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IEH assessment on

ENVIRONMENTAL OESTROGENS: CONSEQUENCES TO HUMAN HEALTH AND WILDLIFE

1995 ASSESSMENT A1

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Executive summary

BACKGROUND

There is currently much concern about possible adverse consequences arising from the release into the environment of man-made oestrogens and other substances with oestrogenic or anti-oestrogenic properties. This concern is directed at both human health effects and effects on wildlife. For humans there is an increasing body of literature suggesting a possible link between environmental chemical pollution and adverse effects on both the male and female reproductive systems. Recent research in fish has identified effects such as production of the egg yolk protein, vitellogenin, in males. This is a protein normally only produced in sexually mature female fish.

The key objectives of this Assessment are to:

> review existing literature and scientific opinion on the evidence for changes in human reproductive health and effects in wildlife, and to examine possible links between the production and release into the environment of man-made chemicals and the observed effects,
>
> *and*
>
> identify the gaps in knowledge, information and research that need to be filled and to make recommendations and establish priorities for future research, addressing in particular those areas which will provide the best information for policy decisions.

The scope of the evidence in this report relating to human health effects is extensive. Areas of concern relating to possible effects of environmental oestrogens on the male reproductive system include sperm counts and sperm quality, cryptorchidism (undescended testes), hypospadias (a congenital malformation of the penis), testicular cancer and male breast cancer. Effects considered in females include breast cancer and impacts on the cardiovascular system. The groups of xenoestrogens discussed include synthetic hormones, phytoestrogens, organochlorine pesticides, polychlorinated biphenyls, dioxins, alkylphenol polyethoxylates and others (including triazine herbicides and bisphenol-A). The report includes an assessment of the relative levels of oestrogen and anti-oestrogen intake, comparing environmental exposures with levels in the diet.

The effects considered in wildlife cover oestrogenic effects in fish, including the occurrence of vitellogenin and the masculinisation of female fish, developmental abnormalities in reptiles, supernormal clutches and female-female pairing in gulls, and imposex in molluscs. The chemicals of concern are similar to those listed above.

REVIEW PROCESS

The Institute for Environment and Health prepared background scientific review documents on the effects of environmental oestrogens in humans and their impact on wildlife, and then invited acknowledged international experts in the field, together with representatives from a number of government and other interested organisations, to discuss these documents at a workshop held in Leicester in January 1995. The purpose of the workshop was to ensure that the scientific reviews were up to date, accurate, comprehensive and balanced. The delegates were also asked to help identify the key issues and gaps in knowledge and make recommendations for further research.

The present report was compiled using the original reviews, together with the extra information supplied and comments received at and after the workshop.

HUMAN HEALTH EFFECTS

The sum total of available evidence concerning effects on the human male reproductive system indicates that there has probably been a fall in the quantity and/or quality of sperm over the last few decades. There is also a strong case suggesting that, in some countries at least, there has been an increase in the incidence of testicular cancer. Based on animal studies, there is a plausible hypothesis that oestrogens, or possibly anti-androgens, disrupt male reproductive function. However, there is as yet no evidence that the effects recognised in the human population are necessarily causally linked to the presence of such chemicals in the environment. Moreover, although a decrease in sperm count, if confirmed, provides some cause for concern, it is not a definitive indicator of decreased human fertility.

There is convincing evidence for an increase in the incidence of breast cancer in women in Westernised countries over the last four decades. A number of environmental and life style factors have been associated with this, amongst which is exposure of the unborn and prepubescent female to environmental oestrogens.

Some chemicals exhibit 'oestrogen disruptive properties', that is they interfere with the metabolism of oestrogens. The naturally occurring hormone 17β-oestradiol is metabolised *via* two separate pathways, one producing a potent oestrogen, which is genotoxic, and the other producing a metabolite with low oestrogenic activity, which is not genotoxic. Women with breast cancer have been found to have a high ratio of potent oestrogenic metabolite to weakly oestrogenic metabolite, and levels of the potent oestrogen in breast cancer tissue are higher than in normal breast tissue. A hypothesis has therefore been proposed whereby an increase in this ratio may increase the susceptibility to breast cancer. However, although it is plausible that oestrogens or oestrogen disrupters are responsible, no definitive evidence yet exists for a causal relation with breast cancer incidence.

IMPACTS ON WILDLIFE

Field and laboratory studies, on a range of different animal species, have demonstrated oestrogenic and anti-oestrogenic effects of chemicals in the environment. However, the importance of these effects to whole ecosystems is not known. Furthermore, it is not possible to make any assumptions about the relevance to humans of even such well documented cases.

RESEARCH PRIORITIES

Identified priorities include:

Human health

- Establishment of the extent of exposure of human populations to putative oestrogens.
- Development of robust test procedures for identifying chemicals with oestrogenic (or similar) properties.
- Development of a biomarker for persistent oestrogenic action.
- Prospective epidemiological studies to investigate testicular cancer and dysfunction and look at geographical differences in incidence.

- A UK prospective study on sperm/semen quality and development of standardised analytical techniques.
- A pan-European study of human reproductive disorders and more extensive investigation of populations highly exposed to putative environmental oestrogens.
- Investigation of the association between environmental oestrogens and breast cancer in women, including geographical variations in cancer incidence.
- Development of experimental models to investigate the relation between susceptibility to breast cancer and environmental factors.
- Investigation of the possible effects of environmental oestrogens on the vascular system in women from different geographical areas.
- Investigation of the bioaccumulation, metabolism, mobilisation and degradation of oestrogenic agents.
- Studies of chemicals with oestrogenic and/or anti-oestrogenic activity for their effects in mothers and on the development of offspring.
- Development of suitable animal models to test for oestrogenic activity, and studies to improve understanding of mechanisms of action.

Wildlife effects

- Consideration of oestrogenicity as a possible factor in future studies of the effects of pollutants on wildlife, including studies on the developing embryo wherever possible.
- Further extension of experimental studies on fish to natural populations.
- Development of biomarkers for oestrogenicity in wildlife and application of tests in addition to the well established induction of vitellogenin.
- Linking studies on the consequences of oestrogen exposure with long-term ecological studies wherever possible.

CONCLUSIONS

There is increasing evidence for adverse trends in several measures of human reproductive health, and various widely distributed environmental contaminants have been shown in the laboratory to possess oestrogenic and related activities. Although information from studies in humans provides no evidence for a causal link between these two observations, and whilst other hypotheses pertaining to trends in human reproductive health are plausible, findings in wildlife increase the concern that a link may indeed exist.

A concerted and co-ordinated programme of research is required to investigate the possible link between demonstrated trends in human reproductive health and human exposure to chemicals in the environment with known oestrogenic or related hormonal activities. Further work is also necessary to refine and clarify the measurement of trends in human reproductive health, especially with respect to sperm quality. The scope of research on wildlife should be extended to cover a range of natural populations and additional markers of effect.

Further research is needed before a robust and reliable assessment can be made of the risk to human health from exposure to environmental oestrogens.

1 Introduction

The impact of environmental oestrogens on human health and wildlife is attracting an ever increasing amount of scientific and public interest (Hileman, 1994; Stone, 1994; Weiss, 1994). Well documented field studies have indicated significant changes occurring in the reproductive physiology of wildlife populations, and there is evidence suggesting changing patterns and trends in human reproductive health. It has been shown that environmental chemicals which mimic endogenous oestrogens, or which act as anti-oestrogens or otherwise affect the balance of sex hormones, may be responsible for the observed changes in wildlife. However, there is still uncertainty as to whether there is a link between observed changes in wildlife populations and the trends identified in humans, and whether there is any evidence for a causal relationship between the presence of oestrogens in the environment and adverse effects in people.

Several major international initiatives in this area have been undertaken recently, including workshops in the USA and Germany and the publication by a Danish research team of a key review paper on male reproductive effects (Toppari *et al.*, 1995). Research on oestrogenic substances has been ongoing for many years. As early as 1938, Dodds and co-workers reported that certain substituted phenols had oestrogenic activity (Dodds & Lawson, 1938; Dodds *et al.*, 1938). This work identified 4, 4'-dihydroxy-αβ-diethylstilbene, later to become known as the drug diethylstilboestrol (DES), as a potent oestrogen. Although scientific interest was directed initially at the pharmaceutical use of synthetic chemicals having oestrogenic activity, attention now largely focuses on the consequences of inadvertent environmental exposure to such substances.

This Institute for Environment and Health (IEH) Assessment reviews the evidence for changes in human reproductive health over the last few decades, examines what role, if any, exogenous substances with known oestrogenic or related activities might be playing, and describes the major concerns relating to effects in wildlife. The focus of this report is environmental oestrogens, although it is recognised that environmental oestrogens are only one of the many possible causes that have been suggested to explain trends in human reproductive health. The report has been compiled with the aid of key experts in the field from Europe and the USA who

participated in a workshop held in Leicester in January 1995. The workshop participants (listed on pp. 105-107) reviewed the most recent data, explained ongoing studies, assessed the available evidence and helped compile the recommendations for future work.

The report deals first with the role of environmental oestrogens in human reproductive disorders, then provides a review of the evidence for wildlife effects. The overall recommendations for future research are outlined in section 4. It is clear that the development of new test methods for screening chemicals for oestrogenic activity will be important in the future; the techniques which have already been developed are described in an appendix to this report.

2 The role of environmental oestrogens in human reproductive disorders

2.1 BACKGROUND

In recent years, several pieces of evidence indicating possible increasing trends for adverse effects on the reproductive capability of animals and man have been forthcoming. The evidence has arisen from disparate observations in wildlife and in humans. In the latter, effects such as decreased sperm count and increased testicular cancer in men and breast cancer in women are of particular concern.

It has been proposed that a common cause for these diverse observations may be the oestrogenic action of certain environmental contaminants. However, attempts to investigate this phenomenon by correlating oestrogenic effects in different species must be undertaken with extreme caution, because of different physiologies. For example, in teleost fish (most fish excluding sharks and rays), unlike other vertebrates, hermaphroditism and spontaneous sex inversion are normal phenomena. The mechanisms of sex changes in fish are not well understood but are clearly different from the physiological control of sex characteristics in mammals (Reinboth, 1980). Vitellogenin formation in fish also has no clear parallel in mammals. In alligators, sex is determined by incubation temperature; eggs incubated below 30°C develop into females and those incubated above 34°C into males (Ferguson & Joanen, 1982).

The situation in human populations therefore needs to be evaluated in terms of exposure to environmental oestrogens and possible effects, through specific mechanisms, on the human reproductive system. There is a need to ascertain whether adverse reproductive effects are increasing or decreasing at this time, whether there are significant global trends and if the observations indicate real changes.

In this section, the evidence for, and significance of, apparent changes in male fertility, and rates of breast cancer and other diseases and disorders are examined critically and possible causes are discussed, focusing on environmental oestrogens. First a brief review of what is known about the male and female reproductive systems is presented.

2.2 OVERVIEW OF THE MALE AND FEMALE REPRODUCTIVE SYSTEMS

The gonads of both sexes function in both the production of germ cells (gametogenesis) and the secretion of sex hormones, including testosterone, which has a masculinising effect (anabolism, hirsutism etc.) and oestrogens, which are feminising (breast development, fat deposition). Both the ovary and testes produce testosterone and oestrogen; the quantities varying according to the sex.

Sexual differentiation into male and female occurs early in fetal development, as a consequence of a genetic signal. The XY chromosome pair bears a gene on the Y chromosome which stimulates development of the testis from the undifferentiated gonadal ridge. In its absence the gonadal ridge develops into an ovary and the female phenotype develops passively. However, once testes have formed, they secrete a peptide (anti-Mullerian hormone), which causes regression of the potential female reproductive tract, and a steroid (testosterone), which stimulates development of the male reproductive tract. Oestrogen does not appear to have a role in sexual differentiation at this time, although there is some evidence that testosterone is aromatised to oestrogen within the brain where it mediates the masculinisation of the central nervous system. In humans, the fetus is protected from high levels of maternal oestrogen by sex-hormone binding globulin (SHBG) which binds to and inactivates the steroid. In addition, the fetus is very efficient at sulphating and therefore inactivating oestrogens (for a review see Knobil & Neill, 1994).

The testis consists of a large number of seminiferous tubules lined with Sertoli cells and germ cells, with interstitial (Leydig) cells lying in the connective tissue between the tubules. The Sertoli cells extend from the basement membrane to the lumen and are linked to form an unbroken ring, dividing the tubular epithelium into basal and adluminal compartments. During spermatogenesis, the germ cells

remain in intimate contact with the Sertoli cells, with the primary germ cells (spermatogonia) in the basal compartment undergoing a number of mitotic divisions to produce daughter cells. Some spermatogonia move away from the basal membrane and enlarge, becoming primary spermatocytes. As they move between Sertoli cells into the adluminal compartment, primary spermatocytes develop through a series of stages to form secondary spermatocytes which themselves undergo meiotic division to form spermatids. Spermatids then pass through a number of maturation steps until they are finally released into the lumen as spermatozoa, which then pass into the epididymus where they undergo further maturation. Spermatogenesis is regulated by testosterone, follicle stimulating hormone (FSH), and various agents produced by the Sertoli cells, although much of the detail remains unclear.

The Sertoli cells in fetal and prepubertal life are able to aromatise testosterone to oestradiol and it is thought that the main function of the latter in the male is to exert a local negative feedback effect on the multiplication and differentiation of Leydig cells and on the capacity of differentiated Leydig cells to synthesise testosterone; oestradiol may also play a role in negatively regulating the synthesis and/or release of gonadotrophins from the anterior pituitary. The gonadotrophins are luteinising hormone (LH), which stimulates the Leydig cells to produce testosterone and FSH, which stimulates various functions of the Sertoli cells, including their multiplication. During puberty, many of these regulatory processes change. The Sertoli cells lose their ability to divide and aromatise testosterone; the latter function is taken over by adult-type Leydig cells. However, oestradiol still appears to be an important local regulator of Leydig cell number and function in the adult testis. Because of these physiological roles of oestradiol, exposure to exogenous oestrogens has the potential to reduce the numbers and/or function of both Sertoli cells and Leydig cells and any other functions dependent on these cells, such as masculinisation and spermatogenesis (Sharpe, 1994).

The most potent oestrogen is 17β-oestradiol, which is the main oestrogen produced by ovarian follicles in women during the menstrual cycle. It has important roles in the maintenance and function of the female reproductive tract as well as having autocrine effects within the ovary. Thus it is important in stimulating proliferation of granulosa cells within the maturing follicle and increasing oviduct motility and secretions necessary for final sperm capacitation. It stimulates proliferation of uterine endometrial cells and sensitises the myometrium to contractile influences. It stimulates breast duct growth and at puberty is important in the female pattern of fat deposition and closure of the epiphyses. It is also important in adulthood for the maintenance of bone structure, by stimulating calcium deposition.

Most circulating oestradiol is bound to protein. Its concentration in plasma varies during the menstrual cycle, reaching a maximum of 0.2 to 0.4ng/ml (equivalent to a circulating total of 0.6 to 1.0mg/day) in the late follicular period near the mid-cycle. Exposure to the levels found in women would have a feminising effect in males, with breast development and fat deposition, reduction in fertility due to suppressed gonadotrophin release and subsequent reduced gonadal function. Oestradiol exerts its effects in tissues *via* a receptor that will also recognise other hormone-like substances or chemicals with hormone-type structures that are able to interact with it.

An important sex difference is in the production of gametes. In the male, the primary germ cells (spermatogonia) are capable of mitotic division throughout life and there is, therefore, an inexhaustible supply into old age. In the female however, all the eggs (ova) are laid down in early fetal development within structures called follicles. Over the fetal, neonatal and childhood periods, as well as in adulthood, cohorts of follicles develop and die before they mature sufficiently to rupture and release the egg ready for fertilisation. By the age of around 50 years, no follicles or eggs remain and ovarian failure, or menopause, occurs. It is possible that exposure to deleterious agents *in utero* may lead to a reduction in egg numbers and thence premature ovarian failure in adulthood.

Key events or sites which may be influenced by external oestrogenic agents include:

- In embryonic development: intra-uterine perturbation of the hormonal secretions or actions necessary for male sexual differentiation.

- In the adult male: antagonism of the release or activity of testosterone leading to feminisation and reduced spermatogenesis.

- In the adult female: abnormally high oestrogenic activity affecting ovarian and oviduct function and fertility, and proliferation of uterine tissue and breast tissue leading to carcinomas.

An important consideration in assessing the potential effects of environmental oestrogens on the endocrine and other systems is the time at which exposure occurs (see also section 3.1). Two general types of effect have been described, ‘organisational effects’, which are developmental effects usually apparent in early life, and ‘activational effects’, which commonly occur in adulthood and are generally transitory in action (Guillette *et al.*, 1995a).

2.3 HEALTH EFFECTS OF CONCERN

2.3.1 EFFECTS ON THE MALE REPRODUCTIVE SYSTEM

In the following sections, effects on sperm count and quality, cryptorchidism (undescended testes), hypospadias (an anomaly in which the urethra opens on the underside of the penis), testicular cancer and breast cancer are examined separately. It has been pointed out, however, that reduced sperm counts in men could be an indicator of other testicular disorders including cancer (Carlsen *et al.*, 1992; Giwercman & Skakkebæk, 1992) and so these effects should not necessarily be considered in isolation.

SPERM COUNTS AND SPERM QUALITY

The subject of male fertility as characterised by falling sperm counts has been commented upon previously (Nelson & Bunge, 1974; James, 1980). Recent information on this subject has catalysed speculation and concern and requires particular examination. It should be appreciated, however, that although a decrease in sperm count *per se* may indicate some cause for concern, it is not a definitive indicator for decreased fertility.

In a study designed to establish control levels of sperm counts, Whorton and Meyer (1984) collated sperm count data from 861 US chemical and agricultural workers from 14 separate studies on testicular function. This group comprised both unexposed controls and exposed men whose sperm counts were not significantly different to those of controls. No correlation was found between age

and sperm count for the group as a whole or when divided by race. Mean and median sperm counts for the population were 107.1 and 83.0 million/ml respectively; 8.7% had sperm counts of less than 20 million/ml semen and were classed as oligospermic.

Substantial intra-individual variation in semen measures within normal men has been reported in the USA. Poland *et al.* (1985) analysed semen samples from 15 healthy subjects collected fortnightly for six months; the length of abstinence prior to sample collection varied between zero and three or more days. Considerable variation was demonstrated for semen volume and sperm count, motility and morphologic features. The quality of semen was significantly affected by short-term abstinence, with a positive linear relationship for semen volume (0.62ml/day), sperm motility (1.2%/day) and sperm count (17.6 million sperm/ml/day) within the study period.

