Hormone disruptors in ecosystems

Aanbiedingsbrief

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to:

The Minister of Housing, Spatial Planning and the Environment

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Executive summary

In recent years effects on reproduction have been identified in a large number of animal species and these effects have been attributed to the influence on hormonal systems of certain substances that are present in the environment. The supposition has been expressed in various publications that such substances also have an impact on human beings. In 1997 the Health Council of the Netherlands reached the conclusion that this supposition has not been verified for the Dutch population. In this advisory report, the Health Council — acting upon the request of the Minister of Housing, Spatial Planning and the Environment (VROM) — describes the current level of knowledge on the effects of hormone disruptors on animal reproduction in Dutch ecosystems.

The Committee focuses primarily on substances that interfere with the sex-hormone balance. It calls a given substance a hormone disruptor if it is capable of disturbing reproductive physiology. The Committee also considers the effects of substances on the thyroid balance, in view of the important role which this system plays in development and reproduction.

In order to chart the implications of hormone disruptors for the situation in the Netherlands, the Committee has conducted an inventory of the field research that has been carried out in this country. In addition, it has classified around 80 pesticides and substances of industrial origin according to their hormone-disrupting capacity in the Dutch environment. The presence and possible effects of a number of natural and synthetic hormones have also been surveyed. These substances are excreted in substantial quantities by humans and especially by livestock.

In contrast to the situation in humans, effects on animal reproduction have definitely either been demonstrated, or else they are likely, in Dutch ecosystems. The majority of studies relate to animals in aquatic ecosystems, (including animals predating in these systems), with much less being known about the effects of these substances on animals that live on the land.

Intersexuality is prominent among the (possible) harmful effects that are associated with the presence of such substances in the water compartment. It is clear, for example, that certain species of snail which inhabit the coastal areas of the North Sea have, to some extent, been affected by a specific substance (tributyltin). It is not known what impact this has on populations of different species that are present in the food chain, and thus on the functioning of the ecosystem as a whole. Unfavourable phenomena have also been identified in fish, which are attributable to the impact of hormone disruptors. What remains unclear, however, is precisely which substances are involved and the scale of the effects in question. According to British research, there is a link between intersexuality, which has been discovered on a large scale in certain fish populations, and the occurrence of increased levels of a specific protein (vitellogenin) in male fish. Such increases point to an oestrogenic effect caused by substances that are present in the environment. Based on research in our country, although as yet limited, it also appears that an increase of vitellogenin in male fish is occurring in the Dutch estuaries.

According to the Committee, there is sufficient evidence of the negative effects of DDE, PCBs and dioxins on reproduction in certain species of fish-eating birds and (marine) mammals. These effects have — especially in the past — led to a decrease in (local) populations. The environmental concentrations of these substances — especially in sedimentation areas of the Rhine, Meuse and Scheldt rivers — are still so high that an adverse impact on the reproduction and development of resident fish-eating top predators can still be expected.

The Committee designates 34 of the 80 or so pesticides and substances of industrial origin in the Netherlands as (potential) hormone disruptors. These are alkylphenols, organochlorine, organobromine and organotin compounds, phthalates and triazines. Some of these substances — for example, the organochlorine compounds — have mainly been used in the past, while in other cases usage — and therefore dispersion in the environment — is more recent. Examples of the latter category are the persistent organobromine compounds, which have already penetrated deep into the food-chain in the oceans. For the majority of substances, data are only available for mammals. For these substances it is therefore impossible to verify the extent to which xenobiotic substances play a role in aquatic animals and invertebrates.

The Committee also regards some natural and synthetic oestrogens as hormone disruptors. These substances are excreted in substantial quantities by humans and, in particular, by livestock and find their way into the surface water by a process of leaching and via sewage treatment works. The concentrations of these extremely potent hormones in the major rivers are, broadly speaking, sufficiently high to give rise to effects on aquatic animals. In this regard, the Committee points out that it is likely that even higher concentrations of natural hormones occur in surface waters in areas of intensive livestock production.

According to the Committee, there are sufficient scientifically founded grounds for concern over the presence of substances — especially in the aquatic environment — which are capable of disrupting the sex-hormone balance in organisms and which might therefore pose a threat to the continued existence of species in ecosystems. In some species, effects on individuals and populations have actually been demonstrated, or else they are likely. Precisely what implications this has for biotic communities and ecosystems as a whole is unknown. However, because only very limited research has been carried out into the effects of the hormone disruptors that are present in the environment, it is quite possible that hormone disruption is more widespread than appears from the present report. It is thus because many substances have been investigated in recent years for their hormone-disrupting action that this list has already grown considerably. Given the large quantity of substances that stand to be investigated over the next few years, it is reasonable to suppose that the number of substances that can be labelled as (potential) hormone disruptors will continue to rise substantially.

In this connection it should also be borne in mind that the Netherlands occupies a unique position in Europe as far as the presence of hormone disruptors in the environment is concerned. Various European rivers bring hormone disruptors into the Netherlands. Because the Netherlands is a sedimentation area, it is precisely the persistent hormone disruptors that remain in the sediments. This country also has an extremely intensive agriculture industry which uses various substances that (possibly) exhibit hormone-disruptive effects. Account also needs to be taken of the presence in this small country of natural hormones as a result of its large numbers of humans and, in particular, livestock.

The Committee recommends that the monitoring programmes should be focused primarily on the water compartment and on manure. As far as the natural hormones are concerned, maximum priority needs to be given to small ditches and manure. Of the other substances, attention needs to be focused principally on the 34 substances which the Committee has classified as (potential) hormone disruptors, with the exception of a number of the organochlorine compounds, for which a successful policy has already been implemented. In view of its conclusion that little field research has been conducted into the effects of hormone disruptors within ecosystems, the Committee advocates that those monitoring programmes that already exist should be extended.

The Committee concludes that the instruments that are already available for monitoring effects on the sex-hormone balance in animals — although limited — are, nevertheless, adequate. These instruments include, amongst others, age structure and sex ratios of populations, transplanted sentinels, *in vitro* tests and chemical monitoring. It recommends extending the existing monitoring programmes with some of these techniques. The Committee emphasises the fact that there is no proven approach and that monitoring requires an iterative process, involving interdisciplinary collaboration, whereby ongoing efforts are made to determine which approach is the most effective.

Chapter 1

Introduction

1.1 Background

The possibility that certain environmental contaminants have hormonal (endocrine) effects on humans and animals has attracted a great deal of attention from the scientific world, the media and other interested parties in recent years. Most significant are effects on reproduction caused by changes in the sex-hormone balance. Abnormalities have been found in the reproductive organs or sexual behaviour of birds, marine mammals, panthers, alligators, fish and snails which have been attributed to exposure to substances capable of influencing hormonal systems. According to various researchers, such substances can also produce effects in humans.

A great deal was published in the 1970s and 1980s about endocrine effects of substances in humans and animals. More recently, both research and public interest in these effects have gained momentum.

By focusing attention on a mechanism of action — namely the disruption of hormonal systems — rather than on the effect, a different picture has emerged of the consequences of the substances that are present in the environment. Three factors play a key role in that picture, namely the intrinsic sensitivity of hormonal systems, the multiplicity of the substances in question and the so-called ‘human-wildlife connection’.

In humans and animals there are, during the development of the organism — both before and after birth — specific periods of great susceptibility to hormone disruption. At such times, even a single, relatively low dose of a hormone-disrupting substance — so low that there need not even be any question of ‘normal toxicity’ — can result in irreparable damage. Due in part to the availability of exceptionally sensitive *in vitro* tests, countless substances are now registered as potential hormone disruptors. This was formulated as follows at a conference held in Wisconsin in 1993:

A large number of man-made chemicals that have been released in 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 chemicals, and some metals...

Both humans and animals are exposed to a (large) number of these compounds simultaneously. This fact, coupled with the observation of abnormalities in feral animals — plus the fact that the human hormonal systems are very similar to those of animals — has led to the concept of the ‘human-wildlife connection’. Central to this concept is the suspicion (based on convincing observations in feral animals) that hormone disruption is also the mechanism of action behind the connections that have been described in various publications between specific diseases or abnormalities in humans and the presence of environmental contaminants. Examples of such phenomena are reductions in sperm quality and quantity, abnormalities of the sexual organs and the development of hormone-related tumours. This problem has been brought to the attention of the general public in the media — with the emphasis on the reduction of male fertility. The question is, to what extent is this suspicion justified? Not only are the concentrations to which animals in heavily contaminated areas are exposed far higher than is the case in humans, but animals are also exposed to different cocktails of substances. It is nevertheless a fact that the ‘human-wildlife connection’ has resulted in an early warning system for problems in humans.

In 1997 the Health Council of the Netherlands published an advisory report examining the extent to which there are grounds for concern over the impact of hormone disruptors on human reproduction and development, especially in the Netherlands (GR97). This report reveals that exposure to a number of these compounds does take place and also that these compounds can also have a (negative) impact on reproduction and development, but that no causal link has been established between exposure and the occurrence of effects in the population. Nevertheless, states the advisory report, the possible impact of hormone disruptors on human health does warrant serious consideration.

As a follow-up to the above-mentioned advisory report, the present advisory report looks into the impact of hormone disruptors on the reproduction of animals in ecosystems in the Netherlands. There are grounds for assuming that the Dutch situation with regard to ecosystems may be different from that in the surrounding countries. Not only is the Netherlands a delta region, which means that a substantial amount of environmental pollution from abroad ends up in our country, but it also occupies a unique position by virtue of its intensive agriculture and livestock production as well as its high population density.

In addition to the industrial chemicals and pesticides, this advisory report also considers a number of hormones that occur in the environment. These are natural and synthetic oestrogens excreted by humans and livestock and phyto-oestrogens which find their way into the environment following the consumption of vegetable material by livestock. Phyto-oestrogens are produced by certain plants and can function as hormone disruptors in animals.

1.2 The request for advice and the Committee

On 29 September 1998 the Minister of Housing, Spatial Planning and the Environment (VROM) asked the Health Council of the Netherlands for advice about the current level of knowledge with regard to the impact of hormone disruptors on ecosystems. Annex A provides the full text of the request for advice. The President of the Health Council instructed a specially co-opted committee — hereinafter referred to as ‘the Committee’ — to prepare the requested advisory report. The composition of the Committee is given in Annex B.

1.3 Structure of this advisory report

In Chapter 2 the Committee considers the sex-hormone balance of animals and the points at which disruption might possibly occur and defines the scope of the subject. In Chapters 3, 4 and 5 the Committee pursues three avenues in order to portray the extent of the threat to (animals in) ecosystems in the Netherlands. In Chapter 3 it surveys the effects on animals that have been identified in the Netherlands. Chapters 4 and 5 are devoted to the compounds and sources that may be to blame for effects on animals. In Chapter 4 the Committee classifies around seventy suspected xenobiotic compounds according to their hormone-disrupting potential. Chapter 5 examines the natural and synthetic hormones and phyto-oestrogens. Finally, Chapter 6 contains the answers to the questions posed by the Minister.

Chapter 2

The sex-hormone balance of vertebrates

In this chapter the Committee discusses the sex-hormone balance of vertebrates (mammals, birds, reptiles, amphibians and fish). It also examines in more detail the thyroid hormone system in the light of its significant influence on both embryonic development and reproduction. After discussing points at which the hormonal balance might possibly be disrupted by substances, the Committee defines the scope of the effects that are caused by substances.

Invertebrates are not discussed in this chapter. The reason for this is that little is known about the sex-hormone balance of most invertebrates — with the exception of the arthropods. In addition, scarcely any data is available about the disruption of the sex-hormone balance by substances. Furthermore, the (steroidal) hormone systems of invertebrates differ markedly from those of vertebrates in a number of cases. However, the Committee in no way wishes to play down the importance of invertebrates: after all, 95 per cent of the animal species in ecosystems are invertebrates.

2.1 What is a hormone?

Hormones are ‘messenger substances’ (both in plants and in animals), whose actions regulate vital functions such as development, growth, reproduction, behaviour and energy balance. They ensure that molecules, cells, tissues and organs within an organism function properly together not only in relation to the internal processes but also as regards their interaction with the environment. A properly functioning hormonal system is essential for life; this applies principally with regard to the individual, but a population also, ultimately, depends on such a system.

The classic view is that hormones are produced by endocrine glands. However, the broad definition of hormones that was given above also includes messenger substances such as cytokines, growth hormones, neuropeptides, etc. It is becoming increasingly clear that messenger substances are released by all manner of tissues and organs. In addition to the hormones secreted, for example, by the thyroid gland and the pancreas, the messenger substances produced by the heart, the lungs or the brain are also essential for life. Hormones are released into the bloodstream and the intercellular fluid (i.e. internal secretion, and not external secretion) as is the case, for example, with milk, sweat or saliva. We therefore also speak of internal, or endocrine, secretion (as opposed to exocrine secretion). The organ system and the accompanying products form the endocrine system.

Hormones are present in the bloodstream either as free molecules, or — and this applies to the majority — bound to transport proteins. Via the bloodstream, hormones reach all living cells, but not all of these cells will react to a particular hormone. Only the so-called target cells do this. These are cells that contain specific substances, the receptor proteins, which are able to bind specific hormones and enclose the cell so that they can elicit their particular effect. The way in which that effect is elicited differs from one type of cell or hormone to another. For example, sex hormones (but also thyroid hormone and retinoids or vitamin A) bind to receptors that are located in the cell nucleus. Within the nucleus the hormone receptor complex binds to a specific site on the DNA, the so-called *hormone responsive element* or HRE, whereupon transcription of one or more genes into messenger RNA takes place. RNA therefore contains the code that is subsequently translated into specific proteins, as a result of which the cell can perform a particular function.

2.2 Sex hormones: classification and synthesis

The sex hormones are generally divided into three functional groups: oestrogens, androgens and progestogens.

The most important representative of the oestrogen group is 17ß-oestradiol. In addition, there are less potent oestrogens, such as oestrone and oestradiol. Oestrogens are, generally speaking, formed in the ovary by cells located around the maturing oocytes (the theca and granulosa cells).

In mammals, the most important of the progestogens is progesterone. In lower invertebrates, derivative forms (such as the dihydro- or trihydroprogesterone) play an important role. Progestogens owe their name to an important function in mammals, namely the maintenance of pregnancy. In mammals, they are produced in the *corpus* *luteum*, a small gland that is formed from the cells around the maturing oocyte once the latter has left the ovary following ovulation.

As far as the androgens — or male sex hormones — are concerned, testosterone and its derivative dihydrotestosterone are the most important forms. Less potent androgens are androstenedione and dihydro-epiandrosterone. In fish (but also in other lower vertebrates) an oxidised variant, 11-ketotestosterone, is frequently the most active androgen. Androgens are produced by the Leydig cells in the testes, but also in the adrenal gland. Sex hormones belong to the steroid group. All sex hormones are biosynthesised from a common basic substance, cholesterol. Cholesterol is ingested with food, but can also be synthesised by the liver. Attached to specific lipoproteins, it is transported by the blood and absorbed (via special receptors) by the cells which use it to produce sex hormones. These cells are located in the gonads, the ovaries and the testis, and in the adrenal gland. The ovaries principally synthesise the oestrogens and progestogens, while the testes synthesise the androgens. The synthesis of sex hormones from cholesterol consists of a series of subtle enzymatic conversions. All of these conversions leave the skeletal structure of the steroid intact, but the groups on the vertices of the structure undergo changes. Methyl groups can be disconnected, -OH groups converted to =O groups and vice versa. This gives rise to a large number of extremely similar products, of which only a limited number are hormonally active. Figure 1 is a highly simplified diagram of steroid synthesis. It shows the synthetic pathways which lead to the three types of sex hormones, in addition to several important enzymes (the so-called P450 enzymes), which catalyse the different steps.

Whether a tissue produces androgens, oestrogens or progestogens from cholesterol depends on which enzymes are expressed in the relevant tissue. These do not always need to be the same ones. Thus, whereas before ovulation the granulosa cells around the maturing oocyte in the ovary produce oestradiol, afterwards they produce progesterone. This is because they then no longer express the enzymes that are required in order to complete the synthetic pathway from progesterone to oestradiol. Sex hormones can undergo important conversions away from the site of origin, either giving rise to hormonally active compounds or, conversely, resulting in inactivation. Thus the conversion of testosterone to dihydrotestosterone is a precondition for the effects of the androgen on the development of the external male sexual organs (the penis and the scrotum) and of the secondary sexual characteristics (such as beard growth).

In fish it has been shown that the testes produce the 11-hydroxyandrostenedione, which in itself has only a weak androgenic action. In the liver, however, it is converted to 11-ketotestosterone, the most important androgen in fish. An extremely important conversion is that of testosterone to oestradiol, which occurs, for example, in the brain.

