CHAPTER 1: INTRODUCTION

Rapid industrial growth after the Second World War resulted in the release of tons of new industrial chemicals without being properly assessed for their toxicological impact on humans and the environment. This also occurred at the same time as the massive commercial production of organochlorine and organophosphate compounds, which were widely used in several areas of modern life. The development of new techniques and the regulation of several new agents to which humans were exposed after Word War II were the basis of modern toxicology. However, the hypothesis that humans and wildlife are harmed by

continuous exposure of chemicals in the environment only became of public interest after the publication of Rachel Carson's "*Silent Spring*" in 1962. In her book, Rachel Carson was the first to present strong evidence that chemicals with the potential to interfere with the endocrine system may cause adverse effects in humans and wildlife at levels found in the environment [Carson, 1962]. Moreover, the tragic thalidomide incident, in which several thousand children were born with serious birth defects, led many scientists to investigate the effects of chemicals during embryonic and fetal development. Since then, new legislation has been passed, new journals have been founded, and the education of toxicologists has spread throughout the USA and Europe.

In the context of human health, this chapter will present relevant aspects and strong evidence that persistent environmental pollutants are interfering with normal endocrine processes. Furthermore, a detailed overview of the flame retardant, polybrominated diphenyl ether (PBDE) will be presented. Finally, the gaps in our knowledge will be presented at the end of the chapter as the main motivation of the present work.

1.1 - ENDOCRINE ACTIVE COMPOUNDS (EACS)

Humans are exposed to many new chemicals in the environment, but the effects of so-called endocrine active compounds (EACs) are currently the main concern in toxicology. EACs include many natural and synthetic chemicals that are thought to share a common mode of action by interfering with the normal molecular circuitry and function of the endocrine system [Silbergeld *et al.*, 2002]. In particular, one difference between reactive chemicals and relatively stable compounds altering physiological signaling systems (e.g. EACs) is the potential of this class of compounds to produce toxicity at a site other than the chemical activity. Normally, the target organ for a chemical is the one in which toxicity is observed: for reactive chemicals, it is the organ in which they react with cellular constituents; for EACs affecting endocrine signaling systems, the site of the action may be distant from that of the observed toxicity. For instance, exposing female Sprague-Dawley rats to atrazine produces lengthening and finally cessation of their estrous cycle [Wetzel *et al.*, 1994; Stevens *et al.*, 1994]. The mechanism appears to involve changes in the brain altering the production of gonadotropin-releasing hormone (GnRH), which produces a decreased LH surge [Cooper *et al.*, 1996]. Thus, this toxicity is observed in the reproductive organs, but the target site for the chemical action is the brain.

Another important issue in EACs toxicity is its effects on the developing organism. It is rather complex to investigate and interpret developmental toxicity as an insult occurring during critical developmental periods may produce permanent adverse effects in the progeny which appear sometimes only later in life. Development is a highly integrated process, in which high rates of proliferation and extensive differentiation are coordinated with each other and with programmed cell death. In the developing organism, feedback mechanisms controlling hormone synthesis and elimination mechanisms controlling hormone degradation are still being developed and may not be fully functional. In this sense, it has been postulated that the impact of EACs on the adult organism probably differs from that on the developing individual. In adults with fully developed physiological processes, exposure to steroids or chemicals with hormone-like effects leads to activational changes that are usually reversible. On the other hand, the existence of critical periods during organogenesis and the sensitivity of developmental processes to relatively small changes in endogenous steroid levels suggest that endocrine disruption during development may have long-lasting deleterious effects (organizational effects) which are often permanent [Guillette, Jr. *et al.*, 1995]. The experience with diethylstilbestrol (DES) is a well-known example of this phenomenon. DES was prescribed to pregnant women from the late 1940's to the early 1970's to prevent miscarriages and pregnancy complications. Treatment with DES during pregnancy was associated with the development of reproductive tract cancer in female offspring from these women [Herbst *et al.*, 1971]. Men exposed to DES *in utero* had significant reductions in sperm concentrations, sperm counts and sperm with normal morphology [Gill *et al.*, 1978]. Moreover, a recent report suggests an increased risk of hypospadias in the grandsons of women treated with DES during pregnancy [Klip *et al.*, 2002]. This underlines the impact of *in utero* epigenetic factors on the development of disease or pathology [Klip *et al.*, 2002]. It is noteworthy that a variety of developmental and reproductive changes associated with environmental contaminants have been reported in both humans and wildlife in the last decades. These include feminization, demasculinization, reduced fertility, reduced hatchability, reduced viability of offspring, impaired hormone secretion or activity and altered sexual behavior [reviewed by Colborn *et al.*, 1993]. However, this chapter will not give a detailed report of EACs toxicity in wildlife but will focus on human effects and experimental toxicological data.

The next section provides a detailed description of the growing body of evidence on EACs interference in human development. The epidemiological data support extensive

