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Response of *Chlamydomonas reinhardtii* to Herbicides: Negative Relationship Between Toxicity and Water Solubility Across Several Herbicide Families

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A cursory look through any textbook describing herbicide effects will quickly reveal a wide variety of mechanisms influencing the amount of herbicide entering a plant, its intracellular concentration or its effectiveness (Duke 1985). It is commonly believed that relatively little herbicide – in the order of a few percent of the total administered dosage – actually binds to the target site. Additionally, the herbicide effect on the organism may be influenced by a host of conditions including the age and physiological state of the organism and environmental conditions before, during and after treatment (Kearney & Kaufman 1988). Given this complexity, it might be concluded that the toxicity of a particular herbicide could be the result of a case-by-case assessment of its effect on any test organism. Alternatively, the Quantitative Structure Activity Relationship (QSAR) approach (Nendza 1991; Urrestarazu Ramos et al 1999) has been developed to help identify major relationships between chemical composition and effectiveness. Thus, in theory, QSAR has potential as a tool to predict toxicity with the limitation that the toxicants for which the QSAR is developed have some comparable basis, most often a similar mode of action.

In this study, 19 different herbicide chemical families embracing 14 different modes of action according to Herbicide Resistance Action Committee classification (Schmidt 1997), were tested. The objective of this study was to search for a general rule that operates above the herbicide family level; a situation where QSAR lacks power. We found that the toxicity of herbicides was more strongly correlated with the general molecular properties of water solubility than with the Octanol / Water partition coefficient K_{ow}. While K_{ow} is usually considered to promote permeability through membranes (Collander & Bärlund 1933) and thus potentially facilitating bio-accumulation, our alternative reasoning is based on the property of water solubility itself : *In vivo*, herbicide molecules must travel through an aqueous environment to reach their target site. Thus, a highly water soluble herbicide should be able to cause injury over a large range of concentrations, while a relatively insoluble herbicide would be limited to much narrower range. Therefore, the less soluble the molecule, the more phytotoxic to the organism it ought to be (xenobiotic). All candidate molecules that are both poorly soluble and of low toxicity would thus never make it through companies'

first screening and further development.

Chlamydomonas reinhardtii, a standard model unicellular chlorophyte, and conventional herbicide dose-response methods were used to explore the relationship between the solubility, molecular weight and the K_{ow} (logarithm of octanol / water partition coefficient) of 29 herbicides. These factors were tested and compared for their individual and combined effectiveness as predictors of the degree of herbicide injury.

MATERIALS AND METHODS

Two *C. reinhardtii* reference strains CC-1010+ and CC-2342- (Duke University code), were used to obtain estimates of genotype-specific effects. Stock populations were maintained in the laboratory as axenic vegetative cultures by serial transfer on minimal carbon source free medium in dim light throughout the experiment. Before use, strains were grown in 20 ml minimal medium under optimal growth conditions, providing aliquots (80 µL) of each genotype from exponential-phase cultures to inoculate the experimental tubes. Following this procedure, we verified that aliquot cell density did not substantially differ from 10^5 living cells/mL over several "dilution / plating / colony counting" measures.

The herbicides used in this study were of technical grade and were stored in the laboratory according to manufacturers' recommendations. Information on molecular weight, maximum solubility in water, partition coefficient and sensibility to autoclaving were taken from "The Pesticide Manual" (1997). When necessary to approach maximum solubility, herbicides were dissolved in dimethylsulfoxyde (DMSO). The volume of DMSO was chosen so that its highest concentration never exceeded 0.1% V/V, a level at least 20 times lower than literature values describing no effect on micro-algae (James et al. 1993; Hess 1980, El Jay 1996). The culture medium was a modification of Bold's medium (Bell 1991), a mixture of inorganic salts. The lack of a carbon source in the medium thus relates the increase of different cell constituents to photosynthetic efficiency (but see Wilson & Levedahl 1964). It is also a rather unfavourable environment for heterotrophs. Autoclaving after the addition of herbicide and pH readjustment was preferred to sterile filtration to ensure axenic conditions throughout the experiment by facilitating the manipulations of relatively large volumes for many tubes. No CO_2 or bicarbonate was added after autoclaving, but tubes with top screws partly opened were vortexed for the daily transmittance growth measure, thus ensuring adequate gas exchanges in the absence of shaker.

