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2,4-D Toxicity: Cause, Effect and Control

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ABSTRACT

The herbicide 2,4-Dichlorophenoxyacetic acid (2,4-D) is one of the many different man-made agrochemicals in use today. This herbicide has a deleterious hormonal effect increasing DNA, RNA and protein synthesis in plants, especially in meristematic tissues of broad-leaved weeds, which are the commonly targeted organisms. This compound can also be used in water reservoirs for the control of algae and it can be toxic for several trophic levels. The mechanism of action for non-targeted organisms is not fully elucidated but the herbicide alters cellular membrane integrity and acts by inhibiting enzymatic complexes involved in electron transfer, stress response to reactive oxygen compounds and oxidative phosphorylation. The herbicide has low to moderate acute toxicity towards humans and animals, but some results suggest a chronicle and/or genotoxic metabolic disturbances. Once applied to the soil the risks of being transported to and by aquatic systems are complex and case-specific, but often associated with several other controlling variables apart from the presence of microorganisms capable of carrying out biodegradation. 2,4-D is commonly degraded by several strains in weeks or in a few months and phylogenetic analyses indicate independent recruitment of the 2,4-D catabolic pathway. However, this gene transference is rarely discussed in the context of biosafety and/or its influence on the microbial ecology of natural communities. Several bioremediation strategies are reported to control 2,4-D contamination and toxicity by commonly altering the indigenous microbial communities' structure and/or metabolism. Policies related to pesticides might also affect the environmental contamination by 2,4-D once this compound can be used as an alternative to replace more toxic ones.

Keywords: 2,4-Dichlorophenoxyacetic acid, environmental contamination, herbicide, toxicity

CONTENTS

INTRODUCTION

The herbicide 2,4-Dichlorophenoxyacetic acid (2,4-D) has been used extensively in modern agriculture and, despite its short half-life in soil or aquatic environments, toxicological studies suggest a great potential for inducing undesirable effects affecting non-targeted organisms (Haward 1991). Phenoxy-hebicides are xenobiotic compounds used to control dicotyledonous weeds and they have been produced and commonly applied at a large scale since the first half of the last century (Haward 1991). On the other hand, little is yet known of its biochemical mode of action despite its important commercial value. Details of the biochemical pathways in which 2,4-D affects the dicot-weeds' metabolism are still lacking and the toxic effects towards several organisms are commonly only described by a series of symptoms or end results. Thus, 2,4-D phytotoxicity is mostly understood by causing symptomatic metabolic abnormalities, and little is yet known about its role during the induction or repression of specific metabolic genes within a common shared pathway in targeted or non-targeted organisms. Therefore, prediction and risk assessments are commonly made using the accumulated knowledge of symptomatic toxic responses as a cut off point. The reasons behind the difficulties of unveiling 2,4-D's toxic mode of action may reside in the fact that it does not only seem to affect several distinct metabolic pathways in a variety of organisms, but it also shows a biphasic effect directly dependent on its initial concentration (Grossmann 2000; Toyoshiba *et al*. 2006). Thus, the attempt of classifying 2,4-D toxicological responses based on concentration ranges is often confusing and it may not be helpful. Similar organisms may have a distinct toxicological response to the same concentration range. As a very crude baseline, for instance, at low concentrations (<0.1 mg l^{-1}), 2,4-D might be used to stimulate growth by cell division and elongation but, in higher concentrations (>0.1 mg l^{-1}), it may cause induced abnormalities culminating with the symptomatic herbicide effect. On the other hand, and even at low concentrations, 2,4-D may also affect non-targeted organisms which often respond by starting a metabolic chain-reaction commonly associated with changes in the cellular membrane integrity and fluidity. Therefore, the purpose of this article is to offer an overview of what is known so far about 2,4-D toxic effects, biodegradation pathways and its potential for impacting microbial communities' structure and function.

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Invited Review

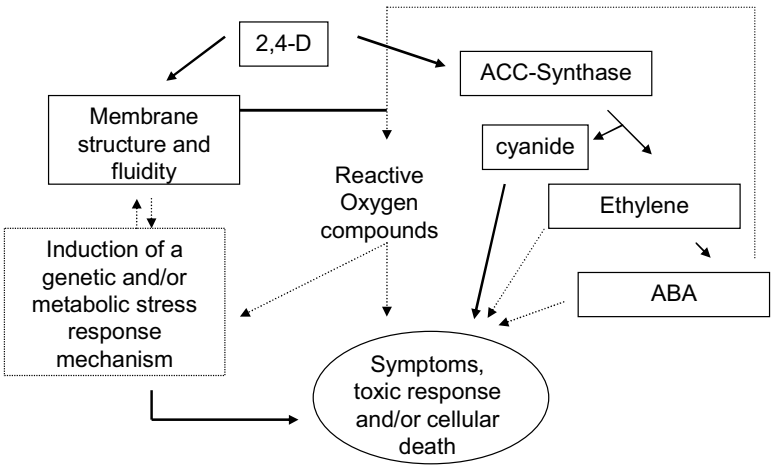

Fig. 1 Schematic representation of 2,4-D mode of action affecting targeted (figure right side) and non-targeted (figure left side) organisms. The main arrows reflect the current hypothesis. For more details please consult text. In short, 2,4-D herbicide acts as an auxin plant hormone for targeted organisms and, despite differences in toxic thresholds, this compounds may affect ethylene pathway directly. Before ethylene concentrations can cause damage, toxic effects might be a resultant of the stimulation of 1-aminocyclopropane-1-carboxylic acid synthase (ACC-synthase) with the selective accumulation of cyanide. The over production of ethylene and the potential intracellular accumulation of abscisic acid (ABA) are known to cause toxic responses directly, but reaching acute concentrations, they may also generate reactive oxygen compounds resulting in the signaling for cellular apoptosis. In non-targeted organisms damage of cellular membrane is believed to be the main mode of action, but it may also affect specific pathways such as of the lipid metabolism. 2,4-D toxicity could also favor the attack by reactive oxygen compounds triggering a metabolic stress response reaction resulting in permanent damage.

