0-2.0%) in males and 1.5% (range 0-3.1%) in females (Poteracki and Walsh, 1998). The incidence of large granular lymphocyte lymphoma in Sprague-Dawley rats was reported to be 0.57% in males and 0.62% in females (Frith, 1988). Abbott et al. (1983) found an incidence of 0.3% for MNCL in aged Sprague-Dawley rats. The 4% leukaemia's and/or lymphoma's in the Donryu rats were all MNCL (Maekawa et al., 1986).

Table 3-2: Combined incidence of lymphomas and leukaemia in other rat strains (%).

	Period	Source	Duration	Males	Females	Reference
Charles-River CD	1982	NTP	26 months	4 (0-12) lymphoreticular	3 (0-6) lymphoreticular	Sher et al., 1982
Sprague-Dawley	1984-1991	Ciba-Geigy	26 months	1.5	0.9	McMartin et al., 1992
Sprague-Dawley	1986-1992	American Cyanamid	26 months	2.0	1.6	Chandra et al., 1992
Donryu	1986	-	28 months	4	4	Maekawa et al., 1986
Wistar	1990-1995	-	26 months	2.4	3.9	Poteracki and Walsh, 1998
Wistar	-1993	Bayer	26 months	1.8	0.8	Bomhard and Rinke, 1994
Wistar	1980-1990	-	26 months	6.0	5.1	Walsh and Poteracki, 1994
Wistar	1979-1987	Bayer	32 months	0.7	1.1	Bomhard, 1992
Wistar	1981	RIVM	31 months	7	4	Kroes et al., 1981

3.3.4 Other species

Haematopoietic tumours in control B6C3F1 mice are mainly malignant lymphoma (male: 7.3% range: 2-20%, female: 20.4% range: 6-44%) and histiocytic sarcoma (0.7% range: 0-6%, female: 2.2% range: 0-10%) (Haseman et al., 1998). The incidence of leukaemia is very low (1 case in 48 studies).
Approximately 30 cases of large granular lymphocyte leukaemia in the dog are described (Ghernati et al., 2000, Helfand et al., 1995, Wellman et al., 1989, McDonough and Moore, 2000). Cases included mainly T-cell but also NK-cell lymphocytosis. A specific antigen present in a high number of cases indicated the splenic red pulp as the origin of the LGL lymphocytosis.
Darbes et al. (1998) described 6 cases of large granular lymphocyte leukaemia/lymphoma in the cat. There was no indication of anaemia in the 3 cats for which a haematological results were available.

3.3.5 Humans

Proliferations of large granular lymphocytes are divided into four distinct entities in a review by Lamy and Loughran (2003): reactive/transient LGL expansion, chronic LGL lymphocytosis, classical indolent LGL leukaemia and aggressive LGL leukaemia. The classification of cases as LGL leukaemia is still difficult but is based on the number of LGL, duration and the clonality. Since the first description of LGL leukaemia in 1985, many studies have been published reporting more than 400 cases. The LGL leukaemia's are divided in either CD3+/T-lineage (>85%) and CD3-/NK lineage (<15%) with different features.

3.3.5.1 CD3+ T-LGL leukaemia

This disease is mainly seen in elderly (median age 60 years with range 4-88) and about one third are asymptomatic at diagnosis. Initial manifestations are mainly related to neutropenia and include fever with recurrent bacterial infections (20-40%). Other features are splenomegaly (20-50%), hepatomegaly (10-20%) and B symptoms (fever, night sweats and weight loss, 20-30%). The haematological features are an increase in LGL in most patients and lymphocytosis (60%) and a decrease in neutrophils (80%), thrombocytes (20%) and haemoglobin (50%). Some patients have no increase in LGL counts but a clonal disorder. The

majority of patients have bone marrow infiltration (69-85%). Infiltration of the sinusoids and portals of the liver and of the red pulp of the spleen is also seen. Several serological factors are correlated with this leukaemia including increases of soluble Fas-ligand (an apoptosis factor). The leukaemias vary in immunological and molecular findings. Clonal expansion is shown in most cases, however, some cases were shown to arise from two separate clones. Several autoimmune diseases are associated with this form of leukaemia including rheumatoid arthritis. Twelve percent of the cases were positive in the direct Coombs test.

3.3.5.2 CD3- NK LGL leukaemia

This form is more aggressive, patients are younger (median age 39 years) and have B symptoms and organomegaly. The majority have massive bone marrow infiltration. Neutropenia is moderate and anaemia and thrombocytopenia are more frequent and severe than in CD3+ cases. It is associated with infection with the Epstein-Barr virus. Outcome is generally poor as most patients die within 2 months after diagnosis.

3.3.6 Conclusion

MNCL as such is only seen at high incidence in F344 and Wistar-Furth rats. In other rat strains, other species and humans, LGL leukaemia or MNCL is seen at much lower incidences. Furthermore, there are differences in the descriptions of these tumours between the F344 rat and the other species. A clear difference is the neutropenia in humans compared to the neutrophilia in F344 rats. Another difference is the absence of reports on erythrophagocytoses in human cases of LGL leukaemia. However, LGL leukaemias in both F344 rats and humans are heterogeneous and full resemblance of tumour types between species should not be expected.

3.4 Substances affecting the rates of MNCL

The current NTP database (2004) contains 59 chemicals causing tumours in the haematopoietic system. Equivocal to clear increases in MNCL were seen in 30 studies with F344 rats. Only 2 out of 24 F344 studies with an increase in MNCL also showed an increase in tumours in the haematopoietic system in the corresponding study in B6C3F1 mice. The haematopoietic tumours seen in the B6C3F1 mice were described as combined lymphocytic, histiocytic and mixed malignant lymphomas for allyl isovalerate and histiocytic sarcomas (all organs) for tetrafluorethylene. Further, ethylene oxide increases MNCL in F344 after inhalatory exposure but not in a diet study in Sprague-Dawley rats. Inhalatory exposure to B6C3F1 mice induces malignant lymphomas. There was limited evidence in humans and the most frequently reported association has been with lymphatic and haematopoietic cancer (IARC, 1994).

Several of the substances inducing MNCL in NTP studies were also tested in F344 rats in additional studies or in other rat strains using comparable dose-ranges. This was used to determine whether the increase in MNCL is repeatable in F344 (Table 3-3) and in other rat strains (Table 3-4). Also comparisons from non-NTP studies are included.

Table 3-3: Substances with a second study in F344 rats.

Substance	NTP study	Effect on MNCL	Reference other study	Effect on MNCL
Mirex	TR-313, 1990	Increased in females	TR-313, 1990	Increased in females
Acetaminophen (paracetamol)	TR-394, 1993	Increased in females	IARC, 1990	None (F344/DuCrj)
Dichlorvos	TR-342, 1989	Increased in males	Bremmer et al., 1988	None
Hydroquinone	TR-366, 1989	Increased in females	Whysner et al., 1995	None
Butyl benzyl phthalate	TR-213, 1982	Increased in females Males not tested	TR-458, 1997 TR-460, 1997	None None
Di-2-ethylhexyl phthalate	Moore et al., 1997 as cited in Caldwell, 1999	Increased in males	TR-217, 1982	None
Diisononyl phthalate	Lington et al., 1997, as cited in Caldwell, 1999	Increased in males and females	Butala et al., 1996, as cited in Caldwell, 1999	Increased in males and females
Olestra	Wood et al., 1991	Increased in males	Wood et al., 1991	None
Ethylene oxide	IARC, 1994	Increased in males and females	IARC, 1994	Increased in males, females not tested

Table 3-4: Substances with a second study in another rat strain.

Substance	NTP study	Effect on MNCL in F344	Other strain	Effect on MNCL or haematopoietic tumours	Reference other study
Mirex	TR-313, 1990	Increased in females	CD	None	IARC, 1979
Acetaminophen	TR-394, 1993	Increased in females	Sprague-Dawley Leeds inbred	None None	IARC, 1990
Dichlorvos	TR-342, 1989	Increased in males	Osborn-Mendel CD	None None	TR-10, 1977 Bremmer et al., 1988
Ethylene oxide	IARC, 1994	Increased in males and females	Sprague-Dawley	None	IARC, 1994
Tetrachloro-ethylene	ECETOC 37, 1990	Increased in males and females	Sprague-Dawley Osborn-Mendel Sprague-Dawley	None None None	ECETOC 37, 1990

3.4.1 Conclusion

Chemically induced increases in MNCL in F344 rats were mostly (6/9) not replicated in a second study in F344 rats, and never in studies in other rat strains or in B6C3F1 mice.

3.5 Opinions of other organisations

No formal opinion on the relevance of MNCL in F344 rats is available from organisations like IARC, EU or the EPA. Looking at the assessments for hazard and risk evaluation for some chemicals with evidence of increased MNCL suggest that in general within the EU and IARC increases in MNCL in F344 were not considered relevant for humans (EU risk assessment reports for existing substances and classification considerations for diisononylphthalate and di-

(ethylhexyl)phthalate) or not sufficient for classification (IARC monographs on: ampicillin (vol. 50, 1990) and allyl isovalerate (vol. 71, 1999 and vol. 36, 1985).

3.6 Assessment and RIVM/SIR strategy

The RIVM strategy is based on the following considerations:

- MNCL is a heterogeneous tumour occurring spontaneously at a high rate in aged F344 rats.
- In other rat strains and other species including humans, also tumours are seen originating from LGL cells. However, those tumours are probably not identical and are seen but at much lower rates.
- The incidence in F344 rats can be increased by treatment with certain substances but for 6 out of 9 substances this effect could not be reproduced in a second study in F344 rats and was never confirmed in another rat strain or in mice.
- The mechanism for the induction of MNCL in F344 is unknown but it was shown that several substances increasing MNCL in chronic studies have a growth stimulating effect on MNCL cells. This indicates that increases in MNCL are at least partly caused by stimulation of proliferation of existing MNCL.

