Opinion of the Scientific Panel on Plant health, Plant protection products and their Residues on a request from the Commission related to the evaluation of alachlor in the context of Council Directive 91/414/EEC[1].

(Question N° EFSA-Q-2004-48)

adopted on 28 October 2004

SUMMARY OF OPINION

The Scientific Panel on Plant health, Plant protection products and their Residues (PPR) concludes that the strength of the evidence suggests that a mode of action other than genotoxicity is involved in the occurrence of nasal turbinate tumours observed in the rat carcinogenicity studies. While the mode of action could be relevant to humans, it is extremely unlikely that concentrations of the active metabolite would be achieved to initiate the chain of events terminating in cancer.

The PPR Panel also concludes that metabolites 65, 54 and 25 have been adequately tested for toxicity, but the toxicity database is inadequate in the case of the soil metabolites 85, 76 and 51. The genotoxicity database is also inadequate for soil metabolites 85, 76 and 51. For metabolite 25 the PPR Panel was unable to conclude that genotoxicity testing was adequate. It is concluded that the information presented for metabolites 65 and 54 is sufficient to demonstrate that they are not relevant; a similar conclusion cannot be reached for metabolites 85, 76, 51 and 25.

Key words : alachlor, acetochlor, chloroacetanilide, 2,6-diethyl aniline, quinoneimine, herbicide, rat, nasal turbinate mucosal tumour, carcinogenicity, genotoxicity, toxicity, metabolism, relevant metabolite, mode of action.

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BACKGROUND[2]

Alachlor is used as an herbicide and is included in the first list of active substances referred to in Article 8(2) of Directive 91/414/EEC[3] concerning the placing of plant protection products on the market. On the basis of the evaluation report prepared by Spain as Rapporteur Member State (RMS), the substance has been peer reviewed with Member State experts and consequently discussed in the working group "Plant Protection Products–Evaluation" on 17/18.9.2003 and in the working group "Plant Protection Products – Legislation" of the Standing Committee on the Food Chain and Animal Health on 2 and 3 October 2003.
A tripartite meeting with the RMS and the main data supplier was organised on 19 December 2003.
The peer review identified several data gaps that were addressed by the notifier. All information submitted has been evaluated and discussed with Member States in the Working Groups "Evaluation".
Some outstanding issues were identified and may trigger a broader discussion.
The notifier provided mechanistic studies, which would confirm that the observed nasal tumours are specific to the rat (2-years rat study) and have no relevant effects to humans.

[2] Background delivered by the European Commission.
[3] OJ No L 230, 19.08.1991, p.1.

However, several Member States consider alachlor and/or some of its metabolites to be genotoxic carcinogens, for which no threshold value can be set. They observed that it cannot be excluded that these observed tumours in rats may be relevant to humans.
Furthermore, studies show that alachlor degrades rapidly in aerobic soil to a large number of metabolites.
Following the applied scenarios for groundwater, alachlor does not leach to shallow groundwater at levels above 0.1 µg/l. However some of the metabolites may exceed significantly this level (metabolites 65, 85, 54, 25, 76, 51).
The Commission Guidance document (SANCO/221/2000 rev 10 final 25 February 2003) on the assessment of the relevance of metabolites in groundwater of substances regulated under Council Directive 91/414/EEC provides that in such cases sufficient information has to be made available to demonstrate that the concerned metabolites leaching above 0.1 µg/l are not relevant.

TERMS OF REFERENCE

Question 1: Is the occurrence of nasal turbinate tumours observed in the rat carcinogenicity study relevant to humans? If so, is a genotoxic mechanism involved?

Question 2: Is the information presented for the metabolites listed above sufficient to demonstrate that they are not relevant ?

ASSESSMENT QUESTION 1

Question 1: Is the occurrence of nasal turbinate tumours observed in the rat carcinogenicity study relevant to humans? If so, is a genotoxic mechanism involved?

1.1. Introduction

Alachlor is one of several chloracetanilide herbicides that have been associated with increased tumour incidences in a number of organs in rodents submitted to long-term, dietary exposure experiments. Epigenetic modes of action have been suggested for this activity, but there has been concern in some Member States that genotoxic mechanisms have not been adequately considered as the more likely mode of action in the development of tumours of the nasal turbinates. This concern is captured in the two, clearly related questions put to the PPR Panel. The opinion of the PPR Panel is based on the experimental data and arguments described under the following headings:
- Kinetic and metabolic studies on alachlor;
- Carcinogenicity studies of alachlor in rats and mice;
- DNA interaction and other genotoxicity studies *in vitro* and *in vivo* with alachlor and its major mammalian metabolites;
- Studies of epigenetic modes of action in the nasal turbinates.

1.2. Kinetic and metabolic studies on alachlor

The absorption, distribution and excretion of alachlor have been studied in rat, mouse, Syrian hamster and rhesus or squirrel monkeys. Metabolism *in vivo* and *in vitro* has been studied in rat, mouse, squirrel monkey and man. Alachlor is well absorbed from the gastro-intestinal tract (GIT) in all species tested. Tissue distribution and whole body autoradiography studies show that alachlor metabolites accumulate in the nasal turbinates of rats (particularly in Long-Evans as opposed to either Sprague-Dawley or F344 strains), but not of CD-1 mice, Syrian hamsters and squirrel monkeys (Ribelin & Wilson, 1985; Hall & Wilson, 1992). Accumulation of radioactivity was found in blood and in the GIT of all species investigated. Haemoglobin binding is stronger in

rats than in other species. Accumulation of radioactivity in the GIT was most pronounced in the rat, as a result of extensive enterohepatic circulation. Excretion in urine and faeces respectively, as a percentage of the dose, was about 45 % and 42 % in rats, about 20 % and 60 % in mice and about 78 % and 17 % in rhesus monkeys. The high faecal elimination in mice is a result of enterohepatic circulation and binding of metabolites to intestinal material, rather than poor absorption. Urinary elimination kinetics in rats are biphasic, the half-life times being about 7 h for the α-phase and about 100 h for the β-phase. The urinary elimination half-life time in rhesus monkeys is about 5 h.

Alachlor is extensively metabolised in rats and mice through a complex network of pathways, whereas metabolism in monkeys appears to be simpler (see Appendix Figures 1, 2 and 3) Studies with liver and kidney homogenates have led to the identification of two major metabolic pathways in all of these species. One of these is oxidative dealkylation (loss of the methoxymethyl group) by cytochrome P450 enzymes to form the secondary chloramide (metabolite 13) (Feng & Patanella, 1988) that is then hydrolysed by microsomal arylamidases to 2,6-diethylaniline (Feng *et al.*, 1990). These reactions can be catalysed by microsomal preparations from rat and mouse liver and nasal fractions and the oxidation products can be conjugated with glucuronic acid or glutathione. The other metabolic pathway requires conjugation with glutathione, with loss of chlorine, a reaction that is mediated by cytosolic glutathione *S*-transferases (Feng & Patanella, 1988). The secondary chloramide product of oxidative dealkylation (metabolite 13) is also a substrate for this conjugation, again with loss of chlorine.

The conjugates are excreted in bile into the GIT, where the corresponding thiol metabolites are formed by cleavage of the C-S bond and then reabsorbed. These thiols are *S*-methylated in the liver to form tertiary and secondary methyl sulphides that undergo further metabolism by *S*-oxidation and side-chain hydroxylation. They are largely eliminated in urine. The influence of sex, dose and route of administration is relatively small.

The specificity of rat liver microsomal arylamidases for several of these potential substrates (6 methylthio compounds, including sulphides, sulphoxides and sulphones, and 2 mercapturates) was examined (Feng *et al.*, 1990). The formation of 2,6-diethylaniline was observed only from the methyl sulphide secondary amide metabolite. Thus, two secondary amides, one formed from each of the metabolic pathways described are substrates for arylamidase. These are the *N*-dealkylated methylsulphide and the *N*-dealkylated chloroacetanilide, which form a common product, 2,6-diethylaniline (Kimmel *et al.*, 1986; Feng *et al.*, 1990). This appears to be a key metabolite in the toxicology of alachlor. Oral dosing of rats and mice with the homologous 2,6-dimethyl-[^{14}C-phenyl]-aniline (a carcinogen of nasal tissue in male and female CD rats) led to a very intense localisation of radioactivity in the nasal mucosa of male and female rats at 24 h. Mice showed only slight localisation in nasal mucosa, but appeared to have a higher level of radioactivity in liver (Hall, 1991). In addition, the administration of radiolabelled metabolites, alachlor-methyl sulphide and 2,6-diethylaniline, also resulted in marked radioactivity in the nasal tissue (Wilson & Hall, 1988), whereas radioactivity did not accumulate in nasal tissue of mice following administration of 2,6-diethylaniline (Hall & Wilson, 1993). A proposed unstable metabolic derivative of 2,6-diethylaniline in rats is 2,6-diethylnitrosobenzene (Kimmel *et al.*, 1986; Wratten *et al.*, 1987), but its significance is unclear. Much more firmly established is the oxidation of 2,6-diethylaniline by hepatic and nasal microsomal (aniline) hydroxylase to 4-amino-3,5-diethylphenol (metabolite 86). The *in vivo* relevance of this pathway was confirmed by the presence of the sulphate conjugate of 4-amino-3,5-diethylphenol in the urine of rats orally administered the methyl sulphide secondary amide metabolite. The initial rate of this hydroxylation reaction in rat preparations is greater when catalysed by the nasal enzyme than when the hepatic enzyme is used, by about seven-fold in one study (Feng *et al.*, 1990) and two-fold in another (Li *et al.*, 1992).

Comparisons of nasal tissue metabolism of alachlor or some of its metabolites to 4-amino-3,5-diethylphenol have been made using tissue from rat, mouse, squirrel monkey and man (Asbury *et al.*, 1994). The activity of rat arylamidase, which metabolises secondary amide metabolites of alachlor to 2,6-diethylaniline has been found to be 20-fold and four-fold higher than that of the mouse or squirrel monkey, respectively while the aryl hydroxylase activity of rat nasal tissue is two-fold and more than seven-fold higher than in nasal tissue from the other two species, respectively. In contrast, species differences in liver enzyme reaction rates with these substrates are small (Feng *et al.*, 1990).

The relative rates of reaction for four important steps in alachlor metabolism in hepatic and nasal tissue of different species can be summarised, as in Table 1.

Table 1: Ratios of initial reaction velocities for hepatic and nasal enzyme preparations from various species *(Asbury et al., 1994; Feng & Patanella, 1988; Li et al., 1992).*

	Rat/Human	Rat/Squirrel monkey	Rat/Mouse
Alachlor GSH* conjugation			
Hepatic	4.0	3.9	0.5
Nasal	32.5	114.3	0.8
Secondary sulphide hydrolysis			
Hepatic	N/A	0.9	2.2
Nasal	5.8	4.0	20.0
Secondary amide hydrolysis			
Hepatic	N/A		
Nasal	3.7		
DEA hydroxylation**			
Hepatic	7.5	3.0	0.3
Nasal	129.8	7.6	1.9

***GSH : reduced glutathione, **DEA : diethylaniline**

These ratios clearly demonstrate that at least for nasal tissue, the rat enzymes have a greater activity than human, squirrel monkey and (except for alachlor conjugation with glutathione) mouse enzymes. This is supportive of a species distinction, with rat nasal tissues forming 4-amino-3,5-diethylphenol more rapidly than the other nasal tissues preparations. It can also be demonstrated that the important 2,6-diethylaniline hydroxylation reaction has higher activity in rat nasal tissue than in rat liver (Table 2).

Table 2 : Ratios of initial reaction velocities for hepatic and nasal enzyme preparations in rats

	Rat hepatic/Rat nasal
Alachlor GSH conjugation	0.45
Secondary sulphide hydrolysis	4.77
Secondary amide hydrolysis	8.0
DEA hydroxylation	0.12

4-Amino-3,5-diethylphenol (metabolite 86) either conjugates with sulphate (metabolite 20) or, upon further oxidation, rearranges to 3,5-diethylbenzoquinone 4-imine (DEIQ), which is suspected of being the metabolite that causes damage in the nasal mucosa. Quinone-imines are electrophilic, can deplete cellular antioxidants (Tee *et al.,* 1987) and covalently bind to reduced glutathione and protein sulphydryl groups (Feng & Wratten, 1987; Feng *et al.*, 1990).