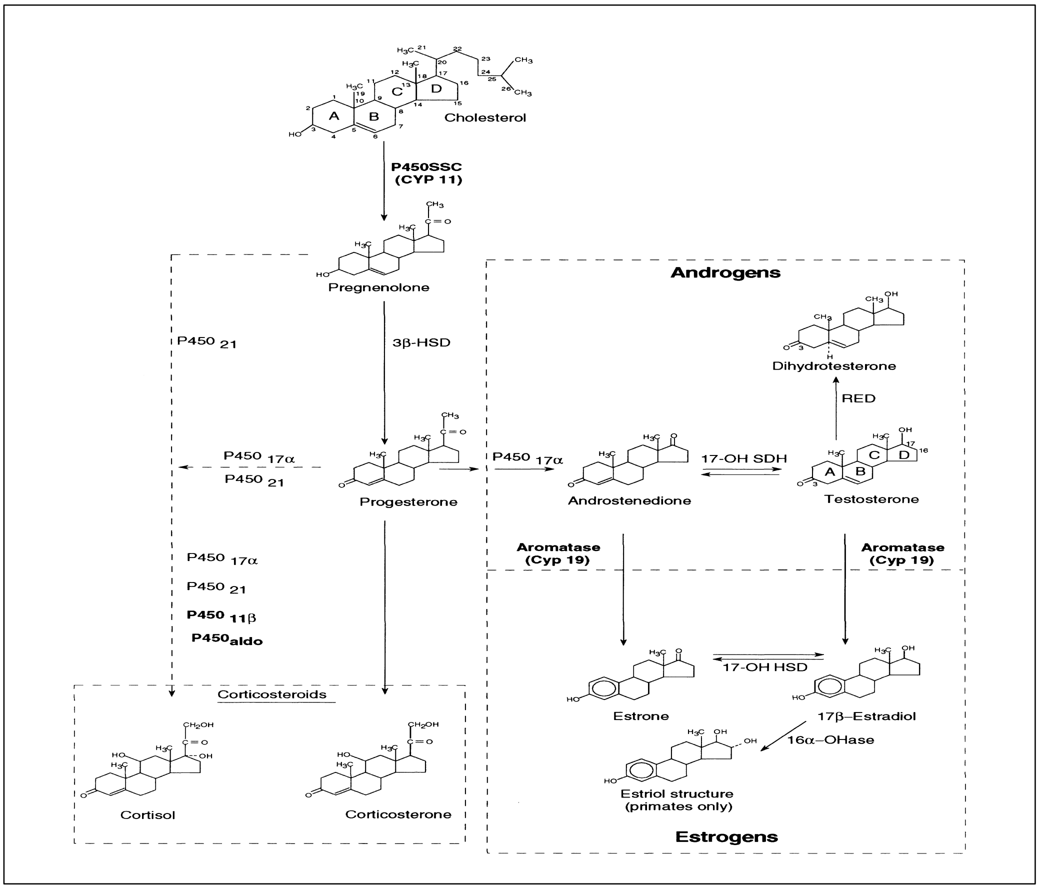

Figure 1 A highly simplified diagram of steroid-hormone synthetic pathways and the associated enzymes.

Virtually all of the effects of testosterone on the female development of the brain, and thus also on female sexual behaviour, come about under the influence of the female hormone oestradiol, which is formed from testosterone. The terms male and female hormone are thus extremely relative. Such conversions probably take place in a large number of tissues, thereby forming products that elicit local or systemic hormonal effects. Thus, steroid conversions have been demonstrated in the lungs, liver, fat, uterus, vagina, aorta, bone tissue, skin and blood. The physiological significance of these processes is still largely unknown.

Steroids are hydrophobic substances and are thus difficult for the kidneys to excrete. They are therefore conjugated to phosphates, sulphates or glucuronides — compounds which are able to pass through the kidneys. It is becoming increasingly clear that conjugation does not take place only in the liver but in all manner of tissues, giving rise to products that do not always have to be regarded as inactivated substances, but rather as other forms of active steroids which allow for intercellular migration in an aqueous environment. Fish are also known to excrete steroidal conjugates into the water, which function as odour attractants (pheromones) and thus allow for communication with other members of the species. This communication may relate to territorial questions, or alternatively to reproductive behaviour.

2.3 Functions of sex hormones

Sex hormones play a role in all developments and processes relating to reproduction. It would be excessive to deal here with all known functions of the sex hormones, but it is possible to outline those functions that apply generally to all vertebrates.

Following fertilisation, the sex hormones are determinative for the expression of the sex of the embryo (sexual differentiation). This means the development of internal and external male sexual organs and the differentiation of the brain towards a male configuration; in mammals, development towards a female configuration is the ‘default’, in so far as it comes about in the absence of male hormones. Thus the foundation is laid for subsequent sexual dimorphism, not only with regard to the external anatomy, secondary sexual characteristics (such as hair growth and vocalisation), but also with regard to reproductive behaviour and the cyclicity of reproduction. Sex hormones are subsequently essential for pubertal development, the maturation of oocytes and the formation of vital spermatozoa, partner identification, sex drive and courtship behaviour, synchronisation between partners and timing of reproduction, and the functioning of the reproductive organs in the broadest sense. In mammals sex hormones also play a role in the initiation and maintenance of pregnancy, the prevention of the immunological rejection of the foetus, the birth and the development of the mammary glands. In non-mammals, where the embryo has to develop outside the mother’s body and therefore requires food reserves in the form of yolk proteins, oestradiol ensures that the liver produces this protein (vitellogenin), which is then incorporated into the oocyte as yolk. As was noted earlier, the adrenal glands also produce sex hormones, notably androgens. Foremost among these is the weak androgen, androstenedione, which can be converted peripherally into a (likewise weak) oestrogenic hormone, oestrone. The functions are not well known. It is generally assumed that adrenal androgens play a role in early pubertal development in both men and women.

Besides having a reproductive function, sex hormones also perform metabolic functions. Thus testosterone is responsible in humans for the prepubertal growth spurt. In more general terms, it is a protein-anabolic hormone which explains why skeletal muscle mass is greater in the majority of men than in women. Oestrogens also have an effect on the development of connective tissue and subcutaneous fat. Furthermore, oestrogens and androgens are mitogenic for certain tissues, i.e. they stimulate cell division. Under normal conditions, this is functional — e.g. for the growth of the endometrium during the first phase of the menstrual cycle, or for the growth of the mammary glands towards the end of pregnancy.

Most of the processes mentioned above are not continuous, but occur cyclically (menstrual cycles, seasonal reproduction). But processes which only occur once in each individual — such as puberty and the menopause — are also determined by sex hormones. This indicates that there is an individual dynamic for hormone production, circulating amounts of hormones, and the degradation and excretion of hormones, which needs to be subject to precise regulatory systems if all of the reproductive processes in the individual are to occur in the correct order and in accordance with the internal and external conditions. It also means that wherever certain threshold values are exceeded, continuous exposure to hormonally active substances will virtually always have a destructive effect on processes that are controlled by sex hormones**.**

2.4 Regulation of sex hormone production

Although the testes and the ovaries are, to a certain extent, capable of autonomous, basal secretion of sex hormones, the fluctuations in their activity are determined to a significant extent by the pituitary gland (hypophysis). It is there that the gonadotropic hormones FSH and LH (follicle-stimulating and luteinising hormone, respectively) are formed. These two hormones regulate the dual function of the sexual organs: the production of the gametes and of sex hormones. Broadly speaking, it is true to say that the principal function of the FSH is to stimulate the formation of gametes and that of LH is the formation of sex hormones — although many subtle distinctions are possible if different species are considered. In humans, for example, this only applies in part: prior to ovulation, FSH provides for the synthesis of oestradiol in conjunction with LH.

The secretion of LH and FSH is regulated both by the brain and by the sex hormones, while secretion of FSH is additionally regulated by two other hormones from the gonads — activin and inhibin. The brain produces neurohormones, including gonadotropin releasing hormone (GnRH). This is a small peptide which reaches the pituitary gland from the hypothalamus via a special connecting blood vessel and from there stimulates the release (and also — in a number of animal species and in humans — the synthesis) of FSH and LH. Besides GnRH, countless other neurohormones have in the mean time been found which influence this process either directly or indirectly, including dopamine, taurine, glutamic acid, GABA, melatonin and neuropeptide Y.

As noted above, the sex hormones, in turn, influence the production of FSH and LH. Depending on the type of sex hormone and on the situation (e.g. the stage of the menstrual cycle), this feedback may either be positive or negative, and a distinction also needs to be made between effects on synthesis and those on release. By virtue of this feedback it is, for example, possible during the menstrual cycle for oestradiol initially to stimulate the synthesis of LH but inhibit release. Via a stimulation of the GnRH, however, a rise in the oestradiol level leads to a sudden release of LH, thereby triggering ovulation. After ovulation, the same cells that initially formed the oestradiol, having become corpus luteum, now produce the progesterone, which in turn exerts a negative feedback on the hypothalamus/hypophysis system and thereby inhibits the secretion of GnRH, resulting in less FSH being released. Only when, at the end of the menstrual cycle, the *corpus luteum* degenerates and stops producing progesterone is FSH once again released and a new cycle begins.

Oestradiol is the most effective of all the sex hormones in the feedback system that is described above. It influences — both directly and via the activity of the neurohormones — both the gene expression of LH and FSH and their release. This applies to both female and male individuals. The testosterone from the testis is converted into oestradiol in the brain and pituitary gland and then has the same effect.

The overall system that regulates reproduction is referred to as the hypothalamic-pituitary-gonadal axis. Because it involves both the nervous system and the endocrine system, we speak of a neurohormonal system. Due to the involvement of the nervous system, the organism is able to react to external stimuli. In many animal species, for example, we find seasonal reproduction: in many cases, the animal is then able to use the length of the day as a type of calendar. The light stimulus reaches the brain via the eyes and there it is brought into contact with the hormonal system.

2.5 Interaction between the sex-hormone balance and other hormonal systems

Besides sex hormones, various other hormones and growth factors play an important role in reproductive physiology, the formation and development of gonads and behaviour. In addition, the nervous system exerts a controlling and regulating influence on the reproductive system. Sensory stimuli (including daylight) can stimulate the release of specific neurotransmitters (dopamine, GABA, glutamic acid) via certain neural networks, and these neurotransmitters subsequently stimulate the release of GnRH by the hypothalamus and therefore the pituitary-gonadal axis (see figure 2).

Of the hormonal systems, it is primarily the thyroid-hormone system that has an important effect on growth in general and on the formation and development of specific organs, such as the brain and gonads, as well as on processes, such as metamorphosis in amphibians. In mammals thyroid-hormone status influences the development of the testes (enlarged testes in connection with a relative deficiency of thyroid hormone and vice versa). In frogs, induction of vitellogenin by oestradiol only occurs if they have first

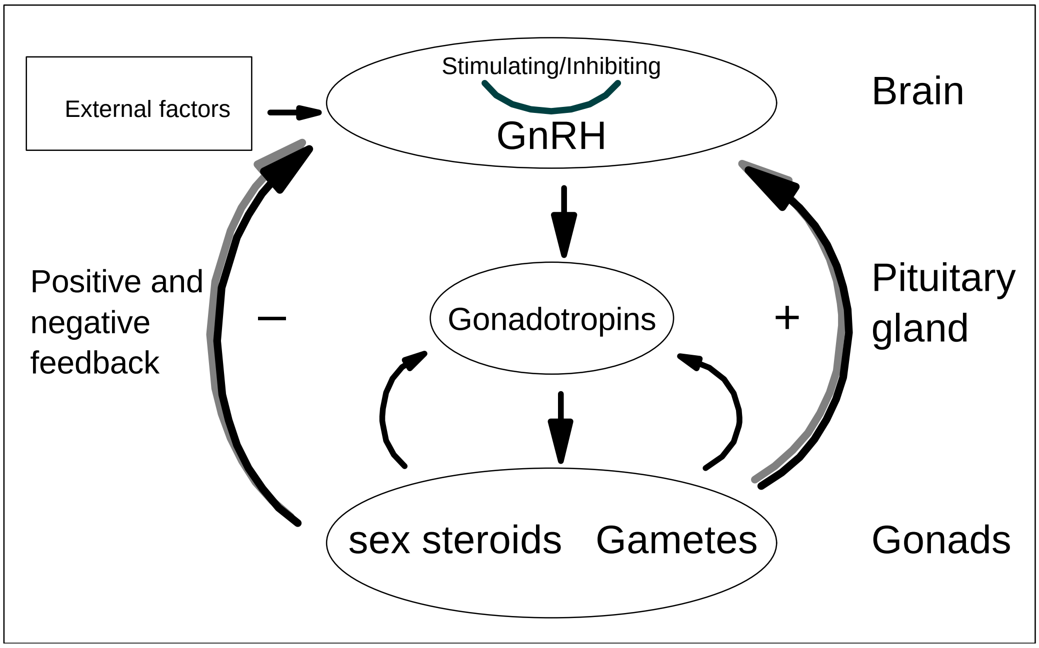

Figure 2 A highly simplified representation of the hypothalamic-pituitary-gonadal axis.

been dosed with a certain amount of thyroid hormone. In fish, thyroid hormones most probably play a role in the maturation of the oocytes.

In addition to reproduction, thyroid hormone is primarily of importance to the normal development of the brain and the cerebral functions, such as motor function and cognition. A thyroid-hormone deficiency during the foetal stage of development in mammals can lead to a severe form of underdevelopment of the brain (cretinism in humans), associated with clearly discernible disturbances in the development of motor and cognitive functions.

The organisation and regulation of the thyroid-hormone system correspond very closely to those of the sex-hormone balance. There is a hypothalamic-pituitary-thyroid axis which is instrumental in stimulating and regulating the production of thyroxine (T4) and triiodothyronine (T3) by the thyroid gland. The hormones produced by the thyroid gland are released into the bloodstream and transported — primarily bound to transport proteins — to the target cells, where the free thyroid hormone (T4) is absorbed and intracellularly activated to T3. T3 then binds to specific thyroid hormone receptors which, after complexation with so-called thyroid-responsive elements (TREs) on the DNA, lead to an increase in the transcription of certain (thyroid-responsive) genes and production of proteins which are involved in the performance of the thyroid-hormone functions.

2.6 Disruption of the sex hormone balance

Points of disruption

The above discussion of the sex-hormone balance is far from complete. It should be evident, however, that this is an extremely complex system, in which many hormones interact in order to make all the facets of reproduction possible. It is precisely because of the highly complex nature of hormonal systems that there are a large number of points at which disruption can occur. Moreover, hormone disruptors can attack various sites simultaneously. The points at which disruption occurs can be classified as follows:

- The substance binds to a sex-hormone receptor and behaves like a sex hormone (hormone mimicry) in so far as, after binding, the receptor is also activated.
- The substance binds to the sex-hormone receptor but no activation occurs. Receptor occupancy by such a substance means that the real hormone can no longer bind to it. The substance then acts as a hormone-antagonist (competitive inhibition). One further type of effect needs to be mentioned in this connection, namely binding of substances to those parts of the DNA where the responsive elements of the sex hormones are located.
- The substance acts upon the metabolism, the transportation and the excretion of the sex hormone, including all associated proteins and enzymes.

In section 1.1 a number of substance groups are named which can result in hormone disruption. In general, the following observations can be made about the points of disruption and the action of these substances. Most of what we know about hormone disruptors from field and laboratory research relates to binding to the oestrogen receptor or to oestrogenic or anti-oestrogenic effects. However, we cannot rule out the possibility (and this is also borne out by preliminary research results) that hormonally active substances in the environment can also have unintended androgenic, progestogenic or thyroidogenic effects because they bind to the relevant receptors. It is also known that some xenobiotic substances acts upon those enzymes that are responsible for the conversion of the sex hormones.

Exposure to natural and synthetic oestrogens will lead to disruption of the sex-hormone balance via binding to the oestrogen receptors followed by activation of the DNA. Oestradiol is the most potent oestrogenic hormone in all vertebrates. Because the hormone-binding specificity of oestrogen receptors of all vertebrates is virtually identical, exposure to low concentrations of oestradiol (or to synthetic oestrogens) leads to disruption in a wide range of animal species.

Numerous xenobiotic substances and phyto-oestrogens are likewise capable of binding to oestrogen receptors, whereupon they can exhibit both hormone mimicry and antagonism. These substances are referred to as pseudo-oestrogens or xeno-oestrogens. Although the majority of them are weakly oestrogenic — i.e. they have a relatively low affinity for oestrogen receptors — they are nevertheless able to disrupt the oestrogen balance due to their constant presence at frequently high concentrations. Moreover, many of the xenobiotic substances referred to here are frequently hydrophobic, as a result of which bioaccumulation is promoted and concentrations are found (in fish, for example) that are orders of magnitude greater than the environmental levels.

Experimentally it has been determined that exposure to weakly oestrogenic substances, which in themselves have little effect, can lead to extra production of oestrogen receptors. This will intensify the response to exposure to hormone disruptors, or to endogenous hormones (synergism). Additivity of the effects has certainly been identified for various xenobiotic substances, but not synergism. There are indications in fish that exposure to oestrone leads to an intensified response to exposure to oestradiol (Boh82).

Disruption of the thyroid-hormone balance

We know of many substances — including both xenobiotic and natural compounds — that can interfere with the thyroid-hormone balance. In contrast to substances which, for example, have an oestrogenic action, the principal influence of the thyroid-hormone system occurs at the level of the metabolism and scarcely at all at the level of direct receptor interaction.