Based on the forgoing, the RIVM/SIR strategy is as follows. Substance induced increases in MNCL in F344 rats are considered not relevant as an indication for carcinogenicity in humans. Increases of MNCL in other rat strains and other species however, are considered to be relevant to humans.

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4. Energy and moisture content and assimilation efficiency of bird and mammal food

Factsheet FSM-009/00, date 20-12-2004

Author: C.E. Smit

Contents

4.1 Introduction

The risk assessment for birds and mammals as performed within the framework of EU Directive 91/414/EC is based on a comparison of the estimated daily uptake of a pesticide with the toxic dose for that compound. The principles of the risk assessment are laid down in a guidance document (EC, 2002). The main exposure route is assumed to be ingestion of food items containing spray residues. Additional exposure may take place by secondary poisoning via eating of contaminated earthworms or fish.
For the exposure via sprayed food, the daily pesticide uptake for a given species is determined by the daily intake of a specific food type (Food Intake Rate, FIR), the concentration of the pesticide in that food, the fraction of the diet that is contaminated, the fraction of a specific food type in the total diet and the potential to avoid contaminated food. The FIR is equal to the daily energy expenditure divided by the energy content of the food with a correction for assimilation efficiency expressed per day. For the standard risk assessment, four typical bird and mammal species are distinguished, in combination with four different food types. In order to establish the FIR for these four indicator species, data on DEE, energy and moisture content and assimilation efficiency have been collected at the Central Science Laboratory in York, United Kingdom (Crocker et al., 2002).
For caloric values, the CSL dataset contains about 2000 data, which are grouped into 15 different food categories. A summary of the data as presented in the CSL report is given in Table 4-1.

Table 4-1: Energy and moisture content of several food sources (Crocker et al., 2002).

Group	Energy content [kJ/g DW]	Moisture content [%]
Dicot crop leaves	11.2	88.6
Grasses and cereal shoots	18.0	76.4
Non-grass herbs	18.0	82.1
Tree leaves	20.7	51.4
Orchard topfruit	11.6	83.7
Cereal seeds	16.7	13.3
Weed seeds	21.0	11.9
Small mammals	21.7	68.6
Bird and mammal carrion	22.6	68.8
Arthropods	21.9	70.5
Caterpillars	21.7	79.4
Soil invertebrates (earthworms and slugs)	19.3	84.6
Fish	20.7	71.1
Aquatic invertebrates	19.6	77.3
Aquatic vegetation	15.0	81.4

For assimilation efficiency of *birds*, the average data as presented by Bairlein (1998) are used by CSL. For assimilation efficiency of *mammals*, the dataset contains 91 individual records. Resulting values are given in Tables 4-2 and 4-3 below.

Table 4-2: Assimilation efficiency [%] of different food types for birds (Crocker et al., 2002; taken from Bairlein, 1998)

Bird order	Representatives	Food type						n	n
		animals	fruits	herbage	seeds	sugars	artificial	species	cases
Struthioniformes	Ostriches			36				2	6
Gruiformes	Cranes, coots, rails	34	45	59			69	1	5
Ralliformes	Coots, rails							1	1
Charadriiformes	Gulls, waders	69					74	7	19
Lariformes	Gulls, terns	79						1	3
Alciformes	Auks	76						1	2
Sphenisiciformes	Penguins	75						7	26
Procellariformes	Petrels	87						2	3
Pelecaniformes	Pelicans, gannets, cormorants	80	76					4	8
Columbiformes	Pigeons						76	4	36
Psittaciiformes	Parrots					96		1	4
Strigiformes	Owls	77						6	45
Falconiformes	Eagles, falcons	84						4	12
Accipitriformes	Hawks	82						11	22
Ciconiiformes	Herons, storks	80						4	8
Anseriformes	Ducks, geese	87		41	83		74	22	98
Galliformes	Fowl	70	57	42	65		67	18	184
Opisthocomiformes	Hoatzin (S. America)						74	1	2
Trochiliformes	Hummingbirds					98		7	16
Coliiformes	Mousebirds (Africa)		56				73	4	14
Piciformes	Woodpeckers	64		61			80	1	14
Passerriformes	Passerines	76	67	76	80	09	72	67	441

Table 4-3: Assimilation efficiency [%] of different food types for mammals (Crocker et al., 2002)

Mammal species	Food type	mean	SD	n
shrews and bats	insects	88	5.9	8
carnivores	vertebrates	85	5.8	16
squirrels	nuts	85	7.5	10
small mammals	seeds and nuts	83	8.5	11
small mammals	grasses	46	10.7	15
small mammals	crops, forbs, mixed vegetation	74	12.3	17
lagomorphs	general vegetation	74	13.5	4
white tailed deer	tree tissue	32	8.4	7
ruminants	hay and browse	80	2.8	3

About 10 years ago, a similar dataset has been established at the RIVM to be used in a food chain model for birds and mammals. The data were published in an RIVM report (Jongbloed et al., 1994) and referred to by Traas et al. (1996). The purpose of the current project was to

combine both datasets to obtain a more complete database which in the future can be used to refine the existing exposure scenarios and to establish scenarios for new indicator species.

4.2 Methods

4.2.1 Caloric values, moisture and ash content

4.2.1.1 Data arrangement

A first comparison the two datasets with respect to caloric value and moisture content indicated that there was little overlap in literature sources. This can be explained by the fact that these data are often published as part of a different type of research, and are thus not found with a keyword based literature search.
Both datasets were available as Excel-spreadsheets in which data for different organism groups were ordered, but not to the same taxonomic level. After combining both files, data were therefore first sorted by scientific species names. Obvious duplicates with the same literature reference were removed. For suspected duplicates, those values that were (nearly) the same but originated from different sources, the original reference was retrieved where possible and the numbers were checked. It appeared that a number of references in both the CSL and the RIVM file were review papers and in addition, various data originated from different papers by the same author(s). Duplicate values could therefore often attributed to citations or self-citations. After removal of duplicates, taxonomic position of the species was checked and/or completed using the Integrated Taxonomic Information System on-line database, http://www.itis.usda.gov, an internet database containing authoritative taxonomic information on plants, animals, fungi and microbes. Data were then ordered into the following main groups: fungi, annelids, molluscs, fish, arthropods, seeds, tree and plant tissue, fruit, birds, mammals, fodder and other. Additional information on life form or habitat was also obtained from the internet.

4.2.1.2 Data treatment and statistical methods

The CSL dataset contained information on caloric content on a dry weight basis (kJ/g DW) and % moisture, the RIVM dataset has additional values for caloric content on the basis of fresh weight (kJ/g FW) and ash-free dry weight (kJ/g AFDW), and for % ash content. After re-arranging the dataset, missing variables were calculated from the other parameters where possible, if kJ/g DW and % ash were available, kJ/g AFDW was calculated, kJ/g FW was calculated from kJ/g DW and % moisture and so on.
Data within each main group were subdivided on the basis of taxonomic level, habitat and/or life stage or because it is anticipated that birds or mammals forage on a specific type of food. Statistical analyses were performed with GraphPad Prism 4.0. Significant differences in caloric content between sub-groups were identified using the dry weight data, because this parameter had the highest number of observations and the smallest variation within subgroups. In case of a comparison between two sub-groups, an unpaired two-sided t-test was used, three or more groups were compared using one-way ANOVA with Tukey's multiple comparison of means. Non-parametric variants were used in case data were not normally distributed and/or variances were not homogeneous. P was 0.05 in all cases.

4.2.2 Assimilation efficiency

4.2.2.1 Birds

For assimilation efficiency of *birds*, Dr. Franz Bairlein of the Institute of Avian Research in Wilhelmshaven, Germany, kindly supplied the underlying data on which the CSL overview was based. It appeared that this dataset, with over 1200 entries, completely covers the RIVM data. This means that for birds, the values as presented in the CSL report (see Table 4-2) remain unchanged.

4.2.2.2 Mammals

For assimilation efficiency of *mammals*, the CSL and RIVM database relied partly on different literature sources. A comparable strategy as presented above was followed, except that food was not classified to the species level but only sorted by category. The following food types were distinguished: fodder, vertebrates, insects, nuts and seeds, grasses, non-grass herbs/crops and mixed plants, and tree tissue. The different food sources were compared taking all mammals together. Where possible, differences between mammal groups for one type of food were analysed.

4.3 Results

4.3.1 Caloric values, moisture and ash content

For each of the main groups, the average, standard deviation, minimum and maximum and number of observations for the respective parameters are given in the summary tables below. The Coefficient of Variation (CV) is the standard deviation expressed as percentage of the mean (= [SD/mean] x 100 %). The results of the statistical analysis of the kJ/g DW data are given in separate tables.

4.3.1.1 Annelids

The annelids were divided into terrestrial and aquatic species. No further division in life stage or habitat (freshwater or marine) was made because too few data were available. Terrestrial and aquatic annelids did not significantly differ in caloric content (two-sided t-test, $P > 0.05$).

Table 4-4: Caloric values, moisture and ash content of annelids

Parameter	Subgroup	mean	SD	CV [%]	min	max	n
kJ/g FW	all values	3.1	1.5	49	0.6	9.4	31
	terrestrial	3.2	0.3	8	2.7	3.5	8
	aquatic	3.1	1.8	57	0.6	9.4	23
kJ/g DW	all values	18.7	4.7	25	8.9	31.5	48
	terrestrial	18.7	3.0	16	13.0	22.2	16
	aquatic	18.6	5.4	29	8.9	31.5	32
kJ/g AFDW	all values	21.6	2.8	13	19.7	23.6	2
	terrestrial	23.6	-	-	-	-	1
	aquatic	19.7	-	-	-	-	1
% H_2O	all values	82.8	5.9	7	62.0	97.6	31
	terrestrial	83.3	1.4	2	80.0	85.0	10
	aquatic	82.5	7.2	9	62.0	97.6	21
% ash	all values	0.8	-	-	-	-	1
	terrestrial	0.8	-	-	-	-	1
	aquatic	-	-	-	-	-	

4.3.1.2 Molluscs

A sub-division was made between terrestrial gastropods and aquatic gastropods, bivalves and cephalopods. Only few data were available for the latter group and they were not included in the statistical analysis. There was a significant difference in caloric content between terrestrial and aquatic gastropods and between aquatic gastropods and bivalves. Bivalves and terrestrial gastropods did not significantly differ, and terrestrial gastropods were not significantly different from terrestrial annelids (see above).