Species differences in the ability to form DEIQ correlate with the ability to form protein adducts in nasal tissue. Phenyl-[^{14}C]-labelled alachlor administered in the diet to female Long-Evans rats (126 mg/kg bw/day) for up to 13 days was covalently bound to proteins in nasal tissue. Hydrolysis and subsequent hplc analysis showed that most of the radioactivity co-eluted with the acid-hydrolysed acetylated cysteine-DEIQ standard. A minor radioactive fraction co-eluted with 2,6-diethylaniline standard. Its source must have been different from cysteine-DEIQ, which was stable under the conditions of acid hydrolysis (Lau *et al.*, 1995). Similar studies with male rhesus monkeys (126 mg/kg bw/day for 14 days) and female CD-1 mice (50 mg/kg bw/day for 14 days) failed to demonstrate any cysteine-DEIQ adduct in proteins from nasal tissue (Mehrsheikh & Lau, 2001 a, b).

1.3. Carcinogenicity studies of alachlor in rats and mice

Alachlor has been studied in six experiments that could give information on its carcinogenic potential: four with rats (Daly *et al.*, 1981a; Stout *et al.*, 1983; Stout *et al.*, 1984; Genter *et al.*, 2000, Genter *et al.*, 2002) and two with mice (Daly *et al.*, 1981b; Rouloff & Thake, 1984).

1.3.1 Rats

It is noted that, although the Long-Evans strain has been used in all of the rat studies, it should not be assumed that this strain is particularly sensitive to the development of the types of lesions described: Sprague-Dawley rats used in studies with the closely related chloroacetanilide, acetochlor, developed the same nasal lesions (Ashby *et al.*, 1996).

1.3.1.1 Study 1:

Long-Evans rats (50/sex/group) were fed alachlor diets delivering doses of 0, 14, 42 and 126 mg/kg bw/day for two years. Of the neoplastic lesions described, there were treatment related increases in adenomas of the nasal turbinate mucosa in males, thyroid follicular cell adenomas in males and adenomas and carcinomas combined in females and various tumours of the glandular stomach in males and females (Table 3). The malignant mixed gastric tumours were unusual and pluripotent. In addition, hepatocellular adenomas were more common, but not significantly elevated, in treated rats (Daly *et al.*, 1981a).

Table 3: Tumour incidences in Long-Evans rats fed alachlor continuously for 2 years (Study 1)

	Males				Females			
Dose (mg/kg bw/day)	**0**	**14**	**42**	**126**	**0**	**14**	**42**	**126**
Stomach								
Any type of tumour	0/50	0/50	0/50	17/50	0/50	0/50	1/50	23/50
Malignant mixed gastric tumour	0/49	0/50	0/50	11/50	0/50	0/50	1/50	17/49
Leiomyosarcoma	0/24	0/33	0/32	1/31	0/17	0/23	0/18	1/29
Osteosarcoma	0/24	0/33	0/32	3/31	0/17	0/23	0/18	4/29
Gastric adenocarcinoma	0/49	0/50	0/50	2/50	0/50	0/50	0/50	1/49
Thyroid follicular epithelium								
Adenoma	1/48	0/50	1/49	11/50	0/49	0/44	2/46	2/49
Carcinoma	0/48	0/48	0/48	0/48	0/48	0/48	0/48	2/49
Nasal turbinate mucosa								
Adenoma	0/46	0/46	10/41	23/42	0/49	0/47	4/45	10/48
Adenocarcinoma	0/46	0/46	1/41	0/46	0/49	0/47	1/45	0/48

Nasal turbinate adenomas showed a dose dependant increase in males and females at doses of 42 and 126 mg/kg bw/day, which was statistically significant in all of these groups except in females at 42 mg/kg bw/day.

1.3.1.2 Study 2:

Long-Evans rats (100/sex/group) were fed alachlor diets delivering 126 mg/kg bw/day. After 5–6 months, 49 females and 19 males were switched to control diet and maintained for 19 additional months (group III). After 7 months, other groups of 10 males and 20 females were killed (group II). The remaining 70 males and 31 females were maintained on the alachlor diet for 2 years (group I). A small control group (6/sex) was also included, but this size is inadequate for any statistical comparisons of tumour incidence. The data from Groups I and III are presented in Table 4, (Stout *et al.*, 1984).

Table 4: Tumour incidences in Long-Evans rats fed alachlor, either continuously for 2 years or for 5 – 6 months followed by 19 months on control diet (Study 2).

	Group I (2 years)		Group III (5-6 months)	
Dose: 126 mg/kg bw/day	**Males**	**Females**	**Males**	**Females**
Stomach				
Malignant tumours	3/68	19/31	0/20	1/49
Thyroid follicular epithelium				
Adenoma	8/69	4/31	1/20	2/49
Carcinoma	10/69	0/31	1/20	2/49
Nasal turbinate mucosa				
Adenoma	42/61	11/25	10/17	19/46
Adenocarcinoma	7/61	2/25	0/17	1/46

Adenomas of the nasal turbinates were increased in male and female rats after 5-6 months exposure to 126 mg/kg bw/day followed by control diet for 19 months, to incidences similar to those observed after two years of continuous exposure. This unusual response was not observed for tumours of the stomach and thyroid. Adenocarcinomas of the nasal turbinates also were increased in male rats after 2 years exposure.

1.3.1.3 Study 3:

Long-Evans rats were fed alachlor diets delivering 0, 0.5, 2.5 or 15 mg/kg bw/day for 25 months. Tumours of the nasal turbinates were not increased at 2.5 mg/kg bw/day and below. Adenomas of this organ were increased in both males (15/45) and females (14/48) in the 15 mg/kg bw/day dose group. There were no carcinomas and there were no increases in tumour incidence in stomach or thyroid (Stout *et al.*, 1983).

1.3.1.4 Study 4:

Male Long-Evans rats (70/group) were fed alachlor diets delivering 0 or 126 mg/kg bw/day for up to 24 months, with sub-groups of about 10-15 killed at 1, 6, 12 and 18 months. In addition, a group of Long-Evans rats was administered the same dose of alachlor for just one month and then maintained on the control diet for five months. In the first study, at 6 months, 50% of the rats treated with alachlor developed ≥ 1 tumours of the ethmoid turbinates, demonstrating that it was not necessary to hold the rats on control diets for several months in order to allow tumours to develop (as might be assumed from the data of Stout *et al.*, 1984, above). The increase in tumour burden was dramatic between 6 and 12 months of alachlor exposure, and by ≥ 12 months of alachlor exposure, rats typically had 5–20 tumours, with a significant portion of the nasal passages occupied by tumours, which ranged from small polyps to vast glandular, often haemorrhagic tumours at any of the ≥ 6 month time points examined. In the second

study, rats treated with alachlor for just one month and then maintained on the control diet for 5 more months had no detectable olfactory mucosal lesions, (Genter *et al.*, 2000; Genter *et al.*, 2002).

In the main study, in addition to these observations, at one month it was found that there was neither any histological abnormality nor evidence of enhanced cell proliferation (assessed by BrdU[4] incorporation) in any region of the nasal cavity; but after 6 months exposure there was proliferation of basal and non-basal cells in the olfactory mucosa. The masses that were recorded (above) ranged from dysplastic plaques to polyploid adenomas that originated in the olfactory regions. Both plaques and neoplasms were associated with regions of respiratory metaplasia and were often covered with a low columnar-to-pseudostratified, poorly ciliated epithelium. The tumour cells no longer expressed characteristics of the olfactory mucosa, including olfactory marker protein (OMP, for mature sensory neurons) and Nma (antibody recognising CYP2A3, an orthologue of human CYP2A6, which is found in the subepithelial Bowman's glands of rats). The sites of plaque and tumour development coincided with regions of Nma immunoreactivity, i.e., lateral and ventral quadrants of the nasal cavity, but not the mucosa lining the dorsal medial meatus and the dorsal septum. These data suggest that local metabolism is important in alachlor-induced tumours and support the concept that regions of altered epithelial differentiation give rise to small raised plaques, which progress to elevated neoplastic polyps and finally to well-differentiated adenomas.

1.3.2 Mice.

1.3.2.1 Study 1:

CD-1 mice (50/sex/group) were fed alachlor diets delivering doses of 0, 26, 78 and 260 mg/kg bw/day for 18 months. Of the neoplastic lesions described, there were insignificant excesses of liver tumours and an excess of lung tumours in high dose females, the latter being within the historical control range, (Daly *et al.*, 1981b).

1.3.2.2 Study 2:

CD-1 mice (60/sex/group) were fed diets containing alachlor at concentrations of 0, 100, 400 or 1600 ppm (M: 0, 16.6, 65.4, 262 mg/kg bw/d, F: 0, 23.7, 90.3, 399 mg/kg bw/d) for up to approximately 18 months. Ten mice per sex/group were killed and examined after 12 months. A number of histological changes were more frequent in the treated groups than in the controls. These included chronic nephritis, centrilobular hypertrophy and eosinophilic foci in the liver and accumulation of eosinophilic globules in the olfactory epithelium of male mice and fibrous osteodystrophy of the sternum in females. Increased tumour incidences were observed in the lungs of mice, which were significantly elevated in males of the 400 ppm group (Table 5), (Roloff & Thake, 1984).

Table 5: Incidences of selected lesions in CD-1 mice exposed to alachlor (expressed as %)

Dose (ppm)	Male Mice				Female Mice			
	0	100	400	1600	0	100	400	1600
Nasal turbinates Eosinophilic globules in olfactory epithelium	0	0	0	11	2	2	0	9
Lung Bronchoalveolar								
Hyperplasia	2	3	2	5	2	2	0	0
Adenoma	7	18	27	22	5	14	10	17
Carcinoma	2	0	5	0	2	2	2	5
All tumours	7	18	32	22	7	15	12	20

[4] BrdU:Bromodeoxyuridine

Thus, in the studies in mice, no tumours of the nasal turbinates were reported.

1.4. Occupational Epidemiology

In the most recent up-date study of an industrial cohort with occupational and environmental exposure to alachlor (Leet *et al.,* 1996), there was no evidence for nasal cancers (or thyroid or gastric cancers), but there was an elevated risk of colorectal cancer. The study conducted in Iowa (USA), followed 943 workers with at least one year of cumulative employment from start up of the alachlor manufacturing process in March 1968 through December 1990. Approximately 96% of all workers were successfully traced to determine their last known residence and cancer status. Eighteen workers were diagnosed with cancer during the follow-up period, based on pathology information from the state-wide cancer registry maintained by the State Health Registry of Iowa. The standardised incidence ratio (SIR) for all cancers was 1.5 (95% CI[5] 0.9-2.4) for all workers exposed to alachlor, which was due primarily to elevated rates for colorectal cancer and chronic myeloid leukemia. Workers with 5 or more years in estimated high alachlor exposure jobs had elevated rates of colorectal cancer (3 cases, SIR = 5.2, 95% CI 1.1-15.1). Interpretation of the study results was limited by the small size of the study population and minimal length of follow-up. Nonetheless, the findings suggest the need for continued evaluation of this and other alachlor-exposed cohorts.

1.5. DNA interaction and other genotoxicity studies *in vitro* and *in vivo* with alachlor and its major mammalian metabolites

In evaluating the mutagenicity data, two general factors have been considered: the complexity of alachlor metabolism in rats in particular, with the likely involvement of metabolites generated in liver that have subsequently undergone entero-hepatic circulation; and the postulated tissue specificity (nasal tissue) for the ultimate metabolite-tissue interactions. These factors detract from the ability of *in vitro* supplemented activation systems to generate the ultimate reactive metabolite(s) and of *in vivo* systems in which organs other than the nasal turbinates are the target to detect any activity. The former problem might be surmountable if intermediate metabolites are studied, rather than alachlor itself.

A selected compilation of the genotoxicity data available on alachlor is summarised in the Appendix in Table 1. Studies not listed included those that are not acceptable for clear, technical reasons. There may, however, be others that should not be included (e.g., commercial preparations).
Most studies (6/10) of alachlor for mutagenic activity in bacteria showed no activity. Two studies, however, reported activity in frameshift-sensitive strains of *S. typhimurium* in the absence of any additional metabolic activation system (Mirkova & Zaikov, 1986; Njagi & Gopalan, 1980). The latter of these was with a commercial preparation. In addition, significant responses were obtained in two other studies with *S. typhimurium* TA100 using additional activation systems, one from plants (Plewa *et al.*, 1984) and others from rat liver and rat olfactory mucosa (Wetmore *et al.*, 1999). This last result contradicts earlier ones in which the activation system was based on nasal turbinate tissue from rats, mice and monkeys (Kier & Stegeman, 1990). In the study by Wetmore *et al.* (1999), the rat liver preparation was effective only at exceedingly high doses of alachlor (8430 and 15000 μg/plate), whereas a significant increase was observed with olfactory epithelium S9 at an alachlor dose of 1250 μg/plate, which was close to a toxic dose. There was no effect of respiratory epithelium S9 at any alachlor dose level in the same study. Since there were no differences in either the strains or dose ranges

[5] CI: Confidence Interval

used between those studies from which significant results were reported and those that did not, the mutagenicity of alachlor in bacteria remains unclear.