2.7 Scope

In estimating the risks posed by substances with a hormone-disruptive action, the Committee will concentrate on effects on reproduction. After all, disruption of reproduction has a potentially major influence on populations, and therefore on the ecosystem. In making its assessment, the Committee will take all sex hormone-mediated effects into consideration, that is to say effects resulting from sex-hormone mimicry and antagonism as a result of receptor binding, and impact on the hormone balance as a result of interference with the synthesis, transportation, degradation and excretion of sex hormones. In addition, it will look at the effects engendered by interaction with the thyroid-hormone system. The choice of the effects on reproduction as the starting point means that a ‘disruptor’ can only be said to exist if the substance in question does, in fact, disrupt the reproductive physiology (or the reproductive behaviour) in individuals or their offspring. The Committee does not regard a change in the hormone level which remains within the limits of normal homeostasis — and therefore does not lead to a physiological response (disruption) — as a disruption.

Chapter 3

Documented effects

In this chapter the Committee surveys the field research performed in the Netherlands in which effects of hormone disruptors on (populations of) animals have been identified. In addition, it provides a brief overview of the research that has been carried out abroad on related animal species. In view of the limited scale on which research has been conducted and the methodological problems involved in such research, it is questionable just how far this survey provides an insight into the full extent of the consequences of exposure to these substances. Moreover, the consequences of effects of substances on populations — in so far as they are already known — for biotic communities and ecosystems* as a whole are unknown.

The field research that has been conducted (on a limited scale, as has already been noted) was in most cases prompted by (chance) observations that something was occurring within a particular population. In most cases, the objective was not to detect sex-hormone disruptors. Moreover, the majority of studies relate to animals in aquatic ecosystems; much less is known about the impact of these substances on terrestrial animals.

From a methodological point of view, it is extremely difficult to demonstrate a causal relationship in field research. The fact is, it is not only substances that can have effects on reproduction. Changes in environmental factors — such as food supply,

* An ecosystem is an integrated and largely self-contained structure of a community of organisms that consists of a number of species, located within a specific, physically limited area, functioning in energy flows and material cycles (Ver95).

disruptive pressure and the fragmentation and destruction of habitats — can likewise play a fundamental role. For the purposes of gathering evidence, it is therefore necessary to undertake a multifactorial interpretation of effects that have been observed in the field.

Both knowledge of the action of a substance itself and the observation of a hormone-disruptive effect in (a population of) animals can, in practice, serve as the point of departure for a field study. In the former case, laboratory research — demonstrating that a substance might be able to elicit a particular effect in certain animals — provides the grounds for conducting field research. Thus, for example, the notion that a common substance induces vitellogenin production in male fish* would justify field research in those locations where this substance occurs environmentally in high concentrations. This applies to (eco)epidemiological research that is designed to establish whether there are effects on a population. For example, it is possible to investigate whether there are disruptions in hormone concentrations in animals, whether there is evidence of reproductive abnormalities and whether pathological abnormalities are linked to disruption of the hormone balance. The next question is what has caused the effect that has been identified. At population level, however, there are many circumstances (both natural and anthropogenic) that can contribute to the effect — as has already been mentioned. It is frequently not possible to unravel these factors.

If effects can be demonstrated on populations, then one must ask what consequences this will have for other populations and consequently for the relevant ecosystem. One approach that could possibly be used lies in the food-web concept. This describes the nutritional relationships between species, i.e. who eats what, and what species eats another species. The food web provides an insight into the possible consequences of one species being attacked by other species. The ultimate purpose of the food-web concept is to quantify the dynamic behaviour of a given biotic community and the associated energy flows and material cycles (GR97). From this it is possible to deduce the consequences of harmful effects by populations on ecosystems. However, such an approach to the assessment of risks for biotic communities and entire ecosystems is still in its infancy.

* The yolk constituent vitellogenin is normally only produced by females. Male fish can produce vitellogenin, but only in small quantities. High production in males indicates exposure to substances with an oestrogenic effect (see also 2.3)

3.1 Effects in birds and (marine) mammals

3.1.1 (Marine) mammals

The Netherlands

In the 1970s the seal population in the Wadden Sea declined sharply as a result of a reduction in reproductive success. The concentrations of PCBs in tissue from seals originating from the Dutch Wadden Sea were found to be seven to ten times higher than those in tissue from seals from the German/Danish Wadden Sea, where reproduction was normal (Reij80). During a study of two groups of common seals fed respectively with relatively contaminated fish from the Dutch part of the Wadden Sea and relatively clean fish from the Atlantic Ocean, lower levels of 17ß-oestradiol were found in the group with the highest PCB uptake, i.e. those animals that received fish from the Wadden Sea (Boo87, Reij86, Reij96b). This distinction only manifested itself around the implantation period in females. Reduced concentrations of thyroid hormones were also detected. That reduction is attributed to a competition between the hormones and hydroxylated PCB metabolites during binding to a transport protein (Bro89). The difference in concentration in the blood of seals fed with fish from the clean reference area and from the Wadden Sea amounted to a factor of five for the PCBs and a factor of two for 4,4'-DDE (Boo87). Seals from the group that had initially received the most contaminated food were subsequently fed with clean fish and, within two years, were found to have returned to their former reproductive level. Furthermore, investigations into the passage of PCBs and PCB-methylsulphonyl metabolites* from mother to pup have demonstrated unimpeded passage of stable metabolites via the maternal milk (Gre96, Gre97). In the case of the unmetabolised PCBs, the passage is selective, in that it is less marked in the more hydrophobic PCBs. Lowering of thyroid-hormone concentrations in seals can impact on early development and subsequent reproductive capacity. According to laboratory studies with mammals, PCBs are likewise capable of influencing mating behaviour (Pet93). However, insufficient data from field research is available about this phenomenon.

It should be noted that the functioning of the immune system is influenced by oestrogens and androgens (among other things). It could thus be possible for disruption of the hormone balance to lead to disturbances of that system. This is principally significant in relation to the maternal response of rejecting the fertilised ovum, which is suppressed by a correct balance between 17ß-oestradiol and progesterone. This could be

* Oxidation of PCBs by the cytochrome P_{450} system may either result in stable hydroxylated PCBs or, alternatively, methylsulphonyl metabolites may be formed after conjugation.

associated with the problems surrounding the implantation of the fertilised ovum in seals, but further research is necessary to confirm this. Between the end of the 1970s and the end of the 1980s, the PCB levels in seals in the Netherlands fell by around 50%. Reproduction has now virtually returned to a normal level (Reij97).

Abroad

Several xenobiotic substances with hormone-disruptive properties have been detected in tissue from many species of marine mammals (Agu95, Boe98, Reij96a, Wag84). According to some studies, abnormalities of reproduction or hormone control are related to specific chlorinated hydrocarbons and their metabolites in the following species: ringed seals (*Phoca hispida botnica*) and grey seals (*Halichoerus grypus*) in the Baltic Sea (Ber85, Hel80), Californian sealions (*Zalophus californianus*) (DeL73) and beluga whales (*Delphinapterus leucas*) (Bél87), among others. PCBs, DDT and the associated metabolites are generally cited as the principal disruptors. In certain Dall porpoises (*Phocoenoides dalli*) a negative correlation has been found between the testosterone level and concentrations of PCBs and DDT (DDE) metabolites (Sub87).

In the Baltic Sea, 30% of examined adult female grey seals and 70% of examined adult female ringed seals have been found to be either partially or completely infertile (Ber85). The infertility was attributed to a blockage of the Fallopian tubes. Follow-up studies have identified methylsulphonyl metabolites of DDE (a metabolite of DDT) and PCBs as the principal causative toxic agents (Ols94).

Beluga whales in Canada are being exposed to high environmental concentrations of PCBs, DDT and polycyclic aromatic hydrocarbons (PAHs), among other compounds (Bél87). Of 94 male Beluga whales examined, one was found to be a hermaphrodite: in addition to obvious external male characteristics, a uterus and small ovaries were visible (DeG94). This abnormality is attributed to a hormonal disturbance in early pregnancy, caused by PCBs or DDT and resulting in the disruption of the normal intra-uterine differentiation of male and female organs. Future investigation will reveal the actual prevalence rate of this phenomenon in the beluga whale population.

Otters

A connection has been made between the decline in otter (*Lutra lutra*) numbers in the industrialised countries and rising PCB contamination in the environment (Kih92, Leo97, Mas89, Ols83). Stable, thriving populations can today only be found on the fringes of Europe, where the lowest concentrations of PCBs are found (Leo97). Feeding tests in the mink, which is related to the otter, have revealed that administration of PCBs via the food has a major influence on reproduction (Boe84, Jen77). The limit value for the occurrence of effects in otters is 0.7 ng TEQ/kg fish (Smi96). Only in fish from the cleanest areas of the Netherlands are levels below this limit value. In the Haringvliet, levels of 40 ng TEQ per kilogram of fish have been found. This suggests that in many areas of the Netherlands, and especially in the areas that are influenced by the Rhine and Meuse rivers, PCB-related effects on the health of otters can be expected upon reintroduction of this species. In otters concentrations have been found that are 16 to 36 times higher than in other Mustelidae (Voo94). This is due to the fact that the otter's diet consists exclusively of fish.

3.1.2 *Birds*

The Netherlands

As long ago as 1970 it was established and documented in the Netherlands by Koeman *et al.* that DDT, PCBs and dioxin-related compounds disrupt the reproduction of fish-eating birds such as the sandwich tern and the eider (Koe69, Koe72). The presence of the 'drins' — endrin and telodrin — in the Wadden Sea (as a result of discharges into the New Waterway Canal) also played a major role in the case of the sandwich tern and the eider (Swe72).

In the Netherlands, effects on reproduction have been best documented in two fish-eating birds, the cormorant (*Phalacrocorax carbo*) and the common tern (*Sterna hirundo*). Since the 1960s, the levels of chlorinated hydrocarbons in Dutch top predators (including fish-eating birds) have been substantially reduced. Nevertheless, field and laboratory research with Dutch common terns and cormorants in the first half of the 1990s has revealed that the levels of all substances (except for the 'drins') in fish are still sufficient to trigger effects (albeit subtle) on reproduction.

Comparative research in a number of Dutch cormorant breeding colonies revealed a relatively poor hatching result in the Biesbosch area. In this study, a positive correlation was found between the DDT metabolites in the egg and the food and the occurrence of thin eggshells, while there was a negative correlation between the PCB levels and the percentage of hatched eggs and hatching success (Dir95). Research with cormorant embryos from two different exposed colonies likewise showed that *in ovo* exposure to the current levels of PCBs and dioxins has a negative effect on embryonic respiration and development (Ber94).

Extensive laboratory research has likewise been conducted with eggs and embryos from the common tern (Bos95, Mur94). It has emerged that in areas with the highest concentration of PCBs and dioxin — e.g. the Haringvliet — the incubation period was reduced by approximately two days (Bos95). Various changes have also been found in cytochrome P_{450} induction and thyroid-hormone and vitamin-A balance, although the physiological and toxicological relevance of this finding is unclear. These effects are associated with the levels of PCBs and dioxins in the yolk sac (Bos95, Mur94, Mur96). Bosveld calculated that a level of around 50 picograms of dioxin equivalents/gram fish (fresh weight) can be regarded as a LOEC (lowest observed effect concentration) for induction of cytochrome P_{450}. Induction of this enzyme is generally considered to be one of the most sensitive biochemical processes for dioxins and related compounds, including some PCBs (Saf90, Saf94).

Abroad

Reporting of effects on the reproduction of birds abroad has been particularly good for North America. As long ago as 1962 Rachel Carson reported that accumulation of chlorinated hydrocarbons was responsible for the thinning of eggshells in songbirds (Car62). The resultant decline in reproduction is to a significant extent attributed to DDE (Ris89). Correlations between high DDE levels and thinner eggshells have been demonstrated in cormorants, herring gulls, terns and in birds of prey such as the bald eagle, prairie falcon and merlin (All91, Gil74, Pea75, Pea88, Rat67, Wes83). Effects on behaviour, nest size, development of the gonads and embryo mortality have also been demonstrated in a large number of feral fish-eating birds (Fry95). In general, these adverse effects on birds in North America have been associated with higher concentrations of chlorinated hydrocarbons, and it is presumed that, alongside DDE, dioxin-related compounds and PCBs also play an essential role (Fry95). Comparison of the situations in the Netherlands and North America underlines, in particular, the association between thin eggshells and DDE in cormorants in the Biesbosch area.

Besides the evidently adverse effects on eggshell thickness and embryonic development, a great deal of research has also been conducted in North America in the past decade into more subtle biochemical variables, which may serve as early indicators of exposure. This has focused above all on the induction of a specific cytochrome, P4501A(1), an enzyme that in birds can be induced even in the presence of low concentrations of dioxin-related compounds and PCBs (Bel90, Bos95, Hof87, Ken96, San94a, San94b). Although the statistical correlation between P4501A(1)-induction and adverse effects of dioxin-related compounds in feral bird species has not been investigated as thoroughly as in rodents, the US and Canadian research with the Foster’s tern, the common tern and the great blue heron has provided sufficient grounds for assuming that this relationship also exists in birds (Ken96, San94). The research results seem to display a close similarity to the findings of the above-mentioned Dutch research with cormorants and common terns (Ber94, Bos95).

For a more extensive review of effects of contaminants on reproduction and development of birds, the Committee refers the reader to a publication by the CSTEE (CTS99).

3.2 Effects in fish

The Netherlands

In the Netherlands, preliminary measurements conducted in flounder from the Lake IJssel and from a number of brackish and estuarine waters reveal significant vitellogenin induction in male fish, especially in the Europoort area and the Nieuwe Waterweg Canal (Bri97). The oestrogenic effects can even be observed to a small extent in the spawning grounds that are situated in the open sea (20 — 40 km off the Dutch North Sea Coast) (All97, Mat98). Since vitellogenin can be present in the blood of male fish for several weeks, it is possible that the spawning populations of flounder in the open sea have largely encountered the exposure in the estuaries (Mat98). However, the possibility of contamination in the open sea cannot be ruled out in advance. In addition, a significant oestrogenicity has been demonstrated *in vitro* in the majority of sediment extracts from estuaries with the aid of receptor/reporter gene assays (Leg98).

In the opinion of the Committee, the fact that the levels of induction of vitellogenin in male flounder from Dutch estuaries and coastal waters are comparable to those in corresponding areas of England could indicate that intersexuality* in fish can also occur in the Netherlands.

Data produced by the Dutch National Institute of Fisheries Research (RIVO) in the 1960s does not reveal any change in sex ratios in flatfish. However, Lang *et al.* have demonstrated somewhat different ratios in dabs from certain areas of the North Sea, sometimes identifying more, and sometimes fewer, female fish (Lan95). There have also been reports since the 1980s of a widespread accurance — and often, in certain areas, a relatively high prevalence — of embryonic abnormalities in several fish species in the coastal waters of the North Sea (Cam92). It is unclear, however, whether these observations are connected with exposure to hormone disruptors.

On the Friesian island of Texel impact studies have been conducted in flounders kept in pools containing moderately polluted dredging spoil (class 2) originating from the Rotterdam Harbour area. Besides an increase in the incidence of a viral skin disease and the induction of liver tumours (Vet97), a 3-4 month delay in maturation has been

* Intersexuality in fish is an abnormality characterised by the development of oocytes in (otherwise normal testes of) male individuals, possibly resulting in reduced fertility and sterility. (As opposed to hermaphroditism, whereby two sexes occur both functionally and naturally in a single individual.)

demonstrated in female fish (Jan97). The precise mechanism of action has not been explained.

Abroad

Reproductive disturbances — including reduced fertility, masculinisation of females and feminisation of males — as a result of hormone disruption have in recent years been observed in the vicinity of pollution sources (effluents from sewage treatment, wood and paper processing and textile plants, etc) in Canada, the US and UK (Gag95, Har96, Har97, Job96, Job97, Job98, Leb97, Lye97, Mun91, Pur94, War96).

The English studies, in particular, may be relevant to the Dutch situation, since they include a detailed investigation of recent effects of hormone disruptors in feral fish populations. According to that research, most of the investigated effluents from sewage treatment works lead to oestrogenic effects in fish (Pur94, Sum95). Oestrogenic activity has also been detected in a number of receiving surface waters (Har97). Caged male rainbow trout (*Oncorhynchus mykiss*) were found to produce vitellogenin. In addition, a delay in testicular growth has been detected. Although alkylphenolic derivatives were the only source identified at first (Job96), it has since been established that the oestrogenic activity of the water samples can be explained also by the presence of natural oestrogens (17ß-oestradiol and oestrone) and, to a lesser extent, synthetic oestrogens originating from the oral contraceptive pill (17α-ethinyl oestradiol) (Des98).

The widespread nature of oestrogenic effects is also evident from a large-scale field study which discovered increased levels of vitellogenin in the roach (*Rutilus rutilus*) in a number of river systems into which liquid effluents are discharged. In addition, the prevalence of intersexuality was found to be extremely high — more than 90% of the population examined in some places (Job98).

The findings outlined above provide an initial indication of a widespread effect of oestrogenically active substances in the aquatic environment. The reproductive disorders identified in fish in the field are consistent with results of laboratory research and indicate an association with discharges from sewage treatment works (STWs), which demonstrably contain oestrogenic steroids (Job98).

