Appendix 8: Reproductive Toxicity

A8.1 Summary

The two reproductive studies of a single test material (carbon black oil) provided to the TG did not comprise a sufficient number of studies on which to assess possible PAC content – toxicity relationships. However, the two reproductive toxicity studies (which involved no exposure during pregnancy) indicated that fertility and sperm production were unaffected at doses at which fetal survival was severely compromised in a developmental toxicity study. Assuming that carbon black oil, which has a high PAC content, is representative of other PAC-containing petroleum streams, it can be reasonably assumed that reproductive effects, such as fertility and sperm production, would not be sensitive effects of PAC-containing materials compared to developmental toxicity effects. In addition, the potential for PAC-containing materials to affect reproductive organs was assessed via 13 week repeat-dose studies in which the testes, accessory sex organs, and epididymides were weighed in males, and the potential for pathological changes was evaluated in microscopic examinations. There was little evidence of reproductive organ effects in the repeat-dose studies of petroleum streams evaluated herein. Across a number of developmental toxicity studies that examined *in utero* or post-natal development, the effects most commonly observed, and statistically significant at the lowest levels, were related to fetal/pup survival and weight gain. There was little evidence of teratogenicity (i.e. malformations) in any of the developmental toxicity studies. As expected, increased incidences of skeletal variations (i.e., delayed ossification) were often observed at dose levels producing decreased fetal/pup body weight. Based on the results of a large number of repeat-dose studies, *in utero* and post-natal studies, and the two reproductive toxicity screening studies of petroleum streams, the most sensitive endpoints related to reproductive and developmental toxicity are those associated with the survival and growth of fetuses and offspring; effects on fertility, sperm production and reproductive organ effects do not appear to be sensitive endpoints for assessment of the potential hazards of PAC-containing petroleum substances. Such information may be used to satisfy the requirements for reproductive toxicity evaluation under the U.S. EPA's High Production Volume (HPV) Challenge Program, as described in the last section of this appendix.

A8.2 Definitions of Reproductive and Developmental Toxicity

Reproductive toxicity is defined as adverse effects on the male and female reproductive systems that result from exposure to chemical substances. Reproductive toxicity studies are designed primarily to assess fertility (i.e., mating behaviour and success) as well as survival and growth of offspring during the perinatal period. Typically, an assessment of the potential for effects on reproductive organs, as well as offspring survival, growth and maturation, is made. Reproductive toxicity studies may be single or multiple generation studies in design. In general, reproductive toxicity studies are not the ideal vehicle for assessing developmental toxicity. In reproductive toxicity studies, developmental effects (e.g., survival, growth and gross appearance of offspring) are evaluated postnatally (rather than at c-section) since the dams are allowed to deliver their litters. However, rodents have a tendency to cannibalize their offspring at birth when there are malformations. Reproductive toxicity may be expressed as alterations in sexual behavior, decreases in fertility, or loss of the fetus during pregnancy. A reproductive toxicant may interfere with the sexual functioning or reproductive ability of exposed individuals from puberty throughout adulthood. Developmental toxicity (adverse effects on the developing offspring, such as birth defects or developmental delays) is a subcategory of reproductive toxicity, but is often treated as a distinct health endpoint for purposes of hazard identification and risk assessment.

Developmental toxicity may be defined as adverse effects on the developing organism that result from exposure prior to conception (either parent), during prenatal development, or postnatally. Developmental toxicity studies are normally designed to assess in utero development. Typically, test material is administered to pregnant female rats throughout the gestational period, followed

by either an assessment of the uterine contents prior to birth (i.e., at the time of cesarean-section) or an evaluation of the offspring shortly after birth. The major manifestations of developmental toxicity include: (1) death of the developing organism, (2) structural abnormality, (3) altered growth (defined below), and (4) functional deficiency. The study design also normally affords some assessment of toxic effects to the dam, but does not typically involve an assessment of mating success or potential effects on reproductive organs.

A8.3 Reproductive Toxicity Studies

Only two studies were submitted to the TG which were specifically identified as reproductive toxicity studies. The two studies consisted of one male and one female reproductive toxicity screening study of carbon black oil (F-179).

In the male reproductive toxicity screen, groups of 10 male rats were given the test substance, F-179, dermally at doses of 0, 0.1, 1, 10, 50 and 250 mg/kg/day. The test material was given for 70 days before a seven-day cohabitation with untreated female rats. Doses of 10 mg/kg/day or higher resulted in reduced body weight and food consumption. Administration of 50 and 250 mg/kg/day caused clinical observations in the male rats. Mating and fertility parameters were unaffected by doses of F-179 as high as 250 mg/kg/day. The test material also had no significant effect on any testicular parameter, including sperm count, concentration, motility, and morphology. The study authors concluded that the NOAEL for fertility and reproductive organ effects in the male rats was higher than 250 mg/kg/day.

