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RESEARCH ARTICLE

Assessment of phenolic herbicide toxicity and mode of action by different assays

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Abstract A phytotoxicity assay based on seed germination/root elongation has been optimized and used to evaluate the toxic effects of some phenolic herbicides. The method has been improved by investigating the influence of experimental conditions. *Lepidium sativum* was chosen as the most suitable species, showing high germinability, good repeatability of root length measurements, and low sensitivity to seed pretreatment. DMSO was the most appropriate solvent carrier for less water-soluble compounds. Three dinitrophenols and three hydroxybenzonitriles were tested: dinoterb, DNOC, 2,4-dinitrophenol, chloroxynil, bromoxynil, and ioxynil. Toxicity was also determined using the *Vibrio fischeri* Microtox® test, and a highly significant correlation was found between EC50 values obtained by the two assays. Dinoterb was the most toxic compound. The toxicity of hydroxybenzonitriles followed the order: ioxynil >bromoxynil >chloroxynil; *L. sativum* exhibited a slightly higher sensitivity than *V. fischeri* to these compounds. A QSAR analysis highlighted the importance of hydrophobic, electronic, and hydrogen-bonding interactions, in accordance with a mechanism of toxic action based on protonophoric uncoupling of oxidative phosphorylation. The results suggest that the seed germination/root elongation assay with *L. sativum* is a valid tool for the assessment of xenobiotic toxicity and can be recommended as part of a test battery.

Keywords Phytotoxicity · Seed germination · Root elongation · Phenolic herbicides · Microtox · QSAR

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Introduction

The knowledge of the toxic effects of pollutants is a key requirement for environmental risk assessment. A fundamental instrument in this field is given by ecotoxicological tests, which use organisms belonging to different trophic levels and involve different endpoints, targets, and modes of action. These bioassays can be used either individually or, preferably, as components of a test battery; this is especially recommended when evaluating the toxic effects of unknown substances, mixtures of chemicals, and samples of complex composition, such as wastes, effluents, and contaminated environmental matrices.

The most widespread ecotoxicological tests use bacteria and aquatic organisms, such as algae, fishes, and invertebrates, particularly crustaceans; for these assays, it is possible to find extensive collections of data for a large number of chemicals. Conversely, ecotoxicity testing with terrestrial organisms is much less common, data are not easily available, and knowledge on target sites and mode of action is still far from complete.

Because of the essential role played by plants in ecosystems and of the widespread use and environmental occurrence of chemicals with herbicidal action, the inclusion of phytotoxicity assays in test batteries could greatly improve their effectiveness and significance (Wang 1991). The relevance of phytotoxicity

testing is also substantiated by its application in environmental assessment and is recognized by the environmental legislation in many countries and by regulatory authorities, which have developed specific guidelines for test conduction (OECD 1984, 2006; US FDA 1987; USEPA 1996). However, both in the aquatic and the terrestrial environment, ecotoxicity tests with higher plants are not widely used, despite their crucial and recognized importance in environmental biomonitoring. Though phytotoxicity tests possess several advantages, their main limitation lies just in the scarcity of literature data, which may hamper a thorough comparative evaluation of methods, protocols, and results (Wang and Freemark 1995; Gong et al. 2001; Wang et al. 2001; Moore and Kröger 2010).

However, in the last years, the interest on phytotoxicity tests has been growing, especially those based on seed germination and/or root elongation of higher plants, as proved by the number of recent studies in which they were applied to environmental samples of varying origin and composition, such as soils, sediments, sludges, wastewater, compost (Beltrami et al. 1999; Gong et al. 2001; Barbero et al. 2001; Banks and Schultz 2005; Valerio et al. 2007; Devesa-Rey et al. 2008; Tiquia 2010; Rodriguez-Ruiz et al. 2014), and to various contaminants of concern, such as nanoparticles, metals, drugs, pesticides, and others (Lin and Xing 2007; Di Salvatore et al. 2008; Jin et al. 2009; Ma et al. 2010; Moore and Kröger 2010; Hillis et al. 2011; Manzo et al. 2011).

These assays are among the most advantageous for these purposes. They are simple and inexpensive, require minimal laboratory equipment, seeds can be stored for rather long periods with minimal costs, and tests can be activated quickly. Different methods and protocols are found in the literature, using different seed species, exposure time, and toxicity endpoint, with root elongation showing in general a higher sensitivity than seed germination. Zucconi et al. (1985) first proposed the use of a germination index, which combines both endpoints and allows thus to take into account the effects of toxicants both on the early phase of seed germination and on initial seedling growth; since then, many authors adopted this approach.

In this work, a seed germination/root elongation assay procedure has been optimized by comparing three seed species, chosen among the most used and recommended ones (*Lepidium sativum, Lactuca sativa,* and *Cucumis sativus*) and by evaluating the effects of experimental conditions, such as seed pre-treatment and the use of organic solvent carriers for less water soluble compounds. It is actually recognized that the results of phytotoxicity assays are often affected by a rather high variability, which could be reduced through a rigorous design and a strict control of testing parameters.

The assay was then applied to determine the toxicity of different phenolic herbicides and to investigate their mechanism of action; the names and the molecular structures of the tested compounds are reported in Table 1.

