Response-Surface Analyses for Toxicity to *Tetrahymena pyriformis*: Reactive Carbonyl-Containing Aliphatic Chemicals

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A response-plane has been developed with *Tetrahymena pyriformis* population growth impairment toxicity data [log 1/50% growth inhibitory concentration (IGC_{50})], the 1-octanol/water partition coefficient (log K_{ow}), and the energy of the lowest unoccupied molecular orbital (E_{lumo}). A statistically robust plane [log $1/IGC_{50}$ = 0.530 (log K_{ow}) − 0.890 (E_{lumo}) − 0.271, $n = 50$, $s = 0.295$, $r^2 = 0.855$, $F = 145$] was found for reactive carbonyl-containing aliphatic chemicals. These compounds had a variety of electrophilic mechanisms of action and included aldehydes acting as Schiff-base formers, α,β-unsaturated aldehydes and α,β-unsaturated ketones acting as Michael-type acceptors, and selected α-diones acting as selective binders to arganine residues; γ-diones acting as selective binders to tubulin; and β-diones with unknown mechanisms of action. Outliers to this model broadly fell into two groups: small reactive molecules (e.g., acrolein) that were more toxic than predicted and molecules in which the reactive center was sterically hindered by an alkyl group (e.g., 2,4-dimethyl-2,6-heptadienal) that were less toxic than predicted.

INTRODUCTION

Aquatic toxicologically based quantitative structure−activity relationships (QSARs) allow for the prediction of the toxic potency of untested chemicals.[1] The use of toxicity data for the effect of chemicals on the freshwater ciliate *Tetrahymena pyriformis* in the development of environmental QSARs is commonplace and includes results of recent investigations from the main author.[2−4] These studies indicate that it is possible to develop statistically significant modes, or mechanisms, of toxic action-based QSARs. However, such QSARs require the a priori identification of the correct mode or mechanism of toxic action from molecular structure.[5] This identification is often a difficult task.[6]

Compounds capable of eliciting their toxic response by a narcosis mode of action account for about 70% of the industrial organic compounds.[7] Chemicals acting by reactive mechanisms of action elicit toxicity in excess of that which would be expected from narcosis alone. In most cases, this is as a result of electrophilic reactions in vivo. Although a priori identification of chemicals that elicit excess toxicity is possible in some cases,[8,9] the specific molecular mechanism of these toxicants is often unclear. Therefore, the prediction of acute toxic potency without regard for toxic mechanism would be advantageous.

The assignment of exact mechanisms of toxic action for electrophilic toxicants a priori is complex. As an example of the task faced in the a priori identification of mechanism of action, the assignment of mechanism to aliphatic compounds containing carbonyl groups may seem trivial. The carbonyl moiety has a dipolar property, because oxygen is more electronegative than carbon and has a greater attraction for electrons than carbon. Furthermore, the π-bond of the carbonyl group is relatively polarizable, and the electron density in this double bond is displaced toward the oxygen atom. In other words, the carbonyl group contains an electron-deficient carbon atom. If unmodulated by the presence of other adjacent groups, carbonyls are relatively unreactive with regard to their toxicity. Thus, basic ketones are classic nonpolar narcotics.[3,10,11] However, when placed in the aldehyde configuration or in an α,β-configuration with another π-bond, the electrophilic reactivity of the carbonyl group and the toxicity of the compound are enhanced and the mechanism of toxic action altered.

One solution to the modeling of acute toxicity without regard to mechanism is the two-parameter QSAR, or response-surface approach. This was proposed initially by Mekenyan and Veith.[12] Efforts to explore further the response-surface approach to modeling ecotoxicological data have been restricted by the paucity of available and reliable toxicity data for chemicals acting by electrophilic mechanisms of toxicity.[5]

Recently, Cronin et al.[13] proposed a response-surface based on hydrophobicity and electrophilicity to describe the toxicity to *T. pyriformis* of multiply substituted mono- and dinitrobenzenes. A highly significant QSAR was developed for the toxicity of these aromatic compounds, without reference to the mechanism of action.