DEFINITION, PURPOSE AND 2,4-D MODE OF ACTION

The man-made herbicide 2,4-D can be found in a variety of water soluble amine salts and in the acid form, but it can also be produced with ester derivatives which strongly enhances its diffusion properties through organic matrices. Because of their chemical characteristics the common commercial 2,4-D formulas dimethyl-amine salt (DMA) and 2-ethylhexyl ester (EHE) accounted for approximately 90% of global use in the decades preceding this century (Charles 2001). According to the International Programme on Chemical Safety (IPCS), alkali or amine salts or esters of 2,4-D are used as herbicides against broad-leaf weeds in cereal crops, as well as on pastures and lawns, at rates of about 0.2-2.0 kg ha^{-1} active ingredient (acid equivalent). Esters are also used at rates of up to 6 kg ha^{-1} (acid equivalent) to suppress weeds, brush, and some trees. Granular formulations are used as aquatic herbicides at rates of 1-122 kg ha^{-1}. At very low foliar application rates (20-40 mg 2,4-D l^{-1} spray water), 2,4-D can also be used as a growth regulator (http://www.inchem.org/documents/hsg/hsg/hsg005.htm). Grötzschel *et al*. (2004) reported that 2,4-D concentrations of 20 mg l^{-1} inhibits permanently 17% of the phototrophic community of hypersaline microbial mats. More complex organisms such as some macrophytes, common in water bodies, are affected by 2,4-D in concentrations around 0.1 mg l^{-1} and, as a consequence of their growth inhibition and dead biomass, their decomposition results in significant changes in the microbial community structure and nutrient cycle within the system (Kobraei and White 1996; Forsyth *et al*. 1997).

In general terms this herbicide causes increased DNA, RNA and protein synthesis in plants, especially in the meristematic tissues of broad-leaf weeds, with some indication of affecting lipid metabolism (Moreland 1980; Hangarter *et al*. 1980). The compound can also be used in plant cell culture as a complementary hormone to induce the process of cell de-differentiation (Davies 1995). The common symptoms of affected plants are accelerated foliar senescence, chloroplast damage and chlorosis with following disruption of the vascular systems. On the other hand, the specific nature of the metabolic response strongly suggests a defined biochemical pathway of action and interaction within targeted organisms (Abel and Theologis 1995). A protein-binding receptor specific for 2,4-D has not yet being characterized, but molecular signalling is assumed to induce a series of events such as aberrations in RNA synthesis, alterations in the cellular membrane and intracellular calcium concentrations. A metabolic pathway causing phytotoxic effect resulting in cellular death has been suggested by Grossmann (1996, 2000). The author reported that auxin-like herbicides induce 1-aminocyclopropane-1-carboxylic acid synthase (ACC-synthase), which is a key enzyme during the production of ethylene. Cyanide is a co-product of ethylene biosynthesis in higher plants via the ACC pathway. To form ethylene ACC is oxidized by ACC-oxidaze generating CO_2 and cyanide. Cyanide is toxic if it accumulates in plant tissues. Therefore it is suggested that cyanide, as the co-product of ethylene biosynthesis, causes phytotoxic effects on plants subjected to auxin-type herbicide treatments. Therefore, despite the uncontrolled production of ethylene which in itself is also a phytotoxic compound capable of causing early tissue senescence, phytotoxicity would be firstly caused by the accumulation of cyanide. On the other hand, Wei *et al*. (2000) pointed out that ACC-synthase might not be the ultimate metabolic pathway targeted by auxin-like herbicides once such compounds would also inhibit root elongation, but the nature of this inhibition is not related to ethylene biosynthesis pathway. Thus, 2,4-D may affect more than one metabolic pathway in sensitive organisms.

Raghvan *et al*. (2005) showed the first comprehensive report on plant genomic expression (*Arabidopsis* sp.) under the exposure of 2,4-D (1 mM). The authors observed that in one hour of exposure 148 genes were induced and 85 repressed. The functional category of the genes indicated that not only the biochemical pathways of ethylene and abscisic acid (ABA) were affected, but also several other genes associated with DNA transcription and metabolic signalling molecules connected to a variety of distinct cellular functions. In addition, other experiments showed that 2,4-D also induces the production of hydrogen peroxide or organic reactive oxygen which also significantly contribute to cellular death (Grossman 2000; Wagner *et al*. 2002). The accumulation of molecular reactive oxygen would be a result of the decrease in photosynthesis because of the closure of the stomata due to the overproduction of ABA. In sequence, the oxidation of cell membrane lipids by organic reactive oxygen would be perceived as molecular signalling inducing apoptosis. It is therefore clear that the complex nature of 2,4-D toxic mode of action makes it very difficult to understand the toxic effects at the molecular level, and while a protein-biding receptor for 2,4-D is not described, it may be very difficult to understanding 2,4-D's toxic mode of action. **Fig. 1** shows a schematic representation of 2,4-D's toxic potential interactions.

Plants and algae show some similarities in symptomatic responses to exposure to 2,4-D. On the other hand, Wong (2000) points out that a proper physiological comparison is lacking and therefore a clear mechanism of 2,4-D toxic mode of action affecting algal communities are still far from being understood. It is reported that 2,4-D at concen-

trations raging from 0.02 to 2 mg l^{-1} is not toxic to algae in general, but it starts to inhibit algae growth at concentrations higher than 200 mg l^{-1} Wong (2000). However, these are very general values and final conclusions should be avoided.