We constructed a geometric dilution series of herbicide concentrations through serial dilution with the aim of encompassing the range zero to normal growth. The unit of growth was 20 ml of culture medium in a 50 ml screw-top glass tube. Two replicate cultures of each genotype-herbicide combination were used. The position of each culture in a rack was assigned at random. Racks were placed under fluorescent light dispensing 4500 lux/cm^2 with a 22 hours light (22°C) / 2

hours dark (20°C) cycle. Cultures were kept under these conditions until the control without herbicide had completed growth (representing 7-8 doublings), giving two identical consecutive daily transmittance measures. The effect of herbicide concentration on growth was then examined by measuring the optical transmittance at 665 nm (Safas 1900 spectrophotometer) of each culture compared with the control of the same genotype. The resulting growth / herbicide concentration relationship was analysed by non-linear regression of the logistic equation : $Y_j = K_j/(1+((K_j/i_j)-1)*\exp(-r_j*D))$ (1)
where Y_j is the transmittance of genotype j ranging from 0 (no growth) to approximately 900 units (maximal growth). As also observed by Ma et al. (2001) and Bell (1991) Y_j within this range is linearly related to algal density. i, r and K are the conventional logistic parameters to be estimated while D is the herbicide concentration expressed as a reciprocal of the dilution ratio, d. The data were analysed using the non linear procedure with Gauss-Newton option in SYSTAT (1994). In acknowledgement of the fact that the herbicide concentration will probably change over time while "dose" refers to what has been introduced, we define our measure as "ED" : ED_{50} being for example the initial concentration of herbicide necessary to reach half the transmittance (cell density) of the control. ED_{50} is estimated from the values generated using equation (1) by their direct implementation in equation : $ED_{50}=D_{max}/d\text{^}[\log((K/i)-1)/r]$ (2)
where D_{max} is the highest concentration tested and d the serial dilution ratio. Wald confidence intervals 95% for ED_{50} were obtained directly from SYSTAT. Except for barban and oryzalin, the gradient also gave an upper estimate of the concentration of herbicide at which no growth was observed. However, this ED_{100} could not be considered as a lethal dose (LD_{100}) as, for example, atrazine delivered at a concentration of 5 µM/L fully inhibited growth, while an inoculum removed after 20 days grew normally in minimal medium. Thus, despite total growth inhibition, the algae were not dead, a similar observation to Cain & Cain (1983) on *C. moewusii*. As Bartlett's test rejected the homogeneity of variances on untransformed data (Bartlett = 230.16, F_{27df} =21.53 and $p<0.0001$), the ED_{50} were successfully log-transformed (Bartlett = 37.43 giving F_{27df} =1.17 and p=0.27) before performing ANOVA. The analysis of the relationship between the ED measures and the values of the physical properties of herbicides (Solubility, Molecular weight and K_{ow}) was performed on the full model $\log_{10}(ED) = a + b*Sol + c*Mol + d*K_{ow} + e*\log_{10}(Sol) + f*\log_{10}(Mol)$ selecting for the last regression using the forward stepwise procedure with "α to enter and remove of 0.15 and 0.05", respectively.

RESULTS AND DISCUSSION

The goodness of fit for the logistic equations over the herbicide concentration gradients, were generally excellent, with a mean R^2 = 0.970 +- se 0.011. All values fell in the range [0.937 , 1] with the single exception of 2,4 D with genotype CC-1010+ for which substantial error variance was maintained between replicates (R^2 = 0.443). For two herbicides, oryzalin and thiazafluron, the gradient did not encompass both extremes of normal and no growth, so we could not

Table 1. The ED_{50} calculated from the logistic equation with Mean, minimum and maximum 95% Wald confidence limits for 29 herbicides (mg/L).