In the female reproductive toxicity screen, groups of 10 female rats were administered F-179 percutaneously at doses of 0, 0.1, 1, 10, 50, and 250 mg/kg/day. The test material was given for 14 days prior to a cohabitation period with untreated male rats and continued until day 0 of presumed gestation. Maternal toxicity was observed at 250 mg/kg/day, including decreased body weight and food consumption. Estrous cycling, mating, and fertility parameters were considered unaffected by administration of the test substance at doses as high as 250 mg/kg/day. No effect on litter parameters was observed at the time of c-section on gestation day 14. Thus, the NOAEL for fertility and reproductive organ effects in the female rats was higher than 250 mg/kg/day.

Interestingly, the same laboratory also conducted a developmental toxicity study of F-179. Groups of 11-18 female rats were given daily dermal doses of 0, 0.05, 10, and 250 mg/kg/day of F-179 starting at 7 days prior to mating through gestation day 20. The dams were allowed to deliver their litters. Maternal toxicity (decreased maternal body weight and food consumption) was observed at 250 mg/kg/day. The test material had a significant effect on the number of litters delivered at 250 mg/kg/day; in fact, no pups were delivered at this dose. It appears that the test material caused all litters to be resorbed at 250 mg/kg/day because the average number of implantation sites per dam was 9, indicating that these females had been pregnant. Thus, the NOAEL for offspring survival in rats was 10 mg/kg/day.

The difference between the results of the developmental and female reproductive toxicity studies of F-179 is readily explained by the difference in the days of dosing. It appears that dosing during gestation is critical to causing an increase in fetal resorptions. In the developmental toxicity study, female rats were given F-179 through the gestation period until gestation day 20 (the normal length of pregnancy in rats is about 22 days). In comparison, the female rats in the female reproduction study were not given the test material during pregnancy; the female rats were given F-179 up until day 0 of gestation.

The results of these studies of F-179 indicate that endpoints of developmental toxicity are a more sensitive than fertility, sperm production or potential effects on male or female reproductive organs for this test material. F-179 is carbon black oil, which is high in PAC content; this test material had one of the highest PAC contents (and lowest NOAELs for developmental toxicity) of the materials evaluated for developmental toxicity. Due to its high PAC content, carbon black oil is probably a reasonable worst case by comparison to other petroleum-derived substances. If F-

179 is representative of other the other PAC test materials, one would expect the developmental toxicity endpoints of PAC test materials to be a more sensitive reflection of overall reproductive effects than male or female fertility or reproductive organ effects.

A8.4 Effects on Reproductive Organs in Repeat-Dose Toxicity Studies of Petroleum Streams

Repeat-dose toxicity studies also provide valuable information about the potential for chemicals to cause reproductive organ effects. The predictive value of these repeat-dose studies as a screening system for potential reproductive toxicants has been reviewed by Morrissey et al. (1988). There is little, if any, evidence of an effect on reproductive organ weights or histopathology in the repeat dose studies of petroleum streams.

Male Reproductive Organs

Testes, Prostate, and Epididymides Weights

Among the repeat-dose studies reviewed by the TG, the testes were weighed in all of the 44 repeat-dose studies evaluated by the TG. There was little evidence of any adverse effect on the absolute or relative weights of the testes in these studies. Testicular weight (absolute) was significantly decreased in only one study (Study No. 63456, Sample No. 86271), and only at the high dose (500 mg/kg/day). Relative testes weights were not significantly decreased at any dose in any study. Testicular weights (relative to body weight) were significantly ***increased*** in five of 44 studies. Decreases, not increases, in testicular weights are usually regarded as an adverse effect. As such, the evidence of an adverse effect on testicular weights was limited to a significant decrease at the high dose group in only one of 44 studies.

Similarly, from the repeat-dose studies included in this project, there is little evidence of an effect of petroleum substances on prostate or epididymides weights. Prostate and epididymides weights (absolute and relative) were reported in 19 of 44 repeat-dose studies; all 19 studies were 13-weeks in duration. Significant decreases in absolute prostate weights were observed in only 2 (Study Nos. 63456 and 63266) of the 19 studies – and only at the high dose in each study (500 and 1000 mg/kg/day). A decrease in relative prostate weight was also seen at the same dose in one of these studies (Study No. 63456). A significant increase in relative prostate weight was noted in one study at one dose only.

Absolute epididymides weights were significantly decreased in only 2 of the 19 studies. Relative epididymides weight was not significantly affected in any study.

Testicular Histopathology

Histological examination of the testes was conducted in 23 of the repeat-dose studies provided to the TG. There was no definitive evidence of any effect on testicular histopathology. There was a possible effect in 4 of the 23 studies, but there was no consistent pattern, and only a small number of animals were potentially affected in any study. The lack of histopathology in the testes provides further support that male reproductive organs are not target organs for the PAC-containing substances evaluated as part of this project.

