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Chapter I

Endocrine Disruptors: New Discoveries and Possible Progress of Evaluation

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Abstract

Life organization requires a sophisticated communication system between and inside cells; it has been well preserved throughout evolution. The hormones are the main leaders of this messenger system, which becomes more and more complex in multi-cellular beings. They act in the nervous and reproductive systems, and are sensitive to environmental interactions. For one half of acentury of intensification of the industrial era, more than 5 million man-made chemicals have been released in the environment without recycling, as if the ecosystems were infinite. These products were often designed either to be stable, as being rather insoluble (plasticizers – phthalates, PCB -, diverse oil residues, inks, insulating or other industrial residues like heavy metals...), and/or to be penetrating and active on the physiology of organisms (drugs, pesticides such as herbicides, insecticides, fungicides, raticides, etc.). These *xenobiotics* become excellent candidates for the disruption of the hormonal messenger system - known as endocrine - in the organisms, as well as the nervous system. They also disturb steroid metabolism, i.e. they are often oxidized through the cytochrome P450 family, making them close to estrogenic structures when they come from polycyclic hydrocarbons. There are also natural families of compounds with estrogenic effects, such as phytoestrogens, in soy and other vegetables, and mycoestrogens. This review will focus on the recent knowledge about these endocrine disruptors (EDs) that are present in all organisms, with recently discovered and unexpected modes of action. They act noticeably on the synthesis,

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storage, production, and transport of hormones themselves (steroidogenesis in particular), but also on metabolism, fixation, action or elimination of hormones, and not only on the direct modification of their effects. EDs also interact on epigenetics, which may influence gene expression over several generations. Moreover, EDs are likely to cause mutations contributing to genetic diseases.

Keywords: Hormones, Endocrine Disruptors, Xenobiotics, Xenoestrogens, Bioaccumulation, Combined effects, Long-term effects, Toxicology limits, Epidemiology limits

Abbreviations

A1254	Aroclor 1254
ABP	Androgen Binding Protein
ADI	Acceptable Daily Intake
AhR	Aryl hydrocarbon Receptor
AMPA	Aminomethylphosphonic Acid
AR	Androgen Receptor
bp	base pair
cAMP	Cyclic adenosine-5'-monophosphate
CAR	Constitutive Androstane Receptor
CBG	Corticosteroid Binding Globulin
CD	Chlordecone
cDNA	Complementary DNA
CIGPC	International Conference on the Management of the Chemicals
CREED	Cluster for Research on Endocrine Disrupters in Europe
CSCC	Cholesterol Side Chain Cleavage
CYP	Cytochrome
DDE	Dichlorodiphenyldichloroethene or,-dichloro-,-bis(-chlorophenyl)ethene
DDT	Dichlorodiphenyltrichloroethane or 1, 1,1-trichloro-2-(2-chlorophényl)-2-(4-chlorophenyl)ethane
DEHP	Di-(2-Ethylhexyl)-Phthalate
DES	Diethylstilbestrol
DHEA	Dehydroepiandrosterone
DNA	Deoxyribonucleic Acid
EC	European Community
ED	Endocrine Disruptors
EDEN	Emerging Diseases in a changing European Environment
EEC	European Economic Community
EFSA	European Food Safety Agency
EFTA	European association of Free Exchange
ER	Estrogen Receptor
EU	European Union
Fa32	Rat hepatic cell line
FAD	Flavin Adenine Dinucleotide
FMN	Flavin Mononucleotide

g	Gram
GMO	Genetically Modified Organism
GSH	Glutathione
GST	Glutathione-S Transferase
hCG	Hormone Chorionic Gonadotropin
HepG2	Human hepatic cell line
IC50	Inhibition Concentration at 50% or concentration inhibiting 50% of the system
IFEN	French Institute for Environment
INSERM	French National Institute of Health and Medical Research
JEG3	Human placental choriocarcinoma cell line
KB	Kilobase
KDa	Kilodalton
Kg	Kilogram
L	Liter
LXR	Liver X Receptor
MA-10	Mouse Leydig Cell Line
MC	Methoxychlore
MDR	Multidrug Resistance proteins
µg	Microgram
Mg	Milligram
mRNA	messenger RNA
MRP	Multidrug Proteins Resistance-associated
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
nM	Nanomolar
NP	Nonylphenol
Nrf2	Nuclear Factor-erythroid 2 p45-related Factor 2
OP	Octylphenol
P450	Cytochrome P450
P450arom	Cytochrome P450 aromatase
P450scc	Cytochrome P450 side chain cleavage
PCB	Polychlorobiphenyls
PCP	Pentachlorophenol
PCRD	Frame program of the European Community for Research, Technological Development and Demonstration
PgP	P-glycoprotein
POP	Persistent Organic Pollutant
ppb	Part per Billion
ppm	Part per Million
PR	Progesterone
PUNE	Program of the United Nations for the Environment
PVC	Polyvinyl chloride
PXR	Pregnane X Receptor
RCEP	Royal Commission on Environmental Pollution
REACH	Registration, Evaluation, and Authorization of Chemical products
RM	Reporter Member

RNA	Ribonucleic Acid
Rshbg	Receptor of the sex hormone binding globulin
RXR	Retinoid X Receptor
SAICM	Strategic Approach to International Chemicals Management
SHBG	Sex Hormone Binding Globulin
StAR	Steroidogenic Acute Regulatory protein
TBT	Tributyltin or (bis)-tributyltin
TBTO	Tributyltin oxide
TCDD	2,3,7,8-Tetrachlorodibenzo- *p* -Dioxin
TDI	Tolerable Daily Intake
TGFβ	Transforming Growth Factor-β
UGT	UDP-Glucuronosyl Transferase
VZ	Vinclozolin
WHO	World Health Organization
WHOROE	World Health Organization Regional Office for Europe
WWF	World Wild Foundation
293	Human Embryonic kidney cell line

Introduction

Life on earth and its organization requires a sophisticated communication system between cells, and inside them, from membranes to genes; it has been well preserved throughout evolution. Hormones are the main leaders of this true "cellular messenger system". It becomes more and more complex in multi-cellular beings. These beings have developed nervous and reproductive systems which carry most environmental interactions towards the main physiological functions, and thus these systems constitute major targets for environmental factors. For one half-century of intensification of the industrial era, more than 5 million man-made chemicals have been released in the environment without recycling [1], as if the ecosystems were infinite. These products were often designed either to be stable, being rather insoluble, and/or to be penetrating and active on the physiology of the organisms. Hence, these artificial agents called "xenobiotics" become excellent candidates for the disruption of the hormonal messenger system - known as endocrine - in the organisms. They act noticeably on the synthesis, storage, production and transport of hormones (steroidogenesis in particular), but also on metabolism, binding, action or elimination of hormones, and not only on the direct modification of hormonal effects. EDs also interact on epigenetic, i.e. on the heritable dressing of the genes, which influences in turn their expression on a long-term basis, for example over several generations. Moreover, EDs possibly cause mutations contributing to genetic diseases.

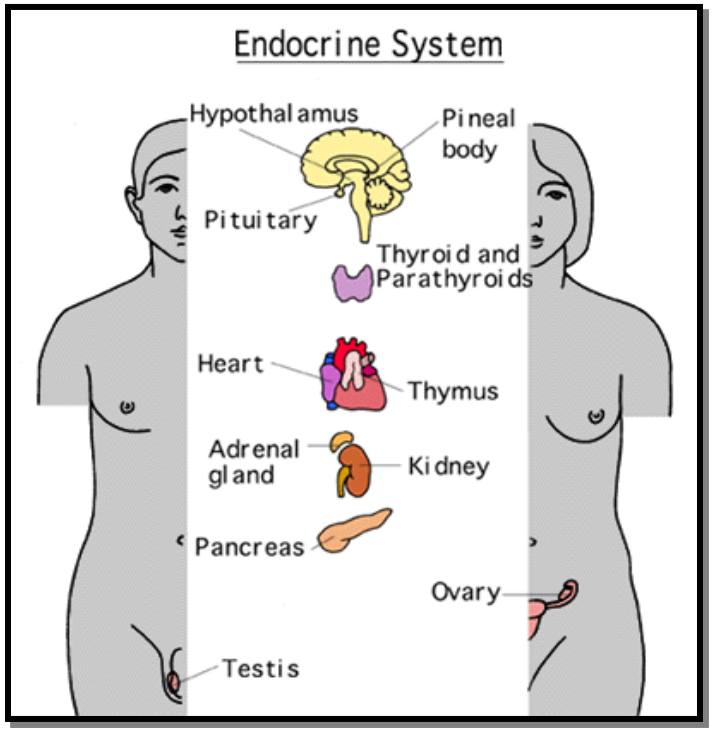


Figure 1. Various human glands secreting hormones. In addition there are: the epiphysis, the mammary gland, the placenta at the time of gestation, and various secreting cells scattered in the digestive system or the skin (according to www.umm.edu/endocrin/anatomy.htm).

I. Fundamental Knowledge on Endocrine Disruptors (EDs)

1. Definitions and Background

A. What Is the Endocrine System? Recent Discoveries

The endocrine system is a complex system, consisting of various secreting glands distributed in the organism, and which is very developed in the human species, at least in ten main tissues (Figure 1).

The hormones act as chemical messengers controlling all the organism functions. A particular subclass excreted out of the body is formed by the pheromones. These are "xenohormones" interfering even on the communications between organisms in animal and vegetable societies, like repulsive or sex appeal agents for example. The knowledge of these hormones in vertebrate species increased a lot recently [2], and the action of ED at this level cannot be excluded. The two hundred different cell types constituting the human body comprise hormonal receptors which partly control hormonal effects. These receptors or interactive molecules are from various types, or subclasses, even for only one hormone. They are both on the membranes and inside the cells. It has been understood that they manage first short-term actions, in particular non genomic ones [3], i.e. not going in first instance to the

genome, such as the opening of ionic channels [4]. Hormones also mediate classical and membrane stimulated genomic effects [5]. One can also note important epigenetic effects discovered more recently [6-8]. Today, endocrinology is considerably enriched by new concepts. Among these are the concept of neurohormones [9, 10], that includes sexual steroids formed by the nervous system, which are able to modify the behavior. There is also intracrinology [11, 12]. This concept, a subspecialty of endocrinology, is different from exocrinology (secretion of hormones out of the cell), paracrinology (regulation of the cells by proximity in a tissue, without the need for the hormones to enter blood circulation), and cryptocrinology (a subclass of paracrinology where cells are so close or inserted one in each other, like the spermatogonia in testicular Sertoli cells). The intracrinology corresponds to the metabolism of precursors (which can come from another organ) in active hormones playing a crucial physiological part, in particular in the same cell. For instance, dehydroepiandrosterone (DHEA) is converted into estradiol or testosterone and dihydrotestosterone in bones or epidermal tissues, to favor important local effects, such as bone densification or male pilosity. Intracrinology research also plays a crucial part in the treatment of hormono-dependent cancers.

B.What is a Definition for ED?

The endocrine disruption generally does not have a direct impact on cell death, and thus there is no acute toxicity; it acts at lower doses. However, it causes changes in cell physiology and communications, and thus possibly on health. A potential ED is, for example, a substance or an external mixture impregnating environment, and human or animal bodies, with the capacity to interfere on the production, metabolism, transport, or the effect of hormones. It will be an active – not only potential - "endocrine disruptor, ED" per se, if it is efficient *in vivo*. Numerous pesticides have been demonstrated to behave as EDs in mammals (herbicides, insecticides, fungicides, raticides, etc), and plasticizers (phthalates) too, but also oil residues and other chemicals, such as medicinal drugs polluting rivers (the Seine river, the Thames, etc.). Dose, time, and even period of exposure are important factors to take into account, i.e. the real exposure to the product. But the amount alone is not very informative, insofar as a negligible amount acting during months or years can be more disordering in a durable way (and even with transgenerational effects) than a short exposure to a high dose. So the concept of "threshold", without taking duration into account, is not really scientific. The World Health Organization (WHO) gave a definition of ED that the European Union (EU) adopted in 1999: *"An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism or its progeny or subpopulations".*

C. Why being Concerned with EDs?

The presence of xenobiotics in the environment causes major concerns. Indeed, up to now there are at least 100.000 substances created by humans deserving a better evaluation according to international agreements and new legislations (REACH), including 1.500 new molecules marketed each year. According to the program of the United Nations for the environment, this number will increase by 80% during the next fifteen years [13], in particular with nanoparticles and new materials. Many of these compounds were demonstrated to degrade the living resources and natural ecosystems, by regularly

contaminating water, the ground, food chains, and the animal or human tissues, even fetal ones [14-17].

In their February 2006 report, the European Food Safety Agency (EFSA) declares that the first concern of Europeans as regards food safety is the presence of pesticides in their food. The adverse effects of contaminating drugs are also a cause of fear; all these substances were indexed like having harmful actions on the reproduction, the growth, and the development of wild species, aquatic, and terrestrial.

Moreover, the frequencies of some disorders in the human reproductive system and some cancers increased recently, even creating the appearance of a new syndrome. It is called the testicular dysgenesis syndrome [18, 19]. Lastly, an international environmental concern is growing, very similar to the one on climatic changes, on the capacity of man to really transmit a planet in good health to its descendants.

Thus, EDs have overall effects which will be initially visible at the level of the physiological functions necessary for the survival of the individual, but also of the species interfering with its environment. Hence, the first end points are the nervous and reproductive systems. This review will primarily take examples based on these two systems.

2. History and Research Advances

In November 1677, thanks to the improvement that he brought about in the microscope, Antoni Van Leeuwenhoek discovered the existence of the "*animalcules*". Subsequently he fought against the concept of spontaneous generation. From then on, the role of microbes in the generation of pathologies began to be understood, until this study reached a sort of peak in the massive exploitation of this idea, together with the progress of Pasteur's medicine in the XX° century. The idea that the body plays a fundamental role in pathologies (notion of "*terrain*" initiated by Claude Bernard), and later the idea that heredity contributes to the development of some diseases, have gained ground since, and in particular with the discovery of the DNA structure in 1953. Consequently, the debate on the respective roles of environment and heredity in pathologies, characters, and behaviors, intervenes almost exclusively in the light of such outstanding discoveries (Figure 2). Hence the role of chemicals, other than directly toxic and/or pharmaceutical, has been neglected in the medical conception for decades until now [20].

To study the link between diseases and the microbiological world, mankind benefited from three centuries of experience, and from a whole century to develop preventions and treatments. However, it has only been a few decades since, at scientific and social levels, we really appreciate the chemical origin of physiological disorders, due to the substances that have just been invented, purified or developed by man (Figure 2). For instance, since 1950, we already knew indeed that the insecticide Dichlorodiphenyltrichloroethane (DDT) interfered in a clear way on the hormonal level by affecting the testicles of the cock [21], and that it was stored in fat, by inducing a deterioration of the mammalian hepatic cells of rats [22] from 1 to 50 ppm. But, it was only in 1962 that, thanks to the American biologist Rachel Carson, a fierce controversy has flared up about pesticides in the environment, with the publication of "*A silent spring*". The book was an indictment of pesticides (among others), accused to kill the wild fauna, to destroy ecological balances and to threaten the human species itself. At the beginning of the 1970s, it was only recognized that the professional or

continuous exposure to some pesticides or pesticide-containing plants acted negatively on fertility or health [15, 18, 23, 24].

However, the first international publications on the role of EDs will relate to the exogenous use of hormones carried out on purpose like prostaglandins in obstetrics [25] or in the control of fertility [26]. Even at the present time, research on the role of hormones or anti-hormones and inhibitors in the treatments of menopause or breast cancers, for example, is still based on the investigations quoted above [27]. The ED compounds involved in these studies can be regarded as drugs voluntarily used as EDs.

According to the 2005 National Research Program described by the French "management of economic surveys and environmental evaluation", there has been a growing realization that many substances likely to disrupt the animal and human endocrine systems are now present in the environment. The new awareness has especially grown since the 1990s. At that time, several studies were published on the decline of sperm quality and quantity [28-31], the increase in abnormalities of genital tract development [32], as well as the growth in the incidence of some human hormono-dependent pathologies from 1950 to 1990 [33, 34], which continues today [35] (Figure 3).