Carlsen *et al.* (1992) analysed the results of sperm counts from men in 61 studies over a period of 52 years (1938-1990). The studies had been undertaken in many countries with variable numbers of subjects per group (e.g. 22 in the USA in 1941, 29 in Sweden in 1971, 4435 in the USA in 1982, 1500 in Libya in 1983 and 104 in the UK in 1988). The authors analysed these diverse data using linear regression and estimated that the average sperm count decreased from 113 million/ml in 1940 to 66 million/ml in 1990, a decrease of over 40% between these two periods. Criticisms such as selection bias (Farrow, 1994) or possible error caused by the distribution of the sperm concentrations being heavily skewed (Bromwich *et al.*, 1994) were discounted by the authors (Keiding *et al.*, 1994a,b).

Further evidence for declining semen quality has been provided by a number of other studies. Irvine (1994) examined 3729 semen samples from donors selected on the basis of an initial sperm concentration greater than 20 million/ml. When samples were grouped by year of donor's birth, median sperm concentration decreased significantly between 1940 and 1969. Van Waeleghem *et al.* (1994) published an abstract of a study of a population of 360 'candidate donors' of sperm in Belgium, which indicated that, from 1977 to 1993, sperm concentrations and motility and the percentage of normal spermatozoa decreased in the donated semen. In a study of sperm donors in Paris, France, the first ejaculates of 1351 men donated at a sperm bank were examined over the period 1973-1992; after adjustment for age and the duration of sexual abstinence, decreases were observed in sperm concentration (2.6% per year) and in percentages of motile (0.3% per year) and normal sperm (0.7% per year), although semen volume was unaffected (Auger *et al.*, 1995).

In contrast, Wittmaack and Shapiro (1992) compared the semen quality of potential sperm donors over a ten year period between 1978 and 1987 in Wisconsin, USA and found no statistically significant trend in sperm concentration or change in sperm counts. A sharp rise in percentage of abnormal sperm between 1982 and 1983 was attributed to a change in identification criteria rather than a true increase in abnormal morphology.

Macleod and Wang (1979) found no significant change in semen volume, mean or median sperm counts or distribution of counts, among groups of US men of 'infertile marriages', when comparing the years 1951, 1966 and 1976 (≥ 1000 men/group). Consistent methodologies were used on similar populations at the same laboratory. In the period 1966-1977, groups of 1000 men were analysed for nine sub-periods (9000 men in total) when presenting for their first semen examination. A second and independent group of 5476 men (up to 500/year) was examined over the same period after referral from previous assessment elsewhere due to 'low' sperm count. The results from the 14 476 men over the 11 year period showed no change in semen volume, mean or median sperm counts or the distribution of sperm counts.

In an earlier study at the same centre (MacLeod & Gold, 1951), it was difficult to distinguish between groups of fertile and infertile men on the basis of mean sperm counts (107 million/ml and 90 million/ml, respectively). The difficulty in distinguishing fertile from infertile men on the basis of mean sperm counts alone is a point taken up by Badenoch *et al.* (1989). MacLeod and Wang (1979) emphasised that semen volume and sperm counts are the least important measures of fertility. Sperm motility and other functional indices are considered to be the most important parameters, and values for these are lacking in most of the surveys reviewed. It has also been noted that the literature regarding changes in male fertility is not based upon prospective studies designed to assess changes among members of the general population but in selected subpopulations of men, including sperm donors and those attending fertility clinics or undergoing vasectomy (Sherins, 1995).

In summary, although a number of studies have drawn attention to the possibility of decreasing sperm counts over the last few decades, the evidence is not entirely convincing. Furthermore, a reanalysis of the data of Carlsen *et al.* (1992) but covering only the last two decades (1970-1990) indicated that sperm counts were not decreasing (Brake & Krause, 1992). In addition, a comprehensive study in the USA, where potential variables were minimised, showed no evidence for decreasing sperm counts or distribution over the period 1951-1977 (MacLeod &

Wang, 1979). However, the most recent study of Parisian sperm donors (Auger *et al.*, 1995) did demonstrate a decrease in the quantity and quality of sperm during a 20 year period (1973-1992) in the study population. Toppari *et al.* (1995) concluded that the available evidence points to a rise, in Europe and many other countries, in male reproductive disorders involving sperm counts and probably sperm quality. Clearly, much more study and analysis is required to establish with certainty the position regarding time-related trends in sperm quantity and quality and their possible impact on male fertility, particularly in the general population.

CRYPTORCHIDISM

The evidence for an increased incidence of cryptorchidism rests primarily on a comprehensive study of male offspring born in the Oxford area over the period 1984-1988 (John Radcliffe Hospital Cryptorchidism Study Group, 1992). Over 7400 boys were examined at birth and at three months, using the same methods utilised in a previous study in the London area (Scorer, 1964). The results were subdivided into those for bodyweight less than 2500g at birth and those greater than 2500g and the means were compared with those of Scorer (1964) obtained in the mid-1950s (Table 1). It can be seen that there is an apparent slight increase in incidence between the two periods for cryptorchidism at birth, and that at three months there is a clear increase from a mean of 0.97% in the mid-1950s in London to 1.78% in the mid-1980s in Oxford. Rates adjusted to the 1980 national birthweight distribution increased between the two periods from 4.0% to 5.4% at birth (an increase of 35.1%, $p = 0.0006$) and from 0.96% to 1.85% at three months (an increase of 92.7%, $p = 0.0002$).

In contrast to the above findings, Berkowitz *et al.* (1993) examined 6935 male offspring in New York, USA, over the period 1987-1990 and, using the same examination criteria as Scorer, found no increase in the prevalence of cryptorchidism at birth or at three months compared with the results of Scorer (Table 1).

However, it is difficult to draw firm conclusions from these studies because rates were measured at different locations. It is important that some further investigation is undertaken, as increased prevalence of cryptorchidism may be linked with other testicular disorders such as hypospadias and cancer.

Table 1: Incidence of Cryptorchidism (%) at birth and three months

Assessment	Birth Wt(g)	London mid-1950s (n=3612) Scorer (1964)	Oxford mid-1980s (n=7400) JRHCSG*(1992)	New York late-1980s (n=6935) Berkowitz (1993)
At birth	<2500	21.00	22.83	19.83
	>2500	2.70	4.08	2.22
	Mean	4.20	5.01	3.68
At 3 months	<2500	1.71	5.16	1.94
	>2500	0.91	1.61	0.91
	Mean	0.97	1.78	1.00

*John Radcliffe Hospital Cryptorchidism Study Group

HYPOSPADIAS

The prevalence of hypospadias at birth in different countries reported in the literature varies by a factor of more than 100 (from 0.37 to 41 per 10 000 infants). A number of factors make the figures difficult to compare however, including variations in definition and verification of the condition and ethnic origin of the population (Toppari *et al.*, 1995).

Since the mid-1960s, the prevalence of hypospadias at birth in England and Wales has increased substantially (WHO, 1991). Furthermore on the basis of notifications, the incidence of hypospadias in England and Wales is reported to have increased with time from approximately 14 per 10 000 total births in 1965 to almost 36 per 10 000 in 1983 (Matlai & Beral, 1985). However, more recent data indicate that, between 1980 and 1985, the incidence of hypospadias/epispadias was similar at 15 per 10 000 live births and 16.2 per 10 000 live births, respectively (On the State of The Public Health, 1993). Comparing these levels with the data for 1992 (8.0 per 10 000 live births), it appears that the incidence has fallen recently. However, from January 1990, certain minor malformations were no longer notified and this may largely account for the decrease.

Increasing incidences of hypospadias have been reported in other countries including Sweden (Källén & Winberg, 1982), Norway, Denmark, Finland, Spain, New Zealand, Australia and the former Czechoslovakia (WHO, 1991).

TESTICULAR CANCER

An increased incidence of testicular cancer has been described in white males in three areas of the USA (Brown *et al.*, 1986). The data were presented in the form of rates only; numbers of cases and statistical analyses were not described. When assessing the time periods 1947-1948, 1969-1971, 1973-1981 and an overall period of 1935-1979, the rates for the most affected age group (25-34 years) rose from 3.8 per 100 000 in 1947 to 11.6 per 100 000 in 1979-1981. The rates for the wider age group (15-44 years) rose from 2.4 per 100 000 in the late 1930s to 4.2 per 100 000 in the late 1950s, remained stable for ten years and then rose to 6.6 per 100 000 in the late 1970s. Feuer (1995) reported some preliminary data from the National Cancer Institute's Surveillance, Epidemiology, and End Results Program in the USA. The data suggested that the incidence of testicular cancer in white males in the USA has risen over the last two decades while the rate in black males, in whom testicular cancer is rare, did not appear to have risen.

A similar increase has been seen in the Nordic countries, Denmark, Sweden, Norway, Finland and Iceland for the period 1943-1980 (Hakulinen *et al.*, 1986). The data were obtained from the national cancer registries, which had collected statistics on 1.7 million cancer cases diagnosed over the time period. In Denmark, which had the highest overall testicular cancer rate, the increase was progressive from approximately 3 per 100 000 in 1945 to about 7.5 per 100 000 in 1980 and an estimated 8.9 per 100 000 in 1990 (Møller, 1993), that is a trebling over 45 years for this rare form of cancer. It was noted that the increasing trend in testicular cancer risk temporarily reversed for the cohort born during the second World War.

In an extension of this survey, covering five Baltic countries (Estonia, Latvia, Lithuania, former East Germany and Poland), the average annual increase was at least 2.3% in all populations studied (Adami *et al.*, 1994).

Comparisons between Nordic countries show some significant differences in overall testicular cancer rates, despite similarities in population types and environment. Most notable is the five times greater rate in Denmark than Finland on an age standardised basis.

Data from England and Wales have also shown increasing trends in testicular cancer, particularly in younger men. The incidence increased by approximately 35% from 1979 to 1987 (On the State of The Public Health, 1992).

In summary, it appears that the incidence of testicular cancer, particularly in younger white men, has increased significantly over the past 50 years and that this may represent a widespread situation, particularly in westernised countries.

It is thought that testicular cancer is initiated by factors acting early on in life, possibly before birth. In assessing possible causes, it may be pertinent to look at life style factors which could explain the disparate rates that exist, for example, between the two Nordic countries Denmark and Finland. It has been suggested that the temporary decline in testicular cancer in the cohort born during the war in these countries may have been due to changes in dietary and other life style factors (Hakulinen *et al.*, 1986; Møller, 1993).

Brown *et al.* (1986) suggested that the increasing rate in the USA may be due to exposure to a new environmental carcinogen or to increased exposure to one already present.

MALE BREAST CANCER

There are few studies on geographical and/or temporal trends in the incidence of this disease, which is rare in men. A survey of breast cancer rates in men in four Nordic countries indicated an increased incidence in Denmark between 1955 and 1980, but showed no discernable trend in Sweden, Norway or Finland (Ewertz *et al.*, 1989).

2.3.2 EFFECTS ON THE FEMALE REPRODUCTIVE SYSTEM

In contrast to reports of increases in male urogenital tract abnormalities in the UK, those for females tend to be falling (Matlai & Beral, 1985). However, there are reports that endometriosis (aberrant location of uterine mucous membrane), is increasing in US women (Hileman, 1994). The subject of most concern at

present appears to be the increasing breast cancer rates in women, although there is also some interest regarding the relation between cardiovascular disease and oestrogen status.

BREAST CANCER

There is evidence to show that breast cancer incidences have been increasing steadily over the past few decades in a number of countries. Data from the cancer registries of five Nordic countries showed that the incidence in Finland, the country with the lowest rates, rose from about 25 per 100 000 in 1953 to more than 40 per 100 000 in 1980. In Denmark, the country with the highest rates, the increase was from 40 per 100 000 in 1945 to about 60 per 100 000 in 1980 (Hakulinen *et al.*, 1986). Between 1973 and 1980, breast cancer incidences in the USA increased by 8% in women less than 50 years old and by 32% in those 50 years old or more (Ries *et al.*, 1991; Wolff *et al.*, 1993). Some of the increase may reflect improved detection with passage of time, but it has been estimated that a 1% annual increase in breast cancer has occurred in the USA since the 1940s (Feuer & Wun, 1992).

In contrast to the above trends, a study in Israel showed an 8% decrease in breast cancer mortality between 1976 and 1986. This fall has been ascribed to the steep decrease in concentrations of α–hexachlorocyclohexane (α-HCH), lindane (γ-HCH) and dichlorodiphenyldichloroethylene (DDE), which is a metabolite of dichlorodiphenyltrichloroethane (DDT), in cows milk following restrictions on the use of these pesticides in 1978 (Westin & Richter, 1990). The authors reported that, although breast cancer incidence data, which were not provided, did not allow the determination of whether the fall was in fact due to improved treatment, this was considered unlikely. However, Shames *et al.* (1994) considered the above study and other data relating to breast cancer mortality and incidence in Israel and concluded that the data provided no evidence for a causal relation between pesticide exposure and risk of breast cancer.

Interest has increasingly focused not only on changes in breast cancer rates *per se*, but also on the underlying causes. Known risk factors for breast cancer, which may account for 30% of cases, are linked with total lifetime exposure to reproductive hormones. The main risk factors include age at menarche, menopause and first full-term pregnancy, total calorie intake, family history, radiation exposure and alcohol intake (Davis *et al.*, 1993). Dietary factors may also be relevant; for example, Western women given a soy protein enriched diet

showed changes in hormonal status and regulation of menstrual cycle, both predisposing towards a lowered risk for breast cancer (Cassidy *et al.*, 1994). Differences in diet would help explain the significant differences in breast cancer rates between Western and Japanese/Chinese women, the incidence among the latter being considerably lower. There is continuing debate on the influence of the use of oral contraceptives on breast cancer incidence. One study found a two-fold increase in incidence after 12 years of oral contraceptive use, although a number of other studies found no evidence for an increase (Anon., 1986).

Recent emphasis has been directed towards the role of environmental contaminants in the causation of breast cancer. In particular, studies have been undertaken to find possible correlations between tissue concentrations of organochlorine compounds, such as DDT and polychlorinated biphenyls (PCBs), and breast cancer incidence (see sections 2.4.4 and 2.4.5). It has also been proposed that DDE may cause lactation failure in women (section 2.4.4).

EFFECTS ON THE CARDIOVASCULAR SYSTEM

Although young and middle aged women have a lower risk of atherosclerosis and its complications than men of similar age, cardiovascular disease remains a major cause of death in post-menopausal women. The observed sex and age differences in coronary heart disease provide considerable evidence for a protective effect of oestrogens in this disease. Oestrogen replacement therapy is commonly used to treat post-menopausal women for symptoms of the menopause, and rates of cardiovascular and cerebrovascular mortality in women who receive the therapy are a third to one half of those in untreated women (WHO, 1995). The natural oestrogen 17β-oestradiol affects cholesterol metabolism and deposition and the plasma lipid profile, inducing a less atherogenic blood pattern and inhibiting the formation of atherosclerotic plaques on arterial walls. In addition, *in vitro* studies using human umbilical vein endothelial cells and an *in vivo* mouse model have demonstrated a role for 17β-oestradiol in angiogenesis (new blood vessel formation), an effect potentially important in protecting against diseases of the circulatory system (Morales *et al.*, 1995).

The beneficial effects of oestrogens on the plasma lipid profile account for up to 30% of the observed reduction in mortality from cardiovascular disease. Studies in animals have demonstrated that oestradiol can exert direct effects on the cardiovascular system, such as increased cardiac output and arterial flow velocity and decreased vascular resistance and blood pressure (Magness & Rosenfield,

1989). Single administrations of 17β-oestradiol have demonstrated an antagonistic effect on exercise-induced myocardial ischaemia in women (Rosano *et al.*, 1993).

Whether such effects of oestrogens extend to xenoestrogens is not known. Epidemiological evidence has linked phytoestrogen-rich diets with a reduced incidence of cardiovascular disease (Adlercreutz *et al.*, 1992), but there is no information on either the acute or chronic vascular effects of many other environmental oestrogenic agents either *in vivo* or *in vitro*.

2.4 XENOESTROGENS

As described above, there is compelling evidence that testicular and female breast cancer rates have been increasing during the last four decades, certainly in westernised countries. The position regarding decreases in male fertility and increases in male urogenital abnormalities is not so clear, although trends may be evident in certain countries or circumstances.

It is not clear whether there is a common cause (mediated by either a single agent or a group of factors) for these changes. Scientific investigations have focused particularly on assessing the influences on the hormonal status of individuals. It is considered that factors which could alter this status early in life, and possibly before birth, could lead to adverse changes in the male and female urogenital tissues and mammary glands. Many factors could have effected changes in hormonal status of populations in recent times, including changes in life style, dietary intakes and the use of oral contraceptives.

The role of environmental contaminants has also been considered in this context. A number of chemical contaminants such as organochlorine compounds have demonstrated effects on hormonal systems in studies *in vitro* and in laboratory animals, but it is not clear what relevance such findings have to human populations. Furthermore, some natural constituents of human foodstuffs have also shown these properties. The possible roles for these substances are examined below.

2.4.1 SOURCES AND MECHANISMS OF ACTION

17 β-Oestradiol and other endogenous hormones occur naturally within the body and control reproductive and other functions as outlined briefly in section 2.2. Xenoestrogens are those chemicals entering the body from the external environment which may mimic or interfere with the action of endogenous oestrogenic hormones. The biological effects of xenoestrogens depend largely on their availability and concentration within tissues and body fluids, their binding to serum proteins such as SHBG and, relative to oestradiol, their binding to the oestrogen receptor. Additionally, recent work demonstrating interactions between growth factor receptors and the oestrogen receptor (Ignar-Trowbridge *et al.*, 1993) as well as aryl hydrocarbon (Ah) receptor and oestrogen receptor associations (Harper *et al*., 1994), raises the possibility that xenoestrogens may act through non-classical signalling pathways. Furthermore, some xenobiotic chemicals have been shown to have an effect on oestrogen metabolism. 17β-Oestradiol is metabolised by two separate pathways, producing either 2-hydroxyoestrone (2-OHE) or 16α-hydroxyoestrone (16α-OHE1). The former is non-genotoxic with low oestrogenic activity, while the latter is a potent oestrogen and is genotoxic. Women with breast cancer have been found to have high 16α-OHE1:2-OHE ratios, and levels of 16α-OHE1 in breast cancer tissue are higher than in normal breast tissue (Hileman, 1994). The risk of developing breast cancer has therefore been linked with these two pathways; substances that elevate the 16a-OHE1 pathway or inhibit the 2-OHE pathway are believed to increase the risk, while substances having the opposite effect reduce the risk (Davis *et al*., 1993). A number of polycyclic aromatic hydrocarbons (PAHs), including dimethylbenzanthracene and benzo[*a*]pyrene, have been shown to inhibit the 2-OHE pathway and also induce mammary tumours in experimental animals, while indole-3-carbinol increases the 2-OHE pathway and decreases the incidence of mammary tumours. Bradlow *et al*. (1995) studied a number of chemicals for their effect on the 16α-OHE1:2-OHE ratio in human breast cell cultures and suggested that compounds that increase the ratio should be regarded as potential breast carcinogens.

