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Herbicides: The Face and the Reverse of the Coin. An *in vitro* Approach to the Toxicity of Herbicides in Non-Target Organisms

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1. Introduction

I. Historical aspects, benefits and disadvantages of herbicide use

During thousands of years up to about a hundred years ago, man expended most of required energy in arable farming with mechanical operations aiming to remove weeds and providing suitable conditions for the efficient growth of crop plants, considering that weeds compete with beneficial and desired vegetation, which means that weeds are plants growing where man does not which them to grow. With the dawn of industrialization, labor to the factories decreased manpower on the farms, which forced to think of more efficient mechanical means of weed control.

The need of weed management is as old as agriculture itself. Six stages in the evolution of weed control practices can be considered: 1) 10,000 B.C. — removing weeds by hand; 2) 6,000 B.C. — the use of primitive hand tools to till the land and destroy weeds; 3) 1,000 B.C. — animal-powered implements like harrows; 4) 1920 A.D. — mechanically-powered implements like cultivators, blades, harrows, finger-weeders, rotary-hoes, rod-weeders, etc.; 5) 1930 A.D. — biological control and; 6) 1947 A.D. — chemical control, with the commercial development of organic herbicides such as 2,4-D and MCPA (Hay, 1974).

Especially in the last century, for various reasons among which the population explosion, in his effort to produce adequate supplies of food, man needed to combat efficiently the attacks of various pests on agricultural and horticultural crops. Pesticides, falling into three major classes: insecticides, fungicides, and herbicides (or weed killers), are required. Herbicides, specifically, are used for control of weeds.

At the end of the twentieth century, with an estimated world population of 6 billion people, some 700 million were undernourished and 1.3 billion exist on an inadequate diet. In 2009 FAO says that 1.02 billion people are undernourished, corresponding to 15 percent of the estimated world population of 6.8 billion. Undoubtedly, the first problem of Humankind is the lack of food, which affects especially underdeveloped countries. So, the urgent need for much greater application of herbicides and other agrochemicals is essential to increase food supply. Crops can duplicate or increase even more at the expenses of the agrochemicals use.

Another advantage of the greater efficiency of modern agricultural practice is the liberation of land. This can be of major importance in countries where the space dedicated to agriculture is limited, and where there is a very high population density or soil is inadequate for crop yields.

For more than a century chemicals have been employed for total weed control. For the removal of all plants from railway tracks, timber yards and car roads, crude chemicals such as rock salts, crushed arsenical ores, creosote, oil wastes, sulfuric acid, and copper salts were used (Whitten, 1966; Green et al., 1987). Under these conditions, all plants were killed and these compounds all function as total herbicides and treated areas remain toxic to plants for months or even years. However, for agricultural purposes, it was preferred that chemicals would selectively kill the weeds, but not harm the crop plants.

Selectivity can be based, for instance, in the fact that the larger rougher surfaces of weed leaves are more effectively wetted by the spray than the narrow, smooth cereal leaves in which there was much greater run-off of the toxicant. In a few cases selectivity is based on biochemical differences between weeds and crop plants, such that the latter have a unique defense mechanism. The absence of β-oxidases from leguminous plants enables 2,4-DB to be applied safely for selective weed control in these crops. Maize detoxifies the triazine herbicides (e.g. atrazine and simazine) by enzymic hydrolysis, but weeds do not possess this enzyme. Physicochemical properties may be important for selectivity. The soil-applied herbicide, triazine, have low aqueous solubility meaning that the chemical only penetrates some 5 cm downwords in the soil. Hence, germinating weed seeds are killed but more deep-rooted crops are unaffected. The phenoxyacetic acids were the first really effective selective herbicides, being the product of war-direct research. They came into use at a time when the maximum food production with the minimum labor force was a vital factor in the war effort (Cremlyn, 1991).

Herbicides and research related to their biochemical mechanisms of action have helped to unravel details of several of the biochemical pathways in plants. Historically, effects of various chemicals on photosynthesis and respiration have been studied since around 1878. Then, chloroform was described as reversibly inhibiting photosynthesis at concentrations that did not affect respiration (Moreland, 1993).

In France, in 1933, the first important discovery in the field of selective weed control was the use of DNOC, a contact herbicide that killed the majority of annual weeds infesting the crop without causing appreciable damage to the cereals. Its selectivity is explained by the fact that DNOC is not translocated in plants and perennial weeds were not killed because, although their top growth was desiccated, their extensive root systems survived and in due course sent up further shoots (Cremlyn, 1991). DNOC was developed for use in Europe and dinoseb (DNBP) for use in USA (Moreland, 1993). DNOC and other dinitro compounds played a big part in increasing food production during World War II as herbicides. They can also be used as insecticides, especially as winter wash for fruit trees (Cremlyn, 1991).

On field observations conducted in 1940 and 1941 by Templeman and Sexton, phenoxyacetic acids were toxic to dicotyledonous, but not monocotyledonous plants. Eventually, 2,4-D was commercially developed for use in U.S.A. and MCPA for use in Europe from 1947 (Rao, 2000). The discovery of phenoxyalcanoates revolutionized practices and the success achieved in their use probably stimulated industry to invest in research that led to the discovery of the large variety of herbicides that has been used untill now (Moreland, 1993).

Templeman and Sexton also reported, on field observations made in 1940 with esters of arylcarbamates and thiocarbamates, that they were toxic to monocotyledonous but not to dicotyledonous species (Moreland, 1993).

In 1949, Macdowall reported inhibition of the Hill reaction, in illuminated chloroplasts, by phenylurethane (I_{50}=2 mM) and dinitrophenol (DNP) (I_{50}=0.63 mM). However, based on I_{50}, arylcarbamates and phenylureas were up to 2500 times more active than phenylurethane, as was detected by Wessels and van der Veen in 1956 (Moreland, 1993).

Herbicides are the most widely used class of pesticides accounting for more than 60% of all pesticides applied in agriculture (Zimdahl, 2002). One of the main concerns about the use of herbicides is their effects on non-target organisms, especially mammalian toxicity.

Most modern herbicides have low mammalian toxicity, because research for new herbicides often rejects chemicals affecting metabolic pathways that are shared by mammals. In many cases, the low mammalian toxicity has to do with the fact that these chemicals interfere with biochemical pathways that do not exist in mammals, such as photosynthesis, essential amino acids biosynthesis or chlorophyll biosynthesis (Shaner, 2003).

There are about 20 mechanisms of action that have been elucidated for herbicides. Of these, some do share common target sites with mammalians. However, the consequence of inhibiting a common target site in plants can be quite different than in animals. What may be a lethal event in plants, e.g. inhibition of 4-hydroxyphenylpyruvate dioxygenase (HPPD), can even have a beneficial effect in mammals, e.g. treatment for tyrosinemia type I (Shaner, 2003). Also a common enzyme existing in plants and mammals, e. g. acetyl-CoA carboxilase (ACCase), can be inhibited by the herbicides cyclohexanediones and the aryloxyphenoxypropionates, killing plants, and not affecting ACCase in mammals. ACCase plays the same role in the fatty acid biosynthesis of the different organisms, despite vast differences in its regulation and protein structure (Incledon & Hall, 1997; Shaner, 2003). These herbicides suppress de novo synthesis of fatty acids in sensitive species by inhibiting the activity of plastid-associated ACCase. Aryloxyphenoxypropionates were reported by Hoechst AG in 1971 (Nester, 1982), and cyclohexanediones (alloxydim and sethoxydim) were introduced by Nippon Soda in 1977 and 1978 (Iwataki & Hirono, 1979; Ishikawa et al., 1985), being used as postmergence herbicides for the control of annual and perennial grasses in broadleaf crops.

Other commercially available herbicides that affect similar target sites in plants and mammals, with the exception of the bipyridiliums, have minimal toxicity to mammals because they are rapidly metabolized and excreted by mammals. As the herbicides do not accumulate in mammalian tissue, they cannot affect the biosynthetic pathways (Shaner, 2003).

On the other hand, the effects of inhibiting a particular enzyme in plants can be quite different from those observed in mammals. For example, considering that protoporphyrin biosynthesis is critical in plants and animals for the production of chlorophyll and heme, PROTOX (protoporphyrinogen IX oxidase) inhibitors cause the accumulation of Proto IX (protoporphyrin IX), which then absorbs light energy and produces reactive oxygen species that disrupt membranes by lipid peroxidation. In mammals, the accumulation of Proto IX also occurs in the liver and bile, but these organs are not normally exposed to light intensities like plant leaves. So, there is little opportunity for the Proto IX to produce degrading reactive oxygen species in animals (Shaner, 2003). Peroxidizing herbicides include diphenylether herbicides and oxodiazoles causing accumulation of large amounts of Proto IX, which generates singlet oxygen by light. If this oxygen free radical is not quenched, lipid peroxidation can be initiated (Lydon & Duke, 1988; Matringe & Scalla, 1988; Camadro et al., 1991).

It was observed in 1980 that glyphosate was a highly specific inhibitor of shikimate pathway enzyme 5-enolpyruvylshikimate 3-phosphate synthase (Steinrucken & Amrhein, 1980). Afterwards, sulfonylureas and imidazolinones were introduced as herbicides interfering with biosynthesis of branched-chain amino acids (leucine, isoleucine, and valine). It was shown that both herbicides inhibited the activity of acetolactate synthase (Ray, 1984; Shaner et al., 1984)

Herbicides can also inhibit carotenoid synthesis. As carotenoids are free radical scavengers, they protect chlorophyll from photooxidation (bleaching herbicides). One of the first compounds shown to function in this way was the pyridinazinone SAN 6706 (Bartels & McCullough, 1972). Afterwards, other compounds identified with similar functions include fluridone, difunon, dichlormate, and aminotriazole (Moreland, 1993).

Some times, e.g. glufosinate in suicide attempts, much of the toxicity has been related to the associated surfactant that is included in the formulation of the herbicide rather than to the active herbicide (Koyama et al., 1997).

From all the information here considered, it is obvious that the use of herbicides implies the existence of advantages and disadvantages resulting therefrom, which, in general, are listed in Table 1.

ADVANTAGES	DISADVANTAGES
— They kill unwanted plants. — They help the crops grow by destroying the weed that is robbing the crops water, nutrients and sunlight. — They can be safely used whereas in some cases manually or mechanically removing weeds can destroy the crop. — They can be used on closely planted crops where other methods cannot be used. — Most of the time one application of the herbicide is enough whereas other methods have to be continually used. — They are easy to use. — They work fast. — Herbicides are relatively cheap, and cheaper than manual weeding. — Non-selective herbicides can effectively clear fields, where houses and roads can then be built. — They can destroy plants bearing diseases. — Some are biodegradable, and become relatively harmless after decay.	— Some herbicides are non-biodegradable being harmful for a long period of time. — They are all at least slightly toxic. — They can cause illnesses, and even cause accidental or suicidal death (like paraquat). — They can be carried into streams by runoff rainwater or leached into underground water supplies polluting them. — Herbivores may eat the plants treated with herbicides and then carnivores eat the herbivores. The toxic herbicide would be passed up the food chain increasing in concentration, being harmful for those animals and man.

Table 1. Advantages and disadvantages of herbicides.

Scientists have made phenomenal progresses in understanding the selective action of hundreds of herbicides by studying their absorption and translocation patterns, mechanisms of action in plants, degradative and detoxification mechanisms in plant and soil, interactions

with other pesticides and chemicals, etc. All this has helped in making more effective, economical and safe recommendations for control of numerous weeds (Rao, 2000).

Lately, scientists have already researched on natural product-based herbicides, which are generally considered safer than synthetic herbicides. However, relatively few commercial herbicidal agents have been derived from naturally occurring compounds. One of the indirect and important benefits of the chemical composition and structural characteristics of natural products is that most of these compounds are rapidly degraded in the natural environment. Thus, this accounts for the perception that most natural products are environmentally benign (Dayan et al., 1999).

An alternative approach of natural products-based weed management consists of having crops producing their own phytotoxins to prevent or suppress the growth of competing weeds in its immediate surroundings (allelopathy). Although some species strongly repress the development of other plants, allelopatic crops have had little success so far (Dayan et al., 1999).

Weed scientists are now facing new challenges, particularly in the light of emergence of weeds resistant to herbicides and concerns and questions about herbicide residues in food, soil, groundwater and atmosphere (Rao, 2000).

Finally, when scientists were capable of getting genes that encode target site enzymes cloned, modified and expressed in transgenic plants, they could confer herbicide tolerance. The possibility of developing herbicide-resistant crops began a new era in the use of herbicides, stimulating work on the genetics of herbicide resistance.

II. Classification of herbicides

By 1976, the herbicides that interfered with electron transport and phosphorylation in both chloroplasts and mitochondria were separated into five classes: a) electron transport inhibitors, b) uncouplers, c) energy transfer inhibitors, d) inhibitory uncouplers (multiple types of inhibition), and e) electron acceptors (Moreland, 1993).

Electron transport inhibitors include the Hill reaction inhibitors. Uncouplers dissociate electron transport from ATP formation by permeabilization to protons collapsing ΔpH. Energy transfer inhibitors interact with the coupling factor complex. Inhibitory uncouplers are those herbicides that act both as electron transport inhibitors and uncouplers. Electron acceptors are herbicides like bipiridilliums that intercept electron flow from photosystem I.

With isolated mitochondria, the inhibitory uncouplers uncouple phosphorylation at low molecular concentrations and inhibit electron transport at higher concentrations (Moreland, 1993).

The pure electron transport inhibitors also have been called diuron-type and include chlorinated phenylureas, pyridazones, s-triazines, triazinones, uracils, and ureacarbamates. This type of herbicides inhibits the Hill reaction but do not inhibit mitochondrial electron transport. Hence, light was known to be required for the expression of toxicity, i.e., plants maintained in the dark revealed no signs of toxicity (Moreland, 1993).

Inhibitory uncouplers were divided into two groups: dinoseb-type and dicryl-type. Included as dinoseb-type are dinitrophenols, benzimidazoles, benzonitriles, bromophenoxim, and thiadiazoles. Dicryl-type includes arylanilides, dinitroanilines, diphenylethers, bis-carbamates and perfluidone (Moreland & Novitzky, 1988).

Herbicides are designated by common names approved by the Weed Science Society of America or the British Standards Institution (Moreland, 1980), and organic herbicides are classified on the basis of: a) method of application; b) chemical affinity and structure similarity, and; c) mode of action (Rao, 2000).

On the basis of the method of application, herbicides are distributed in two groups: 1) soil-applied and 2) foliage-applied. All herbicides applied at preplanting (surface or incorporation) and preemergence (to crop, weeds, or both) are included in the soil-applied group; those applied at postemergence on the plant parts are included in the foliage-applied group.

Here we also present two general classifications of herbicides according to the mode of action and according to the chemical composition.

According to the mode of action, we present in Table 2 the classification extracted and adapted from a review (Moreland, 1980).

Classes of herbicides	Examples of herbicides

*CHLOROPLAST-ASSOCIATED REACTIONS
TYPES OF INHIBITORS
    Electron transport inhibitors
    Uncouplers
    Energy transfer inhibitors
    Inhibitory uncouplers
    Electron acceptors
    Carotenoid synthesis inhibitors
	Diuron, atrazine, pyrazon, etc.
	Perfluidone.
	1,2,3-thiadiazolyl-phenylureas.
	Dinitrophenols, acylanilines, etc.
	Paraquat, diquat.
	Amitrole, dichlormate, etc.

*MITOCHONDRIAL ELECTRON TRANSPORT AND PHOSPHORYLATION
TYPES OF INHIBITORES
    Electron transport inhibitors
    Uncouplers
    Energy transfer inhibitors
    Inhibitory uncouplers
	Pyriclor.
	Isipropyl ester of glyphosate.
	No herbicide.
	Dinitrophenols, acylanilines, etc.

*MEMBRANE INTERACTIONS
    Composition alterations
    Permeability and integrity actions
	Dinoben, perfluidone, ioxynil.
	Paraquat, diclofop-methyl.

*CELL DIVISION
    Energy relations
    Microtubules action
	Chlorpropham, trifluralin.
	Prophan, amiprofos-methyl.

*NUCLEIC ACID METABOLISM DNA and RNA action	Trifluralin.
PROTEIN SYNTHESIS Protein synthesis inhibitors	Glyphosate.

Table 2. Classification of herbicides according to the mode of action at the level of the main metabolic systems in the cell.