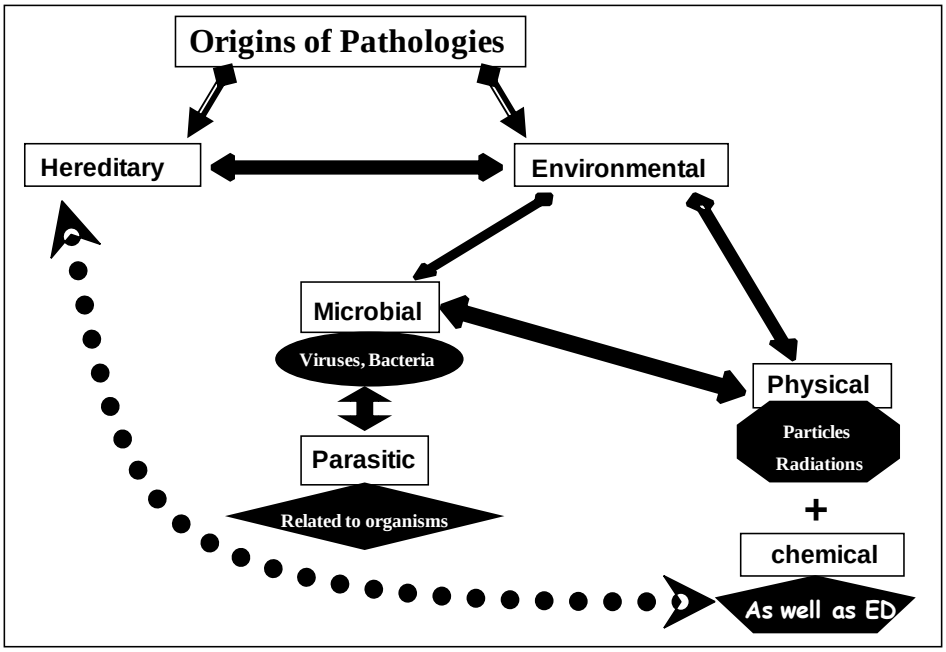


Figure 2. Origins of pathologies (see boxes above). : The various origins of diseases were not discovered simultaneously. As a matter of fact, two centuries and a half elapsed between the first discovery of the microbial origin of some pathologies and the understanding of the effects of (non highly toxic) chemical agents on life, in which process the Endocrine Disruptors play an important role.

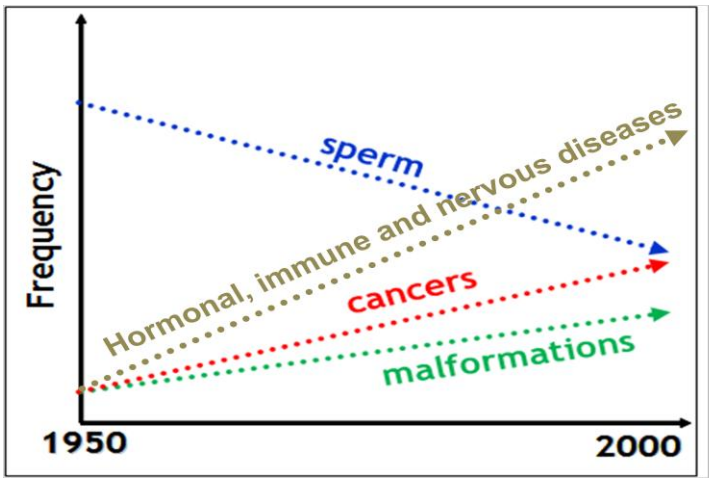


Figure 3. Incidence of environmentally-linked diseases. The fall in quantity and quality of human sperm, geographically variable, and the increase in testicular cancers, among others, together with the raise in neonatal genital malformations, constitute a new testicular dysgenesis syndrome. One can also note in industrialized countries an increase in all cancers, especially in children, as well as in hormonal, immune (including allergies) and nervous diseases.

As a matter of fact, new advances in our knowledge on the matter have been paralleled by the development of technical tools. In the 1990s, the scientific community progressed considerably in the understanding of hormonal receptors functioning (Estrogen and Androgen Receptors, ER and AR) and their binding to substances having mimetic effects to hormones, but also on chemical assays (36, 37].

In the last forty years, the new findings on the endocrine sophisticated networks led to an increased development of research in this field (38]; and some substances, which seemed the most likely to cause problems were withdrawn from the market. However some bioaccumulating pesticides or other persistent organic pollutants (POP) are still dreadful contaminants. Thus, many stable pollutants are still detected not only in wild animals, but also in all the food chain (17, 39, 40]. It is true in human milk, blood, and urine (41-43] through the whole planet, as in foods and many ground waters, in places where these substances are neither in use nor produced. Moreover, even if the short-term toxicological effects can be easily studied, the actual effects of EDs which are synergistic, additive, bioaccumulative, agonist or antagonist, long-term or differed, remain very controversial, little studied, and thus often neglected. Yet all these effects are different from the short term ones, at least *in vitro*, as we demonstrated in our laboratory (44-49].

3. Classification of EDs

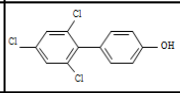
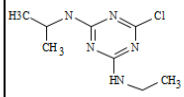
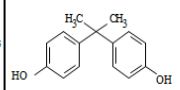
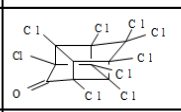
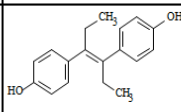
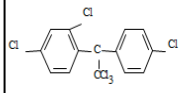
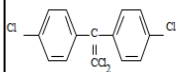
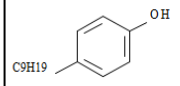
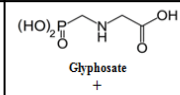
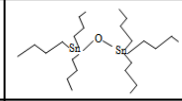
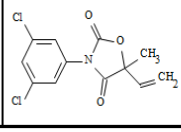
Because of the great number of xenobiotics, and their variety of structures and activities, several classifications are available. A large list of these products is known or suspected to have hormonal activities. We drew up a non-exhaustive list of the substances identified in many environments (Table 1).

Table 1. Non exhaustive list of suspected EDs, in particular on reproduction

Fongicides			
Benomyl	Hexachlorobenzene	Nabam	**Vinclozolin**
Etridiazole	Mancozeb	Pentachloronitrobenzene	Zineb
Fenarimol	Maneb	Triadimefon	Zira
Fembuconazole	Metiram	**Tributyltin Oxide (TBTO)**	
Herbicides			
Acetochlor	Ethiozine	Oxyacetamide	Triazines
Alachlor	Glufosinate-ammonium	Paraquat	**Atrazine**
Amitrole (aminotriazol)	Loxynil	Picloram	Simazine
Bromacil	Molinate	Prodiamin	2,4,5-Trichlorophenol
Bromoxynil	Nitrofen	Terbutryn	Trochlorobenzene
2,4-Dichlorophenol	Oryzalin	Thiazopyr	**Roundup**
Insecticides			
Aldicard	p,p'-DDD	**Endosulfan**	b-HCH
Aldrin	o,p'-DDE	**α-Endosulfan**	**Lindane** (g-HCH)
Bifenthrin	**p,p'-DDE**	**β-Endosulfan**	Methoxychlor
Carbaryl	**o,p'-DDT**	Endrin	Pyrethrines
Chlordecone (Kepone)	p,p'-DDT	fenvalerate	Ronnel (fenchlorfos)
Dibromochloropropane (DBCP)	Dimethoate	Heptachlor et H-epoxide	Toxaphene
DDT and its metabolites	Dinitrophenol	Hexachlorocyclohexane (HCH)	Transnonachlor
Other pesticides			
Ethylene thiourea	Pentachlorobenzene	Pentachlorophenol	Piperonyl butoxide
Industrial Products			
Alkylphenols		Dioxins (dibenzo-p-dioxin polychlorated, DDPC)	
4-OH-Alkylphenol		Furanes	
Nonylphenol (NP)		Hydroxy hydroquinones	
Nonylphenol ethoxylate (NP2EO)		Methylcolanthrene (MCA)	
Nonylphenol ethoxylate carboxylate (NP1EC)		Phthalates:	
Pentaphenol		Benzylbutylphthalate (DIBP-DEHP)	
p-tert pentylphenol (TPP)		Di-n-butylphthalate (DBP)	
Benzopyrene		Phenol	
Biphenyls polybromes (BPB)		Diphenyl ether polychlor	
polychlorated Biphenyls (PCB)		Phenylphenols	
Aroclor 1221		Resorcinol	
Aroclor 1254		Tetrachloro-benzyltoluenes	
Bisphenol-A (BPA)		Thiocyanate	
BPA dimethacrylate		Vinylacetate	
t-Butylhydroxyanisole (BHA)			
Vegetable Substances (mycoestrogens and phytoestrogens) and natural estrogens			
Coumestanes (coumestrol)		Diadzein	Genistein
Isoflavones (leguminous, soya)		Equol	Lignanes (lin, lentil)
Synthetic drugs and other estrogens			
Cimetidin		**Estrogens in the cow's milk and these derivatives**	
Diethylstilbestrol (DES)		Estrogens recycled in water	
Ethinylestradiol-17α (EE2)		Estrogens of the cosmetic products and shampoos	
Estrogens promoting the growth of the meats/poultry		Contraceptive Estrogens	
Heavy metals and others			
Aluminium	Cadmium	Mercury	Lead

Hereafter one finds many xenobiotics, besides natural hormones in medicinal drugs or in food (compiled from [14, 50, 51]. Some of the substances used in our experiments are in bold characters.

Table 2. Examples of various xenobiotic structures known as EDs on mammalian reproduction

Xenobiotics	Class	Chemical Structure	Activities and Effects	Studied models	References	ADI/TDI	NOEL	Half life	France Autorisation
Aroclor 1254 (A1254) MW 325.5	PCB: electrical insulator the cosmitique products		Estrogenic Aromatase Inhibitor	Receptors: ER Cells: 293, JEG3, Animals: Tortoise, Trout	Flouriot *et al.*, 1995; Willingham & Crews, 1999; Benachour *et al.*, 2007	0.0001 mg/Kg/D	0.02 mg/Kg/D	5 years in ground	**Still authorized in some cases**
Atrazine (AZ) MW 215.69	Herbicide		Estrogenic, Carcinogen, Aromatase Inhibitor	Receptors: ER Cells: H295R, 293, JEG3, Animals: Rat	Sanderson *et al.*, 2000; Fukamachi *et al.*, 2004; Benachour *et al.*, 2007	0.0005 mg/Kg/D	0.003 mg/Kg/D	> 100 D in ground	**Interdict in 2003**
Bisphenol A (BPA) MW 228.29	Industrial product Adjuvant of pesticides Plastic monomer Dental Cement		Estrogenic Anti-Estrogenic Anti-Androgenic Aromatase Inhibitor	Receptors: hER, hAR Cells: MCF7, 293, JEG3, Animals : Yeast, Salmon, Carp, Rat	Krishnan *et al.*, 1993; Sohoni & Sumpter, 1998; Rehmann *et al.*, 1999; Benachour *et al.*, 2007	0.05 mg/Kg/D	5-50 mg/Kg/D	182 D in ground and water	**Still authorized**
Chlordecone (CD) MW 490.64	Insecticide Fongicide		Estrogenic Anti-Estrogenic Anti-Androgenic Aromatase Inhibitor	Receptors: ER Cells: MCF7, HeLa, 293, JEG3, Hep Animals: Chicken, Hamster, Mouse	Gray, 1982; Shelby *et al.*, 1996; Blair *et al.*, 2000; Benachour *et al.*, 2007	0.0005 mg/Kg/D	0.05 mg/Kg/D	165 D in human blood 46 years in water	**Interdict in 1993**
Diethylstilbestrol (DES) MW 268.36	Drug Synthetic Estrogene		Estrogenic Carcinogen Aromatase Inhibitor	Cells: HEK 293, JEG3 Women exposed in utero	Brackbill & Berendes, 1978; Gill *et al.*, 1979; Benachour *et al.*, 2007	Drug 0.1 mg, 0.5 mg, 1 mg & 100 mg	5 mg/Kg/D	25 years inhuman reaches 2nd and 3rd generatio	**Interdict in 1977**
o,p'-DDT (DDT) MW 354.5	Organochlorinated insecticide		Estrogenic Anti-Estrogenic Anti-Androgenic Aromatase Inhibitor	Receptors: hER, hAR Cells: MCF7, 293, JEG3, Hep, Animals : Yeast, Rat, Salmon, Bird, Mouse	Bigsby et al., 1997; Sohoni & Sumpter, 1998; Monteverdi & Di Giulio, 1999; Lascombe *et al.*, 2000; Blair *et al.*, 2000; Benachour *et al.*, 2007	0.02 mg/Kg/D	0.3 mg/Kg/D	> 15 years in ground	**Interdict in 1972**
p,p'-DDE (DDE) MW 318.03	Insecticide DDT Metabolite		Estrogenic Anti-Estrogenic Androgenic Anti-Androgenic Aromatase Inhibitor	Receptors: hER, hAR Cells: 293, JEG3, Animals : Tortoise, Yeast					
Nonylphenol (NP) MW 220.34	Detergent Cosmetic Products		Estrogenic Androgenic Aromatase Inhibitor	Receptors: hER, hAR Cells: MCF7, 293, JEG3, HeLa, Animals: Yeast, Rat Salmon, Trout, Mouse,	Odum *et al.*, 1997; Sohoni & Sumpter, 1998; Benachour *et al.*, 2007	0.055 mg/Kg/D	50-100 mg/Kg/D	28-104 D in ground 150 D in water	**Still authorized**
Glyphosate (G)-based Pesticides MW 169.07 Roundup (R)	Herbicide very tolerated by GMO food	 **Glyphosate + different adjuvants**	Anti-Progesterone CYP450scc & Aromatase Inhibitors	Cells: 293, JEG3, Animals : egg of sea urchin, Rat, horse	Walsh *et al.*, 2000; Marc *et al.*, 2002, 2004; Richard *et al.*, 2005; Benachour *et al.*, 2007	0.3 mg/Kg/D	30-20,000 mg/Kg/D	> 200 D in ground 60 D in water	**Still authorized and very much used in the world associated with cultivated GMO**
Tributyltin Oxide (TBTO) MW 596	Biocide antifolling		Androgenic Aromatase Inhibitor	Cells: 293, JEG3, Animals : Oyster, horse	Le Curieux-Belfond *et al.*, 2001; Shimasaki *et al.*, 2003; Benachour *et al.*, 2007	0.00025 mg/Kg/D	0.025 mg/Kg/D	0.5-20 years in sediments	**Interdict in 1982 in some cases and completely in 2006**
Vinclozolin (VZ) MW 268.11	Fongicide		Androgenic Anti-Androgenic Aromatase Inhibitor	Receptors: hER, hAR Cells: 293, JEG3, Animals : Yeast, Rat	Gray *et al.*, 1999; Monosson *et al.*, 1999; Benachour *et al.*, 2007	0,025 mg/Kg/J	2.5-25 mg/Kg/D	Directly metabolized in two metabolites in ground	**Interdict since December 2007**

There are some very different structures from steroids (e.g. Glyphosate), with at least a common mechanism of action, interference with aromatase (according to [45]).

In our laboratory, we recently tested a great number of these products on a new pathway leading to ED that is the deregulation of steroidogenesis. We show, for instance, in Table 2 that steroid disruptors are absolutely not limited to compounds which structurally mimic steroid hormones. They may interfere with the cellular messenger system, hormonal transport,

or the genetic transcription factors, RNA, DNA, or even with unknown secondary metabolites.

4. Possible Action Mechanisms of EDs

It is difficult to determine accurately the various action modes for EDs and to check whether they can be simultaneous: concerning synthesis, release, storage, transport, metabolism of hormones, or receptor binding, activation, or modulation. It is also complicated to predict what will be the efficient *in vivo* ED dose, the minimum exposure level, also at which time of the development or position in the physiological cycle, and on which sex, on how many generations the action will take place. Moreover, as the hormonal system forms a complex network of interactions, we hereafter summarize both direct and indirect effects of EDs, all being non-exclusive of one another (Figure 4).