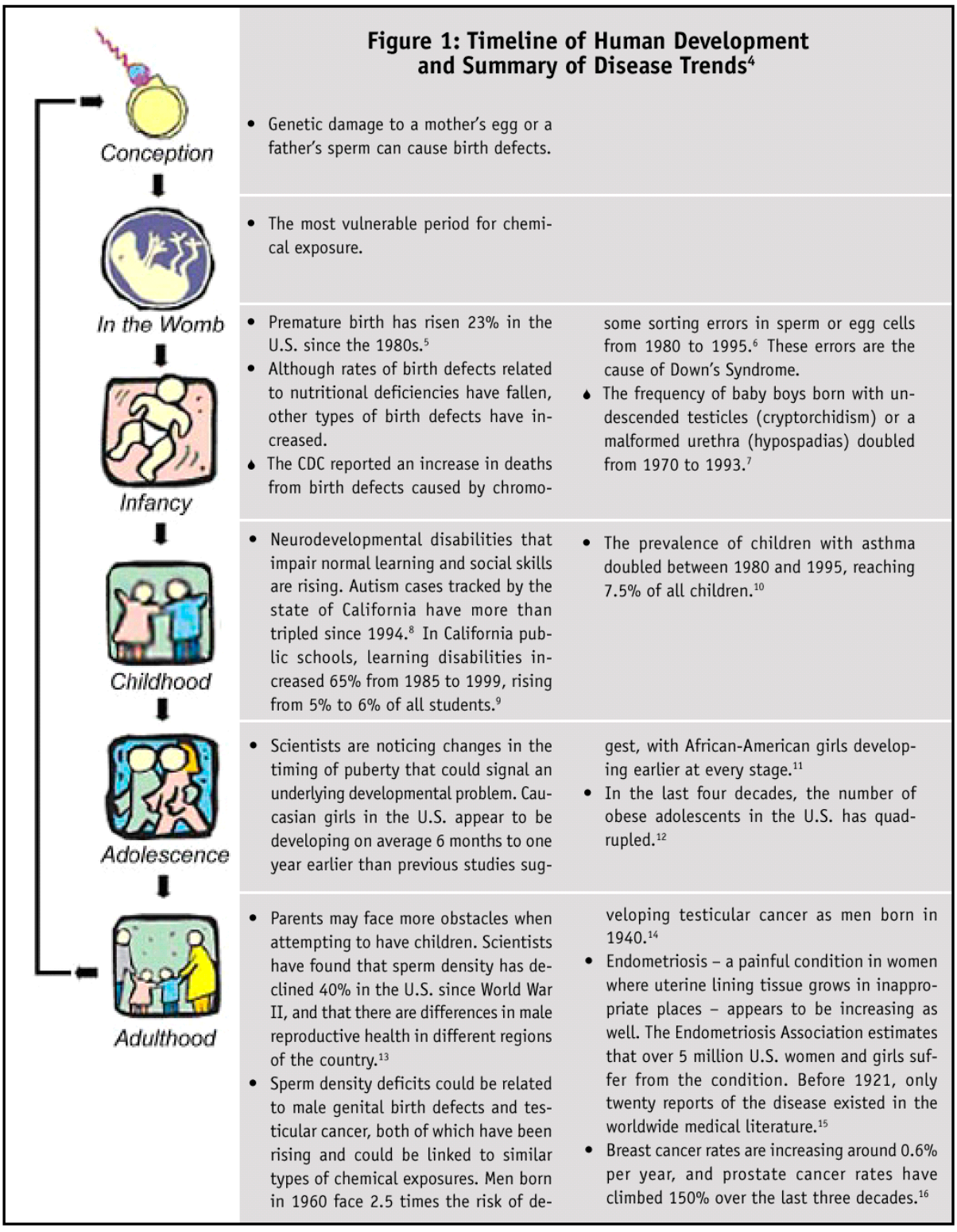
discussions and encourage scientists to elucidate the role of environmental pollutants on human health.

1.2 - HUMAN EVIDENCE OF EAC TOXICITY

There is increasing evidence suggesting that humans are facing different obstacles to healthy development, from the moment of conception until the time to conceive. Problems like premature birth, male genital defects, learning, attention and emotional disturbances, early puberty, obesity; and low sperm quality have been increasing over the last decades, impacting every stage of growth from conception to adulthood (Environment California Research and Policy Center, 2004) (Figure 1). Although many factors may contribute to one of these trends, an increasing amount of research suggests that toxic environmental chemicals play a significant role. Modern techniques with the ability to detect minimal levels of exposure are revealing a wide variety of persistent man-made compounds in human tissue. Parallel to these findings, an association has been reported between chemical levels and human disabilities and diseases, demonstrating toxic effects at surprisingly low exposure levels. Of the thousands of different compounds to which humans are exposed, only a few chemicals have been extensively assessed for their toxicological effects. While a clear correlation has been established between health risks and compounds like lead, mercury, dioxins and polychlorinated biphenyls (PCBs) [Kimbrough *et al.*, 2003; Silbergeld, 2003; Counter *et al.*, 2004; Steenland *et al.*, 2004], other compounds found at high levels in human tissue must still be properly assessed. They include polybrominated diphenyl ethers (PBDEs), which are the subject of the present investigation.

Figure 1 summarizes the actual trends in diseases and disabilities occurring throughout life, from conception to time to conceive. Exposure to chemicals during critical periods of development may play an important role in the etiology of such diseases. For example, one of these trends is the increasing rate of preterm delivery from 1975 to 1995 in the USA, which has risen by 22% among Caucasian woman [Ananth *et al.*, 2001; Branum *et al.*, 2002]. As some experimental studies suggest, this increase may be associated with exposure to environmental chemicals. Women with the highest serum levels of the pesticide dichlorodiphenyltrichloroethane (DDT) were more than three times more likely to give birth to a premature child [Longnecker *et al.*, 2001]. The same correlation was found for

Figure 1: Timeline of human development and summary of disease trends. *



* Extracted from "*Growing Up Toxic - Chemical Exposure and Increases in developmental Disease*"*Environmental California Research and Policy Center, June 2004*

plasticizer phthalates and their breakdown products, in which neonates with high levels of these substances were born one week earlier than those with lower exposure [Latini *et al.*,

2003]. The implications of premature birth are subtle, but may have serious consequences for children, since they face a greater risk of reduced intelligence and behavioral problems, including attention deficit hyperactivity disorder (ADHD), a disability that affects between 3% and 7% of children [Bhutta *et al.*, 2002].