The aim of this study was to develop a *T. pyriformis* toxic response-surface that represented accurately the potency for aliphatic industrial organic compounds. The response-surface was sought based on readily available, mechanistically comprehendible, molecular-based parameters of hydrophobicity and electrophilicity. More specifically, the toxicities

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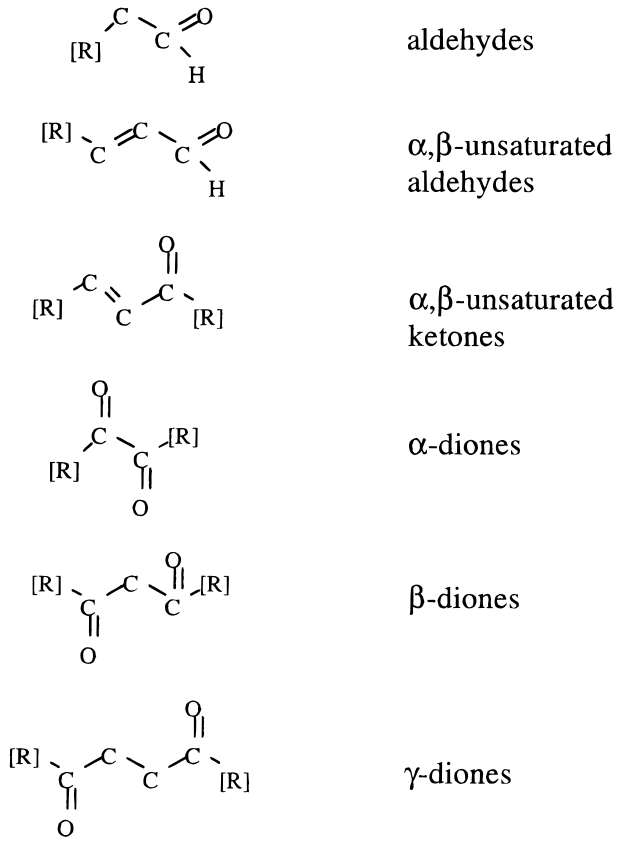

Figure 1. Aliphatic carbonyl structure considered in this study.

of six groups of chemicals, each containing a carbonyl group (i.e., carbon−oxygen double bond), were assessed in this study. These six groups were all considered to be capable of acting as electrophiles and included aldehydes, α,*β*-unsaturated aldehydes, α,*β*-unsaturated ketones, α-diones, *β*-diones, and *γ*-diones (see Figure 1). The aldehydes are formers of Schiff bases, whereas α,*β*-unsaturated aldehydes and α,*β*-unsaturated ketones are Michael-type acceptors.[9,14,15] The α-diones form specific covalent bonds to arginine residues.[16] The *γ*-diones bind specifically to tublin.[17] The mechanism of action of *β*-diones is currently unknown.

METHODS

Chemicals. The toxicity of 56 chemicals consisting of 18 aldehydes, 16 α,*β*-alkenals, 12 unsaturated alkanones, and 10 diones was assessed. The 10 diones included five α-diones, four *β*-diones, and a single *γ*-dione. The chemicals were obtained commercially (Aldrich Chemical Co., Milwaukee, WI; MTM Research Chemicals or Lancaster Synthesis Inc., Windham, NH) at sufficient purity (>95%) that made further purification unnecessary. The chemicals are listed in Table 1.

Tetrahymena Population Growth Impairment Testing. Ciliate (*T. pyriformis* strain GL-C) population growth impairment assessment was conducted as described by Schultz.[18] This static 40-h assay used population density quantitated spectrophotometrically with absorbence at 540 nM as the endpoint. Test conditions allowed for 8−9 cell cycles in controls. Following a range-finder exercise, each chemical was evaluated for three additional replicates. Controls that had no toxicant but had been inoculated with *T. pyriformis*, and blanks that had neither toxicant nor ciliates were used to provide a measure of the acceptability of the replicate and a basis for interpreting toxicant treatment data. Each replicate consisted of six to eight different concentrations of toxicant with duplicate flasks of each concentration. Only replicates with control absorbence values >0.6 but <0.75 were used in the analyses.

The 50% growth inhibitory concentration (IGC_{50}) was determined for each compound by Probit Analysis of Statistical Analysis System (SAS) software[19] with the dependent variable the absorbence normalized as percentage of control and independent variable the toxicant concentration in milligrams per liter.

Molecular Descriptors. The 1-octanol/water partition coefficient (log K_{ow}) values for each toxicant were secured as either by preference measured or, when required, calculated from the ClogP for Windows software (BIOBYTE Corp., Claremont, CA).