Among steroidal effects recently characterized are, thus, nervous effects (through neurosteroids) and membrane ones [52-54].

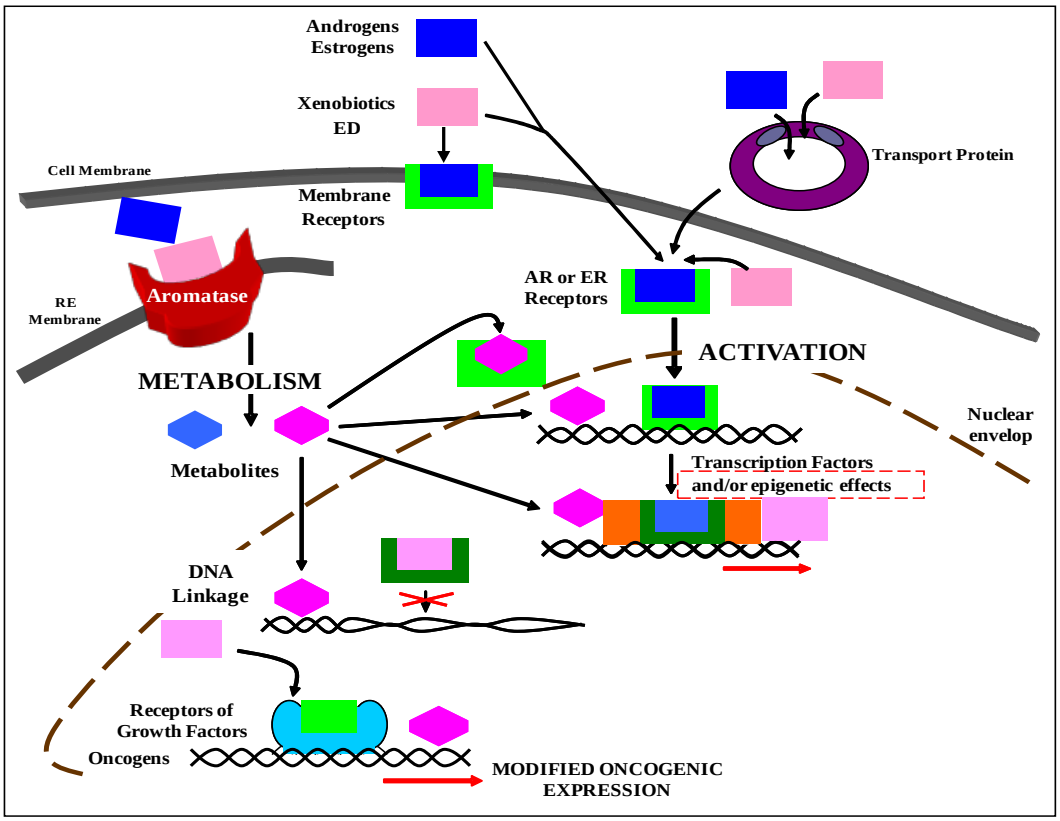


Figure 4. Potential effects of xenobiotics on the endocrine system. They are androgenic or estrogenic-dependent in particular. Membrane or nuclear receptors, transport proteins but also steroidogenic enzymes, including aromatase, are potential targets for xenobiotics. Moreover, enzymes can metabolize these into compounds susceptible to transactivate receptors (for androgens, estrogens or growth factors...) or to disrupt other pathways able to modify gene expressions, implied for instance in cancers. Xenobiotics can also directly bind DNA, or change its chemical “dressing” through epigenetic effects more recently characterized.

The comparative detailed knowledge of molecular ED actions in various species was significantly developed during the last years. Comparative endocrinology dissected the cellular and genetic reasons of differences between species [55-57]. This work rendered possible today to trace parallels or not between the various hormonal disruptions caused by the same family of products on wild and farm faunas and on humans.

A. Disruption of Hormone Receptor Activation

The ED action on hormonal receptors is the most classically studied (Figure 4). Through direct receptor binding, EDs can have a competitive effect with the endogenous hormones and start the usual cascade of intracellular signals stimulating gene expressions and protein synthesis. As it is shown in Table 2, with references, the toxic substances called in this case "agonists" are generally described as estrogenic compounds if they bind ER, and androgenic ones if they bind AR. If they do not transmit the signal, but simply block the receptors or their pathway, they are called anti-estrogens or anti-androgens. Several actions are possible at the same time, estrogenic and anti-androgenic for example. Among a number of estrogenic toxic substances, are Bisphenol-A (BPA), Chlordecone (CD), Methoxychlore (MC), Octylphenol (OP), and Nonylphenol (NP). Various *in vitro* tests can be used to measure ED activity, for example the ER binding test, the test of proliferation of breast cancer cells and transcriptional activation. There are also natural families of products with estrogenic action, such as phytoestrogens like isoflavonoids present in a variety of plants like soya, or in bays, fruits, seeds, and vegetables (lignanes).

In addition, Vinclozolin (VZ), a fungicide, which is an anti-androgen, and its metabolites, inhibit competitively the androgen-AR binding. By contrast to MC, VZ and its metabolites do not act on ER, but are anti-androgens, acting on Androgen Receptor (AR) [58]. Other toxic substances wear also an anti-androgenic activity such as Dichlorodiphenyldichloroethane DDE (the metabolite of DDT), an MC metabolite, but also Fenitrothion (an organophosphate) and the fungicide Procymidone [58]. To quote some additional activities, some Polychlorobiphenyls (PCB) and dioxins (for example the 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, TCDD) act on the cytoplasmic Aryl hydrocarbon receptor, AhR, and stimulate the signaling pathways, the expression of growth factors and the enzymatic activities.

Other toxic substances start up signaling cascades which modify the receptor biochemical structure. The phosphorylation refers to the addition of phosphate groups by means of an enzyme called protein kinase. Phosphorylation of a compound, e.g. a hormone receptor, modifies its interactions with other molecules, its binding properties and its functions. For example, Phenobarbital controls the transcriptional activity of the Constitutive Androstane Receptor (CAR) by increasing its shift from the cytoplasm to the nucleus. The use of okadaic acid, an inhibitor of phosphatase, showed that Phenobarbital increased the phosphorylation of CAR. In humans, the activation of CAR is associated with an induction of genes coding for CYP such as CYP2B6, CYP2C9, CYP2C19 [59]. Indeed, CAR interacts with two endogenous metabolites of testosterone: androstanol and androstenol [60].

B. Disruption of Transport Proteins

Many EDs are able to bind the Sex Hormone Binding Globulin (SHBG) (Figure 4), and even the Corticosteroid Binding Globulin (CBG), as these serum proteins are from hepatic origin and in charge of the transport and activity modulation of the vast majority of steroids circulating towards their action sites [61]. This is true for DDT, PCB and their derivatives,

MC, Atrazine, Lindane, Pentachlorophenol, BPA, OP, and NP [62]. Some phytoestrogens like Genistein have also the capacity to bind SHBG [63]. The various studies quoted above show that xenobiotics bind SHBG with either a higher or lower affinity than steroids; however, even in the case of a lower affinity, they can paradoxically quite well amplify its disruption. They can be fixed to the free SHBG neo-synthesized by the liver, where they abound since it is the major gland of detoxification. Then, SHBG is inevitably conducted by the blood flow to the gonads, where it is loaded with steroids. The xenobiotic parasites will then be driven out by them, going to sensitive organs which will then be contaminated [63], for example by means of cellular infiltration, then junction to the DNA [20]. In addition, the SHBG also intervenes within the context of an endocrine answer independent of the entry of the hormone in the cell [64]. It can bind to a membrane receptor (Rshbg) coupled with the G-proteins. The steroid/SHBG/Rshbg complex then activates the G-protein and the adenylate cyclase. The AMPc produced this way can affect the activity of the hormono-dependent genes [64]. When entering in competition with the endogenous steroids in this system, the EDs could disrupt the hormonal answers.

The xenobiotics can bind not only to SHBG but also to the Androgen Binding Protein (ABP) synthesized by the same gene [65], which remains intra-testicular or intra-cerebral. Hence they have potentially a capacity to modify actions, productions, even concentrations, in particular, of testosterone in the seminiferous tubules and, through this mechanism, to alter spermatogenesis [62].

C. Disruption of Steroid Biosynthesis

Steroidogenesis is the biosynthesis in particular of sexual steroids (estrogens and androgens), this could be a target for environmental compounds [66]. For a long time, estrogens were regarded as female sex hormones and androgens as male ones. However, the two types of steroids are present in both sexes [67-69]. In fact, the sexual differences are more quantitative than qualitative since in the female, the blood rates of estrogens are higher than those of the male, but lower than found for intra-testicular concentrations [70]. There are even cases, in the young horse, where the synthesis of testicular estrogens is more elevated than in the filly, even if they are then conjugated [71, 72]. However, the estrogen-dependent phenomena appear with lower concentrations in the male than in the female (Figure 5), and the naturally-inhibiting effects of estrogens are generally reached in the male at rates which are still stimulating in the female [73].

Therefore, the role of estrogens is major relative to the reproductive function. Taking into account the global physiology of the cells which are sensitive to estrogens, it appears that the androgen/estrogen ratio is more important than the action of a sole hormone in both sexes [74]. Thus, the androgen-estrogen balance (Figure 5) is crucial for functions such as oocyte maturation [75] or spermatogenesis [76], and otherwise ossification, or brain function. The androgen/estrogen ratio is under the control of a key enzyme at the last step of steroidogenesis, the aromatase, which catalyses the irreversible conversion of androgens into estrogens [77, 78]. It represents the estrogen action limiting factor in a normal physiology. This conversion is the reaction known as aromatization [79]. This enzyme was selected for this reason by our laboratory. Consequently it constitutes a new ED target for xenobiotics, and happens to have not yet been largely studied.

Aromatase is an enzymatic complex (Figure 6) localised in the endoplasmic reticulum membrane of steroidogenic cells. It is composed of two coupled enzymes: the cytochrome

P450 aromatase or P450arom [80, 81] and the ubiquitous NADPH reductase [82]. The nature of the estrogen produced on each site of biosynthesis depends on the nature of the androgen mainly available. The two moieties are anchored in the membrane by their N-terminal end [77]. Hence, P450arom is a hemic protein belonging to the vast enzymatic family of cytochromes P450, involved in particular in detoxification. It lies more precisely within the sub-group of steroid hydroxylases [83]. The members of this super family have a cysteine preserved, responsible for a characteristic absorption spectrum at 450 nm. It serves as the fifth ligand with the hemic iron. The reductase does not belong to the super family of the cytochromes P450, but it is a ubiquitous flavo-protein containing two flavins, FAD and FMN [84, 85]. It ensures the electron transfers, necessary in particular for aromatization, from NADPH, via its two flavins up to the P450arom, or to any other microsomal cytochrome P450 with which it comes into contact.

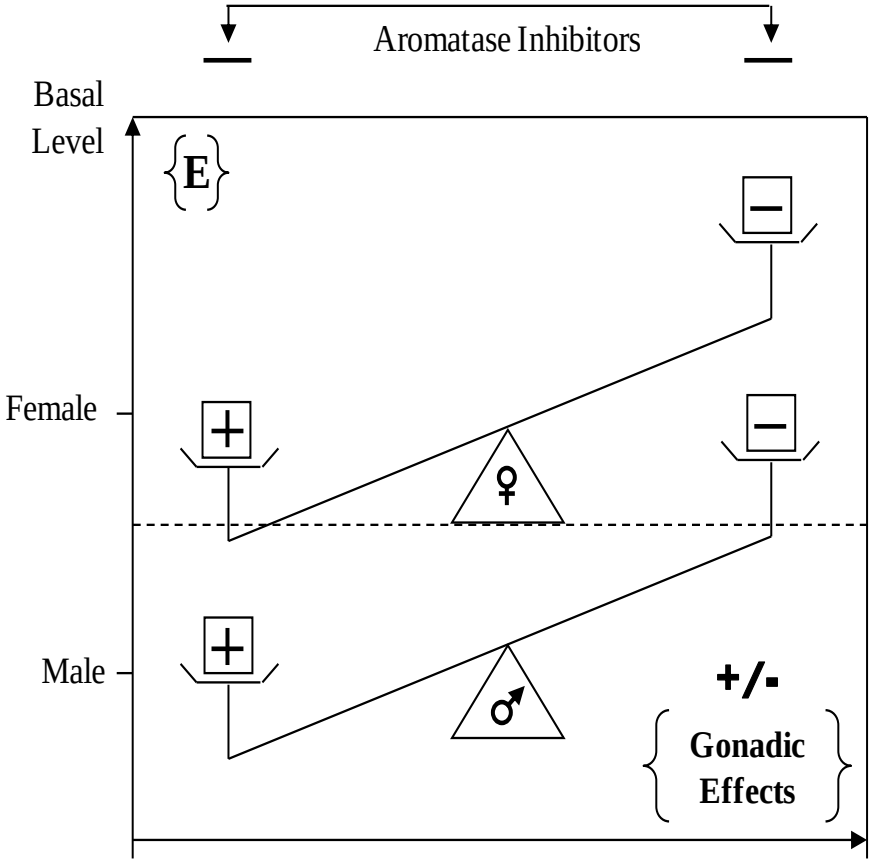


Figure 5. Estrogenic balanced effects in male and female mammals. In the majority of the mammalian species, males have lower estrogen [E] levels than females have. The positive effects of estrogens on gonadic physiology (mainly spermatogenesis or ovulation) are reached at lower levels in the male than in the female, just like the inhibiting effects. The inhibitors of aromatase can modulate the endocrine balance. Above the dotted lines is the representation of the traditional vision; below, news discoveries. Serum levels of estrogens and the gonadic function (a stimulation: + or an inhibition: -) are schematized.

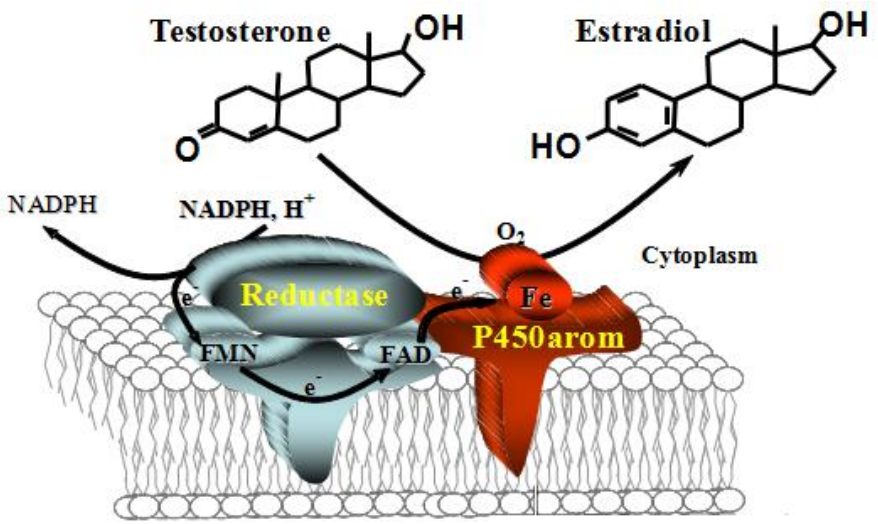


Figure 6. Cellular aromatase localised in steroidogenic cells. This complex is constituted by the cytochrome P450 aromatase, in charge for the binding and conversion of androgens into estrogens, such as testosterone into estradiol (above example). It also involves the flavo-protein NADPH-reductase which transfers the electrons necessary for this reaction (according to [86]) within or at the surface of the endoplasmic reticulum.

