4.1.2.5.2 Respiratory tract

No data are available, although it can be predicted from its low chemical reactivity that nonylphenol is unlikely to be a respiratory allergen.

4.1.2.5.3 Summary of sensitisation

No human data are available. The results of several guinea pig maximisation tests suggest that nonylphenol does not have significant skin sensitising potential. No information on respiratory tract sensitisation is available, although it can be predicted from its low chemical reactivity that nonylphenol is unlikely to be a respiratory allergen.

4.1.2.6 Repeated dose toxicity

4.1.2.6.1 Animal data

There are no data for the inhalation or dermal routes. Two high-quality oral repeated dose studies in rats, of 28 and 90 days duration, have been conducted. The studies followed OECD guidelines and were in compliance with GLP. Additionally, the influence of nonylphenol on growth and cell proliferation and of the mammary gland has been investigated in the rat in a non-standard study involving subcutaneous administration.

In the 28-day study, groups of five male and five female Sprague-Dawley rats were exposed to nonylphenol via incorporation in the diet at nominal dose levels of 0, 25, 100 or 400 mg/kg/day (Hüls 1989). Clinical signs of toxicity, bodyweights and food consumption were recorded and towards the end of the study routine haematology, blood clinical chemistry and urinalysis examinations were made. A full necropsy was performed on all animals at termination. Adrenals, liver, kidneys and testes with epididymides were weighed and a limited range of major organs was examined microscopically.

There were no mortalities or treatment related clinical signs of toxicity. At 400 mg/kg/day, bodyweight gain was significantly reduced throughout the study, and by week four mean bodyweights were 26% and 13% less than the controls for males and females, respectively. The amount of food consumed and food utilisation was also reduced at 400 mg/kg/day for both sexes. For males only at 400 mg/kg/day there were slight differences in comparison with the controls for certain clinical chemistry parameters; urea and cholesterol levels were increased and glucose levels were reduced. Also, there were increases in the group mean relative kidney, liver and testes weights (all by about 20% compared with controls). Histopathological examination revealed hyaline droplet accumulation in the renal proximal tubules (an effect considered to be of no relevance to human health) and a minor vacuolation in the periportal hepatocytes for males at 400 mg/kg/day. Among the females at this level, there were no treatment-related changes in the organs.

For males and females at 25 and 100 mg/kg/day, there were no differences in any of the parameters examined that could be conclusively related to treatment. It should be noted that minor increases in comparison with the concurrent control group were reported for kidney, adrenal and liver weights and for the incidence of minimal hyaline droplet formation in the kidney among males at 25 and 100 mg/kg/day. However, all values were within the laboratory's historical control range (personal communication with study sponsor) and confirmatory changes were not seen for adrenal and liver weight or hyaline droplet formation in the 90-day study (see

below). Consequently these marginal changes could not be reliably attributed to nonylphenol treatment. Overall, this study identifies a NOAEL of 100 mg/kg/day for 28-day exposure.

In the 90-day study, groups of fifteen male and fifteen female Sprague-Dawley rats were exposed to nonylphenol via incorporation in the diet at concentrations of 0 (control), 200, 650 or 2000 ppm (Chemical Manufacturers Association 1997a, Cunny et al., 1997). Calculated nonylphenol intakes were about 0, 15, 50 and 140 mg/kg/day, respectively. Additionally, control and high dose satellite groups of ten animals of each sex were included; these were given a 28 day recovery period at the end of the 90-day exposure. Clinical signs of toxicity, bodyweights and food consumption were recorded and towards the end of the study routine haematology, blood clinical chemistry and ophthalmoscopy examinations were made. A full necropsy was performed on all animals at termination. A number of organs were weighed and histopathological examinations were conducted on a comprehensive range of organs and tissues. Also, oestrous cycles were monitored during week 8 and sperm motility, sperm number (in epididymis) and sperm morphology were evaluated at necropsy.

There were no treatment-related mortalities or clinical signs of toxicity. At 140 mg/kg/day only, there were adverse effects on bodyweight gain, the amount of food consumed and food utilisation throughout the dosing period for both males and females. At 90 days, the mean bodyweights for both sexes at this exposure level were about 7% less than the controls. In the satellite group, some recovery of bodyweight and food consumption values was seen after exposure was discontinued. Haematology and ophthalmoscopy findings and oestrous cycle patterns were not affected by treatment. There was no evidence of effects on spermatogenesis. However, one interesting clinical chemistry change was seen among females from the 140 mg/kg/day group. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were markedly elevated in two females, which correlated with some histopathological changes reported in the liver (see below).