The other classification, distributing herbicides by groups with chemical affinities (Cremlyn, 1991; Rao, 2000), is presented in Table 3.

Classes of herbicides	Examples of herbicides	Mode of action
ALIPHATICS	[1]Dalapon, [2]TCA.	[1]Interference with RNA synthesis; [1,2]modifications of protein structure.
AMIDES Acylanilides	Propanil, pentanochlor.	Inhibition of Hill reation in photosynthetic electron transport.
Chloroacetanilides	[1]Allidochlor, [2]propachlor, [2]alachlor, [2]butachlor, [2]pretilachlor, [2]diethatylether, [2]metolachlor, [2]metazochlor.	[1] Inhibition of protein synthesis, respiration; [2] Inhibition of cell division and protein synthesis.
Benzamides	Benzoylpropethyl, flamprop-isopropyl, fampropmethyl, isoxaben.	Unknown.
Other amides	[1]Naptalam, diphenamid, [2]propyzamide, butam, [3]diflufenican.	[1]Alteration of auxin transport; [2]interference with cell division; [3]inhibition of carotenoid biosynthesis.
AROMATIC CARBAMATES AND THIOCARBAMATES Arylcarbamates	Propham, chloropropham, barban.	Interference with photosynthesis; alteration of meristematic activity; desorganization of microtubular assembly, inhibiting mitosis.
Thiocarbamates	EPTC, butylate, cycloate, pebulate, diallate, triallate, benthiocarb, asulam, nisulam.	Interference with lipid biosynthesis.
ARSENICALS	DSMA, MSMA, cacodylic acid.	They are uncouplers and inhibit respiration.
BENZOTHIADIAZOLES	Bentazon.	Inhibition of photosynthesis and induces lipid peroxation.
BIPYRIDILIUMS	Diquat, paraquat.	Induce lipid peroxidation by reactive oxygen species production.
CARBOXYLIC ACIDS Phenoxyacetic acids	[1]2,4-D, MCPA, 2,4,5-T, etc.	Inhibition of normal plant growth mimicking auxins (IAA); [1] Inhibition of oxidative phosphorylation.
Phenoxybutiric acids	2,4-DB, MCPB.	Inhibition of normal plant growth mimicking auxins (IAA).

Classes of herbicides	Examples of herbicides	Mode of action
Benzoic acids	TBA, dicamba.	Inhibition of normal plant growth mimicking auxins (IAA)
Pyridine derivatives	Picloram, clopyralid, triclopyr.	Inhibition of normal plant growth mimicking auxins (IAA).
Propionic acid derivatives	Dchlorprop, mecoprop, fenoprop, haloxyfop, fenoxaprop, fluazifop-P.	Inhibition of normal plant growth mimicking auxins (IAA).
CHLOROALIPHATIC ACIDS	[1]Dalapon, [2]TCA.	[1]Interference with RNA synthesis; [1,2]modifications of protein structure.
CINEOLES	*Cinmethylin.	Inhibition of asparagine synthetase.
CYCLOHEXANEDIONES	Clethodim, cycloxidim, sethoxydim, tralkoxydim.	Interference with lipid metabolism.
DINITROANILINES	Trifluralin, benfluralin, profluralin, ethalfluralin, fluchloralin, oryzalin, nitralin, butralin, pendimethalin, dipropalin.	Inhibition of cell division.
DIPHENYL ETHERS	Nitrofen, fluorodifen, bifenox, [1]oxyfluorfen, fomesafen.	Inhibition of Hill reation in photosynthesis and photophosphorylation; [1] inhibition of protoporphyrinogen oxidase.
IMIDAZOLIDINONES	Buthidazole.	Inhibition of r espiration and photosynthesis.
ISOXAZOLIDINONES	Clomazone.	− −
IMIDAZOLINES	Imazaquin, imazethapyr.	Inhibition of cell division by action on the enzyme acetolactate synthase (ALS) involved in valine and isoleucine biosynthesis.
NITRILES	[1]Dichlobenil, [2]bromoxynil, [2]ioxynil.	[1]Inhibition of cellulose biosynthesis; [2]uncoupling of oxidative phosphorylation and Hill reation inhibition.
ORGANOPHOSPHORUS COMPOUNDS	[1]Glyphosate, bensulide, **tributyl phosphorotrithiolate.	[1]Inhibition of biosynthesis of phenylalanine.

Classes of herbicides	Examples of herbicides	Mode of action
OXADIAZOLES	Oxadiazon.	Causes rapid peroxidative damage.
PHENOLS	Dinoseb, DNOC.	Uncouplers of oxidative phosphorylation.
N-PHENYLPHTHALAMIDES	Flumiclorac.	Inhibition of protoporphyrinogen oxidase.
PHENYL TRIAZINONES	Sulfentrazone.	Inhibition of protoporphyrinogen oxidase.
PHTHALAMATES	Naptalam.	It is an auxin inhibitor.
PYRAZOLIUMS	Difenzoquat.	It affects nucleic acid biosynthesis, photostynthesis, ATP production, K+ absorption and P incorporation in phospholipids and DNA.
PYRIDAZINES	[1]Chloridazon, [2]metflurazon, [2]norflurazon, [1]pyridate.	[1]Inhibition of Hill reation in photosynthesis; [2]Inhibition of carotenoid biosynthesis.
PYRIDINONES	Fluridone.	Bloks carotenoid biosynthesis.
PYRIMIDINYL THIOBENZOATES	Pyrithiobac.	Inhibition of cell division by action on the enzyme acetolactate synthase (ALS) involved in valine and isoleucine biosynthesis.
QUINOLINE CARBOXYLIC ACIDS	Quinclorac.	Auxin-type herbicide. Accumulates ACC, cyanide and ethylene.
SULPHONYLUREAS	Sulfometuron methyl, DPX-F 6025, DPX-F 5384, chlorsulfuron, DPX-L 5300, bensulfuron methyl.	Inhibition of cell division by action on the enzyme acetolactate synthase (ALS) involved in valine and isoleucine biosynthesis.
TRIAZINONES	Metribuzin.	— —
TRIAZOLOPYRIMIDINE SULFONANILIDES	Flumetsulam.	Inhibition of cell division by action on the enzyme acetolactate synthase (ALS) involved in valine and isoleucine biosynthesis.

Classes of herbicides	Examples of herbicides	Mode of action
TRIAZINES	Simazine, atrazine, prometryne, desmetryn, methoprotryne, terbutryn, cyanazine, eglinazine ethyl, aziprotryne, metrybuzin, metamitron, prometron, terbuthylazine, methroprotryne, aziprotryne.	Inhibition of Hill reation in photosynthetic electron transport.
TRIAZOLES	***Amitrole.	Probable interference with carotenoid biosynthesis leading to photooxidation of chlorophyll.
URACILS (PYRIMIDINES)	Bromacil, terbacil, lenacil, UCC-C4243.	Inhibition of Hill reation in photosynthetic electron transport.
UREAS	Monuron, diuron, linuron, monolinuron, chlorotoluron, isoproturon, difenoxuron, thiazafluron, terbuthiuron, ethidimuron, fluometuron, S3552.	Inhibition of Hill reation in photosynthetic electron transport.
OTHER HETEROCYCLIC HERBICIDES	[1]Endothal, [2]ethofumesate, [3]benzolin, [4]oxadiazon, [4,5]difenzoquat.	[1]Inhibition of lipid and protein biosynthesis; [2]interference with plant cuticular wax; [3]interence with cell growth and development; [4]inhibition of carbon dioxide fixation and electron transport in photosynthesis; [5]alteration of DNA and RNA synthesis.

*Is a member of the herbicide group called cineoles (monoterpenic cyclic ethers) (Romagni et al., 2000)
**Defoliant, induces early leaf abscission through changes in the levels of plant hormones (Matolcsy, 1988)
***Its use in fodder and food crops has been banned worldwide (suspicious of carcinogenicity)

Table 3. Classification of herbicides according to chemical classes.

2. *In vitro* toxicological assays: some biological model-systems

In this section, we will address some of the experimental strategies used by our group to evaluate the toxic potential of pesticides upon interaction with biological systems, using *in vitro* assays. The authors are aware that many other models have been successfully used with the same objectives, some of which will be discussed in confront with authors results in the next section. However, an exhaustive description of *in vitro* model systems used to herbicide toxicological assessment is out of the scope of this chapter.

I. Mitochondria, a key organelle to push cells towards survival or death

Mitochondria are efficient power plants of energy generation for eukaryotic cells. Moreover, mitochondria play a crucial role in several physiological processes and are involved in cell replication, differentiation and apoptosis (Boelsterli, 2003). In this way, mitochondria endowed eukaryotic cells with a broad spectrum of functionalities that prokaryotic cells do not have, but, concomitantly, they brought with them a multiplicity of vulnerable points to host cells. In fact, these organelles are a key target for xenobiotics to induce dysfunction at a cell, organ or organism level and the impairment of energy production is not the only harm of mitochondrial poisoning.

There are several means by which xenobiotics interfere with mitochondrial function. However, as most xenobiotics considered in this chapter are lipophilic molecules, their effects on the activities of mitochondrial matrix soluble enzymes involved in the fatty acid β–oxidation and tricarboxylic acid oxidation pathways will not be directly addressed. The focus of this section will be mainly the xenobiotic-induced alterations of mitochondrial membrane-related processes, such as electron transport, transmembrane potential ($\Delta\Psi$) generation, oxidative phosphorylation, oxygen reactive species generation, permeability transition and the release of pro-apoptotic proteins.

Three important features make mitochondria vulnerable to membrane-active compounds. The first one is the unique lipid composition of the inner membrane (Daum, 1985), with virtually no cholesterol and a high content of cardiolipin (CL), a phospholipid that is common in bacterial membranes but, in eukaryotic cells, only exists in membranes of mitochondria and chloroplasts (Gennis, 1989). Several xenobiotics have a high affinity for CL, which explains their mitochondriotropic effects (Boelsterli, 2003). A very well known example is given by adryamicin-like anthraquinones (Wallace & Starkov, 2000). The accumulation of these xenobiotics at the inner mitochondrial membrane may have different consequences. In the case of adryamicin, for example, it leads to the generation of oxidative stress due to redox cycling of quinone-semiquinone moiety (Boelsterli, 2003). Considering the important roles CL plays in mitochondria (see below), xenobiotic incorporation in specific CL-enriched domains of the inner mitochondrial membrane may predictably have disastrous outcomes for mitochondrial bioenergetics and also for the fate of cells towards survival or death. Cardiolipin is included in the quaternary structure of the complexes II, IV and V (Eble et al., 1990; Fry & Green, 1981; Sedlak et al., 2006) of the mitochondrial electron transport system. Besides those complexes, the complex I also requires cardiolipin for exhibiting optimal activity (Fry & Green, 1981). Furthermore, cytochrome c (cyt c) establishes specific interactions with CL (Rytomaa et al., 1992), which have been suggested to participate in a well coordinated mechanism of programmed cell death, involving cyt c-catalyzed peroxidation of CL, permeabilization of the outer mitochondrial membrane and release of cyt c and other apoptogenic factors from the mitochondria (Gonzalvez & Gottlieb, 2007; Basova et al., 2007). As proposed by Basova et al. (2007), CL association with cyt c could be a decisive step in the early stages of apoptosis, turning off the normal functions of this hemoprotein in mitochondrial electron transport and turning on its peroxidase activity. Therefore, xenobiotics, directly interacting with CL or modulating the bilayer properties of the mitochondrial membranes, may have drastic consequences in mitochondrial bioenergetics and/or apoptosis (Gonzalvez & Gottlieb, 2007).

The second feature which makes mitochondria vulnerable to xenobiotics is related with the high potential across the inner mitochondrial membrane, typically in the range of -180 to -220 mV. The anisotropic distribution of both charges and pH (the matrix side being

negatively charged and slightly alkaline in opposition to the outer side of the membrane, facing the intermembrane space) confers to mitochondria a high susceptibility to accumulate large amounts of positively charged lipophilic compounds. The accumulation of these compounds into mitochondria, at concentrations that may exceed those of the cytosol by several orders of magnitude, exposes these organelles to potentially toxic compounds at much higher levels than any other cellular compartment.

The third reason why mitochondria are prone to be poisoned by lipophilic compounds concerns with the protein-regulated permeability of the inner mitochondrial membrane, which has a crucial importance for the functional integrity of mitochondria. Therefore, drastic consequences for mitochondrial intactness and bioenergetic functioning can be predicted whenever compounds promote an increase of the passive permeability of mitochondrial inner membrane, impair the activity of transport proteins embedded within it or interfere with the assembling of protein components of the mitochondrial permeability transition pore (MPTP). Xenobiotics that cause the opening of MPTP may also promote the release of apoptosis mediators, inducing apoptosis.

On the other hand, xenobiotics that interact with one or several complexes of the mitochondrial electron transport system, impairing the normal electron flow, may enhance the generation of reactive oxygen species (ROS), leading to an imbalance between prooxidant species and the cellular antioxidants. The complex I (NADH: ubiquinone oxidoreductase) is often a site of interference of xenobiotics acting as electron acceptors in reductase-catalyzed reactions. Redox cycling compounds, as the herbicide paraquat, may be particularly harmful. The generation of ROS, amplified by the cycling process, may lead to oxidative DNA damage and lipid peroxidation (Boelsterli, 2003). Additionally, xenobiotics that promote oxidative stress as well as those that induce a sustained increase of mitochondrial calcium concentration may disrupt mitochondrial function by opening the MPTP. The consequent uncoupling of oxidative phosphorylation, due to the collapse of mitochondrial membrane potential, and the release of pro-apoptotic factors are, in this case, the main cause of cellular damage (Boelsterli, 2003).

Although rat liver mitochondria constitute a common model for toxicity assessment, plant mitochondria can be an alternative model, offering several advantages, namely high activities preserved for much longer periods of time, easier availability and low cost (Vicente & Madeira, 2000). In the context of herbicide toxicity evaluation, the use of plant mitochondria is particularly important since useful plants (crops) are often target of the non-selective toxic effects of those compounds.

II. Unicellular microorganisms, a simple model system to study the complex routes of cell/membrane toxicity

A great number of compounds produced by agrochemical industries, including herbicides, are lipophilic molecules. In the case of biocide compounds, this feature is important to increase their ability to overcome cell membrane barriers. In a toxicokinetic context, the disposition of a foreign molecule in a biological system necessarily involves the passage across cell membranes. Thus, lipophilicity is a major determinant of compound capacity to exert noxious effects on target organisms. However, cellular compartmentalization by phospholipid-based membranes is a feature common to all living organisms and, then, all share a high vulnerability to lipophilic compounds. The selective effects of these compounds on specific organisms may arise from factors that affect compound metabolism, distribution or excretion (toxicokinetics) and from differences in the existence, accessibility or structural

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singularities of the molecular target of these compounds (toxicodynamics). The level of concern increases when the interaction of lipophilic compounds with membranes results in unspecific alterations of the physical properties of the lipid bilayer, compromising cell normal functioning. In fact, the lipid bilayer, besides defining a permeability barrier and providing a matrix for embedded proteins, endows the membrane with the capacity of regulating protein function (Lundbaek et al., 2010). Bilayer material properties such as thickness, intrinsic curvature, and the elastic compression and bending moduli, have been shown as having a key role on protein folding, trafficking, distribution in the plane of the membrane and function (McIntosh & Simon, 2006). Therefore xenobiotic-induced bilayer deformations or perturbation of their physical properties may cause drastic effects on a great diversity of vital cell functions. In this case, the interactivity with membrane lipid bilayer is involved in both toxicokinetics and toxicodynamics of the compounds.

In microbial cells, most vital functions are ascribed to the plasma membrane, e.g. signal and energy transductions, regulation of the intracellular environment and transport of solutes (Kaback, 1972; Stock et al., 1990; Trumpower & Gennis, 1994). Not surprisingly, perturbations of membrane structure and function by pollutants will end in more or less severe disturbance of cell growth and viability. Therefore, these simple systems can offer the opportunity to screen the cytotoxic potential of membrane-active compounds. Measurements of growth parameters provided by bacterial cell cultures in liquid media (e.g. specific growth rate, final bacterial yield), the counting of colony forming units in solid growth media and the evaluation of bacterial respiratory activity or other metabolic parameters have been extensively used as short-term screening tests for *in vitro* assessment of the toxicity of several environmental pollutants (Hernando et al., 2003; Sikkema et al., 1995; Monteiro et al., 2005).