Genotoxic activity was observed in one study of alachlor with mouse lymphoma cells and using rat olfactory mucosal S9 (Wetmore *et al.*, 1999). The significant response occurred at a single, toxic dose (5.6 μg/ml, however, cloning efficiency was not unduly affected) and was mainly attributable to an increase in small colonies (indicating damage greater than that due to a few base changes). No effect was seen in another study with the same test system, but using rat liver S9 (Enninga *et al.*, 1987). In contrast to these uncertain results, several studies of chromosomal aberration in cultured mammalian cells have yielded significant responses to alachlor exposure. It is likely that this activity is dependent upon the chloroacetamide function. Studies with another chloroacetamide, acetochlor, have shown that it is clastogenic too, whereas des-chloro-acetochlor, which is, other than the lack of the chlorine atom, the identical molecule, has no clastogenic activity (Ashby *et al.*, 1996). The same group survives in *N*-dealkylated chloracetanilide, which could therefore be reactive in nasal tissue before it is metabolised to 4-hydroxy-3,5-diethylaniline by arylamidase. However, alachlor clastogenicity has not been reliably reproduced *in vivo*, (only one study reporting a positive response, while five others did not) and since the metabolites retaining chlorine are generated in liver, any of them (in addition to unmetabolised alachlor) that are potentially clastogenic in the nose should also be available for similar activity in the bone marrow, the usual target for such assays. The absence of demonstrable clastogenic activity *in vivo* suggests either a lack of sensitivity of the assays (because cellular dose levels easily reached *in vitro* cannot be reached *in vivo*) or the function of protective mechanisms that are not normally available in the *in vitro* assays. Alkaline elution assays conducted *in vivo*, including a single-cell alkaline elution assay on rat nasal epithelium have not demonstrated significant responses, but significant responses were obtained in two of three assays for unscheduled DNA synthesis (UDS) in rat liver following oral dosing by gavage that probably depleted glutathione reserves (by analogy with acetochlor, Ashby *et al.*, 1996). A potentially important result is the finding of binding to DNA in the nasal turbinates, but not in the liver of rats dosed orally with alachlor (Asbury & Wilson, 1994); however, the mean radioactivity for DNA from the hepatic and nasal tissues were reported to be only 92.83 ± 8.63 and 205 ± 58.90 fmol alachlor equivalent/mg DNA, respectively.
The covalent binding indices (CBI)[5] after correction for protein contamination was –0.13 ± 0.89 for liver DNA and 1.66 ± 1.24 for nasal DNA. This is a very low CBI and is unlikely to account for the neoplasic response observed. Of much greater toxicological significance is the protein binding in nasal tissue. In contrast to this low level (if real) of DNA adducts, feeding of female Long-Evans rats with diets delivering 126 mg/kg bw/day for up to 13 days resulted in average levels of DEIQ-cysteine adducts in nasal tissue of 70, 88 and 218 pmoles/mg protein after 3, 7 and 13 days, respectively (Lau *et al.*, 1995). On the other hand, similar studies with male rhesus monkeys (126 mg/kg bw/day for 14 days) and female CD-1 mice (50 mg/kg bw/day for 14 days) failed to demonstrate any cysteine-DEIQ adduct in proteins from nasal tissue (Mehrsheikh & Lau, 2001 a, b).

Because of the complexity of alachlor metabolism, of special interest are genotoxicity studies on mammalian metabolites. Bacterial tests have generally been restricted to the use of *S. typhimurium* TA100 and TA 98 in the presence and absence of S9 preparations from rat liver, although other strains were also used in some cases. Weak or very weak positive results have been obtained in TA100 with CP101384 (35), CP97230 and CP101394 (27); in TA100 and TA1535 with 2,6-diethylaniline and in TA1535 with 2,6-diethyl-2-methylthioacetanilide. Apart from CP97230, the positive responses occurred in both the presence and absence of S9. Other tests conducted with these metabolites were: CP101394 (27), bone marrow micronucleus test

[5] CBI = μmole chemical bound per mole DNA phosphate/mmole chemical administered per kg bw. CBI values in rat liver for strong hepatocarcinogens are > 1000, e.g., dimethylnitrosamine, aflatoxin B1; moderate hepatocarcinogens CBI of 150 – 600, e.g., 2-acetylaminofluorene, *N*-nitrosopyrrolidine; weak hepatocarcinogens CBI of 10 –240, e.g., urethane, 4-dimethylaminoazobenzene, vinyl chloride; non-hepatocarcinogens CBI 1 – 20, benzene, benzo(a)pyrene; doubtful or non-carcinogens CBI of < 0.05 – 1.5, e.g., saccharin, toluene, ethinyloestradiol, oestrone (Lutz & Schlatter, 1979).

in mice, negative (Flowers, 1990); 2,6-diethylaniline, gene mutation assay in Chinese hamster ovary (CHO) cells (*hprt* locus), inconsistent results in four experiments (Flowers, 1987); 2,6-diethylaniline, *in vivo* alkaline elution assay in rat liver, negative (Taningher *et al.*, 1993).

1.6. Studies of epigenetic modes of action in the nasal turbinates

The *in vitro* cytotoxicity effects of alachlor, DEA, sec-amide methyl sulphide, and sec-amide chloride were assessed by evaluating the leakage of acid phosphatase from olfactory and respiratory explant cells. Results showed increased acid phosphatase leakage from olfactory but not respiratory cells following exposure to alachlor; and from both olfactory and respiratory cells following exposure to 2,6-diethylaniline, but not from either olfactory or respiratory cells following exposure to the sec-amide methyl sulphide or sec-amide chloride (Asbury *et al.,* 1995).

Cell proliferation assays were performed in the respiratory and olfactory epithelium of rats and mice exposed to alachlor. Results showed a dose-related increase in cell proliferation in olfactory but not respiratory epithelium in rats administered 42 or 126 mg/kg bw/day. This cell proliferation was reversible after 60-day recovery period. On the other hand, there was no cell proliferation in mice.

The effects of alachlor upon cellular stress response genes in rat nasal turbinate tissue were also evaluated. A significantly increased expression of NMO[6] and HSP70[7] was observed in rat nasal epithelium after 60 days exposure at 126 mg/kg/day. This response was not observed at 30 days (Curtiss *et al*., 1995)

Alachlor doses of about 1000 mg/kg bw, which are close to those causing lethality, are required to deplete reduced glutathione (GSH) in rat liver (Heydens *et al.,* 1999). In contrast, Burman *et al.* (2003) found that both reduced glutathione and ascorbic acid concentrations in olfactory mucosa from male Long-Evans rats rapidly decreased following alachlor exposure for up to 10 days (10 – 126 mg/kg bw/day), with a subsequent increase in both antioxidants to ~ 160% of control levels in the highest dose group and recovery to control levels in all groups by 10 days. These changes in GSH concentrations are associated with up-regulation in olfactory mucosa of glutamine-cysteine ligase. This is the rate-limiting enzyme in GSH biosynthesis and it remained elevated throughout the 10-day dosing period. While GSH was not depleted at all doses, ascorbate concentrations were, and they did not return to normal levels. Ascorbate is important in the maintenance of extracellular matrix proteins, including collagen IV (Chernousov *et al.,* 1998; Hospelhorn *et al*., 1992; Kalcheim *et al.,* 1985; Kim & Peterkofsky, 1997). Alachlor disrupts basal cell orientation in the olfactory mucosa (Genter *et al*., 2000), possibly due to the partial loss of collagen IV following ascorbate depletion, but also possibly due to up-regulated matrix metalloproteinases (Genter *et al*., 2002) resulting in a more general and sustained degradation of the extracellular matrix.

1.7. Discussion (based on the IPCS Mode of Action Framework)

Alachlor reproducibly induces tumours of the olfactory mucosa in rats, but not in mice. The incidence is higher in males than in females. Adenocarcinomas were induced in one of the three experiments, otherwise, progression did not continue beyond the generation of adenomas.

The proposed mode of action for production of nasal tumours in rats is the local generation of cytotoxic metabolite(s) that can interact with cellular macromolecules and induce a sustained

[6] NMO : NAD(P)H Menadione Oxidoreductase 1
[7] HSP70 : Heat Shock Protein 70

cell proliferation, neoplasia arising out of this proliferating cell population. Mutagenesis induced by the cytotoxic metabolite(s) may or may not be part of this process.

The following important steps are involved in the process.
The generation of 2,6-diethylaniline *via* two metabolic pathways, one involving conjugation with glutathione, with the subsequent degradation of the conjugate to a methyl sulphide secondary amide, while the other involves oxidative dealkylation by cytochrome P450 enzymes to a secondary chloramide (metabolite 13). Both of these products are substrates for microsomal arylamidases (the methyl sulphide being the only reactive substrate among several methylthio-metabolites tested) resulting in the formation of their common metabolite, 2,6-diethylaniline.

Both the methyl sulphide metabolite and 2,6-diethylaniline become strongly localised in nasal tissue.

2,6-Diethylaniline can be further oxidised by microsomal aniline hydroxylase to 4-amino-3,5-diethylphenol (metabolite 86). It is proposed that this is a local, nasal reaction. This phenol can rearrange to the sulphydryl-reacting, cytotoxic DEIQ.

At this point, in the sequence of events, there is a reduction in nasal ascorbate concentrations, changes in glutathione concentrations and cytotoxicity followed by cell proliferation, which is presumably a reparative/regenerative response. It has also been demonstrated in one study that mutagenesis can occur, at least *in vitro,* with alachlor as the substrate in the presence of olfactory tissue preparations. This observation seems to undermine the significance of all the earlier, extra-nasal steps in metabolism, since the mutagenic metabolite has not been identified and while it could be DEIQ, it may equally be another metabolite or even reactive oxygen species, the concentrations of which could increase subsequent to perturbations in antioxidant status: quinoneimines can deplete cellular antioxidants. However, while mutagenesis may occur, it does not necessarily mean that this is involved in the neoplastic mechanism.
From within this proliferating cell population, neoplastic transformation occurs, giving rise to adenomas and, in some experiments, carcinomas in the olfactory tissue. Whether this neoplastic transformation is a genotoxic or an epigenetic event is not known. One possibility is that the accelerated cell cycle time reduces the opportunity for repair of genetic damage, which may be either "spontaneous," induced by some alachlor metabolite or as a result of reactive oxygen species. However, it is clear that the dominant reaction with macromolecules in nasal tissue is not with DNA (there is a lack of significant covalent binding with DNA) but with sulphur in proteins or glutathione. The latter would be a detoxifying reaction, but arylation of proteins could lead to a number of biologically relevant responses. Among these, adduction to proteins in chromatin could result in altered gene expression and differentiation control. It is known that alachlor interferes with the maintenance of extracellular matrix proteins and disrupts basal cell orientation in the olfactory mucosa. These possible effects are consistent with the transdifferentiation that is observed in olfactory epithelium (to respiratory epithelium). Other, but unstudied changes that could occur would be a loss of genetic stability or ability to repair the spontaneous genetic damage that occurs daily in all cells.

Adenomas are reproducibly induced by exposure to diets delivering 126 mg/kg bw/day. The incidence is lower when exposure is reduced to 42 mg/kg bw/day. Studies that have demonstrated cell proliferation in the rat nasal turbinates have only used exposures of 126 mg/kg bw/day. Lower doses were not tested and so there has been no demonstration that accelerated cell proliferation always occurs at doses that induce tumours.

No evidence of cell proliferation or histological change was found after exposure for 1 month. The earliest tumours of the nasal turbinates of rats were found after 6 months exposure, with rapid expansion in incidence at later times. Also at 6 months, transdifferentiated tissue and dysplasia were evident in the olfactory region, as was cell proliferation.

The appearance of preneoplastic lesions, as well as neoplasia, are consistent findings in rats, and they have not been observed in mice. The putative proximate metabolite, 2,6-diethylaniline, is strongly localised in rat nasal tissue, but not in mouse nasal tissue, and is therefore consistent with the species difference in neoplastic response. In addition, the metabolic steps leading to the proximate metabolite can occur *in vitro* with hepatic as well as nasal tissue from rats, but not from mice, and is therefore consistent with the species specificity of the carcinogenic response. While this observation does not support an absolute tissue specificity, the enzymatic activity that produces the penultimate reactive metabolite (4-amino-3,5-diethylphenol) from 2,6-diethylaniline is much greater in rat nasal tissue than in rat hepatic tissue. DEIQ, the putative ultimate metabolite, reacts strongly with proteins in nasal tissue, the dominant adduct being DEIQ-cysteine.