Female Reproductive Organs

The petroleum streams evaluated by the TG did not affect female reproductive organ weights or histopathology in the repeat-dose studies.

Ovary and Uterus Weights

In the repeat-dose studies available to the TG, the petroleum substances demonstrated minimal potential to alter the weights of the ovaries and uterus. The ovaries were weighed in 42 of the repeat-dose studies with female rats.[1] Absolute (but not relative) ovary weights were significantly decreased in one study (Study No. ATX-860007) at the two highest doses (1084 and 2710 mg/kg/day). In another study (Study No. 20525), absolute and relative ovary weights were significantly decreased at 125 mg/kg/day, but at no other dose. A significant increase in relative weight of the ovaries was observed in one study (Study No. 20535) at 500 mg/kg/day. There were no other statistically significant differences in absolute or relative ovary weight in any of the other studies.

The uterus was weighed in 18 repeat-dose studies, which were all of 13-week duration. Absolute uterine weights were significantly decreased in 2 studies, but in one case, the decrease was not dose-related. No dose-related, statistically significant decrease in relative uterine weight was seen in any of the 18 studies.

Ovarian Histology

The histopathology of the ovaries was evaluated in twenty-two 13-week studies evaluated by the TG. Based on these studies, ovarian histology does not appear to be a sensitive endpoint of toxicity for the tested materials. Ovarian histology was affected in 2 of the 22 studies, and effects were seen only at the highest dose level. In both studies, the effects on ovarian histology were associated with a high incidence of mortality (i.e., 30-40%).

In summary, there was minimal evidence of female reproductive toxicity in the repeat-dose studies evaluated by the TG, but female reproductive toxicity was not a sensitive endpoint. It occurred only at high doses (i.e., doses much higher than those required to produce developmental toxicity).

A8.5 Developmental Toxicity Studies

The TG had available for evaluation 56 studies which assessed developmental toxicity. Of these, 23 were studies which assessed embryonal and fetal development at the time of caesarean section (i.e., the dams were sacrificed near the end of the gestational period and the uterine contents were examined). For purposes of this report, these studies were termed “prenatal” studies. The principal effects observed in these studies related to fetal growth and survival. In the remaining 33 studies, the dams were allowed to give birth, permitting an assessment of survival and growth during postnatal days 0 through 4. For purposes of this report, these were termed “postnatal” studies. In both the prenatal and postnatal studies, the dams were exposed to the test material during pregnancy. The principal effects observed in these studies were decreased pup growth and survival. **Appendix 3** provides a description of the frequency of effects that was seen at the LOEL in these studies.

Among the prenatal studies, the endpoints most commonly affected were those of fetal survival and weight gain. Among the postnatal studies, the endpoints most commonly affected were those of offspring growth and survival (to day 4 which was the scheduled termination of these studies).

[1] Two of the 44 repeat-dose studies used only male rats. Therefore, there are 42 repeat-dose studies involving female rats.

In both the prenatal and postnatal developmental toxicity studies evaluated by the TG, many of the petroleum substances demonstrated the potential to cause an increase in fetal resorptions and a decrease in litter size. In extreme cases, these effects manifested themselves as decreased female fertility due to the loss of the entire litter. However, the loss of the entire litter was not typically the most sensitive endpoint in the developmental toxicity studies of the petroleum substances. The NOAELs and LOAELs in the in utero studies were defined by more subtle effects (e.g., decreased fetal/pup body weight, decreased litter size), rather than the loss of the entire litter.

The two petroleum substances associated with ovarian histology findings in the 13-week studies, were also used as the test materials in "prenatal" developmental toxicity studies. In at least one case, the same lot number of the petroleum stream was used for both the 13-week and developmental toxicity studies. This affords the opportunity to compare the sensitivity of developmental toxicity endpoints to reproductive organ toxicity. In both of these cases, serious developmental toxicity was observed at a dose level less than or equal to the dose levels associated with histological changes in the ovaries in the 13-week studies. In other words, histological changes in the ovaries did not represent a more sensitive endpoint than did fetal effects.

More specifically, for both test materials that affected the histology of the ovaries, histological changes were noted at 125 mg/kg/day in the 13-week study, with 30 mg/kg/day being a NOAEL. In the developmental toxicity studies, the first test material (CSO) produced marked developmental toxicity (70% resorptions, decreased mean number of live fetuses, and decreased fetal body weight) at 30 mg/kg/day; the NOAEL for developmental toxicity was 8 mg/kg/day. The second test material (HCGO) produced developmental toxicity at 125 mg/kg/day, including 55% resorptions, decreased mean live fetuses and decreased fetal body weight; the NOAEL for developmental toxicity for this material, HCGO, was 30 mg/kg/day.