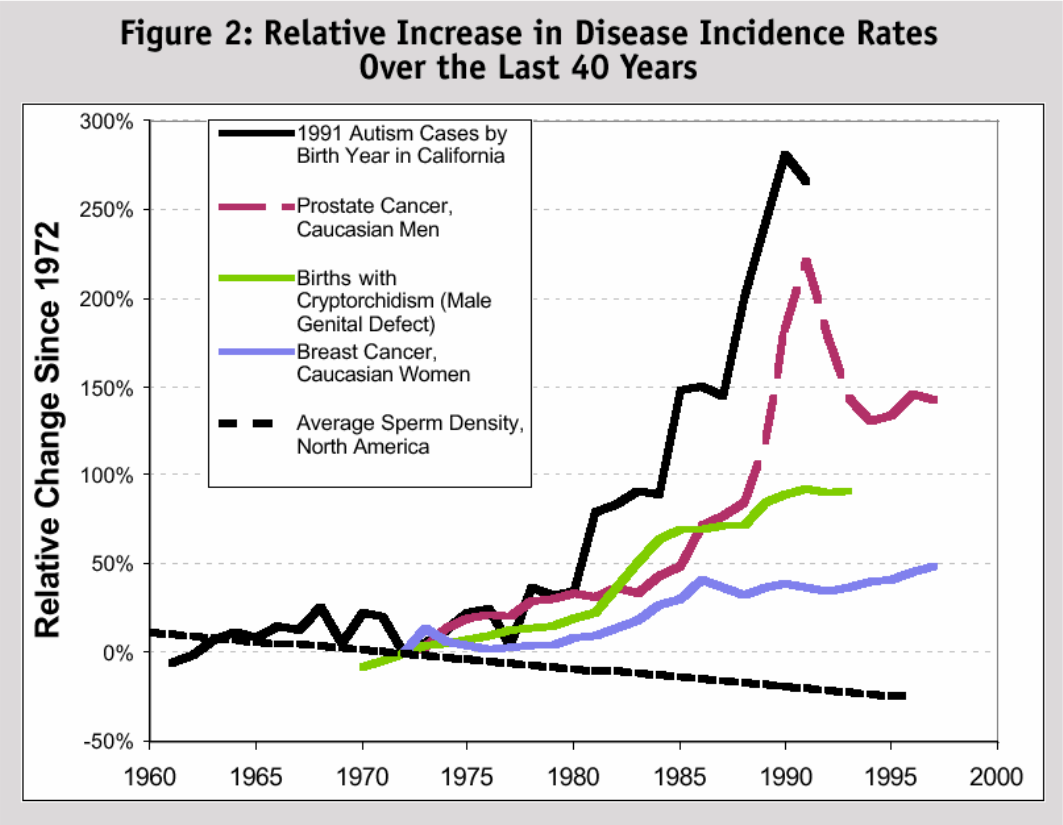
Since the overall data point mainly towards neurodevelopment and reproductive health effects, these two systems will be reported separately. Consistent clinical and experimental data support the hypothesis that environmental pollutants play an important role in some diseases, while there is no evidence for other diseases and disabilities despite the increase of this trend over time. We will report the relevant health impairment trends that have attracted scientific and public attention.

1.2.1 – NEURODEVELOPMENTAL EFFECTS

Neurological impairment and the increase in neurobehavioral disabilities are a cause for concern. Although it is extremely difficult to characterize the cause(s) of such disorders, a body of epidemiological data and experimental research indicate that environmental pollutants play a significant role in the etiology of such diseases. One of the most alarming trends is the dramatic increase in autism cases since 1980 without any generally agreed-upon explanation (Figure 2). There is also no consensus about the exact cause of autism, but an event occurring during fetal development might be involved in the etiology together with predisposing genetic factors. Signs of autism can be detected in the womb even though behavioral symptoms normally do not become fully apparent until after birth. Children with autism show delayed brain growth *in utero* and accelerated brain growth in the months after birth [Courchesne *et al.*, 2003]. Not only is there a higher incidence of autism but general neurological impairments have also been observed in an increasing number of children with learning deficit, psychosocial problems and ADHD [Kelleher *et al.*, 2000; Robison *et al.*, 2002]. Even though there is no plausible explanation for such an increase, it has been postulated that environmental pollutants may play a role in the occurrence of such disorders [Environment California Research and Policy Center, 2004].

Brain development is a complex and lengthy process. Development begins in the third week of pregnancy and continues until the second year of life. This a time when the developing brain is most vulnerable and subject to permanent damage. A complex signaling system coordinates nerve cell replication, growth, differentiation and cell-to-cell

Figure 2: Relative increase in disease incidence rates over the last 40 years. *



* Extracted from "Growing Up Toxic - Chemical Exposure and Increases in Developmental Disease " Environmental California Research and Policy Center, June 2004

communication. Thyroid hormone signals are particularly important, since disruptions in thyroid levels from as early as the 8th week of gestation up to the second year of life can disrupt children's brain development and impair their intelligence and coordination [Pop *et al.*, 1999; Haddow *et al.*, 1999; Howdeshell, 2002]. This signaling system is particularly important, since many environmental chemicals have the ability to disrupt thyroid hormone homeostasis at significantly low levels. For example, experimental data on low-dose exposure through drinking water to perchlorate, a rocket fuel used by numerous aerospace contractors, road-flare manufacturers and pyrotechnic companies, has been shown to be a potent thyroid disruptor. Rats exposed to perchlorate at levels as low as 10 nanogram per kg body weight per day show changes in thyroid hormone levels, brain structure, thyroid morphology, and behavior [see Environment California Research and Policy Center, 2004]. Moreover, experimental studies have consistently demonstrated that monkeys exposed to lead or PCBs during development show symptoms similar to those of ADHD in humans, including an inability to plan and perform tasks in an efficient or sensible sequence, a short

attention span, and deficiencies in learning [Rice, 2000]. In humans, PCBs have also been found to be a potent neurotoxicant. For example, children, whose mothers were exposed to PCBs by accidental poisoning in 1978, showed signs of irreparable damage including delayed brain development and increased social dysfunction like hyperactivity and behavioral problems at school [Rogan *et al.*, 1988; Chen *et al.*, 1992]. Subsequent studies in North Carolina, Michigan, New York, and the Netherlands confirmed the results from accidental poisoning at far lower levels of exposure. In general, it was suggested that as the level of PCB exposure rose before birth, the mental and physical abilities of infants declined after birth. Even at very low levels, prenatal PCB exposure contributed to hyperactivity and attention problems discovered later in childhood [George *et al.*, 1982; Jacobson *et al.*, 1990].