On the other hand, bacteria have one, in maximal two, membranes surrounding the cellular content, which can be easily isolated providing a pure membrane fraction. Two experimental approaches may be developed by using bacterial membranes as a biological model system: a) evaluation of the influence of membrane biophysical properties, perturbed by the accumulation of alien lipophilic molecules, on cellular toxic effects; b) identification of bacterial mechanisms of regulation of the membrane lipid composition triggered by xenobiotic incorporation and its impact on counteracting cell toxicity. In fact, alterations of membrane lipid content (the relative proportions of different lipid classes and the acyl chain composition of phospho- and glycolipids) are common strategies for adaptation and responses to stress in bacteria (Jurado et al., 1991; Sikkema et al., 1995; Weber et al., 1996). Since bacterial lipid regulatory system is highly precise and sensitive, studies of the molecular mechanisms of membrane adaptation, together with determinations of growth inhibition and impairment of bacterial metabolic activities, can provide an alternative methodology for toxicity assessment. Thus, microorganisms offer the advantage of allowing the assessment of xenobiotic toxic effects ranging from the molecular to the cell/whole organism level. These data, together with the knowledge of physicochemical characteristics of the compounds, can be useful to establish structure-activity relationships.

In the field of ecotoxicology, two main reasons explain the considerable attention effects of lipophilic pollutants on microorganisms have drawn: firstly, microorganisms are responsible for recycling organic material in soil ecosystems and, then, are valuable indicators of pollutant ecological impact. Thus, toxicological data concerning soil bacteria may be used to define upper limits for concentration of pollutants and to predict environmental toxicity risks; secondly, several of those microorganisms have shown to be

able to thrive in high concentrations of organic pollutants converting them into non-toxic metabolites (Dejonghe et al., 2003; Sorensen et al., 2003; Neumann et al., 2004). The interest here is to use these microorganisms for the removal of pollutants from the environment (bioremediation).

However, in the present work, the emphasis will be on the use of microorganisms as models to correlate xenobiotic-induced perturbations at a molecular level with the impairment of physiological processes studied *in vitro*. Different microorganisms have been used for this purpose such as the yeast *Sacharomyces cerevisiae* (Cardoso & Leão, 1992) and *E. coli* (Heipieper et al., 1991) as the most studied bacterial model. Microorganisms offer over multicellular organisms several advantages: (i) yeast/bacterial growth in laboratory controlled conditions is easy and economic; (ii) these models avoid ethical issues associated with animal use, providing alternative *in vitro* toxicity tests; (iii) microorganisms allow to correlating in the same system the toxic effects exerted by xenobiotics on the organism as a whole, assessed *in vivo* (e.g. growth disturbances), and their toxic action on cell functions assayed *in vitro* (e.g. protein/channel activities or passive membrane permeability).

In our laboratory, a strain of the thermophile *Bacillus stearothermophilus*, grown in a liquid medium (L-Broth) at 65°C (within the optimal temperature range), has been extensively used as a model system for assessing the cytotoxicity of different groups of environmental pollutants, as herbicides (Pereira et al., 2009; 2010), insecticides (Donato et al., 1997a,b; Martins et al., 2003; Monteiro et al., 2005) and organometals (Martins et al., 2005). Toxicity indicators have been taken from parameters of growth (lag time, specific growth rate and bacterial yield in the stationary phase) when growth is followed by turbidity measurements or by counting the colony-forming units (c.f.u.) after plating serial dilutions of the liquid cultures on agar plates. Other indicators of toxicity have been provided by monitoring the respiratory activity (oxygen consumption rate supported by different respiratory substrates). Studies of growth or respiration performed in a broad concentration range have allowed the determination of toxicity indices as EC50, NOAEL and/or LOEC. A very good correlation has been observed between toxicity data provided by *B. stearothermophilus* and by other model systems, namely rat liver mitochondria (Monteiro et al., 2008; Pereira et al., 2009).

B. stearothermophilus presents several advantages to be used as a toxicological model system, in addition to the general ones of bacterial cells. Firstly, cultures of this bacterium with high cell densities are rapidly obtained at optimal conditions (temperature and pH) of growth (doubling times around 15 min in the absence of toxicants). Thus, a significant amount of cells (around 10^8 colony forming units per ml of growth medium) may be obtained in a few hours. Hence, in comparison with other time-consuming assays for toxicological assessment, *B. stearothermophilus* growth experiments are very advantageous. Moreover, it is also possible in a short time to isolate homogeneous membrane preparations for lipid extraction or for monitoring enzymatic reactions. Additionally, *B. stearothermophilus* growth showed to be very sensitive to perturbations of membrane physical properties and efficient molecular mechanisms of membrane adaptation (involving changes of membrane lipid composition) are triggered by adverse growth conditions (Jurado et al., 1991; Donato et al., 1997a and 2000; Luxo et al., 1998; Rosa et al., 2000). For these reasons this model is particularly useful for drug toxicity assessment at the membrane level.

On the other hand, the growth of this bacterium occurs at high temperatures (optimally at 60-65°C), preventing contamination, simplifying sterilization procedures and also assuring safety for the operator. Concerning the respiration assays, protoplasts are advantageous in comparison with other systems, as animal mitochondria, for several reasons: economics,

easier manipulation and high performance during much longer periods (several days). Additionally, the respiratory system of this bacterium shows a great similarity with that of mitochondria. However, an accurate determination of the levels at which xenobiotics act in the bacterial oxidative phosphorylation system is technically difficult, since there are not known inhibitors for the respiratory complexes, except for the cytochrome oxidase (CN- as inhibitor). In spite of this limitation and the restricted number of substrates that efficiently support the respiration of protoplasts, very good correlations have been obtained regarding the concentration range of xenobiotics that impair the electron transfer system in both bacterial and eukaryotic models (Donato et al., 1997b; Monteiro et al., 2005; 2008; Pereira et al., 2009).

Taking together growth, respiration and membrane physical data collected with *B. stearothermophilus*, a remarkable parallelism may be established with results from studies carried out with other toxicological model systems, revealing differential toxic effects exerted by xenobiotics and their metabolites (Donato et al., 1997b and 2000), xenobiotic isomers (Martins et al., 2003; Monteiro et al., 2003) or different molecules of the same family of compounds (Pereira et al., 2009 and 2010).

III. Cell cultures of plant tissues, an economical model to evaluate phytotoxicity of chemical agents of stress

Plant and animal cell cultures have proven to be a useful tool for assessing the toxicity of chemical compounds *in vitro* (Steward, 1983; Babich et al., 1986). Diverse cytotoxicity endpoints can be considered. The most used are the cell viability, membrane integrity, changes in the content of vital macromolecules (DNA, proteins) or small molecules involved in cell metabolism (ATP, NADH), and antioxidant defenses (e.g. GSH).

Studies with plant tissue cultures are particularly meaningful when the goal is to screen the general toxicity of herbicides in non-target organisms. Useful plants (crops) can be susceptible organisms to the toxic action of those compounds, not only because they coexist with the target-plants (weeds), but also because weeds and crops do share a great number of features putatively affected by the toxic action of the herbicides.

Potato (*Solanum tuberosum* L.) is one of the most important crops in the temperate climate regions of the world (Zuba & Binding, 1989). It has been cultivated in Europe, but its production has been also increasing in China, India and Indonesia, as this crop tolerates a great variety of climates, from temperate to tropic, and from humid to arid. As a food crop, its value results from the accumulation of high amounts of starch in the tuber in combination with proteins, lipids and minerals. Therefore, the interest in studying herbicide toxic effects on potato tissue cultures *in vitro* is dual: a) to appraise the susceptibility or eventual resistance of this important food crop to herbicide toxicity and b) to use these accessible cultures as model systems for screening the toxicity of herbicides *in vitro*.

Tubers, roots, leaves, and other parts of the *Solanum tuberosum* plant have been extensively used as explants for initiating callus (friable, pale-brown lumps) formation in the presence of adequate culture media. The fully mature cells of potato tubers preserve, even after a long period of quiescence, the ability to behave as growing cells when appropriately stimulated, showing activities such as respiration supported by the carbohydrates from starch hydrolysis, protein synthesis, uptake of water and inorganic salts harnessing its metabolism and available energy (Steward, 1983). Plant cell cultures constitute a true clonal growth of the original plant, retaining not only the inheritance and totipotency of their nuclei but also the competence of the cytoplasm to regulate gene expression (Steward, 1983).

The use of plant tissue cultures has several advantages over other methods for toxicity assessment: a) high sensitivity, versatility, speed and simplicity; b) minimal costs for the equipment; c) technical expertise is easy to obtain; d) growth conditions are easily controlled in laboratory. Additionally, in callus tissue cultures, the simplicity of the structural organization and the absence of cellular wall barrier amenable to an effective herbicide penetration and translocation (Mumma & Davidonis, 1983; Magalhães et al., 1989; Nellessen & Fletcher, 1993) make this sensitive model a useful tool to study herbicide toxicity and metabolism in plants (Sandermann et al., 1984; Smeda & Weller, 1991). The growth of non-green callus tissue in the dark precludes the analysis of herbicide effects on the photosynthetic system, but allows to address studies to other putative targets of the toxic action of those compounds, such as mitochondria which have a crucial role in ATP synthesis in these cells devoid of chlorophyl. Thus, potato callus tissue has been used as a relevant material source for *in vivo* and *ex vivo* studies of herbicide toxicity at the mitochondrial level (Peixoto et al., 2008; 2009a).

In summary, plant cell cultures have been established as a good alternative model for ecotoxicological evaluation and also as a means to study herbicide mechanisms of action and metabolism in plants (Harms, 1992).

IV. Fish as an economical vertebrate model for toxicity assessment

Fish are currently used as toxicological models with a major importance in hazard identification, environmental risk assessment, and biomedical research in human development and diseases (Hill et al., 2005; Schmale et al., 2007). Fish are the most numerous, the most diverse and the oldest group of vertebrates, allowing the investigation of fundamental biological principles that can be extrapolated to humans. For example, studies on renal physiology of fishes led to diagnostic methods remaining in use in medicine today (Beyenbach, 2004). In a toxicological context, fish are also potential target for action of toxic compounds, such as herbicides, since aquatic ecosystems are the final reservoir of numerous chemicals produced and used by man (Pritchard, 1993). Thus, they are used in acute and chronic studies to evaluate the susceptibility of individual species to a wide variety of pollutants (*e.g.* herbicides).

Acute toxicity assessment, the first step of chemical toxicological evaluation, allows to determining the LC_{50} (96 h) value (e.g., according to OECD guideline 203, *i.e.*, *as the concentration of the test substance resulting in 50% mortality of the experimental fish over a period of 96 hours*; OECD203, 1992). Although death is the endpoint in acute toxicological assays and it represents an unambiguous parameter, its environmental significance is questionable. The information concerning the way how the chemical compounds exert their acute toxicity in fish is scarce (Nagel, 2002). On the other hand, the chronic effects resulting from long-term exposure to low concentrations are environmentally relevant, allowing the comprehension of the molecular mechanisms underlying the toxic effects and the establishment of structure-activity relationships of several classes of xenobiotics (Pritchard, 1993). Therefore, it is important to understand chronic effects that arise from chemical exposure of fish at all life stages, i.e. embryonic, larval, juvenile and adult (Lawrence & Hemingway, 2003).

The research strategies used to assess chronic toxicity depend on the research team experience, the fish species used and also the physico-chemical properties of the compounds to be evaluated. The main goals are: firstly, identification of the main route of entry of the

toxic substance in the body (skin, gills, food), determination of toxic distribution between various organs and the pathways of biotransformation and elimination (toxicokinetics); secondly, identification of the organs and/or tissues more sensitive to the toxic action, and the cellular and biochemical pathways underlying the toxicity of the chemical compound (toxicodynamics).

Economic advantages together with the general properties shared by many species of fish such as high fecundity, external fertilization of transparent eggs and relatively brief generation times make fish a valuable model for toxicological research. Small size fish species, such us medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) have transparent externally developed embryos, facilitating experiments in developmental toxicology, carcinogenesis, mutagenesis and organ-specific toxicity assays (Wakamatsu et al., 2001; Wiegand et al., 2001; Nagel, 2002; Hill et al., 2005). Other species like rainbow trout (*Oncorhynchus mykiss*), feathead (*Pimephales promeals*) and tilapia (*Oreochromis niloticus*) having large size are more appropriate for the experiments requiring large amounts of tissue, to pursue gene expression profiles, proteomics and lipidomics. Their size also allows better assessment for the histological and anatomical investigation. However, the main focus of the toxicological studies is still the biochemical changes associated with toxic exposure, namely, in proteins involved in xenobiotic detoxification, metabolism and excretion (Lawrence & Hemingway, 2003). The endpoint test includes the measurement of the quantities/activities of cytochrome P450 (CYP) monooxygenase system, UDP glucuronosyl, glutathione transferases and metallothioneins. To assess the possible correlation between oxidative stress induced by xenobiotics and the toxic effects, the presence of intracellular oxidative reactive species are usually investigated, quantifying the lipid peroxidation, the reduced glutathione and α-tocopherol intracellular stores and the activity of enzymes like glutathione reductase, glutathione peroxidase, catalase and superoxide dismutase (Figueiredo-Fernandes, 2006; Peixoto et al., 2006). Additionally, direct damage to DNA, including chromosomal aberrations such as micronucleus formation, DNA adducts (covalent attachment of a chemical to DNA) and strand breakage are also evaluated to indentify genotoxic compounds (Çavas & Könen, 2007).

3. Results

I. Studies with plant and animal mitochondria

There are only a small number of toxicological studies using isolated plant mitochondria. However, to understand the mechanism of action of the herbicides on plants, it will be important to study their effects on plant mitochondria considered as the main target for many of them. Some plant mitochondrial fractions exhibit activities higher than animal preparations, which are preserved over several days, especially when obtained by purification with a Percoll gradient (Neuburger et al., 1982; Vicente & Madeira, 2000). These fractions exhibit higher degrees of purity, as compared with mitochondria of animal origin, namely rat liver mitochondria, avoiding interferences with contaminants. In spite of this, the toxicological evaluation aiming environmental security for man, comprehensively prefers the use of mammalian mitochondria, considering the differences between plant and animal mitochondrial structure and function.

Mitochondria are known as the main intracellular organelle responsible for cell ROS production and we should bear in mind that compounds interacting with mitochondrial membranes putatively disturb the coupling efficiency between oxidation and

phosphorylation and also exacerbate mitochondrial ROS production. A range of biotic and abiotic stresses also raise ROS levels in plants due to both defense responses and perturbations of chloroplastic and mitochondrial metabolism (Van Camp et al., 1998).

Several studies were performed in our laboratories, with isolated plant and animal mitochondria, showing that this organelle could represent a target for several well known herbicides. Here we describe and discuss the main results obtained in our studies, considering other studies presented in literature.

1. DNOC

The compound DNOC (4,6-Dinitro-*o*-cresol) is an herbicide included in the phenols, being also used as fungicide and insecticide. DNOC in the concentration range of 10-50 μM, acted as a classical uncoupler of oxidative phosphorylation in rat liver mitochondria, promoting an increase in succinate-supported mitochondrial respiration in state 4 and a dissipation of ΔΨ. The protonophoric activity of DNOC was evidenced by the induction of mitochondrial swelling in hyposmotic K$^+$-acetate medium, in the presence of valinomycin. At higher concentrations (≥ 50 μM), DNOC induced an inhibition of succinate-supported respiration, and a decrease in the activity of the succinate-dehydrogenase. Treatment of Ca^{2+}-loaded mitochondria with uncoupling concentrations of DNOC resulted in mitochondrial permeability transition (MPT), associated with membrane protein thiol oxidation by ROS, as evidenced by mitochondrial swelling in isosmotic sucrose medium (Castilho et al., 1997). Similarly to rat liver mitochondria, DNOC acts as a classical uncoupler of oxidative phosphorylation in potato tuber mitochondria at low concentrations (≤ 100), and as an inhibitor of mitochondrial respiration at high concentrations (≥ 100 μM) (Vicente et al., 1998). This mode of action is in agreement with a type of herbicides named inhibitory uncouplers, according to a general classification (Moreland, 1980; 1993). Thus, low concentrations (up to about 100 μM) only induce uncoupling effect and higher concentrations also inhibit the mitochondrial electron transport chain.