For the determination of the energy of the lowest unoccupied molecular orbital (E_{lumo}), each toxicant was constructed and its energy minimized in NEMESIS for PC software (Oxford Molecular Limited, Oxford, UK). The structure was then converted into a MOPAC internal file via the BABEL shareware file conversion program. Molecular orbital quantum chemical estimations were performed with use of the MOPAC6 program and the AM1 Hamiltonian.[20]

Development of the Response-Surface. Regression analysis with the SAS statistical package was used for QSARs development. Toxicity, in the form of the inverse of the millimolar concentration that produced the defined response, acted as the dependent variable. The descriptors of hydrophobicity and electrophilicity acted as the independent variables. Model adequacy was quantified with the r^2 value (coefficient of determination adjusted for degrees of freedom). The number of observations (n), root of the mean square for error (s), and the Fisher statistic (F) were noted also.

RESULTS

The toxicants evaluated, their potency, and molecular descriptor values are catalogued in Table 1. Toxicities ranged over nearly 3.5 orders of magnitude from 2,5-hexanedione (log $1/IGC_{50} = -1.40$) to 3-butyne-2-one (log $1/IGC_{50} = 1.97$). Attempts to model the toxicity of all compounds with hydrophobicity failed to produce a significant QSAR. Furthermore, an examination of Figure 2 shows that most of these compounds have toxicity in excess of that associated with nonpolar narcosis.

Response-plane analyses of toxic potency by the hydrophobic term log K_{ow} and the reactivity term E_{lumo} produced the following QSAR:

$$\log 1/IGC_{50} = 0.418\,(\log K_{ow}) - 0.864\,(E_{lumo}) - 0.005 \quad (1)$$

for which $n = 56$, $s = 0.591$, $r^2 = 0.489$, $F = 27.4$.

An examination of residual values for eq 1 (data not shown) revealed outliers fell into two groups. All outliers had residuals that were greater than two times the standard deviation. The outliers with the largest residuals were the small reactive molecules 2-propenal (acrolein), 3-buten-2-one, and 3-butyn-2-one. A further compound, 1-octene-3-one, was also found to be an outlier, this compound is unique to the data set because of the alkene double bond in the

Table 1. Toxicity, Hydrophobicity, and Electrophilicity of Selected Keto Derivatives

name	CAS number	log 1/IGC$_{50}$	log K_{ow}	E_{lumo}
2,3-butanedione	431-03-8	−0.23	−1.34m[a]	−0.5171
3-butyn-2-one	1423-60-5	1.97	−0.89	0.0921
2,3-pentanedione	600-14-6	−0.16	−0.84	−0.4860
3,4-hexanedione	4437-51-8	−0.01	−0.31	−0.4775
2,3-hexanedione	3848-24-6	−0.21	−0.31	−0.4820
2,5-hexanedione	110-13-4	−1.40	−0.27m	0.6633
2-propenal	107-02-8	1.41	−0.01m	−0.1389
3-buten-2-one	78-94-4	1.51	0.12	0.0503
2,3-heptanedione	96-04-8	0.04	0.22	−0.4805
2,4-pentanedione	123-54-6	−0.27	0.40m	0.4707
3-penten-2-one	625-33-2	0.54	0.52m	0.0542
2-butenal	123-73-9	0.70	0.52	−0.1411
propionaldehyde	123-38-6	−0.65	0.59m	0.8663
3,5-heptanedione	7424-54-6	−0.38	0.60	0.3805
isobutyraldehyde	78-84-2	−0.43	0.61	0.8970
butyraldehyde	123-72-8	−0.55	0.88m	0.8746
2-methyl-2-butenal	497-03-0	−0.14	0.83	−0.1144
3-methyl-2-butenal	107-86-8	0.09	0.92	−0.1612
5-hexen-2-one	109-49-9	−1.14	1.02m	0.8210
4-methyl-3-penten-2-one	141-79-7	−0.64	0.92	0.0585
2-pentenal	1576-87-0	0.66	1.05	−0.1179
2,4-octanedione	14090-87-0	0.13	1.13	0.4742
2-methylbuteraldehyde	96-17-3	−0.39	1.14	0.8686
2,4-hexadienal	142-83-6	0.75	1.15	−0.5950
isovaleraldehyde	590-86-3	−0.33	1.23	0.8762
5-methyl-5-hexen-2-one	3240-09-3	−0.87	1.29	0.9163
valeraldehyde	110-62-3	−0.13	1.36	0.8697
2-methyl-2-pentenal	623-36-9	−0.02	1.36	−0.0930
3-hepten-2-one	1119-44-4	0.70	1.57	0.0669
2-hexenal	6728-26-3	0.76	1.58	−0.1151
2,4-nonanedione	6175-23-1	0.51	1.65	0.4830
2-ethylbutyraldehyde	97-96-1	−0.05	1.67	0.9166
2-methylvaleraldehyde	123-15-9	−0.47	1.67	0.9009
2,4-heptadienal	4313-03-5	0.86	1.68	−0.5831
4-hexen-3-one	2497-21-4	0.93	1.04	0.0782
hexylaldehyde	66-25-1	−0.17	1.78m	0.8689
2-heptenal	18829-55-5	1.05	2.11	−0.1143
6-methyl-5-hepten-2-one	101-93-0	−0.45	1.82	0.8638
3-octen-2-one	1669-44-9	0.74	2.10	0.0692
1-octen-3-one	4312-99-6	1.91	2.10	0.0782
2,4-dimethyl-2,6-heptadienal		0.08	2.33	−0.1014
heptaldehyde	111-71-7	−0.00	2.42	0.8668
3-nonen-2-one	14309-57-0	0.98	2.63	0.0708
2-octenal	2548-87-0	1.20	2.64	−0.1145
2,6-nonadienal	557-48-2	1.34	2.68	−0.1256
2-ethylhexylaldehyde	123-05-7	0.16	2.73	0.9142
2,4-nonadienal	5910-87-2	1.22	2.74	−0.5816
octylaldehyde	124-13-0	0.45	2.95	0.8663
2-nonenal	18829-56-6	1.60	3.16	−0.1144
nonylaldehyde	124-19-6	0.81	3.48	0.8665
7-decenaldehyde	21661-97-2	0.95	3.52	0.8565
2-decenal	3913-81-3	1.85	3.69	−0.1151
decylaldehyde	112-31-2	1.28	4.01	0.8668
4-decenaldehyde	65405-70-1	1.21	4.05	0.8233
undecylaldehyde	112-44-7	1.60	4.54	0.8649
dodecylaldehyde	112-54-9	1.76	5.07	0.8656