Human P450arom is coded by the CYP19 gene (Figure 7), the only member of the 19th family of cytochromes P450 rich of 267 families, which contain in total more than 5000 genes [87]. The name CYP19 is used to point out that it is the angular methyl group on C19 of the substrate which is attacked by oxygen. The CYP19 gene is localised on the human chromosome 15 at q21.2 [88]. It extends on more than 123 kb, it is one of the longest CYP genes and a good regulation model for differential tissue expression. The coding area contains 9 exons (II to X), like other cytochromes P450, which have in general between 8 and 10 exons [89-91]. The hemic region, in particular, is localised in the last exon. Only the area of 30 kb in 3'-end codes for P450arom, whereas the area of 93 kb in the 5'-end is mainly useful for regulation.

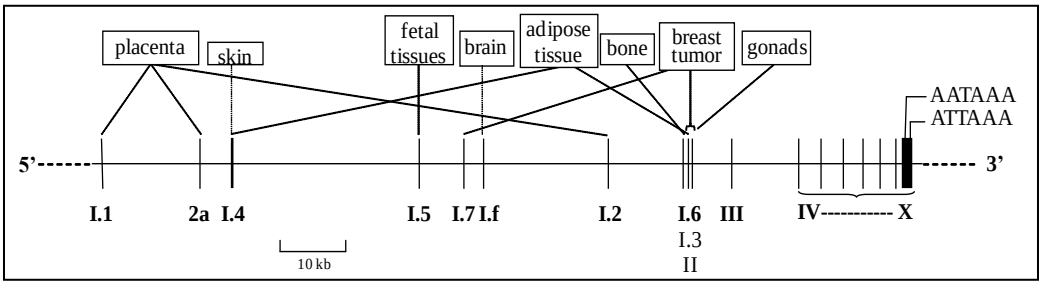


Figure 7. Human aromatase gene (CYP19). Its overall length is more than 123 kb. The horizontal feature represents a fragment of the long arm of the chromosome 15, on which the coding parts (exons) are dispersed, depicted as vertical bars, with their numbers. The sequences indicated on the right are the termination signals, and the sequences where the gene begins to be used in various organs are indicated, which underlines the multiplicity of the possible regulations. Also worth noticing is the shortness of the sequences (90-1580 bp) which will be translated into protein (primarily vertical bars II with X), as compared to the whole gene (according to(20); compiled from literature references).

In spite of multiple tissue localization, which could illustrate a ubiquitous character, the aromatase expression is controlled in a very precise way by the differential use of various promoters in each tissue, and according to physiological conditions. Indeed, the factors and signaling pathways which stimulate or inhibit the aromatase expression vary according to tissues. Ten primary exons controlled by distinct promoters were identified by the untranslated 5'-mRNA end of P450arom. The alternative use of each promoter determines the rates of each mRNA. There is a splicing of the primary exon to a common junction on all transcripts, located 38 bp upstream from the initiation site of the translation (ATG), which is located in the first coding exon. It is the molecular regulation mechanism of the aromatase expression in different ways according to tissues, age or physiological conditions, but then leading to a protein which should in principle be identical in all tissues [77, 78, 92, 93].

Then, the aromatase could interact with environmental pollutants able to disrupt the androgen-estrogen balance (Figure 4) and its modification could be at the origin of pathological processes. As a matter of fact, the contribution of environmental factors, in brain or breast cancers in particular, can be determining [94, 95]. Moreover, under or over-expression of aromatase contributes to the development of various pathological processes, involving abnormalities of bone development, reproduction, and in particular, hormono-dependent cancers, as was shown by research on animal models or on human genetic mutations [27]. With this in mind, we have developed some specific aromatase inhibitors in order to decrease the impact of hormono-dependent cancers. These inhibitors are effective on human cells [27].

Actually, some studies, and in particular our work on aromatase, highlight the interactions of xenobiotics with steroidogenesis [44-46, 48, 96-99]. The effects measured on cells or on living organisms will be exemplified in paragraph II.1.D.

D. Other ED Effects

As we saw in Figure 4, the ED effects cannot be limited to the three above-mentioned steps. For example, independently of the interference between the hormone and the receptor, the ED can interfere on the signaling pathway or on the receptor action, or on another way, or with a cofactor, or directly with the DNA itself. In the last case, for example, it could have a mutagenic action, or simply be inserted between the bases. Consequently it can even be brought towards specific genes, targeted via the polluted receptors. The issue of ED action on the external dressing and imprinting that is on the form of genes (II.2.A) will be further addressed in the following review. It does not exclude the connection with matrix proteins or carbohydrates, as this issue is little researched. The above-mentioned list is not exhaustive. It extends of course to different hormonal systems, like the glucocorticoid one, or to non-steroidal ones like the thyroid, pancreatic, or adrenal systems. All the hormonal diseases have fundamental molecular mechanisms that can be affected by the action of the listed EDs.

5. Xenobiotic Receptors and ED Metabolism

In the same way as all endogenous molecules do, xenohormones may have several roles besides the ED one, i.e. cytotoxic or physiological. Which means the xenobiotics will be eliminated by the immune system, but only if they are large enough, or if they form a complex with other molecules [100], who will play the part of haptens. They will also

generally be metabolized by another system of defence, to be specific, the intracellular system, which has evolved in a more and more sophisticated way during billions years of evolution. It was set up by living beings in order to neutralize and eliminate not only endogenous but also foreign substances relative to their physiology. To do so, a cell will ceaselessly endeavor to make the complex artificial molecules soluble, and in particular by generating hydroxyle groups, via a grouping of detoxification enzymes often located in the endoplasmic reticulum. Among them, the main ones are the cytochromes P450, formed by more than 481 genes [101, 102]. The nuclear receptors of xenobiotics are numerous. Among them, one can find Aryl hydrocarbon Receptor (AhR), Constitutive Androstane Receptor (CAR), the Liver X Receptor (LXR), Pregnane X Receptor (PXR), Retinoid X Receptor (RXR) and Nuclear Factor-erythroid 2 p45-related Factor 2 (Nrf2). They were shown to play a role of key mediators for the intervening enzymes in phases I and II of the xenobiotic metabolism, and also for the protein-conveyors intervening in phase III in the mechanisms of efflux and expulsion [103]. It was highlighted that the cytochromes like CYP1 and CYP3A are co-induced with transport proteins MDR and MRP, in particular via the activation of receptors PXR and CAR [104-106]. The organs which have the major content in these molecules play the major role in detoxification; in the mammals they are the liver, the kidneys, the intestines, the skin, and the lungs. They have also the advantage of being excretion bodies and possessing an interface with the environment in most cases. This metabolism is generally carried out in 3 phases (Figure 8):

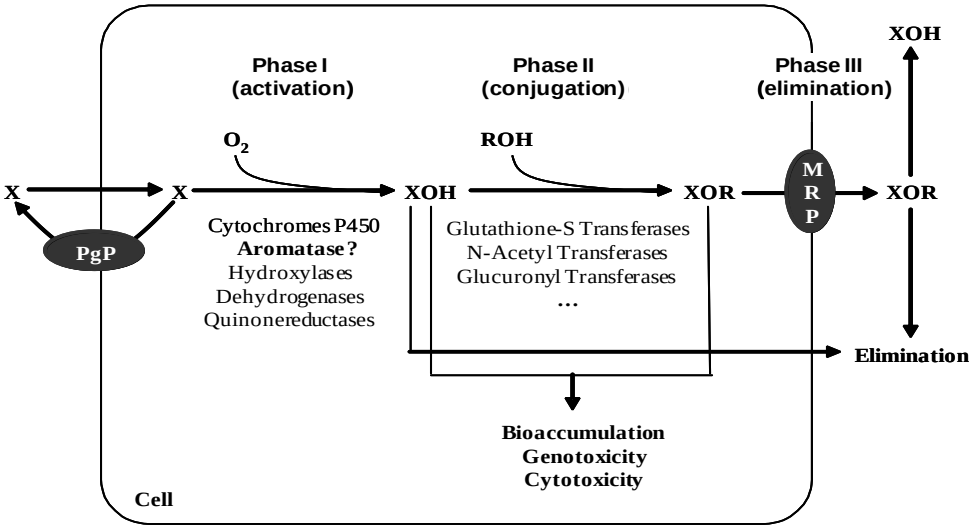


Figure 8. Cellular metabolism of the xenobiotics. The xenobiotic (x) can be eliminated directly by proteins like PgP (P-glycoprotein) or after the metabolism by transport proteins from phase III, the MRP (Multidrug Resistant-associated Protein) or they can be activated by enzymes of phase I, such as cytochromes P450, in hydroxylated compounds XOH after/or combined by enzymes of phase II in XOR with addition of acetyl or monosaccharide radicals R. the products of this metabolism can be partly eliminated or bioaccumulated. Paradoxically, solubilisation and elimination systems, if they are saturated or put out of order, can lead to toxic activations, by forming adducts for example.

Table 3. Non-exhaustive list of the principal human cytochromes, taking part in the xenobiotic metabolism (according to [108, 109, 111])

Family Subfamily	Genic expression	Localization of protein	Principal function
CYP1			
CYP1A1	induced by the xenobiotics like the TCDD and the components of the cigarette smoke	extra-hepatic tissues	metabolism of the carcinogenic compounds (activation procancerigen)
CYP1A2		liver (13% of the CYP of the liver)	metabolism of drugs, activation procancerigen
CYP1B1		several tissues	activation mutagen procancerigen and, hydroxylation of E2
CYP2			
CYP2A6 CYP2A7	expression of the CYP2A6 and 2A7 induced by the xenobiotics	liver	hydroxylation of coumarin
CYP2B6 CYP2B7		slightly in the liver	CYP2B6 is implied in the metabolism of coumarin and lidocaine, the hydroxy-lation of testosterone in 16α- and 17β-
CYP2C8 CYP2C9 CYP2C18 CYP2C19	expression of the CYP2C induced by phenobarbital and the rifampicine	liver (20% of the CYP)	metabolism of drugs, CYP2C8 is implied in the metabolism of the retinol and the retinoic acid
CYP2D6		liver (1 to 2% of the CYP)	metabolism of drugs
CYP2E1	induced by the ethanol	adult liver, lung, brain, cells endothéliales of the umbilical vein	activation of diseases (alcoholism, cancer), metabolism of drugs
CYP2F1		lung	activation of the pneumotoxin 3-methyl-indol
CYP2J2		liver, gastro-intestinal tract, kidney	hypoxidation of the endogenous acid arachidonic
CYP3			
CYP3A4	induced by the glucocorticoids, the antibiotics (phenobarbital, rifampine), inhibited the ketaconazole, the cimetidine and grape juice	adult liver (30-40% of the CYP)	oxidation of drugs, activation procancerigen, metabolism of the endogenous steroids
CYP3A5		liver, kidney, lung, stomach	oxidation of drugs and the endogenous substrates, activation procancerigen
CYP3A7		fetal liver (30% of the CYP)	hydroxylation of testosterone in 6β

A. Phase I: Activation

Phase I, known as the activation phase, implies hepatic enzymes of oxidation whose majority belong to the super-family of cytochromes P450, such as CYP1, CYP2, and CYP3 families, whose number indicates the carbon onto which they are attacked in the molecule. The other enzymes are hydrolases or dehydrogenases and quinonereductases. The phase consists in adding an electrophilic group to the substances to be eliminated, such as the ED in the case studied [102, 107-112]. For example, CYP1A1, CYP1A2, and CYP1B1 families detoxify or activate many environmental pro-carcinogens, toxins, drugs, and medicines (Table 3).

B. Phase II: Conjugation

Phase II, or conjugation phase, implies conjugation enzymes, among which we find the sulfotransferases, the N-acetyl transferases, and the glucuronyl transferases [111, 113]. The metabolites formed during phase I are combined with an absorbent grouping and in particular

a sulphated, acetylated, or glucuronylated one, the purpose of this being to support their solubility and their cellular elimination. The secondary metabolites thus formed can sometimes be more cytotoxic than their primary molecules, or carcinogens, immune-toxic or mutagen by their covalent interactions with the cellular macromolecules (DNA, RNA, or others). It is the case of the DDE which is the main metabolite of the DDT insecticide. It was shown that this metabolite induces a reduction in the expression of the CYP1A during phase I of the metabolism, and during phase II it causes also a phase shift involving UDP-glucuronosyl transferases (UGT) [114]. The study of Dierickx in 1999 [115] shows that the Alachlor, Metolachlor and Propachlor herbicides induce an expression of the CYP1A1/2 for two hepatic cell types, human HepG2 or rat Fa32 cells. However, they cause a very strong reduction of endogenous glutathione (GSH) by increasing the activity of the glutathione-S transferases (GST), only on rat Fa32 cells.

C. Phase III: Elimination

Phase III is simply a phase of elimination which can be carried out thanks to transport proteins P-glycoprotein (PgP) and Multidrug Resistance Proteins (MRP or MDR), which transport the xenobiotics through membranes, as well as their combined metabolites, and especially their derivatives [59]. These conveyors have been shown to be of great importance, owing to the fact that they significantly contribute to the pharmacokinetics of the drugs [116]. They increase after ingestion of a xenobiotic compound. In addition, it is reported that the MDR and the MRP play also a vital role in the elimination of the endogenous compounds by the liver, and in the regulation of the biliary acids. In particular, they can detoxify the xenobiotics combined with glucuronic acids, sulphates and glutathion acids [106, 117]. It was noted that PgP are the first conveyors activated for the outgoing flow of unmodified xenobiotics (hence, not combined), whereas the MRP are rather predominant, and directed against the products of phases I and II; they appear in combined soluble forms [104, 118, 119]. For instance, a study highlighted the differential effect of the DDT pesticide with respect to its metabolite DDE on the expression of PgP [120].

II. Physio-pathological ED Effects

1. Pathological Evidence

A. Concerning Reproduction

Last century was marked by production, use, and release in the environment of significant quantities of chemicals, thus disrupting the cellular messenger system. That was required, but only on some levels of the ecosystem (to kill the plants and insects as in the case of the pesticides), without envisaging the persistent contaminations of the grounds, water, or the side effects on other levels (birds, fish, batrachians, and mammals, for example). Or even the disruption caused can be inherent to the desired effect (i.e. electrical insulators which continue to play this role once introduced). However, among the most sensitive cells are those which depend on the sex hormones and multiply in great quantities, like the spermatogonia, the sexual stem and embryonic cells. The spermatogenesis represents undoubtedly the most fantastic cell multiplication in an organization. Ovogenesis is also extremely significant at

one key period of the development, and their integrity depends on fine mechanisms of correction of the DNA, and balance between apoptosis and cell differentiation. That is controlled by hormones, just like the formation of glands and genitals, and any disruption of their action will ultimately manifest itself by an effect on reproduction.

In Males

The number of abnormalities of sexual development in men, such as malformations of the genital and urinary tracts, has increased since the 1960s [15, 28, 29]. Moreover, the number of certain infertilities grew between 1970 and 1993, such as hypospadias, a neonatal anomaly in which the urethra does not open at the end of penis [121], as well as the cryptorchidism, which is when testicles fail to go down into the scrotum. This has happened in the United States in particular [34]. It has also been reported that the quantity and the quality of human sperm, have declined since 1930 in several countries. The reduction is mainly characterized by a decrease in the volume of the ejaculate [28, 31] and in the concentration in spermatozoa, but also by the deterioration of their morphology and their motility [30, 31]. As a matter of fact, the reduction in the density of human sperm is a cause for infertility in 43% of a Danish male population [122]. The decline is generally linked to disruptions of the environment. For example, it was shown that men having consumed contaminated fish, in particular when PCB was involved, were not able to conceive when tested for 12 months [123]. Other studies indicated that an environmental or professional exposure to high concentrations of pesticides or PCB can result into less birth of boys than girls. In addition, the sons of women exposed to Diethylstilbestrol (or DES) also presented alarming abnormalities. DES is a synthetic estrogen which has been used as a medicine to avoid miscarriages for more than thirty years, without proving to be effective [124]. At puberty, the second generation showed strong rates of malformations of the genital and urinary tracts [32]; moreover, in some cases, an associated sterility, and even nervous or psychological disorders. One wonders as well about the effects on the third generation. These discoveries show that antenatal exposure at EDs, or exposure during critical periods of the development, can affect, among other things, the reproductive system of adults [125], and cause genital malformations [35]. For example, the BPA impairs the development of the male reproductive tract in the rodents with such low doses as a few parts per billion (ppb), amount to which the humans are usually exposed [125]. BPA exposure also caused detrimental effects to human placental cells [126].