The dinitrophenols 2,4-DNP (2,4-dinitrophenol), DNOC (4,6-dinitro-*o*-cresol), and dinoterb (2-*tert*-butyl-4,6-dinitrophenol) are among the first organic chemicals patented for selective weed control and, thanks to their broad spectrum of activity, were widely used in the past as dye intermediates, wood preservatives, herbicides, insecticides, and sliming drugs (Kearney and Kaufman 1975; Gasiewicz 1991). They are typical contact herbicides, but their use in agriculture is now restricted or banned in many countries, mostly because of their persistence and of their high toxicity towards non-target organisms; however, their presence is still detected in the environment (Fobbe et al. 2006).

The benzonitriles ioxynil (3,5-diiodo,4-hydroxybenzonitrile), bromoxynil (3,5-dibromo,4-hydroxybenzonitrile), and chloroxynil (3,5-dichloro,4-hydroxybenzonitrile) are also long-known compounds with herbicidal activity (Wain 1963; Carpenter and Heywood 1963); bromoxymil and ioxynil are currently used worldwide as selective post-emergence agents to control broadleaf weeds in cereal crops (Gasiewicz 1991; Fobbe et al. 2006). However, while plenty of studies on the ecotoxicological behavior of dinitrophenols can be found in the literature, information about benzonitriles is not so well documented, and few toxicity data are available.

With the purpose of a further validation of the seed germination/root elongation assay, toxicity was measured also by the *Vibrio fischeri* Microtox® test, a well-established bioassay based on bacterial bioluminescence inhibition; in addition, the acquired data were compared with those obtained in a previous study by a short-term in vitro assay based on mitochondrial respiratory functions (Argese et al. 2005) and with literature data for fish acute toxicity from standard tests.

Finally, quantitative structure-activity relationships (QSARs) were developed and discussed, with the aims of investigating the mechanisms of action of the examined compounds and highlighting the structural properties responsible for their toxic effects in the different testing systems.

Materials and methods

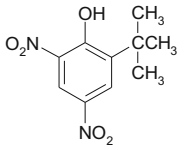
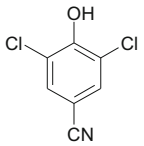
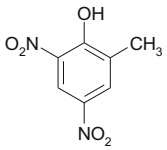
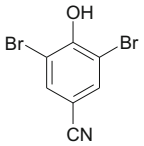
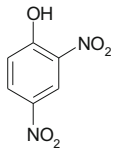
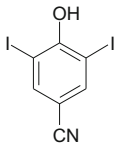
Materials

The examined herbicides were of Pestanal® grade from Riedel-de-Haën (Milan, Italy). Ethanol and dimethyl sulfoxide (DMSO) were of analytical grade from Sigma-Aldrich (Milan, Italy). Deionized water was prepared using a Milli-Q® system (Millipore, Bedford, MA).

Seeds of *L. sativum, L. sativa* and *C. sativus* were supplied by F.lli Ingegnoli (Milan, Italy) in single, closed bags.

The microrganism *V. fischeri* was supplied as lyophilized cells by Azur Environmental (Carlsbad, CA, USA), together with all materials and reagents required for conducting the

Table 1 Names and structural formulas of the examined compounds

Name	Formula	Name	Formula
dinoterb 2-*tert*-butyl-4,6-dinitrophenol		chloroxynil 3,5–dichloro, 4-hydroxybenzonitrile	
DNOC 2-methyl-4,6-dinitrophenol		bromoxynil 3,5-dibromo, 4-hydroxybenzonitrile	
DNP 2,4-dinitrophenol		ioxynil 3,5–diiodo, 4-hydroxybenzonitrile	

Microtox® assay, including reagent, reconstituent solution and diluent (2 % sterile NaCl solution).

Seed germination and root elongation assay

The tests were conducted using glass petri dishes (100-mm diameter) and Whatmann N° 1 filter paper. Ten undamaged seeds of similar size were placed uniformly on the surface of the filter paper, at the bottom of each dish, which contained 5 ml of test solution (or deionized water for the control). Dishes were then closed and incubated in the dark at 25 ±0.5 °C. Four replicates were carried out for each concentration of test solution, as well as for the control. After 72 hours of incubation, the germinated seeds were counted, and the root length was measured on all seedlings. Seeds were considered germinated when the radicle was longer than 1 mm.

The two endpoints were combined into a germination index (GI), calculated according to the following equation:

$$GI = 100 \cdot \frac{Gs \cdot Ls}{Gc \cdot Lc}$$

where Gs and Ls are seed germination and root elongation (mm) values for the test solution, whereas Gc and Lc are the corresponding values for the control (Zucconi et al. 1985; Barbero et al. 2001). For each compound, toxicity was expressed as EC50 value, i.e., the concentration that reduces the germination index by 50 % with respect to the control. EC50 values with their 95 % confidence limits were calculated by probit analysis.

The effect of seed pretreatment with sodium hypochlorite (NaClO) was evaluated using two solutions with different concentrations (0.5 and 4 %, respectively). Seeds were soaked for 20 min in the solutions and then carefully rinsed with deionized water; after this treatment, they were allowed to germinate using deionized water as growth medium, under the conditions described above. Germination and root elongation were then compared to those of non-pretreated seeds.

DNOC was dissolved in deionized water, whereas the concentrated stock solutions of the other compounds, which had a lower water solubility, were prepared using an organic solvent. Dimethyl sulfoxide (DMSO) and ethanol were tested, and DMSO was chosen as the most suitable solvent. Dilute test solutions were prepared using deionized water, and final concentrations of DMSO were always below 0.5 %.

Repeatability experiments using Cr(VI) and 2,4-dinitrophenol as reference toxicants were performed by carrying out five replicates over a 6-month period.