Due to the widespread use of 2,4-D in agricultural and urban areas to control broad-leaf weeds, this compound has been documented as occurring in several terrestrial and aquatic ecosystems such as natural forests, lakes, rivers, sediments and groundwater (Paasivirta *et al*. 1990; Mangat and Elefsiniotis *et al*. 1999; Tuxen *et al*. 2000). The risk assessment of pesticides being introduced into any environment is complex, but unveiling its potential effects towards non-targeted organisms is of significant importance in this process.

TOXICITY, COMMON EFFECTS AND SYMPTOMS AFFECTING NON-TARGETED ORGANISMS

The observation and documentation of 2,4-D toxicity can be very confusing at first. It is common to come across several reports exhorting the harmful toxic effects of such herbicides towards a variety of biosensors, but there is also a fair amount of work suggesting non-harmful consequences at low concentrations. Charles *et al*. (2001), for instance, reported that this phenoxy-herbicide has shown to be non-mutagenic, non-neurotoxic, non-carcinogenic and of moderate toxicity after sub-chronic administration. Therefore, apart from the influence of experimental conditions, which include type of biosensor and time of exposure, there are also other important aspects affecting the results and interpretation of toxic responses. Some of the factors compromising toxicity assessments are the biphasic concentration thresholds and clear understanding of 2,4-D's mode of action to non-target organisms. In addition, it should be stressed at this point that 2,4-D is a widespread, commercially used herbicide, thus, its industrial and economic relevance should not be ignored. It is not concealed that industrial task forces are campaigning for its use once such a compound has undoubtedly improved food production and financial profits at a large scale. Therefore, there is a reasonable amount of pressure for the continuation of its widespread utilization.

Toxicity is defined as the adverse effects caused by the interference of specific agents to the structure and/or processes which are essential for survival and proliferation of a particular organism (Hayes 2001). This basic definition and the need of a clear system for measuring and predicting toxic effects of a particular compound to a specific group or environment resulted in the creation of standardized *in vitro* experimental trials which have often been used to produce environmental risk assessments. Rarely such studies address the primary effect at the molecular level such as changes in gene expression, and most interpretations are based on symptomatic responses. Therefore, with this approach, effects preceding the majority of biological outcomes are often ignored (Aardema and MacGregor 2002). Rao *et al*. (2006) suggested that in order to understand 2,4-D's mode of action it is imperative that mortality should not be considered the endpoint to assess toxicity. It should be appreciated the fact that differences in the mechanisms of toxicity might be at molecular level responses and under the control of distinct concentration thresholds in low and higher doses (Toyshiba *et al*. 2006).

According to Hayes and Laws (1991) phenoxyherbicides have been placed in the third and forth class in a list of toxic compounds and their effect may be acute to animals between concentrations doses of 100 to 1200 mg kg^{-1}. Depending on the organisms, concentration and time of exposure, 2,4-D may produce a toxic effect ranging from embryotoxicity and teratogenicity to neuro-, immuno- and hepatotoxicity (Tuschl *et al*. 2003). A brief comparison between some toxicity data dealing with 2,4-D toxicity is given in **Table 1**. It is clear from such a small sample that concentrations characterising 2,4-D toxicity depend significantly on the type of biosensor and the biological variable assessed. The variation of toxicity thresholds is wider than that observed for its chlorinated intermediate 2,4-DCP (Bukowska 2003).The fact that 2,4-D affects first the membrane structure with a non-specific mode of action may eclipse the identification of a single phenomenon effect. The charged oxygen moiety of 2,4-D can interact with charged or polar groups at the membrane and water interface. The hydrophobic benzene ring and chlorine substitutes can migrate into the hydrophobic part weakening the interactions that stabilize the membrane structure (Argese *et al*. 2005). It has been demonstrated, for example, that such compounds also cause lipid peroxidation in human erythrocyte membranes (Balagué *et al*. 2002; Duchnowicz 2003). Consequently, exposed human erythrocytes showed an increase in the glutathione peroxidase activity causing depletion in glutathione concentrations which in long term can affect the cell protection against reactive oxygen (Bukowska 2003). Consequently, the toxic responses of non-targeted organisms are often related to symptoms resultant of cellular membrane damage causing changes in membrane fluidity with consequent susceptibility to the attack of free radicals. Interestingly, 2,4-D seems not to be directly related to the specific inhibition of any enzyme involved in the membrane related reverse-electron transfer process (van Wezel and Opperhuizen 1995). 2,4-D shows an uncoupling effect in sub-mitochondrial particles at concentrations of 7

Table 1 Few examples of 2,4-D toxic effects illustrating the large range of acute concentrations in which the herbicide affects the different organisms or biosensors (0.249×10^{-3} to 3984 mg l^{-1}). Toxic concentrations are directly related to biosensor sensibility and specific metabolic responses.