In the case of laboratory animals, rats exposed *in utero* to a dioxin, the TCDD, have a spermatozoa numeration 74% lower than the controls [127]. The industrial chemical compounds of the phthalate family induce a reduction of the number of spermatozoa and fertility in rodents [128, 129]. In the same way, the male alligators living in a Florida lake, which are contaminated by pesticides, have 3 times at least less testosterone than their fellow creatures of the uncontaminated lakes, and also present testis malformations, as well as a small size penis [130]. Moreover, male mice, exposed before birth to low doses of BPA (the estrogenic compound), present a larger prostate than that of the non-exposed mice. The BPA is a polycarbonate compound, a plasticizer used in food industry packaging and in dental prosthesis. Some suggest a role of the pollutants in the benign hyperplasia of the prostate or its cancer [131, 132].

In Females

A reduction in the duration of the menstrual cycle in New-York women was linked to the consumption of fish contaminated by PCB and other chemicals [133]. In addition,

endometriosis is characterized by an abnormal development of uterine tissue in various parts of the abdomen, and often causes pains and sterility among women consuming estrogenic drugs (DES) or exposed to dioxins. Mothers whose lactation period was abnormally shortened exhibited significant rates of DDE in their milk and blood [134, 135]. In the same way an exposure to the BPA *in utero* leads to an early puberty and slows down the growth of female mice [136]. An early exposure of female mice or rats to compounds exhibiting estrogenic effects like NP, DES, DDT, Coumestrol, Equol, MC, CD, or the PCB leads to an increase in uterine volume [137] whereas the TCDD involves a reduction in the uterine weight [95]. It is therefore demonstrated that these disruptors have an impact on reproduction. However, we do not know well the effects of the hormonal physiological disorders that they can cause during adult life or during the development, involving for example other hormones (insulin, glucagon, glucocorticoid, thyroxin, etc.), or relative to the sexual or nervous behavior.

B. Concerning the Nervous System

The synthesis of steroids takes place in the gonads, ovaries and testicles, but also in many peripheral tissues like the placenta, mammary gland, prostate, fat tissue, and bone tissue. It was more recently discovered that it takes place as well, quite significantly, in various areas of the brain, an organ which is sensitive to sexual steroids [138] ─ and this has only been known and studied since the seventies.

The results of studies carried out on laboratory animals and on humans clearly show that the exposure to EDs can harm the nervous system, either by affecting the neuroendocrine function or the general behavior. Some harmful effects observed seem to be due to thyroid dysfunction. It can be also the action of substances ensuring the transmission of messages between the nervous cells. The EDs can modify the role of the steroids in the neurotransmission, interfere with neurotransmitters, or even act in other ways on the neurological development of the child [139]. In France, a study suggests negative effects on the cognitive functions of adults subjected to chronic exposure of low doses of pesticides used in vine growing [140]. Other studies show that the neurocognitive effects of the organophosphate pesticides as the DDT on the professionally exposed populations are the disorders of the memory, the anxiety, irritability, and depression [141]. Moreover, the exposure to pesticides seems also related to a greater risk to develop Parkinson's disease and Alzheimer’s [142-144]. In French farmers, the risk to develop Parkinson's disease is multiplied by 5.6 and the risk to develop Alzheimer’s disease by 2.4 [145]. BPA was also proposed in an ED theory of schizophrenia [146]. Does that imply on some level that it is an ED effect of the pollutant? The action mechanisms are still to be investigated.

C. Concerning the Immune System

The immune system is composed of lymphoid structures implied in natural and inducible defences, bringing into play cell mechanisms such as phagocytosis, or the formation of antibodies. The disruption of this system, due to exposure to xenobiotics, can induce a state of immunodeficiency, even immunosuppression, inflammation, hypersensitivity, or allergy [147, 148]. For example, xenobiotics such as metal ions, or surfactants or adjuvants of the pesticides, involve immune reactions in humans or in laboratory animals [149, 150]. The system can be saturated due to attacks of many pollutants within the environment, in addition to bacteria and viruses effects. Therefore of the research topics will have, on the one hand, to

check whether the immunological disruption by the pollutants can modify antimicrobial defences of the organism, and on the other hand, whether it is linked to ED effects, since the steroidal hormones act upon the immune system [151], for example via the cytokines [152]. However, ED effects at these levels must not be confused with the disruptions of the body's defences known as cellular, constituted in particular by the cytochromes P450.

D. Concerning the Hormonal System

As mentioned above, EDs can hamper the development and the regulation of the reproductive, nervous and immunological functions. Moreover, morphological or metabolic abnormalities can be generated and cause cancers [95].

As a matter of fact, ED effects on the hormonal system are quite numerous. They act, as seen in Figure 4, among other things, by miming the natural endogenous hormones, and are thus called "agonists", or by reversing their actions, thus called "antagonists" [50] and inverse agonists. The EDs probably affect all endocrine glands, and even exocrine ones, but for historical reasons and due to the more significant physiological visibility of the disruptions of animal reproduction functions (it is worth mentioning that EDs were discovered following this lead) many studies were especially focused on the disruptions linked to (sexual) steroidal hormones, which play, on top of that, a significant role in some cancers, which are justly called hormone-dependent cancers. This topic will be addressed in the next paragraph.

Yet, as already mentioned, not all toxic substances disrupt the endocrine system through acting directly upon the hormonal receptors; indeed some of them inhibit the synthesis, the transport or the metabolism of the hormones. Some other can even act by using all types of mechanisms. Various studies indicate that the xenobiotics showing an ED potential can deteriorate the steroid biosynthesis *in vivo*. Thus, a chronic exposure to the Lindane pesticide involves a reduction in the serum testosterone rates in the male rats [153] and in the serum estrogen and progesterone rates in the female mice [154]. In 1997, Crain et al. [155] also reported a reduction in the plasmatic testosterone rates of youthful alligators of a Florida lake, which was contaminated by several pesticides, and an increase in the aromatase activity. In 2000, Walsh et al. [156] showed that the glyphosate based herbicide called Roundup [157, 158] at 25 µg/mL decreased the production of progesterone in response to cAMP by 84%, and the activity of P450scc by 61% relative to the mice Leydig cells, MA-10. The P450scc is the enzyme responsible for the first stage of steroid hormones synthesis since it catalyses the cleavage of the cholesterol side chain form the pregnenolone. It belongs to the enzymatic system CSCC "Cholesterol Side Chain Cleavage", localized in the mitochondrial intern membrane [159]. This effect could be related to a 90% reduction in the expression of the Steroidogenic Acute Regulatory protein (StAR), which induces the transfer of cholesterol to the mitochondrial intern membrane [156].

Moreover, the disruption of steroidogenesis by aromatase inhibition *in vitro* and *in vivo* has also been reported. The Fenarimol fungicide inhibits the aromatase activity in the nervous tissue of the male rat [97], in the human placenta and in a culture of JEG3 cells [99]. The TBT is also an *in vivo* aromatase specific inhibitor for a marine gastropod [96] probably leading to the increase in the A rates observed [98] and to the generated sexual disruptions. In the same way, in our laboratory, Le Curieux-Belfond et al., in 2001 [160], showed that 80 nM of Oxide TBT (TBTO) inhibited 90% the *in vitro* aromatase activity of the oyster *Crassostrea gigas*. In 2005, Richard et al. [46], as in 2007, Benachour et al. [48] and Gasnier et al. [49], showed that the Roundup is a potential ED through inhibiting the aromatase on the placental

level of the JEG3cells and in the human embryonic cells 293, which was unexpected and unknown for this type of compound. We will come back to that, as an example of ED action, in the paragraph about combined effects.

E. Concerning Hormone-dependent Cancers

Recent work shows that some "ED-action-like" chemicals are major triggering factors for malignant tumors, rather by weakening the organism in its entirety [161], and it is generally difficult to connect a given tumor to a specific pesticide. This comes from the fact that their effects are not detectable as simply by the epidemiologic tests as they would be for a bacterial or viral infection, as the pollutants are not multiplying and are more difficult to detect. We will come back to that idea. But we must keep in mind that the lipophilic xenobiotics, by themselves, or those which become lipophilic by forming lipid blisters thanks to their adjuvants, as in the case of the pesticides, accumulate slowly in the organisms, act as a mixture and can be transmitted from one generation to the next, through the mother's milk or during pregnancy; and their epigenetic effects too. The famous example of DES, which was taken by pregnant women, and caused cancers of the vagina to their daughters after puberty, became a "classic" one [124, 162]. And what is more, today it is known that the formation of the brain or the urogenital system are very good models for the study of the ED effects [163]. Other studies showed even more directly the carcinogenicity of environmental estrogens, such as the DDT and the AZ in *in vitro* or *in vivo* experiments [95, 164]. Non mutagenic ED may even promote or induce cancers, as it was underlined recently according to the tissue organization field theory [165]. Indeed, several pathways are prone to be involved in non mutagenic-induced carcinogenesis, such as AhR mediated effects, disruption of endocrine signaling, apoptotic resistance, reactive oxygen species actions, and epigenetic effects [166].

In Males

The number of testis cancers has increased by a factor 2 to 4 for the last fifty years in the industrialized countries [167]. The cause of this increase is not known but it was speculated that a disruption of the male's endocrine system can be implied [15, 168]. Ohlson & Hardell (2000) [169] highlighted an increase in the risk of seminal carcinoma in workmen exposed to polyvinyl chloride (PVC), containing phthalates, the estrogenic properties of which could support the formation of the cancerous cells. This example and the previous ones do not constitute an exhaustive list. However, BPA has been admitted recently as an ED since its use was limited or forbidden according to the countries, at least in baby bottles in France and European Union (2010-11). As a matter of fact, in males, it was shown to have a role in hormone dependent prostate cancers [170-172]. Even indirectly by non mutagenic actions, prenatal BPA exposure disturbed histological organization of the mammary gland of rats, increasing susceptibility to other carcinogens [173].

In Females

As already mentioned, it was indicated that DES caused cancers of the vagina to the daughters of women treated during their pregnancy, even though the pathology itself is considered rare [162]. Herbicide AZ was also associated to several cancers, in particular the ovary cancer [174]. Studies showed that an exposure of Rhesus monkeys to TCDD entailed endometriosis [175]. In the United States, exposure to the organochlorinated pesticide

Dieldrine, which has estrogenic properties, was associated to the increased risk of mammary cancer [176] and to a decreased longevity of the women affected [177]. The DDT was also detected in the fat and blood tissues of women with a mammary cancer [178-180]. This is true even if this does not constitute a direct proof by itself, because the initiation of cancer by a product can take place a few decades before the detection of the developed tumor itself. On the other hand, some studies showed that the Genistein had a beneficial role to prevent mammary cancers in rats [181, 182], whereas in 1999 Hilakivi-Clarke et al. [183] reported that the phytoestrogen in question increased the risk to develop a mammary cancer. About 30% of female mice develop cancers of the uterus during their life [184] if they are treated at birth with 50 mg/kg/D of Genistein, a plant estrogen extracted from soya. This, concentration is of the same order of magnitude as the one relative to human consumption, comparable to 1 µg/kg/D DES. Therefore, all depends on the hormonal balance already established in animals or human beings, and according to their age.

2. Differential Effects of EDs

A. Period of Exposure, Delayed Effects and Long Term or Transgenerational Impacts will Alter the Notion of Dose/response Proportionality

The period of exposure can crucially change the hormone effects as well as the ED ones. The genes exposed in the affected cells will not be the same ones, and neither the metabolic "equipment" of the tissues. The genes are dressed with histones, methylations, which strongly influence the inhibition of their constitutive expression in a cell, and the phenomenon is hormone-dependent, and, thus, ED-dependent. However, this "dressing" or "equipment" changes according to the specific period of the development, and induces various phenomena which will have an impact on the whole life, e.g. the formation of limbs, glands, spermatozoa, or of a cancer. The epigenetic mechanisms and the genomic imprinting will explain it, or partly at least. We will come back to that. The information storage of the effect as a function of time will also differ according to the time exposure, the most critical period being generally the embryonic or foetal one. But the initiation of a cancerous cell by a specific ED can also, as we have just mentioned, manifest readily in new-born babies or young people.

The scientific literature offers many examples of age as a factor of risk of cancer or disordered state. Hence, as the formation of the brain is so sensitive, an ED will be able to alter the behavior with permanent consequences in the adult [185, 186]. On the contrary, a similar exposure could have no effect at all on a fully developed brain. Generally speaking, the forming organs are quite sensitive to attacks by chemical substances, and so the critical periods of exposure include the development of the foetus, childhood, adolescence and other periods like the installation of lactation, menopause, or anti-cancer treatments. Several years can elapse between the exposure to a toxic substance and the demonstration of a detectable effect: hence the so-called delayed or deferred effects. The recovering of organic functions after a first toxic exposure can also open the way for long-term purposes, which will have been programmed. A great number of epidemiologic studies try to determine how and how much a foetus was exposed to toxic substances, by means of retrospective questionnaires that the mothers fill many years after the exposure, and only after the demonstration of an adverse effect in their descendants, which complicates the related research work a lot [187]. Treating

the children by anti-cancer drugs, which play the role of pharmacological xenobiotics, can have harmful effects on the long-term endocrine disruption [188]. On top of this, the aromatase inhibitors used in the treatments of the brain or breast cancers constitute a striking example of endocrine disruption used for pharmacological purposes [27]. For years now, some authors [16] have been warning us against the long-term ED-like effects of drugs, or of natural and/or polluting compounds. This is all truer since we find today many drug residues in rivers and surface water, which are not decomposed by water purification plants or human settlements.

In the 1950s and the 1960s, the unfortunate Thalidomide experience illustrated the importance of time exposure in the long-term effects. This drug, prescribed for pregnant women to fight morning nauseas caused malformations in hundreds of children. The time of ingestion proved to be more significant than the total drug quantity since only 2 to 3 pills had been ingested during the pregnancy. The product was responsible for serious malformations of the babies, because it was taken during the 5^{th} and 8^{th} weeks of amenorrhoea, a crucial period of limbs formation. In the same way, gestating female rats which had been fed TCDD on the 15^{th} day of gestation, that is the period of sexual differentiation of the foetus, gave birth to males presenting sexual abnormalities, like a reduction in size of testicles and epididymis, and weakened spermatogenesis [127, 189, 190]. All these effects are not morphologically visible. By exposing foetuses *in utero* to Flutamide, an anti-androgen, Benahmed's team clearly showed that testicular germinal cells in adult rats were prone to cellular death, through a durable deterioration either of mitochondrial metabolism [191].

Other studies highlighted these multi- or transgenerational effects, from F1 or from F3 respectively [192], caused by several EDs or their metabolites (phthalates, Chlorpyrifos, DDT, DDE, BPA, NP...) in various pathologies: testicular dysgenesis syndrome, hypospadias, cryptorchidism, cancer of the brain, and deteriorations of the nervous system, and other problems of infertilities which appear especially during adulthood [6, 39, 193-198].

Moreover, Charles Sultan's team (INSERM Institute, Montpellier) demonstrated an increase in congenital malformations of the penis and cryptorchidism in farmers' new-born babies. Therefore, the boys exhibit obvious organic abnormalities at birth, whereas girls are affected later in life by very early puberties [35, 199, 200]. The increase in genetic abnormalities could at least be due in part to the congenital effects of the studied compounds: that is, not entirely caused by inherited changes. What is more, the question remains to check whether some inherited changes might also be linked to environmental pollutants. We have recently studied this question in a family with a father exposed to numerous pesticides, which has two boys on three with anal and genital malformations [201].

The experimental toxicological studies were often carried out with high amounts of molecules, whereas some EDs can act *in vivo* at very low doses, immediately causing apparent effects or not, but causing also very significant ones later on, when the genes first targeted once are prompted again, after puberty for example. In this manner, estrogens can produce an effect down to amounts as small as a few parts per trillion. Some synthetic compounds are present in human tissues in amounts which are hundreds, or even thousands of times higher. However, in the majority of cases, synthetic compounds are less effective than hormones themselves, but can be metabolized in more active compounds, or stored much longer, as it is the case for hydroxylated PCBs.