At necropsy, no treatment-related macroscopic findings were reported. Among the males killed at 90 days, there was a dose-related increase in group mean absolute (by 6, 9 and 13%, relative to controls, at 15, 50 and 140 mg/kg/day respectively) and bodyweight-related (by 8, 11 and 24%, respectively) kidney weight. In the recovery group, the bodyweight-related kidney weight among males was also increased, although the effect was less marked. However, this organ weight increase could not be correlated with any clinical chemistry or histopathological change and consequently this finding was considered unlikely to be of toxicological significance, particularly at 15 and 50 mg/kg/day where magnitude of the change was small. Also, ovary weight was slightly decreased in females from the 140 mg/kg/day group, in comparison with the controls, at 90 days. In contrast, the weight of this organ was slightly increased in the recovery group. Again, this difference could not be correlated with any histopathological change which, together with the inconsistency between the findings for the main and satellite groups, makes the interpretation of this finding uncertain. Bodyweight-related liver weight was increased at 90 days only in males at 50 and 140 mg/kg/day and females at 140 mg/kg/day, by about 10% compared with controls. This was considered likely to be an adaptive rather than toxicological response.

The only noteworthy microscopic changes were seen in the kidneys and liver. Among males at 140 mg/kg/day in both the main and satellite groups there was a decrease in the occurrence of renal tubular hyaline droplets/globules in comparison with the control group. The biological significance of this change is uncertain. Also, a lack of correlation with the findings of the 28-day repeated dose study, in which an actual increase in the incidence of renal hyaline droplets occurred, casts doubt on whether these changes should be considered to be related to treatment. Slight or moderate individual hepatic cell necrosis was seen in three females at 140 mg/kg/day; two of the affected females also had raised serum ALT and AST. This provides evidence that the liver may be a target organ for nonylphenol toxicity, although this evidence is weak in view of the mild nature of response and small number of animals affected.

The renal histopathological findings have been reviewed by a pathologist not involved in the original investigation (Hard 1998), because of a lack of coherence between the results of this study and a multigeneration study summarised below (NTP 1997). An increased incidence of deposits of intratubular mineralisation in the P3 (straight) segment of the proximal tubule at the outer stripe of the outer medulla/inner stripe of outer medulla (OSOM/ISOM) junction was seen in males at 140 mg/kg/day; 11 out of 25 from this group were affected, compared with 1 out of 25 control males.

Overall, a NOAEL of about 50 mg/kg/day can be derived from this study. At 140 mg/kg day there were reductions in bodyweight gain, food consumption and food utilisation together with evidence of morphological changes in the liver and possibly kidneys.

Further information on repeated dose toxicity can be derived from a good-quality multigeneration study (NTP 1997, see section 4.1.2.9.2 for full details of this study, including information on any findings in reproductive organs). Groups of thirty male and thirty female Sprague-Dawley rats were exposed to nonylphenol via incorporation in the diet at concentrations of 0 (control) 200, 650 or 2000 ppm over three generations. Calculated nonylphenol intakes were, respectively, about 0, 15, 50 and 160 mg/kg/day during non-reproductive phases. The F_0 generation were exposed for 15 weeks, the F_1 and F_2 generations from soon after birth to about 20 weeks of age and the F3 generation from birth to about 8 weeks of age.

Evidence of general toxicity was seen in adults of all generations, although there were no treatment-related clinical signs, mortalities or adverse effects on food consumption. At 160 mg/kg/day, bodyweight gain was reduced in comparison with controls in adults across all generations, with the terminal bodyweights being about 10% lower than the controls. Similar reductions in bodyweight gain were also seen at 50 mg/kg/day in F_1 females, F_2 males and F_3 females. Relative kidney weights were increased at 50 and/or 160 mg/kg/day in adult males of the F_0, F_1 and F_2 generations and also at 160 mg/kg/day in F_1 adult females. Histopathological examination revealed an increase, although often without a convincing dose-response relationship, in the incidence of renal tubular degeneration and/or dilatation in adult males from all generations and all nonylphenol treated groups; similar findings were reported for adult females at 160 mg/kg/day in the F_1, F_2 and F_3 generations and at 15 and 50 mg/kg/day in the F_3 generation. These data are given in **Table 4.13.**