It is not only in the English rivers that oestrogenic effects have been found, but also in the estuaries. Examination of flounders in five estuaries along the coast of Wales and England also revealed that nearly all male specimens exhibited elevated vitellogenin concentrations, and intersexuality was detected in up to 20% in the most polluted estuaries (All97, Mat98). The causes of these phenomena in the flounder are unknown, but industrial effluents could play a greater role than is the case in freshwater fish (Mat98).

Relationship between the English and Dutch situations

A disturbing aspect with regard to the Dutch situation is the association that has been established in the English study between the occurrence of intersexuality and the vitellogenin levels that have been detected. In the Netherlands, where no research has yet been conducted into intersexuality, vitellogenin levels have been detected in estuarine fish which correspond to the levels found in English estuaries.

3.3 Results of in vitro tests in the Netherlands

In vitro measurements reveal that oestrogenic activity is occurring in many waters. This applies not only in the case of effluents from STWs, industrial purification plants and the water from large rivers (Bel99), but also, in particular, in sediments from estuaries with major industrial activities, such as the Europoort and IJmuiden harbour (Leg98). Measurements performed on Rotterdam harbour sludge and contaminated sediments in general lead one to suspect that these are, in most cases, compounds that are readily soluble in water (Leg97, Leg98). What has emerged is that the oestrogenic activity is to be found for the most part in the polar acetone extracts from the sediments and scarcely at all in the more non-polar hexane extracts (Leg97). It is not known at present which substances are involved. It has been established, however, that oestrogenic activity and concentrations of natural and synthetic hormones have been measured in the vicinity of purification plants — but also in water from the Rhine and Meuse rivers — that might be expected to trigger effects on fish. In Chapter 5 the Committee takes a closer look at the presence of natural and synthetic hormones in the Dutch environment.

3.4 Field observations of effects in invertebrates

Whelks and dogwhelks

In coastal areas all over the world, abnormalities have been found in prosobranch snails belonging to the sub-classes Mesogastropoda and Neogastropoda which are attributed to tributyltin (TBT) in anti-fouling ship paints (Ell90, Foa93, Hor94, Saa90). The phenomena in question are imposex and intersexuality. Imposex is the occurrence of an entire or partial male organ in a female individual, while the primary female sexual organs are completely intact. The male organ is, as it were, superimposed. In the case of intersexuality in snails, on the other hand, some female organs have been transformed into a male organ.

All populations of the dogwhelk (*Nucella lapillus*) and common periwinkle (*Littorina littorea*) in the coastal areas of the North Sea have been affected to a greater or lesser extent (NST93). The whelk (*Buccinum undatum*) has disappeared from the Wadden Sea and the populations in the Dutch part of the North Sea have declined. However, in this case, damage inflicted by trawling is also playing an important role in this problem.

Imposex in whelks outside the coastal areas has been discovered for the first time in the southern part of the North Sea (Hal94). There was an extremely strong correlation between the prevalence of imposex and the intensity of shipping traffic in the area concerned. Since then, imposex has also been identified in over 90% of female whelks in the Eastern Scheldt (Men96b). Despite this high prevalence, these animals continue to reproduce — as evidenced by the presence of egg packets on the sea floor and sperm around the oocytes of female whelks with evident imposex. This is not the case with the dogwhelk, since here — unlike in the whelk — imposex leads to the closure of the female sexual opening by the developing sperm duct (Mer88).

Intersexuality in the common periwinkle has been detected in the German Wadden Sea (Bau95, Bau97). Substantially reduced fertility had in particular been identified in harbour areas.

Alongside the strong correlation that has been found to exist between the intensity of shipping traffic and the prevalence of imposex, results of laboratory research confirm that this is a dose-dependent effect of TBT. The concentrations of TBT in areas where imposex occurs are sufficiently high to explain the phenomenon. The precise mechanism is not known. There are two hypotheses regarding the action of TBT. One hypothesis is based on a disruption of the regulation of sex hormones. The fact is that exposure to TBT leads to an increase in testosterone levels (Oeh93). The other hypothesis seeks the cause in a neuroendocrine mechanism (Fer87, LeG83). This hypothesis is supported by the fact that it is extremely young whelks, whose primary sexual organs have not yet developed, that are most sensitive to TBT. It appears from laboratory research that imposex can start to occur in the presence of extremely low-level exposure to TBT: 1 nanogram tin/litre in the dogwhelk and 7 ng/L in the whelk (Gib87, Men97). Intersexuality in the common periwinkle occurs at concentrations of 10-15 nanograms/litre.

The occurrence of imposex as a result of the presence of tributyltin has been detected in around a hundred species of prosobranch sea snails (Fio91). Intersexuality, on the other hand, has only been found in the common periwinkle. Tributyltin is, for example, also extremely toxic for oyster larvae. TBT illustrates that substances exist which even at extremely low concentrations can disrupt the sex-hormone balance of only a certain number of animal species. Effects of such substances can easily be overlooked in both field and laboratory studies.

Besides tributyltin, anti-fouling paints also sometimes contain triphenyltin (up to 10%). Triphenyltin (TPT) is also used on a large scale as a fungicide in potato production. No experimental evidence has yet been produced that TPT can also cause imposex. Some researchers detect imposex, others do not. Laboratory studies have, however, revealed that TPT promotes the occurrence of imposex. Imposex has been found in Japanese waters, where the intensity of shipping traffic is low, but where TPT concentrations are, nevertheless, high due to the presence of agriculture (Hor94). In the Netherlands, too, TPT concentrations are frequently high (Stä95).

Effects of TBT on biotic communities

Little is known about what effects TBT has on biotic communities. On the Isle of Man, researchers removed all of the dogwhelks in order to simulate the consequences of dogwhelk imposex for the biotic communities (Spe90). The result was an increase in the numbers of certain of the dogwhelk's prey, e.g. certain species of shellfish — including the mussel and the acorn barnacle. This increase led to reduced numbers of macrophytes, on which these prey animals feed.

It is presumed — but not yet proven — that effects on the whelk have repercussions on the biotic community which the whelk inhabits. The whelk is a fairly active hunter which lives off bivalve shellfish, worms, small crustaceans and eggs. Whelks also feed on dead material. They, in turn, are eaten by lobsters, crabs and larger fish such as rays and cod. The shell of the whelk fulfils a special role in the ecosystem. It is used by coelenterates, sponges and sea anenomes as an attachment site. The hermit crab (*Pagarus bernardus*) protects itself against predators by using the shell as its shelter. In the southern part of the North Sea the whelk is the only provider of shells that are large enough to accommodate fully-grown hermit crabs (Hal93). Effects on hermit crabs can therefore also be expected in addition to a decline in the organisms that are grazed or hunted by the whelk's prey animals.

3.5 Conclusions

In Table 1 the Committee has summarised the evidence for the causal link between sex-hormone disruptors and effects that have been observed in the Dutch environment. In doing so, it has applied the criteria that were established by Vethaak *et al.* (Vet97). These criteria are a modification of the widely used general criteria formulated by Fox and Hill (Fox91, Hil65).

Table 1 Evidence of the causal relationship between identified effects and xenobiotic substances, with reference to causality criteria.

	Seal	Otter	Cormorant/ common tern	Flounder	Whelk/ dogwhelk
effects identified in individual animals	+	+	+	+	+
adverse effects identified in populations	+	+/-	?	-	+
degree of coherency between effects observed in populations and those in individuals	+	+/-	+	-	+
identification of a plausible mechanism of action consistent with effects observed in individuals	+/-	-	+	+	+/-
consistent with effects observed in individuals	+/-	-	+	+	+/-
positive identification of specific contaminants operative through via this mechanism	+/-	-	+	+	+
likelihood that exposure occurs, in terms of individual -level effects occurring via the mechanism of actionunder consideration	+	+	+	+	+
evidence of recovery of populations or individuals upon removal of chemical stressor or identified concentration-dependent effect from the environment	+	?	?	-	+

Top predators

In the opinion of the Committee, it has been adequately demonstrated that negative effects have been caused on the reproduction of fish-eating mammals and birds in the Dutch environment by DDE, PCBs and dioxins. The Committee refers the reader to section 4.2 for evidence from laboratory research. The effects observed in the field have in the past certainly led to reductions in (local) populations of seals, and probably otters and cormorants. Especially in sedimentation areas of the Rhine, Meuse and Scheldt rivers, the contamination by the substances mentioned is still so high that adverse effects can still be expected on the reproduction and development of resident fish-eating top predators. The Committee notes that research into the possible effects of the above substances (especially PCBs and dioxins) in babies has provided sufficient indications that these substances can influence the development and behaviour of human beings in the early stage of life (Hui95, Jac93, Sau94). No comparable information is available for Dutch wild fauna. Nevertheless, effects of this kind can occur in wild fauna, as demonstrated in research conducted in North America with herring gulls (among other species).

Aquatic animals

The Committee is still in some doubt as to which substances are responsible for the effects that have been identified in fish and in *in vitro* tests. Field research into the disruption of the sex-hormone balance in aquatic organisms has still only been conducted on a limited scale. In general, the Committee believes that, because of the route of exposure and the high concentrations in the aquatic environment, aquatic organisms run greater risks than species that are located higher in the food chain. In the chapters that follow, the Committee will consider which substances might be involved in this process. In the opinion of the Committee, convincing evidence has indeed been produced of a hormone-disruptive effect (on individuals and populations) in whelks and dogwhelks in Dutch coastal waters and the North Sea, as has been demonstrated for TBT. Field and experimental research has revealed that the effects of TBT on the reproduction of these invertebrates are already occurring at extremely low concentrations (a few nanograms per litre). This illustrates the fact that invertebrates can likewise be extremely sensitive to hormone-disruptive effects elicited by substances. Effects on invertebrates therefore also deserve further consideration.

Chapter 4

Classification of xenobiotic potential hormone disruptors in the Dutch environment

There are in the Netherlands various sources of xenobiotic substances that could be capable of disrupting the sex-hormone balance of animals. Sources of these substances are: STWs, agriculture, cross-border rivers and atmospheric deposition. Frequently, this involves undefined mixtures. In addition, sufficient knowledge is not always available about the sex-hormone disruptive effect. In this chapter the Committee will therefore concentrate not on sources, but on individual pesticides and substances emanating from industrial production processes. There are 77 substances for which effects on hormone balance have been reported or suspected in the literature.

After the substances have been tested against a number of criteria relating to exposure, effect concentrations and mechanism of action, they are assigned to three classes based on the ‘weight of evidence’. This is greatest in the case of substances from class 1.

The substances assigned to class 1 are considered separately. In the concluding section, the Committee draws attention to facts and considerations relating to the inevitable uncertainties in the data and the assumptions on which its substance classification is based. It also attempts to link its findings to the consequences which these substances have for ecosystems in the Netherlands.

4.1 Criteria and data

The most important selection criterion is the so-called PEC/N(O)EC ratio. Besides this ratio, the selection process also involves using information about the mechanism of action from *in vitro* tests.

4.1.1 The PEC/N(O)EC ratio

The ratio between the exposure (*predicted environmental concentration, PEC*) and the maximum concentration at which no effect occurs (*no (observed) effect concentration**) is a widely used means of expressing the risk that is posed by a given substance. This advisory report uses only measured exposure concentrations. Where the PEC/N(O)EC ratio is greater than 1, effects can be expected to occur. As a result of interactions between hormone disruptors, however, the concentration at which effects occur may be substantially lower than the PEC that has been determined for each individual substance (synergism, additivity). Account also needs to be taken of longer exposure times in the field, exposure spanning all stages of life and differences in sensitivity between animal species. For this reason the Committee has employed an arbitrarily chosen safety factor of 100. This means that it considers a risk to be present if the PEC/N(O)EC ratio of a given substance is greater than 0.01.

The procedure for determining the PEC/N(O)EC ratio is as follows:

- The direct exposure (PEC) — e.g. the concentration of a substance in water — is divided by the N(O)EC for an aquatic organism
- For top predators, if the substance is able to accumulate in organisms, the PEC is defined as the measured or estimated concentration of a substance in food (e.g. fish). The concentration of a substance in fish can therefore be estimated from the concentration in the sediment or suspended solids. The application of a partition coefficient to a concentration in water also gives an estimate (albeit less reliable) of the concentration in fish. The PEC is then divided by the N(O)EC (determined in food) for a mammal. Even a concentration in the top predator itself, or in its eggs, can be used as an estimate of the PEC. Such a concentration is then divided by the *internal* N(O)EC (the concentration *in* the organism at which no effect is observed).

Organisms may be exposed to a substance in an environmental compartment both *directly* and *indirectly* via food. The conversion of a substance by an organism produces metabolites in that organism. When the organism is eaten by an animal, there is thus

* An NOEC value is determined experimentally; an NEC value is calculated from a dose-effect curve.

exposure both to the substance and to its metabolites. Usually, information is only available about the concentration of the substance in an organism, whereas there is no such data for the metabolites.

Exposure of gill-breathing animals primarily results directly from the presence of the substance in water and (usually to a lesser extent) indirectly from the presence of the substance and its metabolites in their food. Top predators such as birds and mammals are exposed principally to substances and their metabolites which are present in the food. The Committee assumes that *indirect* exposure is only of importance for top predators, and then only for substances that accumulate in (food) organisms. Besides directly obtained results of measurements relating to the potential of a substance to accumulate in organisms, the hydrophobicity of a substance can also serve as a measure of its potential to accumulate. The Committee assumes that a substance can accumulate in organisms if the $\log K_{ow}$ value (K_{ow} = partition coefficient n-octanol/water*) is greater than 3. It should be noted that this measure says nothing about the conversion that the substance may undergo in an organism.

4.1.2 Selection of N(O)EC values

The basic principle applied in selecting N(O)EC values is that the effect must be measured *in vivo* and must indicate a disruption of the sex-hormone balance (see Chapter 2). It is not always possible to determine unequivocally whether an observed effect is the result of hormone disruption or of cytotoxicity (cell toxicity). Damage to the testes can, for example, result from both mechanisms of action. Other information about the mechanism of action (e.g. from *in vitro* binding assays for a hormone receptor) or information about the disruption of the testosterone metabolism can provide a greater insight into the most likely mechanism of action. Sometimes, the Committee has based its choice of the N(O)EC value in part on such *in vitro* data.

A further selection criterion is that hormone disruption must be the primary effect of a given substance. In the case of death due to high-concentration exposure to a substance, there has often been a cascade of (secondary) effects on a large number of systems in an organism. Disruption of the hormone balance in relation to such concentrations is then probably a secondary effect. The level of exposure indicates the likelihood of this being a primary or secondary effect. Theoretically, binding to the receptor is already significant in connection with extremely low concentrations. Disturbance of the metabolism frequently occurs at a somewhat higher concentration, because the substance must then act against the homeostatic mechanism. Toxicity for cells, and subsequently for the whole organism, generally occurs at even higher levels.

* The K_{ow} value describes the partition of a substance between octanol and water. This partition serves as a model for the partition of a substance between (the fat of) an organism and the surrounding water.

Identified N(O)EC values are not accepted, therefore, if other studies reveal that other effects also occur at that concentration which are not directly related to hormones.

It was stated in an earlier chapter that we only speak of a ‘disruptor’ if the physiological conditions have, indeed, been disrupted. In several studies, biochemical effects (such as enzyme induction) have been measured which do not necessarily need to involve a physiological disturbance. In the case of dioxins, it is known that there is a factor-10 difference between the lowest concentrations at which (*in vivo*) biochemical effects occur and the lowest concentrations that have adverse physiological effects. If no N(O)ECs have been found other than those based on a biochemical endpoint, then the Committee will use this factor ten as an estimate in order to extrapolate an N(O)EC value from a biochemical effect within a single species.

Selection criteria for data from *in vitro* tests

In vitro tests can provide supplementary information about the possible mechanism of action and the disruptive potential of a substance. The Committee emphatically believes that the results of an *in vivo* test are determinative for a classification. *In vitro* tests are necessary for confirmatory purposes in order to definitively designate a substance as a hormone disruptor. The data from *in vitro* binding tests for oestrogens, androgens and thyroid hormones are included in the classification.

In this respect, the Committee has employed the following two selection criteria. Because of the substance-specific nature of affinity for binding to a receptor, the affinity of a substance is expressed in relation to 17ß-oestradiol. Substances whose affinity for the receptor is greater than one millionth of that of 17ß-oestradiol are classified as positive. The second criterion is that the concentration of the substance in the *in vitro* test must not have been higher than 10 micromoles. Above such concentrations, in fact, there may be a question of toxicity for the cells.

4.1.3 *Classification number*

The Committee has assigned the 77 organic substances that are mentioned in the scientific literature in connection with hormone disruption to specific classes, as follows:

Class 1. Hormone disruptors in the Netherlands

The Committee assigns a substance to class 1 if the PEC/N(O)EC ratio is greater than 0.01. Furthermore, the substance must score positively in an *in vitro* test. At the concentrations that are found in the Netherlands, this substance can, in all probability, be designated as a hormone disruptor.