2,4-D Concentrations (mg l^{-1})	Biosensor	Toxic effect	Reference
0.249 x 10^{-3} to 249	HepG2 cells	Changes in gene expression triggering a stress response, effecting the system controlling cell cycle, DNA repair and immune response. Decrease of viable cells.	Bharadwaj 2005
0.250	*Sinapis arvensis*	Inhibition of root and hypocotyls elongation.	Wei *et al*. 2000
3.3 to 180*	Mouse	Aberration in the chromosome. Aberration in the chromosome and sperm-head abnormalities.	Amer and Aly 2001
41.08	Yeast cells	Decrease of viable cells and increase of membrane fatty acid saturation.	Viegas *et al*. 2005
59.76	*Rana ridibunda* (Nerve)	Toxicity response.	Zafeiridou *et al*. 2006
75.0	*Clarias baratrachus* (Erythrocytes)	membrane structural perturbations.	Ateeq *et al*. 2002
75 to 100	*Biomphalaria glabata*	Malformed and/or nonviable embryos.	Estevam *et al*. 2006
249	*Arabidopsis* sp.	Changes in the expression of functional genes such as: transcription, metabolism, cellular communication and signal transduction.	Raghavan *et al*. 2005
500	Human Erythrocythes	Decreases the concentration levels of glutathione.	Bukowska 2003
3984	HepG2 cells	Affects cell cycle.	Tuschl and Schwab 2004

*(mg Kg^{-1})

mg l^{-1} (Argese *et al*. 2005), but the disruption of electron transportation by the membrane seems so far to be a result of a non-specific mode of action. On the other hand, the activity of an organic anion transporter may be directly involved with 2,4-D toxicity mitigation by actively diminishing herbicide concentration within the cell (Sweet *et al*. 2005). Intracellular acidification as a result of 2,4-D accumulation and dissociation in neutral cytosol are also among the deleterious effects of this herbicide (Simões *et al*. 2003). An experiment showed that the process of 2,4-D accumulation within the cell was significantly reduced in the absence of Na^+ and stimulated by an outwardly increasing gradient of glutarate (Villalobos *et al*. 1996). Therefore, the enzymatic disruption or the inexistence of such a membrane transporter would enhance intracellular toxicity and affect significantly the toxicity response tolerance levels of different organisms. This is an example of a potential mechanism for physiological adaptation to 2,4-D toxic concentrations which should also be considered during toxicity risk assessments. **Fig. 1** shows a schematic representation of 2,4-D toxic potential interactions for non-targeted organisms.

Saccharomyces cerevisiae exposed to 2,4-D showed a consistent response characterized by the disruption on membrane selectivity and/or permeability barrier, with a potential for recovery associated to changes in fatty acids composition (Viegas *et al*. 2005). Yeast response to 2,4-D include the transcriptional activation of *PDR* and *TPO1* genes encoding plasma membrane multi-drug resistance transporters of the ARP-binding cassette (Teixeira and Sá-Correa 2002). Therefore this is a strong evidence of the important role of active exporters of anionic forms of this lipophilic weak acid. On the other hand, rearrangements in the membrane structure might also represent a physiological strategy against 2,4-D toxicity. Viegas *et al*. (2005) reported that the expression of the *OLE*1 gene may be regulated in response to 2,4-D induced stress. This gene encodes a desaturase that catalyses the conversion of saturated fatty acids to monounsaturated species. 2,4-D probably affects the transcription of *OLE*1 mRNA by stimulating the enzyme DCp1p, which removes the 5′ cap of the *OLE*1 mRNA, exposing it to enzymatic digestion. Thus, the result is a change in the ratios of saturated to unsaturated fatty acids (C18:0+C16:0/C18:1+C16:1) within the cellular membrane increasing its structure stability in the presence of the herbicide (Viegas *et al*. 2005). Therefore, such a physiological response directly affects 2,4-D toxicity concentrations thresholds, making the process of risk assessment even more confusing. It is not known if a similar mechanism is a wide spread phenomena and if such a physiological response would have long-lasting emergent effects to the individual populations and, consequently, to the microbial communities.

The fact that toxicity of 2,4-D mainly occurs due to changes on the cellular membrane structure resulting also in lipid peroxidation suggests that another important mode of action is the cellular exposition to pro-oxidant agents (Teixeira *et al*. 2004). Significant increase in antioxidant enzyme activity of superoxide dismutase (SOD) and glutathione *S*-transferase (GST) was observed in the grill and kidney tissue of fish exposed to 3 μM of 2,4-D (Oruch *et al*. 2004). Therefore, as 2,4-D starts a chain reaction of stress response allied to the disruption of mitochondrial membranes this whole process might also trigger the signaling for cellular apoptosis (Bukowaska and Hutnik 2006). di Paolo *et al*. (2001) suggested a significant effect of 2,4-D on cellular apoptosis as a result of the inhibition of mitochondrial functions. The authors demonstrated the apoptosis-inducing effect of 2,4-D in hepatoma cells at 4 mM. However, little is also known about chemical interactions affecting 2,4-D toxicity effects and physiological responses. 2,4-D toxic thresholds may not only vary as a consequence of distinct sensibility, potential adaptability but also as a resultant of chemical interactions with other potential toxic compounds (Bharadwaj *et al*. 2005).

A number of reviews have attempted to summarize and discuss toxicity data dealing with chemical mixtures and Deneer (2000) examined some data published from 1972 to 1998 and described how the toxic effects of several mixtures containing 2,4-D were the result of an additive effect of their parts. Carpy *et al*. (2000) however, recognized that the additive toxicity effect is clearly observed for high doses of the studied compounds, but the authors were not sure if that would be the case at lower concentrations (<0.1 mg l^{-1}) or below acute toxic effect. Therefore, the available data does not support the use of additive models for chemical mixtures at concentrations below an acute effect. Based on the additive model to predict toxicity, Carpy *et al*. (2000) concluded that the toxic risk of human exposure to mixtures containing pesticides such as 2,4-D at low doses of their individual constituents does not represent a potential source of concern. On the other hand, risk assessments based on such an assumption lacks the understanding of the overall biochemical process of 2,4-D toxicity. Besides, McCarty *et al*. (2006) point out that toxicity-testing of mixed chemicals has often been carried out with chemicals of a similar category. Testing with mixtures containing widely divergent chemicals is uncommon and the additional effect of non-chemical stressor such as changes in temperature and/or moisture is very rare. In an innovative experiment Bécaert *et al*. (2006) showed that the response of soil microbial community to contamination by 2,4-D was increasingly aggravated by another stressing agent. The authors proposed a Soil Stability Index (SSI) to assess this effect. The index was calculated using the comparative response of several enzymatic activity (acid phosphatese, alkaline phosphatase, arylsulfatase, β-glucosidase, protease and urease) and they observed that heat-stress had a significant effect on the microbial community's response to 2,4-D contamination. It was not clear, however, if such reduction was a product of the direct effect on the enzyme activity or caused by the reduction on bacterial densities originally present in the soil. In addition, the authors also found it impossible to ascertain a clear response pattern of enzymatic activity suggesting that 2,4-D's toxic mode of action was case-specific regarding the distinct enzymes.