The commonly named "dose/response" curve defines for example the link between the amount of xenobiotics and the response, assuming that the response should be proportional to

the administered dose, or that a threshold is to be found, i.e. an amount below which there is no effect. It is thus generally advanced that if high amounts do not present harmful effects, low doses should not cause any effect. Whereas this simplistic model is based on toxicology data, it is today generally admitted that it is quite antiquated for studying EDs. The threshold value, as well as the effect intensity, vary considerably as a function of time, time exposure, presence of other products, and on top of this, the effects are not proportional to the ED amount, except when using a restricted "parameter window". There can be antagonistic or contrary effects, even new ones, as for any kind of hormone targeting different genes or desensitizing cells, depending on the dose. Therefore, very low ED concentrations are able to have long-term effects, which cannot be detected at all by "traditional" dose / response studies, as they work only in the short term. It even becomes scientifically inaccurate to discard some toxicological effects on the principle that the "dose / response" relationship does not apply, in particular if a hormonal disruption is considered to be a toxicological problem, as it should be nowadays [202, 203]. For all these reasons, and for several years now, we have been developing a series of miscellaneous tests [47-49, 204], in order to understand as much as possible not only short-term ED effects, but also the long term ones.

Studies showed that low and high concentrations of hormones can exhibit opposite effects. For example, Tamoxifene, an anti-cancer drug, can act as agonist or antagonist according to the tissue involved. It is indeed an agonist in the uterus, where it increases the risk of cancer of the endometrium [205], in the bones, the mineral density of which it keeps steady [206], and in the liver where it increases the risk of hepatic cancers [207], but an antagonist in other tissues like the breast. In mice, another study showed that a 50% estradiol increase in male foetuses involved an increase in the size of the adult prostate, whereas a 200 to 800% increase was associated to a reduction in the size of the prostate [208]. In the same way, estradiol in low doses has a very important positive effect on male reproduction [70], and as it has been shown since, on the spermatogenesis of mammals, since it is formed by germinal cells via the aromatase [209]. On the other hand, it has a more inhibiting effect in males, even when using physiological amounts of the female [27]. It is easy to understand that the impact of an ED, on this finely controlled equilibrium, makes the mechanisms and the effects all fuzzy, in particular during the development.

Some recent studies highlighted the long-term effects of EDs. For example, the implication of some low-dose EDs (DDT, DDE, PCB, BPA, VZ, etc) in men is quite obvious in the development of testicular cancers, hypospadias, cryptorchidism, syndrome of testicular dysgenesis [191, 210-214]. In women, the same applies to the development of congenital malformations, the breast cancer and endometriosis [215]. This seems to be the consequence of a longer-term effect, owing to the fact that the exposure was either *in utero* or during breast feeding, which differs notably from the testicular descent or masculinization problems, the latter consequences being rather immediate. In adults, the long-term exposure to Pentachlorophenols (PCP) can cause chronic tiredness, neuropsychiatric problems, and infections of the skin, respiratory disorders, neuralgic pains, hypothyroidism, and hypo-fertility [216].

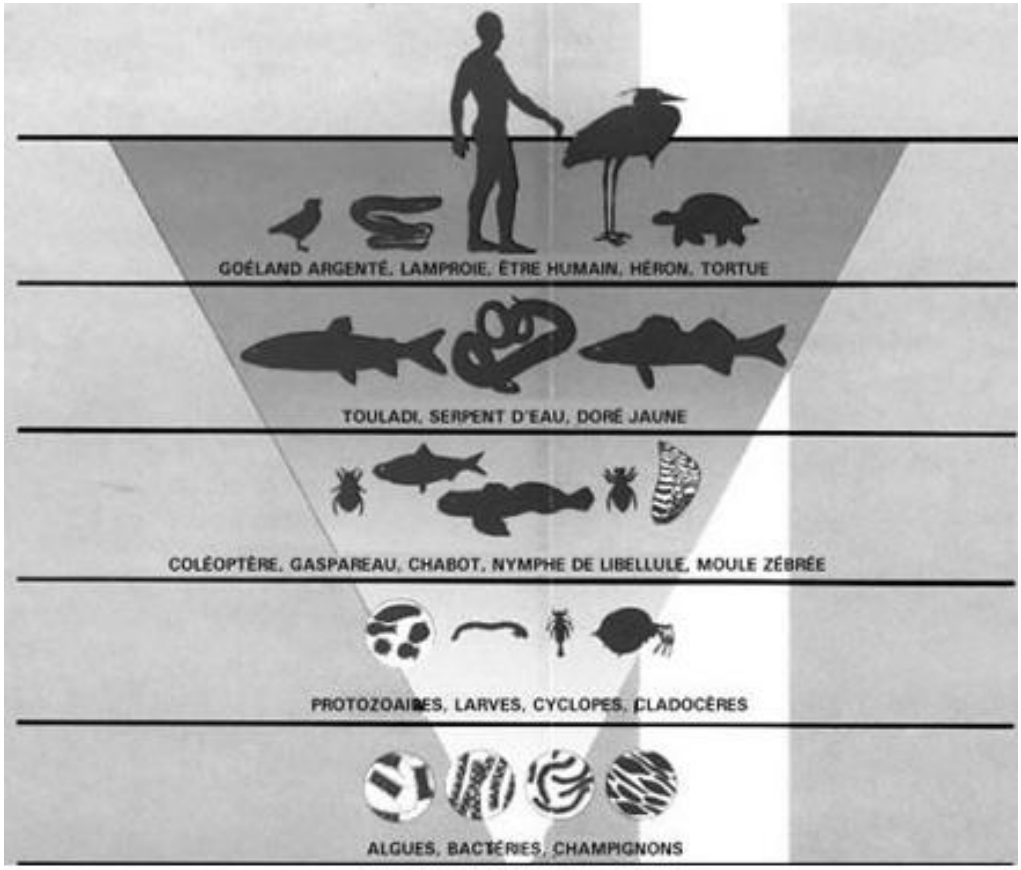


Figure 9. Accumulation of toxic substances in the food chain (according to the International Joint Committee of Canada).

New kinds of disruptions are being highlighted, such as multi- or transgenerational effects. They do not have visible impact on the genomic sequence, but are nevertheless transmitted to the descendants. In fact, the observed disorders are due to defects of the genetic functioning itself and of the regulation of the genetic expression. The said defects are called epigenetic disruptions [217]. Two American teams, led by Michael Skinner of the Washington State University, have just highlighted precisely these invisible however quite real, mechanisms. The researchers showed indeed that the male descendants of rats exposed *in utero* to a fungicide VZ are avoided by the females, which prefer to copulate with the males, the great-grandfathers of which were not contaminated. The VZ has been prohibited in Europe since December 2006, whereas in France it has still been marketed until December 2007. This active substance contains a compound having anti-androgenic effects, which counters the effects of testosterone.

Skinner and Anway already published [218] about the transgenerational effects of VZ and another estrogenic pesticide, MC (Methoxychlorine), relative to the capacity of reproduction in males. Both EDs interfere with the embryonic formation of the testicular cord, and accelerate the apoptosis of spermatic cells in adults. The *in vivo* exposure to the EDs, during the male sex determination caused the apoptosis of spermatic cells and infertility, one to four generations later. This epigenetic mechanism implies, as is now well-known,

DNA methylation and the permanent re-programming of the male germinal line. Therefore, these new observations [219] of the epigenetic actions which create transgenerational effects of EDs on reproduction underline the risks of these environmental toxins in the very long term.

Very recent advances in toxicology conclude that disruptions of cellular communications can induce environmental related disorders. A well-documented example is the bystander effect from damaged cells to intact ones [220]. It was considered as an exception of radiobiology, rather than a general mechanism of environmental disruption first. Historically, the classical paradigm was that all effects of ionizing radiations are caused by direct actions in the heart of the cells. Irradiated cells showed genetic and cytogenetic abnormalities; but then this was also observed in the neighboring non-irradiated cells. Bystander effect is caused by the disruption of cell-cell communications. This was also observed with nanoparticles [221]. The authors showed that Cobalt-Chromium nanoparticles caused DNA damages and chromosome aberrations in fibroblasts across the placental cell barrier. Underlying mechanisms involved cell communications through connexin gap junctions, or through hemichannels and pannexin channels. This suggests indirect effects of nanoparticles; these will have to be considered in safety assessment. Similar bystander mechanisms may be proposed for endocrine disruption.

B. Human Bioaccumulation and Exposures

The human organism can be exposed voluntarily (drugs, spreading of pesticides, etc.) or involuntarily to EDs. It may be due to consuming contaminated water, food or air. There is also the use of detergents, food additives, supplements based on medicinal herbs, cosmetics, BPA-containing plastic wrapping, or phthalates which can be found in food; all these should not be discarded. Other chemicals can be inhaled or absorbed by the skin. Persistent products like the PCBs or the organochlorinated insecticide DDT can contaminate plants and small organisms, which will be then consumed by larger ones and so on. This progression along the food chain, according to which each animal consumes directly at first, and then indirectly, increasing quantities of contaminated species coming from the lower links, causes the concentration of the consumed contaminant to be amplified: this is what is called the bioaccumulation. So this later indicates the process by which the contamination of the environment by persistent chemical substances (including EDs) leads to the bioamplification or the bioconcentration of these chemical substances in the whole of an ecosystem [222, 223], or of an organism. Food rich in animal grease like meat, fish, or eggs, often contains great quantities of contaminants [224]. In humans, the body mass index and waist circumference were recently associated to serum POPs levels, making the chemicals plausible contributors to the obesity epidemic. Indeed, new hypotheses postulate that POPs and obesity are no longer associated by only correlation due to the hydrophilic nature of POPs, but by causation links due to the endocrine disrupting mechanism of POPs [225].

The long-term or transgenerational effects on several levels of the ecosystem are not always explained by the genomic or the epigenetic imprinting. The bioaccumulation of toxic residues in the food chain or the highly lipophilic bodies, like the breast or the brain, has a role in the ED effects.

For instance, in the case of the aquatic environment, the contamination will initially involve the accumulation of such chemical substances (Figure 9) into the sediments, then into the plants, then into the small aquatic organisms, and so on right up to the human body [226].

In fact, the greatest risk of bioaccumulation comes from the chemical substances which are stable in the environment, and the half-life of which is relatively long. Their capacity to be liposolubilized will largely help as well, not only because, in this manner, the substances will much more readily penetrate the cells, which all have lipid membranes, but also because they will consequently be stored in the greasy tissues of the organism. In addition, the chemical substance can have a certain resistance to being fully metabolized inside the organism [222], which would cause a simple elimination through hydrosolubility via urine, sweat, saliva, or pulmonary steam; unless the metabolite itself is not toxic anymore, or prone to form compounds which would be more stable than the initial compound. It can be related to the well-known "traditional" DDE versus DDT case. The capacities of liposolubility, stability and membrane penetration can be provided to rather hydrophilic compounds, thanks to adjuvants like with one of the major herbicides we have studied, Roundup [46], which are classically mixed with the product labelled "active" for marketing purposes. These diluting adjuvants have also in fact synergistic and surfactant-like properties, often associated to detergent properties (besides, certain polluting agents, such as plasticizers or detergents, can incidentally play this role). In general, they form penetrating blisters, which allow entrance and storage of the active ingredients in the organism, and this should be taken into account [227]. Therefore, their chronic toxicity is likely to increase considerably, and even as of a few days, as we showed with Roundup compared to Glyphosate on human embryonic, placental and umbilical cord cells as well as hepatocytes [48, 47, 49, 204]. Besides, loopholes are thus created in the legislation, since only the active ingredient will be evaluated *in vivo* relative to sub-chronic or chronic toxicity [202, 227]. We will come back to that in the chapter on regulation.

Chemical substances such as AZ, CD, DDT, DDE, NP, VZ, and PCBs, which are stable, lipophilic, and persistent in the food chains, sometimes with a half-life of several years or far more, present today, as far as we know, the highest risk of bioaccumulation in animals, and then, later on, in humans [39, 40]. For example, AZ can concentrate approximately 10 times from the water to the liver in fish [222]. In the study of Lewis & Lech (1996) [228], it was shown that the factor of bioaccumulation of NP in trout's varies from 40 to 100, as compared to its content in contaminated water, while PCB and DDE accumulate approximately 170 times, starting from leaves, to caterpillars, and finally to eggs of birds [222]. The DDE bioaccumulation factor reaches values of 200 to 1.000 times in salmons [229], and up to 5.000 to 12.500 in mammary greases and mother's milk [135], which is generally more than in the liver or the blood. In the report of the United Nations Program for the Environment (2005), it was reported for instance that CD concentrates 9.000 times in oysters and 60.000 times in the Atlantic fish *Capucette* species, which lives in estuaries.

Consequently, the diets rich in animal fats can contribute to the accumulation of chemical substances like the organochlorinated compounds (DDT) in humans. Moreover, the colon and breast cancers have been internationally linked to excess fat supply in diet [230]. Nursed babies could receive daily 10 to 60 times more dioxins and PCBs than adults, according to the WHOROE (World Health Organization Regional Office for Europe) in 1989. Recently, a great number of studies in several populations (Japan, Argentina, United Kingdom, Turkey, etc.) reported the presence of such chemical substances in mother's milk, serum, blood, hair and nails [41-43, 231, 232]. Another study highlighted the bioaccumulation of BPA and NP in human urines [233].

In 2003, the WWF (World Wild Foundation) suggested that 47 members of European Parliament, coming from 17 countries, should undergo blood analyses in order to check about a hundred commonly found pollutants. On average, 41 different substances were found per person, including 13 identical ones, like phthalates and perfluorinated flame retardant compounds, but also substances such as DDT, DDE and PCBs. In June 2005, the WWF supplemented this type of study by having the blood of 13 European families tested over three generations, in the 1 to 92 years of age range. Up to 73 chemicals were found in their blood! The highest number of products was noted in grandmothers (63 substances), against 49 in mothers. More worrying, it was found that young people were poisoned by 59 products [13] and that they bioconcentrate the new ones. Another study financed by Greenpeace and WWF proved that the foetuses are already in contact *in utero* with dangerous substances. It was carried out in the Netherlands, based on 42 samples of maternal blood and 27 samples of blood of umbilical cords. A score of toxic substances, pertaining to 8 different chemical groups, was identified. Those found in the blood of umbilical cords intervene for the majority in the making of current consumer goods, such as cans of food, electronic instruments, deodorants, and toothpaste (www.wwf.be/detox 2005). During pregnancy tens of pollutants cross the placenta barrier and bind to the DNA of the foetuses [234].

C. Biphasic or Multiple Effects

The effects of the pollutants are not always linear, as for the hormones or some drugs, contrary to what is known for the short-term actions in traditional toxicology. For example, a 50% increase of estradiol in foetuses of male mice entails an increase in the size of the prostate, whereas a 200 to 800% increase entails a decrease in size [208]. Comparable biphasic effects exist for NP modifying the metabolism of steroids by cytochromes P450 [235]. There are surprises sometimes. Thus, Letrozole, an inhibitor of type II aromatase, indeed cuts in half the aromatase activity for 1 nM, but it stimulates it for 5 nM (in fibroblasts of mammary fat tissue) after an 18 hr incubation [236].

Recently, a study by Andrade et al. (2006) [237] showed non-monotonous effects of the plasticizer Di-(2-ethylhexyl)-phthalate (DEHP) on the activity of the aromatase of the brain in male and female rats. In low doses, it plays the role of an inhibitor, whereas in high doses it stimulates its activity in males, contrary to females. That is characterized by curves in "J" or "U" [208, 238].