Class 2. Potential hormone disruptors in the Netherlands

If it is not possible to determine the PEC/N(O)EC ratio, then the Committee relies on data concerning use (in addition to emission and production) and physico-chemical properties (hydrophobicity, potential to accumulate and persistence in the environment). If the substance is hydrophobic, if its use in the Netherlands exceeds 10 tonnes per year and, in addition, if the substance scores positively in *in vitro* tests, then it is designated as a potential hormone disruptor in the Netherlands.

Class 3. Substances suspected of hormone disruption in the Netherlands

There is less evidence to suggest that these substance are hormone disruptors in the Dutch situation. There may be various reasons for this. Either the PEC/N(O)EC ratio is below 0.01, or else it is not possible to determine this ratio, but there are indications from the literature that the substance is a hormone disruptor. There may also be a question of reproductive toxicity without any clear evidence that the substance interferes with the sex-hormone balance. The capacity to disrupt the thyroid-hormone balance at high levels of exposure is a further reason for assignment to this class.

As far as the remaining substances are concerned, either there is no indication (or insufficient indication) that they disrupt the sex-hormone balance, or they have not been detected environmentally in the Netherlands.

Annex C provides an overview of the classification status of all of the substances. It can be summarised as follows:

- Class 1 : 13 substances
- Class 2 :   6 substances
- Class 3 : 15 substances
- Substances not assigned to any of the three classes: 43

The Committee will now discuss the hormone disruptors that have been assigned to class 1.

Alkylphenols

Alkylphenols find their way into the environment either directly, or in the form of degradation products of alkylphenol-ethoxylates.

4-nonylphenol

Nonylphenol is used as a mixture of isomers. In the literature it is often unclear which mixture is being referred to.

At nonylphenol concentrations of 10-30 µg/L, production of vitellogenin has been detected in male fish (Job96, Lec96, Ren96). Reduction of testicular growth and disturbance of the testosterone metabolism have also been observed at these concentrations, as have changes in the sex ratio at concentrations of 100 µg/L (Gra97, Job96). According to the results of *in vitro* tests, the substance binds to the oestrogen receptor. The binding ratio in relation to 17ß-oestradiol varies from $1{:}10^{-3}$ to $9{:}10^{-6}$. In another *in vitro* test (ER-CALUX) this ratio was $3{:}10^{-5}$. Concentrations of 0.14 µg/L to 10 µg/L have been detected in surface water. The PEC/N(O)EC ratio for fish is substantially greater than 0.01. As demonstrated by the effects, the substance is clearly a disruptor of the sex-hormone balance.

Octylphenol

In rainbow trout, effects on vitellogenin production and a decrease in testicular growth have been detected (as with 4-nonylphenol) at concentrations of 5 and 30 µg/L (Ash95, Job96). The *in vitro*-measured binding to the oestrogen receptor is five times higher than the binding of 4-nonylphenol. The concentrations in the environment are probably a factor of 10 lower than for 4-nonylphenol. The PEC/N(O)EC ratio for octylphenol is greater than 0.01.

DDT

p,p’-DDT

In the Netherlands concentrations of 9.2 mg/kg fat and 171 µg/kg fresh weight have been detected in cormorant eggs. These internal concentrations are probably more than a factor of 100 below the concentrations that elicit effects in rats, chickens and quail (95 mg/kg bodyweight in the rat (Bit70) and 136 and 190 mg/kg bodyweight, respectively, in the chicken and quail (Bit68)), but due to the conversion to the strongly hormone-disruptive metabolite p,p'-DDE (see below), the Committee is nevertheless assigning p,p'-DDT to class 1.

o,p'-DDT

Effects have been detected on the sexual differentiation of birds at concentrations in the eggs of 2 mg/kg fresh weight (Fry81). These concentrations are less than a factor of 100 higher than the levels measured in cormorant eggs in the Netherlands (<0.2 — 282 µg/kg). An *in vitro* test revealed binding in relation to 17ß-oestradiol to be $3{:}10^{-5}$. The PEC/N(O)EC ratio is greater than 0.01. This substance is clearly a disruptor of the sex-hormone balance.

p,p'-DDE

The concentrations in bird eggs (20 mg/kg fresh weight) at which effects on sexual differentiation have been detected (Fry81) are equivalent, in order of magnitude, to the levels found in cormorant eggs in the Netherlands (388-4460 µg/kg fresh weight, IVM93). The concentrations at which Fry found feminisation of male embryos (Fry81) also have the same order of magnitude. One would therefore expect p,p'-DDE to elicit effects at the levels currently prevailing in the environment in the Netherlands.

Chlordane

Technical chlordane is a mixture of chlordane and (among other substances) nonachlor and heptachlor. In the rat, enlargement of the uterus (uterotropy) has been observed in connection with an injected dose of 75 mg chlordane per kg bodyweight (Wel71). *In vitro,* chlordane does not exhibit any binding to the oestrogen receptor (Von96). Concentrations of 1270 µg/kg fresh weight (5217 µg/kg fat) have been found in fatty tissue from seals that have been washed ashore. Direct assessment of this value is hindered by the fact that the concentration in fat is measured for the sum of all components of the mixture and the metabolites, while the above-mentioned injected dose related to chlordane alone. Direct comparison reveals the PEC/N(O)EC ratio to be greater than 0.01. The substance is clearly a disruptor of the sex-hormone balance.

Dieldrin

In rats, an injection of 21 mg/kg bodyweight into the abdominal cavity elicited an inhibition of oestrone-induced uterotropy (Wel71). In the light of the levels that have been found in cormorant eggs in the Netherlands (0.66 mg/kg fresh weight), the

PEC/N(O)EC ratio is greater than 0.01. The *in vitro* results are, to some extent, contradictory. Although cell division (proliferation) has been detected in mammary-gland cells (MCF-7 cells) — which is regarded as an oestrogenic effect — a further *in vitro* test did not demonstrate any binding to the oestrogen receptor. However, neither test provides any certainty with regard to the capacity of a substance to bind and activate the oestrogen receptor. The relative potency displayed in an *in vitro* test (ER-CALUX) in comparison with oestradiol was $3{:}10^{-6}$. In view of the PEC/N(O)EC ratio and the effects that have been observed (including a reduction in the thickness of the eggshell), dieldrin is clearly a disruptor of the sex-hormone balance.

Endosulphane

Damage to the testes and ovaries has been identified upon exposure of fish to 0.2-1 µg/L (Kim95). As in the case of dieldrin, the *in vitro* results are, to some extent, contradictory. Although proliferation has been identified in MCF7 cells, there is no strong binding to the oestrogen receptor. A relative binding of $3{:}10^{-6}$ has been detected in comparison with oestradiol. In the Netherlands, as far as we are aware, the concentrations in surface water are between <0.001 and 0.005 µg/L. The PEC/N(O)EC ratio may thus be greater than 0.01 and the Committee therefore considers endosulphane to be a disruptor of the sex-hormone balance.

Lindane (γ-HCH)

Following exposure of pregnant rats, effects on testicular weight, sperm count and testosterone levels in the plasma have been found in male offspring. The concentration in the liver of the male offspring was 0.6 mg/kg (Dal97a). Concentrations in bird eggs and fat from eels are less than a factor of 10 lower. In fish, damage to the ovaries and a decrease in gonadotropin concentration in the plasma have been found in connection with an exposure concentration of 5-10 µg/L (Hir75). These levels are around 100 times higher than those found in water in the Netherlands. The PEC/N(O)EC ratio is greater than 0.01.

ß-HCH

Exposure of rats to 50 mg/kg via food resulted in uterotropy (Loe84). In the Netherlands, ß-HCH concentrations of up to 5.1 mg/kg have been found in cormorant eggs; in fish, levels of up to 8.1 µg/kg fresh weight and 989 µg/kg fat (IVM93) and 69 µg/kg fresh weight have been detected (1996, RIVO-DLO). Based on this data, the PEC/N(O)EC ratio is, in any case, greater than 0.01.

In male fish, production of vitellogenin has been observed following exposure to 32 µg/L ß-HCH (Wes85).

Polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs) and biphenyls (PCBs)

Effects demonstrated in the field in both birds and (marine) mammals have been associated with the occurrence of PCBs (see Chapter 3). In order to demonstrate this causal relationship, the Committee will take a closer look at the mechanism of action and laboratory findings of the effects of PCBs and substances with a corresponding action.

During the past 15-20 years it has been shown via countless laboratory experiments that, even at extremely low concentrations, PCDDs, PCDFs and PCBs exert an adverse effect on the reproduction and development of mammals, birds and fish. In general, these effects manifest themselves even at exposure levels of 10^{-9} gram TEQ per kg bodyweight.

Despite extensive research over the past decade, the mechanism of action via which these compounds exert their negative effect on reproduction and development is still unclear. There is, however, some degree of consensus within the scientific world about the role of the Ah receptor. This protein is presumed to be the initial mediator for the majority of the toxic and biological effects that are engendered by compounds of this type (Pol82, Saf86, Saf90, Saf94).

It has been found both *in vivo* and *in vitro* that dioxins and related compounds (including some PCBs) can have an anti-oestrogenic effect. At present it is assumed that this effect stems from interactions at DNA level between the Ah and oestrogen receptors. The majority of potent anti-oestrogenic PCDDs and PCDFs have a 2,3,7,8 chlorine substitution pattern. They accumulate strongly in the food chain and are most toxic for mammals, fish and birds (Ber94, Bos92, Saf90, Wal91).

Little research has been conducted to date into the direct relationship between the anti-oestrogenic action of dioxin-related compounds and adverse effects on reproduction. This action is most evident from *in vitro* research with liver cells from the rainbow trout, whereby it has been shown that the production of vitellogenin can be inhibited through the presence of chlorinated dioxins and dibenzofurans. The individual capacity of these substances to inhibit production appears to correspond to that for other toxic and biochemical effects (And96). From a mechanistic standpoint this could explain the marked effect of PCDDs, PCDFs and PCBs on the reproduction and development of certain fish species.

Besides the anti-oestrogenic effect of PCDDs, PCDFs and PCBs, an oestrogenic effect has been detected, in particular, for a number of metabolites of PCBs (Con97, Kram97, Moo97, Ram97). This applies especially to certain hydroxylated metabolites, which are able to bind to the oestrogen receptor. Metabolites have been determined in humans and seals in serum concentrations which are of the same order of magnitude as those of the congeners from which they are formed. In general, the oestrogenic response in the test systems for these specific hydroxylated metabolites is between three and five orders of magnitude weaker than that of oestradiol (Con97, Ram97).

The 2,3,7,8-substituted PCDDs and PCDFs and several dioxin-related PCBs must be reckoned as being among the most toxic substances in relation to the reproduction and development of young organisms.

In view of the effects detected in the field, the high levels in the environment and the low effect concentrations, the PEC/N(O)EC ratio is markedly higher than 0.01. Although it is unclear whether interaction with the oestrogen receptor plays a key role in this respect, it is certain that the substances in question are disruptors of the sex-hormone balance.

Toxaphene

Technical toxaphene is a mixture of around a thousand compounds. All of these compounds behave differently. In rats, uterotropy has been detected following administration of a total of 100 mg/kg bodyweight (Wel71). In the white-beaked dolphin, levels of 19 mg/kg bodyweight have been measured (Boe93). The final internal concentration in the rat experiment can be assumed to have been considerably below 100 mg/kg as a result of conversion of toxaphene in the rat. In a direct comparison of the level in the dolphin and the concentration of toxaphene in the rat, the PEC/N(O)EC ratio is substantially higher than 0.01. It is questionable, however, whether that ratio can be accurately determined via this route, since in the research with rats, toxaphene has been administered as a technical mixture, whereas the levels of toxaphene in the dolphin involve a mixture with a much less complex composition. The Committee is provisionally classifying this substance as a hormone disruptor.

Organotin compounds

There is an extremely strong association between the frequency and intensity of shipping traffic and the incidence of imposex in the North Sea (see Chapter 3). Results of laboratory research indicate that this is a dose-dependent effect of TBT. Imposex has been found at TBT concentrations of 1 nanogram tin per litre (Gib87). The concentrations of TBT in areas where imposex occurs are sufficiently high to explain this phenomenon. This substance therefore also belongs in class 1.

4.2 Discussion

The Committee is anxious, in this section, to qualify its classification of substances. Not only was it sometimes confronted with a marked shortage of research data, but also the available results are open to different interpretations.

The list of substances considered is not exhaustive, since by no means all industrially produced substances have been tested for their hormone-disruptive effect. Where this has occurred, it has related principally to the oestrogen balance; barely any research has been conducted into disruption of the androgen balance. For many substances, insufficient data is available about the concentrations in the environment. Some data are not particularly useful, since they have been measured in a less relevant environmental compartment, e.g. concentrations of hydrophobic substances that have been measured in water. The number of animal species that have been tested is extremely limited. The majority of data relate to the rat. The virtually complete absence of data about invertebrates is a significant shortcoming, given that 95% of the animal species in an ecosystem fall into this category and it therefore forms an important part of the food chain.

Despite the fact that the hormonal systems of vertebrates exhibit major similarities, there are notable differences in detail between species, which means that effects determined in one species cannot automatically be extrapolated to other species. Our knowledge of hormonal systems is confined to a few animal species, and even then it is still incomplete.

The consequences of disruption are dependent on the stage of life at which this disruption occurs. Major differences exist between vertebrate species with regard to the critical phase. It is frequently not known whether a certain exposure has occurred during a critical phase. In addition, the results of laboratory experiments often only allow us to make extremely limited predictions as regards what will be the consequences at population level.

In its definition of the term hormone disruptor, the Committee has stated that it must involve an effect on reproduction which results from a disruption of the sex-hormone balance. However, some of the observed effects cannot unequivocally be attributed to a disruption of the hormone balance, but may be a result of some other type of effect. For example, testicular abnormalities can result both from a direct effect of a substance on the organ itself and from a disruption of the sex-hormone balance.

Nevertheless, the Committee believes — based on the current level of knowledge — that its classification adequately reflects which industrially produced substances, through their action on the sex-hormone balance, pose a potential threat to the reproduction of organisms. It also feels that, in making this classification, it has indicated which substances need to be given priority for closer investigation and where there are gaps in our knowledge.

As has been stated, the data has — in the overwhelming majority of cases — been obtained from research with rats. This means that the classification is primarily a (qualitative) reflection of the (qualitative) risks for birds and mammals. These animals are principally exposed to hormone disruptors via their food. It is therefore not surprising that these are primarily substances which accumulate strongly in the food chain.

Besides the PCBs and DDT metabolites that have already been mentioned in Chapter 3, class 1 also includes many other organochlorine compounds that have likewise been widely used in the past. For some, obvious cases of hormone disruption have been identified in the field. The evidence is not so strong, however, as it is for PCBs and DDT-metabolites.

However, the survey also brings to light other persistent substances with a hormone-disruptive effect which have been used much more recently. Examples are the organobromine compounds, which are used as flame retardants. These substances now already occur in substantial concentrations in higher organisms. Relatively little is known about their action and effective concentrations. The fact that they are capable of causing a world-wide problem, by virtue of their persistence, is evident from a recent publication about the occurrence of polybrominated diphenyl ethers in sperm whales (among other species) (Boe98). Sperm whales feed on cephalopods, which occur at depths of up to two kilometres. Evidently these flame retardants — which have not occurred in the environment for as long as the PCBs, for example — have already penetrated deep into the marine food chain.

Data about effects on aquatic organisms are only available for very few substances. Due to their effects on gill-breathing organisms, alkylphenols and organotin compounds are assigned to class 1. The effects of tributyltin on saltwater snails in the Netherlands have been described in Chapter 3. It is unclear which substances are responsible for the other effects that were mentioned in Chapter 3. In view of the fact that the sex-hormone balance of mammals closely resembles that of aquatic vertebrates, the existence of a hormone-disruptive effect in mammals (the rat) does, indeed, lead one to assume that the substance(s) in question can also engender hormone disruption in aquatic organisms.

Chapter 5

Natural and synthetic hormones in the environment

In this chapter the Committee will consider the consequences for ecosystems of emissions of natural and synthetic hormones (in the oral contraceptive pill). Attention is focused principally on hormones with an oestrogenic effect.

Oestradiol is the most potent oestrogenic hormone in all vertebrates. The binding specificity of oestrogen receptors for oestradiol is virtually identical in highly diverse species — for example, the trout and the human being. In other words: in vertebrates, the system of hormonal and accompanying receptors has undergone little change in the course of evolution. Because of the extremely high binding specificity of natural (and also synthetic) hormones, exposure to low concentrations results in effects on reproduction in all species. Synthetic hormones, such as those in the oral contraceptive pill, are specifically intended to prevent reproduction at low concentrations.

By their very nature, oestrogens will be present in ecosystems at extremely low concentrations as a result of the excretion of these hormones by vertebrates. Such concentrations are not expected to have any effects on animals. Due to the large numbers of human beings and livestock in the Netherlands and the surrounding countries, the extent of the emission of natural hormones can no longer be regarded as natural. So the question is: what implications does this have for ecosystems? The same applies to phyto-oestrogens — substances which are produced by plants and which exert an oestrogenic effect in animals. These substances mainly find their way into the environment via the use of animal feeds.

Because of the manner in which natural and synthetic hormones find their way into the environment — i.e. via sewage treatment works (STWs) or via leaching in areas of arable and livestock farming — aquatic ecosystems, in particular, are being polluted with these substances.