Despite the fact that a single stress effect is rarely found in the real world, studies on multiple stress trials with 2,4-D are very rare. Besides, apart from such stressing interactions affecting the toxicity response of a distinct organism, it is also not known if 2,4-D would interfere with chemically mediated signals connecting several microbial populations and ecological functions within an ecosystem once cellular membranes are the main reactive sites.

2,4-D BIODEGRADATION

As suggested by distinct experiments investigating biochemical characteristics of bacterial isolates there are several strains reported as 2,4-D degraders using at least two main different pathways, α-ketoglutarate or dehalogenase pathways (Amy *et al*. 1985; Chaudhry and Huang 1988; Perkins *et al*. 1990; Ka *et al*. 1994; Vallaeys *et al*. 1996, 1999; Laemmli *et al*. 2000; Itoh *et al*. 2002; Kitagawa *et al*. 2002). This is an intriguing phenomena once 2,4-D is a xenobiotic compound which was only introduced into the environment in the latter half of the last century. van de Meer (1994) suggests that metabolic activity towards xenobiotics such as 2,4-D may have emerged from altered enzymatic specificity of existing enzymatic systems, which are effective for the biodegradation of naturally occurring aromatic compounds. On the other hand, evidences of independently acquired genes indicate an active selective process in the evolution of this complex 2,4-D biodegradation pathway (Dejonghe *et al*. 2000). The first attempt to describe a catabolic pathway for 2,4-D biodegradation was made using *Arthrobacter* spp. (Loos *et al*. 1980). On the other hand, the most studied catabolic pathway until today follows the model encoded by the plasmid pJP4 first isolated from *Ralstonia eutropha* JMP134, formerly known as *Alcaligenes eutrophus* (Cavalca *et al*. 1999; Hotopp and Hausenger 2001). *R. eutropha*

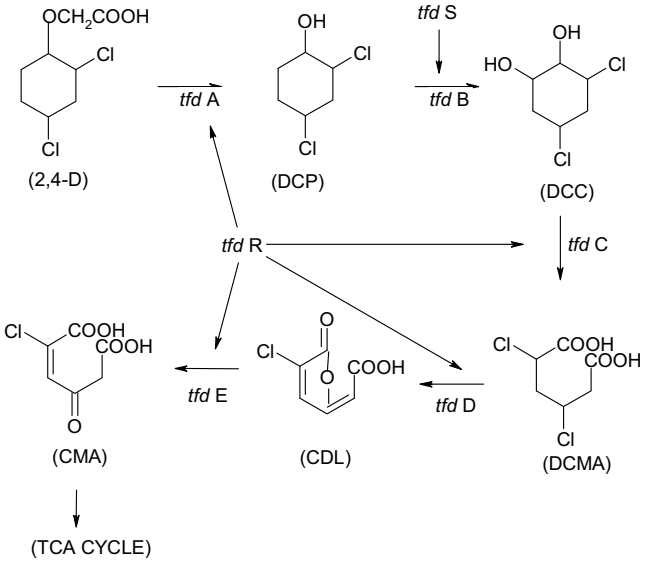

Fig. 2 2,4-D degradation pathway showing the main intermediates: dichlorophenol DCP, dichlorocatechol DCC, dichloro-*cis*-muconate DCMA, trans-2-chlorodienelactone CDL, and 2-chloromaleylacetate CMA), and the respective enzymes of the *tfd* Pathway. The known regulatory genes coding for the respective regulatory proteins R and S are also displayed in the diagram.

JMP134 harbours at least ten known genes directly involved with metabolism of 2,4-D. Six genes are responsible for converting 2,4-D to an intermediate of central metabolism (Streber *et al*. 1987; Perkins *et al*. 1990; Daugherty and Karel 1994), and another three act as regulatory genes and one encoding an active transporters (Bhat *et al*. 1994; Matrubutham *et al*. 1994; You and Ghosal 1995; Leveau and van der Meer 1996, 1997; Leveau *et al*. 1998). The pathway is summarised in **Fig. 2**. Some alternative pathways have been described, but there are strong evidence suggesting that they are not as common in the environment as the one described for pJP4. For instance, *Azotobacter chroococcum* degrades 2,4-D to 4-chlorocatecol, instead of 3,5-chlorocatechol produced in the pJP4 pathway, and then this compound is degraded to an intermediate of general metabolism (Balajee and Mahadevan 1990).

Despite some differences in the upper stream pathway, chlorinated aromatics are commonly transformed into chlorocatecol, which is further transformed by the modified *ortho*-cleavage pathway (Don and Pemberton 1981; Reineke and Knackmuss 1988). The modified *meta*-cleavage of chlorocatechol often leads to dead-end products (Klecka and Gibson 1981).