Table 4.13 Number of animals with histopathological abnormalities in the kidney (n=10)

Males

Generation	Finding	Dose level (mg/kg/day)			
		0	15	50	160
F_0	Renal tubule degeneration	1	3	5	5
	Renal tubule dilatation	0	1	0	0
F_1	Renal tubule degeneration	1	2	7	8
	Renal tubule dilatation	1	1	0	2
F_2	Renal tubule degeneration	3	6	6	6
	Renal tubule dilatation	1	2	0	4
F_3	Renal tubule degeneration	0	7	10	2
	Renal tubule dilatation	0	0	3	3

Females

Generation	Finding	Dose level (mg/kg/day)			
		0	15	50	160
F_0	Renal tubule degeneration	3	3	0	0
	Renal tubule dilatation	0	0	1	0
F_1	Renal tubule degeneration	0	1	1	6
	Renal tubule dilatation	0	0	0	3
F_2	Renal tubule degeneration	1	2	0	4
	Renal tubule dilatation	0	0	0	1
F_3	Renal tubule degeneration	0	8	9	7
	Renal tubule dilatation	0	0	1	1

It is difficult to decide for certain whether or not this increased incidence of renal tubular degeneration and/or dilatation is related to treatment because these changes were not seen to the same extent in the 90-day study, which was conducted using the same strain of rats, and because a dose-dependent trend was not apparent in all generations/sexes. The lack of concordance between the studies cannot be explained on the basis of a slightly longer exposure period in the multigeneration study because kidney effects were seen in the F_3 generation which was exposed for only 8 weeks, nor on the basis of *in utero* and neonatal exposure because the effect also occurred in the F_0 generation. Giving special emphasis to the fact that the increased incidence occurred consistently across all four generations in the multigeneration study, it is considered that this cannot be dismissed as background variation. Consequently, a conclusion has been drawn from this study that there is a LOAEL for repeated exposure of 15 mg/kg/day, based on histopathological changes in the kidneys; this value will be taken forward to the risk characterisation.

The renal histopathological findings have been reviewed by a pathologist not involved in the original investigation (Hard, 1998). The presence of renal lesions in all nonylphenol exposed groups was confirmed, as was the lack of a consistent dose-dependent trend in all generations. The

predominant renal lesions were described as tubular mineralisation at the OSOM/ISOM junction, cystic tubules surrounded by fibrosis, or granular cast formation at the OSOM/ISOM junction.

A briefly reported oral (gavage) study investigating the testicular toxicity of nonylphenol (de Jager et al., 1999a) is summarised in the toxicity to reproduction section. In this study, mortality was observed at 100 (the lowest dose level tested), 250 and 400 mg/kg/day; 3, 15 and 18, respectively, out of 20 animals in each group died during a 10-week dosing period. No further information on these mortalities is available. The presence of mortality at such dose levels contrasts with the findings of the dietary administration studies (Hüls, 1989; Chemical Manufacturers Association, 1997a; NTP, 1997). The differences can probably be accounted for by the method of administration; gavage dosing is likely to produce higher peak concentrations of nonylphenol in the blood than dietary administration.

The influence of nonylphenol on growth and cell proliferation and of the mammary gland has been investigated in rats of the Nobel strain in two studies using non-standard methods. The Nobel strain of rat is particularly sensitive to oestrogenic activity. In the original study, groups of six female juvenile rats were exposed to nonylphenol by the subcutaneous route for 11 days, administered via osmotic minipumps implanted in the dorsal cervical region (Colerangle and Roy, 1996). The dose levels were 0 (DMSO vehicle control), 0.01 and 7.12 mg/day (0.05 and 35.6 mg/kg/day, assuming a bodyweight of 200 g). An additional group received diethylstilbestrol (DES) at 0.01 mg/day (0.05 mg/kg/day) for 11 days by an unspecified route. At the end of the exposure period the rats were killed and the abdominal mammary glands removed for evaluation. Mammary gland growth was assessed by counting the number of mammary structures (terminal ducts, terminal end buds or lobules) and cells in 16 mm^2 areas of the mammary gland. Cell proliferation and cell-cycle kinetics were evaluated using immunohistochemical techniques (reaction with antiproliferating cell nuclear antigen (PCNA)) which allowed cells in S, G1 and G0 phases to be identified. The labelling index (LI, proportion of cells in S phase) growth fraction (GF, proportion of cells in the G1 or S phase) were calculated.