5.1 Emission of natural hormones of human and animal origin

The natural sex hormones that are produced endogenously by humans and other mammals are, in part, excreted in urine and in faeces. This applies also to the synthetic hormones in the oral contraceptive pill. In order to survey the extent of the emissions into the environment, the Committee has — in view of the numbers involved — confined itself to humans, cattle, pigs and chickens. It further limits its attention to pregnant women and animals and egg-laying chickens, since it is they who excrete the largest quantities of sex hormones. In the majority of mammals, excretion occurs primarily via the urine. In ruminants such as cattle and sheep, on the other hand, excretion takes place principally via the faeces (Vel76). In each case, the Committee will confine itself to the most important excretion route.

5.1.1 Cattle

The gestation period of cows normally varies between 277 and 286 days. The production and excretion of oestrogens (primarily via the faeces) begins to increase markedly after approximately 110 days. The compounds in question are 17α-oestradiol, 17ß-oestradiol and oestrone in unconjugated form (56%, 32% and 11%, respectively) (Hof97). According to Hoffman, the total oestrogen concentration in the faeces at the end of pregnancy is, on average, 0.5mg/kg faeces (Hof97). After only 28 weeks, Desaulniers already found average (total oestrogen) concentrations of around 1 mg/kg faeces (Des89). Between day 115 and parturition, the average excretion of oestrogens is 83 µg/kg faeces, according to Hoffman (Hof97). This estimate appears to be on the low side compared with other data. For example, Bamberg and Desaulniers (among others) found average concentrations of 187 µg (17α-oestradiol) and 947 µg (total oestrogens)/kg faeces between 20 and 28 weeks (no measurements were taken after 28 weeks) (Bam84, Des89). The Committee bases its calculation of emissions in the Netherlands on the data produced by Hoffmann*, whilst noting that they are probably an underestimate.

* The concentration of 83 µg/kg faeces applies to the period from 115 days to parturition. Spread out over the entire pregnancy, this gives 51.5 µg/kg faeces.

Table 2 Overview of the production of oestrogens in the Netherlands by female cattle over 1 year of age and dairy cows.

population	number of animals ('000s)	faeces production (kg/animal/day)	conc. of oestrogens in faeces μg/kg faeces)	total oestrogen excretion via faeces (in grams/day)
young cattle, female 1 year and over	508	12.5	51.5	202
dairy cows & cows in calf	1 600	25	51.5	1 277

Table 2 is an overview of the production of oestrogens in the Netherlands by cows over 1 year of age and dairy cows, these being the two groups of cattle which are pregnant for a significant amount of time*.

5.1.2 *Pigs*

The normal gestation period for pigs is between 110 and 120 days. Excretion of oestrogens occurs principally via the urine, and takes the form of oestrone sulphate. Oestrogen production and excretion rise sharply during the first 27 days of pregnancy to values of 1.6 mg oestrone sulphate/litre urine (Atk87). There is then a sharp fall to values slightly above the level prior to pregnancy. Between around day 50 and parturition, the production and excretion rise considerably once again, reaching concentrations of 5 mg per litre urine (Rae63).

It is difficult to estimate the emissions due to the poor correspondence between the quantities given in the Atkins and Reaside studies. Thus Atkins only gives the concentrations during the first 30 days. During the same period of pregnancy, Reaside found concentrations of 0.2 mg/L, expressed in terms of total oestrogens. It is possible that the values given by Reaside, which date back to 1963, are structurally too low on account of the analytical techniques that were used at that time. It would appear both from the Reaside study and from that of Edgerton (Edg71) that the concentration of oestrogens in the urine at the end of the pregnancy is higher than the maximum from the first 30 days by a factor of between 10 and 20. Application of these factors to the Atkins data would give concentrations considerably in excess of 5 mg/L at the end of the pregnancy. Based on an estimate from the curves presented by Reaside and Atkins, the Committee has arrived at an average concentration of oestrogens in the urine throughout

* On average, cows calve for the first time at 2.2 years of age and are slaughtered at 4.6 years. Given a gestation period of 270 days, this means that they are pregnant for 62% of their lifespan. This percentage has been used to calculate the total oestrogen production.

Table 3 Emission of oestrogens by sows in the Netherlands.

population	no. of animals (x 1000)	urine production (litres/animal/day)	conc. of oestrogens in urine (in mg/L)	total oestrogen emission per day (in kg)
sows	1.61	5	0.5-1	4-8

the pregnancy of 0.5 to 1 mg/L. From this it has produced an estimate of the total emissions of oestrogens by sows in the Netherlands (Table 3).

5.1.3 Chickens

Besides oestrogens, chickens also excrete fairly large quantities of testosterone. Table 4 gives the concentrations of both hormones in litter from chicks, layers and cocks, as published by Shore (Sho93).

Table 4 Concentrations of testosterone and oestrogen hormones in poultry litter from chicks, layers and cocks (Sho93).

	testosterone (µg/kg litter)	oestrogens (µg/kg litter)
chicks (f)	133	65
chicks (m)	133	14
layers	254	533
cocks	670	93

Oestrogens are primarily excreted in the form of 17ß-oestradiol, oestrone and 17α-oestradiol (Bis91).

The Committee has made an estimate of the total quantities of hormones that are excreted in poultry litter for layers only (see Table 5), because these birds both account for the greater part of the poultry population in the Netherlands and, furthermore, by far the largest quantities are excreted per bird — as shown in Table 4.

Table 5 Estimate of the emission of oestrogens and testosterone via poultry litter.

population	no. of birds (1000s)	litter production (g/bird)	concentration of oestrogens in litter (nanograms/ gram litter)	total excretion of oestrogens (grams/day)	concentration testosterone in litter (µg/kg litter)	total excretion testosterone (grams/day)
layers	41 000	50	533	1 090	254	520

5.1.4 *Humans*

Endogenously produced oestrogens

During the final period of pregnancy, women excrete approximately 30 mg oestrogens per day via the urine (Adl76, Fot87). This consists mainly of conjugated 17ß-oestradiol, oestriol and oestrone. Based on an estimated average concentration of 10 mg oestrogens per day throughout the entire pregnancy and approximately 180,000 pregnant women (in 1998), the total excretion is approximately 2 kilograms per day. The number of pregnant women in 1998 has been equated with the number of births in that year.

The oral contraceptive pill

The active substance in the oral contraceptive pill (mainly ethinyl oestradiol) is excreted via urine and faeces. A study by Reed revealed that following administration of labelled ethinyl oestradiol, 22 to 50% found its way into the urine and 30% into the faeces (Ree72). What was found in the faeces was principally the unconjugated form. In the urine, 16% of the total dose was found in unconjugated form.

There are estimated to be 1.4 million oral contraceptive pill users in the Netherlands (Statistics Netherlands). The Committee estimates the average daily dose of ethinyl oestradiol via 'the pill' to be 35 µg and the total excretion of ethinyl oestradiol in the Netherlands at 50 grams per day. This is a maximum value, since it is based on total excretion of (unmetabolised) ethinyl oestradiol.

5.1.5 *Conclusion*

According to the above data, the total emissions of endogenous oestrogens by human beings and animals in the Netherlands can be estimated at approximately 10 kilograms per day. This figure is possibly an underestimate, since the contribution made by cattle could be substantially higher than the estimated 1,400 grams. Moreover, the Committee has included neither other livestock — such as rabbits, ducks, sheep, goats, horses, etc — nor companion animals, since these can be regarded as lesser sources. Emissions of ethinyl oestradiol ('the pill') are estimated at 50 grams per day. This is probably an overestimate, in view of the fact that excretion has been equated with ingestion. In any case, this contribution pales into insignificance compared with the estimated total emissions of natural oestrogens (10 kg or more per day). It should be noted that emissions from countries surrounding the Netherlands have not been taken into consideration.

5.2 Fate of natural and synthetic hormone emissions

Ultimately, it is the degradation of natural hormones and the mobility of these substances which determine their concentration in the surface water. Degradation occurs principally via bacteria. Mobility is dependent, among other things, on the properties of the substances in question and on the binding characteristics of the substrates in the different environmental compartments.

5.2.1 Manner of emission

The fate of the hormones that are emitted into the environment is also dependent on the manner in which emission occurs. Thus, for example, hormones that are excreted by humans mainly find their way into the water via sewage treatment works (STWs). However, the sewage treatment system in the Netherlands is arranged in such a way that, in the event of heavy rainfall, the content of the sewers is discharged directly into the surface water. Consequently, degradation by bacteria in the STWs does not take place.

In the case of livestock, too, the route via which the hormones find their way into the environment determines the fate of the substance in question. Pregnant cows spend a large part of the year at pasture. The natural hormones then find their way directly on to the land via the faeces, and subsequently, by a process of leaching, into the surface water. In contrast to pregnant cows, pregnant sows are virtually always in a stall. The emission into the environment will therefore also depend on the degradation by bacteria during manure storage. The manure from the sows is regularly spread on to the land between spring and October. Because this is not permitted between September and the beginning of spring, the manure will remain in storage during that period. Poultry litter is not suitable for use on grassland and is therefore mainly used in arable farming. Poultry litter is partly used in horticulture in the Netherlands and abroad (in some cases in a dried and pelletted form). However, the exportation of pelletted poultry litter is relatively limited due to environmental regulations abroad.

The manner in which the manure is applied to the land can also determine the fate of the hormones. Livestock manure is currently for the most part injected or ploughed into the ground. The natural hormones are then protected from the sunlight, and end up several centimetres below the ground, so that photodegradation has no role to play.

5.2.2 Degradation

The degradation rate of hormones has been measured in various matrices and environmental compartments. In bacterial cultures, for example, Tabak detected a percentage degradation of 70 — 94% for oestradiol, oestriol and oestrone after one week (Tab70). Broadly comparable degradation rates have been found in STWs. Stumpf identified a decrease of 75% for oestradiol and 89% for ethinyl oestradiol after five days (Stu96). In a modern STW in Israel, lower degradation rates of between 20 and 88% have been found for oestrogens after five days (Sho93). In view of the fact that degradation mainly occurs via the bacterial route, it seems likely that the degradation rate in surface water is lower than in bacterial cultures or STWs. According to as yet unpublished results, under aerobic conditions oestradiol in river water is converted into oestrone within the space of a few days (Jur99). The further degradation of oestrone would likewise take a few days. James also detected a conversion of oestradiol to oestrone in river water (Jam98). In that study it took 20 days for oestradiol and oestrone and 17-ethinyl oestradiol to be substantially degraded. Under anaerobic conditions in sediment, the conversion is considerably slower (Jur99). Moreover, it appeared that the conversion of oestradiol to oestrone was reversible. Under both sets of conditions, ethinyl oestradiol was considerably more persistent than oestradiol (Jur99).

In poultry litter, degradation of oestrogens and testosterone also appears to be virtually or completely non-existent after several months. Even industrial processing of poultry litter, or heating to 100 °C for 24 hours, did not appear to influence the hormone concentration to any significant extent (Sho93). Nicols investigated the runoff into ditches of oestrogens from poultry litter that has been applied to pasture (Nic97). This leaching process was still taking place after the pasture had been irrigated for a week.

Although the literature is limited, degradation of hormones in the environment appears to take several days under the most favourable circumstances. Under less favourable circumstances, however — e.g. deficiency of oxygen and micro-organisms — the degradation will be considerably slower. In a substrate such as poultry litter, there is — as was stated above — only minimal degradation even after several months. It is not known to what extent this also applies in the case of pig and cattle manure.

5.2.3 *Mobility*

For the distribution coefficients of oestradiol between water and various sediments, Jurgens detected values of 20-67 L/kg (Jur99). For (labelled) oestradiol and oestrone, respectively, Shore detected 56% and 59% binding to the soil (removable only with organic solvents). Testosterone, however, proved extremely easy to leach out (Sho93). According to the above-mentioned Nicols study, oestradiol is mobile in the soil — judging by the concentrations that have been found in ditches (Nic97).

It appears from the above findings that oestrogens do not bind strongly to soil or sediments. The Nicols study may possibly relate in part to oestradiol in conjugated form.

The conjugated form is considerably more soluble in water than the ‘free’ form, and it might therefore be expected to be extremely mobile.

5.3 Environmental concentrations in the Netherlands

As far as the Committee is aware, concentrations of hormones in the Dutch environment have to date only been measured in one study (Bel99). This relates to concentrations in large rivers, estuaries and in the influent and effluent from STWs and industrial purification plants. Table 6 provides an overview of the results obtained for urban areas.

In both influent and effluent from STWs and industrial purification plants the researchers only found hormones in unconjugated form. Deconjugation of substances by bacteria in purification plants is a known phenomenon and even serves as a measure of the functioning of the purification plant. This deconjugation also evidently takes place before the hormones find their way into the STW. The data presented above provide a picture of the hormone concentrations in large rivers and estuaries near to urban areas. The concentrations are in the order of nanograms per litre. As section 5.1 shows, the emission of hormones by human beings represents only a fraction of the amounts excreted by livestock.

Based on current knowledge of the fate of these hormones, it is not possible, in the Committee’s opinion, to estimate concentrations in the surface water from emission data. The Committee would like to point out, however, that natural hormones emitted by livestock do not, in general, find their way into STWs. Moreover, there are indications, which were discussed earlier, that hormones in the environment are not degraded very rapidly and may be mobile in the soil. It is therefore likely that concentrations in the

Table 6 Measured concentration ranges of hormones in various matrices in ng/L (Source: Bel99).

environmental matrices	17ß-oestradiol	17α-oestradiol	17α-ethinyl oestradiol	oestrone
surface water	<d.l. -5.5	<d.l.-1.1	<d.l.-4.3	<d.l.-5.3
urban waste water:				
influent/sewage	10-48	<d.l.-9	<d.l.10	10-140
sewage sludge	<d.l.-98	<d.l.	<d.l.	<d.l.
effluent	<d.l.-12	<d.l.-5	<d.l.-8	<d.l.-47
industrial waste water:				
influent/sewage	1-25	<d.l.-8	<d.l.-8	3-92
sewage sludge	<d.l.	<d.l.	<d.l.	<d.l.

surface water in areas of intensive livestock production may be considerably higher than in the vicinity of urban areas.

5.4 Effects of natural and synthetic hormones on aquatic organisms

It has been shown that a 17-ethinyl oestradiol concentration of 0.1 nanograms per litre in male rainbow trout leads to production of vitellogenin (Pur94).

Following exposure to 17-oestradiol for three weeks, the limit value for induction of vitellogenin in male rainbow trout is between 1 and 10 nanograms per litre (Rou98). In fathead minnows (*Pimephales promelas*) concentrations above 100 ng/L lead to a decrease in testicular growth (Pan98). Exposure to oestrone only results in vitellogenin induction at concentrations in excess of 25 ng/L (Tyl98). It is known that oestrone can potentiate the action of oestradiol in fish (Boh82). This raises the question of whether this also occurs in connection with combined exposure to oestrone and xenobiotic substances which also possess an oestrogenic action. If this is the case then the risks of these substances for fish could be considerably greater.

The conjugated forms of oestradiol also exhibit an oestrogenic activity in fish. Oestradiol glucuronide has 10% of the activity of oestradiol, and oestrone sulphate, 100% (Pel93).

5.5 Phyto-oestrogens

Many plants appear to naturally contain phyto-oestrogens — substances which are capable of disrupting the hormone balance in humans and animals. In particular, those plant species which, with the aid of bacteria, are able to absorb nitrogen from the air — such as clovers and lucerne — contain high concentrations of these substances. They produce these substances in order to attract the requisite bacteria and to stimulate the germination of certain mycorrhiza fungi (Bak95, Fri98). The extent to which they also produce these substances as a form of defence against herbivores is unclear. In at least one case, plants appear to use an isoflavonoid as a means of birth control in a particular herbivore. The plant in question is eaten by quails and, in years of abundant rainfall (and therefore excessive growth), barely produces any isoflavonoids. In years with little rainfall, however, an oestrogenic effect is elicited in the quails, as a result of which the clutch size is reduced by almost half (Bak95).

The phyto-oestrogens can be subdivided into four groups of substances: the isoflavonoids (genistein, daidzein | glycetein], daidzin, formononetin, biochanin A, glycetein), the coumestrans (coumestrol), the resorcyclic acid lactones and the lignans (enterolactone, enterodiol). In order to investigate whether, broadly speaking, phyto-oestrogens — as a component of animal feeds that is used on a large scale in intensive livestock production — cause problems in ecosystems, the Committee will now consider the emissions, degradation, mobility and effects of these substances.

5.5.1 *Sources and amounts*

In Australia, researchers have identified effects of subterranean clover (*Trifolium subterraneum*) on reproduction in sheep. Plants occurring in the Netherlands which can contain high concentrations of phyto-oestrogens are red clover and lucerne. Since the amount of clover and lucerne that is grown in the Netherlands is fairly small, the Committee considers this source to be of less importance to ecosystems than another source — namely the consumption of soya by livestock. However, the Committee does not discount the possibility that clovers may be a significant source in some locations. In 1997 1.7 million tonnes of soya beans, extracted soyameal and soyameal expellers were processed into animal feeds in the Netherlands (PD98). The most important phyto-oestrogens in soya are daidzin, daidzein, genistein, formononetin and coumestrol. The first four substances occur in soyameal, for example, in concentrations ranging from several hundred mg to 1 g/kg meal. Coumestrol occurs in fresh seedlings in a concentration tens of mg/kg, and in dried beans at just a few mg/kg (Ald97, Pri85). The most important phyto-oestrogens in red clover are biochanin A and formononetin, while in lucerne the most important is coumestrol.