1.2.2 – MALE REPRODUCTIVE EFFECTS

Male reproductive health has declined in the last decades as demonstrated by a decrease in semen quality and density and an increase in testicular cancer. Moreover, some authors have reported an increasing number of genital malformations in children born for the same period (Figure 1 and 2) [see review Toppari *et al.*, 1996; Environment California Research and Policy Center, 2004]. The number of children born with hypospadias (a birth defect causing the opening of the urinary tract to develop on the underside of the penis) and/or cryptorchidism (a birth defect disrupting the descent of the testicles into the scrotum) has doubled in the last three decades [Paulozzi, 1999]. While several factors may contribute to this trend, a significant amount of research has pointed to a possible role of EACs in the disruption of the male reproductive system. Experimental studies support this hypothesis, since they found a clear relationship between genital malformations and chemical exposure in rodents. For example, reproductive defects were reported when rats were exposed to three types of commonly used phthalates (DEHP, BBP and DINP), an additive mixed into plastics to improve their flexibility and malleability. Male pups exposed *in utero* to phthalates showed reduced body mass, hypospadias, cleft phallus, reduced testis weight, and cryptorchidism [Gray, Jr. *et al.*, 2000]. It seems that DEHP reduces testosterone production in the developing testis, altering the signal cascade for normal male reproductive development [Parks *et al.*, 2000]. In another study, a high incidence of hypospadias, cryptorchidism, infertility and other testicular defects were found in male pups exposed *in utero* to 500 mg of dibutyl phthalate (DBP) per kg body weight [Fisher *et al.*, 2003]. Not only plasticizers but also pesticides have a strong impact on reproductive development in

laboratory animals. At dose levels far below those found in lakes, rivers, streams, and even drinking water, the pesticide atrazine causes the development of ovaries and abnormal testicles in male frogs and induces demasculinization [Hayes *et al.*, 2002]. Since Hayes et al. found a 10-fold decrease in the testosterone concentration of male frogs exposed to atrazine, they hypothesized that atrazine may induce aromatase (a P450 enzyme involved in the conversion of testosterone to estrogen) activity, reducing testosterone levels in these animals [Hayes *et al*., 2002]. Even though experimental animal data cannot be unreservedly applied to human risk assessment, they are good indicators that some persistent environmental pollutants may be involved in the increasing incidence of male reproductive defects.

Recent epidemiological studies have also provided valuable information about time-trend changes in human male reproductive health. From 1973 to 1995, the incidence of testicular cancer increased 51%, making it the most common malignancy in young men [McKiernan *et al.*, 1999]. Additionally, this increase correlates to the year of birth, i.e., men who were born in the same period share a common disease risk. This suggests that some early developmental event or prolonged exposure to an environmental contaminant may be the trigger in disease development [Bergstrom *et al.*, 1996]. Carlsen et al. (1992) published a study indicating that human semen quality had declined by ~50% from 1930 to 1991. Many subsequent studies criticized or re-analyzed the original metanalysis, but the trends and conclusions were reaffirmed [Swan *et al.*, 1997; Swan *et al.*, 2000; Skakkebaek *et al.*, 2001]. Several epidemiological studies have provided strong evidence showing that environmental chemicals interfere with male reproductive health. Some of them will be given as examples in the following:

- A recent study carried out in the USA has reported geographical differences in sperm quality with the mean sperm count significantly lower in Columbia, Missouri (58.7 X 10^6 / ml) than in New York, New York (102.9 X 10^6 / ml), Minneapolis, Minnesota (98.6 X 10^6 / ml) or Los Angeles, California (80.0 X 10^6 / ml) [Swan *et al.*, 2003a]. Comparing the levels of urinary metabolites of several pesticides in men from Missouri (rural area) to those from Minnesota (urban area), it was found that those who lived in rural areas had higher levels than those from the city. A statistical correlation between poor semen quality and high levels of alachlor, diazinon, atrazine, metolachlor and 2,4-D (2,3-dichlorophenoxyacetic acid) was observed, indicating that pesticide residues may be a factor interfering with semen quality in men from rural areas in the USA [Swan *et al.*, 2003b].

- In another recent study, phthalate exposure was associated with decreased human reproductive health [Duty *et al.*, 2003]. A negative correlation was found between levels of

monobutyl and monobenzyl phthalate (two phthalate metabolites) and sperm counts in 150 men from the Boston area, suggesting that phthalate exposure might be involved in such a decline.

- A correlation between PCB levels and human reproductive disorders has also been demonstrated. PCBs were widely used as a dielectric fluid in transformers, flame retardants and many other applications banned from the U.S. market in 1972. Between 1978 and 1979, the Taiwanese accidentally ingested rice oil contaminated with PCBs and their pyrolytic products (mainly PCDFs) [Hsu *et al.*, 1984]. Men exposed to contaminated rice oil had a higher percentage of oligospermia, abnormal sperm morphology and reduced sperm binding capability and penetration [Hsu *et al.*, 2003]. Not only men who were directly exposure but also those who were prenatally exposed to contaminated rice oil had a higher percentage of abnormal sperm and reduced sperm penetration of hamster oocytes [Guo *et al.*, 2000]. From an environmental exposure standpoint, it has been postulated that PCBs and their metabolite levels negatively correlate with human semen quality [Rozati *et al.*, 2000; Rozati *et al.*, 2002; Dallinga *et al.*, 2002; Hauser *et al.*, 2002] and positively correlate with the incidence of testicular cancer [Hardell *et al.*, 2003].

The biological consequences of reduced semen quality are of great concern. To illustrate the scientific awareness of such a trend, we used a Danish study evaluating the medical examinations of men considered for compulsory military service. Andersen et al. (2000) [Andersen *et al.*, 2000] found that men tested between 1996 and 1998 had a high percentage of suboptimal semen. The mean sperm concentration was 57.4 X 10^6/ml, but 48% of men had a sperm concentration below 40 X 10^6/ml and 25% a sperm count of <20 X 10^6/ml [Andersen *et al.*, 2000]. While sperm counts below 40 X 10^6/ml have been associated with decreased fecundity and increased time to pregnancy [Bonde *et al.*, 1998], sperm concentrations of <20 X 10^6/ml are considered abnormal according to World Health Organization guidelines [World Health Organization, 1992]. Although the etiology of the decline in human reproductive health is not clear, it is plausible that environmental pollutants play a role in this trend.