Microtox® assay

The tests were carried out according to standard protocols supplied by Azur environmental (Azur environmental 1998). DNOC was dissolved in the supplied 2 % NaCl aqueous solution, while the other compounds were dissolved in 2 % NaCl with 0.4 % (*v*/*v*) ethanol. This concentration is below the maximum allowable concentration (MAC) of 1 % (*v*/*v*), reported to exhibit no significant effects (Salizzato et al. 1998).

Bacterial suspension light outputs were detected by a Microtox® analyzer (model M500) after 5 and 15 min of exposure. The toxicity endpoint (EC50), which represents the concentration of the test compound that caused a 50 % reduction in light output, was calculated using the instrument software.

Quantitative structure-activity relationships (QSAR)

Toxicity values for the various assays were expressed as the logarithm of the inverse molar concentration. The hydrophobicity of the tested compounds is described by the octanol/water partition coefficient (log K_{ow}), whereas the acid dissociation constant (pK_a), the dipole moment (μ), and the Hammet sigma (Σσ) are used as descriptors of the electronic effects. Σσ was calculated as the sum of Hammett σ constants of the individual substituents present on the phenolic ring (values taken from Hansch et al. 1991); *para* substituent constants were used also for *ortho* substituents. The molecular volume (V_{mc}) is used to evaluate the influence of the steric properties of the compounds. E_{HOMO} (the energy of the highest occupied molecular orbital) is used as a descriptor for hydrogen bonding acceptor capacity, which increases with increasing E_{HOMO}. The hydrogen-bonding donor capacity is described by E_{LUMO} (the energy of the lowest unoccupied molecular orbital) and increases with decreasing E_{LUMO} (Urrestarazu Ramos et al. 1998).

E_{HOMO} and E_{LUMO} values were calculated using the Spartan program package (version 4.0. Wave Function, Irvine, CA, USA) running on an Indy workstation. Geometry optimization was performed using the semiempirical PM3 procedure, and energies and partial charges (Mulliken) were recalculated at the ab initio 6–31 G level. V_{mc}, and μ values were calculated using the HyperChem program (version 6.01, Hypercube, Gainesville, FL, USA).

Linear regression analysis was used to produce quantitative structure-activity relationships, by using the Statistica software (version 6.0, StatSoft Inc., Tulsa, OK, USA). The quality of the models was estimated from the coefficient of determination (R^2), the standard error of estimate (s), the ANOVA ratio for regression (F), and the *p* value (*p*).

Results and discussion

Optimization and evaluation of the phytotoxicity testing protocol

The effect of surface seed sterilization with sodium hypochlorite and the response variability of the three seed species are illustrated in Fig. 1. As can be seen, seed pretreatment increased the germination rate only in *L. sativa*, while root growth was negatively affected in all the tested species. This suggests that seed sterilization, often carried out routinely, should be carefully evaluated and avoided if not favorable.

Based on the relative response of the three plants, *L. sativum* was the most suitable test species, showing a maximum germination rate, an optimal root growth, and a lower variability. In addition, the repeatability of germination rate and root length measurements, assessed in a control test (five repetitions, with four replicated dishes containing ten seeds each, no seed pretreatment), was very satisfactory: germination rate was always 100 %, and the average root length was 43.6 ± 2.3 mm, with a low variability within each repetition (coefficient of variation between 4 and 12 %).

The effect of organic solvent carriers was also evaluated, since information about maximum allowed concentrations in phytotoxicity testing is scarce. The EPA and OECD guidelines do not suggest any preferred solvent carrier, but checking for their effect is recommended (OECD 1984; USEPA 1996). Two solvents commonly used as delivery vehicles in toxicity assays, ethanol and dimethyl sulfoxide (DMSO), were therefore tested at four final concentrations: 0.01, 0.1, 1, and 2 % *v/v*. Ethanol caused a reduction of the germination index even at the lowest concentration, yielding an EC50 value of 0.45 % *v/v*. Conversely, DMSO showed a slight negative effect only at 2 % *v/v* (GI = 82 % of the control). These results are comparable to the few available data on plants. Ethanol at a 117-mM concentration (corresponding to about 0.68 % *v/v*), was shown to reduce the germination of *L. sativa* seeds by 50 % of the control (Reynolds 1977), while root length growth in the *Allium* test was found to be 33.8 % of the control at 2 % *v/v* ethanol (Fiskesjö 1985). On the other hand, root growth of germinated seedlings of *L. sativa*, exposed to 1 % *v/v* DMSO, was not significantly affected (Sawhney and Srivastava 1977). Unfortunately, recent phytotoxicity studies mostly lack an evaluation on the effects of organic solvent carriers, which are simply included in the controls. Our results are also in accordance with both acute and chronic toxicity to aquatic organisms (Hutchinson et al. 2006). Specifically, in a 96-h growth bioassay with green algae, DMSO in the range 0.05–1 % *v/v* showed no significant effects, while ethanol at the same concentrations caused a significant growth inhibition (El Jay 1996); additional evidence of the lower toxicity of DMSO is reported for nine species of microalgae (Okumura et al. 2001). DMSO was therefore selected for phytotoxicity testing and was always employed at a final concentration below 0.5 % (*v/v*).

The repeatability and stability of the *L. sativum* assay was then assessed over a 6-month period, using 2,4-DNP—one of the phenolic compounds examined in this study—and Cr (VI), recommended as a reference toxicant (Wang 1987a; Beltrami et al. 1999). As shown by the results reported in Table 2, the variability of EC50 measurements undoubtedly satisfies acceptability criteria in ecotoxicity testing.