The oestrogen concentration in soya depends largely on the part of the plant and the way in which the soya is processed. The seedlings and the seed of the soya plant contain the highest concentrations. The oil which is pressed from the seeds contains virtually no phyto-oestrogens. In the protein-rich fraction that is used for animal feeds, the concentrations are high.

5.5.2 *Metabolism of isoflavonoids in livestock*

In ruminants, genistein is largely converted and then excreted in the form of p-ethylphenol, which does not — as far as we know — possess any oestrogenic activity. Only a few per cent of the genistein is found in the urine.

In monogastric mammals (animals with one stomach) and in ruminants, formononetin, daidzein and daidzin are, in part, converted into equol. Other conversions are probably possible, but quantitatively insignificant. Both the starting materials and the conversion product equol exhibit oestrogenic activity. The excretion of equol in urine varies in different animal species between 20 and 70% (Lun95, Shu70) of the quantities that were administered. The rest is made up of the starting materials.

5.5.3 *Emission and fate*

With annual consumption of soya in animal feed standing at approximately two million tonnes, the daily emission of phyto-oestrogens is around 5,000 kilograms (see 5.5.1). This does not include the consumption of soya production by humans, which can also be considerable. Virtually nothing is known about the degradation of these substances in the environment. The fact that the metabolite equol is not further metabolised in mammals could indicate that this substance is relatively persistent. One week after formononetin had been added to a non-sterile soil, 60% was found still to be present (Oza97). It has not been established which conversions occurred. Given the chemical structure of equol, one would expect this substance to be mobile in the soil. It is possible that binding to humic acids limits mobility. However, the data about the fate of the substances in question is too limited to derive concentrations in surface water from the estimated emission levels.

5.5.4 *Effects*

In vitro, the phyto-oestrogens genistein, formononetin and daidzein and the metabolite equol exhibit an affinity with the oestrogen receptor which is smaller than that of 17ß-oestradiol by a factor of between 1,000 and 100,000. A binding affinity does not, in itself, say a great deal about effects that occur *in vivo*. It is known that the action of the above phyto-oestrogens in mammals is based principally on a disturbance of the hormone metabolism (Ald97, Bak95). Considerably less is known about the consequences for aquatic organisms of exposure to phyto-oestrogens. Injection of genistein, equol or coumestrol in the Siberian sturgeon *(acipenser baeri)* led to the production of vitellogenin (Pel91). This was not the case upon injection of formononetin. In the polyp *Hydra vulgaris*, exposure to 3 micrograms of genistein per litre led to developmental abnormalities (Per94). Whether this is caused by hormone disruption is unclear.

5.6 Conclusions

Concentrations of natural and synthetic hormones measured in the Rhine, Meuse and Scheldt rivers are of an order of magnitude at which effects (albeit subtle) can be expected in fish. It is therefore likely that the said substances are partly responsible for the effects that have been observed in fish in the large rivers and in the coastal areas and *in vitro* tests. It is also likely that the concentrations in ditches in areas of intensive livestock production are considerably higher than the concentrations that have been measured in the surface water, especially in the vicinity of urban areas. The estimated emissions of oestrogens by livestock are many times greater than the amounts excreted by humans. There is great uncertainty, however, about the fate of the oestrogens that are introduced into the environment by livestock. Because the (albeit limited) data in the literature indicates that natural oestrogens may be relatively persistent (several days or considerably longer) and — moreover — mobile, the Committee considers it likely that in ditches concentrations of natural hormones could occur which are sufficiently high to elicit effects on aquatic organisms.

Although emissions of phyto-oestrogens are considerable, too little is known about their fate in the environment to be able to estimate concentrations in surface water. Moreover, there is insufficient data for a risk assessment of the effects of these substances. Because aquatic plants do not — as far as the Committee is aware — produce phyto-oestrogens, it is feasible that aquatic organisms are relatively sensitive to these substances.

Chapter 6

Answers to the questions posed by the Minister

Four (related) questions have been submitted to the Committee for a response. Two of these questions concern the current state of scientific knowledge, while the other two relate more to the way in which our knowledge might be extended. In order to maintain the clarity of arrangement and cohesion of this advisory report, the Committee has, in the preceding chapters, focused its attention exclusively on current scientific knowledge. This means that the answers that need to be formulated in this chapter to the first two questions may be considerably more succinct than those to the two remaining questions.

Question 1

Is the Health Council of the Netherlands of the opinion that pollution of the environment with substances that impact on the endocrine system poses a serious threat to populations and, consequently, possibly also to ecosystems?

Answer

In the Committee’s opinion, there are sufficient grounds for concern about the presence — especially in the aquatic environment — of substances which might disrupt the sex-hormone balance of organisms, and therefore endanger the survival of species in ecosystems. In some species, effects on individuals and populations have actually been demonstrated, or else they are likely. The consequences for entire ecosystems are unknown. Because only very limited applied research has been conducted into the consequences of the hormone disruptors that are present in the environment, it is quite possible that hormone disruption is widespread in vertebrates and, in particular, invertebrates.

Prominent among the adverse effects possibly associated with the presence of such substances in the environment is intersexuality (observed in certain fish and snail species in the North Sea). From a methodological point of view, however, it is extremely difficult to correlate an observed decline in a population with a specific substance (or substances), because other factors might also be involved. For certain species of snail it is evident that populations in the coastal areas of the North Sea have, to a greater or lesser extent, been affected by a single substance (tributyltin). It is likely that other species in the food chain are experiencing the consequences of this.

Viewed at national level, the disruption of reproduction in birds and seals that has been observed in decades gone by appears to be largely a thing of the past. Effects can still be identified, however, in heavily polluted locations — such as the sedimentation areas of the Rhine and Meuse rivers. The Committee notes that the reintroduction of species which have disappeared from the Netherlands (in part as a result of the presence of these substances) — such as the otter — will encounter problems in these areas on account of the prevailing concentrations of hormone disruptors.

Question 2

If so, is it yet possible to indicate which substances (or sources, where applicable) principally warrant attention in this respect?

Answer

Of the 77 pesticides and industrial substances mentioned in the scientific literature in this connection, on which the Committee has focused its attention, it designates 13 as being, in all probability, hormone disruptors. For a further 21 substances, it considers that there are indications to this effect, but there is insufficient evidence about the effects or the environmental concentrations. About the remaining 43 substances — some of which, incidentally, have not been detected in the environment in the Netherlands — it is unable to make any assertions. The 34 substances which the Committee has classified as (potential) hormone disruptors are alkylphenols, organochlorine, organobromine and organotin compounds, phthalates and triazines. The Committee recommends that attention should, in the first instance, be focused on these substances, with the exception of a number of the organochlorine compounds, for which a successful policy is already in place. It notes that, due to the rapid advance of knowledge, the above list is merely a ‘snapshot’. The list has already grown considerably due to the fact that a number of substances have been investigated for hormone-disruptive action in recent years. In view of the large number of substances that will be investigated in the years to come, it is likely that the number of substances designated as (potential) hormone disruptors will continue to increase substantially.

Besides the pesticides and industrial substances, the Committee has looked at natural and synthetic hormones. It regards natural oestrogens (e.g. 17ß-oestradiol), oral contraceptives (e.g. 17α-ethinyl oestradiol) and phyto-oestrogens (e.g. genistein) as probable hormone disruptors in the Netherlands.

The Committee designates industrial production, STWs, agriculture, cross-border rivers and atmospheric deposition as the most important sources of industrial hormone disruptors. For the natural hormones, the key sources are livestock production (this principally involves emissions of substances produced endogenously by livestock), agriculture (vegetable oestrogens — especially via the use of soya in animal feeds) and the use of contraceptives. In the case of the phyto-oestrogens, it is still unclear what exactly the position is with regard to persistence and mobility in the environment.

In view of the possible sources, the Committee recommends that the monitoring programmes should be focused primarily on fresh, brackish and salt waters (especially small ditches) and on manure. As far as natural hormones are concerned, top priority should be given to small ditches and manure.

Question 3

Is it possible to investigate, via a programme of measurements (monitoring), whether there are effects on the sex-hormone balance of animals?

Answer

No proven strategy has yet been developed for systematically measuring the effects of substances which disrupt the sex-hormone balance of animals in the field. In the Committee's opinion, the most effective approach would be to expand monitoring programmes that are currently being applied in the Netherlands with a number of 'tools' specifically designed to track these effects. A preliminary initiative for monitoring the effects of oestrogenically active substances in the Dutch aquatic environment is currently in progress under the auspices of the Government Department of Public Works and Water Management. The National Study of Oestrogenic Substances (LOES) commenced at the start of 1999. In addition, there are research programmes targeting the effects of known hormone disruptors on natural mammal populations and oestrogenic effects in fish populations.

Populations and individuals

An initial indication of a disruption in the reproduction of populations is an abnormal age structure and sex ratios. For individuals, the size of the sexual organs is determinative. In individuals, the secondary sexual characteristics, growth and development are also considered by means of anatomical, external and histological examination. Histological examination of the liver and the thyroid gland is advisable. In the case of individuals, various laboratory tests can also be performed. Recent research has shown the benefit of determining the following biochemical parameters relating to reproductive disorders: vitellogenin production, steroid-hormone concentrations, thyroid-hormone concentrations and vitamin-A levels.

Effects on populations are most easily detected if there is a gradient in the concentrations of substances in the environment. Knowledge of natural background levels of different variables is required. Apart from historical research into sex ratios, it can also be useful to conduct a renewed evaluation of archived samples for new toxicological endpoints.

Relocation of individuals

One of the most successful methods of monitoring the effects of hormone disruptors that has been found to date involves relocating animals: animals (snails, oysters, fish) from clean areas (known as ‘transplanted sentinels’) are placed in cages and exposed to potentially contaminated locations. In the Netherlands, unique inbred lines of carp are being produced under controlled conditions, yielding offspring which are either 100% male or 100% female, as desired (Bon98, Bon99). Because the sex is known in advance, these animals are suitable for monitoring sex hormone-disruptive effects (Gim96). The use of transplanted sentinels has a number of advantages compared with research on feral animals that inhabit a given location. They can be positioned close to the source of pollution; the sex can be determined in advance; and the severity and progression of the abnormality can be closely monitored. Consequently, a good picture can be formed of the extent of the problem. This approach also makes it possible to test the effectiveness of the measures that are adopted.

In vitro tests and transgenic fish

The Committee notes that *in vitro* tests do not provide an unequivocal explanation of the expected effects on reproduction. This requires *in vivo* tests. The available *in vitro* tests have primarily been developed to detect oestrogenic — and sometimes also anti-oestrogenic — effects. Each of these tests is tailored to specific aspects of the effect chain which leads to oestrogenic effects. There are, for example, receptor tests with cells which easily show, via a reporter gene, whether the oestrogen receptor is being activated, or (in some systems) inhibited, by a given substance. An example of this is a yeast-cell system (the so-called YES assay (Rou96)), which is equipped both with a human oestrogen receptor and an accompanying reporter gene. A disadvantage of this test is that it provides no information about anti-oestrogenic action and no opportunity for bioactivation of suspected substances. In addition, substances that are large and relatively hydrophobic are unable to pass through the yeast cell wall, and consequently these substances cannot produce a response (Leg99a). A further example is the ER-CALUX-assay, which employs a human cell line incorporating both the natural oestrogen receptor and a certain biotransformation capacity. Luciferase is used as the reporter. Using this test system it is possible to indicate both the anti-oestrogenic and the oestrogenic effect of substances, either with or without biotransformation. Due to the fact that mammalian cells have a different structure, both hydrophobic and hydrophilic substances can pass through the cell wall (Leg99a).

In vitro tests with primary cell cultures from endometrial cells make some allowance for the possible presence of active biotransformation products in the samples that are to be measured. The so-called E-screen (MCF7-assay (Sot92)) is based on the oestrogen-dependent proliferation of breast-cancer cells. However, these tests are not particularly specific because non-hormonally active substances can also stimulate or (conversely) inhibit cell division.

Tests targeting the capacity of a substance to induce yolk protein synthesis (vitellogenin synthesis) mainly utilise primary cell cultures from fish livers. These tests are not easy to perform, but they are highly specific.

Very recently, a transgenic (genetically manipulated) zebra fish was developed in the Netherlands which promises to be quite useful for monitoring effects of oestrogenic substances in the environment. These fish have a built-in oestrogen reporter gene (luciferase). It is possible to rapidly measure an oestrogenic response in physiologically relevant oestrogenic target organs such as the male and female gonads. The response is determined partly by all of the metabolic steps that are present in the animal and may therefore be invaluable in predicting the hormone-disruptive action of substances in the environment (Leg99b).

Chemical monitoring

In order to explain effects observed in the field, it is necessary to analyse relevant area-specific contaminants. The suspect chemicals can then be characterised by means of ‘toxicity, identification and evaluation (TIE)’ procedures. In doing so, it is helpful to separate an environmental sample into a number of fractions of varying polarity with the aid of high-pressure liquid chromatography (HPLC). Only the fractions which subsequently score positively in *in vitro* tests are subjected to closer analysis. This procedure has been successfully applied in England (Des98).

In the opinion of the Committee, the available monitoring instruments are few in number, but sufficient. It is important to conduct measurements in species representing different trophic levels — such as mammals, birds, reptiles, amphibians, fish and invertebrates. The indicator species which the Committee believes are most suitable for the Netherlands are listed in Annex D. Prerequisites for the selection of species are a sound knowledge of the population dynamics, lifestyle, behaviour and physiology (endocrinology) of the species in question, and it is likewise essential to gain a good picture of 'normal functioning' via viable reference populations and background data. Some of this information is still unavailable. In particular, too little is known about the hormone balance in invertebrates.

Monitoring strategy

In order to unravel complex multicausal connections and visualise monocausal connections with regard to sex-hormone disruptors, it is necessary to conduct a *weight of evidence* analysis of the experimental results. The specific causality criteria for such an analysis originate from Hill and Fox, and have been adapted for hormone disruptors by Vethaak amongst others (Fox91, Hil65, Vet97). The causality is becoming increasingly probable through the gathering of evidence of correlations via a host of different techniques. This not only involves research in the field, but also laboratory and semi-field research.

Often part of the knowledge is already available and it is not necessary to deploy the entire battery of monitoring options. The Committee wishes to emphasise that monitoring must be an iterative process involving continuous collaboration between different disciplines in an effort to investigate which strategy is the most effective. Thus, for example, a limited form of monitoring may suffice if hormone disruption is suspected, or if it is required to investigate whether there is a question of specific hormone disruption in a suspect environment or a sensitive ecosystem. This limited monitoring could, for example, consist in evaluating the reproductive capacity and external characteristics of important species. If hormone disruption is identified, then what is required is clearly either a more elaborate form of monitoring — whereby a large number of characteristics are measured at individual and population level — or monitoring that is directed at specific endpoints in appropriate and sensitive sentinel species.

Finally, the field observations need to be validated — for example via the use of in-situ bioassays with entire organisms or exposure in experimental situations.

Question 4

Is it possible to indicate which parameters need to be included in the process of deriving recommended toxicological exposure limits for hormone-disrupting substances, and does the Committee have any comments as regards the sensibility of these parameters to hormonal influence compared with the classical risk-assessment parameters?

Answer

The classical parameters and accompanying tests that are used in deriving recommended toxicological exposure limits are not tailored to risk assessment with regard to hormone disruptors. Various international committees (OECD, EPA and EC) are currently investigating whether existing test protocols can be adapted for the purposes of evaluating hormone disruptors. Many experts (OECD-EDTA; US-EPA-EDSTAC; EU-CSTEE) advocate a *weight of evidence* approach, which seeks to achieve a multi-stage method of evaluating the possible hormone-disruptive action of a substance, progressing from *in vitro* tests (receptor binding, gene transcription, metabolic enzymes) via short-term *in vivo* tests (uterotropic action, hormone balance, vitellogenin induction) to long-term, two-generation tests investigating the effects during embryonic/foetal development and the associated implications with regard to the reproductive capacity of offspring. Such comprehensive test protocols have been in use for some time with rats for the purposes of evaluating human health risks in connection with the authorisation of new compounds. A significant shortcoming of this standard approach is the short period over which exposure takes place. Effects of a substance will be missed if the most sensitive stage of life lies outside the exposure period.

In general, less sophisticated parameters are used as endpoints in deriving recommended ecotoxicological exposure limits, e.g. impact on growth, mortality and reproductive capacity. This is because for humans, protection is sought at individual level, whereas in the field of ecotoxicology, protection at population level is considered sufficient.