In the group receiving the highest dose of nonylphenol there was a 1.5-fold increase in the number of mammary structures and a 4-fold increase in the number cells/16 mm^2 area, compared with the vehicle control group. At the lowest level the number of structures was similar to the controls, but there was a 2-fold increase in the number of cells. DES caused a 6-fold increase in the number of cells. The LI was increased by 1.3 and 1.8 fold and GF by 1.2 and 2 fold in the nonylphenol low and high dose groups, respectively, in comparison with the vehicle control. DES had a much greater influence on the indices, with increases of 4 and 5 fold for the LI and GF. Cell cycle time was unchanged in the low dose group, slightly decreased (by about 10%) in the high dose group and markedly deceased (by more than half) in the DES group. This study shows that nonylphenol at dose levels of 0.05 and 35.6 mg/kg/day increases growth and proliferation activity in a dose-related manner in the mammary gland of the Nobel rat, although the effects at 0.05 mg/kg/day are marginal. The significance for human health of such a finding is unknown. Furthermore, the use of the subcutaneous route of administration and selection of the oestrogen-sensitive Nobel rat as the model casts doubts about the relevance of these findings to humans. Ashby and Odum (1998) draw attention to the fact that same positive control (DES) data reported for this study also appear in two other reports by Colerangle and Roy (1995 and 1997), and that the vehicle control data of the nonylphenol study is duplicated in the 1997 study. This raises some uncertainties as whether the control data were generated concurrently with the nonylphenol data and questions the validity of this study.

The Colerangle and Roy (1996) study was duplicated by Odum et al. (1999). Groups of ten female OVR^+ Noble rats were exposed to nonylphenol at dose levels of 0 (DMSO vehicle control), 0.073 or 53.2 mg/kg/day or DES at 0.076 mg/kg/day by the subcutaneous route for 11 days, administered via osmotic minipumps implanted in the dorsal cervical region. Mammary gland differentiation and mammary gland cell proliferation were assessed following similar methodology to Colerangle and Roy (1996), except that BRDU as well as PNCA staining was used (BRDU incorporation was considered to be a more sensitive and robust technique) and a more objective method was used to quantitate mammary gland changes. The quantitative determination of the numbers and areas of mammary gland structures showed no differences between the vehicle control and nonylphenol exposed groups, in contrast to the findings of the original study. DES, however, had a marked influence of the differentiation of mammary structures. Terminal ducts were completely absent and terminal end buds were present only in peripheral regions. Also, the numbers and areas of lobules were markedly increased in peripheral and central areas. The mammary gland cell proliferation assessment revealed, in comparison with the vehicle control group, no changes in the nonylphenol exposed groups, and a marked increase (about 4 fold in the lobules) in the DES group. This study shows the DES can induce growth and proliferation activity in the mammary gland of the Nobel rat, but failed to confirm the observation in the Colerangle and Roy (1996) study of such activity following nonylphenol exposure at similar dose levels.

4.1.2.6.2 Human data

The effects of nonylphenol exposure have not been evaluated in humans. There are two isolated case reports of leucoderma on the hands and forearms, with subsequent spreading to other areas, among Japanese workers exposed to alkaline detergents containing polyethylene alkylphenylether (Ikeda et al., 1970). The authors speculated that this might be caused by free octylphenol or nonylphenol, which were found to be present in the detergents. However, in the absence of corroborative reports from elsewhere, no firm conclusions regarding causality can be made.

4.1.2.6.3 Summary of repeated dose toxicity

No useful human data are available. In a multigeneration study in the rat involving oral exposure via the diet for up to 20 weeks, a LOAEL for repeated dose toxicity of 15 mg/kg/day was identified, based on histopathological changes in the kidneys (tubular degeneration or dilatation), although such changes were not apparent at this dose level in a 90-day dietary exposure rat study. At higher dose levels the liver may also be a target organ; minor histopathological changes in the liver (vacuolation in the periportal hepatocytes or occasional individual cell necrosis) were seen at doses of 140 mg/kg/day and above in some dietary studies. The oral toxicity of nonylphenol appears to be enhanced when dosed by gavage, with mortalities being reported at dose levels of 100 mg/kg/day and above. No studies involving dermal or inhalation exposure have been conducted. Nonylphenol has been reported to induce cell proliferation in the mammary gland of the Nobel rat following subcutaneous exposure at levels down to 0.05 mg/kg/day, but this finding could not be reproduced in a duplicate study; furthermore, there are doubts about the relevance of this finding to humans and regarding the validity of the original study.