A comparison with literature data (Table 3) shows that the average EC50 value for Cr (VI) is of the same order of magnitude of those found in similar conditions for *L. sativum* and for other species, such as *L. sativa* and *Panicum miliaceum*. Conversely, *C. sativus* and *Sinapis alba* seem to exhibit a lower sensitivity. No data were found on 2,4-DNP phytotoxicity to *L. sativum*; anyhow, the EC50 value determined in this study is comparable to most data for commonly used species,

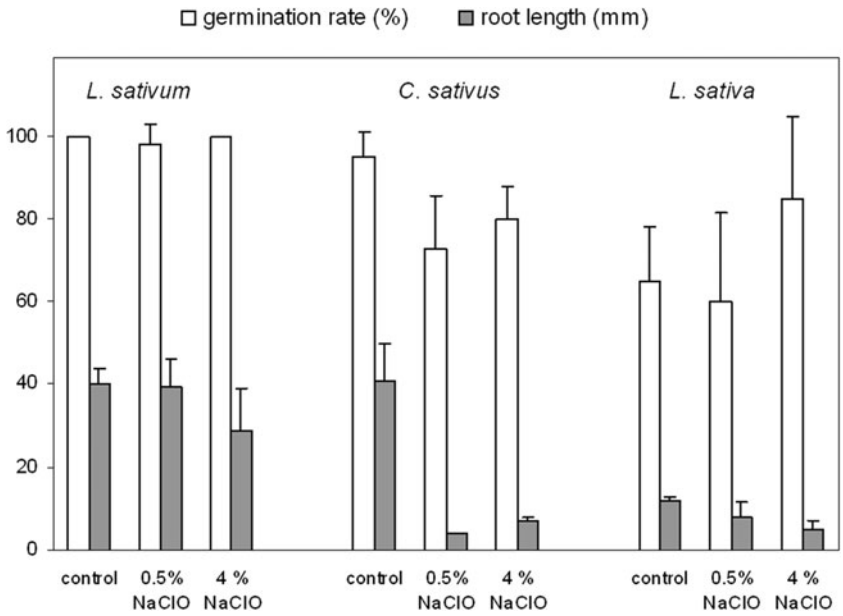


Fig 1 Effect of seed pretreatment with sodium hypochlorite on germination and root elongation in *Lepidium sativum, Cucumis sativus,* and *Lactuca sativa.* Results are from four replicated dishes, each containing ten seeds. Deionized water was used as growth medium. *Error bars* represent the coefficients of variation (CV)

including *C. sativus*, especially for root elongation inhibition, which is in general the most sensitive endpoint. Only *Glycine max*, in a seed germination/root elongation assay, showed a significantly lower sensitivity.

Phenolic herbicide toxicity

After the optimization of experimental conditions and the evaluation of test performance, the seed germination/root elongation assay with *L. sativum* was used to determine the phytotoxic effects of the six phenolic herbicides shown in Table 1. Table 4 reports the resultant EC50 values, together with those obtained with the Microtox® assay.

Dinoterb was found to be the most toxic compound with both assays, which yielded very similar EC50 values. In general, the *L. sativum* assay exhibited a slightly higher sensitivity, especially for hydroxybenzonitriles; the phytotoxicity of these structurally related chemicals varied with the type of halogen substituent, decreasing in the order I >Br >Cl. The phytotoxicity of dinitrophenols followed the order dinoterb >DNOC >2,4-DNP, showing that toxic effects are increased by the presence of an alkyl *ortho* substituent on the benzene ring; moreover, the larger the alkyl group, the higher the toxicity. The Microtox® assay showed the same trend.

Table 2 Repeatability of the *Lepidium sativum* assay, with Cr (VI) and 2,4-DNP as reference toxicants

Test	Cr (VI) EC50 (95 % C.I.) *mg/L*	2,4-DNP EC50 (95 % C.I.) *mg/L*
1	9.7 (7.1–12.4)	4.15 (3.92–4.36)
2	11.5 (9.8–13.5)	3.71 (3.02–4.42)
3	9.5 (7.0–12.9)	3.77 (3.12–4.43)
4	8.2 (5.9–10.5)	4.55 (3.85–5.29)
5	10.2 (8.1–12.3)	4.99 (4.31–5.73)
Mean ± SD	9.8 ± 1.2	4.23 ± 0.54
CV	12 %	13 %

The results of the two assays were compared quantitatively by means of linear regression analysis; a further comparison was made using literature data on fish acute toxicity (96-h LC50 for the fathead minnow *Pimephales promelas*, retrieved from the US EPA ECOTOX database, http://www.epa.gov/ecotox) and EC50 values determined by a mitochondrial in vitro assay (SMP assay) in a previous study (Argese et al. 2005). Unfortunately, other consistent datasets for the whole series of compounds were not found, both for terrestrial and aquatic organisms.