There is evidence to support the idea of a common enzymatic gene ancestor for several chloroaromatic degradative pathways in distinct strains (Daubaras and Chakrabarty 1992; Schlomann 1994), but some bacteria exhibit an independent origin for the chlorocatechol *ortho*-cleavage genes (Eulberg *et al*. 1998). Thus, this is in fact used to explain the worldwide distribution of chloroaromatic degrading bacteria since the short period of first introducing 2,4-D in the environment (Fulthorpe *et al*. 1995; Leander *et al*. 1998). For these reasons, 2,4-D has become a model compound for studying the phenomena behind the aggregation and evolution of catabolic genes, and their manifestation and spreading mechanisms within microbial populations (Amy *et al*. 1985; Leveau and van de Meer 1997; Droge *et al*. 1998; Top *et al*. 1998; McGowan *et al*. 1998; Ogawa and Miyashita 1999). These former studies compared DNA homologies between the genes encoding for the main enzymes of the 2,4-D biodegradation pathway. These scientific reports show strong indication that the first enzymes of the pathway were acquired by some 2,4-D degraders from a different source than the remaining complementing enzymes comprising the pathway. Therefore, horizontal transference of genes might have occurred in more than one step and by more than one process. In addition, it is also not known which selective process has driven such process in an allegedly short period.

In the environment, some soil bacteria may mineralise 2,4-D in several weeks or a few months (Loos *et al*. 1980; Ghani and Wardle 2001). The reason for such large variation in time-scale characterizing 2,4-D biodegradation is case specific but, often, it is related to bioavailability and/or biodegrading organisms. An important factor is the rate of sorption and desorption of 2,4-D into the organic and inorganic matter present in the environmental matrix (Benoit *et al*. 1996). Thus, the adsorbed pesticide may not be bioavailable to degrading microorganisms (Alexander 1994). If a compound is not bioavailable means that it cannot be taken up by the living cell and therefore metabolized. There are several factors affecting bioavailability, chemical reactions which binds 2,4-D to the environmental matrix, and/or chemical or physical processes hindering its diffusion through the environmental matrix. This seems to be an important process affecting 2,4-D biodegradation in soils (Boivin *et al*. 2005) rather than in sediment (Chinalia and Killham 2006), possibly because of the presence of a continuous aqueous matrix favouring the herbicide diffusion. Apart from the bioavailability phenomena, there are other factors such as the effects of mixed complex growth substrates, competition between different microbial species and the toxic effect that the herbicide might have on the natural microbial community affecting important inter-specific interactions (Kilpi 1980; Lappin *et al*. 1985; Alexander 1994; Wikstrom *et al*. 1996). Complex mixtures of organic substrate often favour the biodegradation of readily-metabolized compounds with high energy values such as sugars-and/or fatty acids-like compounds. Xenobiotic compounds are often less readily-metabolized. Competition between bacterial strains commonly favours endogenous populations affecting environmental colonization by the most efficient exogenous biodegraders. Therefore, contrary to the lag phase, it has been shown that 2,4-D degradation rates do not correspond to the initial population density of degrading microorganisms (Lewis *et al*. 1983; Button 1985; Goulder 1987). Microorganisms of complex assemblages are kinetically heterogeneous and they might be distinctly favoured by other factors apart from initial concentrations of the herbicide (Williams 1973; Jones and Alexander 1986). Therefore, bacterial density and degradation rates of distinct 2,4-D concentrations in different environments may be case-specific and they may not be interconnected (Fournier 1980; Ruben *et al*. 1982; Soulas 1993; Chinalia and Killham 2006). In other words, high density of 2,4-D degraders may not necessary correspond to higher biodegradation rates due bacterial metabolic heterogeneity.

EFFECTS ON THE MICROBIAL COMMUNITY

The effect of 2,4-D on the environmental community was investigated in different environments at distinct concentrations (Olson and Lindwall 1991; Chinalia and Kilham 2006; Macur *et al*. 2007), *in situ* (Xia *et al*. 1995; Gonod *et al*. 2006; Macur *et al*. 2007), in laboratorial conditions (Wardle and Parkinson 1990; Prado and Airoldi 2000; Chinalia and Kilham 2006; Gonod *et al*. 2006), alone, or in mixtures with other herbicides (Wardle *et al*. 1994). Olson and Lindwall (1991) reported that 2,4-D concentrations 10 times higher than the applied field ratios had no effect on microbial biomass, carbon mineralization and nitrification. The authors have also suggested high biodegradation rates following 2,4-D application. Fast mineralization of partial 2,4-D concentrations is often reported to take place in the first days of contamination. Boivin *et al*. (2005) reported that in experiments carried out using three different soils (clay, loam and sand) in equivalent concentrations of field treatment dose of 1800 g ha^{-1}, about half of applied 2,4-D was mineralized within 10 days. Bioavailability affected the mineralization of the remaining 2,4-D concentrations. The experiment last