4.1.2.7 Mutagenicity

Only data from *in vitro* test systems and animals are available.

4.1.2.7.1 Studies in vitro

Two pre-incubation bacterial mutagenicity (Ames) tests have been conducted. Both were negative. The first cannot be fully appraised because only a summary report is available (Hüls 1984). *Salmonella typhimurium* strains TA1537, TA 1538, TA 98 and TA 100 were exposed to nonylphenol at concentrations to 5000 µg/plate, both in the presence and absence of metabolic activation (Aroclor induced rat liver S9). The same *Salmonella* strains were used in the second study, together with *Escherichia coli* strain WP2urvA (Shimizu et al., 1985). Concentrations up to 100 µg/plate were tested, both in the presence and absence of metabolic activation (polychlorinated biphenyl induced rat liver S9), and toxicity was reported at the highest concentrations tested. A limitation of both studies is that the results of neither appeared to have been confirmed by a second independent experiment.

In a well-conducted *in vitro* mammalian cell gene mutation test, following OECD guideline 476 and in compliance with GLP, the potential for nonylphenol to induce mutations at the HPRT-locus was investigated in Chinese hamster V79 cells (Hüls, 1990). The exposure period was 5 hours, and a range of concentrations up to 2.5 µg/ml (without metabolic activation) or 1.25 µg/ml (with metabolic activation) were tested. At higher concentrations there was no cell survival. The results were confirmed by independent experiment. The test was negative.

4.1.2.7.2 Studies in vivo

Two micronucleus studies are available.

In the most recent study, conducted according to OECD guideline 474, groups of 5 male and 5 female NMRI strain mice received a single intraperitoneal dose of 50, 150 or 300 mg/kg (Hüls, 1999b). Appropriate positive (cyclophosphamide) and negative (vehicle) control groups were included. The highest treatment level was chosen as the maximum tolerated dose, based on the results of a preliminary study. Bone marrow was sampled 24 hours after treatment. There was a second sampling time of 48 hours for additional groups receiving either nonylphenol at 300 mg/kg or only the vehicle. Toxicity was elicited at 150 and 300 mg/kg, seen as clinical signs such as sedation, squatting posture, abnormal gait and piloerection. There was no consistent effect on the polychromatic to normochromatic erythrocyte (PCE/NCE) ratio. No increases in the frequency of micronucleated PCEs were seen in the nonylphenol exposed groups; thus the tests is considered to be negative. The anticipated response was seen in the positive control group. Although the PCE/NCE ratio was not affected, the fact that the study was conducted at the maximum tolerated dose and using the intraperitoneal route of administration, it can be presumed that exposure of the bone marrow to nonyl phenol was maximised. Accordingly, a high level of confidence can be given to this negative result.

An earlier micronucleus test was conducted using the oral route of administration (Hüls, 1988). In accordance with the OECD guideline, groups of five male and five female mice of the NMRI strain received a single oral dose of nonylphenol at 500 mg/kg. The dose level was chosen as the maximum tolerated dose. No evidence was presented to support this choice, but it is noted that it is greater that a reported oral LD_{50} of 307 mg/kg/day for mice. Appropriate positive and negative controls were included. Bone marrow was sampled at 18, 48 and 72 hours. There were no increases in the frequency of micronuclei at any of the sampling times and the test was declared negative. The PCE/NCE ratio was not affected by nonylphenol, which raises concerns about adequacy of exposure of the bone marrow to the test substance. The toxicokinetic information suggests that the systemic bioavailability of nonylphenol following oral administration is

restricted, which adds to this concern. Overall, because of doubts regarding the extent of exposure of the target tissue, only limited significance can be given to this negative result.