Table 4-5: Caloric values, moisture and ash content of molluscs

Parameter	Subgroup	mean	SD	CV [%]	min	max	n
kJ/g FW	all values	2.2	1.5	69	0.6	6.9	68
	terrestrial gastropods	-	-	-	-	-	-
	aquatic gastropods	2.1	1.6	73	0.8	6.9	34
	bivalves	1.9	1.1	61	0.6	4.9	29
	cephalopods	4.7	0.8	17	3.6	5.6	5
kJ/g DW	all values	18.2	3.6	20	3.8	27.7	95
	terrestrial gastropods	20.0	1.3	7	17.2	21.9	9
	aquatic gastropods	16.8	4.2	25	3.8	27.7	49
	bivalves	19.3	2.0	10	14.3	25.5	35
	cephalopods	23.8	0.4	2	23.5	24.0	2
kJ/g AFDW	all values	25.2	7.5	30	14.6	54.6	24
	terrestrial gastropods	22.5	2.6	12	19.8	25.0	3
	aquatic gastropods	26.8	7.9	30	20.7	54.6	18
	bivalves	18.6	3.5	19	14.6	21.3	3
	cephalopods	-	-	-	-	-	-
% H_2O	all values	86.8	10.7	12	42.8	96.9	77
	terrestrial gastropods	85.7	3.2	4	80.2	90.6	17
	aquatic gastropods	84.5	14.5	17	42.8	96.0	34
	bivalves	91.7	5.5	6	75.6	96.9	24
	cephalopods	78.9	4.0	5	76.0	81.7	2
% ash	all values	35.0	19.5	56	1.0	74.6	21
	terrestrial	22.8	-	-	-	-	1
	aquatic	37.3	20.0	54	1.0	74.6	18
	bivalves	19.8	10.7	54	12.2	27.3	2
	cephalopods	-	-	-	-	-	-

Table 4-6: Comparison of mean caloric content (kJ/g DW) for molluscs

	all values	terrestrial gastropods	aquatic gastropods	bivalves	cephalopods
all values					
terrestrial gastropods			*	n.s.	
aquatic gastropods		*		**	
bivalves		n.s.	**		
cephalopods					

**	significant, one-way ANOVA with Tukey's, P < 0.01
*	significant, one-way ANOVA with Tukey's, P < 0.05
n.s.	not significant, one-way ANOVA
	not tested

4.3.1.3 Arthropods

The arthropods were divided into aquatic and terrestrial species, and for each group a subdivision was made between larvae or sub-adults on the one hand, and adults, mixed or non-

specified life-stages on the other hand. The aquatic species were also divided into marine and freshwater species. It should be noted that for most of the freshwater species only the larval stage is truly aquatic. The adults often have a wet habitat, but do not actually live in the water. Caloric values are presented in Table 4-7, moisture and ash-content in Table 4-8.

Table 4-7: Caloric values of arthropods

Parameter	Subgroup	mean	SD	CV [%]	min	max	n
kJ/g FW	all values	5.6	2.8	50	0.9	22.5	166
	aquatic and terrestrial, larvae	6.3	3.2	51	1.9	14.7	28
	aquatic and terrestrial, adults	5.4	2.7	50	0.9	22.5	138
	aquatic, freshwater and marine	4.9	2.6	52	0.9	22.5	113
	aquatic, freshwater	5.1	2.7	53	0.9	22.5	98
	aquatic, freshwater, larvae	3.6	0.8	22	2.9	4.7	4
	aquatic, freshwater, adults	5.1	2.7	53	0.9	22.5	94
	aquatic, marine, adults	4.0	1.3	32	1.6	5.5	14
	terrestrial	7.0	2.7	39	1.9	14.7	53
	terrestrial, larvae	6.8	3.2	47	1.9	14.7	23
	terrestrial, adults	7.1	2.4	34	3.1	14.0	30
kJ/g DW	all values	21.7	3.8	17	7.4	31.0	582
	aquatic and terrestrial, larvae	22.4	3.2	14	10.3	31.0	185
	aquatic and terrestrial, adults	21.4	4.0	19	7.4	30.9	397
	aquatic, freshwater and marine	20.1	4.3	21	7.4	29.2	232
	aquatic, freshwater	20.9	3.5	17	9.0	29.2	202
	aquatic, freshwater, larvae	20.9	3.7	18	10.3	29.2	49
	aquatic, freshwater, adults	20.9	3.5	17	9.0	28.0	153
	aquatic, marine, adults	15.3	5.5	36	7.4	25.2	29
	terrestrial	22.7	3.0	13	10.3	31.0	350
	terrestrial, larvae	23.0	2.8	12	11.8	31.0	135
	terrestrial, adults	22.6	3.2	14	10.3	30.9	215
kJ/g AFDW	all values	23.7	2.5	10	16.0	31.6	257
	aquatic and terrestrial, larvae	23.5	2.1	9	18.3	29.8	80
	aquatic and terrestrial, adults	23.7	2.6	11	16.0	31.6	177
	aquatic, freshwater and marine	22.9	2.6	12	16.0	31.1	118
	aquatic, freshwater	23.0	2.7	12	16.0	31.1	110
	aquatic, freshwater, larvae	23.3	2.4	10	19.1	29.8	34
	aquatic, freshwater, adults	22.8	2.8	12	16.0	31.1	76
	aquatic, marine, adults	21.6	1.9	9	19.1	24.4	8
	terrestrial	24.4	2.1	9	18.3	31.6	139
	terrestrial, larvae	23.7	1.9	8	18.3	29.2	46
	terrestrial, adults	24.7	2.1	9	19.2	31.6	93

The relatively low dry weight based value for marine arthropods (15.3 kJ/g DW) is caused by the inclusion of crabs in this dataset, which all had a lower energy content as compared to the other groups (mainly shrimps). The most probable explanation for this is that the exoskeleton was included in the analysis. The dataset for ash free dry weight energy content only contained shrimps and the resulting mean value is comparable with that of the other arthropod groups.

Table 4-8: Moisture and ash content of arthropods

Parameter	Subgroup	mean	SD	CV [%]	min	max	n
% H_2O	all values	71.5	9.4	13	38.1	96.0	265
	aquatic and terrestrial, larvae	72.7	10.0	14	46.6	92.0	57
	aquatic and terrestrial, adults	71.1	9.2	13	38.1	96.0	206
	aquatic, freshwater and marine	75.9	8.8	12	38.1	96.0	99
	aquatic, freshwater	76.3	8.0	10	61.0	96.0	83
	aquatic, freshwater, larvae	79.9	8.3	10	74.0	85.8	2
	aquatic, freshwater, adults	76.2	8.0	11	61.0	96.0	81
	aquatic, marine, adults	74.0	12.7	17	38.1	89.8	15
	terrestrial	68.8	8.7	13	44.2	92.0	166
	terrestrial, larvae	72.4	10.1	14	46.6	92.0	54
	terrestrial, adults	67.0	7.4	11	44.2	82.8	110
% ash	all values	7.6	9.4	124	0.0	56.0	219
	aquatic and terrestrial, larvae	8.8	10.1	114	0.0	48.0	65
	aquatic and terrestrial, adults	7.1	9.1	129	0.1	56.0	154
	aquatic, freshwater and marine	11.9	11.1	93	0.0	56.0	96
	aquatic, freshwater	11.1	10.4	94	0.0	48.0	88
	aquatic, freshwater, larvae	13.7	12.8	93	0.0	48.0	31
	aquatic, freshwater, adults	9.6	8.5	89	0.8	31.7	57
	aquatic, marine, adults	21.0	15.0	71	7.0	56.0	8
	terrestrial	4.2	6.1	145	0.1	55.0	123
	terrestrial, larvae	4.4	2.2	50	0.5	8.9	34
	terrestrial, adults	4.2	7.1	169	0.1	55.0	89

There was no significant difference in caloric content of adults and larvae within the terrestrial and freshwater groups, the marine group contained only one value for the larval stage. There was a significant difference between the caloric content of marine and freshwater adults, the same was found for the grouped means of freshwater and terrestrial arthropods (Table 4-9).

Table 4-9: Comparison of mean caloric content (kJ/g DW) for arthropods

	aq. + terr. all	aq. + terr. larvae	aq. + terr. adults	freshwater, all	freshwater, larvae	freshwater, adults	marine, adults	terrestrial, all	terrestrial, larvae	terrestrial, adults
aquatic + terrestrial, all										
aquatic + terrestrial, larvae			n.s.							
aquatic + terrestrial, adults		n.s.								
freshwater, all								***		
freshwater, larvae						n.s.				
freshwater, adults					n.s.		***			
marine, adults						***				
terrestrial, all				***						
terrestrial, larvae										n.s.
terrestrial, adults									n.s.	

***	significant, t-test, P < 0.001
n.s.	not significant, t-test, P > 0.05
	not tested

4.3.1.4 Tree and plant tissue

Tree and plant tissue data were divided on the basis of life form (trees or plants) and taxonomy (*Poaceae* and other plants) and for plants, a subdivision was made on the basis of the plant parts analysed. Caloric content of various plant parts was not significantly different, as was the case for the difference between cereals and other grasses. Caloric content is given in Table 4-10, moisture and ash content of tree and plant tissue is given in Table 4-11.