Xenoestrogens derive from many sources. They may, for example, be deliberately administered as pharmaceuticals or as oral contraceptives such as the ethynyl derivatives of oestradiol. Oestrogenic substances occur naturally in foodstuffs,

particularly vegetables, and may be found in food moulds (e.g. on grain). Several synthetic chemicals (e.g. organochlorine compounds), present as environmental contaminants, have also shown oestrogenic properties.

In considering the effects of exposure to xenoestrogens in humans, it is important to take into account the possibility of additive, synergistic or inhibitory effects, since it is likely that most exposures are to combinations of compounds with varying oestrogenic and/or anti-oestrogenic activities.

A number of chemicals have been demonstrated to possess anti-oestrogenic activity. Examples include Ah receptor agonists such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related chlorinated aromatic compounds (see section 2.4.6), various PAH combustion products, and a number of compounds found in cruciferous vegetables such as indole-3-carbinol. In addition, many chemicals which are weakly oestrogenic are also anti-oestrogenic at certain levels, an example being the bioflavonoids found widely in foods. Because of these often contrasting activities it is important to consider the total combined effect of xenoestrogens and anti-oestrogens, in the diet and in the environment (Safe, 1995).

In the following sections the potential effects of xenoestrogens on humans are examined. Particular attention is given to the amounts entering the body, and their relative oestrogenic or anti-oestrogenic potency and toxic action.

2.4.2 NATURAL AND SYNTHETIC HORMONES

Direct administration of hormones to animals and humans has been undertaken for a variety of purposes for many years. The best known example, apart from the contraceptive pill, is that of DES used to prevent miscarriages in women from the early 1950s until its use was stopped in the early 1970s. During this period an estimated 2.3 million women in the USA were prescribed DES during pregnancy, exposing approximately two to three million progeny to the drug *in utero*. A significant proportion of female offspring suffered reproductive organ dysfunction, abnormal pregnancies and disrupted menstrual cycles. In a small number, vaginal clear cell adenocarcinoma appeared when they became young adults, a rare cancer for women of this age. Abnormalities in males, found at an

increased incidence in DES-exposed offspring, included cryptorchidism, microphallus and epididymal cysts. There was also some evidence for decreased sperm count and motility, although the numbers of subjects were small (Stillman, 1982; Linn *et al.*, 1988). These and other effects, including testicular cancer, have been reproduced in animal studies with DES (Arai *et al.*, 1983; Newbold *et al.*, 1987; Bullock *et al.*, 1988). It should be noted that although DES binds to oestrogen receptors in cells, it does not bind to SHBG. Thus it is able to enter cells more freely than endogenous oestrogens and, as a result, has greater oestrogenic activity at relatively similar blood concentrations. The action of DES in humans and animals is taken as a model for what might be expected from exposure to any active oestrogenic substance.

Another use for DES for many years was as a growth promoter in cattle, thus exposing humans to residues of the hormone in meat products (McLachlan *et al.*, 1984). A variety of oestrogenic effects have been observed among operators in the manufacture and formulation of DES and oral contraceptives such as ethinyloestradiol after inhalation and skin absorption. Feminising effects have been observed among male workers in a DES plant in the USA (Steeno & Pangkahila, 1984).

More recent attention has been directed to exposure to natural and synthetic hormones in the environment. Significant concentrations of oestradiol and oestrone are naturally excreted by pregnant women and female animals. It is also suspected that constituents and breakdown products of the contraceptive pill may enter the environment through sewage effluent. Oestrogens appear to be persistent in the environment and it has been reported that oestrogens occur in measurable concentrations (24-48ng/l) in sewage effluent used for irrigation in Tel Aviv, Israel (Shore *et al.*, 1993).

A study by Aherne *et al.* (1985) failed to detect ethynyloestradiol, the principal component of the contraceptive pill, in either river or potable water in the UK (limit of detection 5ng/l). In a later study by the same group (Aherne & Briggs, 1989) it was reported that detectable levels of ethynyloestradiol were found in both river and potable water samples with a detection limit of 1ng/l; however, these findings were questioned by Harries *et al.* (1995). Norethisterone, another oral contraceptive constituent, was detected in river but not potable water in both studies (limit of detection 10ng/l, Aherne *et al.*, 1985; limit of detection 2ng/l, Aherne & Briggs, 1989).

In assessing the impact of exposure of humans to synthetic hormones, distinction must be made between the direct and deliberate application of pharmacologically

effective doses and exposure to trace amounts present in food, milk and perhaps water. The former has resulted in adverse effects directly attributable to oestrogenic action in the case of DES. The quantities of DES, and of hormones used in oral contraceptives, will result in vastly greater exposures than any expected from residual or environmental intakes; however the timing as well as the level of exposure is important.

2.4.3 PHYTOESTROGENS

Phytoestrogens are compounds with oestrogenic activity that are naturally present in edible plant material. They are constituents of many human foodstuffs such as beans, peas, sprouts, cabbage, spinach, soyabean, grains and hops. Phytoestrogens include classes of compounds such as the isoflavones, lignans, coumestans (e.g. coumestrol), and the resorcylic acid lactones (e.g. zearalenone), which are fungal metabolites found in foodstuffs such as wheat. Although the levels of dietary oestrogens in plants are thought to be generally low, several plants contain high concentrations of phytoestrogens (Price & Fenwick, 1985; Setchell, 1985) and, if ingested in large quantities, these may evoke significant biological effects. In the last 50 years, several important examples of the way in which non-steroidal dietary oestrogens can influence reproductive physiology in animals have been described (Bennetts *et al.*, 1946; Shutt, 1976; Setchell *et al.*, 1987).

Estimated oestrogenic potency can vary according to the test used and the species tested. The phytoestrogens are structurally similar to 17β-oestradiol and bind to isolated oestrogen receptors with affinities ranging from 500 to 1000 times less than that of 17β-oestradiol (Shutt & Cox, 1972; Martin *et al.*, 1978; Verdeal *et al.*, 1980; Juniewicz *et al.*, 1988). They produce typical and predictable oestrogenic responses when administered to animals (Shutt & Cox, 1972; Shemesh *et al.*, 1972; Evans *et al.*, 1991). Coumestrol is about one tenth as potent as oestradiol in binding to the oestrogen receptor in human tumour cell line MCF-7 cells, while genistein and zearalenone show about 30-50 times less potency than oestradiol. Their potency for inducing uterine growth in ovariectomised rodents, the classical test for assessing oestrogenic action, is much less than in the binding assay; coumestrol was about 3000 times less potent than DES, while genistein was about 100 000 times less effective than DES (Verdeal & Ryan, 1979). More recent studies in MCF-7 cells measuring oestrogen responsive cell proliferation or oestrogen specific protein induction have suggested different potencies for several

phytoestrogens relative to oestradiol; coumesterol is 900 to 100 000 times less potent, zearalenone is 25 to 120 times less potent and genistein is approximately 10 000 times less potent (Welshons *et al.*, 1990*;* Mayr *et al.*, 1992; 1990; Soto *et al.*, 1992).

In addition to an oestrogenic action, some phytoestrogens show anti-oestrogenic activity. For example, anti-oestrogenic action has been shown *in vitro* by indole-3-carbinol, a compound present in cruciferous vegetables such as sprouts and cabbage. This has been ascribed to induction of liver microsomal enzymes and consequent increased hydroxylation of oestrogens, thus protecting target cells by decreased formation of 16α-OHE1. This may have a protective action on the initiation and promotion of mammalian cell transformation, hence decreasing the risk of mammary carcinogenesis (Jellinck *et al.*, 1993). A recent study, however, indicated that the anti-oestrogenic activity of indole-3-carbinol does not require cytochrome P450-induced metabolism of oestradiol (Liu *et al.*, 1994). Indolo[3,2-*b*]carbazole is an Ah receptor agonist derived from indole-3-carbinol and has been shown to exhibit anti-oestrogenic activity in MCF-7 cells. It is also weakly oestrogenic in that it binds with low affinity to the oestrogen receptor (Liu *et al.*, 1994).

A distinction should be made between phytoestrogenic agonist/antagonist effects elicited *via* the oestrogen receptor and those acting through non-receptor-mediated mechanisms; Sheahan (1995) suggested that it is unlikely that all the effects of phytoestrogens are oestrogen-receptor mediated. Partial agonist/antagonist activity is a common feature of many weak oestrogens and it is difficult to estimate precisely the risks and benefits to humans. Some anti-oestrogenic compounds may be beneficial in the prevention of hormone-dependent cancers, as described below.

Zearalenone has been effective in treating the symptoms of post-menopausal syndrome in women at a dose of 75-100mg/day (Verdeal & Ryan, 1979; Price & Fenwick, 1985). Barnes *et al.* (1990) showed that soya protein, a rich source of isoflavones, reduced, in a dose-dependent manner, the number of tumours in animal models of chemically-induced mammary carcinoma. Premenopausal women given a soya protein enriched diet containing 45mg isoflavones daily for one month showed increased follicular phase length and/or delayed menstruation. Mid-cycle surges of LH and FSH were suppressed. These modifications may be potentially beneficial for decreasing risk factors for breast cancer (Cassidy *et al.*, 1994). Evidence substantiating this includes a recent epidemiological trial which showed that a high soya intake was associated with a decreased risk for breast cancer in premenopausal women in China and Singapore (Lee *et al.*, 1991).

In the Far East where hormone-dependent cancer rates are low, a typical daily consumption of isoflavones is 50-100mg (Barnes *et al.*, 1990; Adlercreutz *et al.*, 1991; Coward *et al.*, 1993). In contrast, in Britain the dietary intake of isoflavones has been estimated to be 1mg/day (Jones *et al.*, 1989). Vegetarians have a reduced risk of developing heart disease and hormone-dependent cancers (Phillips, 1975; Oliver, 1982; Frentzel-Beyme *et al.*, 1988). One study found that vegetarians excrete high concentrations of lignans; mean urinary levels of enterolactone and enterodiol were 1165µg/day and 168µg/day respectively. This compares to mean lignan excretion in omnivores of 156µg/day of enterolactone and 62.3µg/day of enterodiol (Adlercreutz *et al.*, 1986; Cassidy *et al.*, 1991).

The fact that plants and animals have co-evolved for millions of years has led to the suggestion that humans, like animals, may have adapted to exposure to some phytoestrogens (Guillette *et al.*, 1995a).

Taking into account the lifelong exposure to phytoestrogens, it is probable that the potential adverse properties are more than counterbalanced by the anti-oestrogenic and other benefits of eating a high plant food diet.

2.4.4 ORGANOCHLORINE PESTICIDES

The use of organochlorine compounds such as DDT, dieldrin, lindane, chlordecone and many others as pesticides was once extensive but has decreased in recent decades (IARC, 1991). A major characteristic of some of these compounds is their ability to accumulate and concentrate in biological systems. Because of slow breakdown and elimination, their residues can remain for long periods in human and animal tissue. A vast scientific literature exists on the uptake, metabolism and toxicology of the organochlorine pesticides in man and animals and it is against this background that their oestrogenic properties are reviewed.

This section focuses on DDT as the main compound in this class since it has been one of the most used and more persistent of this group of compounds. Commercial DDT comprises several isomers, the most prevalent being the *p,p*'-

form, which makes up about 75-80% of the total. In studies of competitive binding of DDT isomers or [^{3}H]-oestradiol to uterine cytoplasmic oestrogen receptors, *p,p'*-DDT and its main metabolite *p,p'*-DDE had no significant effect, whereas the *o,p'*-DDT isomer decreased [^{3}H]-oestradiol binding by about 40%. The oestrogenic action of this latter isomer was confirmed by administering 1.5mg *o,p'*-DDT to immature ovariectomised female rats by intraperitoneal injection and subsequently measuring the ability of uterine cytosol to bind [^{3}H]-oestradiol. Binding was reduced by 50-60% following the *o,p'*-DDT administration. In comparison, unlabelled oestradiol, at a dose 1000 times lower than that of *o,p'*-DDT, completely eliminated the [^{3}H]-labelled binding; thus *o,p'*-DDT was 1000-10 000 times less potent than oestradiol in this test (Forster *et al.*, 1975). Similar oestrogenic potencies have been shown for *o,p'*-DDT in other studies (Robison *et al.*, 1985; Galand *et al.*, 1987). However, the *o,p'*- isomers of DDT are relatively unstable and are rarely found in the environment.

In a recently published study, Kelce *et al.* (1995) confirmed that while *p,p'*-DDE, the major and persistent DDT metabolite, has little ability to bind with the oestrogen receptor, it does bind strongly with the androgen receptor and inhibits androgen receptor action *via* the inhibition of androgen-induced transcription. The authors suggested that *p,p'*-DDE can cross the placenta to the developing human fetus and reach levels shown, in rats, to induce anti-androgenic effects *in vivo*.

In terms of reproductive toxicology, DDT has been tested in several rat and mouse multigeneration studies (Smith, 1991). In one study of mice over six generations, a dietary level of 100mg/kg (ppm) DDT produced slight reductions in lactation and survival in some but not all generations. A dietary concentration of 25mg/kg DDT was the no-effect level for reproductive effects, equivalent to 3.3mg/kg bw/day in non-lactating adult females (Keplinger *et al.*, 1970). It has been speculated that organochlorine pesticide exposure may result in lactation failure in women (Rogan *et al.*, 1987). The authors studied the effects of PCBs and DDE in breast milk on children from birth to one year of age. Although no effect on child morbidity was demonstrated, higher levels of DDE (but not PCBs) were associated with higher rates of lactational failure in the mothers, defined as short duration of lactation with final weaning within two months.

Because of concerns about DDT storage in fat, several attempts have been made to estimate the relation between DDT levels in breast fat and breast cancer incidence. Falck *et al.* (1992) showed that women with malignant breast cancer had significantly higher mammary adipose tissue levels of *p,p'*-DDT and the

metabolite *p,p'*-DDE than women with benign changes. Since the number (20) in the two groups was small, no definite conclusion was reached. Using serum levels as an indicator, Wolff *et al.* (1993) found that the concentration of *p,p'*-DDE was greater in women with breast cancer (mean 11ng/ml, n=58) than in matched controls (mean 7.7ng/ml, n=171). Although this indicated a strong relation between DDE levels and breast cancer, the authors suggested that DDE was merely a surrogate for another, unknown active agent. In contrast, Unger *et al.* (1984), also using small numbers of subjects, found no significant difference in DDE levels in breast fat from women with or without breast cancer from autopsy or biopsy samples. The results of Falk *et al.* (1992) and Wolff *et al.* (1993) are also countered by another small case-control study of 150 case patients and 150 matched control subjects (Krieger *et al.*, 1994). The notable feature of this study was that serum samples were obtained many years before the patients were diagnosed with breast cancer and during the period before restrictions were placed on DDT use. The mean results showed that there was no difference in serum *p,p'*-DDE levels between the two groups. Although subgroup analyses suggested a positive association between breast cancer risk and serum DDE levels in black women, the authors reported that this could have been the result of chance. In a study of women with breast disease, which also used only very small numbers of patients, Dewailly *et al.* (1994) compared the organochlorine body burden in control women with benign breast disease (n=17) with that in breast cancer patients (n=20). Levels were measured in breast adipose tissue and in plasma and, although most levels were higher in breast cancer patients, a significant difference was observed only for hexachlorobenzene in plasma. When the oestrogen receptor status of cases was measured however, oestrogen receptor-positive cases (n=9) had significantly elevated levels of DDE in adipose tissue and plasma compared with receptor-negative cases and controls. The results suggested that the body burden of DDE in women with hormone-responsive breast cancer, but not hormone-nonresponsive breast cancer, was higher than that in women with benign breast diseases. Key and Reeves (1994) concluded that it is unlikely that DDT increases the risk of breast cancer, but since the current evidence is based upon a relatively small number of subjects, some further investigation is warranted.

When assessing potential adverse effects of DDT in man it should be noted that in the earlier period of its use millions of people were exposed to relatively high quantities of the compound. It was used, for example, for mass de-lousing, where clothing and exposed skin were dusted with the compound. Also, hundreds of millions of homes worldwide have been sprayed with DDT in anti-malarial campaigns. Workers manufacturing and formulating the insecticide have been exposed to much higher levels than members of the general population, where the

main intake is through food residues. Observations and medical surveys over many years have revealed a number of minor effects in occupationally exposed individuals, but no evidence of significant ill-health. The levels of DDT and DDE in tissues and body fluids of these exposed groups were far higher than in the general population. Workers involved with the manufacture and formulation of DDT, for example, showed fat levels in excess of 100mg/kg (ppm) as total DDT (i.e. *p,p'*-DDT, *p,p'*-DDE and other metabolites and isomers). By comparison, during 1950-1980, total DDT in the fat of members of general populations, in a number of countries worldwide, was mostly in the range of 2-10mg/kg (ppm) (Smith, 1991). Because of bans or restrictions on the use of DDT in many countries, the concentrations of DDT in fat and body fluids are steadily declining. In the USA, body fat concentrations declined from a high of approximately 15mg/kg (ppm) in 1955 to less than 5mg/kg in 1980 (Levine, 1991). Similar decreases have been shown for DDT in human breast milk (Somogyi & Beck, 1993).

The daily intake of DDT from the environment (mainly food) has been very small for a number of years. For example, the daily intake per person in the USA in 1980 was estimated to be 34.0ng/kg bw/day, and for infants in 1979 was 113.0ng/kg bw/day. By 1990, intakes estimated from the Food and Drug Administration's total diet study were 10.3, 26.0 and 77.0ng/kg bw/day for 60-65 year old females, 14-16 year old males and 6-11 month old infants, respectively (Winter, 1992). It should be noted that the acceptable daily intake, set by the Joint Meeting on Pesticide Residues in 1984, is 20µg/kg bw/day (FAO/WHO, 1985). In view of the negligible oestrogenic potency of *p,p'* DDT and DDE, and the very low and declining intake of these compounds, it would appear unlikely that any oestrogenic action would occur in the general population. The possibility of rapid release of DDT from fat depots, during fasting or prolonged exercise for example, would not seem likely to alter this conclusion, since studies have shown that this is not accompanied by any significant effect based upon pathological and biochemical examination (Mitjavila *et al.*, 1981). However, it should be noted that *p,p'*-DDE has been ascribed an anti-androgenic activity and may therefore require further consideration (Kelce *et al.*, 1995).