In conclusion, DNOC affected similarly plant and animal mitochondria, with a higher sensitivity for rat liver mitochondria, requiring lower concentrations to produce the same results.

2. Dinoseb; 2,4-D; 2,4,5-T; 2,4,5-TP and MCPA

Studies on phenoxyacetic acid herbicides are here considered and compared with the phenol herbicide, dinoseb. The herbicide dinoseb (2-*sec*-butyl-4,6-dinitrophenol) partially inhibited both FCCP (carbonylcyanide p-trifluoromethoxyphenylhydrazone)-uncoupled respiration and state 3 respiration, indicating its limited interaction with the mitochondrial redox chain at the level of the complexes II and III, as reflected by the low inhibition induced by 500 nM dinoseb on succinate dehydrogenase (10%) and cytochrome *c* reductase (20%), respectively. Additionally, it increased the rate of state 4 oxygen consumption, stimulated ATPase activity, induced mitochondrial membrane permeabilization to protons (H$^+$), and depressed ΔΨ. Thus, dinoseb has a limited effect on the activities of the redox chain complexes at the same time it acts as an uncoupler of oxidative phosphorylation in rat liver mitochondria (Palmeira et al., 1994a). In fact, dinitrophenols, e.g., dinoseb, were previously described as uncouplers of oxidative phosphorylation in mung bean mitochondria (Moreland & Novitzky, 1988). Differently to dinoseb, the herbicide 2,4-D (2,4-dichlorophenoxyacetic acid) has a more pronounced inhibitory action on the redox chain and acts as uncoupler only at relatively high concentrations. Thus, 2,4-D, in the

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concentration range of up to 800 μM, decreased ΔΨ as a function of concentration. State 3 and FCCP-uncoupled respiration were depressed by approximately the same extent (60% at 700 μM), ruling out direct interactions on phosphorylation assembly independent of the redox chain. In fact, the herbicide, at 600 μM, strongly inhibited succinate dehydrogenase (50%) and cytochrome *c* reductase (75%). Therefore, 2,4-D specifically affects the redox chain at the mitochondrial complexes II and III, as opposed to a limited effect of dinoseb whose action is essentially related with uncoupling. Additionally, 2,4-D also uncoupled oxidative phosphorylation in rat liver mitochondria at concentrations 1000-fold higher than those required for a similar dinoseb effect (≥150 μM) (Palmeira et al., 1994a). Just as 2,4-D, 2,4,5-T (2,4,5-trichlorophenoxyacetic acid), MCPA (4-chloro-2-methylphenoxyacetic acid) and 2,4,5-TP [2-(2,4,5-trichlorophenoxy)propionic acid], at high concentrations, alter energy metabolism in rat liver mitochondria by uncoupling oxidative phosphorylation. The herbicide 2,4,5-TP possesses the strongest uncoupling properties followed by 2,4,5-T, MCPA and 2,4-D (Zychlinski & Zolnierowicz, 1990). *In vivo* treatment of rats with 2,4,5-T and 2,4-D also causes damage to mitochondrial and cellular membranes, leading to cellular toxicity. This implies that, *in vivo*, these herbicides also have the potential to inhibit the oxidative phosphorylation of mitochondria, affecting energy production in cells (Sulik et al., 1998). Similarly to rat liver mitochondria, in potato tuber mitochondria 2,4-D has an uncoupling effect which causes the complete loss of phosphorylation by permeabilizing the inner mitochondrial membrane to H$^+$. In plant mitochondria, simultaneously with uncoupling, 2,4-D also inhibited the electron transport chain by blocking the activity of dehydrogenases (succinate dehydrogenase, malate dehydrogenase and NAD-dependent malic enzyme) linked to the respiratory chain (Pireaux et al., 1992). As with DNOC, inhibitory action in plant mitochondria with 2,4-D also required higher concentrations (>3mM) to obtain the same range of inhibition, as compared with animal mitochondria.

3. Dicamba

Dicamba (2-methoxy-3,6-dichlorobenzoic acid) is included in the group of benzoic acid herbicides (classe of carboxylic acids). In the concentration range of 4-30 μmol/mg protein (2-15 mM), promoted an increase in state 4 succinate-supported respiration in rat liver mitochondria. Nevertheless, at the concentration of 50 μmol/mg protein (25 mM), it strongly inhibited succinate-supported mitochondrial respiration (state 4). These results indicate not only an uncoupling effect, as a consequence of an increase on the permeability of inner mitochondrial membranes to protons, but also a strong inhibitory effect on the redox complexes of the mitochondrial respiratory chain. The protonophoric activity of dicamba was evidenced by the induction of mitochondrial swelling in hyposmotic K$^+$-acetate medium, in the presence of valinomycin. The inhibitory effect of dicamba on the respiratory complexes was evidenced by: 1) the inhibition, at the same extent, of state 3 respiration and FCCP uncoupled respiration with parallel dissipation of the ΔΨ, and 2) the strong inhibition of the mitochondrial complexes II and III, as reflected from the decrease in the enzymatic activity of the succinate dehydrogenase and cytochrome c reductase. Dicamba, in the concentration range of 4-30 μmol/mg protein, also inhibited the the activities of the ATPase and ATP synthase. From these results it was concluded that dicamba decreases oxidative phosphorylation by a dual effect on the redox chain (inhibition of redox complexes, and stimulation of proton leakage through the mitochondrial inner membrane) (Peixoto et al., 2003a). Similarly to rat liver mitochondria, dicamba interfered with potato tuber mitochondria by inhibiting the activities of the respiratory complexes II

and III, of ATP synthase, and also stimulating the proton leakage through the mitochondrial inner membrane (Peixoto et al., 2003b). In turn, comparative effects of dicamba and a related compound, 2-chlorobenzoic acid, on potato tuber mitochondria showed that dicamba is a stronger mitochondrial respiratory chain inhibitor and uncoupler. So, considering the results, it was suggested that differences in the lipophilicity due to their chemical structures are related to the different sensitivities of mitochondrial bioenergetics to the referred compounds (Peixoto et al., 2003b).

4. Metolachlor and Alachlor

We also studied chloroacetanilide herbicides included in the larger group of amides for their effects on mitochondria. Metolachlor [2-chloro-N-(2-ethyl-6-methyl-phenyl)-N-(2-methoxy-1-methylethyl) acetamida], in the concentration range of 400-1000 nmol/mg protein (0.4-1.0 mM), interferes with rat liver mitochondrial bioenergetics, inhibiting state 3 and FCCP-uncoupled respirations supported by either malate/glutamate or succinate as the respiratory substrates. These results demonstrated that both complex I and complex II are sensitive to metolachlor. Accordingly, metolachlor-induced $\Delta\Psi$ dissipation and depression of the phosphorylation rates of rat liver mitochondria can be explained by the inhibitory action of the herbicide exerted in complex I- and complex II-dependent respirations. This assumption was supported by the parallel depressive effect on state 3 and FCCP-uncoupled respirations dependent on both malate/glutamate and succinate and the absence of effects when a mixture of ascorbate+TMPD was used as substrate (complex IV). The low stimulatory effect of state 4 respiration suggest a low ability of the compound to induce inner membrane permeabilization. Therefore, the toxicological effects of metolachlor on rat liver mitochondrial bioenergetics, expressed by its inhibitory action on mitochondrial respiratory chain for complexes I and II, may be relevant to understand the mechanism responsible for its toxic action (Pereira et al., 2009). Just as metolachlor, the herbicide alachlor [2-chloro-N-(2,6-diethyphenyl)-N-(methoxymethyl acetamide)], in the concentration range of 400-1200 nmol/mg protein (0.4-1.2 mM), interferes with rat liver mitochondrial bioenergetics by inhibiting mitochondrial respiratory chain activity at complexes I and II levels (Pereira et al., 2010).

5. Linuron

Linuron [N´- (3,4-dichlorophenyl)-N-metoxy-N-methylurea] is an herbicide included in the group of ureas. In the concentration range of up to 160 μM, linuron interferes with rat liver mitochondrial bioenergetics, inhibiting state 3 respiration supported by either malate/glutamate (65%) and succinate (8%) as the respiratory substrates. The FCCP-uncoupled respiration supported by malate/glutamate was inhibited by 40%. Nevertheless, succinate-supported FCCP-uncoupled respiration was not affected by the tested linuron concentrations. These results indicate that complex I of the mitochondrial respiratory chain is more sensitive to linuron than complex II. This assumption was also supported by a higher linuron-induced dissipation of $\Delta\Psi$ (11%) and depression of the phosphorylation rate (70%) of rat liver mitochondria respiring malate/glutamate as compared with succinate-dependent respiration (7% and 33%, respectively). The same concentration range of linuron progressively stimulated state 4 respiration using either malate/glutamate or succinate as respiratory substrates. However, at 120 μM linuron, a higher stimulation of state 4 supported by succinate (100%) was detected, as compared with that supported by

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malate/glutamate (20%) reinforcing the idea that the inhibitory effect of linuron is mainly exerted at the level of complex I. On the other hand, this result indicates that linuron has also some ability to induce inner membrane permeabilization to cations. This explains the strong inhibitory effect of succinate-supported state 3 respiration, taking into account that complex II is note very sensitive to linuron.

The toxicological effects of linuron on rat liver mitochondrial bioenergetics, expressed by its inhibitory action on mitochondrial respiratory chain for complex I and membrane permeabilization, may be relevant to understand the mechanism responsible for its toxic action (unpublished results).

6. Paraquat

Paraquat (1,1´-dimethyl-4,4´-bipyridylium dichloride), included in the group of bipyridiliums, in the concentration range of up to 10 mM, promoted an increase in succinate-supported mitochondrial respiration (state 4) on rat liver mitochondria, indicating some energy uncoupling due to some permeabilization of mitochondrial membrane to H^+. The deleterious effect of the herbicide on membrane organization was confirmed by its ability to induce lipid peroxidation (Palmeira et al., 1995a). So, this must be the main cause of permeabilization. State 3 respiration is a little depressed by about 15% at 10 mM paraquat, whereas CCCP-uncoupled respiration is depressed by about 30%. The inhibitory effect of paraquat on uncoupled respiration reflects its interaction with the mitochondrial respiratory chain. In fact, succinate cytochrome-*c* reductase and cytochrome-c oxidase are inhibited by 35 and 39% respectively, at 10 mM paraquat, whereas succinate dehydrogenase is not significantly affected, indicating that paraquat partially inhibits the redox chain at mitochondrial complexes III and IV. Furthermore, paraquat partially inhibits the ATPase activity for concentrations in the range of 1-2 mM, suggesting a direct effect on this enzyme complex. However, at higher concentrations (5-10 mM), the ATPase activity is stimulated, probably as a consequence of the described uncoupling effect. Paraquat depresses $\Delta\Psi$ of rat liver mitochondria as a function of herbicide concentration. In addition, the depolarization induced by ADP is decreased and repolarization is biphasic suggesting a double effect. Repolarization resumes at a level consistently higher than the initial level before ADP addition, for concentrations up to 10 mM. This particular effect is clear at 1 mM paraquat and tends to fade out with increasing concentrations of the herbicide. Based on these results, it was concluded that paraquat uncouples oxidative phosphorylation of rat liver mitochondria by inducing lipid peroxidation and, also, by inhibiting redox chain and ATPase/synthase activity (Palmeira et al., 1995a). Comparative studies of the effect of paraquat on mitochondrial bioenergetics of rat liver and potato mitochondria confirmed the protonophoric action of herbicide on both mitochondrial membranes. However, the sensitivity of potato tuber mitochondria to paraquat was lower than that of rat liver mitochondria (Vicente et al., 2001). Differences between rat liver and potato tuber mitochondria were also observed on the effects of paraquat on both $\Delta\Psi$ and oxygen consumption of complex-I-dependent respiration. Thus, paraquat (20 mM) considerably dissipated complex-I-dependent $\Delta\Psi$ and stimulated state 4 oxygen consumption of liver mitochondria, but not potato tuber mitochondria, even at 40 mM paraquat (Vicente et al., 2001). According to these results potato tuber mitochondria, in contrast to rat liver mitochondria, are protected against paraquat radical (PQ^+) afforded by complex I redox activity (Vicente et al., 2001). Interestingly, the complex I may afford protection for the paraquat effects on plant mitochondria, explained by Nagata et al. (1987). Working with

bovine heart and yeast mitochondria, the reduction of NAD^+ to NADH can be catalyzed at the expense of the paraquat radical, avoiding its degradative effects. Studies performed to understand the different sensitivity of plant and animal mitochondria to paraquat toxicity revealed that it may also be related with antioxidant agents (Vicente et al., 2001; Peixoto et al., 2004) and different native aliphatic contents of the mitochondria membrane phospholipids (Peixoto et al., 2004). In fact, the levels of superoxide dismutase (SOD), glutathione reductase and α-tocopheral content in potato tuber mitochondria were significantly higher than in rat liver mitochondria, which, in turn, revealed higher values of lipid peroxidation and protein oxidation induced by paraquat (Vicente et al., 2001; Peixoto et al., 2004). Also, the total number of double bonds (unsaturated index) in rat liver mitochondrial membranes was higher than in potato tuber mitochondrial membranes (Peixoto et al., 2004). From these studies it was also concluded that peroxides are not major intermediates in paraquat toxicity at the mitochondrial level since the activity of the glutathione peroxidase and catalase are much higher in rat liver than in potato tuber mitochondria (Peixoto et al., 2004).

A summary of the results of the effects of some herbicides on plant mitochondria is depicted in Figure 1.
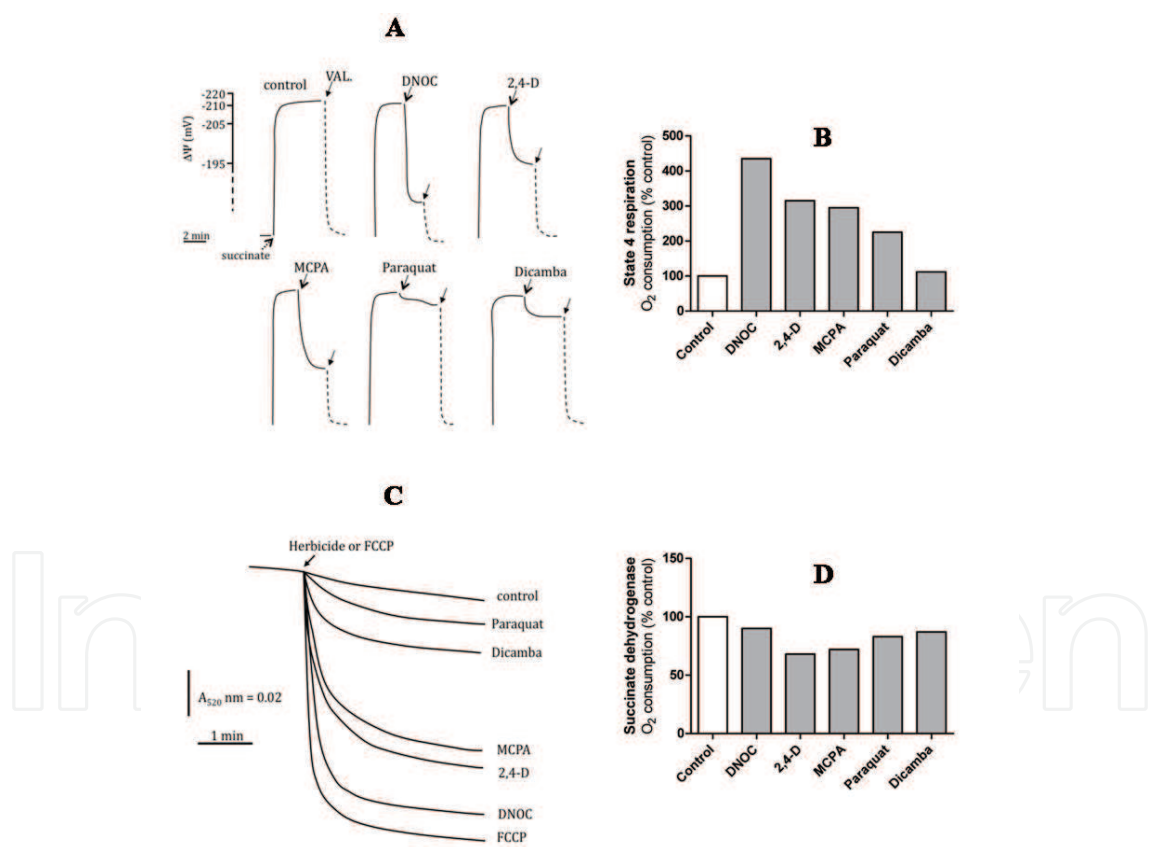

Fig. 1. Effects of DNOC (80 μM), 2,4-D (3 mM), MCPA (3 mM), paraquat (40 mM) and dicamba (2 mM) on succinate-supported transmembrane potencial ($\Delta\Psi$) (**A**), state 4 O_2 consumption (**B**), swelling in hypo-osmotic K^+-acetate medium (**C**), and succinate dehydrogenase activity (**D**) of potato tuber mitochondria. Val., valinomycin; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone. The traces represent typical recordings from several experiments with different mitochondrial preparations.