Table 4-10: Caloric values of tree and plant tissue

Parameter	Subgroup	mean	SD	CV [%]	min	max	n
kJ/g FW	tree tissue	9.9	0.8	8	9.0	11.1	5
	conifer needles	9.5	0.0	0	9.5	9.6	2
	crop leaves (incl pods)	1.1	0.6	53	0.5	2.3	20
	cereals and grasses	3.9	1.7	43	2.3	6.1	6
	cereals	2.4	0.1	4	2.3	2.4	2
	other grasses	4.	1.6	34	2.5	6.1	4
	plants, all values	1.9	1.1	56	0.8	3.5	8
	plants, leaves	-	-	-	-	-	-
	plants, roots	1.9	1.0	55	0.8	3.0	4
	plants, stems and branches	-	-	-	-	-	-
	plants, miscellaneous	2.6	1.1	41	1.2	3.5	4
kJ/g DW	tree tissue	20.2	0.9	4	18.9	21.9	16
	conifer needles	21.2	0.8	4	20.0	22.3	13
	crop leaves (incl pods)	11.4	3.0	26	6.3	16.7	21
	cereals and grasses	17.6	1.5	8	12.7	20.9	68
	cereals	16.9	2.1	13	12.7	19.6	11
	other grasses	17.8	1.3	7	13.5	20.9	57
	plants, all values	17.8	1.9	11	11.7	23.2	146
	plants, leaves	17.8	1.6	9	14.0	20.0	24
	plants, roots	17.1	1.5	9	13.0	19.8	15
	plants, stems and branches	17.4	1.1	6	16.1	19.4	10
	plants, miscellaneous	18.0	2.1	12	11.7	23.2	98
kJ/g AFDW	tree tissue	-	-	-	-	-	-
	conifer needles	56.2	1.1	2	21.4	43.3	5
	crop leaves (incl pods)	-	-	-	-	-	-
	cereals and grasses	19.1	0.9	5	17.6	20.3	10
	cereals	-	-	-	-	-	-
	other grasses	19.1	0.9	5	17.6	20.3	10
	plants, all values	20.1	1.1	6	18.1	23.6	26
	plants, leaves	20.1	0.8	4	19.3	21.4	7
	plants, roots	19.4	-	-	-	-	1
	plants, stems and branches	-	-	-	-	-	-
	plants, miscellaneous	20.1	1.3	6	18.1	23.6	17

Table 4-11: Moisture and ash content of tree and plant tissue

Parameter	Subgroup	mean	SD	CV [%]	min	max	n
% H_2O	tree tissue	49.5	4.4	9	42.7	54.7	5
	conifer needles	56.2	1.1	2	55.4	56.9	2
	crop leaves (incl pods)	88.5	4.6	5	79.7	95.3	31
	cereals and grasses	76.4	5.7	7	68.5	87.6	11
	cereals	82.2	5.3	6	77.0	87.6	3
	other grasses	74.2	4.3	6	68.5	81.5	8
	plants, all values	88.1	5.4	6	80.0	95.0	8
	plants, leaves	-	-	-	-	-	-
	plants, roots	88.4	5.8	7	81.9	95.0	4
	plants, stems and branches	-	-	-	-	-	-
	plants, miscellaneous	84.7	4.4	5	80.0	90.0	4
% ash	tree tissue	-	-	-	-	-	-
	conifer needles	18.2	26.7	147	2.3	49.0	3
	crop leaves (incl pods)	-	-	-	-	-	-
	cereals and grasses	4.2	1.6	38	1.6	6.1	9
	cereals	-	-	-	-	-	-
	other grasses	4.2	1.6	38	1.6	6.1	9
	plants, all values	7.2	4.0	57	0.5	18.0	21
	plants, leaves	8.8	0.6		8.2	10.0	7
	plants, roots	1.4	-	-	-	-	1
	plants, stems and branches	-	-	-	-	-	-
	plants, miscellaneous	7.1	4.7	67	0.5	18.0	12

Pooled means for plants and for cereals and other grasses were not significantly different from each other, there was also no significant difference between tree tissue and conifer needles. Other groups differed significantly (Table 4-12).

Table 4-12: Comparison of mean caloric content (kJ/g DW) for tree and plant tissue

	tree tissue	conifer needles	crop leaves	cereals/grasses	cereals	other grasses	plants	plants, leaves	plants, roots	plants, stems	plants, misc.
tree tissue		n.s.	***	***							
conifer needles	n.s.		***	***							
crop leaves	***	***		***							
cereals/grasses	***	***	***								
cereals						n.s.					
other grasses					n.s.						
plants, all values	***	***	***	n.s.							
plants, leaves									n.s.	n.s.	n.s.
plants, roots								n.s.		n.s.	n.s.
plants, stems and branches								n.s.	n.s.		n.s.
plants, miscellaneous								n.s.	n.s.	n.s.	

***	significant, one-way ANOVA with Tukey's test or Kruskal-Wallis with Dunn's test, P < 0.001
n.s.	not significant, one-way ANOVA with Tukey's test or Kruskal-Wallis with Dunn's test, P > 0.05
n.s.	not significant, Mann-Whitney test, P > 0.05
	not tested

4.3.1.5 Seeds

For seeds, a similar division was made as for tree and plant tissue, and a distinction was made between kernels and whole seeds. Non-specified values were added to the dataset for whole seeds. Caloric content is given in Table 4-13, moisture and ash content in Table 4-14.

Table 4-13: Caloric values of tree and plant seeds

Parameter	Subgroup	mean	SD	CV [%]	min	max	n
kJ/g FW	all seeds	18.8	6.3	33	2.4	31.8	57
	kernels	22.3	6.4	29	6.9	31.8	20
	whole/not specified	16.9	5.3	32	2.4	31.0	37
	cereals	13.2	3.7	28	2.4	17.1	14
	grasses (incl sedges)	16.8	0.6	3	16.4	17.2	2
	grasses, kernels						
	grasses, whole/not specified	16.4	17.2	105	16.8	0.6	2
	non-grass plants	18.6	5.8	31	12.4	29.4	6
	non-grass plants, kernels						
	non-grass plants, whole/not specified	18.6	5.8	31	12.4	29.4	6
	non-conifer trees	20.5	6.2	30	6.9	31.8	30
	non-conifer trees, kernels	21.5	7.2	33	6.9	31.8	15
	non-conifer trees, whole/not specified	19.6	5.0	26	12.1	31.0	15
	conifers	24.8	2.2	9	22.8	28.4	5
	conifers, kernels	24.8	2.2	9	22.8	28.4	5
	conifers, whole/not specified						
kJ/g DW	all seeds	21.6	4.1	19	9.5	33.6	292
	kernels	24.8	4.8	19	15.0	33.6	66
	whole/not specified	20.7	3.5	17	9.5	32.8	226
	cereals, whole/not specified	17.6	1.8	10	12.8	19.7	41
	grasses (incl sedges)	19.1	1.0	5	16.8	21.8	42
	grasses, kernels	19.7	1.0	5	18.5	21.2	6
	grasses, whole/not specified	19.0	1.0	5	16.8	21.8	36
	non-grass plants	21.7	3.3	15	9.5	31.4	109
	non-grass plants, kernels	23.3	3.4	14	19.0	30.8	11
	non-grass plants, whole/not specified	21.5	3.2	15	9.5	31.4	98
	non-conifer trees	22.9	4.5	19	15.0	33.6	67
	non-conifer trees, kernels	24.1	5.0	21	15.0	33.6	31
	non-conifer trees, whole/not specified	21.9	3.7	17	15.9	32.8	36
	conifers	27.2	3.1	11	18.6	32.4	33
	conifers, kernels	28.4	3.1	11	18.6	32.4	5
	conifers, whole/not specified	25.7	2.4	9	19.7	29.8	15
kJ/g AFDW	all seeds	25.6	4.8	19	17.4	34.0	51
	kernels	28.1	4.3	15	18.7	34.0	25
	whole/not specified	23.2	3.9	17	17.4	33.6	26
	cereals	19.4	1.3	7	18.4	20.3	2
	grasses (incl sedges)						
	grasses, kernels						
	grasses, whole/not specified	11.6	1.8	16	10.3	12.9	2
	non-grass plants	22.7	2.8	12	20.7	24.7	2
	non-grass plants, kernels						
	non-grass plants, whole/not specified						
	non-conifer trees	23.9	4.3	18	17.4	34.0	28
	non-conifer trees, kernels	25.3	4.5	18	18.7	34.0	11
	non-conifer trees, whole/not specified	23.0	4.0	17	17.4	33.6	17
	conifers	29.6	2.7	9	23.6	33.3	18
	conifers, kernels	30.7	2.1	7	26.1	33.3	13
	conifers, whole/not specified	26.7	2.1	8	23.6	28.8	5

Table 4-14: Moisture and ash content of seeds

Parameter	Subgroup	mean	SD	CV [%]	min	max	n
% H_2O	all seeds	14.6	12.0	82	2.8	87.6	70
	kernels	15.5	11.7	76	5.0	54.0	21
	whole/not specified	14.3	12.2	85	2.8	87.6	49
	cereals	17.7	18.1	102	5.8	87.6	17
	grasses (incl sedges)	11.6	1.8	16	10.3	12.9	2
	grasses, kernels						
	grasses, whole/not specified						
	non-grass plants	9.9	2.8	29	6.0	13.0	7
	non-grass plants, kernels						
	non-grass plants, whole/not specified	9.6	2.9	31	6.0	13.0	6
	non-conifer trees	15.0	11.0	74	2.8	54.0	34
	non-conifer trees, kernels	16.8	13.6	81	5.0	54.0	15
	non-conifer trees, whole/not specified	13.5	8.6	64	2.8	34.6	19
	conifers	12.2	4.5	37	6.9	19.0	5
	conifers, kernels	12.2	4.5	37	6.9	19.0	5
	conifers, whole/not specified						
% ash	all seeds	4.2	2.8	68	0.4	19.4	47
	kernels	4.2	1.4	34	1.6	6.9	24
	whole/not specified	4.1	3.8	93	0.4	19.4	23
	cereals	1.0	0.8	82	0.4	1.5	2
	grasses (incl sedges)						
	grasses, kernels						
	grasses, whole/not specified						
	non-grass plants						
	non-grass plants, kernels						
	non-grass plants, whole/not specified						
	non-conifer trees	3.9	1.8	46	1.6	7.1	25
	non-conifer trees, kernels	3.9	1.7	44	1.6	6.9	11
	non-conifer trees, whole/not specified	3.9	1.9	49	1.6	7.1	14
	conifers	4.0	1.5	37	0.9	6.1	18
	conifers, kernels	4.5	1.1	26	2.3	6.1	13
	conifers, whole/not specified	2.8	1.6	59	0.9	4.9	5

It was first tested whether kernels and whole seeds were different, this was the case when all seeds were combined, for conifers and trees, but not for grasses and non-grass plants (t-test). Thereafter, differences in caloric content of kernels and whole seeds between groups were tested (one-way ANOVA). Results are summarised in Table 4-15.