Other organochlorine pesticides showing oestrogenic properties *in vitro* and *in vivo* are methoxychlor and chlordecone (Kepone) (Bulger *et al.*, 1978, 1979). Methoxychlor (a closely related derivative of DDT), unlike DDT, is cleared quickly from the body and does not accumulate in fat. Chlordecone represents a somewhat different case in that its oestrogen-like activity has been demonstrated in humans. Workers exposed to high levels of the compound by various routes during its manufacture suffered a variety of effects including abnormal sperm,

decreased sperm motility and oligospermia (Guzelian, 1982). Studies on the kinetics of chlordecone elimination from these workers showed that, unlike DDT, equilibrium is rapidly attained between blood and other tissues and that the average half-life of the compound is similar in fat and blood (125-153 days). Although highly exposed subgroups might be affected by the oestrogenic effects of chlordecone, it is thought that the exposure of the general population is likely to be minimal (WHO, 1984).

2.4.5 POLYCHLORINATED BIPHENYLS

PCBs have been used industrially since 1929 as heat transfer and hydraulic fluids, solvent extenders, flame retardants and dielectric fluids. By 1970 hundreds of millions of kilograms had been produced. Despite decreasing use, and increasing restrictions on the production of PCBs, large quantities have been dispersed in the environment and remain there. They are generally very stable materials which can accumulate and persist in biological systems. PCBs are represented by 209 closely related structures (congeners); commercial products contain mixtures of many of them. This creates difficulties in the analysis of environmental and biological samples and in the interpretation of results (WHO, 1992a).

The current European Council definition of a PCB is a fluid containing more than 0.005% PCB (Environmental Protection Regulations, 1992). The continued use of transformers and capacitors containing PCBs to the end of their useful lives is permitted by the EC only if they were constructed before the middle of 1986 (EEC,1985). A Disposal Directive to include phased disposal of PCBs is currently under discussion. It is anticipated that these measures will lead to a fall in the levels of PCBs present in the environment.

Early studies have shown that some PCB congeners possess oestrogenic properties but with a potency only about one millionth that of oestradiol (Lione, 1988). Oestrogenic activity appears to decrease as the degree of chlorination increases. Bitman & Cecil (1970) tested the oestrogenic activity of a number of PCBs *in vivo* by comparing the subcutaneous dose required to increase significantly the level of glycogen produced the by rat uterus over an 18 hour period. The authors found moderate oestrogenic activity for Aroclors 1221, 1232, 1242 and 1248, but no

activity for Aroclors 1254, 1260, 1262 and 1268. The last two figures in the Aroclor nomenclature indicate the percentage by weight of chlorine in the mixture, thus the more highly chlorinated PCBs, which are the most stable, were the least active.

Several hydroxylated PCBs bind to the oestrogen receptor and it is possible that *p*-hydroxylated PCB metabolites may be the active oestrogenic components of certain PCB mixtures. Using a mouse uterine oestrogen receptor binding assay, Korach *et al.* (1988) have shown that some of the hydroxybiphenyls are active oestrogenic compounds. Three compounds with activities of 1/42 to 1/90 of oestradiol were 4-hydroxy-2',4',6'-trichlorobiphenyl, 4-hydroxy-2',3',4',5'-tetrachlorobiphenyl and 4,4'-dihydroxy-2'-chlorobiphenyl. The hydroxy metabolites are formed by the mixed function oxidase system. However, although these derivatives are more active than the parent compounds, they are also more water soluble and more readily excreted.

Immature female rats given intraperitoneal doses of Aroclor 1242, at levels of 80 or 320µg/rat, showed increased cell proliferation in the uterus (Jansen *et al.*, 1993). The oestrogenic activity was reduced by administration of another congener, 3,4,3',4'-tetrachlorobiphenyl, indicating that this form showed anti-oestrogenic properties. This finding is consistent with other studies showing that this congener and some of its most closely related derivatives inhibited oestradiol-induced secretion of the 52-kDa protein from MCF-7 human breast tumour cells, a further indicator of anti-oestrogenic action (Krishnan & Safe, 1993). It was shown that the anti-oestrogenic potency of these congeners was related to their ability to bind to the Ah receptor, indicating that the Ah receptor mediates the anti-oestrogenic activity of these compounds (see also section 2.4.6).

A recent study identified several hydroxylated PCB congeners (mostly *p*-hydroxylated) in human serum, all of which were substituted with chlorine atoms at both neighbouring carbon positions (therefore mostly *m*-chlorinated); most congeners found were also *o*-chlorinated (Bergman *et al.*, 1994). Based on results of structure-activity studies for hydroxylated PCBs, *o*-chlorine substitution leads to the greatest oestrogenic activity, as measured by oestrogen receptor binding (Korach *et al.*, 1988).

Drenth *et al.* (1994) reviewed the reproductive effects of PCBs and suggested a number of mechanisms of action. In addition to functioning naturally as both agonists and antagonists of hormone action, PCBs may also be involved in the induction of a number of cytochrome P450 *iso*-enzymes responsible for the

metabolism of steroid hormones. Studies *in vitro* have demonstrated effects of PCBs on the metabolism of testosterone, progesterone and oestradiol, but little information is available *in vivo*. A third suggested mechanism involves changes in hormonal responses due to the suppression of oestrogen and progesterone receptors in the liver and uterus. Exposure to PCBs during the stages of pre- and early post-natal development is likely to lead to permanent disruption of reproductive function due to effects on sex-dependent behavioural patterns and hormone secretions of the brain, the response of gonads to hormones *via* receptors and changes in sex-dependent cytochrome P450 *iso*-enzyme expression in the liver.

Attempts have been made to correlate PCB tissue levels with breast cancer incidence in women (see p. 30 for methodology and design of these studies). As with DDT and DDE levels, the two studies utilising breast fat for PCB analysis showed conflicting outcomes. Falck *et al.* (1992) showed a correlation between PCB concentration and breast cancer, while Unger *et al.* (1984) could find no such relation. When blood serum levels were examined, Wolff *et al.* (1993) found PCBs at a greater but not statistically significant level in patients with breast cancer. Krieger *et al.* (1994) found no difference in PCB levels in serum taken from women before the onset of breast cancer compared with those of matched controls. Analysis by subgroups of white, black and Asian women suggested a negative association of breast cancer risk with serum levels of PCBs among white women, but this could have resulted by chance (Krieger *et al.*, 1994). Serum levels were markedly higher among the black and Asian women compared with white women. It should also be noted, however, that an epidemiological study of PCB-exposed workers did not show an increased incidence of breast cancer (Brown, 1987). Key & Reeves (1994) summed up the position by stating that they could see no evidence for an association of PCBs with breast cancer risk.

A similar exercise has been undertaken with the examination of semen samples from 33 fertile men compared with 50 subfertile and 50 infertile men and 25 post-vasectomy subjects (Bush *et al.*, 1986). In the analysis of 74 PCB congeners there was an indication that the concentrations of three congeners were inversely related to sperm motility in samples where the sperm count was less than 20 million/ml. Because of the small number of subjects and the possibility that the PCBs may have been indicators for more potent unknown agents, no firm conclusion could be drawn from this study.

The possible oestrogenic effects of PCBs in human populations are difficult to evaluate because of the theoretical presence of many different congeners and the

countering anti-oestrogenic activity of some of them. However, some rough estimate can be made by comparing exposures in animal reproduction studies with human exposures. Reproductive toxicity assessments in several animal species have shown the monkey to be the most sensitive. Feeding dietary levels of 2.5 or 5mg/kg (ppm) Aroclor 1248 extended the duration and degree of menstrual bleeding, while 5mg/kg (ppm) for seven months decreased the conception rate and gave rise to smaller offspring (Lione, 1988; Jones, 1989). The daily intake of approximately 200µg/kg bw/day in these studies was one to two orders of magnitude greater than the tolerable dose of of 1-3µg/kg bw/day used by some authorities when considering lifetime human PCB intake (Somogyi & Beck, 1993). It is possible, however, that more highly exposed populations could be at risk. For example, mothers eating 11.8kg or more of PCB-contaminated fish from Lake Michigan, USA for six years before delivery produced infants with lower bodyweights and smaller head sizes than those not so exposed (Fein *et al.*, 1984). It is not certain, however, if the reproductive effects in animals and humans associated with exposure to PCBs are due to oestrogenic activity.

Relevant observations for PCB exposures in humans have been obtained from two major accidental poisonings in the Far East where a large number of people ate contaminated rice oil: the Yusho (Japan) and Yu-Cheng (Taiwan) incidents. In the follow-up, women showed increased fetal loss. In surviving fetuses the most consistent findings were low birth weight and a dark brown pigmentation of the skin, both defects resolving within about six months. The direct contribution of PCBs to these effects is difficult to determine however, since the rice oil was contaminated with other chlorinated products including chlorinated quaterphenyls and polychlorinated dibenzofurans (PCDF) (Lione, 1988; Jones, 1989).

2.4.6 DIOXINS

The polychlorinated dibenzo-*p*-dioxins (PCDD) and PCDFs present a rather different situation to the other environmental contaminants reviewed here. They are not commercial products but arise indirectly from incineration processes and as waste products from the chemical manufacturing and pulp and paper industries. In the environment they are present in nanogram (10^{-9}g) or picogram (10^{-12}g) quantities and the total amount entering the environment is very small (e.g. about 15kg annually in the USA (US EPA, 1994)). The toxicity of some of

the more active compounds is extremely high; for example, the LD_{50} for TCDD in guinea pigs is 0.6µg/kg bw.

There are 75 PCDD isomers and 135 PCDF isomers. In environmental and toxicological considerations the 2,3,7,8-isomers appear to be the most important. Most information is available for TCDD and the data for this isomer are usually utilised when comparative assessments are made for other isomers. Normal human exposure is *via* food, notably meat, milk and fish.

In contrast to the discussions on the previous groups of compounds, the major characteristic of the dioxins is their anti-oestrogenic activity. For example, TCDD (at 20 or 80µg/kg bw) was shown to decrease hepatic and uterine weight in the classical *in vivo* bioassay for oestrogenic activity, while oestradiol (at 5 or 15g/kg bw) caused an increase in both these parameters. Competitive binding studies showed that TCDD did not bind to oestrogen receptors, but was strongly bound to the Ah receptor. Oestradiol did not bind to this receptor (Romkes *et al*., 1987). In further studies it was also shown that TCDD inhibited oestradiol-induced peroxidase activity, progesterone receptor levels and epidermal growth factor receptor binding. In MCF-7 human breast tumour cells, TCDD inhibited the oestradiol-induced proliferation and secretion of a number of proteins; pre-treatment with TCDD caused a rapid decrease in nuclear oestradiol receptor binding activity (Safe *et al*., 1991). In a long-term rat study with TCDD at dose levels of 0.001-0.1µg/kg bw/day, the incidences of pituitary, uterine and mammalian gland tumours were significantly decreased (WHO, 1989). TCDD has also been demonstrated to inhibit the growth of mammary tumours in female rats after initiation with 7,12-dimethylbenzanthracene (Holcombe & Safe, 1994).

The potency of the anti-oestrogenic properties of PCBs and dioxins has been tested in MCF-7 human breast tumour cell lines by their ability to inhibit oestradiol-induced secretion of a 52-kDa protein (Krishnan & Safe, 1993). With few exceptions, the order of their potencies, headed by TCDD, paralleled their activity as agonists for other Ah receptor-mediated responses. The ubiquity of the Ah receptor-mediated action of dioxins and the relation between the receptor and toxic potency has led to the concepts of Toxicity Equivalence Factors (TEF) for grading the toxic action of these compounds (Safe, 1990). It should be noted, however, that there is not complete agreement that dioxin toxicity is receptor-mediated (WHO, 1989; ECETOC 1992).

TCDD has been shown to have adverse effects on sexual behaviour, spermatogenesis and reproductive capability in male rats at levels as low as

0.064g/kg bw. This was ascribed to an effect on the androgenic status of the male rats (Malby *et al.*, 1992a,b,c). In a variety of reproductive toxicity studies, the lowest effect levels occurred at or below 0.001µg TCDD/kg bw/day in the rat and monkey (Bowman *et al.*, 1989; ECETOC, 1992). One study has associated dioxin exposure with reproductive abnormalities in female rhesus monkeys (Rier *et al.*, 1993). Ten years following treatment of three groups of animals (8 per group) to levels of 0, 5 and 25ng/kg (ppt) dioxin in the diet for a five year period, the incidence of endometriosis, a condition characterised by growth and proliferation of endometrial cells at sites outside the uterus, was determined. Both the incidence and severity of disease were correlated with dioxin exposure, although no mechanism of action was suggested.

Human exposures to high levels of dioxins have occurred, notably at Seveso, Italy in 1976, when an estimated 300g of material was discharged over an area of 2.2km^2 as a result of an industrial accident. Observations and medical follow-up since that time have shown that the only confirmed effect in humans is chloracne, which has regressed in the majority of cases (Bertazzi, *et al.*, 1989; WHO, 1989). In the most recent epidemiological study on this accident, it was reported that breast cancer incidence among females was below expectation in the most contaminated zones and there was a clear decrease for endometrial cancer in the less contaminated zones (Bertazzi *et al.*, 1993).

Although dioxins exhibit a variety of effects in animal studies, it would appear that these are not caused by oestrogenic activities. In fact their action is strongly anti-oestrogenic and it has been suggested that this should be taken into account when assessing the overall risk from environmental oestrogens (Safe, 1994). Existing and projected regulatory action is designed to ensure a progressive reduction in dioxin exposure (EEC, 1992; US EPA, 1994).

2.4.7 ALKYLPHENOL POLYETHOXYLATES

The alkylphenol polyethoxylates (APEs) are non-ionic surfactants introduced into the UK in the 1940s and used extensively in cleaning products, paints, herbicides, pesticides and as industrial process aids in pulp and paper production and textile manufacturing. The annual world consumption in 1988

was over 360 000 tons (Ahel *et al.*, 1993). About 18 000 metric tonnes of nonylphenol ethoxylate was used in the UK in 1992; it is estimated that 37% is discharged to the aquatic environment, while 46% is applied to or disposed of to the land and is assumed not to enter the aquatic environment in any significant quantity (CES, 1993). The basic structure consists of an alkylphenol with a side chain of several ethoxylate groups. Although there are many different compounds with 8-12 ethoxylate groups, some 80% are the nonylphenol ethoxylates. Most of the ethoxylate groups are readily removed in biological degradation systems, but the remaining groups and the alkylphenols, generally *p*-nonylphenol, are resistant to further breakdown. Concern about the lack of complete degradation, the appearance of the residual products in rivers and their toxicity to aquatic species has led to a planned phased withdrawal from some uses by the end of the century (CES, 1993; Warhurst, 1995). Although highly toxic to aquatic species, mammalian toxicity is low; for example, the oral LD_{50} in rodents is 2-4g/kg bw (Benson & Nimrod, 1994). Exposure to APEs may occur not only *via* the water supply but by other routes, such as through the use of cosmetics.

The oestrogenic properties of the alkylphenols have been studied *in vitro* and *in vivo*. In human breast tumour MCF-7 cells, *p*-nonylphenol induced cell proliferation and increased progesterone receptor levels, but was about 300 000 times less potent than oestradiol. The compound was also given at 1-50mg quantities to ovariectomised female rats and the effect on endometrial mitosis was compared with animals given oestradiol. The mitotic index in animals given 50mg *p*-nonylphenol was less than that in those given 1.25µg oestradiol (Soto *et al.*, 1991). The oestrogenic activity of this compound and of *p*-octylphenol was demonstrated by White *et al.* (1994), using a series of *in vitro* assays including proliferation of human breast tumour cells (including MCF-7 cells), gene transcription in transfected chickens embryo fibroblasts, and vitellogenin gene expression in trout hepatocytes. Octylphenol was more potent than nonylphenol in these assays, but was still 1000 times less active than oestradiol. The effects of these alkylphenols appeared to be mediated *via* the oestrogen receptor.

Direct extrapolation of the above results to man is difficult since other toxicological data on the APEs and their degradation products is sparse. The concerns for man arise mainly from possible intake *via* drinking water or by eating contaminated fish. Analysis of drinking water in New Jersey, USA showed the presence of ten nonylphenol ethoxylates and one octylphenol ethoxylate at concentrations of 15-29ng/l (Clark *et al.*, 1992). Given the low oestrogenic potency shown for the alkylphenols in drinking water, it would seem unlikely that such small concentrations of ethoxylates would result in any detectable

oestrogenic effect in man, although the possibility of additive and/or synergistic effects even at subthreshold levels must be taken into account. Nonylphenol and two of its ethoxylates have been measured in edible portions of four species of freshwater fish at levels ranging from 0.13 to 3.1mg/kg dry weight (Ahel *et al.*, 1993).

In a recent environmental assessment of APEs and alkylphenols, Warhurst (1995) claimed that these chemicals do not degrade adequately in sewage treatment, they persist in the environment, break down to form toxic intermediates and may have oestrogenic effects at current environmental concentrations. As a contracting party to the Paris Convention, the UK has already agreed to phase out the use of nonylphenol ethoxylates as industrial cleaning agents by the year 2000, and to reduce all discharges to the environment (CES, 1993).

2.4.8 OTHER XENOESTROGENS

With the expansion of testing chemicals for oestrogenic potency, more potential oestrogens continue to be identified. Recently, a constituent leached from polycarbonate flasks, bisphenol-A, has been shown to be oestrogenic in *in vitro* tests (Krishnan *et al.*, 1993).

Eldridge *et al.* (1994) demonstrated that two strains of female rats given the chlorotriazine herbicides atrazine and simazine daily by oral gavage for two weeks had significant reductions in bodyweight, ovarian and uterine weight and decreased circulating levels of oestradiol. Although these effects were observed with both chemicals, the oral dosing levels were extremely high (100 and 300mg/kg/day), at or above the maximum tolerated doses for these strains. Further studies have demonstrated that neither of these compounds has intrinsic oestrogenic activity. Although the chlorotriazine herbicides were capable of weak inhibition of oestrogen-stimulated responses in the rat uterus, this occurred only at very high dose levels (Tennant *et al.*, 1994a). This weak anti-oestrogenic activity appears to be non-receptor mediated, as neither compound demonstrated an ability to compete with the binding of radiolabelled oestradiol to rat uterine oestrogen receptors (Tennant *et al.*, 1994b). Atrazine has, however, been demonstrated to depress the 2-OHE pathway of oestradiol metabolism and elevate the 16α-OHE1 pathway, an effect hypothesised to increase the risk of breast cancer in women (Davis *et al.*, 1993; Bradlow *et al.*, 1995).

Surveys undertaken by the Ministry of Agriculture, Fisheries and Foods (MAFF), in which levels of atrazine and simazine were measured in sea water samples from around the UK, showed that concentrations and distribution patterns were similar in 1990, 1991 and 1992 (MAFF, 1994). Residues of both compounds were detected at the majority of inshore stations, with levels decreasing rapidly but still detectable offshore. The highest concentrations of simazine and atrazine were found around the Humber and Mersey estuaries, at concentrations of 37ng/l and 42ng/l, respectively.