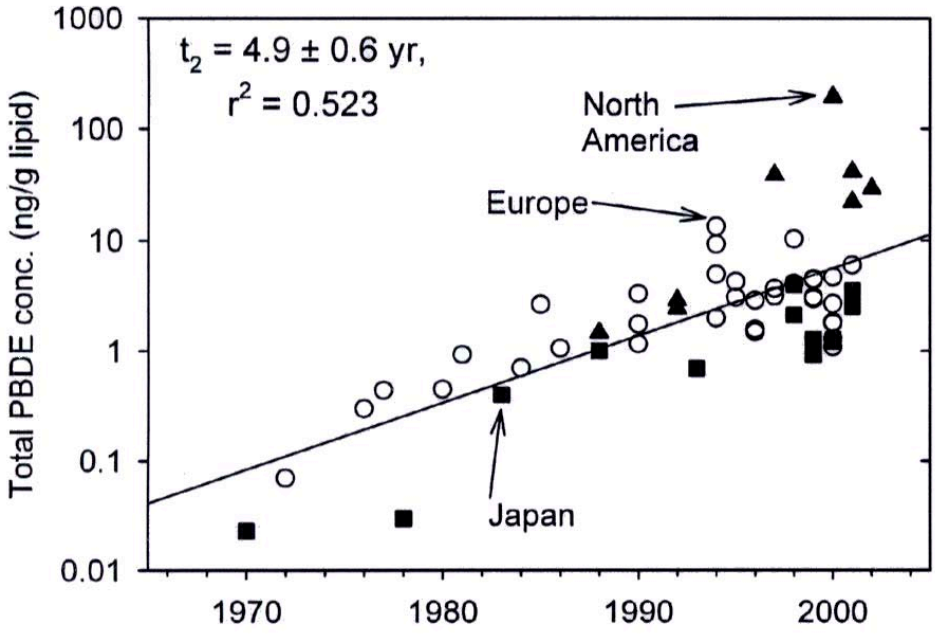
An overview of human health problems in the last 50 years was presented above. This was supported by several studies showing that exposure to environmental pollutants may play an important role. The next session will discuss the important aspects of chemistry, toxicology and biota levels for a new environmental threat PBDE, a brominated flame retardant. Since there has been an alarming increase in PBDE levels in the last years, the

paucity of data on their harmful effects in humans warrants further experimental investigation.

1.3 – A NEW ENVIRONMENTAL THREAT: POLYBROMINATED DIPHENYL ETHERS (PBDES)

Consistent information supporting the hypothesis that environmental pollutants are interfering with normal development was presented in the previous sections. However, there is a lack of data, which requires further experimental and clinical investigation. Of the thousands of chemicals, to which human are exposed, there has been a growing concern about PBDEs toxicity in the last decade because increasing levels have been detected in human tissue and in breast milk. PBDEs are different from many currently banned organochlorines, since they are still being added to manufactured products (e.g. mattress, computer, textiles) as a flame retardant. While dioxin and PCB levels (two halogenated compounds present in every level of the food chain) have decreased over the last 10 years, an exponential increase in brominated flame retardant levels has been observed for the same period (Figure 3). A general overview of PBDEs will be presented in this section.

Figure 3: Time trend of PBDE concentration in human samples. *

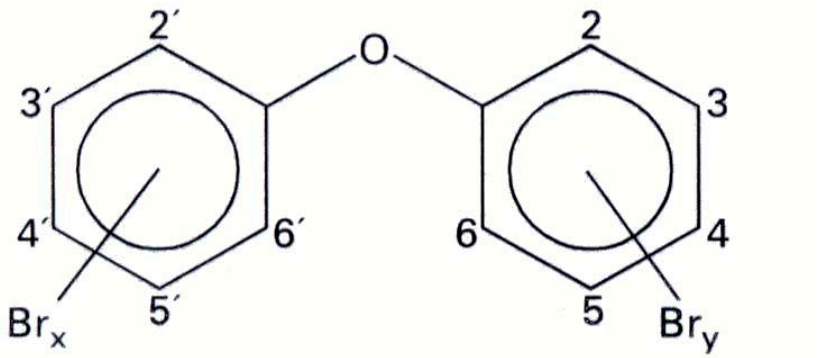


* from Hites RA, Environ Science and Technol 38:(4), 945-956, 2004

1.3.1 – CHEMICAL PROPERTIES, WORLD PRODUCTION AND ENVIRONMENTAL LEVELS

Brominated flame retardants (BFRs), which also include PBDEs, are added to flammable materials to retard and/or inhibit the initial phase of fire. They are incorporated into various plastic components in electronic devices (e.g. cabinets, circuit boards, cables, switches, capacitors) and cars as well as building materials and textiles. Chemically, the bromine component of the compound is responsible for the molecule's flame retardant activity and is unique in its ability to provide flame retardancy in the gas phase [Hardy, 2002]. Depending on their use, these chemicals can be divided into two groups: reactive and additive flame retardants. Reactive BFRs are mixed with the plastic before polymerization to form covalent bonds and become part of the polymer matrix. Additive BFRs, however, are mixed with the polymer that makes these BFRs more likely to leach out of goods and products during their lifetime [World Health Organization, 1994]. Besides PBDEs, polybrominated biphenyls (PBBs) and hexabromocyclododecane (HBCDD) are examples of additive BFRs detected in biota samples at high levels.