Table 4-15. Comparison of mean caloric content (kJ/g DW) for seeds

	all seeds	kernels	whole/not spec.	cereals, whole	grasses	grasses, kernels	grasses, whole	non-grass plants	non-grass plants, kernels	non-grass plants, whole	non-conifer trees	non-conifer trees, kernels	non-conifer trees, whole	conifers	conifers, kernels	conifers, whole
all seeds																
kernels			***													
whole/not specified		***														
cereals (only whole/not spec.)					n.s.		n.s.	***		***	***		***	***		***
grasses				n.s.				***			***			***		
grasses, kernels							n.s.		n.s.			n.s.			***	
grasses, whole				n.s.		n.s.				***			***			***
non-grass plants				***	***						n.s.			***		
non-grass plants, kernels						n.s.				n.s.		n.s.			**	
non-grass plants, whole				***			***		n.s.				***			***
non-conifer trees				***	***			n.s.						***		
non-conifer trees, kernels						n.s.			n.s.				n.s.		**	
non-conifer trees, whole				***			***			***		n.s.				***
conifers				***	***			***			***					
conifers, kernels						***			**			**				**
conifers, whole				***			***			***			***		**	

***	significant, one-way ANOVA with Tukey's test or Kruskal-Wallis with Dunn's test, P < 0.001
n.s.	not significant, one-way ANOVA with Tukey's test or Kruskal-Wallis with Dunn's test, P > 0.05
***	significant, Mann Whitney test, P < 0.001
**	significant, t-test, P < 0.01
n.s.	not significant, t-test or Mann Whitney test, P > 0.05
	not tested

4.3.1.6 Vertebrates

A summary of vertebrate food sources fish, birds and mammals (including meat) is given in Table 4-16. Dry weight caloric content of birds and mammals and of mammals and fish did not significantly differ, the difference between fish and birds was significant (Kruskal-Wallis with Dunn's test, P < 0.001).

Table 4-16: Caloric values of vertebrate food and fodder

Parameter	Subgroup	mean	SD	Cv [%]	min	max	n
kJ/g FW	fish	6.1	2.1	35	2.9	11.2	66
	birds	7.7	2.4	31	3.5	17.7	57
	mammals	7.1	1.8	25	3.2	11.5	64
kJ/g DW	fish	21.0	3.7	18	12.0	30.5	60
	birds	24.2	5.2	21	16.8	38.6	141
	mammals	22.2	2.9	13	16.5	28.3	109
kJ/g AFDW	fish	-	-	-	-	-	-
	birds	27.2	5.4	20	19.1	38.8	68
	mammals	25.8	2.9	11	20.9	30.9	39
% H_2O	fish	73.7	5.4	7	62.3	81.8	43
	birds	67.2	7.7	11	44.0	84.6	54
	mammals	69.6	5.7	8	58.8	84.5	66
% ash	fish	-	-	-	-	-	
	birds	8.5	5.0	59	0.3	16.2	64
	mammals	9.0	4.0	44	1.2	13.4	23

4.3.1.7 Fruit and fodder

The last group contains data of fruit and of commercial fodder. The data for fodder are mainly for bird fodder (22) with only two for mammal fodder (2). Data are summarised in Table 4-17.

Table 4-17: Caloric values of fodder

Parameter	Subgroup	mean	SD	CV [%]	min	max	n
kJ/g FW	fruit	2.2	1.3	57	1.0	5.8	19
	fodder	15.7	3.9	25	11.8	22.8	7
kJ/g DW	fruit	14.8	4.9	33	7.2	22.2	24
	fodder	15.1	2.5	17	12.6	19.7	21
kJ/g AFDW	fruit	-	-	-	-	-	-
	fodder	20.3	1.2		19.4	21.1	2
% H_2O	fruit	83.9	4.1	5	74.0	88.0	19
	fodder	8.0	1.7	22	6.0	9.3	3
% ash	fruit	-	-	-	-	-	-
	fodder	-	-	-	-	-	-

4.3.2 Assimilation efficiency of mammals

In Table 4-18, summary statistics are given for the different food types. Assimilation efficiency of grasses and tree-tissue are significantly lower as compared to other food types (one-way ANOVA with Dunnett's test, $P < 0.005$). For tree tissue, this may be caused by deers having lower efficiencies than other ruminants. The number of data for the latter group, however, is too small to draw conclusions on this. For mammals eating seeds and nuts, there was no difference between squirrels and mice. The same goes for the assimilation efficiency of non-grass herbs/crops and mixed plants by either small mammals or hares and rabbits.

Table 4-18: Assimilation efficiency [%] of different food types for mammals

Mammal species	Food type	mean	SD	CV [%]	min	max	n
mouse, rabbit, squirrel, badger	fodder	85.5	10.2	12.0	71.2	95.0	6
shrew, bat	insects	87.4	6.3	7.2	78.0	94.9	8
shrew, otter, bobcat, fox, weasel	vertebrates	80.8	7.3	9.1	62.7	91.0	21
mouse, vole, squirrel	seeds and nuts						
	all mammals	84.3	7.6	9.1	65.2	94.0	23
	squirrels	85.2	7.5	8.8	72.0	94.0	10
	mice	83.6	8.0	9.6	65.2	91.0	13
vole, lemming	grasses	46.8	12.8	27.3	19.0	79.0	35
mouse, vole, hare	non-grass herbs[1]						
	all mammals	75.5	11.0	14.5	50.7	91.4	26
	lagomorphs	74.3	13.5	18.2	60.0	91.3	4
	small mammals	75.7	10.8	14.3	50.7	91.4	22
deer, ruminants	tree tissue						
	all mammals	42.1	21.9	52.1	24.0	80.6	9
	deer	31.7	8.4	26.4	24.0	45.9	7
	other ruminants	78.5	-	-	76.4	80.6	2

1: including crops and mixed vegetation

4.4 Discussion and conclusions

4.4.1 Caloric values, moisture and ash content

From the above presented tables it appears that variation in energy content within subgroups is reduced when values are expressed on the basis of dry weight or ash free dry weight. As for the latter far less data are available, dry weight data are preferred. For most groups, the greater variation in caloric content expressed on a fresh weight basis cannot be explained by a variation

in moisture content. The variation in moisture content is remarkably low, with CV almost always < 15 %. Only for seeds, a large variation in moisture content is found, indicating that the usually applied drying period of 24 hours at 80 or 105 °C may not be sufficient for this type of material. It is suggested by Cummins and Wuycheck (1971) that freeze drying followed by desiccation over P_2O_5 should be used for material with a high lipid content.
From the statistical comparison, it appeared that for a number of subgroups data can be pooled, and that for other groups a subdivision should be applied.

Based on the division in food sources as made by Crocker et al. (2002), which is presented in Table 1, the values as proposed on the basis of the combined dataset are given in Table 4-19.

Table 4-19. Energy and moisture content of several food sources (combined dataset)

Group	Energy content [kJ/g DW]	Moisture content [%]
Dicot crops	11.4	88.5
Grasses and cereal shoots	17.6	76.4
Non-grass herbs	17.8	88.1
Tree and conifer tissue	20.7	52.9
Fruit	14.8	83.9
Grass and cereal seeds	18.4	14.7
Weed seeds	21.7	9.9
Tree seeds	22.9	15.0
Conifer seeds	27.2	12.2
Terrestrial vertebrates	23.2	68.4
Fish	21.0	73.7
Bivalves	19.3	91.7
Freshwater arthropods	20.9	76.3
Terrestrial arthropods	22.7	68.8
Soil invertebrates (earthworms and slugs)	19.4	84.3
Aquatic vegetation[1]	15.0	81.4

1: value taken from Crocker et al. (2002), no new data available

4.4.2 Assimilation efficiency

4.4.2.1 Mammals

Relatively few data on assimilation efficiency by mammals are available. Especially for insects and tree tissue, the dataset is limited. For the latter group, this is not considered problematic, as the intake of contaminated tree tissue is not assumed to be a major exposure route. Contaminated insects, however, are considered to represent a major uptake route. The present dataset consists of only eight values, seven of which are for shrews, and of those seven, four values are obtained with the sawfly as prey species. To obtain a more reliable estimate, more data on other insect species and arthropods in general should become available. The assimilation efficiencies as proposed on the basis of the combined dataset are given in Table 4-20.

Table 4-20. Assimilation efficiency [%] of different food types for mammals

Mammal species	Food type	mean	SD	n
small and medium mammals	fodder	85.5	10.2	6
shrews and bats	insects	87.4	6.3	8
carnivores	vertebrates	80.8	7.3	21
small mammals	seeds and nuts	84.3	7.6	23
small mammals	grasses	46.8	12.8	35
small and medium mammals	non-grass herbs[1]	75.5	11.0	26
deer, ruminants	tree tissue	42.1	21.9	9

1: including crops and mixed vegetation

4.4.2.2 Birds

As already stated in section 2.2.1, the values for birds as presented by Crocker et al. (2002) and summarised in Table 4-2, remain unchanged.