A8.6 Discussion of the Potential Effects of Petroleum Substances on Reproductive Structure and Function

Data from the repeat-dose studies evaluated in this project, as well as the two reproductive toxicity screening studies, suggest that PAC-containing petroleum substances have limited potential to affect fertility or male or female reproductive organs. Based on these data, these manifestations of reproductive toxicity do not appear to be sensitive endpoints for the petroleum substances included in this project, particularly when compared to the endpoints of developmental toxicity.

This position is supported by a recent review of the literature on the effect of PACs on developmental and reproductive toxicity. The review suggests that statistical analysis and modelling would be best directed at the fetal and pup effects observed in developmental toxicity studies, not fertility or reproductive organ effects. This review was conducted by Dr. Jamie Benedict of the USEPA whose work was presented at the Annual Meeting of the Society of Toxicology in March 2006. The animal studies reviewed by Dr. Benedict were studies of benzo[a]pyrene. Dr. Benedict drew the following conclusion in her summary of the available literature on reproductive and developmental effects of PAHs in laboratory animals:

> "Animal data also suggest an increased susceptibility to PAH-induced developmental effects following *in utero* exposures."

Dr. Benedict's assessment of the animal studies of benzo[a]pyrene is consistent with the observation of developmental toxicity in the studies of petroleum substance studies evaluated herein. The most sensitive endpoints of developmental toxicity were endpoints of decreased survival and decreased growth among fetuses/pups. These endpoints are typically evaluated in reproductive toxicity studies in which exposure occurred during gestation, as well as in

developmental toxicity studies. Since little evidence of changes in male and female reproductive organs was observed in the repeat-dose studies of petroleum substances, it is likely that the endpoints affected in the developmental toxicity studies, particularly fetal and/or offspring survival and growth, would be the most useful endpoints for assessing hazard potential, for risk assessment, and for satisfying other regulatory needs.

It is possible that an endpoint that was not observed in either the repeat dose or developmental toxicity studies might be affected by petroleum substances. In other words, there is a chance that petroleum substances could cause an important change in a reproductive toxicity endpoint that would not have been discerned in the existing studies. But, this is unlikely. For example, the repeat-dose and developmental toxicity studies would not have picked up a delay in sexual maturation. However, delays in sexual maturation are often due to changes in sex hormone levels. If a petroleum substance had caused a change in sex hormone levels, histological changes in the reproductive organs of adult animals in the repeat-dose studies would have been expected.

In summary, based on the results of a large number of repeat-dose and developmental toxicity studies as well as two reproductive toxicity screening studies of petroleum substances, sensitive endpoints are more likely to be associated with developmental effects rather than with fertility or reproductive organ effects.

A8.7 High Production Volume (HPV) Challenge Program and Category Closure

Under the High Production Volume (HPV) Challenge Program, U.S. EPA has provided guidance on the requirements for evaluating reproductive toxicity. The EPA guidance indicates a reproductive toxicity study may not be required for certain petroleum substances if there is (1) a 90-day repeat-dose study, and (2) a developmental toxicity study. Based on this guidance, the TG does not believe reproductive toxicity studies are required to evaluate the potential for reproductive toxicity among the classes of petroleum substances included in this project.

Specifically, for the reproduction toxicity endpoint, U.S. EPA states:

- when a 90-day repeated dose study is available and is sufficiently documented with respect to studying effects on the reproductive organs and a developmental study is available, the requirements for the reproduction toxicity endpoint are satisfied;
- when either a 90-day or 28-day repeated-dose study is the only repeated dose study available, it is recommended that the reproduction/developmental toxicity screening test (e.g. OECD Test Guideline 421) be carried out in order to satisfy the requirements for the reproduction toxicity endpoint; and
- when a 90-day repeated dose study is available and demonstrates no effect on reproductive organs, in particular the testes, then a developmental study (e.g. OECD Test Guidelines 414) can be considered as an adequate test to complete information on reproduction/developmental effect.
- OECD Test Guideline 415 - 416. The screening tests [OECD Test Guideline 422: Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test, (for when repeated dose toxicity is not available) and OECD Test Guideline 421: Reproduction/Developmental Toxicity Screening Test (for when repeated dose toxicity is available)] are also acceptable.[2]

Since no significant adverse effects on reproductive organs were observed in the 90-day repeat-dose studies of petroleum streams, the most sensitive endpoints of reproductive toxicity of petroleum streams are likely to be the sensitive endpoints observed in the developmental toxicity studies. Therefore, the PDx for developmental toxicity is likely to be a reasonably good predictor

[2] http://www.epa.gov/chemrtk/sidsappb.htm

of the PDx for reproductive toxicity, as well. Such information may be used to satisfy the requirements for reproductive toxicity evaluation under the U.S. EPA's High Production Volume (HPV) Challenge Program.