4.1.2.7.3 Summary of mutagenicity

No human data are available. Nonylphenol tested negative in two bacterial assays and an *in vitro* mammalian cell gene mutation assay. An *in vivo* micronucleus test, conducted using the intraperitoneal route, was negative. A second in vivo micronucleus test, which used the oral route, was also negative, although there were methodological weaknesses in this study. These results show that nonylphenol is not mutagenic.

4.1.2.8 Carcinogenicity

Carcinogenicity has not been studied directly in humans or animals. However, some information on the carcinogenic potential can be derived from other data. On the basis of the information currently available it is considered unlikely that that nonylphenol is mutagenic, so concerns for cancer caused by a genotoxic mechanism are low. Considering the potential for carcinogenicity by a non-genotoxic mechanism, no evidence of sustained cell proliferation or hyperplasia was seen in the standard repeated exposure toxicity studies. Nonylphenol has been reported to induce cell proliferation in the mammary gland of the Nobel rat following subcutaneous exposure at levels down to 0.05 mg/kg/day, but this finding could not be reproduced in a duplicated study; furthermore there are doubts about the relevance of this model to humans because of the route of exposure and sensitivity of the strain selected. Overall, there are low concerns for carcinogenicity by a non-genotoxic mechanism.

4.1.2.9 Toxicity to reproduction

Only data from animals or *in vitro* test systems are available.

4.1.2.9.1 Studies investigating oestrogenic activity

The oestrogenic activity of nonylphenol has been investigated in a number of studies using either recombinant yeast, oestrogen sensitive MCF-7 cells or a rodent uterotrophic assay response. None of these assays have been validated as an internationally accepted toxicity test method, although the MCF-7 and uterotrophic assays have been established for a number of years as standard assays for oestrogenic activity. It should be noted that the significance to human health of oestrogenic activity detected in these assays has yet to be established.

In vitro systems

4-Nonylphenol was one of a number of alkyl phenols tested in a yeast assay in a study which looked at the structural features important for oestrogenic activity in this chemical group (Routledge and Sumpter, 1997). The assay uses a recombinant strain of yeast (*Saccharomyces cerevisiae*) which contains an oestrogen-inducible expression system. In the presence of oestrogens a reporter gene (Lac-Z) encoding for the enzyme ß-galactosidase is expressed, which can be monitored by measuring a colour change reaction in the culture medium. The oestrogenic activity of the test substances was expressed as a potency relative to 17ß-oestradiol by comparing the molar concentrations required to produce the same response. 17ß-oestradiol was found to be about 30 000 times more potent than nonylphenol. Tamoxifen, an oestrogen antagonist known to act via the oestrogen receptor, was shown to inhibit the activity of the alkyl

phenols, demonstrating that the assay response was due to interaction with the oestrogen receptor.

The oestrogenic activity of nonylphenol has also been assessed in an *in vitro* assay involving oestrogen sensitive human breast tumour MCF-7 cells (Soto et al., 1991). The cells are cultured in the presence of charcoal-stripped (to remove endogenous oestrogens) human serum which inhibits cell proliferation. Substances with oestrogenic activity can then overcome this inhibition. The MCF-7 cells were cultured 17ß-oestradiol and nonylphenol at several concentrations were each cultured in triplicate in multiwell plates and cell proliferation was assessed after a six-day exposure period by counting nuclei from lysed cells. Nonylphenol at a concentration of 10 µM elicited a similar proliferative response to oestradiol at a concentration of 30 pM; thus, on a molar basis the oestrogenic potency of oestradiol, as measured in this assay, is 3 000 000 times greater than that of nonylphenol. At concentrations of 1 and 0.1 µM the proliferative response produced by nonylphenol was similar to that observed in negative control cultures.

In another similar *in vitro* assay, MCF-7 and ZR-75 human breast cancer cell lines were used (White et al., 1994). Cells were cultured in quadruplicate in the presence of nonylphenol at concentrations ranging from 0.1 nM to 10 µm or 17ß-oestradiol at 10 nM. No oestrogenic activity was detected at nonylphenol concentration of 100 nM and less. At 1 and 10 µM nonylphenol elicited a proliferative response which at the higher concentration was similar to that produced by oestradiol. Thus, 17ß-oestradiol was 1000 times more potent than nonylphenol in this assay. In a further investigation, the ability of nonylphenol to stimulate transcriptional activity was determined in MCF-7 and chicken cell fibroblasts (CEFs) transfected with reporter gene pEREBLCAT and a mouse oestrogen receptor. Nonylphenol stimulated transcription at culture concentrations of 1 and 10 µM.