The proposed chain of events – metabolism to a proximate metabolite that is strongly localised in nasal turbinates of the susceptible species, but not of non-susceptible species, rearrangement to a strongly electrophilic substance that can then react with both glutathione and macromolecules in the target tissue, thereby causing impairment of antioxidant status and cytoxicity and other tissue damage that can lead to neoplasia – is a plausible mechanism and the available data do not conflict with it. Precisely how the damage leads to neoplasia is not indicated by the data, although plausible hypotheses are available and have been described above.

Other modes of action should be considered, one of which is genotoxicity of alachlor or its metabolites generated either in the liver or locally, in the olfactory nasal mucosa. One study has reported mutagenic responses *in vitro* in bacteria and, at a single concentration, in a mammalian cell line when the incubations were with alachlor and a metabolic activation system based on rat olfactory epithelial tissue, but either not at all or only at extremely high dose levels when rat liver was used. The result with rat nasal tissue contradicts another study that found no mutagenic effect in bacteria when the activation system was based on nasal turbinate tissue from rats, mice and monkeys. It is possible, however, that in the negative study, the active olfactory epithelium may have been diluted by respiratory epithelium (presumed to be inactive). The evidence for a mutagenic effect on the target tissue is, therefore, unclear. Other evidence for or against a mutagenic effect *in vivo* comes from alkaline elution and UDS assays in rats. Alkaline elution assays, including a single-cell (comet) assay with rat nasal epithelium have not provided any evidence for genotoxicity, but while two of three UDS assays in rat liver have given significant results, the doses applied were predicted also to cause significant glutathione depletion. There is a lack of significant covalent binding with DNA. Hence a direct genotoxic mode of action is not sustained by the available evidence.

It is concluded, therefore, that the data are consistent with the proposed mechanism of action, this being as follows. The occurrence of a concentration of a metabolite of alachlor in the olfactory mucosa and its further metabolism to a sulphydryl-reacting, cytoytoxic product that induced cell proliferation and changes in gene expression. Neoplasia arises from this metaplastic or transdifferentiated tissue. The evidence in favour of a genotoxic mode of action is weak.
While the mode of action could be relevant to humans, it is extremely unlikely (based on considerations discussed in section 1.2) that concentrations of the active metabolite would be achieved to initiate the chain of events terminating in cancer.

Conclusions and Recommendations

The Scientific Panel on Plant health, Plant protection products and their Residues (PPR) concludes that the strength of the evidence suggests that a mode of action other than genotoxicity is involved in the occurrence of nasal turbinate tumours observed in the rat carcinogenicity studies. While the mode of action could be relevant to humans, it is extremely

unlikely that concentrations of the active metabolite would be achieved to initiate the chain of events terminating in cancer.

ASSESSMENT QUESTION 2

Question 2: Is the information presented for the metabolites listed above sufficient to demonstrate that they are not relevant ?

2.1. Introduction

In responding to this question, the PPR Panel took notice of "Guidance Document on the Assessment of the Relevance of Metabolites in Groundwater of Substances Regulated under Council Directive 91/414/EEC (Sanco/221/2000–rev.10–final, 25 February 2003)". The opinion of the PPR Panel is based on the available experimental data obtained with the significant, aerobic soil metabolites of alachlor that address the headings listed in this document:

- Screening for biological activity
- Screening for genotoxicity
- Screening for toxicity

It has been brought to the attention of the PPR Panel that two additional alachlor metabolites, notably alachlor *t*-oxanilic acid (metabolite 70) and metabolite 39, that were not mentioned in the background delivered by the Commission, may exceed significantly 0.1 µg/l level in groundwater.

2.2. Screening for biological activity

Data on the biological activity of metabolites 65, 85, 54, 25, 76 and, 51 of alachlor are available from two field studies, which permit this issue to be addressed. In these studies, eight warm season plant species and eight cool season plant species were tested (i.e., conditions related to the growing of maize and soybean on one hand and to the growing of wheat and oilseed rape on the other). The dose rate tested was 3.36 kg/ha, which is in excess of the dose of metabolite that might be anticipated from the metabolism of alachlor in field conditions. None of the tested metabolites had any biologically relevant effect on terrestrial plant species in either study (Prosch, 2001; Moran, 2002).

2.3. Screening for genotoxicity

Relevant metabolites are to be screened for their genotoxicity by at least the following *in vitro* tests: Ames' test, gene mutation test with mammalian cells and a chromosomal aberration test. Should any of these give equivocal results, then they should be tested by *in vivo* experiments. Where data on DNA interactions and other genotoxicity studies exist for the six listed soil metabolites, they are negative (Table 5). Only for the *t*-sulphonic acid metabolite, (65), is there a study *in vivo*: a mouse bone marrow micronucleus induction test. In addition, this metabolite (65) and the *t*-sulphinylacetic acid metabolite (54) have been tested for gene mutation induction in bacteria and mammalian cells *in vitro* and for clastogenicity in mammalian cells *in vitro*. It is considered that the level of testing exercised is adequate with regard to metabolites (65) and (54).

Of the remaining four soil metabolites of concern, the *t*-methylsulphoxide metabolite (25) has only been tested in *S. typhimurium* TA100 and TA98, although with negative results. Direct testing is therefore inadequate. Although metabolite 25 is a product of alachlor in rats in the absence of information on the extent of formation of this metabolite in rats, the PPR Panel was unable to conclude that genotoxicity testing of metabolite 25 was adequate.

While the *s*-sulphonic acid metabolite (85) has not been tested at all, it was argued in the dossier that it is structurally sufficientlysimilar to the *t*-sulphonic acid metabolite (65) - which has been well tested and has shown no mutagenic activity - as to provide sufficient confidence that (85) also is likely to be non-mutagenic. While this may appear to be a reasonable position,

as noted in the next section there may be toxicological differences between these metabolites, metabolite 85 having an acute LD_{50} value about one-quarter that of metabolite 65.

Similarly, the *s*-hydroxyalachlor metabolite (76) has not been tested at all, but it has been argued that it is structurally similar to the *t*-hydroxyalachlor metabolite (39). This metabolite (39) is included in the Table 6 to provide information, but is not a metabolite of concern and gave negative results in three assays *in vitro*. While it might be predicted that metabolite (76) would also give negative results in these assays, it has a possibly reactive hydrogen atom bonded to the nitrogen. Metabolite 76 is also similar to metabolite 51.

In the case of *s*-norchloroalachlor metabolite (51), recourse has been made to *t*-norchloracetochlor for comparison (Ashby *et al.*, 1996) This compound is referred to as the des-chloro-analogue of acetochlor that, like (51), lacks the chlorine atom of the parent compound, but unlike metabolite 51 does not possess a possibly reactive hydrogen bonded to the nitrogen atom; however, there is no information available regarding the activity of metabolite 51 in tests with mammalian cells for the induction of either gene mutations or chromosomal aberrations. Testing is therefore inadequate with this metabolite.

Table 6: Genotoxicity data on aerobic soil metabolites of alachlor predicted to occur at toxicologically significant concentrations

Assay			Result	Reference
S-acid class				
***t*-sulphonic acid (65): major in soil, groundwater PEC > 0.1 μg/L**				
In vitro	Gene mutation	Ames test in *S. typhimurium* strains	Negative	Kier, 1984 (IIA, 5.8.1/11)
	Gene mutation	Mouse lymphoma/TK test	Negative	Cifone, 2000 (IIA, 5.8.1/22)
	Chromosome aberrations	Cytogenetic test in human lymphocytes	Negative	Murli, 2000 (IIA, 5.8.1/26)
In vivo	Chromosome aberrations	Micronucleus test in mouse bone marrow	Negative	Stegeman *et al.*, 1995 (IIA, 5.8.1/32)
***s*-sulphonic acid (85): minor in soil, groundwater PEC > 0.1 μg/L**				
*Data might be extrapolated from its N-alkylated structural analogue, alachlor t-sulphonic acid (**65**).*				
***t*-sulphinylacetic acid (54): minor in soil, groundwater PEC > 0.1μg/L**				
In vitro	Gene mutation	Ames test in *S. typhimurium* and *E. coli* strains.	Negative	Stankowski, 2001 (IIA, 5.8.1/33)
	Gene mutation	Mouse lymphoma/TK test	Negative	Cifone, 2000 (IIA, 5.8.1/21)
	Chromosome aberrations	Cytogenetic test in human lymphocytes	Negative	Murli, 2000 (IIA, 5.8.1/28)
S-methyl class				
t-methylsulphoxide (25): very minor in soil, groundwater PEC > 0.1 μg/L				

Assay			Result	Reference
In vitro	Gene mutation	Ames test in *S. typhimurium* TA98 and TA100 strains	Negative	Kier, 1985 (IIA, 5.8.1/12)
Alachlor class				
***t*-hydroxyalachlor (39): very minor in soil (Here for comparison with 76), groundwater PEC > 0.1 µg/L**				
	Gene mutation	Ames test in *S. typhimurium* strains	Negative	Kier, 1984 (IIA, 5.8.1/10)
In vitro	Gene mutation	CHO/HPRT test	Negative	Stankowski, 2001 (IIA, 5.8.1/38)
	Chromosome aberrations	Cytogenetic test in human lymphocytes	Negative	Murli, 2001 (IIA, 5.8.1/37)
***s*-hydroxyalachlor (76): very minor in soil, groundwater PEC> 0.1 µg/L**				
*Data might be extrapolated from its N-alkylated structural analogue, t-hydroxyalachlor (**39**).*				
***s*-norchloroalachlor (51): very minor in soil, groundwater PEC > 0.1 µg/L**				
In vitro	*Chromosome aberrations*	*Cytogenetics test in human lymphocytes*	*Negative*	*Bridged from **acetochlor** t-NCA study by Ashby et al., 1996, (Monsanto IIA, 5.4/34; Human & Exp. Toxicol. 15: 702-735 - Table 7)*

2.4. Screening for toxicity

Metabolites are considered "relevant" if their toxicological properties lead to a classification as toxic or very toxic (T or T+) according to Directive 67/548/EEC. The available toxicity data are summarised in Table 7.

Table 7: Toxicity data on aerobic soil metabolites of alachlor predicted to occur at toxicologically significant concentrations

Assay		Result (mg/kg bw or mg/kg bw/day)	Reference
S-acid class			
***t*-sulphonic acid (65): major in soil, groundwater PEC > 0.1 µg/L**			
Oral LD_{50}	Rat	> 6000	Bonette, 1993 (Monsanto report report SB-92-131)
90-day feeding	Rat	NOAEL 157-207	Siglin, 1993 (Monsanto report SB-92-383)
Developmental toxicity	Rat	Parental NOAEL 1000 Developmental NOAEL 1000	Holson, 1995 (Monsanto report WI-95-068)

Assay		Result (mg/kg bw or mg/kg bw/day)	Reference
PCNA staining (nasal turbinates)	Rat	Negative at 2000	
s-sulphonic acid (85): minor in soil, groundwater PEC > 0.1 μg/L			
Oral LD_{50}	Rat	1548	Blaszcak, 1993 (Monsanto report PL-94-191)
90-day feeding	Rat	No data	
Developmental toxicity	Rat	No data	
t-sulphinylacetic acid (54): minor in soil, groundwater PEC > 0.1μg/L			
Oral LD_{50}	Rat	> 5000	Blaszcak, 1993 (Monsanto report PL-94-192)
28-day feeding	Rat	738-776	Stout & Thake, 2000 (Monsanto report MSL-16608)
90-day feeding	Rat	240-296	Bechtel *et al*., 2001 (Monsanto report MSL-17122)
Developmental toxicity	Rat	No data	
S-methyl class			
t-methylsulphoxide (25): very minor in soil, groundwater PEC > 0.1 μg/L			
		No data	
Alachlor class			
s-hydroxyalachlor (76): very minor in soil, groundwater PEC > 0.1 μg/L			
		No data	
s-norchloroalachlor (51): very minor in soil, groundwater PEC > 0.1 μg/L)			
		No data	

None of the tested metabolites was classified as toxic or very toxic.

Studies that have been conducted with metabolite 65, the alachlor *t*-sulphonic acid, show that it is very poorly absorbed and excreted much more quickly than alachlor, with only minimal metabolism. In contrast to alachlor, it does not accumulate in nasal tissues. When administered to rats in a 91-day study (Siglin, 1993), *t*-sulphonic acid (65) produced adverse effects only at the highest tested dose (10000 ppm, equivalent to 896 and 1108 mg/kg bw/day in males and females, respectively). No alachlor-related changes in thyroid weights or pathology were noted. In the absence of toxicity in these tissues, a neoplastic response secondary to target organ toxicity seems unlikely.