4.5 References

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5. Sorption of dissociating compounds

Factsheet FSM-010/00, date 04-04-2005

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Contents

5.1 Introduction

The sorption to soil and sediment components is a determining factor for the mobility of chemicals. This property accounts for the distribution among soil, sediment and water phases, as well as for volatilisation from soil surfaces, and influences the chemicals bioavailability and hence e.g. its transformation by soil microbes. The extent of sorption to soil and sediment is governed by a variety of physico-chemical properties of both the soil and the contaminant. The heterogeneous soil chemistry and physics due to the variant proportions of the major components - mineral and organic matter, water, air and (micro)organisms - account for the differences in the binding capacity of different soils. The relevant parameters comprise organic carbon content, clay content, humidity, pH-value, cation exchange capacity, temperature, etc. Sorption occurs when the free energy of the interaction between an environmental solid and an organic chemical and an organic chemical sorbate is negative. The sorption process can be either enthalpy or entropy driven, depending on the properties of the solid and chemical solute. Enthalpy-related forces include van der Waals interactions, electrostatic interactions, hydrogen bonding, charge transfer, ligand exchange, direct and induced dipole-dipole interactions, and chemisorption, while hydrophobic bonding or partitioning is considered the primary entropy driven force. The complex and heterogeneous nature of environmental solids makes it difficult, if not impossible, to identify specific sorption mechanisms for most solid-chemical combinations and in most situations, several mechanisms operate simultaneously. Fortunately, for many solid-organic chemical interactions, one or two mechanisms dominate the sorption process and generalizations regarding sorption behaviour can be made. The sorption of most neutral, hydrophobic organic chemicals by environmental solids correlated highly with the organic matter content of the solid. Another generalization is that cationic organics and weak bases protonated at low pH, are highly sorbed on negatively charged soils, while the sorption of weak acids on soil is generally greater at low pH when the molecular form of the acid dominates (Boethling and Mackay, 2001).
Sorption coefficient quantitatively describe the extent to which an organic chemical distributes itself between an environmental solid (i.e., soil, sediment, suspended sediment, wastewater solids, etc.) and the aqueous phase that it is in contact with at equilibrium.

The reversible sorptive exchange of chemicals between the water phase and a solid-phase sorbent is represented by the sorption coefficient, which quantitatively describes the extent to which an organic chemical distributes itself between an environmental solid (i.e., soil, sediment, suspended sediment, wastewater solids, etc.) and the aqueous phase that it is in contact with at equilibrium. Sorption coefficients generally are determined from an isotherm, a diagram that depicts the distribution of the substance between a solid sorbent and the solution in equilibrium with it over a range of concentrations. In many cases, sorption isotherms are linear at low concentration but tend to become nonlinear (sorption tends to decrease) as the concentration of chemical in the aqueous phase increases, especially for polar or ionisable chemicals or soils that are low in organic carbon and high in clay (Boethling and Mackay, 2001). If the sorption isotherm is linear, the concentration of chemical sorbed by solids is directly proportional to the concentration of the chemical in water, and the slope of the isotherm is referred to as the linear sorption coefficient, so that the sorption coefficient is defined as follows:

$$Kd = \frac{\textit{Concentration of chemical sorbed to soil or sediment}}{\textit{Mean concentration of chemical in aqueous solution}}$$

Due to the different composition of soils, their sorption capacity varies considerably and hence the adsorption coefficients measured for the same compound may extend over several orders of magnitude. Therefore, a normalisation to the organic carbon fraction (%OC/100), the principal interaction site for hydrophobic compounds, is used to reduce the variance of sorption coefficients measured in different soils and to arrive at a carbon normalised partition coefficient (Koc).

$$Koc = Kd \cdot (100 / \%OC)$$

The adsorption of a substance can be obtained or estimated from:
- Direct measurement
- Simulating testing
- Koc measured by adsorption studies (OECD 106)
- Koc measured by the HPLC method (OECD 121)
- Adsorption control within an inherent biodegradability test
- If no Koc is available, it may be estimated from octanol-water partitioning coefficient (Kow).

In the manual of decisions for implementation of the sixth and seventh amendments to Directive 67/548/EEC on dangerous substances (Directives 79/831/EEC and 92/32/EEC) it is stated that for the base set notification of new substances (Annex VII A) determination of the Koc by HLPC analysis (Annex V test method C19) would normally be applicable. At higher tier notification (Annex VIII, levels 1 and 2) or in case of hazard concern, determination of adsorption/desorption on soils, including Koc, using the batch equilibrium method (Annex V test method C18) would be applicable. In REACH an adsorption/desorption screening study like the HPLC method is required for all substances manufactured or imported in quantities of 10 tonnes or more, where at tonnage above 100 tonnes further studies are required depending on the result of the screening study.

When no measured data are available for a specific adsorbing material, QSARs can be used to estimate the Koc from the Kow. In general, these QSARs are only applicable to non-ionic organic chemicals and are not valid for ionic substances as for these substances other parameters than the organic matter will determine the sorption. For ionic substances, the EU TGD perscribes that a measured adsorption coefficient is needed, or it may be possible to first investigate how significant the value might be by using a high value of Koc in the assessment. For the same reason the HPLC method is also considered less applicable for ionic substances.

This document sets out some of the main principles of the QSAR models and the HPLC method and describes the limitation they have in the treatment of organic ionisable substances with respect to adsorption coefficients. In addition pragmatic approaches will be given for the risk assessment of organic ionisable substances. It is not a full discussion of every aspect of the subject, but is intended to give guidance, emphasising some issues that fall outside current EU guidance.

5.2 The use of QSARs

For estimation of the adsorption coefficient several QSAR models are available. In all these models a Koc value is estimated from the Kow. The influence of Kow is logical because hydrophobic interactions are the most dominant type of interactions between non-polar organic chemicals and the soil organic carbon. On the other hand, however, it is also obvious that chemicals with more polar groups may interact with the soil via more specific (electronic type) interactions. In those cases, Kow will not be the only crucial parameter in the estimation of Koc. Because of this, different models are developed and proposed for different classes of chemicals. In appendix I the different QSARs with the chemical domains are presented.
As mentioned by Posthumus & Slooff in their evaluation of QSARs (2001) these QSARs are not applicable for salt, surfactants, organometallic or ionised chemicals. Van Beelen (2000) showed that the Koc of a cationic pesticide like diquat, zwitterions like glyphosate or an organometallic pesticide like meterian is underestimated by QSARs with a factor between 10,000 and 100,000. Also for veterinary drugs, the prediction of log Koc by log Kow leads to significant underestimation of log Koc and log Kd, DOM values (Tolls, 2001). This suggests that mechanisms other than hydrophobic partitioning play a significant role in sorption of ionisable pesticides and veterinary drugs. A number of hydrophobicity-independent mechanisms such as cation exchange, cation bridging at clay surfaces, surface complexation, and hydrogen bonding appear to be involved. These processes are not accounted for by organic carbon normalization, suggesting that this data treatment is conceptually inappropriate and fails to describe the sorption behaviour for ionic substances. Moreover, prediction of log Koc based on the hydrophobicity parameter log Kow is not successful.
Depending on the pKa, weak and moderately strong organic acids could be present in a neutral form within an environmentally relevant pH range. For these chemicals the log Kow of the neutral acid form of the molecule could be used to predict soil sorption. This aspect will be further discussed at the end of section 3.

5.3 The use of the HPLC method

The principle of the HPLC method is that a linear relationship exists between chromatographic parameters (retention times or capacity factors) of solutes and the Koc determined by batch equilibrium adsorption studies in soil, sediment and sludge. The chromatographic parameters are determined on an analytical column packed with cyanopropyl-modified silica gel stationary phase:

- O – Si	- CH2 - CH2 - CH2	- CN
silica	non-polar spacer	polar moiety

The dual composition of the stationary phase having polar and non-polar sites allows for interaction of polar and non-polar groups of a molecule in a similar way as is the case for organic matter in soil or sewage sludge matrices. This enables the relationship between the retention time on the column and the adsorption coefficient on organic matter to be established. The elution is performed under isocratic conditions with methanol:water mixture for neutral substances or methanol:citrate buffer (pH 6.0) mixtures for ionisable substances. This is done for the test substances and six reference substances for which Koc values should be available. The OECD guideline 121 states that these substances should be preferably structurally related to the test substance. The Koc of the test substances is then estimated via correlation between the retention times and Koc values of the test compounds. However, it will usually be difficult to find reference substances that are structurally related and have well known Koc values. In any case there will be considerable uncertainty because Koc is estimated via correlation based on a property in an artificial system and not on studies with soils.

The method is considered applicable to a variety of other chemicals belonging to the following chemical classes:

- aromatic amines (e.g. trifluralin, 4-chloroaniline, 3,5-dinitroaniline, 4-methylaniline, N-methylaniline, 1-naphthylamine);
- aromatic carbonic acid esters (e.g. benzoic acid methylester, 3,5-dinitrobenzoic acid ethylester);
- aromatic hydrocarbons (e.g. toluene, xylene, ethylbenzene, nitrobenzene, 1,2,3-trichlorobenzene);
- aryloxyphenoxypropionic acid esters (e.g. diclofop-methyl, fenoxaprop-ethyl, fenoxaprop-P-ethyl);
- benzimidazole and imidazole fungicides (e.g. carbendazim, fuberidazole, triazoxide);
- carbonic acid amides (e.g. 2-chlorobenzamide, N,N-dimethylbenzamide, 3,5-dinitrobenzamide N-methylbenzamide, 2-nitrobenzamide, 3-nitrobenzamide);
- chlorinated hydrocarbons (e.g. endosulfan, DDT, hexachlorobenzene, quintozene);
- organophosphorus insecticides (e.g. azinphos-methyl, disulfoton, fenamiphos, isofenphos, pyrazophos, sulprofos, triazophos);
- phenols (e.g. phenol, 2-nitrophenol, 4-nitrophenol, pentachlorophenol, 2,4,6-trichlorophenol, 1-naphthol);
- phenylurea derivatives (e.g. isoproturon, monolinuron, pencycuron);
- pigment dyestuffs (e.g. Acid Yellow 219, Basic Blue 41, Direct Red 81);
- polyaromatic hydrocarbons (e.g. acenaphthene, naphthalene);
- 1,3,5-triazine herbicides (e.g. prometryn, propazine, simazine, terbutryn);
- triazole derivatives (e.g. tebuconazole, triadimefon, tradimenol, triapenthenol).