Given the high degree of correlation between Microtox® EC50 values at 5-min and 15-min ($R^2 = 0.986$, $R^2_{adj} = 0.983$, $s = 0.076$, $F = 289$, $p < 0.00007$), with slope and intercept of the regression line close to 1 and 0, respectively, the following analyses included only 15-min EC50 values. Figure 2 a–d illustrates the results of regression analysis. A good correlation exists between *L. sativum* and Microtox® EC50 values ($R^2 = 0.918$, $R^2_{adj} = 0.898$, $s = 0.189$, $F = 44.84$, $p < 0.003$); only DNOC is slightly displaced from the regression line (Fig. 2a). On the other hand, the correlation between *L. sativum* EC50s and fish LC50s is weaker, but still significant ($R^2 = 0.668$, $R^2_{adj} = 0.584$, $s = 0.718$, $F = 8.03$, $p < 0.05$). As can be seen in Fig. 2b, fish LC50 values span over a wider range (more than three orders of magnitude), with *L. sativum* showing a higher sensitivity to hydroxybenzonitriles and 2,4-DNP than *P. promelas*. The Microtox® assay (Fig. 2d) displayed a more robust correlation with fish acute toxicity ($R^2 = 0.872$, $R^2_{adj} = 0.840$, $s = 0.445$, $F = 27.2$, $p < 0.006$), with

Table 3 Literature data on phytotoxicity of CrVI and 2,4-DNP, determined by seed germination and root elongation assays

Toxicant	Test species	Concentration (95 % C.I.) (mg/L)	Endpoint	Reference
CrVI	*Cucumis sativus*	43.6 (38.7–50.0)	EC50 seed germination/root elongation 72 h	Beltrami et al. (1999)
	Lactuca sativa	7.5 (4.0–12.8)		
	Lepidium sativum	4.4 (3.1–5.8)		
	Cucumis sativus	44 (36–60)	IC50 root elongation 120 h	Wang (1987b)
	Lactuca sativa	3.7 (1.9–5.2)		
	Panicum miliaceum	17 (7.8–36)		
	Lepidium sativum	1.8 (1.5–1.9)	EC50 root elongation 48 h	Montvydienė and Marčiulionienė (2004)
	Sinapis alba	100 (89.76–103.85)	EC50 seed germination 72 h	Fargašová (1994)
	Sinapis alba	45.71 (38.65–50.46)	EC50 root elongation 72 h	Fargašová (1994)
2,4-DNP	*Glycine max*	143 (n.r.)[a]	IC50 seed germination/root elongation 96 h	Gramatica et al. (2002)
	Cucumis sativus	10.7 (n.r.)	RC50 root elongation 48 h	Wang et al. (2000)
	Cucumis sativus	100.6 (n.r.)	GC50 seed germination 48 h	Wang et al. (2002a)
	Brassica spp.	17 (n.r.) [b]	EC50 root elongation 120 h	Siddiqui et al. (2011)
	Cucumis spp.	11 (n.r.) [b]		
	Panicum spp.	3.7 (n.r.) [b]		
	Phaseolus spp.	15 (n.r.) [b]		

n.r. not reported

[a] calculated from log(1/IC50) = 0.11 (concentration values in mmol/L)

[b] calculated from EC50 values in mol/L

lower differences in sensitivity between the two assays, except for dinoterb. No significant correlations were found between the SMP assay and both *L. sativum* (Fig. 2c) and *V. fisheri* (results not shown), but the SMP assay exhibited a higher sensitivity for all compounds.

These results are consistent with the current knowledge about the examined compounds. While dinitrophenols are well known as potent protonophoric uncouplers of oxidative and photosynthetic phosphorylation, the mode of action of the hydroxybenzonitrile halogeno-derivates appears more complex; these compounds are also reported to be uncouplers, but they exert most of their herbicidal action through the inhibition of electron transport in photosystem II (Fedtke and Duke 2005; Fobbe et al. 2006; Casida 2009). However, photosynthetic pathways are not involved in seed germination and early seedling growth, which are heterotrophic processes that rely on respiratory metabolism. Thus, both dinitrophenols and hydroxybenzonitriles are supposed to exert their toxic effects by uncoupling ATP production from electron transport in seed mitochondria. This in turn can affect a number of ATP-requiring processes that are fundamental for germination and radicle growth, resulting in a reduction of GI in the phytotoxicity assay used in this study.

An uncoupling action can account also for the inhibition of bioluminescence in the Microtox® assay, since light production in *V. fischeri* requires ATP and is tied directly to cell respiration. This can explain the high degree of correlation between the two assays.

On the other hand, the lack of correlation with the SMP assay is most likely due to the different structural properties of

Table 4 EC50 values obtained by the *Lepidium sativum* and the Microtox® assays (95 % confidence intervals are given in parentheses)

	L. sativum EC50		Microtox® 5-min EC50		Microtox® 15-min EC50	
	mg/L	μM	mg/L	μM	mg/L	μM
2,4-DNP	4.15 (3.92–4.36)	22.5	14.3 (13.1–15.6)	77.7	13.8 (12.3–15.6)	75.0
2-DNOC	3.88 (3.59–4.18)	19.6	4.17 (3.94–4.42)	21.0	5.67 (5.16–6.22)	28.6
Dinoterb	0.384 (0.283–0.485)	1.60	0.407 (0.365–0.453)	1.69	0.371 (0.355–0.440)	1.54
chloroxynil	2.87 (2.28–3.05)	15.3	8.58 (7.51–10.9)	45.6	7.85 (5.72–10.6)	41.8
bromoxynil	2.41 (2.24–3.01)	8.70	7.69 (6.10–8.53)	27.8	7.30 (6.69–8.51)	26.4
ioxynil	1.85 (1.56–2.36)	4.99	4.26 (4.16–4.36)	11.5	4.82 (4.41–5.32)	13.0