In a study by Hotz (1995) alachlor *t*-sulphonic acid (65) did not show any increase in PCNA[8] staining of the olfactory septum or turbinates of male rats administered 2000 ppm alachlor *t*-sulphonic acid (65) in the above-mentioned 91-day study (Siglin, 1993), as compared with those of rats from the control group, suggesting that alachlor *t*-sulphonic acid (65) did not induce an

[8] PCNA : Proliferating Cell Nuclear Antigen

increase in cell proliferation. Given that, in the case of alachlor, the central process in the formation of nasal tumours is local regenerative cell proliferation after the death of cells whose structure and function is disturbed by protein binding of 3,5-diethylbenzoquinone 4-imine, the absence of proliferation in nasal tissues of alachlor *t*-sulphonic acid (65)-treated rats suggests that no oncogenic potential exists for this metabolite in nasal tissues.
Study of developmental toxicity resulted in NOAEL values for both parental toxicity and developmental toxicity of 1000 mg/kg bw. All of these data indicate that metabolite 65 has been tested adequately.

Of the remaining metabolites, *t*-sulphinylacetic acid (54) also shows no evidence of significant toxicity in acute and repeated dose studies of up to 90 days duration. This also has been adequately tested, although not so thoroughly as metabolite 65.

The *s*-sulphonic acid metabolite (85) has only been tested in an acute oral toxicity assay. Arguments were presented in the dossier for toxicity in other assays similar to metabolite 65. However, it is noted that the LD_{50} value was 1548 mg/kg bw for 85, whereas it was > 6000 mg/kg bw for metabolite 65. Although the acute toxicity of 85 remains low, it is clearly greater than for 65, suggesting that close comparisons should not be drawn.

There are insufficient toxicological data for metabolites 76 and 51. In the case of metabolite 25, it has most probably been adequately tested because it is a metabolite of alachlor found in the urine of rats.

CONCLUSIONS AND RECOMMENDATIONS

The Scientific Panel on Plant health, Plant protection products and their Residues (PPR) concludes on the basis of the reasons stated above that metabolites 65, 54 and 25 have been adequately tested for toxicity, but the toxicity database is inadequate in the case of the soil metabolites 85, 76 and 51. The genotoxicity database is also inadequate for soil metabolites 85, 76 and 51. For metabolite 25 the PPR Panel was unable to conclude that genotoxicity testing was adequate. It is concluded that the information presented for metabolites 65 and 54 is sufficient to demonstrate that they are not relevant; a similar conclusion cannot be reached for metabolites 85, 76, 51 and 25.

DOCUMENTATION PROVIDED TO EFSA

1. Letter from Mr A. Checchi-Lang from the Health & Consumer Protection Directorate-General requesting a consultation EFSA on alachlor, with ref. E1/DVB D/510337(04), 23 March 2004.
2. Guidance document (SANCO/221/2000 rev 10 final 25 February 2003 on the assessment of the relevance of metabolites in groundwater of substances regulated under Council Directive 91/414/EEC. pp 1-14.
3. Scientific Committee on Plants (SCP) opinion on the use of alachlor as an herbicide, adopted on 25 October 1988, pp 53-77.
4. Draft Assessment Report on alachlor, annex B, Volume III, Chapter 5: Toxicology and metabolism, April 1999, pp 134-413.
5. Addendum to the Draft Assessment Report on alachlor, annex B, Volume III Chapter 6, Toxicology and metabolism. May 2001, pp 1-266.

6. Addendum to the Draft Assessment Report on alachlor, annex B, Volume III Chapter 1, Identity. May 2001, pp 1-266.
7. Addendum to the Draft Assessment Report on alachlor, Volume I, Relevant metabolites, July 2003, pp 1-22.
8. Addendum to the Draft Assessment Report on alachlor, annex B, Volume III, Chapter 5: Toxicology and metabolism, July 2003, pp 1-75.
9. Addendum to the Draft Assessment Report on alachlor, annex B, Volume III Chapter 6, Toxicology and metabolism. November 2001, pp 4-27.
10. Report summary of the Ministerio de Agricultura, Pesca y Alimentacion on the available informations on metabolites of alachlor, October 2003, pp 1-17.
11. Belgian comments on the Addendum to annex B 6, 30 October 2001, pp 1-2.
12. German comments on the Addendum of November 2001, section toxicology, 30 October 2001, pp 1-2.
13. Danish comments on the draft report of April 1999, impact on human health and animal health, 08 January 2001, pp 1-2.
14. Danish comments on the toxicology of alachlor for evaluation meeting, 22 August 2003, p 1.
15. An evaluation of the genotoxic and carcinogenic potential of alachlor and its environment–Monsanto response to the tripartite meeting on alachlor held in Madrid, Spain on 19 December 2003, 11 February 2004, pp 1-89.
16. Brewster, D.W. & Holtz, K.J. 1991 A study of the effect of alachlor on cell proliferation in specific tissues of the rat and mouse. Annex II 5.8.2/02. Monsanto report MSL-10744 (ML-88-23, ML-90-422, ML-90-423/EHL 87112, 90059, 90114), pp 1-95. Unpublished data.
17. Brewster, D.W. & Holtz, K.J. 1991 A study of the effect of alachlor on cell proliferation in specific tissues of the rat and mouse. Annex II 5.8.2/02. Monsanto report MSL-10744 (ML-88-23, ML-90-422, ML-90-423/EHL 87112, 90059, 90114), pp 1-95. Unpublished data.
18. Coleman, S., Liu, S., Linderman, R., Hodgson, E. & Rose, R.L. 1999 *In vitro* metabolism of alachlor by human liver microsomes and human cytochrome P450 isoforms. Elsevier Chemico-biological Interactions, pp27-39
19. Coleman, S., Linderman, R., Hodgson, E. & Rose, R.L. 2000 Comparative metabolism of chloroacetamide herbicides and selected metabolites in human and rat liver microsomes. *Environmental Health Perspectives*, 108, 12, 1151-1157.
20. Feng, P. & Patanella, J.E. 1988. *In vitro* metabolism of alachlor by rat and mouse liver and nasal enzyme. Annex II 5.1/41. Monsanto report MSL-7168 (202400), pp 1-30. Unpublished data.
21. Green, T., Lee, R., Moore, R.B., Ashby, J., Willis, G.A., Lund, V.J. & Calpp, M.J.L. 2000. Acetochlor – induced rata nasal tumors : further studies on the mode of action and relevance to humans. Annex II 5.1/45. *Regulatory Toxicology and Pharmacology* 32, 127-133.
22. Green, T. 2001. The *in vitro* metabolism of the sulphoxide metabolite of alachlor in rat and human nasal tissues. Annex II 5.1/44. Monsanto report CTL/R/1499, ZE-2000-159, pp 1-28. Unpublished data.
23. Green, T. 2001. Supplement to the *in vitro* metabolism of the sulphoxide metabolite of alachlor in rat and human nasal tissues. Annex II 5.1/44 bis. Monsanto report CTL/024408, n°ZE-2000-159 (addendum), pp 1-10. Unpublished data.

24. Heydens W., Wilson A., Kier L., Lau H., Thake D. & Martens M. 1999. An evaluation of the carcinogenic potential of the herbicide alachlor to man. *Human and experimental toxicology*, 18, 363-391.

25. Heydens W., Wilson A., Kraus L., Hopkins I & Hotz K. 2000. Ethane sulfonate metabolite of alachlor: assessment of oncogenic potential based on metabolic and mechanistic considerations. *Toxicological sciences*, 55, 36-43.

26. Kraus, L.J., Hopkins, W.E. , Kinnett, M.L. & Wilson, A.G.E. 1995. Elimination, absorption, tissue distribution and metabolism of alachlor ethane sulfonate (MON 5775) in Long-Evans rats following oral administration. Annex II 5.8.1/31. Monsanto report MSL-14489, PL-95-066, EHL 95031, pp 1-49. Unpublished data.

27. Lau, H. Asbury, K.J., Hopkins, W.E. & Wilson A.G.E. 1994. Alachlor and metabolites. *In vitro* metabolism of Alachlor, 2,6-Diethyl-2-Methylthioacetanilide by rat and human nasal turbinates and liver. Annex II 5.1/36. Monsanto report MSL-13837 (ML-93-14/EHL 92214), pp 1-39. Unpublished data.

28. Li, A.A, Asbury, K.J. & Hopkins, W.E. 1990. *In vitro* metabolism of alachlor, alachlor Secundary Methylsulfide (2,6-Diethyl-2methylthioacetanilide), & 2,6-Diethylaniline by rat and monkey nasal turbinate tissue. Annex II 5.1/43. Monsanto report MSL-10729 (ML-90-428/EHL 90160), pp 1-27. Unpublished data.

29. McClanahan, R.H., 1998. Metabolism of alachlor methyl sulphide in Long-Evans rats. Annex II 5.1/29. Monsanto report MSL-7585 (202400) pp 1-41. Unpublished data.

30. Patanella, J.E., Feng, P., & Wratten, S.J. 1985. *In vitro* metabolism of alachlor by rat, mouse and monkey liver and kidney homogenates. Annex II 5.1/37. Monsanto report MSL-4915 (7824), pp 1-117. Unpublished data.

31. Patanella, J.E. & Feng, P. 1987. *In vitro* metabolism of alachlor by rat liver, kidney, lung, nasal and stomach homogenates. Annex II 5.1/40. Monsanto report MSL-6677 (202476), pp 1-50. Unpublished data.

32. Ridley, W.P., Warren, J. & Mueller, J.E. 1986. A study of the *in vitro* liver slice metabolism of alachlor in the male rat, mouse and monkey. Annex II 5.1/38. Monsanto report MSL-5392 (ML-84-195/EHL 840079, 840080), pp 1-65. Unpublished data.

33. Stout, L. D. 1983. Chronic study of alachlor administered in feed to Long-Evans rats (2 volumes.) Annex 5.5/03. Monsanto report MSL-3284 (ML-80-186/ehl 800218.) Addendum Evaluation of nasal cavity of make and female rats, Pathology summary (October 12, 1984), pp 1-866. Unpublished data.

34. More Monsanto reports were available, which are listed in the following reference list.

REFERENCES

Aguirrezabala-ga, I., Santamaría, I. and Comendador, M.A. (1994) The *w/w+* SMART is a useful tool for the evaluation of pesticides. *Mutagenesis,* 9, 341-346.

Arnold, D. (1972) Mutagenicity study with Lasso in albino mice. Mosanto report BTL-72-11, unpublished data.

Asbury, K.J., Lau, H.H.S., Hopkins, W.E. & Wilson, A.G.E. (1994) *In vitro* metabolism of alachlor, 2,6-diethyl-2-methylthioacetanilide (alachlor secondary sulfide), alachlor sec-amide, and 2,6-diethylaniline by rat and human nasal turbinates and liver. Monsanto report MSL-13837, unpublished data.

Asbury, K.J., Lau, H.H.S. & Wilson, A.G.E. (1995) Alachlor and its metabolites: A study of the effect of alachlor and selected metabolites on cytotoxicity markers in nasal tissue of the Long-Evans rat. Monsanto report MSL-14187, unpublished data.

Ashby, J, Kier, L, Wilson A.G.E., Green T, Lefevre PA, Tinwell H, Willis GA, Heydens WF & Clapp MJL (1996). Evaluation of the potential carcinogenicity and genetic toxicity to humans of the herbicide acetochlor. *Hum. Exp. Toxicol.,* 15, 702-735.

Bechtel, C.L. & Thake, D.C. (2001) Three month study of MON 5768 administered by dietary admix to rats. Monsanto report MSL-17122, unpublished data.

Blaszcak, D. L. (1993a) Acute oral toxicity in rats with MON 5767. Monsanto report PL-94-191, unpublished data.

Blaszcak, D. L. (1993b) Acute oral toxicity in rats with MON 5768. Monsanto report PL-94-192, unpublished data.

Bofanti, M., Taverna, P., Chiapetta, L., Villa, P, D'Incalci, M., Bagnati, R. & Fanello, R. (1992) DNA damage induced by alachlor after *in vitro* activation by rat hepatocytes. *Toxicology,* 72, 207-219.

Bonette, A. (1993) Monsanto report SB-92-131, IIA, 5.5.1/05; unpublished data.

Burman, D.M., Shertzer, H.G., Senft, A.P., Dalton, T.P. & Genter, M.B. (2003) Antioxidant perturbations in the olfactory mucosa of alachlor-treated rats. *Biochem. Pharmacol.*, 66, 1707-1715.

Chesters, G., Simeiman, G.V., Levy, J., Alhajjar, B.J., Fathulla, R.N. & Harkin, J.M. (1989). *Environm. Contam. Toxicol.*, 110, 1-74.

Chernousov, M.A., Stahl, R.C. & Carey, D.J. (1998) Schwann cells use a novel collagen-dependent mechanism for fibronectin fibril assembly. *J. Cell Sci.*, 111, 2763-2777.