Figure 4: General structure formula of PBDEs



There have been no reports of naturally occurring PBDEs in the environment, and commercial PBDEs are produced by the bromination of diphenyl oxide under certain conditions, resulting in products containing mixtures of brominated diphenyl ethers [World Health Organization, 1994]. Although it is theoretically possible to form 209 different congeners (depending on the number and position of the bromine atoms on the two phenyl rings) (Figure 4), PBDEs are commercially available as technical mixtures. Commercial PBDEs are produced with three degrees of bromination, a penta-BDE, an octa-BDE and a deca-BDE-mixture, which indicate the average bromine content of congener composition. Of

the theoretical 209 congeners, technical commercial PBDE mixtures contain a limited number of congeners that are less complex than those of PCBs. For instance, the penta-BDE mixture is composed of two major congeners (PBDE 47 and PBDE 99), representing ~80% of total product weight [de Wit, 2002]. In 1999, the global demand for PBDEs has been estimated to be close to 70,000 metric tones, 13% being penta-, 6% octa- and 81% of deca-BDE mixtures [Bromine Science and Environmental Forum, 2004]. The percentage of worldwide demand used in North America in 1999 corresponded to 98% of penta-BDE, 36% of octa-BDE and 44% of deca-BDE. Since tetra- and penta-BDE congeners (most predominant in penta mixtures) are the most persistent, it is not surprising that much higher levels of PBDEs are found in U.S. biota samples (including human samples). In the regulatory field, the two commercial technical mixtures, penta- and octa-BDE, have been phased out in Europe with a final ban in all applications for the EU market to take effect in 2004 [Bromine Science and Environmental Forum, 2004]. However, a U.S. ban of penta- and octa-BDE is still under consideration at the federal level [Bromine Science and Environmental Forum, 2004].

Constant monitoring of the environment has revealed an increase in the levels of PBDE over the last decades [Hites, 2004]. The few studies analyzing air samples report outdoor levels of 5-300 pg/m³, while indoor concentrations are higher, ranging up to ~1800 pg/m³. However, the concentration can range up to 67000 pg/m³ in some occupational settings. This value was observed in an electronics shredding plant [see review Hites, 2004]. Several studies in marine mammals have found a difference between mammals monitored in the Canadian Artic and those living in the coastal waters of mostly industrialized countries. The sum of all PBDEs in samples from the Canadian Artic was below 5 ng/g lipid, whereas the levels from other countries ranged from ~700 to ~5000 ng/g lipid during the 1990's [see review Hites, 2004]. Interestingly, the authors found that the doubling time of Artic samples was ~7 years, while that for the rest of the world was ~5 years. A similar trend was also observed in bird eggs collected from the Great Lakes region and Sweden from 1970 to 2000 [see review Hites, 2004]. In fish, there is almost no correlation between PBDE concentrations and sampling time, although most samples were collected during the 1990's with some in 2000 and 2001. There is no information on the concentrations in fish during the 1970's and 1980's, which may create a bias when determining the time-trend concentration. Nevertheless, concentrations vary greatly depending on the type of fish and collection site. For example, whitefish from the Columbia River have PBDE concentration ranging from 12

to 1060 ng/g lipid, depending on where they were caught [Hites, 2004]. In general, fish are less contaminated in Europe than in North America, with arithmetic and geometric means for the sum of PBDE being 120 and 49 ng/g lipid in Europe versus 1050 and 310 ng/g lipid in North America.

Environmental contamination levels are a reliable indicator of human exposure/contamination, since we are a top predator in the food chain. In fact, risks posed by brominated flame retardants have sparked a long debate after a constant increase in the levels found in human tissue. The next section provides a general overview of the PBDE levels to which humans are exposed.

1.3.2 - PBDE HUMAN LEVELS: A CAUSE OF CONCERN

Synthetic halogenated compounds including chlorinated dioxins, dibenzofurans, and PCBs have been identified as global environmental and human contaminants over the past 30 years. Their harmful effects on wildlife and humans have been extensively reviewed, although their mechanism(s) of action remains unclear. Continuous monitoring of human samples has revealed that PBDE levels have exponentially increased in the last decades (Figure 3). For example, an analysis of human breast milk between 1972 and 1997 showed a 60-fold increase in the PBDE levels in Swedish women [Meironyte *et al.*, 1999], and recent studies have reported much higher levels in human breast adipose tissue [She *et al.*, 2002] and human breast milk [Schecter *et al.*, 2003]. In the last years, a large number of articles have been published reporting very high levels of this compound in milk, blood and adipose tissue. Like environmental levels, an exponential increase with a doubling time of ~5 years has also been observed in human tissue, and the levels have risen by a factor of ~100 over the last 30 years according to Hites, 2004 [Hites, 2004]. Another interesting factor is that North American samples are always above the regression line by a factor of >10 (when the data from all countries are plotted together) and the Japanese always below by a factor of ~5 with Europe in the middle range. European and Japanese demand for PBDEs are mostly deca-BDE mixtures, whereas there is a massive demand for penta-BDE mixtures in the U.S., accounting for 98% of total world production [Bromine Science and Environmental Forum, 2004]. Deca-BDE congeners in the environment vary from nondetectable to low detectable levels, while tetra- and penta-BDE congeners are very persistent [World Health Organization, 1994]. Thus, it has been suggested that the "quality" of PBDEs has a direct impact on human

and environmental levels. Moreover, widespread use of manufacture products with flame retardancy mandated by federal law may account for the high levels in the U.S.

Congener-specific analysis in human samples should not be taken literally into account because only a few congeners have been measured thus far. However, it gives a rough, but extremely valuable, estimation of congener distribution. The most predominant PBDE congener is the tetra-BDE 47 (accounting for more than 50% of total PBDE), followed by penta-BDE 99 (the subject of the present investigation), hexa-BDE153 and penta-BDE 100 [Krupp *et al.*, 1988; de Wit, 2002; Kalantzi *et al.*, 2004; Sjodin *et al.*, 2004]. A recent study estimated the half-life of the most predominant congeners, PBDE 47, PBDE 99, PBDE 100, PBDE 154 and PBDE 153, which reported a very high half-life of 1.8, 2.9, 1.6, 3.3 and 6.5 years, respectively [Geyer *et al.*, 2004]. The predominance of these congeners with long half-lives in commercial mixtures is the key factor for their persistence in environmental and human samples. Given that PBDE are used in plastics and in electronic products, one would expect that workers involved in assembling or disassembling these products would have a higher burden. In a few publications, PBDE serum concentrations from occupationally exposed employees (involved in dismantling electronics like computers) were compared to those of non-exposed employees in the same facility. Although the data for occupational exposure are not as complete as than those for environmental exposure, a congener-specific analysis has shown that blood levels of exposed workers were twice those of the controls [Sjodin *et al.*, 1999; Thomsen *et al.*, 2001; Lado-Abeal *et al.*, 2003].