In our laboratory [44], we characterized the biphasic effects of Lindane, an isomer of the most toxic hexachlorocyclohexane [239], and of BPA. These products were tested in nontoxic amounts on the natural aromatase of placental human cells (JEG3), and on human cells of embryonic kidney [293] transfected with aromatase cDNA. Incubations of short duration (from 10 min to 6 h) involve an increase in aromatase activity, whereas longer durations (18 h) entailed its inhibition. These results illustrate the variability of action of these compounds as a function of dose and duration, with actions on various levels, or modifications of the metabolism of the products.

Similar results were reported for these two compounds on the progesterone formation by the Leydig cells in mice [240, 241]. In the same way, an exposure of the Leydig cells in rats or mice to Lindane concentrations of the order of one µM during 2 to 4 h inhibits the steroidogenesis stimulated by the hCG [242, 243], but multiplies by two the basal testosterone concentrations [243].

D. Combined Effects

The related studies are generally interested in the effects of only one chemical at the same time, based on previous understanding of the microbial effects in the short term, effects which were studied one by one. However, humans are daily exposed, simultaneously, to a great number of xenobiotics in their environment (food, air, water, ground and products for human consumption) and to their metabolites [223]. However, some mixtures clearly have synergistic or multiplicative effects, or sometimes compensatory ones, as was shown in our laboratory [45].

Therefore, establishing a product homologation which would be strictly scientifically based appears to be a very complicated challenge. As a matter of fact, action modes for mixtures are quite varied: the receptors can be dissimilar, affinity for the substrate can differ and several interactions are possible between the various compounds. The most typical case will be the one of the pesticide adjuvants which stabilize the active ingredient, and greatly amplify its cellular penetration. We will come back to that, but it becomes clear that the auxiliary and active ingredients, which together are named "commercial formulation" of a given product, must imperatively be tested together for the above reasons, and using the *in vivo* tests, which is far from being always the case. Because in farming active ingredients are most generally not used on their own.

Various modes of combination exist (potentiating, additive, synergistic, or antagonistic effects); moreover, the medium in which the ED acts changes its activity because the mixture does not have the same effect, depending on the medium in which it is disseminated. For example, AZ increases the toxicity of Chlorpyrifos by a factor 7 on the ground, and by a factor 4 in water [244]. For some, the combined effects of the pesticides could be predicted if they are of the same class, i.e. if their action mechanisms are known, but this is really not the majority of the cases [245].

In midges, AZ alone does not have an effect on the activity of acetylcholine esterase. However, in combination with Chlorpyrifos, it is reduced significantly [246]. It was noted that the mixture of Aroclor 1254 (A1254) with TBT could have a synergistic effect and would involve a significant fall of weight in some fish like carp [247]. In Njiwa et al.'s study, in 2004, a synergistic effect of A1254 with DDT was shown, affecting the spermatic release [248].

Roundup and its active ingredient, Glyphosate, are currently studied in our laboratory as previously underlined. We showed that Roundup reduces the viability of cells resulting from human placenta (JEG3) and of embryonic cells [293] in a more effective way than Glyphosate alone. This effect depends on the amount, and is detectable with concentrations from 100 to 200 times lower than the amounts recommended in agriculture, and that is largely amplified with time [48]. We also showed the impact of the adjuvants. Indeed, the addition of several adjuvants in a proportion of 0.1% to Glyphosate can make it possible to reduce the aromatase activity of the cells (JEG3) in a more effective way than when Glyphosate is alone [46]. Lastly, these studies show that Roundup acts as an ED, with even weaker amounts, and without showing toxicity within the time limits considered, at least for two different species of mammals (horse and man,, as well as for testicles as for fresh placenta), and still inhibiting the activity of the aromatase.

Next, among a list of EDs, classified as such due to other action mechanisms found in the scientific literature, we studied at some length the combined effect of various xenobiotics on the aromatase of human placenta, or in the embryonic line 293 receiving the DNA coding for

this enzyme. Some substances have little or no effect in high doses (500 µM). It is the case of A1254, AZ, VZ, and DDT. We discovered that others have a very significant inhibiting effect that is DDE, CD, NP, and BPA. On the other hand, in a surprising way, our results generally show more significant effects when the substances go in twos, or in 4 to 5 mixtures, with a final concentration of 20 µM only for the whole mix [45], and this even for the first group, which did not present an effect at 500 µM.

However, it was shown that PCBs, DDT and other products contaminate blood, milk and mammary fat tissue throughout the world (Japan, Argentina, United Kingdom, Turkey, etc.) in doses varying from 0.5 to 2.3 µg/g [41-43]. This is of the same order than the acceptable daily intake (ADI), or than in our own experiments just described.

3. Limits of Toxicology and Epidemiology

The previous observations highlight the fact that traditional toxicology is quite a limited science, as far as the study of EDs is concerned, in particular because the biphasic, multiple, or combined effects are seldom linear and create unexpected amplifications. As we have seen, such data invalidate the antiquated notion of "dose/response" proportionality. The detection of differed, transgenerational, or long-term effects after the period of exposure, as well as the observation of very different thresholds of sensitivities according to age, and of varied reactions according to sex, manifestly complicate the toxicological interpretations of the overall data. All these observations are not unusual as far as EDs are concerned. Statutory toxicological studies on pesticides in Europe (CEE/91/414 directive) don't take into account the whole of the hormonal effects.

The reproductive ability is measured based on the litters of rats, whereas in men, the quantity and the quality of spermatozoa are more likely to affect the hypo-fertility or the anomalies of pregnancy. In women, the age of fertility will be possibly affected by EDs, but it is not measured for a laboratory animal. Besides, the toxicological studies on such animals, which are mostly carried out in healthy adults, tend to use the most homogeneous possible stocks of model species. However, human populations have very variable genetics and physiologies, and therefore much more complex reactions, in particular in babies, patients, old people, etc. all this not being taken into account either. Hence the precautionary principle will be wholly meaningful when applied as an active booster for research and preventive actions, in particular when results and knowledge are lacking [203].

Epidemiology is a science which will be accepted as an authority to decide upon the actual risk of a polluting factor. However, it was initially based on microbiological knowledge, i.e. using pathogenic agents which are generally visible under a microscope that is easily identifiable, multiplying very quickly in a few hours, possessing an organic specificity and causing rather precise symptoms and with well-identified actions in a relatively short term. And yet it is just the opposite as far as chemical pollutants are concerned. These agents disrupt the majority of the cellular messenger system into which they penetrate, because they are infinitely small, molecular, not easily measurable nor quantifiable; they do not multiply but accumulate slowly, through multiple and combined actions. The pesticides, for example, are developed and released in the environment in order to have toxic actions on a certain level of the ecosystem; hence they will probably have side effects which are not measured by clinical trials prior to being put on the market (like it is done with drugs).

The factor of risk, taking the level of danger and of exposure into account, should inevitably measure the intra-tissue rates of the various pollutants before associating any pathology to a given agent, or even to a mixture, a procedure which is almost never carried out: so epidemiology, being rarely able to conclude, is a science little adapted to the study of EDs and other chemical pollutants.

It is therefore necessary, in addition, to take the following five parameters much more into account, in order to supplement epidemiology, as they can often make it possible to better conclude if they are concordant:

- "*In vitro*": Test-tube experiments, looking for biochemical or molecular effects, explaining for example enzymatic, genetic actions, or pollutant-hormone-receptor interactions
- From *"in vitro"* to *"ex vivo"*: Cell or tissue culture, or inside the organ itself
- "*In vivo*" effects on several types of laboratory animals, which must give first physiological indications as compared with humans
- "*In situ*" study of impacts on farm animals, and on wild ones as well
- In humans, study of accidents known in some professions or in some contaminated places.

However, it remains true that epidemiologic studies have the advantage of giving useful information about the health hazards which can be directly evaluated in humans. It is extremely difficult, and in general unethical, to research into environmental poisons by deliberately exposing human subjects to toxic substances (except in studies of occupational hazards) to observe the harmful effects which could result from them. This is quite different from the case when the effect of new drugs is tested on voluntary or selected subjects, or the impact of pollutants on animals. Even for an ethical consideration to an animal point of view, it appears ethically responsible to analyze the blood and organs for the first animals exposed to an ED, or to any xenobiotic or new food/feed, rather than to give directly this compound to millions of animals in livestock. In fact, the major part of the empirical data which establish a bond between chemical pollutants (even EDs) and harmful effects on health come from studies on human populations which were accidentally exposed to toxic substances: for example, PCBs and dioxins in Seveso, in Italy, of farmers in particular, or asbestos, etc. Other examples could be given, like studying the survivors of Chernobyl or the populations of fishermen of the Aral Sea.

In addition, since most effects allotted to endocrine disruption like hypofertility, reproduction/fertility problems, sterility, cancers, immune diseases, and neurocognitive disorders cannot easily be extrapolated or transposed from animals to humans due to multiple causes. Research must be considerably deepened in this field.

In February 2006, a conference on the Strategic Approach to International Chemicals Management (SAICM) was held in Dubai. Considering current human health and environmental issues relative to such chemical substances, more than 60 ministers from all over the world met, not only to pursue the purpose of the conference, but also to cover the evaluation of the risks, the harmonization of labelling and the treatment of obsolete stocks. Besides, the UE wishes to modernize the European legislation, however considered already as one of the best in the world. Today, more and more scientists and decision-makers agree that

they should work out an innovating strategy ensuring the protection of human health and of the environment, in a sustainable development context. Within this framework, a unique integrated system of Register, Evaluation and Authorization of Chemicals called "REACH" was set up in 2007. (NB: the pesticides will not be evaluated through this program, but by means of other directives such as 2006/60/CEE). Such new resolutions remain to be internationally adopted and, more importantly, practically enforced in all countries.

In conclusion of these new ideas, epidemiology is not always the pertinent tool to decide about potentially long-term combined toxicological effects. Biochemical and endocrine mechanisms, cellular effects, laboratory, farm or wild animal studies, as well as the observations of human exposures are more relevant.

III. Protective Measures Adopted by the Current European Legislation and Possible Improvements

In this chapter, the EU will especially be shown as an example, since they have one of the best legislations in the world, taking in particular the precautionary principle into account, and also since they adopted recently a whole set of directives regulating the use of the pesticides and other industrial products.

1. Regulations Context

It is well-known that before the Second World War, agriculture used mainly mineral derivatives (copper, lead arsenate, etc.) or plant extracts. After World War II, farming entered the era of synthetic pesticides (phytopharmacological compounds and biocides). Synthesizing chemical weapons during the war stimulated the related knowledge and productions. The intensification of agriculture increased and standardized the use of such compounds, in particular in France, a country which took a significant place among the first world consumers. In fact the European market, in 2002, for instance, was the second world market for pesticides, France was the leader in this matter, that is to say the third world market after the USA and Japan. During the technological post-war era, industrial products have been developed at the same time, research workers planning to mass-produce stabilized insulating or plasticizing products, which, once in the environment, would therefore be potentially harmful to the cellular messenger system, and specifically relative to endocrine disruption. Since then, the number of statutory texts trying to control the marketing of all these substances has only been increasing.

The Community legislation in use was described in a 1999 report of the European Commission entitled: "Community Strategy concerning EDs - a series of substances suspected to influence the hormonal system of men and animals: COM/99/0706". This current legislation on the effects of chemical substances on the environment at large and on human and animal health is based on a three-stage approach. It includes *a stage of identification of the safety hazards*, consisting in determining the harmfulness of a substance for human health and the environment, according to its intrinsic properties. The second stage consists in a *risk evaluation* founded on an evaluation of the potential hazards combined with an evaluation of

the exposure to the chemical substance considered. The third and last stage is *the risk management* stage, during which economically accepted strategies (known as appropriate) will be worked out.

For all three stages, the number of scientific data available for each substance can vary in a significant way. Therefore the precautionary principle is an essential element of this approach. Moreover, the Court of Justice declared (see decision of May 5, 1998, item C 180/96) that "when uncertainties remain as for the existence or the range of health hazards, the institutions in charge can take protective measures without having to wait for the full scientific demonstration of the reality and gravity of the potential hazards in question". There are at least two aspects to take into account to try to determine a suitable policy, on the basis of the precautionary principle. The first one is the need to found the action on a valid scientific evaluation – and the scientific debates are likely to flourish about this concept. The second one is the need to be able to answer the issues at stake in a quick and effective way as scientific knowledge progresses.

It must be pointed out that the European Commission adopted a report in November 1998 on the application of four official texts or "legislative instruments" (directive 67/548/EEC, directive 88/379/EEC, regulation (EEC) n°793/93 and directive 76/769/EEC) relative to the Community policy concerning the chemical substances. One of the aspects highlighted in this report is the need to check that these instruments follow the last scientific developments, in particular with regard to the potential threat of EDs. In December 1998, following this report, the Council underlined the necessity to work for the development of an integrated and coherent approach of the future Community policy relative to the chemical substances, which would duly take the precautionary principle into account. The Council was delighted that the Commission intended to develop such a strategy in consultation with the Member States and the other interested parts. There is no doubt that the current strategy concerning EDs will in the long run constitute an integral part of the general strategy to develop.

Starting from this point, and taking into account some hazards (in an incomplete manner, as was seen for EDs), as well as the exposure of living beings to said hazards, the commission issued a series of directives and regulations, which are applicable today for 27 European countries and approximately 450 million people. A short description of how it all works is now given. The main snag is that the directives must be transcribed into national laws to be directly valid process which can take a certain time, depending on the country involved. Therefore, there are total or partial "political" delays of more than five years, for example for the French transcriptions of the directives regulating the genetically-modified organisms tailored to contain pesticides with possible ED action (2001/18/CE). However, the Commission and the independent organizations involved will be entitled, in the meantime, to win the case against the disobedient Member State after two years of delay. The regulations, on the contrary, are applicable as of the time limit specified in their promulgation, and in all the Member States at the same time, and they have force of law above national laws on the related subjects.

2. Legislative Instruments Covering the Evaluation and the Risk Management of the Chemicals in EUROPE

A. Evaluation of Hazards

Since 1967, the Community has been concerned with (directive 67/548/CEE) classification, packing, labelling of the substances known to be hazardous, however without envisaging an evaluation of EDs. It is only in 1992 (directive 92/32/CEE modifying the above-mentioned one) that hazards such as "carcinogenicity", "toxic for reproduction", "environmental hazard" began to be indexed, this indexation making it possible to take EDs indirectly into account . It would now be necessary to modify the above regulation to introduce the last results of the research in progress.

B. Evaluation of Risks

This normative list for the risk evaluation is declined as follows by chronological order.

- 1976. Directive 76/769/EEC is relative to the limitation of the marketing and the use of some substances and hazardous preparations, for example: Carcinogens, Mutagens, poisons for reproduction, also known as Reprotoxics, the whole set forming the CMR group. A targeted evaluation is planned in case of emergency.
- 1989. Directive 89/109/EEC concerns the materials and objects intended to come into contact with foodstuffs (e.g. soft plastics, phthalates, etc.), for the study of ED effects.
- 1991. Directive 91/414/EEC concerns the marketing of the phytopharmacological products, or pesticides. As for the following ones, all the data available on potential ED effects s are supposed to be studied by the Commissions; but they can call them into question.
- 1993. This regulation (EEC) n° 793/93 plans the evaluation of the risks presented by all the existing substances in contact with humans, possibly with complementary tests. It draws up a list of priority substances.
- 1998. This directive 98/8/EC is relative to the marketing of the products known as biocides, like the pesticides of non agricultural use (for private gardens, anti-lice shampoos, etc.) and their evaluation.