Cifone, M.A. (2000a) L5178Y TK$^{+/-}$ Mouse lymphoma forward mutation assay with a confirmatory assay with MON 5775. Monsanto report CV-2000-97, unpublished data.

Cifone, M.A. (2000b) L5178Y TK$^{+/-}$ Mouse lymphoma forward mutation assay with a confirmatory assay with MON 5768. Monsanto report CV-2000-96, unpublished data.

Curtiss, S., Cabonce, M., Oakley, L. & Kier, L. (1995) Study of the effects of alachlor on cellular stress response genes in rat nasal turbinate tissue. Monsanto report 14116, unpublished data.

Daly, I. W. & Hogan, G.K. (1981a) Alachlor: an 18-month feeding study of alachlor in mice Monsanto report BD-77-413, unpublished data.

Daly, I. W. & Hogan, G.K. (1981b) Alachlor: a chronic feeding study in rats. Monsanto report BDN-77-421, unpublished data.

Enninga, I.C., Verspeek, C.M., Van Oort, G. & Van de Waart, E.J. (1987) Evaluation of the mutagenic activity of Alanex in an *in vitro* mammalian cell gene mutation test with L5178Y mouse lymphoma cells. Makteshim Agan report: NOTOX 0429/EL 121, unpublished data.

Erexon, G.L, Bryant M.F., Doer C.L., Kwanyuen P.G. and Kligerman A.D. (1993) Cytogenetic analysis of human peripheral blood lymphocytes exposed to alachlor *in vitro. Environ. Mol. Mutagen.*

Erexon G. L. (2001) Chromosomal observation *in vivo* in rat bone marrow cells using alachlor. Monsanto report CV-2001-12, unpublished data.

Farrow, M.G. & Cortina, T. (1984) Alachlor: *in vivo* bone marrow chromosome study in rats with alachlor. Monsanto report HL-83-165, unpublished data.

Feng, P.C.C. & Patanella, J.E. (1988) Identification of mercapturic acid pathway metabolites of alachlor formed by liver and kidney homogenates of rats, mice and monkeys. *Pest. Biochem. Physiol.*, 31, 84-90.

Feng, P.C.C. & Wratten, S.J. (1987) *In vitro* oxidation of 2,6-diethylaniline by rat liver microsomes. *J. Agri. Food Chem.*, 35, 491-496.

Feng, P.C.C., Wilson, A.G.E., McClanahan, R.H., Patanella, J.E. & Wratten, S.J. (1990) Metabolism of alachlor by rat and mouse liver and nasal turbinate tissues. *Drug Metab. Distr.*, 18, 373-377.

Flowers, L.J. (1987) CHO/HGPRT gene mutation assay with 2,6-Diethylaniline. Monsanto report MSL-7144, unpublished data.

Flowers, L.J. (1990) Micronucleus assay with CP101394. Monsanto report MSL-10163, unpublished data.

Genter, M.B., Burman, D.M., Dingeldein, M.W., Clough, I. & Bolon, B. (2000) Evolution of alachlor-induced nasal neoplasms in the Long-Evans rat. *Toxicol. Pathol.*, 28, 770-781

Genter MB, Burman DM, & Bolon B (2002) Progression of alachlor-induced olfactory mucosal tumours. *Int. J. Exp. Path.* 83, 303-307.

Georgian, L., Moraru, I., Draghicescu, T., Dinu, I & Ghizelea, G, (1983) Cytogenetic effects of alachlor and manozeb. *Mutation Res.*, 116, 341-348.

Godek, E.G. (1984) Alachlor: CHO/HGPRT mammalian cell forward genemutation assay. Monsanto report PK-83-249, unpublished data.

Hall, L.J. (1991) A study of the distribution and localization of dimethylaniline in rats and mice using whole-body autoradiography. Monsanto report MSL-11227, unpublished data.

Hall, L.J., Wilson, A.G.E. (1992) Comparison of the distribution and excretion of radiolabeled alachlor in the Sprague-Dawley, Fisher 344 and Long-Evans rat and golden Syrian hamster. Monsanto project ML-91-96, unpublished data.

Hall LJ, Wilson, A.G.E. (1993) A study of the distribution and localization of diethylaniline in rats and mice using whole-body autoradiography. Monsanto EHL Project 89081/ML-89-128, unpublished data.

Heydens, W.F., Wilson, A.G., Kier, L.D., Lau, H., Thake, D.C. & Martens, M.A. (1999) An evaluation of the carcinogenic potential of the herbicide alachlor to man. *Hum. Exp. Toxicol.*, 18, 363-391.

Holson, J.F. (1995) A developmental toxicity study of MON 5755 in rats. Monsanto report WI-95-068, unpublished data.

Hospelhorn, A.C, Martin, B.M. & Franzblau, C. (1992) Type IV collagen synthesis and accumulation in neonatal rat aortic smooth muscle cell cultures. *Matrix*, 12, 352-361.

Hotz, K.J. (1995) Effect of MON 5775 on Cell Proliferation in the Nasal Tissue of Male F-344 Rats. Monsanto report ML-95-070, unpublished data.

Jeffries, P. & Barea, J.M., (1994). Biochemical cycling and arbuscular mycorrhizas in the sustainability of plant-soil systems. *In:* Gianinazzi, S., Schuepp, H. (Eds.). Impact of Arbusuclar Mycorrhizas on Sustainable Agriculture and Natural Systems. Birkhauser Verlag, Basel, Switzerland, pp.101-115.

Kalcheim, C. Duksin, D., Bachar, E. & Vogel, Z. (1985) Collagen-stimulating factor from embryonic brain has ascorbate-like activity and stimulates prolyl hydroxylation in cultured muscle cells. *Eur. J. Biochem.*, 146, 227-232.

Kier, L.D. (1984 a) Ames/Salmonella mutagenicity assays of [N-(2,6-diethyl)phenyl-N-methoxymethyl]-2-amino-2-oxoethanesulfonic acid, sodium salt. Monsanto report MSL-3594, unpublished data.

Kier (1984 b) Ames/Salmonella mutagenicity assays of [N-(2,6-diethyl)phenyl-N-methoxymethyl]-2-(hydroxy)acetamide. Monsanto report MSL-3597, unpublished data.

Kier, L. D. (1985) Ames/Salmonella mutagenicity assays of synthesised metabolites (CP76095, CP76097, CP91431 and CP91432). Monsanto report MSL 4879, unpublished data.

Kier, L.D. (1992) Alachlor: rat bone marrow micronucleus assay of alachlor. Monsanto report EHL-91102, unpublished data.

Kier, L.D. & Stegeman, S.D. (1990) Ames/Salmonella mutagenicity assays of alachlor, 2',6'-diethyl-2-methylthioacetanilide (alachlor secondary sulfide) and 2,6-diethylaniline without S9 and with nasal turbinate S9s from rat, mouse and monkey. Monsanto report MSL-10728, unpublished data.

Kim, Y.R & Peterkofsky, B. (1997) Differential effects of ascorbate depletion and α,α'-dipyridyl treatment on the stability, but not on the secretion, of type IV collagen in differentiated F9 cells. *J. Cell Biochem*., 67, 338-352.

Kimmel, E.C., Casida, J.E. & Ruzo, L.O. (1986) Formadine insecticides and chloroacetanilide herbicides: disubstituted aniline and nitrosobenzenes as mammalian metabolites and bacterial mutagens. *J. Agric. Food Chem*., 34, 157-161.

Lau, H.H.S., Asbury, KJ & McClanahan RH (1995) Characteristics of covalent adducts formed with nasal tissue protein following dietary administration of 14C-alachlor to female Long-Evans rats. Annex II 5.82/03.1. Monsanto report n° MSL-14169 (ML-94-161/EHL 94064, pp1-74. Unpublished data

Leet, T., Acquavella, J., Lynch, C., Anne, M., Weiss, N.S., Vaughn, T. & Checkoway, H. (1996) Cancer incidence among alachlor manufacturing workers. *Amer. J. Ind. Med.*, 30, 300-306.

Li, A.A., Asbury, K.J., Hopkins, W.E., Feng, P.C.C. & Wilson, A.G.E. (1992) Metabolism of alachlor by rat and monkey liver and nasal turbinate tissue. *Drug Metab. Distr.*, 20, 616-618.

Lutz W.K., & Schlatter Ch., (1979) *In vivo* covalent binding of chemicals to DNA as a short-term test for carcinogenicity. *Arch. Toxicol. Suppl*. 2, 411-415.

Mehrsheikh, A. & Lau, H.H.S. (2000 a) Characterisation of alachlor binding to rhesus monkey nasal tissue. Monsanto report MSL-17253, unpublished data.

Mehrsheikh A. & Lau, H.H.S. (2000 b) Characterisation of alachlor binding to mouse nasal tissue. Monsanto report MSL-17252, unpublished data.

Meisner, L.F., Belluck, D.A. & Rlloff, B.D. (1992) Cytogenetic effects of alachlor and/or atrizine, *in vivo* and *in vitro*. *Environ. Mol. Mutagen*. 19, 77-82.

Mirkova, E. & Zaikov, K. (1986) Investigation of mutagenic action of alachlor with the microbial test system of Ames. *Khig. Zdraoveopay,* 29, 26-31.

Mirsalis, J.C. & Tyson, K. (1984) Alachlor: Evaluation of the potential of alachlor to induce unscheduled DNA synthesis in the *in vivo- in vitro* hepatocytes DNA repair assay. Monsanto report SR-83-293, unpublished data.

Moran, S.G. (2002) Monsanto report MSL-17972 , *in* Addendum to the Draft Assessment Report on alachlor, Volume I, Relevant metabolites, p 9, July 2003. unpublished data.

Moriya, M. , Ohta T, Watanabe K, Miyazawa T, Kato K, Shirasu Y. (1983) Further mutagenicity studies on pesticides in bacterial reversion assay systems. *Mutation Res.,* 116, 185-216.

Murli, H. (2000 a) Chromosomal aberrations in cultured human peripheral blood lymphocytes with MON 5775. Monsanto report CV-2000-100, unpublished data.

Murli, H. (2000 b) Chromosomal aberrations in cultured human peripheral blood lymphocytes with MON 5768. Monsanto report CV-2000-99, unpublished data.

Murli, H. (2001) Chromosomal aberration in cultured human oeripheral blood lymphocytes with MON 52707. Monsanto report CV-2001-018, unpublished data.

Njagi, G.D.E. & Gopalan, H.N.B. (1980) Mutagenicity testing of some selected food preservatives, herbicides and insecticides Ames test. *Bangladeshi J. Bot.*, 9, 141-146.

OECD (1982) Organisation for Economic Co-operation and Development. Environment monographs No. 76. Guidance document for the development of OECD guidelines for testing of chemicals. OECD, Paris, 1993, pp 3-62.

Paivio A., Jansen, B. & Becker, L.J. (1975) Inter-relationships among variables comprising amphipod production. *Hydrobiol.* 1, 71-74.

Plewa, M.J., Wagner,ED, Gentile, GJ, Gentile, JM. (1984) An evaluation of the genotoxic properties of herbicides following plant and animal activation. *Mutation Res.*, 136, 233-245.

Probst, G.S., McMahon, R.E., Hill, L.E., Thompson, C.Z., Epp, J.K. & Neal, S.B. (1981) Chemically-induced unscheduled DNA synthesis in primary rat hepatocytes: a comparison with bacterial mutagenicity using 218 compounds. *Environ. Mutagen.*, 3, 11-32.

Prosch, A. (2001) Monsanto report MSL-17143, *in* Addendum to the Draft Assessment Report on alachlor, Volume I, Relevant metabolites, p 9, July 2003, unpublished data.

Ribas, G., Frenzilli, G., Barale, R. & Marcos, R. (1995) Herbicide-induced DNA damage in human lymphocytes evaluated by the single-cell gel electrophoresis (SCGE) assay. *Mutation Res.*, 344, 41-54.

Ribas, G., Surrallés, J., Carbonell, E., Xamena, N., Creus, A. & Marcos, R. (1996) Genotoxicity of the herbicides alachlor and maleic hidrazide in cultured human lymphocytes. *Mutagenesis* 11, 221-227.

Ribelin WE, Wilson AGE (1985) Whole-body autoradiography studies on ^{14}C alachlor in rats, mice and monkeys. Monsanto report MSL-5270, unpublished data.

Rouloff, M.V. & Thake, D.C. (1994) Alachlor: oncogenicity study of alachlor administered in feed to CD-1® for 18 months. Monsanto report MSL-13847, unpublished data.

Shirasu, Y., Moniya M. & Ohta T. (1980) Alachlor: microbial mutagenicity study. Monsanto report ET-80-01-01, unpublished data.

Siglin, G. (1993) Subchronic (91 day drinking water) toxicity in rats with MON 5775. Monsanto report SB-92-383, unpublished data.