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Herbicide		Chemical structure	Herbicide		Chemical structure
Log Kp	LD$_{50}$		Log Kp	LD$_{50}$	
DNOC			Dicamba		
2.39	7.0		-1.88	1581	
Dinoseb			Metolachlor		
2.29	25		3.4	1200	
2,4-D			Alachlor		
-0.83	469		3.09	1200	
MCPA			Linuron		
-0.81	962		3.0	1146	
2,4,5-T			Paraquat		
3.72	820		-4.5	110	
2,4,5-TP			Oxyfluorfen		
4.0	500		4.86	> 5000	

Table 4. Chemical structure, Log Kp (octanol-water partition coefficient at pH 7, 20 °C) and
Rat-Acute oral **LD$_{50}$** (mg Kg^{-1}) values of the referred herbicides. (Data of Log Kp and LD$_{50}$
obtained from: IUPAC global availability of information on agrochemicals,
http://sitem.herts.ac.uk/aeru/iupac/index.htm, July 2010).

Comparing the results presented in Figure 1 with the Log Kp values of the herbicides
studied (Table 4), efforts were made to establish a relationship between lipophylicity and
their mechanisms of action.

Exception for paraquat, a good correlation between herbicide Log Kp values (Table 4) and
the transmembrane potencial dissipation, the stimulation of state 4 respiration and the

proton-dependent swelling induced by them can be established, hence, corroborating their effects on mitochondrial membrane permeabilization inducing uncoupling of mitochondrial phosphorylation.

These toxicological effects of the herbicides on mitochondria, putatively related to their incorporation into the mitochondrial membrane, cause an alteration on the surface charge density and a disturbance in the physicochemical and structural properties of the inner membrane, resulting in an unequivocal disturbance of electron delivery between redox complexes and, consequently, affecting the phosphorylative system. Obviously paraquat acts in a completely different way.

The mechanism of action of paraquat has been related to reactive oxygen species produced by paraquat redox cycling. Nevertheless, paraquat affects mitochondrial functions and induces a slight permeabilization of mitochondrial membrane probably due to lipid peroxidation (Peixoto et al., 2004). Mitochondrial protein thiol oxidation was also observed by paraquat treatment (Peixoto et al., 2004). Respiratory complexes from electron respiratory chain can also be negatively affected by all these herbicides as revealed from succinate dehydrogenase activity assays (Figure 1D). In this case, correlation with lipophylicity is not so obvious.

II. Effects on Prokaryotic and Eukaryotic cell cultures

In this section, studies of the effects of herbicides on bacteria and plant cell cultures will be described. If the use of plant cell cultures is easily accepted as an important means to estimate the potential toxicity of those compounds in target and non-target organisms, the interest in performing herbicide studies by using bacterial cells as toxicological model systems is apparently less obvious. However, considering the crucial role microorganisms play in soil as well as in aquatic environments, a significant ecological damage is predictable if these microbial communities are affected by the accumulation of herbicides in soil, upon their application in agriculture fields, or in aquatic systems, as a consequence of herbicide runoff. In fact, microorganisms are of fundamental importance for the vital functioning of any ecosystem. The microflora of the soil, namely in the top soil layer (0-15 cm) where an intense microbiological activity takes place, has a high responsibility for soil fertility because of its capacity to convert potential plant nutrients in active or available forms. On the other hand, microorganisms densely colonizing fresh water and marine sediments promote the transformation of organic matter and constitute food source for organisms of higher trophic levels. Therefore, the adverse effects of chemical agents such as herbicides upon microorganisms may constitute an early warning indicator of ecosystem perturbation.

In the literature, we can find a significant amount of studies showing herbicide-sensitive bacteria (Cserháti et al., 1992; Hernando et al., 2003) and micro-algae (Nystrom et al., 1999; Li et al., 2008), as well as microorganisms that not only tolerate the presence of high concentrations of herbicides but also are able to degrade them (Dejonghe et al., 2003; Sorensen et al., 2003; Neumann et al., 2004). If, on the one hand, the biodegradation constitutes an important factor favoring the removal of herbicides from the environment, on the other hand, the elimination of sensitive microorganisms and the proliferation of the most tolerant ones, due to the selection pressure exerted by those compounds, may cause changes to the existing microbiological balances with potential adverse consequences for the ecosystem. An example of differential sensitivity to herbicides has been found for sulfonylurea herbicides among fresh-water and marine micro-algae (Nystrom et al., 1999), microbial aquatic communities (Thompson et al., 1993) and soil bacteria (Forlani et al., 1995).

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As previously referred, adverse effects may be caused by herbicides on beneficial microorganisms and their associated transformations in soil, affecting plant-nutrient availability. Therefore, the evaluation of herbicide toxicity exerted in soil microorganisms is recommendable to define upper limit concentrations. With the purpose of contributing to collect data concerning herbicide effects on soil microorganisms, three herbicides (the chloroacetamides metolachlor and alachlor and the arylurea linuron) were studied using *B. stearothermophilus* as a bacterial model system.

1. Metolachlor and alachlor toxic effects on bacterial cells

Metolachlor (Pereira et al., 2009) and alachlor (Pereira et al., 2010) inhibit the growth of a strain of *B. stearothermophilus* in the concentration range of 100 to 600 µM. The herbicides added to cultures of this Gram-positive bacterium, grown in a complex liquid growth medium (L-Broth) at 65 ºC, affect the bacterial growth in the dependence of the concentration, inducing an increase of the lag time, decrease of the specific growth rate and reduction of the maximal optical density attained in the stationary phase of growth. The effective concentrations which alter these growth parameters by 50% compared to the control (EC_{50}) are around 400 to 450 µM for metolachlor and slightly higher than 500 µM for alachlor. Comparing these values of EC_{50} with those determined in identical conditions for other pesticides, such as DDT (Donato et al., 1997b), methoprene (Monteiro et al, 2005) or tributyltin (TBT) (Martins et al., 2005), it is clear that the herbicides metolachlor and alachlor have a much lower toxicity. Accordingly, the herbicide effects on the bacterial respiratory activity, evaluated in terms of the oxygen consumption rate in protoplasts, also denote a moderate toxicity. Thus, for oxygen consumption supported by NADH, metolachlor and alachlor EC_{50} of 1.2 µmol/mg protein or 1.2 mM (Pereira et al., 2009) and 2.2 µmol/mg protein or 2.2 mM (Pereira et al., 2010), respectively, are two order of magnitude higher than those found for DDT (Donato et al., 1997b) or the organotin TBT (Martins et al., 2005). The respiratory activity elicited by ascorbate/TMPD showed to be insensitive to both herbicides in the range of 0.5 to 5.0 µmol/mg protein (0.5 to 5.0 mM), suggesting that the herbicides act upstream the cytochrome c oxidase segment. These results are in accordance to those found in rat liver mitochondria for both herbicides at the same concentration range (Pereira et al., 2009; 2010).

Therefore, data obtained for the bacterial model, in terms of growth and respiratory activity, and for rat liver mitochondria, concur with each other and with other results in the literature (Stevens & Sumner, 1991) indicating that the herbicides alachlor and metolachlor exhibit a reduced toxicity in comparison with other tested pesticides or pollutants.

2. Linuron toxic effects on bacterial cells

The toxic effects of linuron on the growth and respiratory activity of *B. stearothermophilus* were also evaluated. Although concentration-dependent effects of linuron on bacterial growth follow the same trend of the tested chloroacetamides, resulting in longer lag times, lower growth rates in the exponential phase and lower bacterial yields, as compared to the control, they revealed to be much more severe. Thus, the inhibition of bacterial growth by linuron occurred at concentrations one order of magnitude lower than those required by the other tested herbicides to promote the same effect. An identical behavior was observed when herbicide effects on the respiratory activity of protoplasts, elicited by NADH, were monitored. The effective concentration of linuron of 98 nmol/mg protein or 98 µM (unpublished data) to reduce for a half the protoplast oxygen consumption rate relatively to

the control (protoplast preparation without herbicide, containing a correspondent amount of DMSO, *i.e.* the herbicide vehicle solvent) is also one order of magnitude lower than the equivalent concentrations of alachlor and metolachlor (see above) and one order of magnitude higher than the concentration of TBT to produce the same effect (Martins et al., 2005). These results are also in accordance with those obtained using the mitochondrial model system (see above).

These findings confirm previous data (Donato et al., 1997b; Monteiro et al., 2005; 2008) showing a good parallelism between the results obtained with the bacterial model and rat liver mitochondria, suggesting a similar sensitivity of both models for chemical toxicity assessment.

3. Paraquat effects on plant cell cultures

Paraquat is a non-selective contact herbicide of the bipyridilium class, whose incorrect use may cause pulmonary injury in humans (Boelsterli, 2003). This organ-selective toxicity results from parquat structural similarity with diamines, such as putrescine, which are actively transported by type I and type II alveolar cells. The toxicodynamics of this compound is related with its high redox-cycling activity (Boelsterli, 2003). In plants, the mechanism of action of this herbicide consists of affecting photosynthesis, being reduced by light reaction I (Cremlyn, 1991). Since this herbicide is absorbed from the foliage, but not from the roots, it is predictable those effects do not occur on non-green cells.

In order to appraise paraquat toxicity in non-target plants, studies were performed using cell cultures from non-green potato (*S. tuberous* L.) tuber calli (Peixoto et al., 2008). Several endpoints were used to evaluate the cytotoxic effects of this herbicide: cell growth, total synthesised protein; cellular integrity; adenine nucleotide content and the activity of several antioxidant enzymes. Paraquat concentration of 50 µM promoted a complete inhibition of the growth of callus tissue cells. Studies of fluorescein release showed that paraquat concentrations which inhibited cellular growth by 50% (5 µM) did not affect the integrity of the cell membrane. Accordingly, the protein/cellular weight ratio did not significantly alter upon incubation of plant cells with this concentration of paraquat (5 µM). This finding is in agreement with studies performed in potato tuber-isolated mitochondria (Vicente et al., 2001) showing that concentrations of paraquat up to 30 mM do not induce swelling depending on permeabilization to H^+.

Since previous studies using plant and animal mitochondrial fractions had shown paraquat exerts deleterious effects on animal mitochondria bioenergetics due to oxidative stress and also disturbs, although less severely, plant mitochondria functioning (Vicente et al., 2001; Peixoto et al., 2004), the adenosine nucleotides in callus tissue cultures exposed to this herbicide were quantified in order to obtain information regarding paraquat effects on oxidative phosphorylation, glycolisis and pentose pathways (Peixoto et al., 2008). Paraquat induced ATP and ADP depletion, together with an increase of AMP, and strongly increased the NAD^+/NADH ratio, revealing decrease of the cellular redox state. The depletion of intracellular ATP and NADH together with the increase of the levels of ADP, AMP and NAD^+, due to paraquat effects, was also reported in isolated rat-hepatocytes (Palmeira et al., 1994b; Palmeira, 1999). The ATP depletion has been related to the interference with the mitochondrial or glycolytic energy pathways in isolated rat hepatocytes (Palmeira, 1999) as well as in non-green callus tissue (Peixoto et al., 2008). On the other hand, the redox cycling of paraquat may justify the depletion of cellular reducing equivalents, in both biological

systems. Both events, depletion of ATP and decrease of the cellular redox state, may lead to the impairment of cellular functions, hence promoting inhibition of potato tuber calli cell growth (Peixoto et al., 2008) and decrease of hepatocyte viability (Palmeira et al., 1995b). It is also suggested that lipid peroxidation induced by superoxide anions generated by paraquat redox cycling, plays an important role in the toxicity mechanism of this herbicide (Krall et al., 1988; Peter et al., 1992). In isolated rat hepatocytes it was demonstrated that paraquat promoted an increase in thiobarbituric acid (TBA)-reactive species as a function of concentration and incubation time (Palmeira et al., 1995b). In parallel, it was shown that at the same concentration range paraquat induced loss of intracellular glutathione (GSH) with a concomitant increase of the oxidised form (GSSG), decrease of protein thiols and cell death (Palmeira et al., 1995b). These events have been suggested as being interrelated.

Antioxidant enzymes can be up-regulated during oxidative stress (adaptive response induced by H_2O_2 as a second messenger) and their activities have been proposed as being involved in mechanisms of herbicide toxicity or resistance (Allen et al., 1997).

In order to clarify the level of protection plant cells exhibit against paraquat-induced oxidative stress, the activity of antioxidant enzymes was determined in crude extracts of potato callus tissue previously exposed to different concentrations of the herbicide (Peixoto et al., 2008). It was observed that paraquat induced a high stimulation of superoxide dismutase (SOD) and glutathione reductase (GR) but did not affect glutathione transferase (GST). Catalase (CAT) was stimulated only at low paraquat concentrations (1µM) and was inhibited at higher concentrations of the herbicide. The high increase of hydrogen peroxide resulting from the dismutation of superoxide anions generated by paraquat redox cycling, which is favoured by the high herbicide-induced stimulation of SOD, likely justifies why CAT is inhibited above 1µM paraquat, since potato CAT is very sensitive to H_2O_2 (Beaumont et al., 1990). These data agree with those reported for tobacco plants, which also showed an increase of SOD activity and CAT inhibition (Furusawa et al., 1984).

GR activity is responsible for the reduction of GSSG back to GSH. Since paraquat promotes a decrease of GSH in potato calli cells (Peixoto et al., 2008), its stimulatory effect on GR activity may result from a cellular response to GSH level decrease. Finally, the GST which has been shown to promote detoxification of several herbicides, as dicamba and 2,4-D (Hatton et al., 1998), by conjugation of the electrophilic herbicides with GSH, seems to have no effect on paraquat, since its activity was not stimulated by the latter herbicide in contrast with the formers.

In conclusion, studies of paraquat toxicity in plant cell cultures concur with those reported in animal cells, showing that the most probable cause of cell death in non-target organisms involves decrease of the cell energy charge, decrease of GSH content and depletion of cellular reducing equivalents needed to assure the normal cell functioning, including energy metabolism and antioxidant defence mechanisms.

4. Dicamba, 2,4-D and MCPA effects on plant cell cultures

The cytotoxic effects of the herbicides dicamba, 2,4-D and MCPA on cell cultures of potato tuber calli (*S. tuberousum* L.) were evaluated upon exposition to the herbicides for a period of 4 weeks, by monitoring the changes in cell growth, membrane integrity, energy charge, cell redox state and activity of several antioxidant enzymes, in studies parallel to those previously referred for paraquat (Peixoto et al., 2008; 2009a).

Regarding the cell growth, the concentrations at which herbicides inhibited growth by 50% (IC_{50}) were 59, 50 and 20 µM for MCPA, dicamba and 2,4-D, respectively. Studies of

fluorescein release to monitor membrane integrity showed that, in contrast to paraquat (see above), the three herbicides tested affected membrane integrity at concentrations at which growth was inhibited by 50%. Unexpectedly, MCPA at the concentration of 120 µM, at which growth was completely inhibited, induced a decrease of fluorescein absorbance at 458 nm, apparently reflecting a decrease of fluorescein release (Peixoto et al., 2009). These results were clarified by using fluorescence microscopy. Cells were loaded with fluorescein diacetate, a non-polar and non-fluorescent molecule, which easily permeates the plasma membrane of intact cells (Guilbaut & Kramer, 1964). Into the cell, the esterified molecules undergo hydrolysis by intracellular esterases, generating fluorescein, which fluoresces by exposure to short wavelength light (Prosperi, 1990). Therefore, viable cells containing fluorescein can be observed by fluorescence microscopy (Peixoto et al., 2009a). When the membrane is disrupted, fluorescein is released occurring an increase of extracellular fluorescence, detected by microscopy or by the spectrometric method. In the case of cells incubated with 120 µM MCPA, fluorescence microscopy showed a strong decrease of fluorescence (Peixoto et al., 2009a). This unexpected result is due to intracellular esterase's inhibition, which in turn avoids fluorescein production. This study served to show that the fluorescein assay, as a method to monitor membrane integrity, should be carefully interpreted. The effects of the three herbicides on membrane integrity are consistent with their capacity to induce H$^+$ permeabilization and $\Delta\Psi$ dissipation in isolated mitochondria from plant as well as animal origin (Zychlinski & Zolnierowicz, 1990; Peixoto et al., 2004).