While there is a considerable amount of information on PBDE levels in human samples, no data has thus far been reported on their toxicological effects in humans. Several studies have only recently published experimental evidence from animal studies suggesting that PBDEs may interfere with normal developmental and physiological processes sometimes at dose levels close to human exposure. Moreover, congener-specific potency and a broad scope of toxic effects have been demonstrated for PBDEs similar to PCB-mediated toxic effects. A short description of the experimental findings will be presented in the next section.

1.3.3 – TOXICOLOGY AND EXPERIMENTAL STUDIES

Most experimental toxicological studies have used commercial PBDE mixtures, but a few investigators did use a single congener. The main criticism of using commercial mixtures is the comparably low purity of the mixtures and lack of knowledge about the

possible interference of other compounds (*e.g.* dioxins). The main toxicological effects and possible modes of action of PBDEs are summarized below.

In rodents, penta-BDE showed a low acute toxicity displaying an LD-50 (lethal dose necessary to kill 50%) in the range of 0.5-5 g/kg body weight (BW) [World Health Organization, 1994]. Clinical signs included reduced growth, diarrhea, piloerection, reduced activity, forelimb tremors, reddening around the eyes and nose and continuous chewing. The porphyrin concentration increased considerably after oral dosing with the commercial penta-BDE mixture, DE-71 (mixture of tetra- penta- and hexa-BDE), at 100 mg/kg BW for 13 weeks [World Health Organization, 1994]. No mutagenic activity was observed in the Ames test using several Salmonella strains under induced and noninduced microsomal activation conditions [World Health Organization, 1994]. Immunological effects were suggested in mice after exposure to DE-71; suppression of the anti-SRBC response was observed as well as decreased thymus weight [Fowles *et al.*, 1994]. The congener PBDE 47 markedly reduced the number of splenocytes in mice (C57BL) after daily oral administration for 14 days [Darnerud *et al.*, 1999].

Both commercial penta-BDE and single congeners have been known to affect thyroid hormone homeostasis by reducing serum thyroxin levels in rats and mice [World Health Organization, 1994; Hallgren *et al.*, 2001; Zhou *et al.*, 2001; Hallgren *et al.*, 2002; Zhou *et al.*, 2002]. This system seems to be one of the most sensitive to PBDE exposure. Effects on thyroxin levels were observed already at single dose of 0.8 mg/kg [Fowles *et al.*,1994]. Tetra-BDE congeners reduced thyroid hormone levels in female rats following 14-day oral administration (18 mg/kg) [Hallgren and Darnerud, 2002]. In another study, commercial technical mixtures were administered for 4 days at dosages of 0, 0.3, 1, 3, 10, 30, 100 and 300 mg/kg BW. Dose-related reductions in serum T4 levels were observed for penta- and octa-BDE but not for deca-BDE mixtures. When pregnant rats were treated orally with 0, 1, 10 and 30 mg penta-BDE mixture /kg BW from gestational day 6 to postnatal day 21, the fetuses and dams on gestational day 21 and the offspring on postnatal day 4 and 14 had lower serum concentrations of T4 in all doses tested [Zhou *et al.*, 2002]. Two mechanisms have thus far been proposed to explain the PBDE-induced hypothyroidism. PBDE metabolites are thought to bind to the thyroxin-transporting protein TTR, thereby decreasing the thyroxin levels in blood [Meerts *et al.*, 2000]. Moreover, PBDEs may induce the phase II enzyme uridine diphosphoglucuronosyl transferase (UDPGT), the enzyme involved in the metabolism of T4 that increases the rate of elimination [Zhou *et al.*, 2001]. However, insufficient

information and a lack of consistency in the set of data reported thus far warrant further investigation on the mode of action in thyroid disruption.

Technical PBDE mixtures as well as some congeners are able to induce both phase I and phase II detoxification enzymes in the liver, which are involved in the metabolism and/or metabolic activation of xenobiotics. Regarding cytochrome P450-mediated phase I metabolism, CYP 1A and CYP 2B families were induced as demonstrated by the increasing activity of liver microsomal ethoxyresurufin-*O*-deethylase (EROD), methoxyresorufin-*O*-demethylase (MROD) and penthoxyresorufin-*O*-despenthylase (PROD) after exposure to Bromkal 70, DE-71, DE-79 (pentaBDE mixtures) and PBDE 47 [Hallgren et al., 2001; Zhou et al., 2001; Zhou et al., 2002; Hallgren and Darnerud, 2002]. Other enzymes used as indicators of microsomal phase I activity were also induced by PBDEs, including benzphetamine N-demethylation, *p*-nitroanisole demethylase, aryl hydrocarbon hydroxylase (AHH) and benzo(a)pyrene hydroxylase [Carlson, 1980a; Carlson, 1980b; Trainor *et al.*, 2003]. In a 14-day study, penta- and octa-BDE mixtures, but not deca-BDE, induced long-lasting UDPGT activity in rats. In two studies (2001, 2002), Hallgren et al. demonstrated that Bromkal 70 and PBDE 47 also increased the UDPGT activity but to a lesser degree [Hallgren and Darnerud, 2002; Hallgren *et al.*, 2001].

Some studies have also shown that PBDE is a potent neurotoxicant displaying effects similar to PCBs. Based on the available data, this system seems to be the most sensitive to PBDE-induced toxicity. For example, mice neonatally and perinatally exposed to PBDEs have shown impaired sensorimotor development, altered locomotor activity and delayed development of spontaneous behavior [Branchi *et al.*, 2002; Branchi *et al.*, 2003; Viberg *et al.*, 2003a; Viberg *et al.*, 2003b]. When a single dose of PBDE 47 (0.7 or 10.5 mg/kg) or PBDE 99 (0.8 or 12 mg/kg) was administered to mice on postnatal day 10, these animals exhibited permanent aberrations in motor behavior. Moreover, learning and memory deficits were also observed in PBDE 99-treated mice [Eriksson *et al.*, 2001; Eriksson *et al.*, 2002]. Two studies suggest that cholinergic nicotinic receptors may also be a target of PBDEs, since a decrease in alpha-bungarotoxin binding in the hippocampus was found in mice neonatally exposed to PBDE 153 and the response of the cholinergic agent nicotine was also altered in mice neonatally exposed to PBDE 99 [Viberg *et al.*, 2002; Viberg *et al.*, 2003a]. In vitro, PBDEs were capable of inducing cell death in cerebellar granule cells in culture [Reistad *et*

al., 2002] and releasing arachidonic acid via the phospholipase A_2 pathway, which is associated with learning and memory [Kodavanti *et al.*, 2002].