In a series of *in vitro* experiments, Kelce *et al.* (1994) demonstrated that the two metabolites of the dicarboximide fungicide vinclozolin were effective antagonists of rat androgen receptor binding and thus acted as anti-androgens. When given orally to pregnant rats from gestational day 14 to post-natal day three (the period of sex differentiation) at doses of 100 and 200mg/kg/day, a number of reproductive abnormalities in male rat pups were induced, including hypospadias, cleft phallus, testicular granulomas and atrophic seminal vesicles (Gray *et al.*, 1994). The authors concluded that similar effects in humans are likely to occur if exposure of the fetus during sex differentiation results in similar tissue levels of metabolites (Gray *et al.*, 1994).

Di(2-ethylhexyl) phthalate (DEHP) is a known reproductive toxicant and carcinogen in animals and may be a potential human health hazard through its use as a plasticiser in medical and food packages and its subsequent leaching out into the product. Davis *et al.* (1994) investigated the effects of DEHP in adult female rats. Animals were dosed daily for a maximum of 12 days and DEHP was found to suppress serum oestradiol levels, leading to hypoestrogenic anovulatory oestrus cycles and the development of polycystic ovaries. When investigating the mechanism of DEHP-induced liver hyperplasia and tumourigenesis in the rat, Eagon *et al.* (1994) observed that male rats fed 1.2% DEHP in the diet had significantly elevated serum oestradiol levels and the activity of liver oestrogen receptors was significantly reduced. Effects of DEHP on the reproductive system of male rats include reduced serum testosterone levels and atrophied seminiferous tubules and testes (WHO, 1992b). Rats and guinea-pigs appear to be particularly sensitive to DEHP-induced testicular atrophy.

Regarding human reproductive effects, occupational exposure to high levels of phthalates has been reported to be associated with an increase in rates of miscarriage and other complications of pregnancy in a Russian study of women factory workers (Aldyreva *et al.*, 1975).

2.4.9 RELATIVE LEVELS OF OESTROGEN INTAKE

Direct exposure to hormones arises, for example, from drinking bovine and human milk. Several hormones, including oestrogens, appear to be synthesised within the mammary gland as well as transported into milk from the maternal circulation. The concentration in bovine mammary gland secretions increases during the week before parturition and declines rapidly during the first two to three days postpartum, whereas levels in human colostrum decrease over the first five days postpartum and then remain constant until about six weeks postpartum. The ratio of oestrogens in milk to that in plasma during late gestation was reported to be generally greater than one, although absolute levels were not given (Grosvenor *et al.*, 1992).

Verdeal and Ryan (1979) contrasted the dose received from exposure to a number of dietary oestrogens with those from various hormone treatments in humans. It was estimated that the daily dose from the intake of 100g beef with a residue of 0.5µg/kg (ppb) DES was at least 10 000 times less than that received daily (as DES equivalents) from hormone treatment. Using the same comparison, daily doses from the intake of 100g wheat with a residue of 2mg/kg (ppm) zearalenone, or 100g *Phaseolus vulgaris* beans containing 2-10µg/kg (ppb) oestradiol, were at least 2500 and 3300 times less, respectively.

In a mass/potency balance analysis of human exposure to oestrogenic chemicals, Safe (1995) estimated the human exposure to dietary and environmental oestrogens as the daily dose in terms of oestrogen equivalents (EQ). The estimated dietary EQ levels of bioflavonoids were 102µg/day, a level 4×10^7 times higher than the corresponding value for oestrogenic pesticides (2.5pg/day). Estimated EQs for post-menopausal therapy, the birth control pill and morning after pill were 3.35, 16.7 and 333mg/day, respectively. An estimate was also given for exposure to anti-oestrogens in the diet and the environment expressed as TCDD anti-oestrogen equivalents (TEQ), one TEQ being approximately equal to one EQ. The TEQ contribution from PAHs in food ranged from 1.2-5.0ng/day and that from an active derivative of indole-3-carbinol from brussel sprouts ranged from 0.25-1.28ng/day. It was concluded that, with the exception of hormone administration, the major human intake of endocrine disruptors associated with the oestrogen-induced response pathways are naturally-occurring oestrogens found in foods.

Several factors should be taken into account when assessing the potential oestrogenic effects of chemicals. The potential for accumulation and persistence, for example in fat, should be compared with that of degradation, as each factor is likely to be different for different xenoestrogens. Pharmacological and pharmacodynamic differences also need to be considered in any overall assessment.

2.5 CONCLUSIONS

There are concerns about the increasing trends in certain human hormonally-mediated effects that have occurred over the last 50 years. The evidence is particularly convincing for breast cancer in women and testicular cancer, and the balance of evidence suggests a downward trend in sperm count. Life styles and dietary intakes, which influence hormonal status, have also changed during this period.

There are several variables to be considered, some of them conflicting, when attempting to assess the role of environmental oestrogens on human health. Firstly, the oestrogenic potency of the contaminants reviewed is low or very low compared to endogenous oestrogens such as oestradiol. These may not be strict comparisons, however, since circulating oestradiol is mainly bound to SHBG in the body while xenoestrogens, although not necessarily binding to SHBG, might bind, to a greater or lesser extent, to other proteins. Many of the commercially-derived contaminants described in this report are the subject of regulatory bans or restrictions. For a number of organochlorine pesticides there has been, at least in developed countries, a progressive and significant decline in their use over the past 20 years. As with all toxicological reactions, it is possible that there may be additive or synergistic effects between individual xenoestrogens. However, such interactions are difficult to define in practice because of the various possible mechanisms of action. For example, some of the contaminants such as dioxins and some PCBs have anti-oestrogenic actions and this needs to be taken into account when assessing the overall oestrogenic impact of chemicals in the environment. Humans have always been exposed to chemicals with oestrogenic and anti-oestrogenic activity in the form of phytoestrogens present in plant foodstuffs. During the last 50 years many people have in addition been exposed to high concentrations of hormonal substances in the form of medical treatments and

oral contraceptives. Against these backgrounds, and considering that the effects of exposure may depend on the stage in life at which exposure occurs, it is currently very difficult to assess whether or not environmental oestrogens play a significant role in reproductive disorders in the general population. As with any chemical exposure there may be particular populations or subgroups more at risk than the general population, for example because of occupational exposure or a limited and/or unbalanced dietary intake.

It is expected that as more substances are tested, a greater number will be shown to have oestrogenic activities. It is important that any potential for activity in humans is assessed in the light of specific toxicological action and actual human exposure, in order to provide a perspective on the degree of risk to human health.

As yet, a causal relationship between exposure to environmental oestrogens and adverse effects on human reproductive health has not been established. Further information from epidemiological and experimental studies is still needed to allow assessments of risk to be made based on a weight of evidence approach.

3 Wildlife effects

3.1 BACKGROUND

A major international workshop held in the USA in 1991 concluded that "A large number of man-made chemicals that have been released into the environment, as well as a few natural ones, have the potential to disrupt the endocrine system of animals, including humans. Among these are the persistent, bioaccumulative organohalogen compounds that include some pesticides (fungicides, herbicides, and insecticides) and industrial chemicals, other synthetic products, and some metals. Many wildlife populations are already affected by these compounds. The impacts include thyroid dysfunction in birds and fish; decreased fertility in birds, fish, shellfish, and mammals; gross birth deformities in birds, fish, and turtles; metabolic abnormalities in birds, fish, and mammals; behavioural abnormalities in birds; demasculinisation and feminization of female fish and birds; and compromised immune systems in birds and mammals"(Colborn & Clement, 1992).

In this report, the field considered is limited to the direct oestrogenic effects of pollutants; thus effects mediated *via* the thyroid, immune system and generalised effects on fertility are not covered. However, two 'masculinisation' effects are included which may not strictly relate to oestrogenic or anti-oestrogenic activity. These are presented for completeness as they are often considered in general discussions of the effects of environmental oestrogens.

It is possible that there are common modes of action for the diverse effects listed above. Evans (1988) described the 'superfamily' of steroid, retinoid and thyroid receptors. He concluded that although animals employ complex and often distinct ways to control their physiology and development, the discovery of receptor-related molecules in a wide range of species suggests that mechanisms underlying morphogenesis and homeostasis may be more ubiquitous than previously expected.

McLachlan (1993) has proposed a simple model which divides the way that exogenous chemicals may act at the hormone receptor site into those that act as hormonal mimics, and thus cause a hormonal response, and those that act

as hormonal blocks and thus stop normal reactions. Using this approach, the present report is concerned with oestrogenic and androgenic mimics.

In assessing the potential effects of chemicals with oestrogenic or anti-oestrogenic activity it is important to consider the particular species exposed, as the roles of steroids are different between species and sexual differentiation and endocrinology may also differ. The developmental stage at which exposure occurs is particularly important. The effects of exposures occurring at a critically sensitive period in the lifetime of an individual, such as during embryonic development, have been termed 'organisational effects' as they may lead to permanent structural modifications of the reproductive, immune or nervous systems. While these effects are usually manifest in early life, they may extend into adulthood. Factors which may alter the response of embryos to environmental oestrogens include bioaccumulation, degradation and secretion of xenoestrogens, and levels of free and bound hormones (Guillette *et al.*, 1995a). In adulthood, the effects on the endocrine system of exposures to environmental oestrogens (or anti-oestrogens) are more commonly transitory in action and have been termed 'activational effects'.

3.2 EFFECTS IN WILDLIFE SPECIES

A number of different effects, ascribed to a wide variety of potentially oestrogenic chemicals, have been reported. Examples from both wildlife and domestic animals are presented in Table 2. The sections below consider in more detail the effects highlighted most recently, concentrating on wildlife.

3.2.1 OESTROGENIC EFFECTS IN FISH

OCCURRENCE OF VITELLOGENIN

The reported effects of environmental oestrogens in fish include hermaphroditism and vitellogenin formation in males. The former is an example of an embryonic or organisational effect whereas the production of vitellogenin (an egg yolk protein) is a mature or activational effect.

Vitellogenin is a protein synthesised in the liver of oviparous fish, amphibians and most egg-laying mammals in response to oestradiol stimulation. Vitellogenin formation is normally specific to the functional female, but can occur in males in response to exogenous oestrogen stimulation (Clemens, 1978). It is transported in the blood to the ovary where it is sequestered in the oocytes to form the yolk of the eggs. It is well established that the induction of vitellogenin in the female fish is under oestrogenic control, specifically 17β–oestradiol (reviewed by Bromage & Cumaranatunga, 1988).

During 1978 and 1980 the incidence of hermaphrodites in roach populations from the river Lea, UK, downstream of two sewage treatment works, was reported as being 'unusually high'. A follow-up study in 1981, in which groups of 100 mature roach from each area were examined, also found hermaphrodite fish; the overall incidence for the age and size range examined was about five percent. The incidence of hermaphroditism was increased in older fish, possibly indicating a cumulative effect. The gonadosomatic index of these fish indicated a decline in male gonad development and an increase in female gonad development with time (Thames Water, 1981).

A survey of fish exposed to sewage treatment effluent in the UK was carried out in 1988 as a consequence of the observation by anglers of hermaphrodite fish in sewage treatment water (STW) lagoons. The study covered 30 STWs throughout England and Wales, including several from each Water Authority (Purdom *et al.*, 1994). Caged rainbow trout (*Oncorhynchus mykiss*) were placed at outfalls of STWs and plasma levels of vitellogenin were measured subsequently. At 12 sites the fish failed to survive the three week exposure and at four others there were

Table 2: Summary of adverse effects suggested as due to environmental oestrogens

Animal (and sex)	Change recorded	Chemical/feed of concern	Key references
Cattle (F)	infertility, nymphomania	alfalfa, oestrogenic pastures, subterranean clover	Moule *et al.* (1963)
	infertility abortion	hay sorghum	Mirocha *et al.* (1968) Mirocha *et al.* (1974)
Sheep (F)	infertility, post-natal mortality of lambs, uterine prolapse, sterility, endometrial hyperplasia, vulvar hypertrophy	subterranean clover	Moule *et al.* (1963)
Sheep (M) (castrated)	hypertrophy of seminal vesicles and mammae, death		
Swine (F)	hypertrophy of vulva, uterus and mammae, vaginal prolapse, infertility, abortion	corn	Mirocha *et al.* (1976)
Swine (M)	hypertrophy of mammae, testicular atrophy		
Turkeys (F)	vent enlargement, infertility	'feeds', sesame meal, corn	Mirocha *et al.* (1971)
Turkeys (M)	vent enlargement	sesame meal, corn	
Chickens	vent enlargement	corn	Mirocha *et al.* (1971)
Quail	impaired reproduction	forbs (a desert plant)	Leopold *et al.* (1976)
Marine gastropods	masculinisation (imposex)	tributyltin	Bryan *et al.* (1986) Ellis & Pattisina (1990)
Alligators	disruption of embryonic development of reproductive system	DDE, dicofol	Guillette *et al.* (1994)
Fish	induction of vitellogenin		Harries *et al.*, (1995) Purdom *et al.* (1994)
Fish (F)	masculinisation	pulp mill effluent	Davies & Bortone (1992)
Gulls (M)	feminisation (super-normal clutches)	organochlorines	Fry & Toone (1981) Fox (1992)

failures due to other causes. Elevated vitellogenin levels were found at all sites for which fish could be tested. In male fish, levels were 2.1-147mg/ml (except for one value of 0.023mg/ml) compared with 0.05-1.80µg/ml in controls; for female fish, the values were 3-112mg/ml (except for one value of 0.47mg/ml), compared with 4.5-88.3µg/ml in controls. Some additional experiments were carried out with carp (*Cyprinus carpio*), but the degree of vitellogenin induction was much lower and the results were less consistent.

A further study was carried out in the summer of 1992 in the River Lea in Hertfordshire. Male rainbow trout (n = 12/cage) were placed in 19 cages at sites immediately at the point of discharge of sewage effluents and at various distances downstream. Vitellogenin levels were compared before placement and after three weeks exposure at each site. Results indicated that the levels were highest in the immediate vicinity of sewage discharges, but were still detectable up to 15km downstream of a discharge. The majority of measurements in fish were in the ng/ml range, with those at the immediate point of discharge reaching µg/ml levels. A more detailed follow-up study involved placing two cages of rainbow trout (n= 10/cage) at nine sites, at the point of entry and downstream of one discharge point, for four weeks in the autumn of 1992. Plasma levels of vitellogenin were significantly increased after exposure only at the point of discharge, although a non-significant increase was observed at the next two sites downstream. The mean vitellogenin concentration at the point of discharge (0.38ng/ml) was lower than that recorded in the previous study (56ng/ml), but this may be explained by greater dilution caused by unusually high river flow during the second study (MAFF, 1994).

Studies by Harries *et al.* (1995) included surveys for oestrogenic activity in five UK rivers in addition to the River Lea. Caged male rainbow trout were placed downstream of STW discharges for a period of four weeks; in stretches of three of the rivers (River Lea, River Aire and River Arun) significant increases in plasma vitellogenin were observed compared to control fish held in a laboratory in water from a bore hole. Variations in magnitude of effect were seen with respect to distance from discharges, between rivers and between seasons. Seasonal variation was suggested to be linked to the speed with which effluent became mixed in the river. Limited studies in wild male roach, exposed to almost neat effluent and downstream of STW discharges in the River Lea, found levels of vitellogenin below 1µg/ml and, in some cases, below the 10ng/ml limit of detection. The authors reported that laboratory studies indicated that roach were much less responsive than trout (Harries *et al.*, 1995)

Vitellogenin levels in female winter flounder (*Pleuronectes americanus*) have been examined from polluted and relatively clean sites in the north-eastern USA (Pereira *et al.*, 1992). Significantly higher serum vitellogenin levels were found at one contaminated site (mean ± SD, 61 ± 7µg/ml) compared with the control site (40 ± 4µg/ml); however, at another site where liver tumour levels were significantly elevated, the vitellogenin levels were significantly lower in winter flounder with tumours than in those without. No examination of pollutant levels was included in this study.

Elevated levels of vitellogenin-like protein were found, outside the reproductive season, in female mosquitofish (*Gambusia affinis*) collected from drainage ditches contaminated with a variety of xenobiotics, relative to fish from uncontaminated ponds (Denison *et al.*, 1981). The authors suggested that the xenobiotics were the cause of the elevation.

Vitellogenin is found in most egg-laying mammals, but there does not appear to be any study related to pollution on its induction in these classes of animal.

MASCULINISATION OF FISH

Masculinised female mosquitofish were found at a number of sites in two streams in Florida, USA (Elevenmile Creek and Fenholloway River; Howell *et al.*, 1980; Davis & Bortone, 1992). The masculinisation effect (or arrhenoidy) involved the development of male secondary sex morphological structures, in particular the modification of the anal fin into a gonopodium-like structure. Behavioural changes were also noted. The authors noted that masculinisation should not be confused with sex reversal.

The changes were found downstream, but not upstream, of paper mills (Kraft process) and have therefore been linked to the effluent. This was confirmed by laboratory studies exposing mosquitofish to Kraft mill effluent (KME). Additional studies were carried out using phytosterols (sitosterol and stigmastanol) and these also produced masculinisation. Tall-oil, a major component of KME, contains 3% plant steroids of which sitosterol and stigmastanol comprise 85% (Howell & Denton, 1989). However, detailed fractionation and examination of the oestrogenic and anti-oestrogenic activity of KME was not carried out in this analysis.

An extensive review by Owens (1991), which assessed the hazards of pulp and paper mill effluents in the aquatic environment, did not mention the

masculinisation of fish, suggesting that this is not a commonly reported phenomenon. The most reliable indicator of exposure to particular effluent compounds was found to be the induction of mixed function oxidase enzymes. It is possible that the induction of mixed function oxidase enzymes and vitellogenin are linked, as treatment with oestradiol causes proliferation of the endoplasmic reticulum in many species.

Davis and Bortone (1992) noted that the two streams studied in Florida represented a worst-case scenario, as the flow of these streams was small and virtually the entire flow was used for discharge of effluent, suggesting that the phenomenon is perhaps seen only at high concentrations of KME.

Leatherland (1992) reviewed detailed studies carried out in salmon from the North American Great Lakes. The responses monitored were gross pathological lesions or major decreases in reproductive success; the main effects seen were those on the thyroid system, attributed to an unknown 'biologically active environmental factor'. A low reproductive success was observed in some species; factors associated with this infertility included lower plasma levels of gonadotrophin and gonadal steroid hormones in both males and females, poor expression of secondary sexual characteristics and a high prevalence of precocious sexual maturation in males, and poor egg quality, low egg thyroid hormone content and a high prevalence of embryo deformities in females.