[a] m indicates a measured value.

1-position. These four chemicals were more toxic than predicted by eq 1. The other outliers were 4-methyl-3-penten-2-one and 2,4-dimethyl-2,6-heptadienal. Both of these chemicals were less toxic than predicted by eq 1. Deletion of these six toxicants and subsequent reanalyzis resulted in the equation:

$$\log 1/\mathrm{IGC}_{50} = 0.530\ (\log K_{ow}) - 0.890\ (E_{lumo}) - 0.271 \quad (2)$$

for which $n = 50$, $s = 0.295$, $r^2 = 0.855$, $F = 145$. A comparison of r^2 and s values for eqs 1 and 2 indicated that, as expected with the deletion of the outliers, the QSAR became statistically more significant. Figure 3 shows a plot of toxicity predicted by eq 2 against the observed toxicity. Toxicity is clearly well predicted by eq 2.

DISCUSSION

Recent studies have made progress in predicting acute toxic potency from molecular structure.[21] Nonreceptor-mediated acute aquatic toxicity have been classified for these purposes into two cause-and-effect categories: reversible narcoses and irreversible electrophilic reactivity. Within each

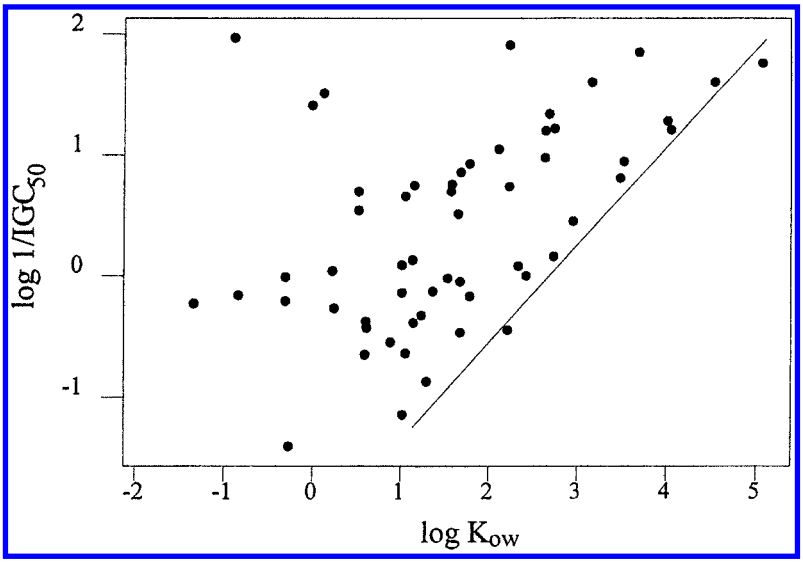

Figure 2. Plot of log $1/IGC_{50}$ (toxicity) versus log K_{ow} (hydrophobicity). The line represents the baseline nonpolar narcosis model.