The protein/cellular weight ratio showed an increase by action of the three herbicides tested (Peixoto et al., 2008; 2009a). This parameter should reflect herbicide effects on cell intactness. This was supported by concentrations of paraquat that did not affect membrane integrity and, concomitantly, did not induce significant alteration of protein/cellular weight ratio (Peixoto et al., 2008). In the case of MCPA, the increase in the protein/cellular weight ratio is more likely to be a consequence of cellular water and electrolyte loss with a decrease of cell volume and weight, rather than the increase of protein synthesis. This conclusion is supported by the observation of increased number of cells per gram of callus and cells becoming harder and dark-brownish with increasing herbicide concentrations (Peixoto et al., 2009a).

The effect of the three herbicides on the intracellular content of adenosine nucleotides, reflected by a decrease of ATP content with a concomitant increase of the levels of ADP and AMP, indicates an inhibitory action in cell metabolism. Since non-green callus tissue was used, ATP generation depends on glycolisis and oxidative phosphorylation. Considering the herbicides of the chlorophenoxyacetic acid group (2,4-D and MCPA), the MCPA seems to have been less efficient in reducing the cell energy charge (taken as [ATP]+0.5 [ADP]/[ATP]+[ADP]+[AMP]) than 2,4-D (Peixoto et al., 2008; 2009a). Dicamba is probably somewhat less efficient yet.

In summary, the inhibition of cell growth promoted by these herbicides may be correlated with the decreased availability of ATP, which is needed for ion translocation, nutrient import and metabolite export. Interestingly, the same order of efficiency (2,4-D>MCPA≈dicamba) was observed in terms of herbicide-induced growth inhibition.

Cell redox state as influenced by the herbicides tested was also evaluated in terms of NAD$^+$/NADH ratio. All the herbicides tested increased NAD$^+$/NADH ratio, revealing a decrease in the cellular redox state. The herbicide 2,4-D promoted a stronger effect as

compared with MCPA or dicamba. Paraquat and 2,4-D showed similar efficiency in promoting the decrease of cell energy charge, but paraquat effect on the cell redox state was the highest of all the herbicides tested in potato callus cells. Consistently, paraquat revealed to be the most potent growth inhibitor, too (Peixoto et al., 2008).

As previously referred for paraquat, the activity of the antioxidant enzymes CAT, GST and GR was evaluated upon incubation of the potato callus tissue with different concentrations of the herbicides 2,4-D, MCPA and dicamba (Peixoto et al., 2008; 2009a). Considering the two herbicides of the phenoxyacetic acid group, it was observed that 2,4-D (Peixoto et al., 2008) strongly stimulated the catalase (150%) and superoxide dismutase (about 50%) activities at the highest concentration assayed (50 μM), whereas MCPA, up to 120 μM, did not induce any significant change (Peixoto et al., 2009a). Apparently, MCPA does not stimulate production of the superoxide anion and hydrogen peroxide, in contrast with 2,4-D and paraquat (Peixoto et al., 2008). Dicamba promoted an increase of CAT and SOD activities, although at a smaller extent than 2,4-D (Peixoto et al., 2008).

Concerning the GST activity, an enzyme involved in the detoxification of a great deal of pollutants, it was stimulated by both phenoxyacetic acid herbicides (2,4-D and MCPA) and by dicamba, in contrast with paraquat, as previously referred. As GST uses GSH for conjugation with electrophilic compounds, it would be expected GR activity to be also stimulated by the herbicides. In fact, it was observed a stimulation of GR activity in callus tissue cultures incubated with all the herbicides tested, paraquat included (Peixoto et al., 2008; 2009a). Although the experimental approach used in these studies do not enable authors to conclude that GST is involved in 2,4-D, MCPA and dicamba bioinactivation in potato callus tissue, their results concur with data in the literature regarding different herbicides and several plant species for which GST activity plays an important role in detoxification (Hatton et al., 1998). In the case of paraquat, GR activity stimulation was probably induced as a cellular response to an intracellular decrease of GSH used to detoxify the reactive oxygen species generated as a consequence of the herbicide redox cycling (see above). Additionally, GSH oxidation could be also related to other cytotoxic events such as lipid peroxidation and decrease in protein thiols, which have been demonstrated in isolated rat hepatocytes treated with 2,4-D and paraquat (Palmeira et al., 1995b).

In conclusion, data obtained in plant and animal cell cultures concur to suggest similar mechanisms of toxicity by 2,4-D and paraquat in non-target organisms, although with different relative potencies. The decrease of GSH/GSSG ratio is probably the primary event in the cytotoxic process leading to death. In the case of MCPA and 2,4-D, the lack of studies in animal cells precludes any comparison. However, it is probable that MCPA cytoxicity results primarily from disturbance of membrane physicochemical properties leading to loss of electrolytes and metabolites, decrease in ATP availability and decrease of the cellular redox state. Dicamba biological activity shows some similarities with both 2,4-D and MCPA. It disrupted the membrane integrity as 2,4-D and MCPA but induced an increase of catalase and SOD activities, in contrast with MCPA, although in a lower extent than 2,4-D. At the mitochondrial level, dicamba uncoupled oxidative phosphorilation by inducing H⁺ permeabilization, as MCPA, but also inhibited the electron transfer in the mitochondrial redox system, as 2,4-D (see above).

Since all the herbicides tested in potato callus tissue promote significant alterations in plant as well as in animal mitochondria functioning, the bioenergetic impairment may be suggested as one of the mechanisms underlying the cytotoxicity of those compounds in non-target organisms.

III. Effects of paraquat and oxifluorfen on tilapia fish (*Oreochromis niloticus*)

Hundreds of herbicides of different chemical structures, extensively used to control a wide variety of agricultural pests, contaminate aquatic habitats due to leaching and runoff water from treated areas. Fish are among the non-target organisms that can be seriously affected by the ecological imbalance imposed through this excessive charge of chemicals. In freshwater, the presence or absence of fish could be used as a bioindicator of the degree of water pollution. Considering that fisheries are an important source of food provided with protein and lipid of high nutritional value, representing an economic benefit for many countries, herbicide contamination of surface waters derived from agricultural practices is a problem of worldwide importance. On the other hand, fish constitute the vertebrate model most used in the field of ecotoxicology.

Mortality data, although being the definitive demonstration of toxicity for the organism in study, are often left out of ecotoxicological studies as being unable to provide reliable information about the environmental hazard resulting from pollutant contamination. Therefore, rather than mortality, physiological parameters (Tortorelli et al., 1990) and biochemical alterations (Stephensen et al., 2002) have been extensively used to perform an integrated evaluation of the risk pollutants may represent for the wildlife.

Toxicity exerted by many herbicides in aquatic organisms has been associated to an increased generation of ROS (Di Giulio et al., 1989; Livingstone et al., 1990). These toxic effects can be promoted via redox cycling, paraquat being the paradigmatic example, or result from herbicide metabolism by phase I, with production of ROS as by-products. Under physiological conditions, antioxidant defenses are available in fish, like in mammals, to scavenge ROS or to prevent its production (Figueiredo-Fernandes et al., 2006; Peixoto et al., 2006). In the presence of pollutants promoting oxidative stress, organisms can adapt by up-regulation of antioxidant defenses such as GSH-related enzymes, which constitute non-specific biomarkers of exposure to pro-oxidative xenobiotics. Thus, alterations in the activity of antioxidant enzymes have been proposed as early indicators of exposure to pollutants, including herbicides (Di Giulio et al., 1989).

Several field studies for biomonitoring environmental pollutants have shown that alterations of GSH-dependent/producing enzymes as well as GSH content may be useful tools to evaluate the ecotoxicological effects exerted by herbicides in fish. Stephensen et al. (2002) showed that the levels of GSH and GSH-related enzymes in the liver of rainbow trout (*Oncorhynchus mykiss*) constitute suitable biomarkers for oxidative stress in fish developed in laboratory. In this context, tilapia (*Oreochromis niloticus*) has been used as a biological model for toxicological assessment, showing several advantages: high growth rates, easy reproduction, effciency in adapting to diverse diets, great resistance to diseases and handling practices, as well as good tolerance to a wide variety of husbandry conditions (Fontaínhas-Fernandes, 1998). On the other hand, tilapia constitutes an indicator species in biomonitoring programs, since an increased level of its biotransformation enzymes may reflect the existence of environmental pollutants.

Figueiredo-Fernandes et al. (2006) carried out studies of the effects of paraquat on the hepatic levels of antioxidant enzymes in tilapia. Studies regarding the toxic action of xenobiotics in animals are often performed in liver, since this organ is a main target of chemical injury. This fact results from its role in xenobiotic biotransformation and its unique position within the circulatory system. Therefore, biochemical and histological alterations in fish hepatocytes have been extensively reported as important biomarkers of exposure to toxic compounds, in laboratory as well as in field studies.

The effects of paraquat on oxidative stress enzymes of tilapia hepatocytes were studied in the dependence of temperature and gender (Figueiredo-Fernandes et al., 2006). Males and females of tilapia were kept in tanks at 17 and 27 °C (breeding non-compatible vs breeding compatible temperatures). A qualitative analysis showed that fish mortality induced by paraquat was accompanied by the appearance of extensive areas of hepatocytic necrosis. Preliminary experiments were carried out to select test concentrations of paraquat corresponding to 25% of the LC50, at which hepatocytes showed no signals of necrosis. SOD and GST activities showed sex-dependence, since males present higher values than females at both temperatures assayed. No significant alterations were found in the dependence of temperature. SOD, GR and GST activities were increased by exposition to paraquat. An increase of GR activity induced by paraquat was also demonstrated (Stephensen et al., 2002) in rainbow trout. The authors justify this fact due to the ability of GR to reduce paraquat to its cation radical, causing a chain reaction with paraquat radical promoting oxidation of GSH, which in turn induced an increased activity of GR.

We have also observed alterations of the condition factor (CF) and the hepatosomatic index (HSI) of tilapia exposed to paraquat (Figueiredo-Fernandes et al., 2006). These parameters serve as indicators of the general organism well being. A high condition factor corresponding to a high body weight/length relationship generally means good environmental conditions. Also a high HSI, defined as the ratio of liver weight to body weight, normally reflects a high reserve of energy. However, an increase of both parameters, CF and HSI, was promoted by paraquat in tilapia. An increase of HSI was also registered in different laboratories upon exposition of fish to different pollutants. This alteration was interpreted (Stephensen et al., 2002) as being due to an increased activity of xenobiotic biotransformation enzymes.

Other studies were conducted in tilapia exposed to the herbicide oxifluorfen (Peixoto et al., 2006). The exposure of tilapia for a period of 14 to 21 days to sub-lethal concentrations (0.3 to 0.6 mg/L) of oxyfluorfen [2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4(trifluoromethyl) benzene], a diphenyl ether herbicide commonly used in agriculture to control broadleaf and grassy weeds with specific recommendations (EPA 1992), resulted in a progressive increase of the activity of catalase and, to a lower extent, of glutathione reductase. These results are consistent with those reported in the literature concerning different aquatic organisms in the presence of pollutants such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (Otto & Moon, 1995). Regarding glutathione S-transferase (GST), an enzyme engaged in the detoxification of many xenobiotics, by conjugation of their phase I metabolites, it was observed an increase of its activity induced by 7 days exposition to oxyfluorfen, but thereafter the activity decreased to levels lower than the control. Data in literature are also variable, reporting an increase (Otto & Moon, 1995) or a decrease (Pedrajas et al., 1995) of GST activity in fishes exposed to PAHs and PCBs. In contrast to the other enzymes, SOD activity showed a progressive decrease along the 21 days of exposition to the herbicide. These data are supported by other studies using rainbow trout injected with paraquat (Pedrajas et al., 1995) but are opposite to results obtained with other species exposed to other pollutants. In conclusion, fish antioxidant enzymes have a limited potential to be used as pollution biomarkers, since very different responses can be observed depending both on the xenobiotic and on the organism under study.

It was also observed that tilapia exposure to oxyfluorfen promoted changes in the hepatic fatty acid profile, with special incidence on the unsaturated acids (Peixoto et al., 2006),

decreasing the oleic acid content and increasing the nervonic acid (24:1). It was shown that oxyfluorfen also induced alterations of the fatty acid composition in plants (Watanabe et al., 2001), promoting an increase of the monounsaturated chains (C16:1 and C18:1). Disturbance of the fatty acid biosynthesis regulatory network by a potential oxyfluorfen-induced oxidative stress may explain those alterations.

Oxyfluorfen (0.3 and 0.6 mg/L) was also evaluated on the mitochondrial hepatopancreas bioenergetics of tilapia (Peixoto et al., 2009b). Although no significant change was registered in respiration supported by piruvate/malate (complex I) or succinate (complex II) as detected by oxygen consumption, the mitochondrial $\Delta\Psi$ was dissipated in some extent. Furthermore, the phosphorylation rate decreased, indicating a perturbation of the phosphorylative mitochondrial system, potentially affecting the ATP availability. The controversial results in herbicide-induced oxygen consumption (no effect), $\Delta\Psi$ dissipation and decrease of the phosphorylation rate can putatively be explained as a limited direct effect on the respiratory complexes, referred in literature as "Biochemical Threshold Effect" (Rossignol et al., 2003). The big size of the fishes used in the study could also explain small effects on the mitochondrial respiration, since bigger and fatter fishes could present higher resistance to xenobiotic, due to its accumulation in fat tissue, particularly when the xenobiotic is lipophilic.

In conclusion, oxyfluorfen induces biochemical changes in fish hepatopancreas regarding the antioxidant enzymatic system, fatty acid metabolism and mitochondrial bioenergetics. However, the relationships between those toxicity parameters have not been completely clarified, yet.

4. Concluding remarks

A great deal of work has been done to assess the environmental risks and health hazards resulting from the extensive and intensive use of herbicides. As other pesticides, the herbicides are common contaminants of surface water and soils. When applied to the agricultural areas, they may have different fates: microbial or non-biological degradation, plant uptake or adsorption and transport by surface water far from the site of application. Therefore, organisms in soil and water niches, from microorganisms to plants and animals, may be exposed to a large number of these pollutants and their metabolites. Additionally, the persistence of these compounds in the environment, associated with their lipophilicity, renders them amenable to progressive accumulation in biological tissues (bioaccumulation) and, consequently, prone to be concentrated along the food chains, increasing the concentration towards the top of the chain (biomagnification). On the other hand, water contamination by agricultural run-off and aerial spraying of herbicides may also have dangerous repercussions on public health even in areas far from those primarily affected. Considering all these risks, including those resulting from a daily consume of eventually contaminated food, it is obvious the general public concern with an unrestricted use of herbicides. The acute and chronic toxic effects of herbicides on useful animals and humans have implemented the research focused on the herbicide mechanisms of action.

A great body of evidence has shown that many environmental contaminants affect transversely the most of the organisms of an ecosystem including man, affecting basic cell functioning and structural properties common to all living cells. In the case of lipophilic compounds, where a great number of herbicides are included, membrane structure and function constitute the first potential target for the toxic effects of the incoming compound,

predictably reflected by perturbations in the bioenergetic process, since this physiological event is on the strict dependence of membrane intactness and stability. On the other hand, this process is a basic mechanism required to drive the activity of all living systems. In prokaryotic cells energy transduction, as well as most vital cell functions, is ascribed to the cytoplasmic membrane. Not surprisingly, perturbations of membrane structure and function by toxic compounds will end in severe perturbation and inhibition of cell growth and viability. On the other hand, mitochondria have a crucial role as energy suppliers in eukaryotic cells, being their functioning strictly dependent on membrane intactness and the proper activity of membrane protein complexes. Thus, these organelles are common targets for the toxic action of lipophilic compounds that, upon incorporation into mitochondrial membranes, can act as uncoupling agents, electron-transport inhibitors or energy-transfer inhibitors.