3.2.2 DEVELOPMENTAL ABNORMALITIES IN REPTILES

A decline in the population of alligators (*Alligator mississippiensis*) was found in Lake Apopka in central Florida, USA in the 1980s (Guillette *et al.*, 1994). This decline was in contrast to population increases throughout the south-eastern USA and was linked to poor reproductive success.

The percentage viability of eggs collected from Lake Apopka was 28.3% compared with 51.5% for those from the less polluted Lake Woodruff National Wildlife Refuge. Clutches tended to produce either many offspring or no offspring. The mortality of young from Lake Apopka was much higher (41% within ten days) than that from the control area (<1%). The survival to six months of the

young from Lake Apopka was also decreased, but bodyweight and total body length of those that did survive were equal to those of controls. Male alligators from Lake Woodruff had four times the testosterone levels of those from Apopka; the latter had levels of testosterone that were not significantly different from those in females from either lake. The levels of plasma oestrogen were significantly higher (118pg/ml compared with 76pg/ml) in females from Lake Apopka (Guillette *et al.*, 1994).

There were also structural differences in alligators from the two sites. Females from Lake Apopka had abnormal ovarian morphology with large numbers of polyovular follicles and polynuclear oocytes. Males had histologically poorly organised testes and abnormally small phalli. It was considered that the gonads of juvenile alligators had been permanently modified *in ovo* and that normal synthesis of steroid hormones was unlikely (Guillette *et al.*, 1994).

In subsequent *in vitro* studies, Guillette *et al.*, (1995b) found that females from Lake Woodruff synthesised oestradiol at significantly higher rates than those from Apopka. Also LH stimulation had little effect on ovaries from alligators from Woodruff, but did cause stimulation of those from Apopka. Unstimulated testes from Apopka males synthesised significantly more oestradiol than those from Woodruff males, but LH did not stimulate synthesis in either case. No difference was observed in testosterone synthesis. Although detailed enzymatic data were not available, it was suggested that the modification of these patterns was due to hepatic degradation of the sex steroid hormones.

3.2.3 SUPERNORMAL CLUTCHES AND FEMALE-FEMALE PAIRING IN GULLS

The term 'supernormal' is used to denote clutches of five or six eggs in the case of birds, such as gulls, that normally lay three eggs. The finding of supernormal clutches (SNCs) of western gulls (*Larus occidentalis*) in California, USA in the 1960s (Hunt & Hunt, 1977) sparked considerable interest and controversy. SNCs were caused by female-female pairing and the percentage fertility of the eggs was low (Fry & Toone, 1981; Fry *et al.*, 1987). The SNCs of the western gull were

found on Santa Barbara Island, an area known to be highly polluted with DDT and PCBs, and this led to the premise that the effect was related to pollution (see Section 3.3.1).

The hypothesis put forward by Fry and co-workers is that feminisation of male gull embryos affects the reproductive behaviour of the birds when they mature (Fry & Toone, 1981). The suppression of normal behaviour could lead to males not migrating to the breeding colonies. In this case the sex ratio would be skewed and there would also be reduced competition for available nest sites. Both conditions are needed for female-female pairing to occur.

The role of reduced competition was shown by studies on the ring-billed gull (*Larus delawarensis*), which found that SNCs were most prevalent in expanding colonies (Fox & Boersma, 1983). On the North American Great Lakes this meant that the lowest incidence was on Lake Ontario and the highest on Lake Superior, which is the reverse of the degree of contamination by organochlorines. The fact that SNCs are not necessarily pollutant related has also been shown by the fact that SNCs were regularly reported in some species of gull before the 1940s (Conover & Hunt, 1984).

The sex ratio of gulls was assessed by examining specimens from a large number of museums in Canada and the USA. There was a statistically significant decrease in the ratio of males to females in the western gull and a non-statistically significant decrease in the ratio in the herring gull when specimens collected before 1940 were compared with those collected after 1950 (Conover & Hunt, 1984). The authors disagreed with the hypothesis put forward by Fry and Toone (1981) that feminisation of male embryos caused males not to breed. They considered that although the feminisation of male embryos could alter the sex ratio at the breeding colonies, it would not affect the population as a whole (contrary to their own findings), and proposed that the low male/female ratio in the western gull stemmed from a high differential male mortality. Fry *et al.* (1987) have dismissed selective mortality of males on the grounds that there is no evidence that male gulls are more sensitive to pollutants.

The two hypotheses put forward to explain SNCs (female-female pairing associated with feminisation of embryos, and differential mortality of males) are not mutually exclusive, although they may represent effects on different life stages. The former is an embryonic organisational effect while the latter appears to be an effect acting in later life. It should be stressed, however, that clear evidence that feminisation explains SNCs is lacking.

3.2.4 IMPOSEX IN MOLLUSCS

Detailed studies have been carried out to investigate the condition known as imposex (females developing male characteristics) in marine gastropods associated with tributyltin (TBT) contamination in marinas. Sites where imposex has been recorded include the north-eastern USA (Smith 1981), the UK (Bryan *et al.*, 1986), the North Sea (Hallers-Tjabbes *et al.*, 1994), Alaska (Short *et al.*, 1989), south-east Asia (Ellis & Pattisina, 1990) and New Zealand (Smith & McVeagh, 1991). Although the studies on molluscs have some similarities to those reported on alligators (section 3.2.2), there does not appear to be any study demonstrating that direct androgenic (or anti-oestrogenic) effects are involved. A major difficulty in producing such information is the low levels of hormones and the poor understanding of the biochemical aspects of reproduction in these species. The work of Wester *et al.* (1990) has demonstrated that neither tributyltin oxide nor tributyltin dichloride causes effects that mimic the action of steroids in fish.

Detailed studies, both in the laboratory and the field, have been carried out by the Plymouth Marine Laboratory, UK since the first finding of imposex in the dogwhelk (*Nucella lapillus*) in Plymouth Sound in 1969. A broader survey around the south-west Peninsula of England revealed that imposex was widespread with the most marked effects along the Channel coast (Bryan *et al.*, 1986). Within Plymouth Sound the degree of imposex increased markedly between 1969 and 1985.

Imposex is typified by the development in female molluscs of a small penis close to the right tentacle. A superficial vas deferens grows between the genital papilla and the penis. In the most extreme cases this occludes the papilla, thus preventing egg liberation and reproduction. Laboratory experiments and *in situ* transfer experiments have indicated that imposex may be initiated in dogwhelks with concentrations of TBT as low as 1ng/l (Bryan *et al.*, 1986). The no observable effect level for development of imposex is reported to be less than 1.5ng/l (WHO, 1990). Sterilisation of some females has occurred at locations with 1-2ng/l and has affected all females in areas averaging 6-8ng/l (Fox, 1992). Affected populations suffer reproductive failure, and local extinction has occurred around marinas.

Effects of TBT on at least 45 species of marine gastropods have been clearly demonstrated in many parts of the world (Ellis & Pattisina, 1990). Gastropods and copepods are much more sensitive to the effects of TBT than are other organisms; effects on other crustaceans and fish are not seen until the concentration is several

orders of magnitude higher (WHO, 1990). In this case, therefore, the problem appears to be clearly related to a specific, known pollutant which has enabled preventative measures to be taken. Bans on the use of TBT in paints used on small boats and aquaculture cages were introduced in the UK in 1987 (Langston *et al.*, 1994). Studies in Northumbria, UK comparing populations of dogwhelks in 1986 and 1989 indicated that the severity of imposex had been reduced and that the recruitment of juveniles had improved (Evans *et al.*, 1991). However, despite local improvements, the findings of Ellis and Pattisina (1990), showing problems over a wide area in south-east Asia, and those of Hallers-Tjabbes *et al*. (1994), showing effects in an open sea situation in the North Sea, suggest that imposex may remain a problem for many marine species.

Toxicological studies using rats have shown the critical effect of tributlytin compounds to be on the immune system. The mechanism is thought to involve uncoupling of mitochondrial oxidative phosphorylation in thymocytes, or cell membrane damage due to either disruption of the bilayer or inhibition of transmembrane enzyme systems (Boyer, 1989). This and other reported adverse effects in mammals are only seen at doses much higher than those causing effects in molluscs (WHO, 1990).

3.3. CHEMICALS OF CONCERN

A large number of different chemicals have been implicated as having possible oestrogenic, anti-oestrogenic, androgenic or anti-androgenic effects, including organochlorines and other pesticides, natural plant sterols, metals and by-products of the contraceptive pill (Colborn & Clement, 1992).

3.3.1 ORGANOCHLORINE PESTICIDES

Guillette (1993) reported that DDE, from a major spill of dicofol contaminated with DDE, was the key agent in the developmental abnormalities of alligators in Lake

Apopka, Florida. In experiments in which alligator eggs were treated with DDE, effects on plasma steroid concentrations similar to those observed in eggs from Lake Apopka were reported at DDE levels comparable to those found in the area by Heinz *et al.* (1991); the range of concentrations was 0.89-29µg/g (ppm; wet weight of egg).

Facemire *et al.* (1995) reported that many members of the free-ranging and captive populations of panthers (*Felis concolor coryi*) in Florida, USA suffer from a number of defects in the reproductive, endocrine and immune systems. Reproductive defects included abnormal sperm, low sperm density and cryptorchidism. Although these effects had been attributed to inbreeding, measurements of serum 17β-oestradiol levels indicated no significant difference between males and females. The authors suggested that the presence of environmental pollutants with oestrogenic activity in the diet of these animals, particularly in raccoons, may have been responsible for the effects. Levels of *p,p'*-DDE and PCB 1254 measured in the liver tissue of three panthers ranged from 5.45-57.65µg/g lipid fresh weight and 7.32-27.06µg/g lipid fresh weight respectively.

The effects of DDT, DDE, methoxychlor and oestradiol injected in corn oil into the yolk of eggs of the California gull (*Larus californicus*) were studied by Fry & Toone (1981). The percentage of eggs that hatched was low, with 45% of embryos dying within two days, leading to small sample sizes for the various (22) injection groups. For this reason the groups were combined before statistical analysis was made. Oestradiol, even at the lowest of the four doses used (0.5 ppm), caused feminisation of the male embryos, indicating the validity of the technique. Injection of *o,p'*-DDT caused feminisation in five out of six of the lower dosed embryos (2 and 5 ppm), no effect was seen with *p,p'*-DDT and half (3/6) of the high *p,p'*-DDE dosed embryos (20 and 100 ppm) showed feminisation. Methoxychlor caused feminisation of all embryos at all doses (2 to 100 ppm), although the total number of embryos was small (only eight for five doses studied).

An organochlorine pesticide that has been shown experimentally to be oestrogenic in fish, as indicated by the induction of vitellogenin, testicular atrophy and hermaphroditism, is β-HCH (Wester, 1991), but concentrations used were high with respect to environmental concentrations.

In addition to the oestrogenic activity of organochlorines, more recent attention has focused on other potential activities including anti-androgenicity (see section 2.4.4).

The organochlorine pesticides are of particular concern as many of them are persistent and bioaccumulate. The *o,p'* isomer of DDT has long been known to be oestrogenic (Bitman *et al.*, 1968), but although this isomer comprises 15-20% of

technical DDT, it is rapidly degraded in the environment. Lamont *et al.* (1970) examining brown pelican (*Pelecanus occidentalis*) eggs (n=10) collected at Ancapa Island, California, USA near to a major source of DDT, found an average of 0.24µg/g (ppm; wet weight of egg), and a maximum of 0.7µg/g (ppm), of the *o,p'*-isomers of DDT and dichlorodiphenyldichlorethane (DDD) combined, although the total concentration of DDT was approximately 80µg/g (ppm). No *o,p'*-isomers were found by Heinz *et al.* (1991) in alligator eggs from Florida.

Hebert *et al.* (1993) measured the levels of a number of organochlorine contaminants in 78 snapping turtles (*Chelydra serpentina*) from various sites in Southern Ontario, Canada. Samples of liver, leg muscle and eggs were analysed for chemicals including PCBs, DDE, DDT, chlordane, mirex and dieldrin. The levels of the contaminants in liver and egg tissues were significantly correlated and it was suggested that liver contaminant levels could therefore provide information regarding egg contamination. In this respect it was noted that the patterns of liver contamination were generally consistent with the developmental success of eggs at the various sites sampled.

3.3.2 POLYCHLORINATED BIPHENYLS

PCBs are of particular concern because of the persistence in the environment and stability of many congeners. Furthermore, little is known about the concentration of the hydroxy derivatives in the environment. The oestrogenic effects of PCBs have been considered in detail in the human health section (p. 33).

Although there is no direct evidence for oestrogenic effects of PCBs in the environment, Facemire *et al.* (1995) suggested that PCBs may have contributed to the reproductive defects seen in Florida panthers (see section 3.3.1). Furthermore, studies in the Baltic indicated that organochlorine pollution was associated with reproductive problems (uterine stenoses and occlusions, adrenocortical hyperplasia and hormonal osteoporosis) in Baltic grey seal (*Halichoerus grypus*) and ringed seal females (*Phoca hispida botnica*) (Bergman & Olsson, 1985). In an earlier study the reproductive success of Baltic seals was correlated with PCB and DDT levels; higher levels of many metabolites (including phenols and methyl sulphones) were identified in the tissue samples of non-reproductive females

compared with pregnant females (Jensen *et al.*, 1979). However, a cause and effect relation has not been established.

Reijnders (1986) also attributed the reproductive failure of seals in the Wadden Sea, The Netherlands, to pollution by PCBs. Fish, caught either in the Wadden Sea or in the north-east Atlantic, were fed to two groups of female common seals (*Phoca vitulina*; 12 per group) for approximately two years. The two diets were comparable with respect to nutritional status although chemical analysis revealed significant differences between the diets for PCBs and *p,p'*-DDE. Average daily intakes were 1.5mg PCBs and 0.4mg *p,p'*-DDE for seals fed fish from the Wadden Sea and 0.22mg and 0.13mg, respectively, for seals fed Atlantic fish. Three male seals fed Atlantic fish were alternated between the groups during the mating season. No significant differences were demonstrated in progesterone or 17β-oestradiol profiles when comparing pregnant or non-pregnant seals from both groups. However, the reproductive success of seals fed Wadden fish was significantly lower than that of seals fed Atlantic fish. Although the mechanism of action was not known, the effect was attributed to disruption by PCBs of the reproductive process around the implantation stage. In a subsequent study, Brouwer *et al.* (1989) suggested that PCB-induced reductions in vitamin A and thyroid hormone levels may have been responsible for the increase in reproductive disorders in seals from these areas.

Bergman *et al.* (1994) analysed coagulated blood from Baltic grey seals for the presence of hydroxylated PCBs. Blood samples, obtained during autopsy, contained total PCB concentrations ranging from 7.9-83µg/g lipid weight (mean 30µg/g lipid weight). At least 13 hydroxylated PCB metabolites were shown to be present, the two most abundant being 4-hydroxy-2,3,5,3',4'-pentachlorobiphenyl and 4-hydroxy-3,5,2',3',4'-pentachlorobiphenyl. Total levels of PCBs in six human plasma samples from Sweden were almost ten-fold lower and the spectrum of metabolites was shown to be different.

Bergeron *et al.* (1994) studied the oestrogenic effect of PCBs in eggs of the turtle *Trachemys scripta*. This species exhibits temperature-dependent sex determination; warm incubation temperatures produce all female hatchlings, cool temperatures all males, and intermediate temperatures varying ratios of males to females. Eggs were spotted with a number of PCB compounds and both positive (17β-oestradiol) and negative (ethanol) controls, incubated until hatching and dissected to determine sex ratios. Nine of the 11 PCBs tested demonstrated no evidence of sex reversal either alone or in various combinations. The two positive compounds 4-hydroxy-2',4',6'-trichlorobiphenyl and 4-hydroxy-2',3',4',5'-

tetrachlorobiphenyl, when tested alone, significantly reversed sex at the temperature that normally produced males and demonstrated a synergistic effect when combined.

3.3.3 DIOXINS

Dioxins have been considered in detail in the human health section (p. 36). While the dioxin equivalents of eggs have been found to be positively correlated with reproductive success in fish-eating birds in the North American Great Lakes, the authors concluded that the major contaminant influence on egg mortality was PCBs (Tillitt *et al.*, 1992).

The levels of dioxins, as PCDDs and PCDFs, have been measured in a number of rivers in England and Wales. Concentrations in water samples were very low (total PCDD/F concentrations <6ng/kg), while those in sediment, although varying considerably between sites, were much higher (total concentration 0.3-16µg/kg). Levels were reported to be comparable to those found in UK soils and in surface river sediments in Europe (Rose *et al.*, 1994).

3.3.4 SYNTHETIC HORMONES

In laboratory studies in which fish were exposed to controlled levels of ethynyloestradiol (the principal oestrogen of the contraceptive pill), a clear dose-response relationship for the induction of vitellogenin was found. Significant increases in vitellogenin in male trout were found at concentrations of 10ng/l; at 50ng/l the level of vitellogenin was of the same order of magnitude as that which had been measured at STWs. Injection experiments showed that the synthetic ethynyloestradiol was much more potent at inducing vitellogenin than natural oestradiol (Purdom *et al.*, 1994).

Sheahan *et al.* (1994) exposed male and female rainbow trout to ethynyloestradiol at nominal concentrations of 0.1, 0.3 and 1ng/l at two temperatures (11.4 and 17.4°C) under controlled conditions in glass aquaria. After two weeks, at the higher temperature only, increases in plasma vitellogenin were evident in fish (sex

not specified) exposed to 1ng/l, relative to levels in control fish. After 12 weeks, at either temperature, levels of vitellogenin in the groups with higher exposure were generally greater than after two weeks. Levels of vitellogenin in excess of 1mg/ml were present in 70% of the fish exposed to ethynyloestradiol at 1ng/l at either temperature, but in only 20-30% of fish in the lower exposure groups and in controls. At 28 weeks, levels of vitellogenin in treated and control females were not different at either temperature. However, levels in males exposed at 1ng/l at either temperature were significantly higher than in controls. Mean vitellogenin levels in males exposed at 0.3ng/l were also higher than in controls, but this was significant only at the lower temperature.

Although it was suggested that ethynyloestradiol may have been at least partly responsible for the observed oestrogenic effects of STW effluent in fish, technical problems precluded its measurement (Purdom *et al.*, 1994). In one study, ethynyloestradiol was reported to be found in UK river water (Aherne & Briggs, 1989), however, other studies have failed to detect it (Aherne *et al.*, 1985; Harries *et al.*, 1995 (see section 2.4.2)). Ethynyloestradiol is excreted in a conjugated form which has been found to be inactive in vitellogenin induction (Sumpter & Jobling, 1995), although it is speculated that the conjugated form may be enzymatically deconjugated by bacteria during sewage treatment, thus releasing the active chemical.