To summarise the *in vitro* oestrogenic data, there is evidence that nonylphenol has oestrogenic activity, of 3-6 orders of magnitude less potent than oestradiol.

In vivo systems

The oestrogenic activity of nonylphenol has been assessed in several studies using an assay based upon the uterotrophic response in the rat.

In the first study, five groups of immature (aged 20 - 22 days) female rats (six in each group) of a Wistar derived strain received single oral gavage doses of nonylphenol in corn oil on each of three consecutive days (ICI, 1996). The dose levels ranged from 9.5 to 285 mg/kg/day. Vehicle and positive (oestradiol benzoate 8 µg/kg, by subcutaneous route) groups were included. One day after the final dose the females were killed and the uterus was removed from each animal and weighed. Absolute uterus weight and bodyweight related uterus weight were statistically significantly increased, in a dose-dependent manner, at levels of 47.5 mg/kg/day and above. The NOAEL was 9.5 mg/kg/day. The uterine response seen in the positive control group was much greater than that of the nonylphenol groups, although a direct comparison of potency is not possible given the differing exposure routes. Similar data from the same laboratory have also been presented in peer-review literature (Odum et al., 1997). This latter report also included oral positive control groups (17ß-oestradiol, 10-400 µg/kg), which indicated that oestradiol was about 1000 times more potent in this assay than nonylphenol.

In a similar assay, groups of ten ovariectomised female Sprague-Dawley rats were dosed once daily for three consecutive days by the oral route with ethanol/oil suspensions of nonylphenol at

levels of 0 (vehicle control), 30, 100 and 300 mg/kg/day (Chemical Manufacturers Association 1997b). Positive control groups received ethynyloestradiol in ethanol at levels of 10, 30 and 80 µg/kg/day according to the same dosing regimen. One day after the final dose the females were killed and the uterus was removed from each animal and weighed. Uterus weights at 300 mg/kg/day were significantly increased (1.5-fold) in comparison with the vehicle control group. A slightly greater response (a 2-fold increase) was seen in the 30 and 80 µg/kg/day positive control groups.

In another uterotrophic assay, groups of three immature (aged 20 - 21 days) Sprague-Dawley rats each received a single intraperitoneal injection of nonylphenol at dose levels of 0, 1, 2 or 4 mg/animal (approximately 25, 50 or 100 mg/kg) (Lee and Lee, 1996). Oestradiol, administered by the same route, served as a positive control. The animals were killed 24 hours later and each uterus was removed, weighed and analysed for protein and DNA content and peroxidase (thought to be a uterotrophic marker enzyme) activity. There was a dose-dependent and statistically significantly increase in uterine weight at all levels, with associated increases in uterine protein and DNA content and uterine peroxidase activity. In further experiments, the uterotrophic activity of nonylphenol was found to be blocked by the co-administration ICI 182,780, an oestrogen antagonist, providing evidence that the effect of nonylphenol is mediated through the oestrogen receptor. Also, the potency was compared with oestradiol; in this assay oestradiol was found to be about 1000 - 2000 times more potent than nonylphenol.

Overall, these *in vitro* and *in vivo* studies show that nonylphenol has oestrogenic activity of a potency that is between 3 to 6 orders of magnitude less than that of oestradiol.

4.1.2.9.2 Effects on fertility

The effects of nonylphenol on fertility and reproductive performance have been investigated in a multigeneration study, and additionally, the testicular toxicity of nonylphenol has been studied in a repeated exposure study.

The multigeneration study was comprehensive, of good quality, and was conducted in compliance with GLP (NTP 1997). The overall study design was based on the OECD two-generation reproduction toxicity study guideline, with an extension to include the production of an F_3 generation. Groups of thirty male and thirty female Sprague-Dawley rats were exposed to nonylphenol via incorporation in the diet at concentrations of 0 (control) 200, 650 or 2000 ppm over three generations. Calculated nonylphenol intakes were, respectively, about 0, 15, 50 and 160 mg/kg/day during non-reproductive phases and rising to around 0, 30, 100 and 300 mg/kg/day during lactation.