The most relevant data on the toxicological effects of PBDEs were given above, leading to the conclusion that a greater effort should be made in the experimental field in the coming years. This is extremely important for identifying and assessing the risks to humans from this new environmental pollutant. The available data indicate that PBDEs display variable dose-related effects, suggesting various modes of action for this compound similar to those of PCBs. The actual gaps in our knowledge of PBDEs will be presented in the last section of this chapter as the motivation for the present investigation.

1.4 – MOTIVATION

There is still a lack of knowledge on penta-BDE, including the dose level employed in the published experimental investigations. Since the studies performed so far used high doses, we examined the effects of early developmental exposure to environmentally relevant levels, i.e. low doses, of penta-BDE 99, one of the most persistent PBDE congeners. Regulatory practices for human risk assessment and hazard identification of such chemicals have been criticized when extrapolation factors in the order of 1000 are used to bridge gaps in knowledge, especially at low dose levels. Regulators must extrapolate results not only from animal studies (typically from mice and rats) to humans but also from the very high doses usually used in animal experiments to the very low doses that are characteristic of human exposure for risk assessment [Calabrese, 2004; Calabrese, 2003]. This often creates biases and uncertainties due to the assumption of threshold dose, below which there is no risk of harm. Standard testing guidelines for chemical risk assessment have been developed by governmental (or intergovernmental) organizations in the U.S., Europe, and Japan to create regulatory approaches for non-carcinogenic and carcinogenic effects based on assumed modes of action leading to these effects. Guidelines for assessing non-carcinogenic effects assume that the mode of action for these effects is linear or threshold. This assumption is based on the existence of known homeostatic, compensatory, adaptive, and repair processes. Therefore, the focus of a quantitative dose-response assessment is the identification of a no observed adverse effect level (NOAEL) and lowest observed adverse effect level (LOAEL). An alternative to the NOAEL is the benchmark dose [Eaton *et al.*, 1995]. It has been suggested that developmental toxicities caused by EACs do not exhibit thresholds, and the use of the current non-carcinogenic methods would thus be inappropriate [Colborn *et al.*,

1996]. Although a review of biological phenomena in the literature reveals that linear and non-linear relationships are reported with about equal frequency [Goldsmith *et al.*, 1993], non-linear dose-response relationships are not considered in risk assessment procedures. Low doses were chosen as the focus here because we felt that substances should be evaluated in the range at which environmental exposure occurs. This was supported by strong evidence that chemicals harm human health, as demonstrated in epidemiological studies (presented at the beginning of this chapter) and because several papers on experimental field have shown that EACs affects at low doses [Howdeshell *et al.*, 1999; Welshons *et al.*, 1999; Markey *et al.*, 2001; Willingham, 2001; Kuriyama *et al.*, 2003, Salama *et al.*, 2003; Mayer *et al.*, 2003; Kuriyama *et al.*, 2004].

PBDE 99 is one of the major constituents of the penta-commercial mixture [Hale *et al.*, 2001] and is one of the predominant congeners found in animals and humans, including breast milk samples [Fernlof *et al.*, 1997; Hooper *et al.*, 2000; Meironyte *et al.*, 2001; She *et al.,* 2002; Zennegg *et al.*, 2003]. Its presence in breast milk highlights the importance of evaluating possible effects of early developmental exposure, since this is a critical period when an organism is extremely sensitive to minor fluctuations in hormone concentrations. It is known that the development of reproductive organs before and after birth is under hormonal regulation that is time- and concentration-dependent. Variations in the exposure time and concentration of regulating hormones can lead to serious developmental abnormalities, i.e., an organizational change, which could result in an irreversible manifestation at a later time in life. To test this hypothesis we administered PBDE 99 once during gestation at the beginning of organogenesis and due to its lipophilicity and long half-life (ca. 41.6 days in female rats) [Geyer et al. 2004]; the offspring should also be exposed through the lactational period.

1.5 – OBJECTIVES

The aim of the present study is to assess the effects of developmental exposure to low dose PBDE-99 on neurodevelopment and male reproductive health in rat offspring. Since this congener has a long half-life (about 41.6 days in rats) [Geyer *et al.*, 2004], we administered a single doses of PBDE-99 (60µg and 300µg PBDE99/kg BW) on gestational day 6, expecting exposure during organogenesis and lactation (about 37 days). Two independent experiments were conducted addressing different questions. In the first experiment, thyroid hormone levels, tissue distribution of the parent compound, and hepatic enzyme activities were measured in dams and offspring at different lactational time points. The second one was designed to evaluate the neurobehavioral and reproductive effects manifested from prepuberty to adulthood. A more detailed description of endpoints employed to evaluate such parameters is given below:

Experiment I

- Thyroid hormone levels in dams and offspring during lactation (postnatal day 1 and 22);
- Tissue distribution of the parent compound in liver and adipose tissue from dams and offspring;
- EROD and UDPGT activity in hepatic microsomes from dams and offspring during lactation.

Experiment II

- Developmental landmarks (time of eye opening, fur development, eruption of incisors, testes descent) and reflexes (cliff-drop aversion and rotating rod);
- Basal locomotor activity in male and female offspring on PNDs 36 and 71;
- Reproductive assessment in adult male offspring
    - Sex steroid hormones;
    - Sexual organ weights;
    - Sperm, spermatid counts and daily sperm production;
    - Testicular morphometry and sertoli cell count;
    - Flow cytometry analysis of testicular cell population;
    - Male reproductive performance;
    - Sexual behavior;