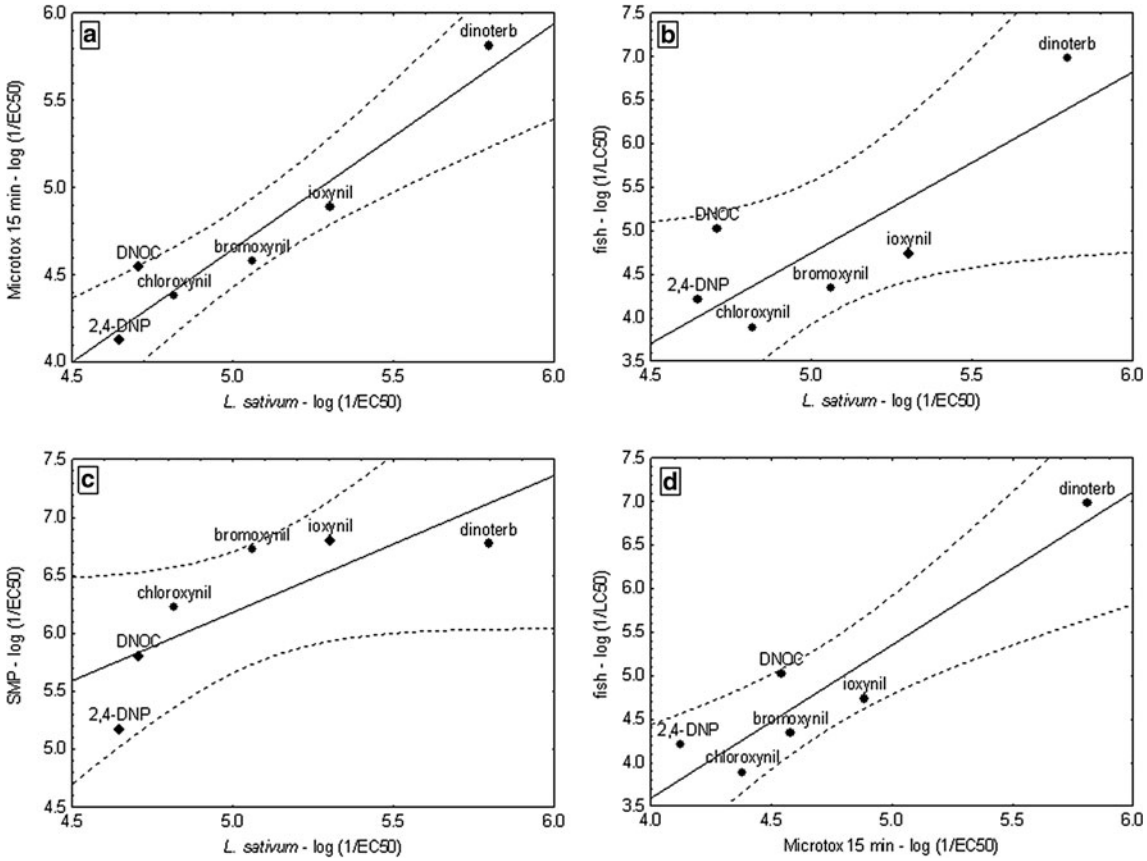


Fig. 2 Results of linear regression analysis between toxicity data. The *dotted lines* represent 95 % confidence limits

the three testing systems. SMPs are artificial structures, obtained by disruption of mammalian mitochondria. They are inside-out vesicles of the inner mitochondrial membrane, where the proteins involved in electron transport and ATP synthesis are directly exposed to toxicants in the assay medium (Argese et al. 2005); this can explain the higher sensitivity shown by the SMP assay. In seeds and bacteria, conversely, toxic molecules have to cross cell wall and membranes before they can reach their targets (the inner mitochondrial membrane in seeds and the plasma membrane in *V. fischeri*), and more complex interactions are necessarily involved in xenobiotic passage across these barriers.

The weak correlation with the fish acute toxicity assay can be ascribed as well to the number of processes that may occur during the 96-h exposure in vivo, such as toxicant absorption, biotransformation, and excretion, which could follow different pathways or have different rates for the two subgroups of phenolic herbicides; this can cause the apparent separation observed in the graphs of Fig. 2b and d, and explain the lower sensitivity of fish to hydroxybenzonitriles, with respect to dinitrophenols.

Quantitative structure-activity relationships

Phytotoxicity assays have proved suitable to investigate the mechanism of toxic action of various organic pollutants by means of quantitative structure-activity relationships, but the number of related studies is very limited compared to those on aquatic toxicity (Hulzebos et al. 1991; Feng et al. 1996; Wang et al. 2000, 2002a, 2002b, 2002c; Gramatica et al. 2002).

A QSAR analysis was performed in order to provide information about the relationships between toxicity and physicochemical properties of the studied phenolic compounds and to highlight the biochemical processes involved.

The molecular descriptors used to establish QSARs, together with toxicity data for the examined compounds are reported in Table 5.

In order to distinguish and clearly estimate the contribution to toxicity of the different types of interactions, the intercorrelations among the various molecular descriptors were calculated. As shown in Table 6, a high degree of correlation exists between $\Sigma\sigma$ and E_{HOMO} and between μ and E_{LUMO}; this should be necessarily taken into account in the discussion of multiple regression analysis results.