3.3.5 ALKYLPHENOL POLYETHOXYLATES

Harries *et al.* (1995) tested octylphenol, nonylphenol, nonylphenol monoethoxylate and nonylphenol diethoxylate at nominal concentrations of 30µg/l for oestrogenic activity in male rainbow trout held in large glass tanks. Significant elevations of plasma vitellogenin were produced in fish exposed for three weeks to all the compounds. Octylphenol, the most potent alkylphenolic compound tested, produced an elevation of a similar magnitude to that of ethynyloestradiol at a concentration of 2ng/l. The increases in vitellogenin produced by the other compounds were three to four orders of magnitude less. Thus the alkylphenolic compounds tested in this study were only weakly oestrogenic compared with ethynyloestradiol. Dose-response studies with octylphenol and nonylphenol demonstrated that the lowest concentrations required to increase plasma vitellogenin levels significantly were 4.8µg/l and

20.3µg/l, respectively. Concomitant with the increases in plasma vitellogenin in nonylphenol-exposed but not octylphenol-exposed fish was the finding of a dose-related reduction in the rate of testicular growth, as determined by the relation of testicular weight to total bodyweight. It has been reported (Blackburn & Waldock, 1995) that concentrations of nonylphenol in river water rarely exceed 10µg/l, although levels as high as 100µg/l have been reached in rivers receiving industrial effluent.

Studies of the oestrogenic capacity of a number of persistent alkylphenolic compounds have been carried out by measuring the secretion of vitellogenin from cultures of trout hepatocytes (Jobling & Sumpter, 1993; White *et al*., 1994); 4-butylphenol and 4-octylphenol were the most oestrogenically active, followed by 4-nonylphenol. Even the most active phenol was a thousand times less active than 17β-oestradiol. Nevertheless, alkylphenols were shown to be oestrogenic in fish, avian and mammalian cells and to mimic the effects of oestradiol by binding to the oestrogen receptor.

The biodegradation of mixtures of APEs has been studied in some detail by Ahel and co-workers (1994a,b). The biodegradation of APEs in wastewater treatment plants can occur aerobically or anaerobically, leading to the formation of alkylphenoxyacetic acids and alkylphenol, respectively. Both pathways can also give rise to alkylphenol mono- and diethoxylates. Following further degradation by anaerobic sludge treatment, either pathway can lead to the formation of alkylphenol (Ahel *et al.*, 1994a).

Ahel *et al.* (1994b) examined the distribution of nonylphenol polyethoxylate and its metabolites in river water following discharge of secondary effluent from sewage plants. The most abundant compounds were the nonylphenoxycarboxylic acids, followed by the lipophilic nonylphenol ethoxylates, while nonylphenol, also lipophilic, was significantly less abundant. Overall, it was found that this group of compounds was one of the most abundant organic pollutants in the river.

In a report commissioned by the Department of the Environment, it was estimated that of the total UK nonylphenol ethoxylate production, 83% was discharged to the aquatic environment and to land (37% and 46% respectively), and 17% was destroyed (CES, 1993). The environmental levels of non-ionic surfactants have been reviewed by Holt *et al.* (1992). In general the concentration in river waters was within the range 0.02-0.1mg/l, well below the concentration of 4-octylphenol of 2mg/l (10^{-5}M) found to be effective at stimulating vitellogenin formation *in vitro* (White *et al.*, 1994). However, it is known that alkylphenols

bioaccumulate; bioconcentration factors for 4-nonylphenol of 1300 in a marine fish (*Gasterosteus aculeatus*) and 3400 in mussels have been reported (Ekelund *et al.*, 1990) and a bioconcentration value of 280 has been reported for salmon (Ahel *et al.*, 1993).

3.3.6 PHYTOESTROGENS

Plant sterols have been considered as the active components of pulp mill effluent (see p. 51). A number of plant sterols present in pulp mill effluent can be converted microbially to C19 steroids, including testosterone. Howell and Denton (1989) demonstrated that stigmastanol, in the presence of a mycobacterium culture, caused masculinisation of fish. Detailed studies on the amounts of plant sterols and their active metabolites in effluents and their oestrogenic potential do not appear to have been made.

Notable effects have been shown in ewes and rams feeding on subterranean clover, which under some conditions contains relatively large amounts of isoflavones. Adverse effects include anatomical changes in the cervix and uterus, infertility and aberrant sexual behaviour in ewes and reduced sperm counts in rams (Moule *et al.*, 1963; Price & Fenwick, 1985; Kaldas & Hughes, 1989). Feeding clover has been associated with infertility in cattle (Moule *et al.*, 1963). Swine appear to be particularly sensitive to the effects of zearalenone, a mycotoxin produced by the fungus *Fusarium graminearum*. Feeds contaminated with zearalenone, at a few mg total dose, have produced a variety of reproductive disorders such as vulvovaginitis, infertility and abortion (Mirocha *et al.*, 1971). Experimental evidence has also implicated zearalenone in the vent enlargement and infertility of turkeys and chickens eating contaminated corn (Mirocha *et al.*, 1971).

Attempts to breed captive cheetahs successfully in the USA have been thwarted by reproductive failure and liver disease. Phytoestrogens in the diet of these animals have been purported to play a major role in these effects (Setchell *et al.*, 1987). Analysis of the diet revealed large amounts of daidzein and genistein, and bioassays of extracts for uterotrophic activity were positive in immature mice and ovariectomised rats.

It should be noted, as described in section 2.4.3, that some phytoestrogens have anti-oestrogenic as well as oestrogenic activities.

3.4 CONCLUSIONS

The induction of vitellogenin in fish in water contaminated by sewage treatment effluent and the feminisation of alligators in a polluted lake in Florida have demonstrated the presence in the environment of man-made chemicals that act as oestrogen mimics. To date these effects have been demonstrated only on a local scale. This is in contrast to the occurrence of imposex in molluscs, which has been reported worldwide. Imposex has been linked to TBT exposure and may reflect the presence of chemicals with anti-oestrogenic or directly androgenic effects (although the mechanism of action has not been established). It is anticipated that studies currently being undertaken will aid in resolving the uncertainty about the scale of the effect of environmental oestrogens in natural populations.

A number of effects that may be due to environmental oestrogens have been reported in wildlife studies. Although a wide variety of chemicals, including organochlorines, synthetic hormones and alkylphenolic compounds, have been shown, to a greater or lesser extent, to act as hormone mimics in experimental studies, it has not been possible to demonstrate conclusively which agents are responsible for the effects observed in field situations. It is proposed that a weight of evidence approach should be used to evaluate field studies in the light of controlled laboratory experiments.

4 Key areas for future research

4.1 RECOMMENDATIONS FOR FUTURE WORK

EXPOSURE OF THE HUMAN POPULATION TO ENVIRONMENTAL OESTROGENS

It is important to establish the extent of human exposure to environmental oestrogens (i.e. how many people are exposed and to what), since there is evidence that male, and perhaps female, reproductive health is at risk.

There are still some uncertainties as to what agents should be considered as environmental oestrogens. Apart from agents with direct oestrogenic activity, there are chemicals with no oestrogenic activity but which possess anti-androgenic properties. Other chemicals with no oestrogenic activity *per se* may affect oestrogen metabolism or catabolism and therefore have 'indirect' oestrogenic properties.

Robust procedures for identifying chemicals in the environment that have oestrogenic activity must be developed*. Further developments should include the establishment of standardised *in vitro* assays to screen for oestrogenic activity. Possible limitations to screening tests should be considered; for example, it may be metabolites rather than the parent compound that are important for oestrogenic action. This work will benefit from international collaboration.

There are now a number of hypotheses concerning associations between environmental oestrogen exposure and adverse reproductive outcomes in humans which need to be investigated (see below); the extent of exposure should be investigated in parallel with these studies. For example, there appear to be

* Test procedures used to date are described in the appendix

large differences in disease rates in Denmark and Finland for some reproductive disorders. However, it is not known whether or not there are similarly large differences in environmental oestrogen exposure.

There is no known biomarker in humans for persistent oestrogenic action; it is of critical importance to develop such a marker.

INVESTIGATION OF ADVERSE HEALTH EFFECTS IN THE HUMAN POPULATION

There are differences in the incidences of various testicular disorders in different countries; notable differences occur between rates in Denmark and Finland. The incidence of testicular cancer may be a surrogate measure for the incidence of testicular dysfunction and this should be investigated further. It is still not established whether or not there is a link between cryptorchidism, hypospadias and testicular cancer. Prospective epidemiological studies should be set up to investigate various aspects of testicular dysfunction and to determine whether qualitatively or quantitatively similar effects are seen in the UK as have been observed elsewhere.

Data on sperm quality from retrospective studies have limited use. Prospective studies on sperm quality, which should investigate different geographical and ethnic groups, are needed. At present in the UK there is no adequate procedure for keeping records concerning sperm quality; this should be rectified and a prospective study on sperm quality should be undertaken in the UK. Standardised methods for analysing sperm quality are required, to allow comparisons to be made between centres. Studies are also required to ascertain the link between sperm quality and fertility.

Irrespective of the environmental oestrogen issue, there is a need to generate a more comprehensive database on the functioning and activity of the human reproductive system. Even if it ultimately transpires that environmental oestrogens are not implicated in human reproductive disorders, there would nonetheless be a better data set against which other hypotheses for causation could be tested. Pan-European studies will be needed to give comprehensive and comparable data.

The suggestion that environmental oestrogens may be associated with breast cancer in women should be further investigated. Again there appear to be

differences between Denmark and Finland. The incidence of male breast cancer in the UK and any possible association with environmental oestrogens should also be studied.

The possible effects of environmental oestrogens on the vascular system should be studied in populations of women from different geographical areas.

The bioaccumulation, metabolism and degradation of oestrogenic agents should be investigated; in particular a better understanding is needed concerning the presence and mobilisation of these agents during pregnancy, when they may affect the developing embryo.

Oestrogenic chemicals may have different effects at different stages in life and this should be investigated. The effects in mothers exposed to chemicals with oestrogenic and/or anti-oestrogenic activity should be studied, as should effects on the development of their offspring. One of the most sensitive sites of action of steroids *in utero*, with respect to sexual differentiation, is the brain. Studies should be conducted to collect data on sexual orientation and sexually controlled behavioural effects. It is noted, however, that stereotypical sexual behaviour, which may be obvious in some experimental animals, is not so readily observable in humans.

Populations highly exposed to putative environmental oestrogens, for example through accidental poisonings, should be the subject of more extensive investigations of reproductive health.

EXPERIMENTAL MODELS

There are many similarities between species in the development of the reproductive system and there is a need to develop suitable animal models to test possible oestrogenic activity of chemicals in the environment.

Studies in some domestic animals (eg. cows, sheep) might prove to be more informative than epidemiological studies in investigating, for example, whether low level *in utero* exposures to oestrogenic compounds have more serious consequences than similar exposures in adults.

Experimental models should be developed to investigate the relation between susceptibility to breast cancer and environmental factors.

Animal models could also be used to study receptors, that is what receptors have to be occupied and for how long to produce an effect. Additivity and synergism between oestrogenic/anti-oestrogenic chemicals could also be tested using animal models.

EFFECTS IN WILDLIFE

Several incidents of oestrogenic effects in wildlife have been demonstrated; there is, therefore, a need to consider such possible outcomes when investigating the effects of pollutants in wildlife. The most critical point is early development and thus studies on the developing embryo (most easily carried out using those species which produce external eggs) should be included in the protocol of investigations.

Studies are ongoing, or planned, to investigate whether fisheries are at risk from oestrogenic chemicals. Studies include whether or not induction of vitellogenin is occurring in wild fish, and if such effects occur in marine fish, for example in estuaries. There is a need to expand studies from the experimental situation to the natural environment to determine whether wild fish populations are suffering, or are likely to suffer, any adverse effects from environmental oestrogens.

Vitellogenin induction is a reaction in mature organisms; since it is likely that developing organisms will be more sensitive to the impact of xenoestrogens, it is important to develop other biomarkers. It would be valuable to look for these biomarkers in fish populations known, by vitellogenin induction, to be affected by oestrogenic pollutants.

Experiments to determine the impact of oestrogenic compounds on wildlife could be carried out on eggs of a variety of species, including birds, amphibians, reptiles and fish. For many species, eggs are available in large numbers, thus studies would have little impact on the environment.

A considerable number of test methods for oestrogenic activity have been developed and these are described in the appendix to this report. It is important that a number of standardised tests be applied in wildlife investigations rather than relying on a single test such as vitellogenin induction. Studies to investigate characteristics that might arise from oestrogen exposure should be linked where possible with long-term ecological studies.

4.2 CONCLUSIONS

A concerted and co-ordinated national programme of research is required to investigate the possible link between demonstrated trends in human reproductive health and human exposure to chemicals in the environment with known oestrogenic or anti-oestrogenic activities. Research should be concentrated, where possible, in UK centres of excellence. A robust and reliable assay for screening chemicals for oestrogenic activity needs to be developed, together with experimental models for investigating mechanisms of action and, if possible, a reliable biomarker for persistent oestrogenic action. Further studies are required on sperm quality, and it is essential for this purpose to develop standardised methods of analysis. Pan-European investigations are needed to strengthen the existing databases.

Future studies of the effects of pollution on wildlife should consider oestrogenic and related activities. Current experimental studies of oestrogenic activity in fish should be extended to natural populations, and other classes of animals should be similarly studied. Additional markers and screening assays for oestrogenic and related activities should be developed.

Proof of a cause-effect relationship between exposure to oestrogens in the environment and adverse effects on human reproductive health is likely to remain elusive. Similarly it may not be possible to identify conclusively which agents, acting singly or in combination, are responsible for adverse effects in wildlife populations. It follows that a weight of evidence approach, which takes into account findings from epidemiological and other studies in humans, field studies in wildlife and experimental studies, will be needed in order to make an appropriate assessment of risk to human health from environmental oestrogens. Further research in all these fields, as recommended above, is essential before such a risk assessment can be made.

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APPENDIX: TEST METHODS FOR OESTROGENIC POTENTIAL

INTRODUCTION

Benson and Nimrod (1994) summarised the test methods currently available for the assessment of oestrogenic activity. Analysis of the oestrogenic potential of compounds can be performed using either *in vivo* or *in vitro* assays, and in both cases the activity is usually expressed relative to that of oestradiol, the most common endogenous oestrogen.

One of the tests for oestrogenic potential *in vitro* involves the use of the human breast tumour MCF-7 cell line. Human serum contains a factor preventing MCF-7 cells from proliferating and oestrogens, either natural or synthetic, are required for proliferation when cells are grown in media containing human serum. This proliferation response is not dependent on interaction of oestrogens with the oestrogen receptor. MCF-7 cells also respond to oestrogens by induction of progesterone receptors (PR), which is an oestrogen receptor-mediated event. This model has been reported to reproduce the effects observed in the female genital tract *in situ* (Soto *et al*., 1991). Another cell line (Ishikawa-Var I) used to assess oestrogenic activity is derived from a human endometrial cancer (Markiewicz *et al*., 1993).

Another *in vitro* assay to measure oestrogenic activity involves the binding of compounds directly with the oestrogen receptor. Examples of receptors used include those isolated from spotted seatrout and mouse uteri.

Vitellogenin is a protein produced in the livers of fish in response to oestrogenic stimulation and thus its synthesis (especially in males) has been used as an indicator of oestrogenic activity. The synthesis of vitellogenin by cultured trout hepatocytes has been developed as an *in vitro* assay for oestrogenicity.

In vivo, the most common bioassay involves the measurement of uterine weight in immature female or ovariectomised rodents.

IN VITRO ASSAYS

CELL PROLIFERATION, INDUCTION OF PROGESTERONE RECEPTORS AND OTHER ASSAYS IN MAMMALIAN CELLS

Soto *et al*. (1991) assessed the oestrogenic potential of a leachate from plastic centrifuge tubes using both *in vitro* and *in vivo* assays. The *in vitro* assay involved the use of MCF-7 human breast tumour cells maintained in culture, the so-called E-screen. The principle underlying the assay is that a molecule in human serum specifically inhibits the proliferation of human oestrogen-sensitive cells; oestrogens induce cell proliferation by neutralising this inhibitory effect. Non-oestrogenic steroids and growth factors do not abolish the inhibitory effect. Thus, only oestrogens induce proliferation of MCF-7 cells and human serum, stripped of endogenous oestrogens by treatment with charcoal-dextran, inhibits the proliferation of these cells in a dose-dependent manner. In addition to cell proliferation, PR induction in MCF-7 cells was determined as a widely accepted marker for functionality of the oestrogen receptor pathway.

Cell proliferation assays were performed by culturing cells in medium supplemented with fetal bovine serum for 24 hours to allow for cell attachment. The medium was then replaced with culture medium containing 10% charcoal-dextran stripped human serum containing either 17β-oestradiol or substances suspected of being oestrogenic. After six days, the cells were lysed and the numbers of nuclei counted on a Coulter Counter. Results were presented as number of cells per well.

For the PR assay, the method was similar except that plates were seeded in duplicate and, following the six day exposure period, one plate was harvested for cell counting while the other was processed for PR determination by removing the media, freezing the cells at -80°C and extracting the PR in another incubation step. Following centrifugation, cell extracts were incubated with a rat anti-human PR monoclonal antibody covalently attached to beads. The beads were then incubated with horseradish peroxidase-coupled rat anti-human PR antibody followed by an incubation with the substrate (*o*-phenylenediamine). The absorbance was read at 492nm and results presented as fmoles PR per 10^6 cells.

Krishnan *et al*. (1993) used MCF-7 human breast tumour cells in culture to determine the oestrogenic activity of bisphenol-A, a chemical released from polycarbonate flasks during autoclaving. MCF-7 cell proliferation was assessed by [^{3}H]-thymidine incorporation. Cells were incubated in culture medium for 48 hours, after which the medium was changed to medium containing 5% charcoal-treated serum. Cells were incubated for a further six days with various concentrations of oestradiol and bisphenol-A. [^{3}H]-Thymidine was added to control and treated cells for one hour, followed by replacement with unlabelled thymidine. Cells were solubilised and aliquots were added to scintillation fluid and counted. DNA levels were determined in other aliquots using the diphenylamine assay. Incorporation of radioactive thymidine was expressed as disintegrations per minute/µg DNA.

PR induction was assessed in cells grown and treated as above. After incubation, cells were removed and sonicated and cytosol was prepared by centrifugation. Aliquots were incubated with [^{3}H]-promegestone. Non-specific binding was assessed in parallel samples by incubation with a 250-fold excess of unlabelled promegestone. Dextran-coated charcoal was used to separate protein-bound hormone from free hormone and levels of receptors were presented as fmoles of ligand bound/mg cytosol protein.