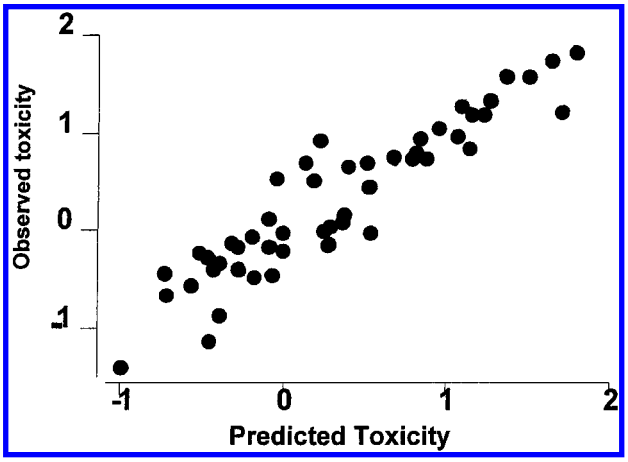

Figure 3. Plot of observed versus predicted (from eq 2) toxicity.

category, different modes or mechanisms can be defined. To simplify mode and mechanism assignment, rule-based systems have been developed.[21,22−25] These systems arrange toxicants into categories based on the presence or absence of selected structural features termed toxicophores. In other words, the a priori assignment of mode of toxic action is based on the presence or absence of specific structural features that correspond, theoretically, to that mode of action. Although this is an empirical approach to toxicity prediction, the assignment of mechanism of toxic action from chemical structure alone is not easy.[6]

To circumvent the problems of developing QSARs for specific mechanisms of action, it can be assumed that the expression of toxicity is a combination of penetration into or through biological membranes and the interaction of the toxicant with the site of action. McFarland[26] represented this principle mathematically by the following generic QSAR:

$$\log (\text{toxicity})^{-1} = A(\log \text{ of penetration}) + B(\log \text{ of interaction}) + C \quad (3)$$

Description of the toxic potency of compounds with a variety of mechanisms of action requires toxicity to be considered by the McFarland model. The penetration of the chemical to the site of action is via passive diffusion and may be modeled by hydrophobicity (log K_{ow}). The interaction of the xenobiotic with the biological systems is mainly via electrophilic reactions and can be described by E_{lumo} (see eq 2). The combination of the two terms in eq 2 is the basis of the surface-response approach to predicting toxicity.[12,13] This approach may allow for predictions to be made regardless of whether the individual mechanism of toxic action has been assigned or is even known.

Although comprehension of the precise biochemistry of toxic action may be lacking, progress is being made in the identification of the toxic mechanisms by which chemicals may act. Most industrial organic chemicals are thought to exhibit a narcosis mode of toxic action.[7] Narcosis is the general term that describes an organismal response to a chemically inert xenobiotic. The cellular membrane is the theoretical site of action. Narcotic effects are thought to result in noncovalent interactions, such as the disruption of van der Waals interactions between lipid and/or protein components, within the membrane.[27,28] The simplest and most fundamental example of such toxicity is nonreactive, nonpolar narcosis.[29] Although hydrophobicity does estimate a toxicant's ability to partition into the cellular membrane, this most basic type of toxicity is not completely understood at the molecular level.[30] A variety of compounds including basic ketones[3,10,11] are classified as nonpolar narcotics.

Chemicals exhibiting toxicity in excess of that associated with narcosis are considered bioreactive.[3] Such chemicals can have a stereoelectronic interaction with a biological system.[31] Most bioreactive toxicants act via electrophilic mechanisms.[14] Electrophilic reactivity can be considered to be the ability of a chemical to interact covalently with a biological system. It may involve substitution or conjugation of electron-rich groups to nucleophilic sites in cellular macromolecules (i.e., amino or nucleic acid polymers).