Simple regression analysis between EC50 values and QSAR parameters showed that no significant relationship occurs for any of the tests and of the molecular descriptors considered in the study, except for the SMP assay, for which a significant relationship exists between EC50 values and log K_{ow} ($R^2 = 0.952$), as previously reported (Argese et al. 2005). Thus, when taken separately, none of the parameters is able to

Table 5 Toxicity data and molecular descriptors used for QSAR analysis

	L. sativum log (1/EC50)	Microtox® 15-min log (1/EC50)	SMP log (1/EC50)	Fish 96-h log (1/LC50)	log K_{ow}	E_{HOMO} (hartree)	E_{LUMO} (hartree)	μ (debye)	pK_a	$\Sigma\sigma$	Vmc (Å^3)
2,4-DNP	4.65	4.13	5.17[b]	4.20	1.67[a]	−0.39194	0.03241	6.6345	3.94[a]	1.56	157
2-DNOC	4.71	4.54	5.80[b]	5.02	2.12[a]	−0.38250	0.03548	7.3110	4.31[a]	1.39	168
dinoterb	5.80	5.81	6.77[b]	6.98	3.54[a]	−0.37732	0.02802	8.2138	4.80[a]	1.36	213
chloroxynil	4.82	4.38	6.23[b]	3.89	2.90[b]	−0.35125	0.07396	3.1397	4.90[c]	1.12	137
bromoxynil	5.06	4.58	6.72[b]	4.34	3.39[b]	−0.34566	0.07485	3.5504	4.30[c]	1.12	186
ioxynil	5.30	4.89	6.80[b]	4.74	3.94[b]	−0.32896	0.07445	3.8446	4.50[c]	1.02	216

[a] Values taken from Escher and Schwarzenbach (1996)

[b] Values taken from Argese et al. (2005)

[c] Values taken from Nolte et al. (1995)

describe the toxicological behavior of the tested herbicides, suggesting that a sum of interactions between molecules and their target sites may be responsible for their toxic action.

Very different results were obtained using a two-parameter approach. Table 7 summarizes the results obtained by multiple regression analysis. For *L. sativum*, Microtox® and fish assays, significant correlations have been obtained whenever the hydrophobic descriptor log Kow was used as first independent variable, and $E_{LUMO,}$ E_{HOMO}, the dipole moment or the Hammet σ as a second independent variable. In contrast, for the SMP assay, no one of the additional descriptors yielded significant correlations (Argese et al. 2005). For the fish assay, other significant QSAR equations, not including log K_{ow}, can be observed in Table 7, but they show a lower significance level and contain independent variables with a certain degree of intercorrelation (see Table 6).

A key consideration to interpret these results as a common basis to explain the mechanism of toxic action is the higher statistical value of the relationships that include log K_{ow}. This parameter is a basic QSAR descriptor, used to characterize the hydrophobicity of chemicals and to model their ability to penetrate through biomembranes; thus, good correlations with log K_{ow} confirm that the toxic action involves membrane processes.

The QSAR analysis suggests that hydrophobicity plays a major role in the interaction between the examined substances and their sites of action in all the assays. Besides hydrophobicity, the molecular interactions best describing the toxicity values are the electronic and hydrogen bonding ones, leading to models without outliers.

These results are consistent with a mechanism of toxicity based on protonophoric uncoupling of oxidative phosphorylation, that is the ability of molecules to translocate protons across the inner mitochondrial membrane (in seeds and fish) and the plasma membrane (in bacteria), thus dissipating the transmembrane proton gradient necessary for ATP synthesis (Argese et al. 1999).

All the examined herbicides are lipophilic weak acids that partition easily into the lipid bilayer of membranes; the shuttle mechanism of protonophoric uncoupling requires that both the neutral protonated and the dissociated form be able to diffuse across the membrane, with the phenoxide ion playing a major role at the pH values used in the assays considered in this study, which resemble ambient pH values (Escher and Schwarzenbach 1996). The mobility of the phenoxide ion in the lipid bilayer is strongly influenced by charge delocalization in the molecule, which can be favored by the presence of electron-withdrawing substituents on the benzene ring; dinitrophenols contain two nitro groups in the *ortho*- and *para*-position to the phenoxide group, while hydroxybenzonitriles exhibit one *para*-nitrile group and two halogen substituents in the *ortho*-position. As can be seen in Fig. 3, these electron-withdrawing substituents contribute to charge delocalization, thus increasing phenoxide mobility in the lipid bilayer. Though presenting an electron-donor group in the *ortho*-position, dinoterb was found to be the most potent uncoupler among the examined phenolic compounds. This is supposed to be to an effect of the bulky alkyl substituent, which not only increases the hydrophobicity of the compound, but also causes

Table 6 Correlation matrix showing correlation coefficients among the different molecular descriptors ($n = 6$)

	logK_{ow}	E_{HOMO}	E_{LUMO}	M	pK_a	$\Sigma\sigma$	V_{mc}
logK_{ow}	1.00						
E_{HOMO}	0.79	1.00					
E_{LUMO}	0.54	0.92*	1.00				
μ	−0.38	−0.83*	−0.97*	1.00			
pK_a	0.60	0.38	0.27	−0.18	1.00		
$\Sigma\sigma$	−0.79	−0.97*	−0.91*	0.81*	−0.51	1.00	
V_{mc}	0.71	0.32	−0.03	0.20	0.11	−0.26	1.00

Correlations marked with an asterisk are significant at $p < 0.05$

Table 7 Results of linear regression analysis between toxicity data and the molecular descriptors used for the QSAR analysis ($n = 6$)