for 60 days and, at this time, less than 2% of the remaining 2,4-D was considered bioavailable to the microbial soil communities. Therefore, mineralization seems to be limited at low 2,4-D concentrations. Prado and Airoldi (2000) measured microbial activity by calorimetrically monitoring Latosol soil contaminated by 2,4-D (0-0.6 mg g^{-1}). These authors reported that microbial community quickly assimilated the herbicide as organic source. However, it affected microbial activity towards naturally occurring organic sources. Therefore, the authors concluded that constant 2,4-D applications would directly affect organic matter turn over in the soil ecosystems. Wardle *et al*. (1994) showed using respirometry assessments that 2,4-D amendments (1.5 kg ha^{-1}) affected microbial decomposition of pasture shoot and root litter differently. Whilst amendments inhibit plant decomposition in some trials, it had the opposite effect in others. The authors suggested that such effect was related to 2,4-D concentrations once the retention of the herbicide by distinct plants vary. Therefore, it might have been an effect of 2,4-D concentrations. As the herbicide may be used as carbon source by several bacterial strains, concentrations may also affect microbial communities differently. For instance, Macur *et al*. (2007) showed that 2,4-D contamination (0-500 mg kg^{-1}) provide a selective advantage for organisms capable of utilizing 2,4-D as carbon source such as *Burkholderia*-like species. In addition, bacterial diversity assessments showed considerable variation according to the method employed. DGGE-profiling showed significant changes only at 2,4-D concentrations higher than 100 mg kg^{-1}, but diversity assessed by cell-culturing showed significant changes at concentrations higher than 10 mg kg^{-1}. It is difficult to establish predictive relationships between soil and sediment characteristics and microbial processes. Such fact is often attributed to soil or sediment heterogeneity which is commonly defined by physico-chemical properties and little attention is paid to microbial communities. Gonod *et al*. (2003) reported a large variability of 2,4-D mineralization potentials at concentrations of 7.8 μg g^{-1} by soil aggregate sizes and classes. The authors suggested that an uneven distribution of degrading organisms in the aggregates would be more effective in controlling the potential of 2,4-D mineralization than carbon source bioavailability. Therefore, biodegradation rates are not only affected by bioavailability of the targeted compound but also directly dependent on microbial species and their distribution within the environmental matrix. Mineralisation rates by sediment microbial communities, for example, have been reported as varying between 5 to 750 μg d^{-1} according to sediment type and 2,4-D initial concentrations (Chinalia and Killham 2006).

It can be stated that the discussed results varied considerably and the confusing and sometimes conflicting analysis reported in the literature may be the consequence of different perspectives or the lack of a common comparative approach for measuring relevant variables as the resultant of 2,4-D contamination. For instance, in early days, the characterization of microbial communities would relay mainly on microbial culturing such as plate culturing and most probable numbers (Breazeale and Camper 1970). Later, these studies were followed by a more functional based strategy such as microbial production, respiration, enzymatic activity and microbial biomass measurements (Wardle and Parkinson 1991; de Lipthay *et al*. 2003; Chinalia and Kilham 2006; Macur *et al*. 2007). Following these wide range of approaches many other scientific reports provide substantial evidence that 2,4-D toxic effects may also vary according to the influence of several environmental factors such as the percentage of organic carbon, inorganic composition, moisture, temperature, pH, oxygen and 2,4-D frequency of application (Thompson *et al*. 1984; Shaler and Klečka 1986; Greer and Shelton 1992; Rai 1992; Han and New 1994; Mulroy and Ou 1997; Welp and Brűmmer 1999). Few are the authors reporting a minimal effect of such variables (Fulthorpe and Schofield 1999). Therefore, it seems inadequate that 2,4-D toxic concentrations are commonly seen as an end point to tentatively measure contamination affects, but it is understandable why it has been used as a comparative baseline. It has been suggested by several authors that 2,4-D concentrations lower than 2 mg kg^{-1} may not significantly affect microbial production, biomass, respiration and nitrification (Biederbeck *et al*. 1987; Hogan and Ward 1998; Wardle and Parkinson 1990, 1991; Olson and Lindwall 1991; Whelp and Brümmer 1999; Lupwayi *et al*. 2004). Concentrations higher than 2 mg kg^{-1} of 2,4-D has shown the potential to cause significant disturbances on the function of distinct microbial communities (Wardle and Parkinson 1990; Olson and Lindwall 1991; Ingham *et al*. 1995; Rath 1998; Welp and Brümmer 1999; Prado and Airoldi 2000).

A challenging point during environmental risk assessment of disturbances caused by 2,4-D inputs has been the process of integrating individual environmental variables or processes in order to characterize a dangerous contamination threshold. Therefore, the end result of 2,4-D environmental contamination may have not yet been fully appreciated. It is difficult to identify and quantify the environmental changes with long lasting effects as a result of 2,4-D contamination. Long term experiments showed a reduction of 46% in the bacterial diversity after four years of uninterrupted 2,4-D soil contamination (Breazeale and Camper 1970). In a 15 years experiment, Rai (1992) reported that 2,4-D affected significantly bacterial density, biomass and the process of denitrification. Therefore, it is clear that environmental impact should not only be measured by assessing the toxic effect of contamination but also by identifying the accumulative effect of such ecological changes. For instance, physiological profiling of the affected microbial community have been suggested as an integrating strategy to quantify the alterations caused by 2,4-D contamination (Lupwayi *et al*. 2004).

The delimitation of toxicity limits have been so far the common approach used for assessing the environmental effects of 2,4-D contamination (St-Laurent *et al*. 1992; Fabra *et al*. 1997; St. Kouts *et al*. 2005). It is also common to find some scientific reports attempting to extrapolate experimental data from few microbial specimens to tentatively characterize the response of the whole community (Colmer 1953; Okay and Gaines 1996). There are some scientific investigations focusing on a more integrated ecological approach for characterizing 2,4-D contamination with distinct history of use and agricultural exploration (Breazeale and Camper 1970; Olsom and Lindwall 1991; Xia *et al*. 1995; Tiedje *et al*. 1999; Chinalia and Kilham 2006). However, it is evident that they are short of unifying criteria supporting a comparative risk assessment approach based in the understanding of emerging functional processes. On the other hand, Rath *et al*. (1998) reported a significant structural change in the microbial community of an anaerobic microbial community of flooded soil contaminated by different 2,4-D concentrations (0.75, 1.5, 7.5 e 15 μg g^{-1}). The authors reported an overall increase in the microbial biomass at lower 2,4-D concentrations (0.75 and 1.5) and a decrease in higher concentrations. However, in the majority of the cases, the number of 2,4-D degraders increased. For instance, it has been reported an increase of 2,4-D degraders from 0.6×10^3 to 5.7×10^5 cfu g^{-1} dry weight of sediment as a result of distinct 2,4-D concentrations (Chinalia and Killham 2006). Microbial identification of 2,4-D degraders is not always reported and it is often assumed as most probable numbers (MPN) or plate-counting in selective medium. On the other hand, some examples are given in **Table 2**. Therefore, an increase of microbial density seems to be a common unifying response in the majority of 2,4-D contaminated environment. It is not known, however, if such variable would support a reliable 2,4-D impact index capable of characterizing or predicting a potential effect on ecosystems' resistance or resilience to ecological perturbations.