According to the guideline, the method is not applicable for substances which react either with the eluent or the stationary phase. It is also not applicable for substances that interact in a specific way with inorganic components (e.g. formation of cluster complexes with clay minerals). The method may not work for surface active substances, inorganic compounds and

moderate or strong organic acids and bases. Log Koc values ranging from 1.5 to 5.0 can be determined.
For ionisable substances it is recommended to use a buffered mobile phase, but care has to be taken to avoid precipitation of buffer components or test substance.
For chemicals that completely dissociate the interpretation of this study is however not easy and it is rather questionable whether the results can be used at all. What is normally observed is that the anion has no retention on the column but that the cation strongly binds to it. For example, for salts that consist of a cationic quinolinium part and an anionic tosylate moiety (see fig. 5-1), the anionic moiety shows at both pH 2 and 7 a low Koc value. The cationic part appeared to have a very high retention at neutral pH (high Koc value).

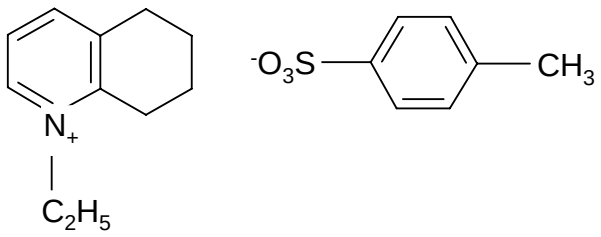

Figure 5-1: Structural formula of quinolium moiety (left) and tosylate moiety (right)

Cations
A plausible explanation is that the cation has a high affinity for the free ion pair on the polar moiety (-CN) of the cyanopropyl matrix. When the pH is reduced the binding of the cation is normally lower due to competition between the H^+ ions and the cation on the binding site of the matrix. It is however unclear how this behaviour on the column can be related to sorption to sludge, sediment and soil. It is not unlikely that cations will bind strongly to sludge, as we see for metals, and that the HPLC study does give some indication of the sorption behaviour.
Some organic cations adsorb strongly to the inorganic components of soil or sludge, especially if they have long alkyl groups in them. This has been shown by full soil measurements, and is also predicted by the SRC program PCKOCWIN (in part). The limited useful data suggests that the HPLC method can pick up some of this adsorption behaviour, but gives Koc values much lower than those actually measured (Steve Robinson, Personal Communication.)
Many of the reported correlations have been developed using a relatively small data set with a limited range of Koc values. Highly polar and highly hydrophobic compounds have not been tested adequately. Moreover, as the binding of the cation is predominantly dictated by the polar moiety of the stationary phase, sorption can not be related to the Koc value of the reference substances which binding is also related to the non-polar spacer. Binding of organic cations may correlate more readily with other properties than organic matter such as CEC and clay content and therefore it is more appropriate to use a Kd (or Kp) value for these chemicals. This can however only be determined with the batch adsorption method and not with the HPLC method.

Anions
It is not to be expected that anions would bind strongly to either organic or inorganic components of soil, unless they fit into one of two special cases:
(a) they are of high molecular weight. Data for dyestuffs and pigments suggest that even multiply charged sulfonates can bind to soil; the HPLC method would not detect this.
(b) they are metal complexing agents, such as EDTA, or polyphosphonates. The metal present in the complex seems to result in a very high adsorption to the inorganic components of soil, which is perhaps not surprising. These kind of substances can bind more than one

metal ion and so interaction with the mineral in soil is strong. Again, the HPLC method and prediction from Kow do not pick this up. Simple anions such as a carboxylate do not appear to be strong enough complexing agents for this to apply to them.

Depending on the pKa of the substances, OECD 121 recommends to study the sorption behaviour at different pHs. It is then proposes to use the Koc value where both parts of the molecule are not charged. Sometimes this is at a pH outside the environmental pH range of 4-9, and therefore has no relevance for the risk assessment. But even when the measurements are done within the pH range of 4-9, it is considered that the behaviour at pH 7 should be the starting point, assuming that this is most relevant for a sewage treatment plant (and also because most of the ecotoxicity study are performed at pH 7-8). For surface water and soil the behaviour at other pHs might be more relevant, though from a pragmatic point of view the same Koc value is considered appropriate.

5.4 Pragmatic approaches for the risk assessment

For ionisable substances it is most appropriate to ask for an adsorption/desorption study with sludge (ISO/FDIS 18749) or soil (OECD 106) to get a better estimate of the sorption behaviour and to determine a Kd value in stead of a Koc value (for pesticides it is assumed that sorption determined in soil is equally valid for sediment and sludge and therefore it is not necessary to determine it separately).
For the risk assessment of substances that are produced or used at low tonnage levels, it is however not always necessary to ask for such a study. In this case at first a so-called dual worst case approach can be followed:

Extreme 1
For substances or moieties with a high mobility it is assumed that no adsorption to sludge will take place. In the absence of degradation and volatilisation, 100% of the substance will end up in waste water and surface water and consequently gives the highest risk for the aquatic environment. For this purpose a Koc value of 10 is recommended as default.
Extreme 2
For substances or moieties with a low mobility, a realistic maximum Koc value will be assumed. An extreme high log Koc value, e.g. 6, will not automatically give the highest risk for the terrestrial environment or for humans indirectly exposed via the environment in case sludge will be applied to land. This is caused by the fact that with increasing Koc values the percentage bound to sludge will level off to a plateau level, while the partitioning from the solid phase to the water phase will always be proportionally related to the Koc value. This means that the pore water concentration in soil follows a hyperbolic curve with an increase in adsorption. When the risk for the terrestrial environment is derived from the aquatic toxicity data via equilibrium partitioning, it will follow the same pattern. In order to determine the Koc value related to the highest PEC/PNEC ratio for the terrestrial compartment and the margin of safety (MOS) for humans indirectly exposed via the environment, the Koc value was varied in EUSES. The following assumptions were made:

- Industrial use category/use category 10/42 and industrial use was used for which an Emission Scenario Document (ESD) is available in the TGD. This scenario is applicable for pigment dyestuffs. EUSES defaults for this scenario were not adjusted. Tonnage level was set to 100 tpa (import).
- The substance is not readily biodegradable.

- A worst case scenario for bioaccumulation was used in which the log Kow was set to 6, which is only of influence for calculating the BCF for earthworms (correspondingly set to 1.2E-4).
- Another parameter which is of significant influence on the concentration in soil/pore water is evaporation. Therefore, the following exercise was done for non-volatile substances (VP was set to 1E-6 Pa) and volatile substances (VP was set to 10 Pa).
- Molecular weight was set to 300 and water solubility to 1000 mg/l, assuming that in most cases the water solubility of ionic compounds is high.

To get fictive PEC/PNEC and Margin of Safety (MOS) -values, acute aquatic toxicity values for fish, daphnia and algae were set to 1 mg/l and NOAEL for mammals was set to 1000 mg/kg bw/day.

Table 5-1: EUSES calculations with variation of Koc values for defining worst-case scenario for the terrestrial compartment

VP [Pa]	Log Kow	Log Koc	BCF worm [l/kg wwt]	[1]PECsoil [mg/kg wwt]	[2]PEC pore water [mg/l]	Conc.worm [mg/kg]	PEC/PNEC soil	MOS
1E-06	6	1	$1.2.10^{-4}$	$1.8.10^{-3}$	$6.0.10^{-3}$	33	84	550
1E-06	6	2	$1.2.10^{-4}$	0.078	0.041	220	440	320
1E-06	6	3	$1.2.10^{-4}$	1.7	0.093	500	940	200
1E-06	6	4	$1.2.10^{-4}$	9.0	0.051	280	510	370
1E-06	6	5	$1.2.10^{-4}$	15	$8.4.10^{-3}$	46	84	$1.9.10^{3}$
1E-06	6	6	$1.2.10^{-4}$	16	$8.9.10^{-4}$	5.6	8.9	$1.1.10^{4}$
10	6	1	$1.2.10^{-4}$	$1.1.10^{-3}$	$3.9.10^{-3}$	21	70	590
10	6	2	$1.2.10^{-4}$	0.039	0.021	110	240	430
10	6	3	$1.2.10^{-4}$	1.4	0.078	420	790	230
10	6	4	$1.2.10^{-4}$	8.8	0.050	270	500	370
10	6	5	$1.2.10^{-4}$	15	$8.3.10^{-3}$	46	83	$1.7.10^{3}$
10	6	6	$1.2.10^{-4}$	16	$8.9.10^{-4}$	5.6	8.9	$6.8.10^{3}$

[1] in agricultural soil averaged over 180 days
[2] in pore water of agricultural soil

From this exercise it can be concluded that using an extreme high log Koc value (e.g. 6) will not lead to the worst case scenario for the terrestrial compartment and also not for man exposed via the environment. The PEC in pore water shows to be the highest for a log Koc value of around 3. It can be concluded from table 5-1 that the Koc value giving the highest risk for the terrestrial environment and man indirectly exposed via the environment is around 1000.
For substances which are (readily or inherently) biodegradable based on screening tests, the estimated half-lives for soil in EUSES depend on the solids/water partition coefficient. As shown in table 5-2, it is assumed that the half-live for substances with a $Kp_{soil} < 100$ (i.e. Koc < 5000) is 10 times lower than for substances with a $Kp_{soil} > 100$. In concomitant, the calculated average soil concentration for substances with a $Kp_{soil} > 100$ is much higher than for substances with a $Kp_{soil} < 100$. Consequently, the highest risks for biodegradable substances will not be found at a Koc of 1000 but just above a Koc value of 5000 (i.e. Kp_{soil} of 101).