Test	Regression equativo	R^2	R^2_{adj}	F	s	p
L. sativum	$\log(1/EC50) = 0.78\ \log K_{ow} - 17.7\ E_{HOMO} - 3.67$	0.986	0.976	104.07	0.067	0.002
	$\log(1/EC50) = 0.58\ \log K_{ow} - 13.3\ E_{LUMO} + 4.05$	0.974	0.957	57.28	0.090	0.004
	$\log(1/EC50) = 0.79\ \log K_{ow} + 2.12\ \Sigma\sigma + 0.06$	0.982	0.970	82.18	0.076	0.002
	$\log(1/EC50) = 0.51\ \log K_{ow} + 0.12\ \mu + 2.88$	0.946	0.911	26.48	0.131	0.01
Microtox®	$\log(1/EC50) = 1.08\ \log K_{ow} - 29.4\ E_{HOMO} - 9.13$	0.973	0.956	55.18	0.124	0.004
	$\log(1/EC50) = 0.76\ \log K_{ow} - 22.4\ E_{LUMO} + 3.69$	0.969	0.949	47.05	0.134	0.005
	$\log(1/EC50) = 1.04\ \log K_{ow} + 3.24\ \Sigma\sigma - 2.43$	0.883	0.806	11.37	0.260	0.04
	$\log(1/EC50) = 0.64\ \log K_{ow} + 0.21\ \mu + 1.66$	0.962	0.937	37.93	0.149	0.007
Fish	$\log(1/LC50) = 1.94\ \log K_{ow} - 67.8\ E_{HOMO} - 25.4$	0.948	0.913	27.39	0.327	0.01
	$\log(1/LC50) = 1.20\ \log K_{ow} - 52.5\ E_{LUMO} + 4.15$	0.971	0.952	50.75	0.243	0.005
	$\log(1/LC50) = 0.93\ \log K_{ow} + 0.51\ \mu - 0.64$	0.977	0.961	62.81	0.219	0.004
	$\log(1/LC50) = -1.08\ E_{LUMO} - 9.91\ \Sigma\sigma + 23.1$	0.901	0.835	13.63	0.452	0.03
	$\log(1/LC50) = 0.80\ \mu - 5.66\ \Sigma\sigma + 7.63$	0.891	0.819	12.29	0.473	0.04
SMP	$\log(1/EC50) = 0.73\ \log K_{ow} + 4.11$	0.952	0.940	78.96	0.161	0.0009

a shielding of the negative charge of the phenoxide ion (Escher and Schwarzenbach 1996).

Conclusions

The results of this study suggest that the toxic action of the examined compounds, though displaying different potency, involves metabolic processes that share common features also in organisms characterized by great physiological and morphological differences, such as seeds, bacteria, and fishes. This proves the effectiveness of phytotoxicity assays based on seed germination/root elongation for the detection of the toxic effect of xenobiotics both on target and non-target organisms and confirms their environmental relevance, making them suitable for the ecotoxicological assessment of environmental samples.

The rather simple and inexpensive testing protocol of the seed germination/root elongation assay employed in this study, together with the use of a species characterized by high sensitivity, such as *L. sativum*, may represent a further advantage for its application as a screening test as well as for inclusion in test batteries; however, the results showed that the optimization of experimental conditions is a basic requirement to improve the reliability and repeatability of toxicity measurements.

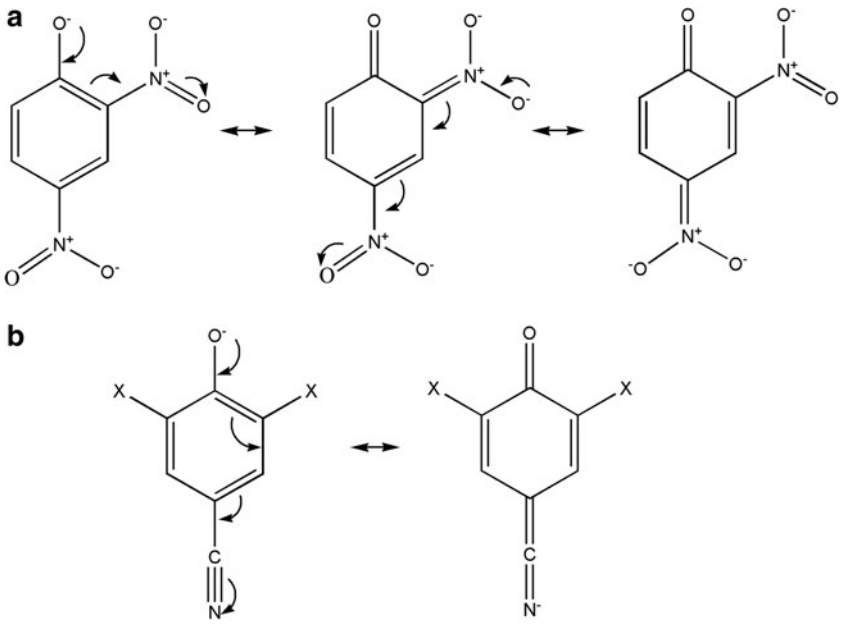


Fig. 3 Main resonance structures of 2,4-dinotrophenol (**a**) and of dihalogenated hydroxybenzonitriles (**b**)

The comparison with the response of other assays and the results of the QSAR analysis support the hypothesis that both in phenolic and in hydroxybenzonitrile herbicides, a mechanism of toxic action based on protonophoric uncoupling of oxidative phosphorylation is operative and, ultimately, highlight the importance of using batteries including both short-term in vitro tests and standard in vivo assays for a thorough assessment of xenobiotic toxicity.

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