Stankowski, L. (2001a) *Salmonella-Escherichia coli/*Mammalian-microsome reverse mutation assay with a confirmatory assay with MON 5768. Monsanto report CV-2001-013, unpublished data.

Stankowski, L. (2001b) CHO HGPRT forward mutation assay with a confirmatory assay and duplicate cultures with MON52707. Monsanto report CV-2001-017, unpublished data.

Stegeman, S.D., Kier, L.D., Garrett, S.L. & Mc Adams, J.G. (1995 a) Mouse bone marrow micronucleus assay of alachlor. Monsanto report MSL-14446, unpublished data.

Stegeman, S.D., Kier, L.D., Garrett, S.L. & Mc Adams, J.G. (1995 b) Mouse bone marrow micronucleus assay of MON 5775. Monsanto report ML-95-069, unpublished data.

Stout, L. D. (1984) Alachlor: special chronic study of alachlor administered in feed to Long-Evans rats. Monsanto report EHL-800219, unpublished data.

Stout, L. D. & Thake, D.C. (2000) 28 day dietary toxicity study in rats with MON 5768. Monsanto report MSL-16608, unpublished data.

Surralés, J., Catalán, J., Creus, A., Norppa, H., Xamena, N. and Marcos, R. (1995) Micronuclei induced by alachlor, mitomycin-C and vinblastine in human lymphocytes: presence of centromeres and kinetochores and influence of staining technique. *Mutagenesis,* 10, 417-423.

Taningher, M., Terranova, MP, Airoldi L, Chiappetta & Parodi S. (1993) Lack of alachlor induced DNA damage as assayed in rodent liver by the alkaline elution test. *Toxicology*, 85, 117-122.

Tee, L.B., Davies, D.S., Seddon, C.E. & Boobis, A.R. (1987) Species differences in the hepatotoxicity of paracetamol are due to differences in the rate of conversion to its cytotoxic metabolite. *Biochem. Phramacol.*, 36, 1041-1052.

Torres, C., Ribas, G., Xamena, N., Creus, A. & Marcos, R. (1992) Genotoxicity of four herbicides in the Drosophila wing spot test. *Mutation Res.*, 280, 291-295.

Wetmore, B.A., Mitchell, A.D., Meyer, S.A. & Genter, M.B. (1999) Evidence for site-specific bioactivation of alachlor in the olfactory mucosa of the Long-Evans rat. *Toxicol. Sci.*, 49, 202-212.

Wilderman, A.G. & Nazar, R.N. (1982) Significance of plant metabolism in the mutagenicity and toxicity of pesticides. *Can. J. Genet. Cytol.*, 24, 437-449.

Wilson, A.G.E., Hall, L.J. (1988) A study of the distribution and localization of alachlor - methylsulfide in rats using whole-body autoradiography. Monsanto report MSL-8268, unpublished data.

Wratten, S.J., Fujiwara, H. & Solsten, R.T. (1987) Properties and decomposition of 2,6-diethylnitrosobenzene. *J. Agric. Food Chem.*, 35, 484-491.

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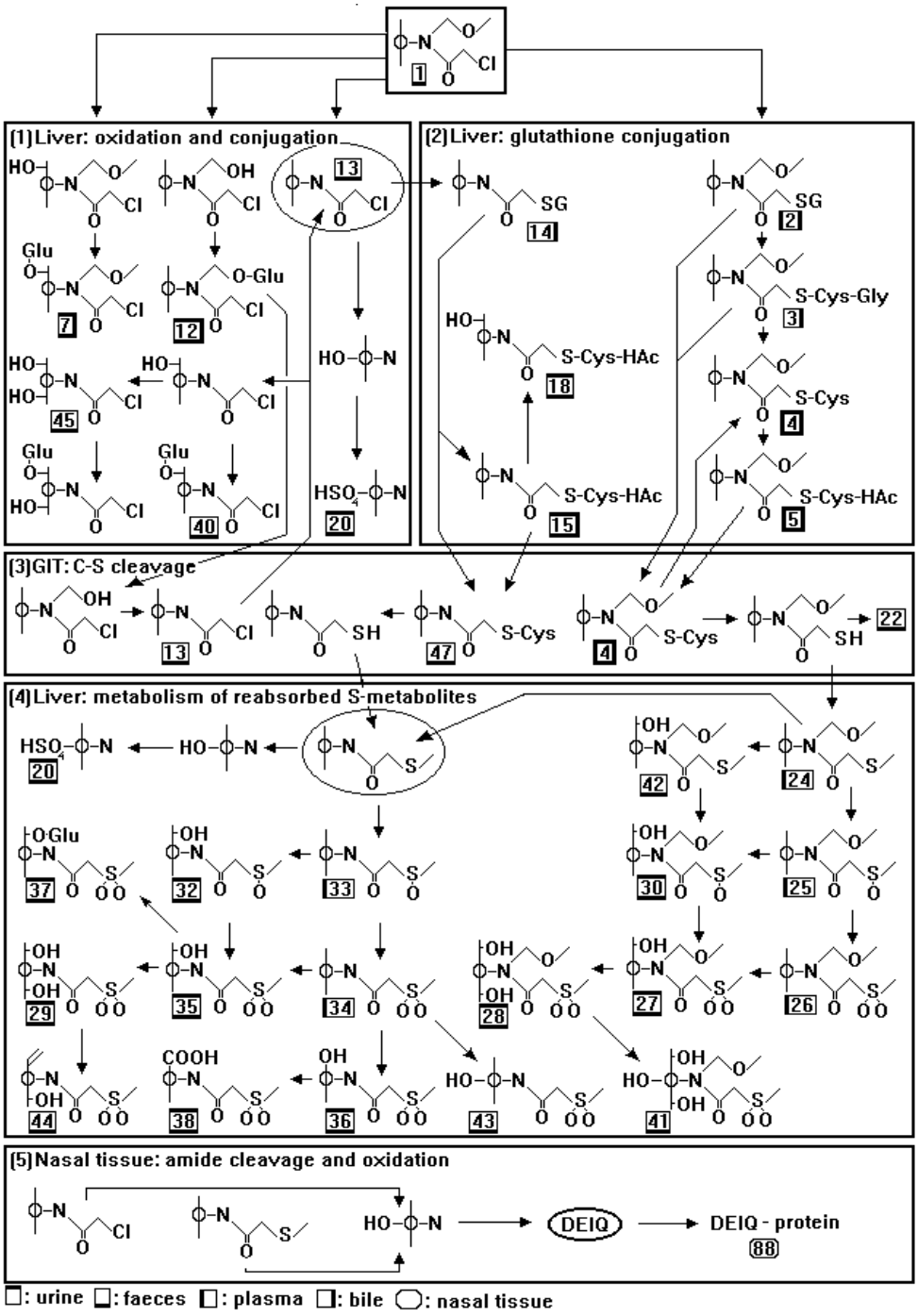
APPENDIX : FIGURES AND TABLES

Figure 1: The metabolic pathway of alachlor in rat



N.B.: The metabolite circled in section 4 is methyl sulphide secondary amide.

Figure 2: **The metabolic pathway of alachlor in mouse**

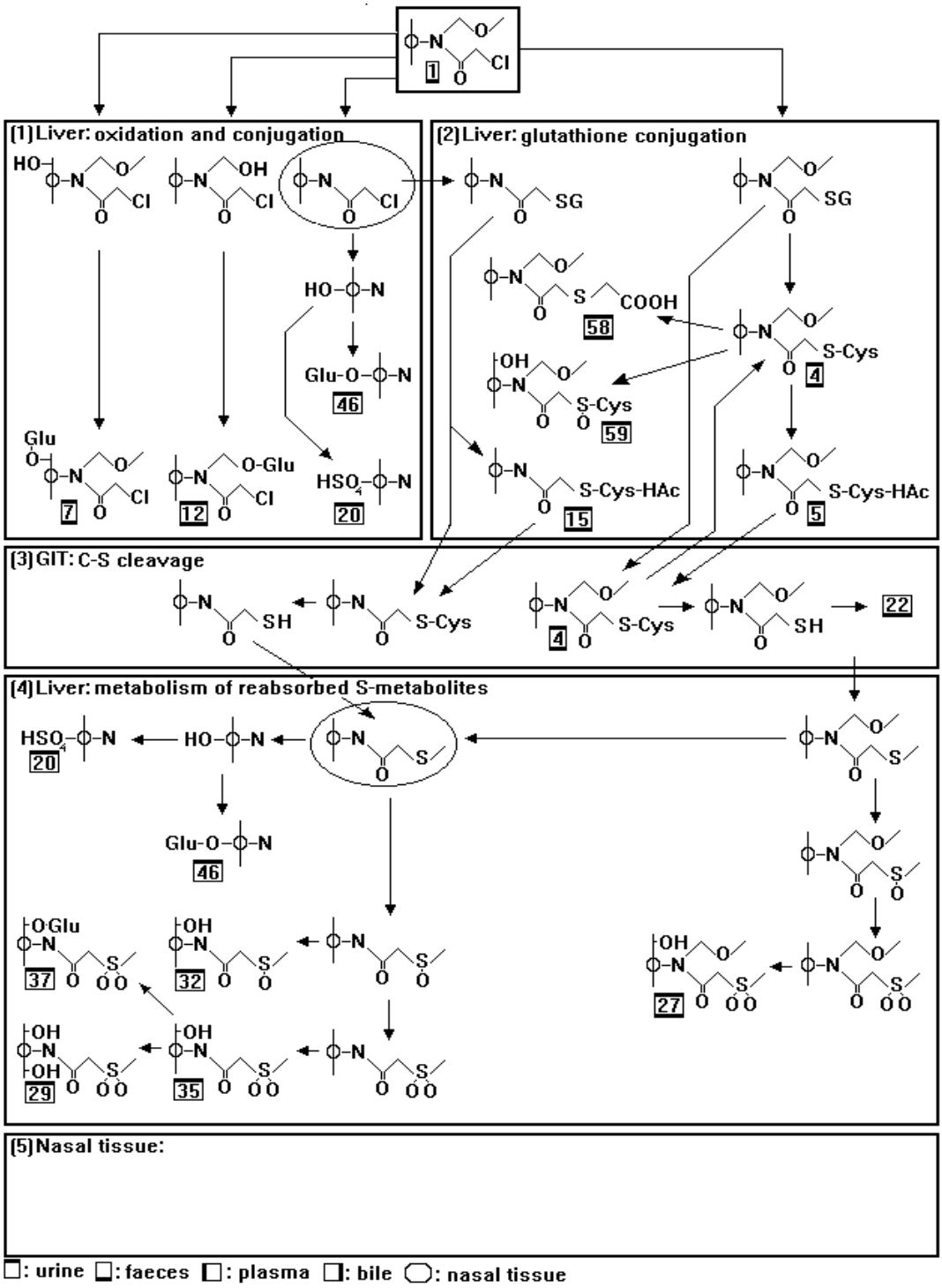
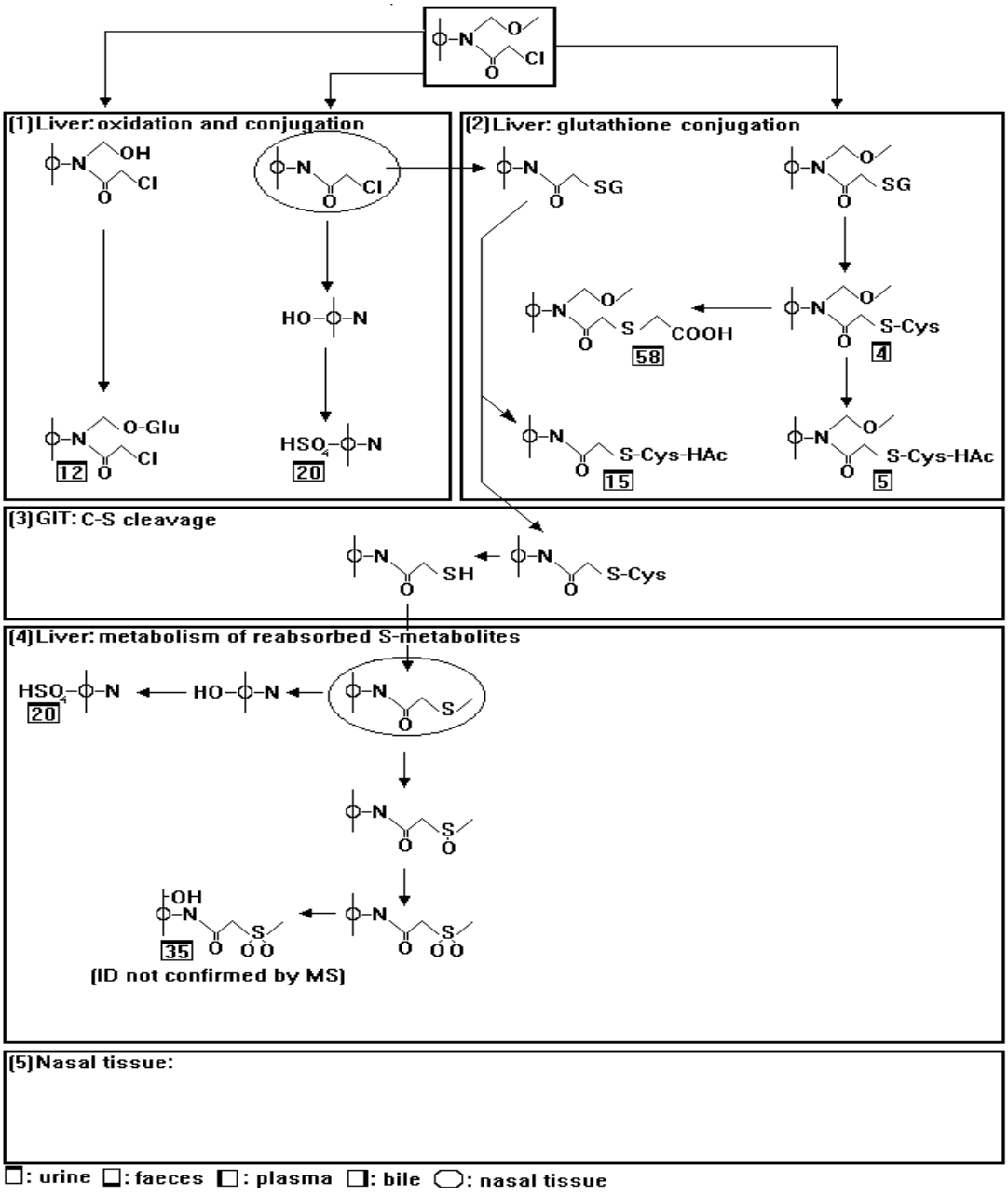