Herbicide	Chemical Class	HRAC Code	Inhibition Target site	ED_{50}	& Wald 95% confidence
Chlorsulfuron	sulfonylureas	B	Acetolactate synthase	22.5	[19.3 -- 25.6]
Metsulfuron methyl				185	[182 – 189]
Triasulfuron				98.8	[88.5 -- 109.1]
Chloridazon	pyridazinone	C1	Photosynthesis PSII	18.3	[14.7 -- 22.0]
Atrazine	triazines			0.15	[0.14 -- 0.16]
Lenacil	uracils			0.05	[0.04 -- 0.06]
Diuron	ureas	C2		0.09	[0.08 -- 0.10]
Isoproturon				0.11	[0.11 -- 0.12]
Thiazafluron				(4.9)	[/ -- /]
Bentazone	Benzothia -diazone	C3		131	[118 – 144]
Acifluorfen	diphenylethers	E	P P Oxydase	0.56	[0.17 -- 0.95]
Norflurazon	pyridazinones	F1	Phytotene DS	0.73	[0.41 -- 1.06]
Aclonifen	diphenylethers	F3	Unknown	0.11	[0.09 -- 0.13]
Amitrole	triazole			963	[197 -- 1729]
Fluometuron	ureas	F3&C2	Unknown + PSII	2.01	[1.46 – 2.57]
Glyphosate	Glycines	G	EPSP synthase	375	[318 – 432]
Glufosinate ammonium	phosphonic acid	H	Glutamine synthase	5100	[4030 – 6170]
Oryzalin	dinitroanilines	K1	Microtubule Assembly	(2.5)	[/ -- /]
Pendimethalin				0.08	[0.07 -- 0.10]
Chlorpropham	carbamates	K2	Microtubule organisation.	0.46	[0.42 -- 0.50]
Barban	carbamates	K2 ?	Microt. org ?	10.5	[9.94 -- 11.02]
Fluthiamide	oxyacetamides	K3	Cell division	1.19	[1.12 -- 1.27]
Alachlor	Chloro -acetamides			2.78	[2.19 -- 3.38]
Dimethenamid				4.41	[3.65 -- 5.16]
Metazachlor				0.86	[0.80 -- 0.93]
Propachlor				8.13	[7.62 -- 8.63]
2,4 D	phenoxy-carb-oxylic acid	O	Synthetic auxins	133	[106 – 161]
Clopyralid	pyridine carb-oxylic acid			326	[311 – 340]
Tridiphane	Epoxide ?	?	GSTransferase ?	0.41	[0.25 -- 0.56]

(Classification is based on target of inhibition according to HRAC (Schmidt 97).
/ denotes that the gradient did not encompassed extremes to fit the logistic)

calculate the ED values in the manner described above, but approximations, not taken further into account in the calculations of regression coefficients, are given between brackets in Table 1.

The analysis of variance for herbicide or genotype effects of the $\log_{10}$ ED_{50} gives a high "herbicide effect" (F-ratio=138.937, df=26, $p<0.0001$) while no "genotype effect" is observed (F-ratio=0.059, df=1 and p=0.810), indicating that, on average, the two strains responded in similar ways to the different herbicides. With a single replicate, there were insufficient degrees of freedom to estimate the herbicide-genotype interaction. However, by examining more closely the overlap between the 95% Wald's confidence limits we observed an apparent increased tolerance of CC-1010+ over CC-2342- for aclonifen, chlorsulfuron, clopyralid, dimethenamid, fluthiamide, isoproturon, metazachlor and triasulfuron, while CC-2342- seemed a more tolerant line to alachlor, atrazine, chlorpropham, diuron, metsulfuron-methyl and pendimethalin. These changes in the pattern of tolerance according to genotype, seem to follow neither described cross-resistance patterns (Galloway & Mets 1984; Főrster et al. 1997) nor general detoxication pathways (Hatton et al 1996; Tommasini et al 1997).