Table 5-2: Half-lives (days) for (bulk) soil based on results form standardised biodegradation test results, according to the EU TGD

Kpsoil [l/kg]	Readily biodegradable	Readily biodegradable, failing 10-d window	Inherently biodegradable
≤ 100	30	90	300
> 100, ≤ 1000	300	900	3000
> 1000, ≤ 10000	3000	9000	30000

It should be noted that the described relationship between the Koc and the risk for the terrestrial compartment and humans indirectly exposed via the environment applies for substances with a relative low Henry's law constant (e.g. high water solubility and low vapour pressure). By varying the water solubility and vapour pressure in EUSES it appears that when the water solubility (mg/l): vapour pressure (Pa) ratio is < 1000 the highest risk will be found at higher Koc values as mentioned above, because then partitioning between air and water also becomes a relevant fate process (e.g WS/VP of 10: Koc ≈ 4000; WS/VP of 1: Koc ≈ 20000). For these substances the Koc value giving the highest risks should be determined case by case.

Both extremes for the anion and cation should be considered in the risk assessment, and the consequences are examined. It may be then shown that the outcome does not actually depend on Koc strongly, in which case there is no compelling need to ask for a study. On the other hand, it may be found that there could be a need for terrestrial testing. Even the terrestrial testing could be easier than doing a Koc study requiring a radiolabelled sample.

When the above described extremes will indicate a risk, a further approach could be, in cases where analysis is feasible, to run a sewage treatment simulation, and then to interpret the results by fitting the outcomes to the inputs (Koc, degradability, volatility) needed for SIMPLETREAT. This then gives a Koc value that can be used in EUSES. If available also a Zahn-Wellens elimination level can be used as an estimate of the extent of adsorption to sludge. If the substance is not degradable, then the removal in a Zahn-Wellens test can be considered as a reasonable estimate of the likely removal through sludge adsorption in a WWTP. This doesn't give a Koc/Kd, but it does give a simple estimate of fate in the STP for the first stage of the risk assessment. In the revised TGD (EU, 2003) the 3h value is recommended. For slowly adsorbing substances, consideration could be given to the hydraulic retention time in a STP (default is 6.8 h). Values beyond 24 h would not normally be used. Where data are not available for adsorption up to 24 hours, data from time scales beyond this can only be used if adsorption is the only removal mechanism, with an upper limit of 7 d. Preferably, a batch test with activated sludge can be requested. The Kd or percentage sorption determined in the ISO test can be used to derive a corresponding Koc value (e.g. via iteration in EUSES), considering that the substances will predominantly bind to organic matter in sludge. This Koc value can then be used to model the partitioning in soil/sediment. If based on equilibrium partitioning a risk can be excluded no further testing is necessary. If a risk can not be excluded, then additional studies can be requested, starting with the toxicity studies.

5.5 Assessment and RIVM/SEC strategy

For non polar organic chemicals different QSARs can be used to determine the Koc value. For salts, surfactants, organometallic or ionised chemicals however no QSARs are available. Likewise the HPLC method (OECD 121) is not applicable for these groups of chemicals either. For ionisable substances these methods could only be used when at a pH of around 7, which is considered most relevant for a STP, the substance is predominantly present in the non-ionised form.
Before a batch adsorption study is requested a dual worst case approach can be followed to determine whether an environmental risk is likely to occur. For substances which are considered to be mobile it is assumed that no adsorption to sludge will take place by using a Koc value of 10. For less mobile substances, like organic cations, dyestuffs, pigments or metal complex agents, a Koc value of 1000 and 5050 for non-biodegradable and biodegradable substances respectively, is recommended, which gives the highest risk for the terrestrial compartment and humans indirectly exposed via the environment. If this approach can still not exclude a risk, available data from the ISO/FDIS 18749 test or (if already available) a Zahn-Wellens test can be used for a better estimate of the adsorption to sludge.

5.6 References

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Annex 1

Table I. QSARs for soil and sediment sorption for different chemical classes (EU TGD, 2003)

Chemical class	Equation	Statistics
Predominantly hydrophobics	logKoc = 0.81 logKow + 0.10	n=81, r^2=0.89, s.e.=0.45
Nonhydrophobics	logKoc = 0.52 logKow + 1.02	n=390,r^2=0.63,s.e.=0.56
Phenols, anilines, benzo-nitriles, nitrobenzenes	logKoc = 0.63 logKow + 0.90	n=54, r^2=0.75, s.e.=0.40
Acetanilides, carbamates, esters, phenylureas, phosphates, triazines, triazoles, uracils	logKoc = 0.47 logKow + 1.09	n=216,r^2=0.68,s.e.=0.43
Alcohols, organic acids	logKoc = 0.47 logKow + 0.50	n=36, r^2=0.72, s.e.=0.39
Acetanilides	logKoc = 0.40 logKow + 1.12	n=21, r^2=0.51 s.e.=0.34
Alcohols	logKoc = 0.39 logKow + 0.50	n=13, r^2=0.77, s.e.=0.40
Amides	logKoc = 0.33 logKow + 1.25	n=28, r^2=0.46, s.e.=0.49
Anilines	logKoc = 0.62 logKow + 0.85	n=20, r^2=0.82, s.e.=0.34
Carbamates	logKoc = 0.37 logKow + 1.14	n=43, r^2=0.58, s.e.=0.41
Dinitroanilines	logKoc = 0.38 logKow + 1.92	n=20, r^2=0.83, s.e.=0.24
Esters	logKoc = 0.49 logKow + 1.05	n=25, r^2=0.76, s.e.=0.46
Nitrobenzenes	logKoc = 0.77 logKow + 0.55	n=10, r^2=0.70, s.e.=0.58
Organic acids	logKoc = 0.60 logKow + 0.32	n=23, r^2=0.75, s.e.=0.34
Phenols, benzonitriles	logKoc = 0.57 logKow + 1.08	n=24, r^2=0.75, s.e.=0.37
Phenylureas	logKoc = 0.49 logKow + 1.05	n=52, r^2=0.62, s.e.=0.34
Phosphates	logKoc = 0.49 logKow + 1.17	n=41, r^2=0.73, s.e.=0.45
Triazines	logKoc = 0.30 logKow + 1.50	n=16, r^2=0.32, s.e.=0.38
Triazoles	logKoc = 0.47 logKow + 1.41	n=15, r^2=0.66, s.e.=0.48

n is the number of data, r^2 is the correlation coefficient, s.e. is the standard error of estimate

Table II . Domain of the sorption models (EU TGD, 2003)

Model	X-variable domain Log Kow in log units	Chemical domain	Substituents or Warnings
Hydrophobics	1 - 7.5	All Chemicals with C, H, F, Cl, Br, and I atoms	
Nonhydrophobics	(-2.0) - 8.0	All Chemicals that are not classified as Hydrophobics	Overestimated n-Alkyl Alcohols (0.9 log units) Organic Acids (0.55 log units) Underestimated Amino-PAHs (1-2 log units) Aliphatic Amines (1-2 log units) Alkyl Ureas (1.0-1.5 log units)
Phenols	1.0 - 5.0	Phenols Anilines Benzonitriles Nitrobenzenes	Cl, Br, CH_3, OH, NO_2, CH_3O Cl, Br, CH_3, CF_3, CH_3O, N-Me Chlorinated Cl, Br, NH_2
Agricultural	(-1.0) - 8.0	Acetanilides Carbamates Esters Phenylureas Phosphates Triazines Uracils	
Alcohols, acids	(-1.0) - 5.0	Alcohols Organic Acids	Alkyl, Phenalkyl, OH All
Acetanilides	0.9 - 5.0	Anilides	CH_3O, Cl, Br, NO_2, CF_3, CH_3
Alcohols	(-1.0) - 5.0	Alcohols	Alkyl, Phenalkyl, OH
Amides	(-1.0) - 4.0	Acetamides Benzamides	F, Cl, Br, CH_3O, Alkyl NO_2, N-Me
Anilines	1.0 - 5.1	Anilines	Cl, Br, CF_3, CH_3, N-Me, N, N-di-Me
Carbamates	(-1.0) - 5.0	Carbamates	Alkyl, Alkenyl, Cl, Br, N-Me, CH_3O
Dinitroanilines	0.5 - 5.5	Dinitroanilines	CF_3, Alkyl-SO_2, NH_2SO_2, CH_3, t-Bu
Esters	1.0 - 8.0	Phthalates Benzoates Phenylacetates Hexanoates Heptanoates Octanoates	alkyl, phenyl, Cl alkyl, phenyl, NO_2,OH,Cl,NH2 alkyl, phenalkyl alkyl alkyl alkyl
Nitrobenzenes	1.0 - 4.5	Nitrobenzenes	Cl, Br, NH_2
Organic Acids	(-0.5) - 4.0	Organic Acids	All
Phenols	0.5 - 5.5	Phenols	Cl, Br, NO_2, CH_3, CH_3O, OH
		Benzonitriles	Cl
Phenylureas	0.5 - 4.2	Phenylureas	CH_3, CH_3O, F, Cl, Br, Cyclo-alkyls, CF_3, PhO
Phosphates	0.0 - 6.5	All Phosphates	
Triazines	1.5 - 4.0	Triazines	Cl, CH_3O, CH_3S, NH_2, N-Alkyl
Triazoles	(-1.0) - 5.0	Triazoles	Alkyl, CH_3O, F, Cl, CF_3, NH_2

* The precision of estimates is higher for the less hydrophobic chemicals and lower for the more hydrophobic chemicals. For chemicals with the logKow data from 1 to 4 the spread of residuals is from 0.2 to 0.5 log units and for the chemicals with the logKow data from 4 to 7.5 the spread of residuals is from 0.6 to 1.0 log units.