The Committee believes that the present endpoints are probably often capable of revealing the hormone-disruptive action of substances, providing the tests for substances are performed using representative and sensitive organisms and providing they embrace exposure over several generations. The tests that are currently being used in order to derive recommended ecotoxicological exposure limits do not meet this requirement. Tests that do satisfy the stipulated requirements will be extremely costly. It is always necessary to be able to relate the effects that are identified in the field to specific substances. The Committee therefore feels that there is a great need to develop test protocols which, on the one hand, provide for a longer exposure period and in which exposure occurs principally in the critical phase, while at the same time also examining endpoints which are sufficiently discriminating for a hormone-disruptive action.

The existing test protocols are often too short-term and the endpoints too limited for the purposes of research in fish, and exposure does not usually take place during the stages of life that are considered to be the most sensitive for sex-hormone disruptors. For amphibians, no test protocols exist for sex hormone-disruptive action, except for the hormone-mediated metamorphosis effects.

The major shortcoming is the lack of test protocols for research in invertebrates. This stems principally from a lack of insight into the normal functioning of sex-hormone systems in invertebrates. It is questionable just how representative the water flea (*Daphnia magna*) that is used in the current tests is of other invertebrates. The fact is that, under laboratory conditions, reproduction in this species generally occurs asexually (parthogenesis), whereas it is probably the sexual phase that is more sensitive to hormone disruptors. Good protocols for invertebrates are extremely important, since — as the Committee noted earlier — they constitute 95% of the animal species in an average ecosystem. As a consequence, they represent an important part of the basis of the food chain. Furthermore, many pesticides are specifically designed to disrupt the hormone balance of these animals. For a comprehensive consideration of potential endpoints and the adaptation of current test protocols, the Committee refers the reader to the results of two international expert workshops organised by the OECD, EC and the SETAC (EMW97, EDI99).

As far as sensitivity to hormone disruption is concerned, it is generally true to say that parameters which are indicative of hormone-mediated molecular or biochemical changes are the most sensitive, followed by cellular and tissue changes and, finally, functional, physiological changes. In addition, characteristics that are measured in the embryonic/foetal life stages are more sensitive than variables in adult life stages. This is due, in part, to the relative inability of an organism in the early stage of life to deal with disruptions up to a certain level via homeostasis. Furthermore, foetal tissues are often more sensitive to disruptions owing to the exceptional rates of differentiation and proliferation at which disruptions cause irreparable damage during embryonic/foetal development.

Because the majority of test protocols for hormone disruptors are still at an experimental stage, it is not possible at this point in time to provide a reasonably definitive list of variables and their respective sensitivities.

The Hague, 22 July 1999,
on behalf of the Committee

(Signed)
JW Dogger (Secretary), AD Vethaak (Chairman)

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A The Request for Advice

B The Committee

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D Indicator species

Annexes

Annex **A**

The request for advice

The President of the Health Council of the Netherlands received the following letter, dated 29 September 1998, from the Minister of Housing, Spatial Planning and the Environment.

I hereby request the Health Council to advise me on substances in the environment which are capable of impacting on the endocrine system and about the effects of these substances on ecosystems.

A stream of scientific publications have appeared in recent years which focus on substances present in the environment which can impact on the endocrine system. At the forefront of the discussion are substances with an effect on the sex hormones. Disruption of the hormone balance, resulting in reproductive problems, is regarded as an important reason for the decline in populations of animals in various polluted locations.

Eggs of birds, fish and amphibians in polluted areas exhibit reduced hatching success. Furthermore, feminisation phenomena are being observed in male offspring, for example an increased oestradiol/testosterone ratio, reduced penis size and the production of vitellogenin. In a number of cases, these field findings have been confirmed by means of laboratory research with substances. Less information is available about mammals, where the decline is generally attributed to a reduction in immunological resistance. Impairment of the developing immune system, under the influence of endocrine disruption, could provide an explanation of these phenomena.

A large number of substances have already been designated as suspect in this regard, especially persistent organochlorine compounds such as DDT, dicofol, PCBs and dioxins, but also substances such as mercury, alkylphenols, natural and synthetic oestrogens and phyto-oestrogens. Because it is highly doubtful whether, given the present systems of substance evaluation, it is possible to detect endocrine effects using the prescribed tests, an initiative has been launched in international circles (EU, OECD) to adapt the testing guidelines.

Exposure to these substances is also relevant for human beings. Acting on its own initiative, the Health Council of the Netherlands has therefore already published an advisory report about the human aspects of hormone disruptors in April 1997. Following on from this report, I would like to request the Health Council to advise me about the state of the art with regard to the ecotoxicological aspects.

I would be grateful for an answer to the following questions:

- Is the Health Council of the Netherlands of the opinion that pollution of the environment with substances that impact on the endocrine system poses a serious threat to populations and, consequently, possibly also to ecosystems?
- If so, is it yet possible to indicate which substances (or sources, where applicable) principally warrant attention in this respect?
- Is it possible to investigate, via a programme of measurements (monitoring), whether there are effects on the sex-hormone balance of animals?
- Is it possible to indicate which parameters need to be included in the process of deriving recommended toxicological exposure limits for hormone-disrupting substances, and does the Committee have any comments as regards the sensibility of these parameters to hormonal influence compared with the classical risk-assessment parameters?

In view of the great public interest in this topic and the fact that the Lower House has been promised a Memorandum on this matter by the end of this year, I would ask the Health Council to give priority to this Ministerial Commission. I would like to receive this advisory report by spring 1999 at the latest.

The Minister of Housing, Spatial Planning and the Environment,

signed JP Pronk

Annex **B**

The Committee

- AD Vethaak, *chairman*
  ecotoxicologist; National Institute for Coastal and Marine Management, Middelburg
- M van den Berg
  professor of toxicology; Research Institute of Toxicology, University Utrecht
- JP Boon
  biochemist; Netherlands Marine Research Institute, Den Burg
- A Brouwer
  toxicologist; Wageningen Agricultural University
- HJTh Goos
  professor of endocrinology; University Utrecht
- P Hagel
  chemist; National Fisheries Research Institute, IJmuiden
- PJH Reijnders
  population ecologist, ecotoxicologist; Institute for Forestry and Nature Research, Den Burg
- MA Vaal, *consultant* (until 1 January 1997)
  National Institute of Public Health and the Environment, Bilthoven
- P de Voogt
  environmental chemist; University of Amsterdam
- SE Wendelaar Bonga
  professor of endocrinology; Catholic University, Nijmegen

- PW Wester, *consultant* (from 1 January 1997)
  National Institute of Public Health and the Environment, Bilthoven
- J van Zorge, *consultant*
  Ministry of Housing, Spatial Planning and the Environment, The Hague
- JW Dogger, *secretary*
  Health Council of the Netherlands, The Hague

Expert consultants:
- WHO Ernst
  professor of ecology and ecotoxicology; Free University, Amsterdam
- AJ Murk
  toxicologist; Wageningen Agricultural University

Annex **C**

Overview of the classification status of 77 substances

Table C1 provides an overview of the classification of 77 industrially produced substances and pesticides, which were discussed in Chapter 4. Recorded in the table are the outcome of the testing of available data on each substance against a number of criteria and the subsequent assignment of that substance to a particular class (see 4.1). The criteria applied in the columns of Table 7 are as follows:

- Production, consumption and emissions in the Netherlands. The Committee established whether the substance has been authorised in the Netherlands and determined the annual production, consumption and emissions of the substance in the Netherlands. A positive score means that the production, consumption or emissions in the Netherlands are in excess of 10 tonnes per annum. This criterion is, incidentally, only significant if no data is available about environmental concentrations.
- Concentrations in water, sediment, suspended matter and in organisms. A positive score means that the substance has been detected in one or more environmental compartments.
- Physical: hydrophobicity of the substance (Kow value), potential to accumulate in organisms (BCF or BSAF value), persistence in the environment. For substances with a log Kow or log BCF value greater than 3, the exposure of top predators via food is significant and results in a positive score
- PEC/N(O)EC ratio >0.01 (see 4.1.2)
- Potency Score for *in vitro* tests for binding to the oestrogen receptor (OR), the androgen receptor (AR) or the thyroid-hormone receptor (T4)(for criteria see 4.1.2)

- Reproductive toxicity. In the absence of data on the hormone-disruptive effects of a substance, the occurrence of reproductive toxicity indicates that the substance could be a hormone disruptor.

The complete collection of data about production, consumption, emissions, concentrations in the environment and the physical properties of substances can be found in two background studies that have been conducted on behalf of the Committee. The first study was carried out by MJ Greve under the supervision of Committee members Dr J Boon and Dr P de Voogt. This data has been supplemented in a follow-up study conducted by R Franse under the supervision of Committee member Dr P de Voogt.

The survey of the effects of these substances, which have been used for the PEC/N(O)EC, potency and reproductive-toxicity criteria, has been carried out by A Bulder under the supervision of Dr AJ Murk and JW Dogger.

Table C1 Overview of the classification status of 77 substances. Meaning of the symbols: + satisfies the relevant criterion; - does not satisfy the relevant criterion; ? insufficient data.

substance	production, consumption, emissions	concen-tration	physical	PEC/NEC	potency ER/AR	potency T4	repro-ductive toxicity	comments	class
A Alkylfenols									
4-nonylphenol	+	+	+	+	+	?		environmental concentrations possibly within effects range	1
4-octylphenol	+	?	+	+	+	?		environmental concentrations possibly within effects range	1
B Anilides									
Alachlor	?	-	-	?	-	+	+	not a convincing hormonedisruptor, only thyroid effects	4
Pronamide	+	?	+	?	?	?	?	cannot be evaluated, probably persistent	4
C Bisphenol A	?	+	+	+?	+	?	-	potential hormone disruptor	2
Tetrachloro						+		potential hormone disruptor	2
Tetrabromo						+			
D Carbamates									
Aldicarb	+	-	-	?	?	?	?	cannot be evaluated, not persistent	4

substance	production, emissions	tration		PEC/NEC		T4	repro- toxicity		class
		+		+		?		reprotoxic, hormone	3
Carbendazim)									
	+		-		?		+		
								disruption unknown	
Carbofuran		?		?		?			3
								the Netherlands, probably	
	+		+-		?		?		
								effects detected	
Fenoxycarb		?		?		?		no concentrations/endocrine	4
	-		-		?		-		
								effects detected	
E Chloorophenoxy-carboni acids									
2,4,5-T		+-		-		+		too little known about	3
	+		-		-		?		
								disruptor	
F Dicarboximides									
Iprodion		+		?		?		no endocrine effect	
Procymidon		?		?		?		no concentrations and	4
	+		+		+		+		2
	+		-		-		+		
								via ETU	
Maneb		?		-		+		“	
Metiram		?		?		+		”	
Molinate		?		?		+		“	
Zineb		?		?		+		reproductive effects on	4
								sufficiently high to cause	

substance	production, consumption, emissions	concen-tration	physical	PEC/NEC	potency		repro-ductive toxicity	comments	class
					ER/AR	T4			
Ziram	+	?	-	?	-	+	-	reproductive effects on administration of doses sufficiently high to cause systemic toxicity	4
H Phenylurea herbicides									
Diflubenzuron	+	?	+	?	?	?	+	no concentrations/endocrine effects	4
Ethylene thiourea	?	?	-	?	?	+	?	thyroid-hormone disruptor	4
Linuron	+	+-	+	-	-	?	+	not a hormone disruptor	4
I Phthalates									
Butylbenzyl-phthalate (BBP)	+	+	+	+	+	+-		PEC/NEC probably below 0,01	3
Di-butyl-phthalate (DBP)	+	+	+	+?	+	+-		potential hormone disruptor	3
Di-ethylhexy-phthalate (DEHP)	?	+	+	?	?	+-		NL risk cannot be evaluated	3
J Nitroanalines									
Oryzalin	+	?	+	?	?	?	-	no concentrations in NL, no endocrine effects	4
Pendimethalin	+	?	+	?	?	?	-	no concentrations in NL, no endocrine effects. Highly toxic for fish and ap. vertebrates.	4
Quintozene	?	-	+	?	?	?	?	no endocrine effects detected	4
Trifluralin	-	-	+	?	?	+	-	no endocrine effects detected	4
K Organobromine compounds									
PBB	+	+	+	+?	?	+	+?	potential hormone disruptor	2
PBDE	+	+	+	+?	?	+	?	potential hormone disruptor	2
L Organochlorine compounds									
Aldrin								mainly converted into Dieldrin	

substance	production, consumption, emissions	concen-tration	physical	PEC/NEC	potency		repro-ductive toxicity	comments	class
					ER/AR	T4			
Dieldrin (+ Aldrin)	?	+	+	+	+	+		environmental concentrations possible within effects range	1
Chlordane	-	+	+	+	-	+		environmental concentrations possible within effects range	1
Heptachlor	?	+	+	?	+	+		no concentrations in NL, below detection level	3
Chloordecone (Kepone)	?	?	+	?	+	?		potential hormone disruptor	2
p,p'-DDT	-	+	+	-	-	+	+	endocrine risk via p,p'-DDT metabolite	1
o,p'-DDT	-	+	+	+	+	+	+	environmental concentrations possible within effects range	1
p,p'-DDE	-	+	+	+	+	+	+	environmental concentrations possible within effects range	1
p,p'-DDD	-	-	+	-	-	+	+	no endocrine effects expected	4
Methoxychlor	-	?	+	+?	+	?	?	no consumption or concentrations in NL	4
Dicofol	-	?	+	?	+-	?	-	no concentration in NL	4
Endosulphan	-	+	+	+	+	?		environmental concentrations possible within effects range	1
HCB	?	+	+	-	-	+	?	no potential risk, effets on T4 via PCP	4
PCP	+	+	+	-	-	+	-	thyroid-hormone disruptor	3
HCH (γ + β)	+	+	+	+	+	?		environmental concentrations possible within effects range	1
Mirex	?	?	+	?	?	?	?	no concentrations in NL, no endocrine effects	4
PCB's, PCDD's, PCDF's	-	+	+	+	+	+	+	environmental concentrations possible within effects range	1
Toxaphene	-	+	+	+?	+	+	-	environmental concentrations possible within effects range	1
M Organophosphorus compounds									
Malathion	-	?	-	?	-	?	?	no endocrine activity expected	4
Parathion (-ethyl)	+		+	?	-	?	?	no endocrine activity expected	4

substance	production, consumption, emissions	concen-tration	physical	PEC/NEC	potency ER/AR	potency T4	repro-ductive toxicity	comments	class
N Organotin compounds									
Tributyltin	+	+	+	+	+	?	?	environmental concentrations possible within effects range	1
Triphenyltin	+	+	+	+	+	?	?	environmental concentrations possible within effects range	1
O Polycar-bonates	?	?	?	?	?	?	?	no effects/concentrations, bisphenol a ingredient	4
P Aromatic hydrocarbons									
Styrenes	+	?	-	?	?	?	?	no concentrations/effects, alkylphenols added to polymer, remove from list	4
Dibenzan-thracene	?	+	+	?	?	+	?	closer investigation required	3
Q Synthetic pyrethroids									
Biphenthrin	-	?	+	?	?	?	?	no concentrations in NL, no effects	4
Cyfluthrin	-	?	+	?	?	?	?	no concentrations in NL, no effects	4
Cypermethrin	-	+	+	?	?	?	+	no endocrine effects	4
Deltamethrin	-	+	+	?	?	?	?	no endocrine effects	4
Esfenvalerate	+	?	+	?	?	?	?	no endocrine effects	4
Fenpropathrin	-	+	+	?	?	?	?	no endocrine effects	4
Permethrin	-	+	+	?	?	?	>	no endocrine effects	4
R Triazines									
Amitrole	+	?	-	?	?	+	-	no concentrations in NL, endocrine effects unknown	4
Atrazine	+	+	-	+?	+	?	+	potential hormone disruptor	2
Clofentezine	+	?	?	?	?	?	?	nothing known	4
Ethiozine	?	?	?	?	?	?	?	”	4
Metribuzin	+	-	-	?	?	?	-	no concentrations/effect data	4

substance	production, consumption, emissions	concen-tration	physical	PEC/NEC	potency		repro-ductive toxicity	comments	class
					ER/AR	T4			
Simazine	+	+	-	?	-	?	?	insufficient data	4
S Other pesticides									
Azadirachtin	?	?	?	?	?	?	?	no data	4
Hexaconazole	?	?	?	?	?	?	?	no data	4
Imidazole	?	?	?	?	?	?	?	no data	4
Nitrofen	?	?	?	?	?	+	-	insufficient data, metabolite is T4 disruptor	4

Annex **D**

Indicator species

Table D1 Species that are suitable for use in monitoring in situations where there may be hormone-disrupting substances.

mammals	bank vole, common shrew/field vole/bat, mole, rabbit, otter, common seal
birds	little owl, kestrel, starling, mallard, black-headed gull, common tern, cormorant
reptiles	grass snake, slowworm
amphibians	brown/green frog, common toad
fish	three-spined stickleback, roach, bream, carp, gudgeon, flounder, dab, eelpout
invertebrates	molluscs, crustaceans, etc.[a]

[a] Invertebrates are a gap in our knowledge. Molluscs exhibit imposex/intersex in association with TBT exposure, and a number of studies have used Daphnia (Gerritsen, 1997) and woodlice (Donker *et al.* 1997) in order to assess risks to the reproductive success of specific hormone disruptors.