Table 2 Some examples of 2,4-D degraders listed in the literature.

Microbial strains reported as 2,4-D degraders	Taxonomic Group	References
Achromobacter xylosoxidans (formerly *Alcaligenes xylosoxidans*)	δ-proteobacteria	Tonso *et al*. 1995
Azotobacter chroococcum	δ-proteobacteria	Balajee and Mahadevan 1990
Pseudomonas spp.	δ-proteobacteria	Musarrat *et al*. 2000
Halomonas sp.	α-proteobacteria	Maltseva *et al*. 1996
Rhodopseudomonas palustris	α-proteobacteria	McGowan *et al*. 1998
Sphingomonas paucimobilis	α-proteobacteria	Ka *et al*. 1994
Bradyrhizobium spp.	α-proteobacteria	Kamagata *et al*. 1997
Burkholderia cepacia CSV90 (formerly *Pseudomonas cepacea* CSV90)	β-proteobacteria	Bhat *et al*. 1994
Burkholderia graminis	β-proteobacteria	Dejonghe *et al*. 2000
Delftia acidovorans MC1 (formerly *Comamonas acidovorans* MC1)	β-proteobacteria	Müller *et al*. 1999
Rhodoferax fermentans	β-proteobacteria	Tonso *et al*. 1995
Variovorax paradoxus	β-proteobacteria	Don and Pemberton 1981
Flavobacterium spp.	Bacteroidetes	Chaundry and Huang 1988
Phanerochaete chrysosporium	Fungus	Yadav and Reddy 1993

CONTROL MECHANISMS AND CONCLUDING REMARKS

Agricultural production has increased significantly with the help of chemical inputs and both practices have caused dramatic changes in the natural ecosystems. Environmental scientists and decision-makers are therefore facing economic and ecological related challenges which are often conflicting. It is common sense that economic growth and ecological sustainability is only possible through the work of regulatory governmental institutions. Regulatory decisions of a certain country can only be fully understood in the framework of the legislation of that country. On the other hand, documents providing general guidelines for the use and setting exposure limits consistent with the protection of human health and the environment are easily available for consultation. An example is the compilation stored in the database of the International Register of Potentially Toxic Chemicals (IRPTC). This international register was established by UNEP in 1976 and it aims to help the world community make a better use of existing global resources and to give information base to managing chemicals effectively (http://www-cger.nies.go.jp/cger-e/db/info-e/InfoDB Web/db/irptc.htm). It is lacking, however, the existence of a unifying international ruling organization not only capable of determining universal guidelines for the use of chemical inputs into the environment, but also with enough power to enforce such rulings.

After the analysis of several controlling strategies, it is a common unifying idea that countries regulatory ruling bodies developed their controlling rules based in ecological risk assessments which in general terms are a very flexible process of gathering toxicological data with a major focus in human health. Therefore, the processes in place to control the use of different herbicides are based mainly in toxicological data. Because of the large range in which distinct concentrations of 2,4-D may affect the acute toxic responses of different testing biosensors this herbicide is commonly classified as having a moderate potential for causing environmental damage. For instance, 2,4-D is a commonly approved systemic herbicide, and specified uses, limitations, and safety precautions are mostly described by concentrations limits regarding human exposure or toxicity testing thresholds. Therefore, the herbicide is often approved for the use in soil, places near water or in water bodies and the spraying of certain 2,4-D preparations is a common practice. Handling of 2,4-D commercial products are not always restricted to agricultural use or users only. On the other hand, a more systematic approach for measuring and controlling 2,4-D contamination is very rare.

Bus and Hammond (2005) report that regulatory agencies in North America and Europe started a process of re-registration/re-evaluation activities for 2,4-D which resulted in the formation of the "Industry Task Force II on 2,4-D Research Data" which is another example of data compilation available for online consultation. Once again, despite being of significant importance and a major step towards a fair balance between economical growth and risk assessment, it is clear that important issues have not been properly addressed. Changes in the microbial communities function, microbial-signalled based processes and the result of genetic exchanges within the environment are not mentioned as important variable during environmental risk assessment. Therefore, in spite of the extensive data dealing with toxicity response, teratogenicity, reproductive toxicity and neurotoxicity in humans and some standard testing biosensors, the indication of an integrating functional approach for assessing changes in a contaminated ecosystem is still rare. Consequently, one possible perspective for the future may also represent a new stage in which environmental contamination is perceived. It seems that the existence of an international ruling body is not the only integrating step towards controlling environmental contaminations. It is true that environmental contamination does not respect international borders, but an update on the process of risk assessment in order to address an integrated view of the microbial communities function is also needed. At what extension 2,4-D use and contamination contributes to upset ecological functions and the interactions within ecosystems is not yet known. The majority of the available data seems not to address this issue directly.

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