Figure 3: The metabolic pathway of alachlor in monkey



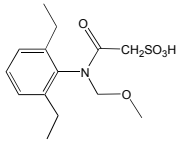
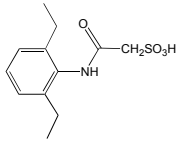
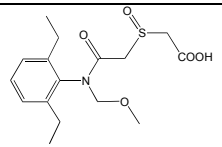
Appendix table 1: Genetic effects of alachlor

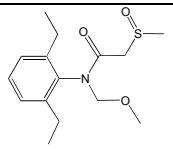
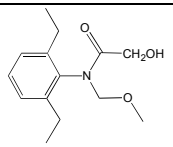
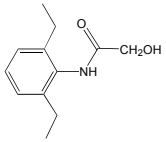
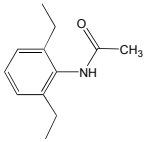
Test system	Result[a]		Test Material and Dose Range	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Salmonella typhimurium TA1535, TA1537, TA1538, TA98 & TA100 ; *Escherichia coli* WP2uvrA, reverse mutation	–	–	10 - 5000 µg/plate	Shirasu *et al.*, 1980
Salmonella typhimurium G46, C3076, D3052, TA1535, TA1537, TA1538, TA98 & TA100 ; *Escherichia coli* WP2 & WP2uvrA, reverse mutation	–	-	NG	Probst *et al.*, 1981
Salmonella typhimurium TA98 & TA100, reverse mutation	–	–	0.2 - 500 µg/plate	Wildeman & Nazar, 1982
Salmonella typhimurium TA1535, TA1537, TA1538, TA98 & TA100 ; *Escherichia coli* WP2uvrA, reverse mutation	–	–	NG	Moriya *et al.*, 1983
Salmonella typhimurium TA1538, reverse mutation	+	–	8 – 100 µg/plate	Mirkova & Zaikov, 1986
Salmonella typhimurium TA1535, TA1537, TA98 & TA100, reverse mutation	–	–	8 – 100 µg/plate	Mirkova & Zaikov, 1986
Salmonella typhimurium TA1537, TA98 & TA1978, reverse mutation	+ ?	+ ?	Commercial preparation 0.01 – 1.0%	Njagi & Gopalan, 1980
Salmonella typhimurium TA1535, TA1538, & TA100, reverse mutation	-	-	Commercial preparation 0.01 1.0%	Njagi & Gopalan, 1980
Salmonella typhimurium TA100, reverse mutation	-	+ (plant activation)	Commercial preparation, NG?	Plewa *et al.*, 1984
Salmonella typhimurium TA1535, TA1537, TA1538 & TA98, reverse mutation	-	–	Commercial preparation, NG	Plewa *et al.*, 1984
Salmonella typhimurium TA1535, TA1537, TA1538, TA98 & TA100 ; *Escherichia coli* WP2uvrA, reverse mutation	-	-	10 – 15000 µg/plate	Chesters *et al.*, 1989
Salmonella typhimurium TA1535, TA1537, TA98 & TA100, reverse mutation	–	- (S9 from nasal turbinates of rat, mouse,	5 – 5000 µg/plate	Kier & Stegeman, 1990

		monkey)		
Salmonella typhimurium TA100, reverse mutation	–	+ (S9 rat olfactory mucosa)	500 – 1500 µg/plate	Wetmore *et al.*, 1999
Drosophila melanogaster, somatic mutation (*white/white+)*	+		1 – 6 mM feed	Aguirrezabalaga *et al.*, 1994
Drosophila melanogaster, somatic mutation (*mwh+/+flr*^{3})	+		1 – 10 mM feed	Torres *et al.*, 1992
DNA strand breaks, rat hepatocytes *in vitro*	+?	NT	10 – 400 mM	Bonfanti *et al.*, 1992
Unscheduled DNA synthesis, male F344 rat primary hepatocytes *in vitro*	–	NT	0.5 – 10 nM ??	Probst *et al.*, 1981
Single-cell alkaline elution, human lymphocytes *in vitro*	+??	+??	5 – 20 µg/ml	Ribas *et al.*, 1995
Gene mutation, mouse lymphoma L5178Y cells, *tk* locus *in vitro*	-	-	1 – 100 µg/ml	Enninga *et al.* 1987 (not published)
Gene mutation, mouse lymphoma L5178Y cells, *tk* locus *in vitro*	-	+ (S9 rat olfactory mucosa)	1 – 5.6 µg/ml	Wetmore *et al.*, 1999
Gene mutation, CHO-K1-BH4 cells, hprt locus, in vitro	-	-	1-330 µg/ml	Godek et al., 1984
Chromosomal aberrations, human lymphocytes *in vitro*	+	??	1 – 40 µg/ml	Georgian *et al.*, 1983
Chromosomal aberrations, human lymphocytes *in vitro*	+	??	10 – 1000 µg/ml	Meisner *et al.*, 1992
Chromosomal aberrations, human lymphocytes *in vitro*	-	NT	2 – 40 mg/ml (or µg/ml??)	Erexon *et al.*, 1993
Chromosomal aberrations, human lymphocytes *in vitro*	+	??	1 – 20 µg/ml	Ribas *et al.*, 1996
Micronucleus induction, human lymphocytes *in vitro*	-	NT	2 – 40 mg/ml (or µg/ml??)	Erexon *et al.*, 1993
Micronucleus induction, human lymphocytes *in vitro*	+	??	1 – 20 µg/ml	Ribas *et al.*, 1996
Micronucleus induction, human lymphocytes *in vitro*	+	±	1 – 320 µg/ml	Surralés *et al.*, 1995
Covalent bidnig to DNA, nasal turbinates of male Fischer 344 rats *in vivo*	+		125 mg/kg bw x 1 (po)	Asbury & Wilson, 1994
Covalent bidnig to DNA, liver of male Fischer 344 rats *in vivo*	-		125 mg/kg bw x 1 (po)	Asbury & Wilson, 1994
Alkaline elution, liver cell nuclei from BALB/c mice *in vivo*	-		1 mmol/kg bw x 1 (ip); 0.5 mmol/kg bw x 5 (ip)	Taningher *et al.*, 1993
Alkaline elution, liver cell nuclei from Sprague-Dawley rats *in vivo*	-		2 mmol/kg bw x 1 (ip); 1 mmol/kg bw x 5 (ip); 1.5 mmol/kg bw x 1 (po)	Taningher *et al.*, 1993
Single-cell alkaline elution, nasal epithelium, male Alpk:ApfSD rats *in vivo*	-		1070 ppm, 7 days diet	Ashby *et al.*, 1997 (not published)

Unscheduled DNA synthesis, Fischer 344 rat liver cells *in vivo*	+	50 – 1000 mg/kg bw x 1 (po)	Mirasalis & Tyson, 1984
Unscheduled DNA synthesis, rat liver cells *in vivo*	w+	1000 mg/kg bw x 1 (po)	Chesters *et al.*, 19894
Unscheduled DNA synthesis, Fischer 344 rat liver cells *in vivo*	-	50 – 1000 mg/kg bw (po)	Hamilton, 1992
Chromosomal aberrations, bone marrow cells, Wistar rats	+	1250 – 5000 mg/kg bw x 1 (ip)	Georgian *et al.*, 1983
Chromosomal aberrations, bone marrow cells, Wistar rats	-	200 ppm, 280 days, diet	Georgian *et al.*, 1983
Chromosomal aberrations, bone marrow cells, Sprague-Dawley rats	-	100 – 1000 mg/kg bw x 1 (po)	Farrow & Cortina 1984
Chromosomal aberrations, bone marrow cells, male & female B6C3F1 mice	-	20 ppm, 30 or 90 days, drinking water	Meisner *et al.*, 1992
Chromosomal aberrations, bone marrow cells, SD rats	-	312.5, 625 & 1250 mg/kg bw x 1 (po)	Erexson, 2001
Micronucleus test, bone marrow cells, male & female Long-Evans rats *in vivo*	-	150 – 600 mg/kg bw × 1 (ip)	Kier, 1992 (not published)
Micronucleus test, bone marrow cells, male CD-1 mice *in vivo*	-	250 – 1000 mg/kg bw × 1 (po)	Stegeman *et al.*, 1995 (not published)
Dominant lethal effects, male albino mice	-	15 & 30 mg/kg bw x 1 (ip)	Arnold, 1972 (not published)

Appendix table 2: Structures of the six soil metabolites of alachlor predicted to be toxicologically important in ground water

Name (EU n°.)	Structure, Formula, MW	Chemical name	Synonyms
t-sulfonic acid (**65**)	 $C_{14}H_{21}NO_5S$ **315.42** **337.4 (Na salt)**	2-[(2,6-diethylphenyl) (methoxymethyl) amino]-2-oxo-ethanesulfonic acid (Sodium salt)	*t*-ESA *tert*-ESA *tert*-amide sulfonic acid CP 108065 (Na salt) MON 5775
s-sulfonic acid (**85**)	 $C_{12}H_{17}NO_4S$ **271.37**	2-[(2,6-diethylphenyl) amino]-2-oxo-ethanesulfonic acid	*s*-ESA *sec*-ESA *sec*-amide sulfonic acid CP 76082 (acid) MON 5767
t-sulfinylacetic acid (**54**)	 $C_{16}H_{23}NO_5S$ **341.43** **363.4 (Na salt, anh.)**	[[[2-[(2,6-diethylphenyl) (methoxymethyl)]amino-2-oxo]ethyl]sulfinyl] acetic acid (Sodium salt)	*t*-SAA *tert*-SAA *tert*-amide sulfinylacetic acid CP 147920 (Na salt) MON 5768 (Na salt)

Name (EU n°.)	**Structure,** **Formula,** **MW**	Chemical name	Synonyms
t-methylsulfoxide (**25**)	 $C_{15}H_{23}NO_3S$ **296.41**	N-(2,6-diethylphenyl)-N-(methoxymethyl)-2-(methylsulfinyl) acetamide	CP 76097
t-hydroxyalachlor (**39**) [included for comparison with (**76**)]	 $C_{14}H_{21}NO_3$ **251.3**	N-(2,6-diethylphenyl)-2-hydroxy-N-(methoxymethyl) acetamide	*t*-OH *tert*-OH *tert*-alachlor alcohol CP 51214 MON 52707
s-hydroxyalachlor (**76**)	 $C_{12}H_{17}NO_2$ **207.59**	N-(2,6-diethylphenyl)-2-hydroxy-acetamide	*s*-OH *sec*-OH CP 51215
s-norchloroalachlor (**51**)	 $C_{12}H_{17}NO$ **191.3**	N-(2,6-diethylphenyl)-acetamide	*s*-NCA *sec*-NCA *sec*-acetamide CP 58997