Electrophiles can cause toxicity via several molecular mechanisms of action.[9] In this study the toxicity of six, chemically closely related groups of compounds was assessed. Each group was postulated to be capable of electrophilic interactions with macromolecules in vivo. However, each group was considered to have its own distinct, and in most cases, identifiable electrophilic mechanism.

Aliphatic aldehydes are considered to elicit their toxic response by forming Schiff bases with amino groups, such

as the ϵ-amino of lysine, that may be present in biological membranes.[9,15]

The α,β-unsaturated alkenals and ketones are compounds with a carbon−carbon π-bond in the α-position to a carbonyl group. As such, they are considered able to undergo Michael-type addition[9,15,32] to nucleophilic macromolecular sites such as thio groups. The toxicity resulting from such Michael-type addition has been modeled previously by the use of hydrophobicity-based QSARs.[3,4]

Aliphatic compounds with carbonyl groups (diones) are capable of undergoing a variety of electrophilic reactions. As previously noted, the α-diones bind selectively to guanidino-containing compounds, e.g., arginine.[16,33] On the other hand, γ-diones act as selective binders to tublin.[17] Specifically, 2,5-hexanedione undergoes initially an irreversible condensation with a primary amine, such as a protein lysyl ϵ-amine. The result of this condensation is a heterocyclic aromatic pyrrole. Because pyrroles are unstable, they react further via oxidative cross-linking. In ciliated systems, the target protein is tubulin.[17] The mechanism of action of β-diones is unknown. However, because both α-diones and γ-diones are toxic by an electrophilic mechanism, it was assumed that β-diones also act by an electrophilic mechanism.

The response-surface developed in this study (eq 2) predicts well the toxicity to *T. pyriformis* across these varied mechanisms of action. Similar to the McFarland model (eq 3), it is based on descriptors to quantify hydrophobicity and electrophilicity. As such, it describes a 'domain' for aliphatic compounds with the 'global' response-surface for *T. pyriformis* toxicity. The difference in the 'domain' described by eq 3 and the domain of the global response-surface described for aromatic electrophiles[13] is characterized by differences in the regression coefficients and slopes.

A consideration of outliers, or compounds deleted from the response-surface, is important to these analyses. The outliers to eq 1 included 2-propenal, 3-buten-2-one, and 3-butyn-3-one. The rationale for the excess toxicity exhibited by these compounds may reside in their molecular size. Smaller reactive molecules are inherently more reactive and thus are relatively more toxic than larger molecules in the same congeneric group. 2-Propenal has been often cited as an outlier to QSAR studies.[4,8,34] Although 1-octen-2-one cannot be considered to be a small reactive compound, it is likely that the toxicity is greater than predicted because this compound is unique to the data set with the alkene double bond in the 1-position. The other outliers to eq 1 were 4-methyl-3-penten-2-one and 2,4-dimethyl-2,6-heptadienal. These two chemicals were less toxic than predicted by eq 2. The substitution of the methyl group α to the carbon−carbon double bond of 4-methyl-3-penten-2-one and 2,4-dimethyl-2,6-heptadienal probably sterically hinders covalent bonding to the activated site.

In the past, the study of electro(nucleo)philic reactivity has been impeded by the lack of both experimental toxicity data and molecular-based descriptors. Historically, QSAR approaches for electrophiles used the Hammett substituent constant σ.[35] More recently, molecular orbital descriptors have been used. Namely the molecule-based descriptor E_{lumo}[31,36] and the atom-centered maximum acceptor superdelocalizability A_{max}.[9] E_{lumo} is a global property that describes the gross electrophilicity of a compound. Although not conclusively demonstrated, E_{lumo} seems to be capable of being applied within different domains of the response-surface (i.e., it is applicable for the response-surface for nitroaromatic compounds[13] and aliphatic compounds proposed herein). Conversely, σ and A_{max} are specific to individual substituents and atoms, respectively. Therefore, they cannot be applied outside of a limited series of related compounds. Because of this greater global applicability, the molecular-based term E_{lumo} is the preferred parameter.

To summarize, the toxicity of six groups of reactive compounds with a carbonyl carbon−oxygen double bond in different molecular environments has been tested with *T. pyriformis*. Response-surface analyses of toxicity using hydrophobic and electrophilic descriptors resulted in a quality QSAR independent of the toxic mechanism.

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