The forward stepwise multiple regression for any physical property effects of herbicides on ED_{50} displayed the very simple relationship illustrated in Figure 1a

$$\log_{10}(ED_{50}) = a + e*\log_{10}(\text{Solubility}) \qquad (3)$$

with a=-1.222+- se 0.193, b=0.779+-se 0.065 and an overall adjusted R^2 of 0.737 ($p<0.0001$). This fit outperforms the relationship to K_{ow} given with many groups of chemicals in aquatic organisms by QSAR literature (Nendza 1991; Urrestarazu Ramos et al 1999) which, applied here, gives $\log_{10}(ED_{50}) = 1.506 - 0.534*K_{ow}$, R^2 = 0.619. The partition coefficient K_{ow} is definitely less correlated with toxicity than solubility and, consequently, is removed by the forward elimination procedure. The most striking feature of this regression is the simple pattern that emerges: more soluble herbicides require higher concentrations for toxicity. By forcing a slope of 1, this strong negative relationship between herbicide solubility and toxicity can be further simplified, yielding from equation (3) 'a' equals -1.777 +/- se 0.112 and corrected R^2 = 0.682. The ED_{50} for any herbicide therefore simply becomes statistically 10^a =1.7% of its maximum solubility in water.

To examine whether the negative relationship between herbicide solubility and toxicity evident in this study is generally reflected in the literature, a "meta analysis" was undertaken by examining similar data sets from three published studies. The log – log correlation between I_{50} and solubility for *C. reinhardtii* from Fedtke (1991) is not significant at the 5% level (N=19, adjusted R^2 = 0.124, p=0.077) but becomes highly significant removing only paraquat (N=18, adjusted R^2 = 0.387, p=0.003) giving $\log_{10}(I_{50}) = -1.339 + 0.459* \log_{10}(\text{Solubility})$ as shown in Figure 1b. With a R^2 = 0.113, K_{ow} is slightly outperformed by maximum solubility as a regressor to toxicity. Hess's (1980) data also examined I_{50} but this time using *C. eugametos*. The I_{50} – solubility negative relationship is very similar (Fig. 1c), and becomes highly significant after removing DCPA (for which the I_{50}

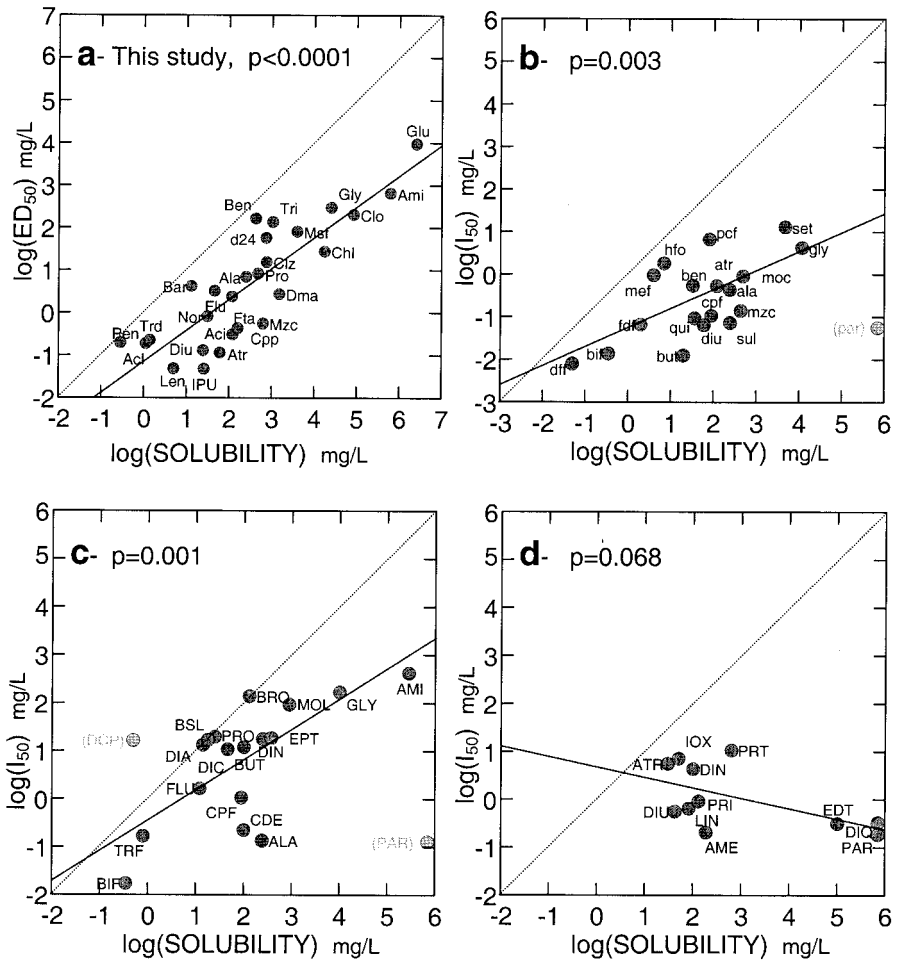

Figure 1. $\log_{10}$ - $\log_{10}$ relationship between herbicide maximum solubility in water and acute or medium term toxicity concentration (mg/L). Dashed line for toxicity effect at the maximum solubility. Graphs refer respectively to :

a- *C. reinhardtii* strain CC-2342. Labels are referring to Nick Name in Table 1.