Huge efforts have been done by our research group to develop predictive tests of the ecological hazards resulting from the exposure to pesticides, assessing in laboratory their toxic effects on model biological systems provided by prokaryotic, animal and plant cells. On the other hand, aiming at elucidating the mechanisms underlying the toxic action of pesticides at a cellular or subcellular level, different experimental approaches have been improved. Three kinds of studies have been implemented in our laboratory to address xenobiotic cytotoxicity *in vitro*: 1) a biophysical approach to lipophilic compound/membrane interactions using membrane lipid models, 2) the use of prokaryotic/eukaryotic cells and 3) the use of mitochondria from plant/animal origin, to evaluate and identify the mechanisms underlying the toxic action of xenobiotics *in vitro*.

A great deal of work has been carried out in our laboratory showing that pesticide membrane incorporation leads to alterations of the structural and physical properties of membrane lipid bilayer, induction of lateral phase separation and generation of lipid microdomains, with predictable impact on protein distribution and sorting in different regions of the membrane (Donato et al., 2000; Videira et al., 2002). Consequent repercussions in membrane permeability and the activity of membrane-associated proteins have been also reported (Videira et al., 2001). One of the biochemical mechanisms that have shown a strong correlation with xenobiotic-induced disturbance of membrane lipid structure and organization is the oxido-reductive systems of bacteria and mitochondria of eukaryotic cells, with obvious impact in all cellular energetic processes (Donato et al., 1997b; Videira et al., 2001; Monteiro et al., 2005 and 2008; Pereira et al., 2009).

A remarkable parallelism has been established between pesticide effects on prokaryotic models (namely the micro-organisms *Bacillus stearothermophilus* and *B. subtilis*) and the other model systems. Thus, partitioning and physical effects have been estimated with bacterial membrane lipids similar to those described for synthetic lipid membrane models and native eukaryotic membranes (Donato et al., 1997c; Donato et al., 2000; Martins et al., 2003; 2005); the impairment of the electron transfer in the bacterial respiratory system has been also demonstrated in rat liver mitochondria (Monteiro et al., 2008; Pereira et al., 2009); the inhibition of bacterial growth and loss of animal cell cultures viability have shown the same dependence on the structural characteristics of chemical related compounds, such as organotin compounds (unpublished data). Mitochondrial fractions of eukaryotic cells have also shown to be plausible alternative *in vitro* model systems for an evaluation of pesticide toxicity, which can simultaneously clarify its mechanisms of action. Data obtained from mitochondria assays have shown to be strongly correlated with cytotoxic parameters

provided by more complex biological systems as whole organisms, like fish (Peixoto et al., 2006). These ones have been widely used as models to evaluate the health of aquatic systems and in studies of xenobiotic-induced pathological effects on vertebrates.

The good correlations established with data provided by these different model systems in studies of herbicide toxicity, as described in this chapter, contribute to validate the use of our models in predictive tests of environmental hazards and toxicity assessment. As also emphasized along this chapter, the most remarkable advantage in using models such as bacteria and plant cells or organelles regards the use of a biological material easily and economically obtained, minimizing or avoiding ethical issues associated with the use of mammals.

5. References

Allen, R.D.; Webb, R.P. & Schake, S.A. (1997). Use of transgenic plants to study antioxidant defenses. *Free radical Biol Med*, 23, 473-479

Babich, H.; Shopsis, C. & Borenfreud, E. (1986). In vitro cytotoxicty testing of aquatic pollutants (cadmium, copper, zinc, nickel) using established fish cell lines. *Ecotoxicol Environ Saf,* 11, 91-99

Bartels, P. G. & McCullough, C. (1972). A new inhibitor of carotenoid synthesis in higher plants: 4-chloro-5-(dimethylamino)-2-(*a,a,a*-trifluoro-*m*-tolyl-3(2H)-pyridazinone. *Biochem Biophys Res Commun*, 48, 16-22

Basova, L.V.; Kurnikov, I.V.; Wang, L.; Ritov, V.B.; Belikova, N.A.; Vlasova, I.I.; Pacheco, A.A.; Winnica, D.E.; Peterson, J.; Bayir, H.; Waldeck, D.H. & Kagan, V.E. (2007). Cardiolipin switch in mitochondria: shutting off the reduction of cytochrome *c* and turning on the peroxidase activity. *Biochemistry*, 46, 3423-3434

Beaumont, F.; Jouve, H.M.; Gagnon, J.; Gaillard, J. & Pelmont, J. (1990). Purification and properties of catalase from potato tubers (*Solanum tuberosum*). *Plant Sci*, 72, 19-26

Beyenbach, K.W. (2004). Kidney sans glomeruli. *Am J Physiol Renal Physiol,* 286, F811-F827

Boelsterli, U.A. (2003). *Mechanistic Toxicology: The Molecular Basis of How Chemicals Disrupt Biological Targets*, Taylor & Francis, London, New York

Camadro, J-M; Matringe, M.; Scalla, R. &Labbe, P. (1991). Kinetic studies on protoporphyrinogen oxidase inhibition by diphenyl ether herbicides. *Biochem J*, 277, 17-21

Cardoso, H. & Leão, C. (1992). Mechanisms underlying the low and high enthalpy death induced by short-chain monocarboxylic acids and ethanol in *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol*, 38, 388-392

Castilho, R.F.; Vicente, J.A.F.; Kowaltowski, A.J. & Vercesi, A.E. (1997). 4,6-dinitro-*o*-cresol uncouples oxidative phosphorylation and induces membrane permeability transition in rat liver mitochondria. *Int J Biochem Cell Biol*, 29,1005-1011

Çavas, T. & Könen, S. (2007). Detection of cytogenetic and DNA damage in peripheral erythrocytes of goldfish (*Carassius auratus*) exposed to a glyphosate formulation using the micronucleus test and the comet assay. *Mutagenesis,* 22, 263–268

Cremlyn, R. J. (1991). *Agrochemicals. Preparation and Mode of Action*, Wiley, Great Britain

Cserháti, T.; Illés, Z. & Nemes-Kósa, S. (1992). Effect of benzonitrile ester herbicides on the growth of some soil bacteria. *J Appl Bacteriol*, 72, 523-528

Daum, G. (1985). Lipids of mitochondria, *Biochim Biophys Acta*, 822, 1-42

Dayan, F.; Romagni, J.; Tellez, M.; Rimando, A. & Duke, S. (1999). Managing weeds with natural products, *Pesticide Outlook*, October, pp.185-188

Dejonghe, W.; Berteloot, E.; Goris, J.; Boon, N.; Crul, K.; Maertens, S.; Hofte, M.; de Vos, P.; Verstraete, W. & Top, E.M. (2003). Synergistic degradation of linuron by a bacterial consortium and isolation of a single linuron-degrading *Variovorax* strain. *Appl Environm Microbiol* 69, 1532-1541

Di Giulio, R.T.; Washburn, P.C.; Wenning, R.J.; Winston, G.W. & Jewell, C.S. (1989). Biochemical responses in aquatic animals: a review of oxidative stress. *Environ Toxicol Chem*, 8, 1103-1123

Donato, M.M.; Jurado, A.S.; Antunes-Madeira, M.C. & Madeira, V.M.C. (1997a). Effects of a lipophilic environmental pollutant (DDT) on the phospholipid and fatty acid contents of *Bacillus stearothermophilus. Arch Environ Contam Toxicol*, 33, 341-349.

Donato, M.M.; Jurado, A.S.; Antunes-Madeira, M.C. & Madeira, V.M.C. (1997b). Comparative study of the toxic actions of DDT and DDE on the growth and respiratory activity of a microorganism used as a model. *Appl Environ Microbiol*, 63, 4948-4951.

Donato, M.M.; Antunes-Madeira, M.C.; Jurado, A.S. & Madeira, V.M.C. (1997c). Partition of DDT and DDE into membranes and extracted lipids of *Bacillus stearothermophilus. Bull Environ Contam Toxicol*, 59, 696-701

Donato, M.M.; Jurado, A.S.; Antunes-Madeira, M.C. & Madeira, V.M.C. (2000). Membrane lipid composition of *Bacillus stearothermophilus* as affected by lipophilic environmental pollutants: an approach to membrane toxicity assessment. *Arch Environ Contam Toxicol*, 39, 145-153

Eble, K.S.; Coleman, W.B.; Hantgan, R.R. & Cunningham, C.C. (1990). Tightly associated cardiolipin in the bovine heart mitochondrial ATP synthase as analysed by 31P nuclear magnetic resonance spectroscopy. *J Biol Chem*, 265, 19434-19440

Figueiredo-Fernandes, A.; Fontaínhas-Fernandes, A.; Peixoto, F.; Rocha, E. & Reis-Henriques, M.A. (2006). Effects of gender and temperature on oxidative stress enzymes in Nile tilapia *Oreochromis niloticus* exposed to paraquat. *Pest Biochem Physiol*, 85, 97–103

Fontaínhas-Fernandes, A.A. (1998). Tilapia production, In: *Aquaculture Handbook*, Reis-Henriques, M.A. (Ed.), pp. 135-150

Forlani, G.; Mantelli, M.; Branzoni, M.; Nielsen, E. & Favilli, F. (1995). Differential sensitivity of plant-associated bacteria to sulfonylurea and imidazolinone herbicides. *Plant Soil*, 176, 243-253

Fry, M. & Green, D.E. (1981). Cardiolipin requirement for electron transfer in complex I and III of the mitochondrial respiratory chain. *J Biol Chem*, 256, 1874-1880

Furusawa, I.; Tanaka, K.; Thanutong, P.; Mizuguchi, A.; Yazaki, M. & Asada, K. (1984). Paraquat resistant tobacco calluses with enhanced superoxide dismutase activity. *Plant Cell Physiol*, 25, 1247-1254

Gennis, R.B. (1989). *Biomembranes: Molecular Structure and Function*. Springer-Verlag, New York

Gonzalvez, F. & Gottlieb, E. (2007). Cardiolipin: Setting the beat of apoptosis. *Apoptosis,* 12, 877-885.

Green, M.B., Hartley, G.S., & West, T.F. (1987) *Chemicals for Crop Improvement and Pest Management*, 3rd Edn, Pergamon Press, Oxford, p. 207

Guilbaut, G.G. & Kramer, D.N. (1964). Fluorometric determination of lipase acylase a- and b-chymotrypsin and inhibitors of these enzymes. *Anal Chem*, 36, 409-412

Harms, H. (1992). In-vitro systems for studying phytotoxicity and metabolic fate of pesticides and herbicides in plants. *Pestic Sci,* 35, 277–281

Hatton, P.J.; Cummins, I.; Price, L.J.; Cole, D.J. & Edwards, R. (1998). Glutathione transferases and herbicide detoxification in suspension-cultured cells of giant foxtail (Setaria faberi). *Pestic Sci*, 53, 209-216

Hay, J. R. (1974). Gains to the grower from weed science. *Weed Sci,* 22, 439-442

Heipieper, H.-J.; Keweloh, H. & Rehm, H.-J. (1991). Influence of phenols on growth and membrane permeability of free and immobilized *Escherichia coli*. *Appl Environ Microbiol*, 57, 1213-1217

Hernando, M.D.; Ejerhoon, M.; Fernández-Alba, A.R. & Chisti, Y. (2003). Combined toxicity effects of MTBE and pesticides measured with *Vibrio fischeri* and *Daphnia magna* bioassays. *Water Res*, 37, 4091-4098

Hill, A.J.; Teraoka, H.; Heideman, W. & Peterson, R.E. (2005). Zebrafish as a model vertebrate for investigating chemical toxicity. *Toxicol Sci,* 86, 6-19

Incledon, B. J. & Hall, J. C. (1997). Acetyl-coenzyme A carboxylase: quarternary structure and inhibition by graminicidal herbicides. *Pestic Biochem Physiol,* 57, 74-83

Ishikawa, I.; Yamada, S.; Hosaka, H.; Kawana, T.; Okunuki, S. & Kohara, K. (1985). Herbicidal properties of sethoxydim for the control of gramineous weeds. *J Pestic Sci,* 10, 187-193

Iwataki, I. & Hirono, Y. (1979). The chemical structure and herbicidal activity of alloxydin-sodium and related compounds. In: *Advances in Pesticide Science*, Geissbuhler, H.; Brooks, G.T. & P.C. Kearney, (Eds.), Part 2, pp. 235-243, Pergamon Press, Oxford

Jurado, A.S.; Pinheiro, T.J.T. & Madeira, V.M.C (1991). Physical studies on membrane lipids of *Bacillus stearothermophilus*. Temperature and calcium effects. *Arch Biochem Biophys*, 289, 167-179

Kaback, H.R. (1972). Transport across isolated bacterial cytoplasmic membranes. *Biochim Biophys Acta*, 265, 367-416

Koyama, K.; Koyama, K. & Goto, K. (1997). Cardiovascular effects of a herbicide containing glufosinate and a surfactant: in vitro and in vivo analysis in rats. *Toxicol Appl Pharmacol,* 145, 409-414

Krall, J.; Bagley, A.C.; Mullenbach, G.T.; Hallewell, R.A. & Lynch, R.E. (1988). Superoxide mediates the toxicity of paraquat for cultured mammalian cells. *J Biol Chem*, 263, 1910-1914

Lawrence, A.J. & Hemingway K.L. (2003). Introduction and Conceptual Models, In: *Effects of Pollution on Fish - Molecular Effects and Population Responses*. Lawrence, A. & Hemingway, K., (Eds.), pp. 1–13, Blackwell Science Ltd, Oxford

Li, X.-Q.; Ng, A.; King, R. & Durnford, D.G. (2008). A rapid and simple bioassay method for herbicide detection. *Biomarker Insights*, 3, 287-291

Livingstone, D.R.; Garcia-Martinez, P.; Michel, X.; Narbonne, J.F.; O´Hara, S.; Ribera, D. & Winston, G.W. (1990). Oxyradical generation as a pollution-mediated mechanism of toxicity in the common mussel, *Mytilus edulis* L. and other molluscs. *Funct Ecol*, 4, 415-424

Lundbaek, J.A.; Collingwood, S.A.; Ingólfsson, H.I.; Kapoor, R. & Andersen, O.S. (2010). Lipid bilayer regulation of membrane protein function: gramicidin channels as molecular force probes. *J R Soc Interface*, 7, 373-395

Luxo, P.C.; Jurado, A.S. & Madeira, V.M.C. (1998). Lipid composition changes induced by tamoxifen in a bacterial model system. *Biochim Biophys Acta*, 1369, 71-84

Lydon, L. & Duke, S.O. (1988). Porphyrin synthesis is required for photobleaching activity of the p-nitrosubstituted diphenyl ether herbicides. *Pestic Biochem Physiol,* 31, 74-83

Magalhães, M.J.A.; Ferreira, J.R.; Frutuoso, L. & Taínha, A. (1989). Study of the disappearance of endosulfan, parathion, trichlorfon and pirimicard from broccoli and Portuguese cabbage. *Pestic Sci,* 27, 23–31

Martins, J.D.; Monteiro, J.P.; Antunes-Madeira, M.C.; Jurado, A.S. & Madeira, V.M.C. (2003). Use of the microorganism *Bacillus stearothermophilus* as a model to evaluate toxicity of the lipophilic environmental pollutant endosulfan. *Toxicol In Vitro*,17, 595-601

Martins, J.D.; Jurado, A.S.; Moreno, A.J.M. & Madeira, V.M.C. (2005). Comparative study of tributyltin toxicity on two bacteria of the genus *Bacillus*. *Toxicol In Vitro,* 19, 943-949

Matolcsy, Gy.; Nádasy, M. & Andriska, V. (1988). *Pesticide Chemistry,* Elsevier, Amsterdam

Matringe, M. & Scalla, R. (1988). Studies on the mode of action of acifluorfen-methyl in nonchlorophyllous soybean cells. *Plant Physiol,* 86, 619-622

McIntosh, T.J. & Simon, S.A. (2006). Roles of bilayer material properties in function and distribution of membrane proteins. *Annu Rev Biophys Biomol Struct*, 35, 177-198

Monteiro, J.P.; Martins, J.D.; Luxo, P.C.; Jurado, A. S. & Madeira, V.M.C. (2003). Molecular mechanisms of the metabolite 4-hydroxytamoxifen of the anticancer drug tamoxifen: use of a model microorganism *Toxicol In Vitro*, 17, 629-634