Nonylphenol exposure commenced for the F_0 generation at about 7 weeks of age and continued until study termination when the F_3 generation were about 8 weeks old. F_0 animals were mated (one male with one female) within each dose group to produce the F_1 generation, selected F_1 animals were similarly mated to produce the F_2 generation and selected F_2 animals were mated to produce the F_3 generation. For the F_0 generation and retained F_1, F_2 and F_3 animals, clinical signs of toxicity, bodyweights and food consumption were reported. Oestrous cycles were monitored prior to mating. At the necropsy of adult animals, sperm samples were taken (but not from the F_3 generation) for analysis of density, motility (using a computer assisted sperm motion analysis system, only conducted on control and high dose group males) and morphology, a number of organs were weighed and selected organs were sampled for histopathology. Additionally, testicular spermatid counts were made. Parameters assessed in the young offspring included litter

size, bodyweights, survival, gross appearance, ano-genital distance, sexual development and, for animals killed at weaning, gross appearance of organs at necropsy and reproductive organ weights.

There was evidence of general toxicity in adults of all generations, seen as a reduction in bodyweight gain at 50 and 160 mg/kg/day and histopathological changes in the kidneys at all dose levels. These aspects are described in greater detail in section 4.1.2.6.1.

Considering the reproduction-related parameters, there were no adverse effects on fertility or mating performance. However, several other parameters were affected. Oestrous cycle length was increased by about 15% in the F_1 and F_2 females at 160 mg/kg/day, in comparison with controls. The timing of vaginal opening was accelerated by 1.5-7 days at 50 mg/kg/day and by 3-6 days at 160 mg/kg/day in females of the F_1, F_2 and F_3 generations. Also, absolute ovarian weights were decreased at 50 mg/kg/day in the F_2 generation and at 160 mg/kg/day in the F_1, F_2 and F_3 generations; however, no effect on ovarian weight was apparent in the F_1 and F_3 generations when analysed as an organ-to-bodyweight ratio. In males, changes in sperm endpoints were seen only in the F_2 generation; epididymal sperm density was decreased by about 10% at 50 and 160 mg/kg/day and spermatid count was decreased by a similar amount at 160 mg/kg/day. However, there may have been methodological problems with the epididymal sperm density measurements, because the density in all F_2 generation groups, including controls, was considerably greater (by about 25-40%) than reported for the F_0 and F_1 generation males; the age of each generation was similar at necropsy, so major differences in the sperm density would not be expected.

To summarise the reproductive aspects of this study, fertility and mating performance were not adversely affected by nonylphenol treatment. However, there were changes, albeit relatively slight, in the oestrous cycle length, timing of vaginal opening, ovarian weight and sperm/spermatid count. The effects on the oestrous cycle were seen in both the F_1 and F_2 generations (not assessed in F_3 females) and the timing of vaginal opening was influenced in all three generations; this consistency provides firm evidence of a relationship with treatment. These effects were possibly related to the oestrogenicity of nonylphenol. There is some uncertainty about the relationship to nonylphenol treatment with respect to the ovarian weight reduction because this effect was apparent after adjusting for bodyweight in only one generation and did not correlate with any histopathological changes; nevertheless, it is compatible with the anticipated direct effects of exogenous oestrogenic activity. Also, there is uncertainty regarding the cause of the apparent reduced sperm/spermatid numbers in the F_2 generation. It has been hypothesised that such changes could result from foetal or neonatal exposure to exogenous oestrogenic activity (Sharpe and Skakkebaek, 1993), but if the hypothesised mechanism were operating, semen/testicular changes should also have occurred in the F_1 generation. Furthermore, the possibility of methodological problems adds to the difficulty in interpreting the sperm/spermatid count data. However, the observation of impaired male reproductive tract development in an intraperitoneal study summarised in section 4.1.2.9.3 provides supporting evidence in favour of the sperm/spermatid count changes being causally related to nonylphenol treatment. Furthermore, the intraperitoneal study indicates that a critical window of exposure for this effect is likely to be the neonatal period. Overall, this study provided evidence that nonylphenol exposure over several generations can cause minor perturbations in the reproductive system of offspring, which are compatible with the predictable or hypothesised effects of exogenous oestrogenic activity, although these perturbations do not cause functional changes in reproduction of the rat at the dose levels tested. A clear NOAEL for these changes of 15 mg/kg/day was identified.