C. Risk Management

- For short-term action of products or in case of emergency

  The directive 92/59/EEC, relative to the general safety of the products, envisages temporary restrictions of some products in emergencies.
- For general action or targeted long-term action of products

  The numbers of the directives and regulations are always for the EEC or the EC (more recent name). The risk management began in 1976 (76/769) with the marketing limitation of some hazardous substances (CMR of the 67/548 directive). It can also be manure (76/116) or cosmetic products (76/768). In 1979 (79/117) the marketing of some active substances of the phytopharmacological products was prohibited; at the same time, it was planned to fix the value of the maximum contents

for the residues of pesticides in the foodstuffs of animal origin, and in some products of vegetable origin, and in the cereals (86/362, 363 and 90/642). In 1988, directive 88/378 concerns the safety of toys. One year later, directives 89/109 and 90/128 are relative to materials and objects intended to come into contact with foodstuffs, and recommendation 89/542 is about the labelling of detergents and cleaning products. In 1990, (regulation 2377/90) the topic was the fixing of maximum limits of residues of veterinary medicinal products in food of animal origin. Among those, hormones or products stimulating growth can be found, and hence quite a logical ED effect. In 1991, two-year tests were proposed (the whole life of the laboratory rats used as models) for the phytopharmacological products to be put on the market (91/414). It is not any more a legislation prohibiting some substances, but a regulation drawing up a list of phytopharmacological products authorized after these tests. Then the next subject (95/2) dealt with the food additives, and more precisely, for a part of the EDs (96/22), it dealt with the prohibition of some substances showing a hormonal or thyreostatic effect, and β-agonist substances in animals. Likewise (96/23), control measurements had to be implemented with regard to some substances and their residues in the live animals and their products. In 1997, a regulation (194/97) again fixes the maximum contents for some contaminants in the foodstuffs. Added to all that, the directives and regulations on the treatments (waste, pollution, etc.) and the air or the quality of water; these treatments or mediums being able to generate or contain EDs.

A directive will usually be modified several times due to the necessary evolution of its content. For example, for the directives relative to pesticides, an appendix is provided, with a list of active substances which can be incorporated in the product. Before inscription, each active substance is entrusted to a Member State which becomes Reporter Member (RM). The RM is in charge of directing and carrying out the toxicological evaluation (or revaluation) of the active substance concerning health and environment. At the end of the process and after the results, it is decided to register or not the active substance in the list of the directive.

It should be noted that following this decision, the Member States can only authorize the marketing of phytopharmacological products composed of active substances included in the appendix of the directive. However, some prohibited substances enlisted may be granted, by special dispensation, a sort of new deadline, until which the marketing of the corresponding phytopharmacological preparations is still allowed. This additional time is granted to help find an effective solution to replace the substance concerned, whenever its employment does not present an unacceptable risk (a very subjective notion indeed), and if its withdrawal could cause a technical difficulty which would have dire economic consequences according to political influences.

Although some chemical substances have already been prohibited by the Community legislation for a certain time, like asbestos, there are still gaps in this legislation. Thus, there are too few data on the effects of many existing substances which were put on the market before 1981, the year when the obligation to test and notify the new substances was founded. These substances account for approximately 99% of the total volume of the substances on the market (!), and although the European Commission started a process of evaluation and management of these substances, it is very much time-consuming, and the existing substances are not submitted to the same test requirements and time limits as the new substances are.

In addition, there are not enough texts about the sale conditions, the diffusion and the use of these products (according to the Commission White book, dated 27th February 2001, relative to the strategy for the future policy in the field of the chemical substances). The installation of the REACH program, the specs of which remain to be precise, does not deny this assertion.

In 2003, the commission adopts a regulation which draws up the list of all the pesticides marketed in 2000, as well as the list of all the substances that any producer or Member State required to be evaluated. There are 354 active substances which must be evaluated. The work is divided into 4 successive phases:

- Evaluation of wood-protective products and rodenticides;
- Evaluation of antifouling products, repulsive products and baits, molluscides, insecticides, and acaricides;
- Evaluation of biocides intended for human hygiene, disinfectants;
- Evaluation of products for the protection of pellucid, fibres, leather, rubber, and anti-mildewed products.

Among the “ED” pesticides, there are also persistent organic pollutants like Dieldrin, DDT, etc. Aimed at controlling the spread of such pollutants, the Convention of Stockholm came into effect on May 17, 2004, within the framework of the Program of the United Nations for Environment (PUNE). On the whole, 151 countries signed it and 127 ratified it, and in particular the majority of the Member States of the EU; the Union itself ratified it in November 2004. In fact, the European regulations transposing the Convention is stricter than the international provisions are in this matter: it envisages the pure and simple suppression of POP substances, which are very toxic for mankind and the fauna: they remain intact and resist degradation in the environment during generations, plus they propagate by air and water on long distances, and they accumulate in the fat tissues of living organisms. This list is likely to lengthen with time. In its first version, the Convention banned 12 chemical substances that the experts ended up calling the "twelve bastards". These substances are: Aldrin, Chlordane, DDT, Dieldrin, Dioxins, Endrin, Furanes, Heptachlore, Hexachlorobenzene, Mirex, PCBs, and Toxaphene.

By the end of 2006, Europe implemented the REACH program (regulation (EC) No 1907/2006), which is a considerable advancing as regards the management of the chemicals in the EU: over an 11-year period, some 30.000 industrial chemical substances (not pesticides) must be evaluated. Within the framework of this recording process, the manufacturers and the importers are led to generate data for all the chemical substances produced or imported in the Union in quantities higher than a ton per year. The informants are also requested to identify suitable measures as regards risk management, and to communicate such safety measures to the users.

Moreover, REACH will allow an additional evaluation of the substances giving cause for concern. This system applies to the substances which cause cancer, sterility, genetic mutations or congenital malformations, and as well to the ones which are persistent and accumulate in the environment. Therefore, EDs are concerned in the first place. The system of authorization will lead the companies to adopt surer replacement substances gradually, when they exist. The current restrictions as regards use will be maintained in the system

REACH. It also guarantees that the animal experimentation is limited to the bare minimum and the recourse to alternative methods is encouraged.

Based on the above action-packed plan, a European agency for chemicals was instituted, modifying directive 1999/45/CE and abrogating regulation (EEC) No 793/93 of the Council, regulation (EC) No 1488/94 of the Commission, as well as directive 76/769/CEE of the Council and directives 91/155/CEE, 93/67/CEE, 93/105/CE and 2000/21/CE of the Commission. Directive 2006/121/CE modifies directive 67/548/CEE in order to adapt it to the REACH regulation.

Lastly, concerning pesticides, the recent directive 2006/60/CE modifies the 90/642 with regard to the maximum contents for the residues of 11 products, including Glyphosate. For example, as regards the latter, which enters the composition of the GMO-modified food, in order for the GMO to tolerate it, the maximum content tolerated is 20 mg/kg in soya beans, which is equivalent to 0.005% of Roundup Grands Travaux (400 g/L of G) content according to our research work, this content kills the human cells after 24 hr of exposure and induce apoptosis or necrosis cell death pathways [47]. In wild mushrooms, concentrations equivalent to 0.014% of Roundup Bioforce® (360 g/L of G) become authorized! As seen already, our last experiments demonstrate that ED effects are in fact noticeable with levels as low as 0.5 ppm ($5x10^{-5}$%) [49]. Such contents suggest an excessive use of the product on the GMO, like the Roundup-tolerating soya especially imported from the American continent, as well as a possible accumulation of this product in the food chain. In this kind of case, the legislation reaches its own limits.

3. Legislation Limits

In the case of ED effect substances, the 1999 Report of the European Parliament Commission duly noticed that the two directives on classification (67/548/CEE) and on evaluation of risks (93/793/CEE) should be amended or modified, precisely to take EDs into account.

However, it cannot be denied, and in particular because of the bioaccumulation and the stability of some residues in the environment and in the food chain, that nowadays many pesticides and industrial products are currently found in the living organisms, as mentioned above, and as seen in chapter II, in spite of the very evolutionary legislation previously detailed. Consequently, the chemical industry should not shun its responsibility. There is a rather systematic time-lag between the scientific knowledge and the effective implementation of corresponding new regulations. This time-lag is more or less long, according to debates and political struggles. It is possible that in a few years, for example, the currently legal use of some products and pesticides could be made illegal in most countries, as was already the case in the past for DDT, Metoxychlore and Dieldrin, and even for AZ which was prohibited as soon as 2003 in France and 2004 in Europe. This way, the maximum limits of residues are fixed for the phytopharmacological products authorized in the European Union, but what about the residues of pesticides detected in imported food of the countries authorizing or tolerating pesticides prohibited in France?

In 2002, following Paul Lannoye's initiative, the European Parliament made several requests which were only partially honoured in 2008:

- That no substance considered carcinogenic, toxic for the reproduction, mutagen, ED, or prone to bioaccumulation should be written down on the positive list
- That the comparative evaluation and the principle of substitution should be resorted to
- That should be taken into account the effects on the health of children and foetuses, but also the additive and synergistic effects of pesticides and their impact on domestic bees
- That pesticides requiring strict restrictions of use which are not easily controllable should be banned
- That metabolites should be evaluated in the same way as primary substances
- That the labelling criteria of the products treated with pesticides should be highly restrictive
- That a reduction programme of the use of pesticides should be set up
- That a good practice code should be developed under the form of a directive
- That residues of pesticides in the products should be strictly checked.

It should be noted, though, that it is still needed to research the subject more thoroughly, and that a validated testing method does not exist as yet, but instead a cluster of indicators to establish if a substance constitutes an ED or not. With this in mind, several initiatives have already been launched, or are being studied within the European Commission. The strategy, founded on existing data, must be flexible enough to be able to adapt to the evolution of scientific knowledge.

The Commission regularly draws up a priority list of the substances to be evaluated in order to determine their role in the endocrine disruption. It will help, among other things, to fill the gaps in the knowledge concerning aspects such as the "dose/response" relationship, the sources and ways of exposure, and the epidemiologic studies on the cause/effect relationships, which will later contribute to direct future research and/or monitoring measurements. In addition, one will try to determine the list of specific utilizations, in particular for the more vulnerable children, who must be studied with a particular emphasis, from the point of view of the consumer policy. For example, according to our publications, Roundup should be registered on this priority list of potential EDs to examine, like numerous formulation pesticides, since it has an effect on the placental [46] and embryonic cells [48], but also on umbilical cord cells [47] or hepatocytes [49], and so can affect pregnancy, as well as embryos or foetuses before affecting the children themselves.

Moreover, the current evaluations are based on studies made via *in vitro* biological tests which consist, for example, in connection tests to the receptors of oestrogens and androgens, tests of gene transactivation, and tests on extracts of crushed testicles or other test tube experiments [249]. These tests do not cover, by far, all the ways in which the synthesis, the metabolism, the transport and the action of the steroidal hormones can be disrupted; moreover, they require the evaluation of the negative effect of the cytotoxicity on biosynthesis by the gonads and the activity of the aromatase, among other things. Due to the limits of the *in vitro* test tube experiments, *in vivo* tests had to be included in the series of screening and biological tests. The laboratory rat is the *in vivo* model usually employed for official toxicological studies and research in endocrinology, as well as for the toxicity tests on development and reproduction, with the aim of determining the potential negative effects on

human and animal health, and evaluating human health risks at large [250]. These tests consist in exposing the animals during the critical stages of their development, and evaluating the reproduction function of the animals exposed *in utero*. Other methods will have now to be developed to detect the disruption relative to estrogens, androgens, and thyroid hormones, among other things. However, these tests are carried out in the short run, and it was previously highlighted that one of the major difficulties associated to the study of environmental poisons are related to the long period which often precedes the outbreak of a disease.

Therefore, in 2010, it can be said that the tests carried out on EDs are both insufficient and incomplete. Research relates to one substance, whereas the exposure to several ones is the rule. In effect, these tests are mainly done on the active ingredient of the product, and on a two-year base. Two major problems of the legislation are exposed here. First, the long-term effects cannot appear, owing to the fact that the study is generally too short. Secondly, there are no tests on the adjuvants of the pesticides, and the mixtures present in the environment are not taken into account, by lack of awareness. Thus, the legislation must evolve by taking these mixtures to which the human are exposed into account. They are partially taken into account via skin adsorption, a process made easier by the adjuvants, but the formulation adjuvants are not added in the chronic *in vivo* tests, whereas they should.

4. The Prague Declaration on Endocrine Disruption

At the beginning of May 2005, the European Commission based a research program on EDs called Emerging Diseases in a changing European Environment (EDEN). An assessment was carried out after two years, in Prague, at the time of a working group of the Cluster for Research on Endocrine Disrupters in Europe (CREED), a consortium of several European laboratories working on the question. This research has already made it possible to better include the role of EDs in the multi-factorial origin of various disorders and diseases, but it was concluded that the role of food, way of life, and stress should also be clarified. Much remains to be done. For example, concerning the reduction in the characteristics of human sperm, the studies undertaken up to now have been retrospective, as they were based on the existing literature and the files of the laboratories. It would now be advisable to set up exploratory studies.

Facing the drastic cuts in research funding, the European researchers wish that the next PCRD ("*Programme Cadre de la communauté européenne pour des actions de Recherche, de Développement technologique et de démonstration*") should continue to support their efforts. There were more than one hundred scientists and international and interdisciplinary experts to sign the declaration of Prague. As a matter of fact, in the May 2005 document, the experts stated: "*Europeans are exposed, on low levels, to a great number of endocrine disruptors which can act in concert. Many of these chemical substances, drugs or natural products are found in human tissues and the mother's milk. The human beings are exposed to these chemical substances as of their youth when the organism under development can be particularly sensitive*". Therefore, a collective appeal requests that the political, financial and regulatory effort should continue. Not only these researchers require the maintenance of their findings, but they also call for the implementation of safety regulations on ED-containing products. They require here and now that the substances which have well-known ED-like

properties should be included in the REACH program. Falling within the scope of biodiversity preservation, the Declaration of Prague reminds us that the scientific challenge relative to EDs supposes a financing scheme, and the organization of research in the long term, in order to get a full understanding of the mechanisms and the interactions involved; and of the consequences on human and natural life. For all these scientists, "*it is that which will take part as well as possible in the protection of the health of the European citizens and their environment*" (Excerpt from the researchers' appeal, Declaration of Prague, May 2005).

Conclusion

The complex functioning of the endocrine system is monitored by numerous regulation mechanisms. The fact that it is essential to the maintenance of the biological equilibrium necessary to support life explains why the consequences of a possible disruption of this overall balance by the contaminants of our environment should be investigated.

Drugs like DES, the contraceptive pill or the doping products, or pesticides like DDT, CD, and other industrial products, have been known for a long time to be factors of hypo-fertility or infertility in humans, and their mode of action is definitely linked to an endocrine disruption. Moreover, observing the effects of very low doses, such as appears today with the Roundup or BPA for example, or the fact that some ED mixtures are toxic whereas the EDs are not when taken individually with the same amounts, leads to proceed with extreme caution in this matter, and to implement the precautionary principle, in order to stimulate the related research. In the case of BPA, after years of wrangling over its toxicity, numerous studies were performed and the resulting debates have definitively brought new insights in environmental-health studies [251]. New standards should be implemented. First, it is proposed to analyze the current tools and their limitations, and then to integrate various approaches into new strategies to revise the assessment [252)]. These should be determined with standardized protocols examining chemicals in a wide range of doses, over long term and in sensitive periods, covering environmental typical exposures and including in vivo data. Therefore, the main points which deserve fuller research should concern:

- The description of the substances which are likely to disrupt various endocrine equilibrium
- The determination of the direct impacts on health
- A better comprehension of the amount, exposure time and genetic factors with respect to the toxicity of these substances
- The consequences of an exposure to multiple EDs and of their possible synergistic effects
- The development of suitable animal models
- The determination of the critical periods of exposure
- The determination of action mechanisms.

Undoubtedly, the list of the substances suspected to be EDs is quite long, and increases with products such as Roundup. Starting from an initial list of 600 substances, the European Commission selected 66 of them on a priority list at the very start of the XXIst century. These

molecules will be the subject of research programs aiming at a better evaluation of their dangerousness and of the threats involved. As far as EDs are concerned, assessing "acceptable risks" for the population at large certainly is quite a delicate task, which requires more data, and more accurate ones, than the ones we have collected so far. However, our current knowledge appears to be sufficient to highlight the limits and loopholes of the legislation, and to suggest faster improvements and stricter bans on specific substances, in order to protect the population at large. We can also wonder if chemicals that have been marketed to be stable (like plasticizers) or toxicants (such as pesticides) are not endocrine or nervous disruptors by nature since they are designed to inhibit the cell communication system. At this stage, to choose beyond xenobiotics or natural/biodegradable substances to an economical point of view will be political by nature.

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