b- *C. reinhardtii* from Fedtke (1991). Labels are: **mef**enacet, **ala**chlor, **but**achlor, **m**eta**z**achlor, **m**eto**l**achlor, **atr**azine, **diu**ron, **chlorprop**ham, **ben**sulfuron, **sul**fometuron, **bif**enox, **fluorodif**en, **diflu**fenican, **par**aquat, **p**enta**chloroph**enol, **qui**nonamid, **h**aloxy**fop**, **seth**oxydim, **gly**phosate.

c- *C. eugametos* from Hess (1980). Labels are: BIFenox, ALAchlor, TRiFluralin, PARaquat, CDEc, FLUridone, DINoseb, BenSuLide, DIAllate, DCPa, DIChlobenil, BROmoxynil, GLYphosate, AMItrol, ChlorProPHam, PROpham, BUTylate, EPTc, MOLinate.

d- *C. moewusii* from Cain & Cain (1983). Labels are: AMEtryne, ATRazine, DINoseb, DIQuat, DIUron, EnDoThall, IOXynil, LINuron, PARaquat and PromeTon and PropanIl.

value exceeds its actual maximum solubility) and again paraquat (N= 17, adjusted R^2 = 0.471, F-ratio= 15.241, p=0.001) giving $\log_{10}(I_{50})$ = -0.475 +0.637* $\log_{10}$(Solubility). Similarly, here again, the relationship to toxicity of K_{ow} (R^2 = 0.265) is outperformed by maximum solubility. In contrast, the data of Cain & Cain (1983) using *C. moewusii* showed a weakly significant correlation $\log_{10}(I_{50})$ = 0.678 -0.217* $\log_{10}$(Solubility), with adjusted R^2 = 0.248, p=0.068 (Figure 1d). This last result appears to be highly dependent on their choice of herbicides: the set of diquat, paraquat and endothall being highly toxic to algae as well as highly soluble. Nevertheless, the expectation that the toxicity of all the herbicides occurs at lower concentrations than their maximum solubilities is upheld. By applying our analysis to other studies we have illustrated that two molecules of similar solubility may still have different levels of toxicity, for example, glufosinate and paraquat. It must be emphasised that the negative relationship between candidate herbicide solubility and toxicity perhaps partially explains which herbicide candidates are ultimately commercialised. This negative relationship suggests that homologation rules would probably seldom allow highly soluble and toxic molecules to be registered as herbicides. Accordingly, within the triangular surface defined under the maximum solubility line, most herbicide molecules are positioned in the upper part of the triangle.

It is striking to observe that irrespective of the mode of action of the herbicides or any other chemical differences among them, maximum solubility in water remains a powerful predictor of their potential toxicity. The superior performance of this parameter over K_{ow} thus questions the simple side effect of membrane affinity usually proposed as the basis for this relationship (Collander & Bärlund 1933) and suggests the existence of another rule or bias.

To improve upon the QSAR approach which is mainly restricted to use within herbicide families, we investigated the relationship between the water solubility and the toxicity to *Chlamydomonas reinhardtii* across 19 different herbicide chemical families. The quality of the relationship obtained using standard dose-response for the 29 herbicides suggests that, in the absence of other information and despite different mechanisms of herbicide action, solubility in water, or to a lesser degree the octanol / water partition coefficient, provides an indication of herbicide potential toxicity. In conclusion, if this simple negative relationship between herbicide solubility and toxicity represents a general "rule", as partially confirmed by the analysis of similar data sets from other published studies, then solubility could be both the simplest and best predictor of ecotoxicological consequences of herbicides on non-target organisms.

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