White *et al*. (1994) studied the effect of alkylphenolic compounds on the growth of cells from the human breast tumour cell lines MCF-7 and ZR-75. Cells were initially maintained in culture medium containing 10% charcoal-stripped fetal calf serum (containing no hormone) for seven days. The medium was then replaced with fresh medium containing 17β-oestradiol or alkylphenolic compounds at various concentrations and cultured for a further five days. Results were quoted as numbers of cells

Mayr *et al*. (1992) tested zearalenone and a number of phytoestrogens and cereal extracts for oestrogenic activity in MCF-7 cells by measuring the induction of an oestrogen-specific exoprotein of molecular mass 52kDa. Cells maintained in oestrogen-free medium were exposed to test compounds for 72 hours at various concentrations, and exoproteins were labelled with [^{35}S]-methionine, separated by SDS-polyacrylamide gel electrophoresis and visualised by autoradiography. The concentrations of compounds causing a half-maximal response (ED_{50}) were estimated by comparing the intensities of the 52kDa bands on the X-ray films following autoradiography.

White *et al*. (1994) tested alkylphenolic compounds for their ability to stimulate the transcriptional activity of the oestrogen receptor directly by investigating their effects in transiently transfected MCF-7 cells. The reporter gene pEREBLCAT, which contains an oestrogen response element linked to the reporter gene chloramphenolacetyl transferase (CAT), was used. MCF-7 cells (containing endogenous receptors) were transfected with DNA including a reporter plasmid pEREBLCAT and an internal control plasmid (luciferase). After transfection, cells were maintained with no hormone, 17β-oestradiol, or alkylphenolic compounds for 48 hours, harvested and assayed for luciferase and CAT activities. Luciferase activity was used to correct for differences in transfection efficiency.

Chicken embryo fibroblasts (lacking endogenous receptors) were also used in these transfection assays. In addition to transfection with pEREBLCAT, the cells were co-transfected with a mouse oestrogen receptor expression vector. The assay was designed to determine whether the effects of alkylphenolic compounds were mediated by the oestrogen receptor, that is whether they could stimulate EREBLCAT transcription in the presence or absence of mouse oestrogen receptor. Results of transcriptional activity were expressed in terms of the induction of the reporter in the absence and presence of each compound.

Mayr *et al*. (1992) also tested zearalenone and a number of phytoestrogens and cereal extracts for oestrogenic activity in mouse LeC-9 cells (an oestrogen-sensitive transformed mouse-cell line). These cells contain an oestrogen-responsive element combined with a thymidine kinase promoter and a bacterial chloramphenicol acetyltransferase (CAT) gene. They also contain a bacterial gene for β-galactosidase (β-GAL) and express the human oestrogen receptor. Chemicals inhibiting transcription and/or translation would affect CAT and β-GAL gene expression to a similar extent. However, β-GAL gene expression is unresponsive to oestrogenic activity and so was used as an indicator of effects on transcription and/or translation. The LeC-9 cell clones chosen for the experiment had low levels of endogenous CAT activity, which was stimulated 200-300 fold by 17β-oestradiol. After exposure to the test compounds, cell lysates were incubated with [^{14}C]-chloramphenicol, following which chloramphenicol and its enzymatically acetylated derivatives were extracted and separated by thin layer chromatography. CAT activity was expressed as the radioactivity of chloramphenicol acetates divided by the total radioactivity expressed as a percentage. β-GAL activity was determined using *o*-nitrophenyl-β-D-galactopyranosid as a substrate and measuring the absorbance of the reaction product at 420nm.

Markiewicz *et al*. (1993) used two different *in vitro* assays to study the oestrogenic activity of non-steroidal phytoestrogens including coumesterol, genistein and daidzein. The first assay involved the use of a variant of the human Ishikawa cell line derived from a well differentiated endometrial adenocarcinoma. The variant, Ishikawa-Var I, is unresponsive to the proliferative action of oestradiol but is sensitive to the stimulatory effect of oestrogens on alkaline phosphatase activity. Cells maintained in culture and seeded in 96-well plates were exposed to solutions of test compounds and incubated for 72 hours. Following washing, *p*-nitrophenyl phosphate was added to the wells and the rate of the alkaline phosphatase-catalysed hydrolysis to *p*-nitrophenol was measured colourimetrically. Concentrations of test compounds required to produce 50% of the maximal increase in alkaline phosphatase activity (EC_{50} values) were calculated. The effects of the anti-oestrogens 4-hydroxytamoxifen and ICI 164,384 on the actions of the above isoflavonoids were evaluated by comparing the levels of alkaline phosphatase activity induced by incubations of isoflavonoid alone or in combination with either anti-oestrogen. Results were presented graphically comparing concentration with optical density.

The second assay used histologically normal human endometrium obtained from biopsy samples or hysterectomy, the end point being the oestrogen-stimulated production of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Tissue samples were cut into fragments and partially immersed in medium in culture dishes to maintain oxygenation. Following a 'settling period' of 24 hours, the medium was replaced with fresh medium containing either oestradiol, equol (a metabolic product of isoflavonoids) or 4-hydroxytamoxifen alone or in combination. Tissues were incubated for a further 24 hours, following which the medium was removed, centrifuged and frozen and the tissues were homogenised and analysed for protein content. Levels of $PGF_{2\alpha}$ in unextracted medium were measured by radioimmunoassay (RIA). Results were expressed as a % of the vehicle control (ethanol).

OESTROGEN RECEPTOR BINDING*

Korach *et al*. (1988) tested a number of hydroxylated PCBs for their ability to bind with oestrogen receptors prepared from mouse uteri. The cytosolic fraction, containing oestrogen receptors, was prepared from the uterine tissue of

*IEH is aware of a paper currently in preparation by Routledge and Sumpter concerning the development of a recombinant yeast strain containing the human oestrogen receptor for the detection of oestrogenic activity *in vitro*.

ovariectomised mice by centrifuging the homogenised uteri and removing the cytosolic supernatant. Aliquots of this fraction were incubated with [^{3}H]-oestradiol together with various concentrations of the unlabelled PCB competitors. The receptor-bound fractions were assayed using a hydroxyapatite absorption procedure and the results were presented as C_{50} values, the concentration of inhibitor yielding half-maximal binding relative to oestradiol.

Thomas and Smith (1993) described an *in vitro* screening assay for potential oestrogenic effects by measuring the binding of a number of xenobiotics, including *o,p'*- and *p,p'*-DDT, *o,p'*- and *p,p'*-DDE, PCB mixtures, chlordecone and non-steroidal anti-oestrogens (including tamoxifen), to the oestrogen receptor of the spotted seatrout (*Cynoscion nebulosus*). The livers of eight adult spotted seatrout with vitellogenic oocytes were homogenised, centrifuged and the cytosolic extracts prepared and pooled. Extracts were incubated in triplicate with [2,4,6,7,^{3}H]-oestradiol alone and in the presence of the competing ligands (in propylene glycol:ethanol (4:1 v/v) vehicle) over a range of concentrations for 24 hours at 4°C. Following incubation, dextran-coated charcoal was added to separate free from bound radiolabelled oestradiol and the percentage binding of [^{3}H]-oestradiol was determined. Results were presented as the concentration of xenobiotic required to cause a 50% displacement of [^{3}H]-oestradiol from its receptor (IC_{50}).

White *et al.* (1994) carried out receptor binding studies using liver cytosol from male and female rainbow trout (*Oncorhyncus mykiss*). This cell fraction contains oestradiol receptor binding sites. The experiments tested the ability of alkylphenolic compounds to compete with radiolabelled oestradiol for binding to the trout oestradiol receptor. Liver cytosol was incubated on ice with dextran-coated charcoal to remove endogenous steroids, centrifuged and the cytosolic supernatant aspirated. Saturation analysis was performed on this fraction to establish the concentration of 17β-[2,3,7-^{3}H]-oestradiol that saturated the receptor preparation. Cytosol samples were incubated with a saturating concentration of [^{3}H]-oestradiol with and without competing ligands over a range of concentrations. The unbound ligand was removed by the addition of charcoal and specific binding was quantified.

INDUCTION OF VITELLOGENIN

Pelissero *et al.* (1993) reported the development of an *in vitro* bioassay to determine the oestrogenic potencies of chemicals in fish. The assay is based on the

ability of rainbow trout hepatocytes to produce vitellogenin in response to oestrogenic stimulation.

The trout used in the study were either males, immature females or sterile and weighed approximately 500g. Hepatocytes were prepared using a routine collagenase digestion procedure following which cell viability was assessed. Cells were maintained in culture for four to six days until cell aggregates formed. Test and control solutions were then added and cells incubated for a further two days, following which the medium was collected and stored until analysis.

Levels of vitellogenin in the wells were determined at the beginning and end of each experiment by RIA as described by Sumpter (1985) and were expressed in ng/ml. This trout hepatocyte assay was also used by Jobling and Sumpter (1993) to assess the oestrogenicity of detergent components of sewage effluent.

White *et al.* (1994) investigated the oestrogenicity of persistent alkylphenolic compounds in rainbow trout hepatocytes prepared and cultured according to the method of Jobling and Sumpter (1993). Cells were treated with 17β-oestradiol, 4-octylphenol, 4-nonylphenol, 4-nonylphenol diethoxylate and 4-nonylphenol carboxylic acid. Following treatment, the medium was assayed for vitellogenin by RIA.

Sumpter and Jobling (1995) reported that this assay is somewhat insensitive, as the freshly prepared trout hepatocytes are exposed for only two days before the response is assessed while in their natural environment fish are exposed continuously to any oestrogenic chemicals present in the water.

IN VIVO ASSAYS

HUMANS

Klug *et al*. (1994) reported on the development of a monoclonal antibody-based enzyme immunoassay (EIA) for the simultaneous quantitation of 2-OHE and 16α-OHE1 in human urine. These two compounds are the major metabolites of oestradiol and have oestrogen antagonist and agonist activities, respectively. The balance of these two pathways determines, to a large extent, the net *in vivo* oestrogenic stimulus, and

several studies have hypothesised that a relative increase in 16α-hydroxylation is a biomarker for the risk of developing breast and other oestrogen-dependent cancers. These metabolites are excreted as glucuronide and sulphate conjugates and so urine samples, controls and standards were initially deconjugated by addition of a diluent containing β-glucuronidase and arylsulphatase for two hours at room temperature. Samples were then diluted by addition of a neutralisation buffer and aliquots were added to the wells of monoclonal antibody-coated anti-2-OHE and anti-16α-OHE1 EIA plates. Following this, aliquots of 2-OHE:alkaline phosphatase and 16α-OHE1:alkaline phosphatase conjugates were added to the respective plates and these were then incubated at room temperature for three hours. *p*-Nitrophenyl phosphate was added to each plate and the absorbance was read kinetically at 420nm. Results were presented as dose-response curves, plotting rates (milli-absorbance units/minute) against concentration of 2-OHE or 16α-OHE1 (in ng/ml). The EIA assays for the two compounds were compared with a GC/MS method and yielded correlation coefficients of approximately 0.94 for both. It was commented, however, that non-steroidal compounds such as phytoestrogens could interfere with the assays and, if present in high concentrations in urine, could affect the results.

EXPERIMENTAL ANIMALS

One of the metabolic changes that oestrogen produces in the rat uterus is an increase in glycogen content. Bitman and Cecil (1970) used this response to measure the oestrogenic activity of a number of DDT analogues and PCBs. Test substances were dissolved in olive oil or an aqueous ethanol solution and injected subcutaneously into immature female Wistar rats (21-23 days old; 36-48g). Animals were killed after 18 hours and the uteri were excised, weighed and analysed for glycogen by the anthrone procedure. The potency of active compounds was reported in terms of the minimal subcutaneous dose (mg) required to increase significantly the level of glycogen above the control level.

Galey *et al*. (1993) modified the classical mouse uterine bioassay for the evaluation of horse and cattle food suspected of being oestrogenic. Solvent extracts of the samples were mixed with mouse feed (free of the oestrogenic mycotoxin zearalenone) and 100g of this was then fed to groups (n=3) of immature female mice over a period of five days. Other feeds included control, DES, oestradiol, coumesterol, feeds with no reported oestrogenic properties and a feed causing hyperoestrogenism in cattle. DES, oestradiol and coumesterol were added to feeds over a range of doses. On day six the mice were killed and uterine

weights determined. Bodyweights were monitored before and after the study and selected uteri were fixed for histological evaluation. Results were presented as uterine weight in grams.

In an *in vivo* assay reported by Soto *et al*. (1991), adult female Sprague-Dawley rats were primed with 17β-oestradiol at 12, 13 and 14 days following ovariectomy. The substances suspected of being oestrogenic were administered by subcutaneous injection on days 19 and 20. Positive (17β-oestradiol) and negative (vehicle only) controls were included. Animals were sacrificed 24 hours after the second injection. Four hours before sacrifice, colchicine was administered to arrest endometrial mitotic figures and assess mitotic indices. Results were presented as mitoses per 1000 cells of the luminal endometrial epithelium.

INDUCTION OF VITELLOGENIN IN FISH

Sumpter (1985) described the development of a RIA for plasma vitellogenin in the rainbow trout (*Salmo gairdneri*). Following a single intramuscular injection of 17β-oestradiol to induce vitellogenin, weekly blood samples were collected from immature male and female rainbow trout. The plasma was gel-filtrated and the fractions containing vitellogenin, assessed by the appearance of a new blood protein, were pooled into one. This was applied to a sepharose column and, following elution, the adsorbed protein was displaced. Trout vitellogenin antibodies were raised in rabbits, all producing high titres. Vitellogenin was iodinated with Iodogen, separated from free iodine and gel-filtered. A standard RIA protocol was followed using anti-rabbit gamma-globulin to precipitate the labelled vitellogenin following incubation. Trout plasma samples were assayed in triplicate at varying dilutions, depending on the vitellogenin level. The antibody concentration used routinely led to a 40% binding of the label. Assay sensitivity, defined as the amount of vitellogenin required to reduce the binding of the label by 10% of the maximum value, was 100pg/tube. Vitellogenin concentrations ranged from ng to mg/ml, depending on sex and sexual maturity.

Tyler and Sumpter (1990) reported on the development of an RIA for carp (*Cyprinus carpio*) vitellogenin, similar to that for rainbow trout reported by Sumpter (1985). Vitellogenin synthesis was induced by injections of 17β-oestradiol and vitellogenin was then isolated using gel filtration and ion exchange. Antibodies were raised in rabbits given intracutaneous injections of vitellogenin in Freund's complete adjuvant and were obtained from the serum of clotted blood samples. Vitellogenin was labelled with ^{125}I using Iodogen to a specific activity of

70-100 mCi/mg. For the RIA, the antibody was used at a concentration at which approximately 50% of the label bound in the absence of unlabelled vitellogenin. In the procedure, vitellogenin (or unknown) was incubated with antibody and labelled vitellogenin, followed by isolation and scintillation counting of the precipitated labelled vitellogenin. The assay was validated using blood samples from males and females at various stages of maturity and was demonstrated to be sensitive, highly specific and robust. Vitellogenin concentrations ranged from non-detectable to mg/ml levels.

Pereira *et al*. (1992) measured the levels of vitellogenin in the blood of winter flounder (*Pleuronectes americanus*) at 'clean' and 'degraded' areas around Long Island Sound and Boston Harbour, USA. The authors also investigated the relation between parental vitellogenin levels and survival of offspring, and the effect of gross liver lesions on vitellogenin production. Vitellogenin levels were estimated indirectly by measuring levels of alkali-labile phosphorus. This method measures the phosphate bound to the vitellogenin molecule and is reported to be a good indicator of serum vitellogenin (i.e. that 'in transit' from the liver to the gonads). The method involved collection of blood from the caudal artery, preparation of serum and analysis of inorganic phosphorus using an automated blood analyser. Results were quoted as µg inorganic phosphate/ml serum.

Purdom *et al*. (1994) used an *in vivo* fish bioassay to study the oestrogenic effects of sewage treatment effluents. Immature rainbow trout (and in one study, carp) were placed in steel cages at or near sewage treatment works in a number of field trials for various periods ranging from 21 to 91 days. Following exposure, during which the fish were not fed, blood was sampled from the caudal sinus after anaesthesia with 2-phenoxyethanol. Fish were then killed, measured for length and sexed. Blood samples were centrifuged and the plasma removed and assayed for vitellogenin using a RIA.

Laboratory tests were carried out to test the potency of contraceptive pill constituents. Trout and carp were held in glass aquaria in flowing water to which solutions of steroids in distilled water were added. One trial involved the administration of test compounds *via* intramuscular injection. Again, levels of vitellogenin were measured by RIA.

Sheahan *et al*. (1994) measured the levels of plasma vitellogenin and gonadosomatic and hepatosomatic indices (organ weight as percent of bodyweight) in groups of rainbow trout held at two different temperatures and exposed to 17α-ethynyloestradiol at concentrations 'which might occur some

distance downstream of sewage treatment works'. Mixed sex groups of one year old fish were placed into light-proofed aquaria, divided into two series of tanks with one held at approximately 11.4°C and the other at 17.4°C. All fish were acclimated for four weeks following which 17α-ethynyloestradiol was dosed to fish in groups of three tanks at 0.1, 0.3 and 1.0ng/l nominal concentrations. The concentrations used were below the limit of detection for the standard analytical technique and were therefore difficult to verify. Blood samples were collected at intervals throughout the study and the plasma was analysed for vitellogenin by RIA (Sumpter, 1985). After 28 weeks the fish were killed and the liver and gonads removed and weighed. Histological sections of liver were classified according to the condition of cytoplasmic storage, and gonads according to the stage of spermatogenesis or oogenesis.

SEX DETERMINATION IN TURTLES

Bergeron *et al*. (1994) used the temperature-dependent sex determination of turtles as a biomarker of environmental contamination with PCBs. In common with other reptiles the red-eared slider turtle (*Trachemys scripta*) is a species in which the incubation temperature of the egg determines the sex of the offspring; a warm temperature (eg. 31°C) produces all female hatchlings, cooler temperatures (e.g. 26°C) produce all males, and intermediate temperatures (29 to 30°C) result in varying ratios of males to females. Application of exogenous oestrogens to the eggshell during the period of sexual differentiation can overcome the effects of temperatures producing males, inducing ovarian development. Eggs were incubated under temperature-controlled conditions (27.8°C or 26°C) and, at the beginning of the gonadal development stage, approximately four weeks after being laid, were assigned to groups and spotted with PCB compounds in 95% ethanol, 17β-oestradiol (positive control) in ethanol, or ethanol alone (negative control). Incubation was continued until hatching, approximately seven weeks later, after which hatchling sex ratios were determined by visualisation under dissection microscope and verified by histology. The assay was described as being a useful system in which to study the oestrogenic activity of xenobiotic compounds.

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LEICESTER, UK, 30 JANUARY 1995

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