Monteiro, J.P.; Jurado, A.S.; Moreno, A.J.M. & Madeira, V.M.C. (2005). Toxicity of methoprene as assessed by the use of a model microorganism. *Toxicol In Vitro,* 19, 951-956

Monteiro, J.P.; Oliveira, P.J.; Moreno, A.J.M. & Jurado, A.S. (2008). Disruption of hepatic mitochondrial bioenergetics is not a primary mechanism for the toxicity of methoprene. Relevance for toxicological assessment. *Chemosphere,* 72, 1347-1354

Moreland, D.E. (1980). Mechanisms of action of herbicides. *Ann Rev Plant Physiol*, 31, 597–638

Moreland, D. E. & Novitzky, W. P. (1988). Effects of inhibitors and herbicides on membrane potential of mung bean mitochondria. *Pestic Biochem Physiol,* 31, 247-260

Moreland, D. E. (1993). Research on biochemistry of herbicides: An historical overview. *Z. Naturforch,* 48c, 121-131

Mumma, R.O. & Davidonis, G.H. 1983. Progress in pesticide biochemistry and toxicology, In: *Plant Tissue Culture and Pesticide Metabolism*, Hutson, D.H. & Roberts, R., (Eds.), Wiley, pp. 255–274, Chichester

Nagata, S.; Günther, H.; Bader, J. & Simon, H. (1987). Mitochondria catalyze the reduction of NAD by reduced methylviologen, FEBS Lett, 210, 66-70

Nagel, R. (2002). DarT: the embryo test with the zebrafish *Danio rerio* – a general model in Ecotoxicology and Toxicology. *Altex, 19,* Suppl 1, 38-48

Nellessen, J.E. & Fletcher, J.S. (1993). Assessment of published literature pertaining to the uptake/accumulation, translocation, adhesion and biotransformation of organic chemicals by vascular plants. *Environ Toxicol Chem*, 12, 2045–2052

Neuburger, M. ; Journet, E.-P. ; Bligny, R. ; Carde, J.-P. & Douce, R. (1982). Purification of plant mitochondria by isopycnic centrifugation in density gradients of percoll. *Arch Biochem Biophys,* 217, 312–323

Neumann, G.; Teras, R.; Monson, L.; Kivisaar, M.; Schauer, F. & Heipieper, H.J. (2004). Simultaneous degradation of atrazine and phenol by *Pseudomonas* sp. strain ADP: effects of toxicity and adaptation *Appl Environm Microbiol*, 70, 1907-1912

Nystrom, B.; Bjornsater, B. & Blanck, H. (1999). Effects of sulfonylurea herbicides on non-target aquatic microorganisms. *Aquat Toxicol*, 47, 9-22

OECD 203 (1992). OECD Guideline for Testing of Chemicals. Test Nº 203: Fish, Acute Toxicity Test. www.oecd.org/dataoecd/17/20/1948241.pdf

Otto, D.M.E. & Moon, T.W. (1995). 3,3',4,4'-tetrachlorobiphenyl effects on antioxidant enzymes and glutathione status in different tissues of rainbow trout. *Pharmacol Toxicol*, 77, 281-287

Palmeira, C.M.; Moreno, A.J.M. & Madeira, V.M.C. (1994a). Interactions of the Herbicides 2,4-D and dinoseb with liver mitochondrial bioenergetics. *Toxicol Appl Pharmacol*, 127, 50-57

Palmeira, C.M.; Moreno, A.J.M. & Madeira, V.M.C. (1994b). Metabolic alterations in hepatocytes pomoted by the herbicides paraquat, dinseb and 2,4-D. *Arch Toxicol*, 68, 24-31

Palmeira, C.M.; Moreno, A.J. & Madeira, V.M.C. (1995a). Mitochondrial bioenergetics is affected by the herbicide paraquat. *Biochim Biophys Acta*, 1229, 187-192

Palmeira, C.M.; Moreno, A.J. & Madeira, V.M.C. (1995b). Thiols metabolism is altered by the herbicides paraquat, dinoseb and 2,4-D: a study in isolated hepatocytes. *Toxicol Lett*, 81, 115-123

Palmeira, C.M (1999). Herbicide-induced mitochondrial and cellular liver toxicity: a review of paraquat, dinoseb, and 2,4-D effects. *Toxic Subst Mech*, 18, 187-204

Pedrajas, J.R.; Peinado, J. & Lopez-Barea, J. (1995). Oxidative stress in fish exposed to model xenobiotics; oxidatively modified forms of Cu,Zn-superoxide dismutase as potential biomarkers. *Chem Biol Interact,* 98, 267-282

Peixoto, F.; Vicente, J.A.F. & Madeira, V.M.C. (2003a). The herbicide dicamba (2-methoxy-3,6-dichlorobenzoic acid) interacts with mitochondrial bioenergetics function. *Arch Toxicol*, 77, 403-409

Peixoto, F.; Vicente, J.A.F. & Madeira, V.M.C. (2003b). Comparative effects of herbicide dicamba and related compound on plant mitochondrial bioenergetics. *J Biochem Mol Toxicol,* 17, 185-192

Peixoto, F.; Vicente, J.A.F. & Madeira V.M.C. (2004). A comparative study between plant and animal mitochondria with paraquat reveal that hydrogen peroxide is not related to the observed toxicity. *Toxicol in Vitro*, 18, 733-739

Peixoto, F.; Alves-Fernandes, D.; Santos, D. & Fontaínhas-Fernandes, A. (2006). Toxicological effects of oxyfluorfen on oxidative stress enzymes in tilapia *Oreochromis niloticus. Pest. Biochem. Physiol, 85: 91–96*

Peixoto, F.; Gomes-Laranjo, J.; Vicente, J.A.F. & Madeira, V.M.C. (2008). Comparative effects of herbicide dicamba, 2,4-D and paraquat on non-green potato tuber calli. *J Plant Physiol*, 165,1125-1133

Peixoto, F.; Lopes, M.L.; Madeira, V.M.C. & Vicente, J.A.F. (2009a). Toxicity of MCPA on non-green potato tuber calli. *Acta Physiol Plant*, 31, 103-109

Peixoto, F.; Santos, D.L.; Vilela, S. & Fontaínhas-Fernandes, A. (2009b). Characterization of liver Nile tilapia (*Oreochromis niloticus*) mitochondria and mitochondrial bioenergetics alterations by exposure to oxyfluorfen herbicide (Goal). *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, 61, 386-392

Pereira, S.P.; Fernandes, M.A.S.; Martins, J.D.; Santos, M.S.; Moreno, A.J.M.; Vicente, J.A.F.; Videira, R.A. & Jurado, A.S. (2009). Toxicity assessment of the herbicide metolachlor: comparative effects on bacterial and mitochondrial model systems. *Toxicol In Vitro*, 23, 1585-1590

Pereira, S.P.; Fernandes, M.A.S.; Martins, J.D.; Moreno, A.J.M.; Vicente, J.A.F.; Videira, R.A. & Jurado, A.S. (2010). Toxicity assessment of the herbicide Alachlor using in vitro model systems. International Courses on Toxicology, May 11-14, p.38, University of Coimbra. Portugal

Peter, B.; Wartena, M.; Kampinga, H.H. & Konings, W.T. (1992). Role of lipid peroxidation and DNA damage in paraquat toxicity and the interaction of paraquat with ionizing radiation. *Biochem Pharmacol*, 43, 705- 715

Pireaux, J.-C.; Dizengremel, P. & Botton, B. (1992). Effects of pesticides on oxidative phosphorylation and electron transfer in potato tuber mitochondria. *Plant Sci*, 83: 23-30

Pritchard, J.B. (1993). Aquatic Toxicology: Past, present and Prospects. *Environ Health Perspect*, 100, 249-257

Prosperi, E. (1990). Intracellular turnover of fluorescein diacetate. Influence of membrane ionic gradients on fluorescein efflux. *Histochem J*, 22, 227-233

Rao, V. S. (2000). *Principles of Weed Science*, 2nd Edn, CRC Press, New Hampshire, USA

Ray, T.B. (1984). Site of action of chlorsulfuron. Inhibition of valine and isoleucine biosynthesis in plants. *Plant Physiol,* 75, 827-831

Romagni, J.G.; Duke, S.O. & Dayan, F.E. (2000). Inhibition of plant asparagine synthetase by monoterpene cineoles. *Plant Physiol,* 123, 725-732

Rosa, S.M.L.J.; Antunes-Madeira, M.C.; Matos,M.J.; Jurado, A.S. & V.M.C. (2000). Lipid composition and dynamics of cell membranes of *Bacillus stearothermophilus* adapted to amiodarone. *Biochim Biophys Acta*, *1487*, 286-295

Rossignol, R.; Faustin, B.; Rocher, C.; Malgat, M. & Letellier T. (2003). Mitochondrial threshold effects. *Biochem J*, 370, 751-762

Rytomaa, M.; Mustonen, P. & Kinnunen, P.K. (1992). Reversible, non-ionic, and pH-dependent association of cytochrome c with cardiolipin-phosphatidylcholine liposomes. *J Biol Chem*, 267, 22243-22248

Sandermann, H.; Scheel, D. & Trenck, T.V.D. (1984). Use of plant cell cultures to study the metabolism of environmental chemicals. *Ecotoxicol Environ Saf*, 8, 167–82

Schmale, M.C; Nairn, R.S. & Winn, R.N. (2007). Aquatic Animal Models of Human *Disease. Comp Biochem Physiol C Toxicol Pharmacol*, 145, 1–4

Sedlak, E.; Panda, M.; Dale, M.P.; Weintraub, S.T. & Robinson, N.C. (2006). Photolabeling of cardiolipin binding subunits within bovine heart cytochrome c oxidase. *Biochemistry*, 45, 746-754

Shaner, D.L.; Anderson, P.C. & Stidham, M.A. (1984). Potent inhibitors of acetohydroxyacid synthase *Plant Physiol*, 76, 545-546

Shaner, D. L. (2003). Herbicide safety relative to common targets in plants and mammals. *Pest Manag Sci*, 60, 17-24

Sikkema, J.; de Bont, J.A.M. & Poolman, B. (1995). Mechanisms of membrane toxicity of hydrocarbons. *Microbiol Rev*, 59, 201-222

Smeda, R.J. & Weller, S.C. (1991). Plant-cell and tissue-culture techniques for weed science research. *Weed Sci*, 39, 497–504

Sorensen, S.; Bending, G.D.; Jacobsen, C.S.; Walker, A. & Aamand, J. (2003). Microbial degradation of isoproturon and related phenylurea herbicides in and below agricultural fields. *FEMS Microbiol Ecol*, 45, 1-11

Steinrucken, H.C. & Amrhein, J. (1980). The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimate 3-phosphate synthase. *Biochem Biophys Res Commun*, 94, 1207-1212

Stephensen, E.; Sturve, J. & Forlin, L. (2002). Effects of redox cycling compounds on glutathione content and activity of glutathione-related enzymes in rainbow trout liver. *Comp Biochem Physiol Part C*, 133, 435-442

Stevens, J.T. & Sumner, D.D. (1991). Herbicides, In: *Handbook of Pesticide Toxicology*, Hayes, W.J. & Laws, E.R. (Eds.), pp. 1317-1408, Academic Press, New York

Steward, F.C. (1983). Reflections on aseptic culture, In: *Handbook of Plant Cell Culture*, Evans, D.A.; Sharp, W.R.; Ammirato, P.V. & Yamada, Y., (Eds.), pp. 1-10, Macmillan Publishing Company, NewYork

Stock, J.B.; Stock AM & Mottonen, J.M. (1990). Signal transduction in bacteria. *Nature*, 344, 395-400

Sulik, M.; Kisielewski, W.; Szynaka, B.; Kemona, A.; Sulik, A.; Sulkowska, M. & Baltaziak, M. (1998). Morphological changes in mitochondria and lysosomes of hepatocytes in acute intoxication with 2,4-dichlorophenoxyacetic acid (2,4-D). *Materia Medica Polonia*, 30, 16-19

Thompson, D.G.; Holmes, S.B.; Thomas, D.; MacDonald, L. & Solomon, K.R. (1993). Impact of hexazinone and metsulfuron methyl on the phytoplankton community of a mixed wood/boreal forest lake. *Environ Toxicol Chem*, 12, 1695-1707

Tortorelli, M.C.; Hernández, D.A.; Vásquez, G.R. & Salibián, A. (1990). Effects of paraquat on mortality and cardiorespiratory function of catfish fry *Plecostomus commersoni*. *Arch Environ Contam Toxicol*, 19, 523-529

Trumpower, B.L. & Gennis, R.B. (1994). Energy transduction by cytochrome complexes in mitochondrial and bacterial respiration: the enzymology of coupling electron transfer reactions to transmembrane proton translocation. *Annu Rev Biochem*, 63, 675-716

Van Camp, W. ; Van Montagu, M. & Inze D. (1998). H_2O_2 and NO: redox signals in disease resistance *Trends Plant Sci*, 3, 330–334

Vicente, J.A.F.; Santos, M.S.; Vercesi, A.E. & Madeira, V.M.C. (1998). Comparative effects of the herbicide dinitro-*o*-cresol on mitochondrial bioenergetics. *Pestic Sci*, 54, 43-51

Vicente, J.A.F. & Madeira, V.M.C. (2000). The excellente of turnip mitochondrial fractions. *Biochem Educ*, 28, 104-106

Vicente, J.A.F.; Peixoto, F.; Lopes, M.L. & Madeira, V.M.C. (2001). Differential sensitivities of plant and animal mitochondria to the herbicide paraquat. *J Biochem Mol Toxicol*, 15, 322-330

Videira, R.A.; Antunes-Madeira, M.C. &. Madeira, V.M.C (2001). Ethylazinphos interaction with membrane lipid organization induzes increase of proton permeability and impairment of mitochondrial bioenergetic functions. *Toxicol Appl Pharmacol,* 175, 209-216

Videira, R.A.; Antunes-Madeira, M.C. &. Madeira, V.M.C. (2002). Differential effects induced by alpha- and beta-endosulfan in lipid bilayer organization are reflected in proton permeability. *Biochim Biophys Acta*, 1564, 140-148.

Wakamatsu, Y.; Pristyazhnyuk, S.; Kinoshita, M.; Tanaka, M. & Ozato, K. (2001). The see-through medaka: a fish model that is transparent throughout life. *Proc Natl Acad Sci USA*, 98, 10046–10050

Wallace, K.B. & Starkov, A.A. (2000). Mitochondrial targets of drug toxicity. *Annu Rev Pharmacol Toxicol*, 40, 353-388

Watanabe, K.; Ohori, Y.; Sato, Y. Boger, P. & Wakabayashi, K. (2001). Changes in fatty acid composition of neutral lipid in mung bean cotyledons by oxyfluorfen-induced peroxidation. *Pestic Biochem Physiol*, 69, 166-173

Weber, F.J. & de Bont, J.A.M. (1996). Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes. *Biochim Biophys Acta*, 1286, 225-245

Whitten, J. L. (1966) *That we may live,* Van Nostrand, Princeton, N.J., p. 31

Wiegand, C., Krause, E., Steinberg, C. & Pflugmacher, S. (2001). Toxicokinetics of atrazine in embryos of the zebrafish (*Danio rerio*). *Ecotoxicol Environ Saf,* 49, 199–205

Zimdahl, R. L. (2002). My view. *Weed Sci*, 50, 687

Zuba, M. & Binding, H. (1989). Isolation and culture of potato protoplasts, In: *Biotechnology in Agriculture and Forestry 8. Plant Protoplasts and Genetic Engineering I*. Bajaj, Y.P.S., (Ed.), pp. 124-146, Springer-Verlag, New York

Zychlinski, L. & Zolnierowicz, S. (1990). Comparison of uncoupling activities of chlorophenoxy herbicides in rat liver mitochondria. *Toxicol Lett*, 52, 25-34

Herbicides and Environment

Edited by Dr Andreas Kortekamp

Herbicides are much more than just weed killers. They may exhibit beneficial or adverse effects on other organisms. Given their toxicological, environmental but also agricultural relevance, herbicides are an interesting field of activity not only for scientists working in the field of agriculture. It seems that the investigation of herbicide-induced effects on weeds, crop plants, ecosystems, microorganisms, and higher organism requires a multidisciplinary approach. Some important aspects regarding the multisided impacts of herbicides on the living world are highlighted in this book. I am sure that the readers will find a lot of helpful information, even if they are only slightly interested in the topic.

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