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Lemna minor L. as a model organism for ecotoxicological studies performing ^{1}H NMR fingerprinting

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ABSTRACT

A validated method applying ^{1}H NMR fingerprinting for the study of metabolic changes caused in *Lemna minor* L. by various phytotoxic substances is presented. ^{1}H NMR spectra of crude extracts from untreated and treated colonies with the herbicides glyphosate, mesotrione, norflurazon, paraquat and the phytotoxin pyrenophorol were subjected to multivariate analyses for detecting differences between groups of treatments. Partial least squares-discriminant analysis (PLS-DA) and hierarchical cluster analysis (HCA) were carried out in order to discriminate and classify treatments according to the observed changes in the metabolome of the plant. Although the compounds at the concentrations used did not cause macroscopically observable symptoms of phytotoxicity, characteristic metabolic changes were detectable by analyzing ^{1}H NMR spectra. Analyses results revealed that metabonomics applying ^{1}H NMR fingerprinting is a potential method for the investigation of toxicological effects of xenobiotics on *L. minor*, and possibly on other duckweed species, helping in the understanding of such interactions.

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1. Introduction

Duckweeds (*Lemna* spp.) are floating, flowering macrophytes, widespread in aquatic environments and easily cultured under laboratory conditions. The plant size is minute facilitating experimentation under limited spatial conditions but large enough to allow macroscopic observations. The morphology, growth habits and sensitivity of *Lemna* species to a wide range of xenobiotics are features which make duckweeds valuable bioassay organisms (Mohan and Hosetti, 1999; Duke et al., 2000; Duke et al., 2002; Michel et al., 2004; Fenske et al., 2006; Vervliet-Scheebaum et al., 2006).

Duckweed bioassays have mainly been used in ecotoxicology for phytotoxicity evaluation of xenobiotics. In such studies the examined attributes include morphological characteristics (Bassi et al., 1990; Li and Xiong, 2004), biochemical parameters (Mohan and Hosetti, 1997; Severi, 1997; Teisseire et al., 1999; Teisseire and Vernet, 2000; Geoffroy et al., 2004; Mitrovic et al., 2005), growth (Severi et al., 1997; Teisseire et al., 1999; Vujević et al., 2000; Verdisson et al., 2001; Pomati et al., 2004; Mitrovic et al., 2005), photosynthesis (Prasad et al., 2001; Frankart et al., 2002; Hulsen et al., 2002; Beninger et al., 2004), and chemical composition (Vujević et al., 2000; Prasad et al., 2001; Verdisson et al., 2001; Pomati et al., 2004). Furthermore, official guidelines (OECD Guidelines for the testing of chemicals, 2006) and advanced systems such as Lemnatec® (http://www.lemnatec.com) have been developed for the estimation of the effects of xenobiotics on *Lemna* spp.

Since the above parameters have been associated with metabolic processes, an experimental protocol unraveling and classifying metabolic changes caused in *Lemna* spp. by bioactive xenobiotics would be useful for standardizing duckweed bioassays and studying in-depth the effect of those substances to plant systems.

Metabonomics is a relatively recent approach to studying biochemical networks in biological systems by obtaining metabolic profiles mainly with utilization of NMR spectroscopy (Ratcliffe and Shachar-Hill, 2001; Bollard et al., 2005; Krishnan et al., 2005; Ward et al., 2007). Although metabonomic approaches regarding toxicology have attracted the attention of scientists (Robertson, 2005; Lindon et al., 2007), the method has limited application in ecotoxicological studies (Bundy et al., 2002; Viant et al., 2006a,b; Miller, 2007).

Since duckweeds have been valuable organisms in assessing environmental health, in the present work a metabonomic strategy for the evaluation and classification of impacts of xenobiotics on the metabolome of *Lemna minor* applying ^{1}H NMR fingerprinting is presented.

In order to evaluate the applicability of the method to intact *L. minor* plants, ^{1}H NMR spectra of crude extracts from colonies, untreated and treated with phytotoxic substances, were analyzed and subjected to multivariate analysis. The ^{1}H NMR fingerprinting pro-

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tocol applied in the present study was based on the method which has given satisfactory results in studies on the mode of action of synthetic and natural phytotoxic compounds on graminaceous species (Aranibar et al., 2001; Ott et al., 2003; Aliferis and Chrysayi-Tokousbalides, 2006).

2. Materials and methods

2.1. Plant material

L. minor L. cultures were kindly provided by LemnaTec GmbH, Germany, and were maintained on Steinberg medium (International Organization for Standardization, 2003).

2.2. Chemicals and reagents

The phytotoxin (5*S*,8*R*,13*S*,16*R*)-(−)-pyrenophorol was isolated from cultures of a pathotype of *Drechslera avenae* (Kastanias and Chrysayi-Tokousbalides, 2000). Paraquat (1,1′-dimethyl-4,4′-bipyridinium) (33.5%), mesotrione [2-(4-mesyl-2-nitrobenzoyl) cyclohexane-1,3-dione] (99.7%), and norflurazon [4-chloro-5-methylamino-2-(α,α,α-trifluoro-*m*-tolyl)pyridazin-3(2*H*)-one] (98.0%) were kindly provided by Syngenta Hellas SA. Glyphosate [N-(phosphonomethyl)glycine] (99.5%) was courtesy of Agan Chemical Manufacturers Ltd. Chemical structures of compounds are given in Fig. 1. Deuterium oxide (D_2O) (99.9%) and trimethylsilyl-2,2,3,3-d_4-propionic acid (TSP) were obtained from Euriso-Top SA.

2.3. Duckweed treatment and sample preparation

L. minor cultures were grown on Steinberg medium in plastic beakers of 25 mL capacity and 4 cm in height covered with plastic lids with five punctures each. For the experiments, 10 colonies (15.0 ± 0.5 mg) of the same age and overall appearance were used per treatment. The selected colonies were placed in plastic 12-well plates and kept in a growth chamber at 22 ± 1 °C, relative humidity of 85 ± 5% and a photoperiod of 16 h light and 8 h dark. The light source used was cool white fluorescent lamps (Sylvania) providing illumination of approximately 45 μmol m^{-2} s^{-1}. In each well 3 mL of Steinberg medium or solutions of the phytotoxic compounds in Steinberg medium were added. The doses used were the highest concentrations which did not cause phytotoxicity symptoms on Lemna fronds 72 h after treatments under the conditions set. Glyphosate, mesotrione, norflurazon, paraquat, and pyrenophorol were used at a final concentration of 296, 152, 111, 0.19, and 1280 μM, respectively. Lemna colonies were collected 72 h after treatments and immediately pulverized with a pestle in a mortar under liquid nitrogen. Pulverized samples were stored at −80 °C not longer than 4 days in 15 mL screwcap glass vials. The samples were lyophilized for 24 h and the lyophilized preparations were suspended in D_2O (1.0 mL) containing TSP (0.05 w/v) and centrifuged at 11 500*g* for 60 min at 4 °C. The supernatants were then collected and further purified by centrifugation at 11 500*g* for 30 min. The NMR samples were prepared from the supernatants (0.8 mL). In order to add as much variation as possible and examine

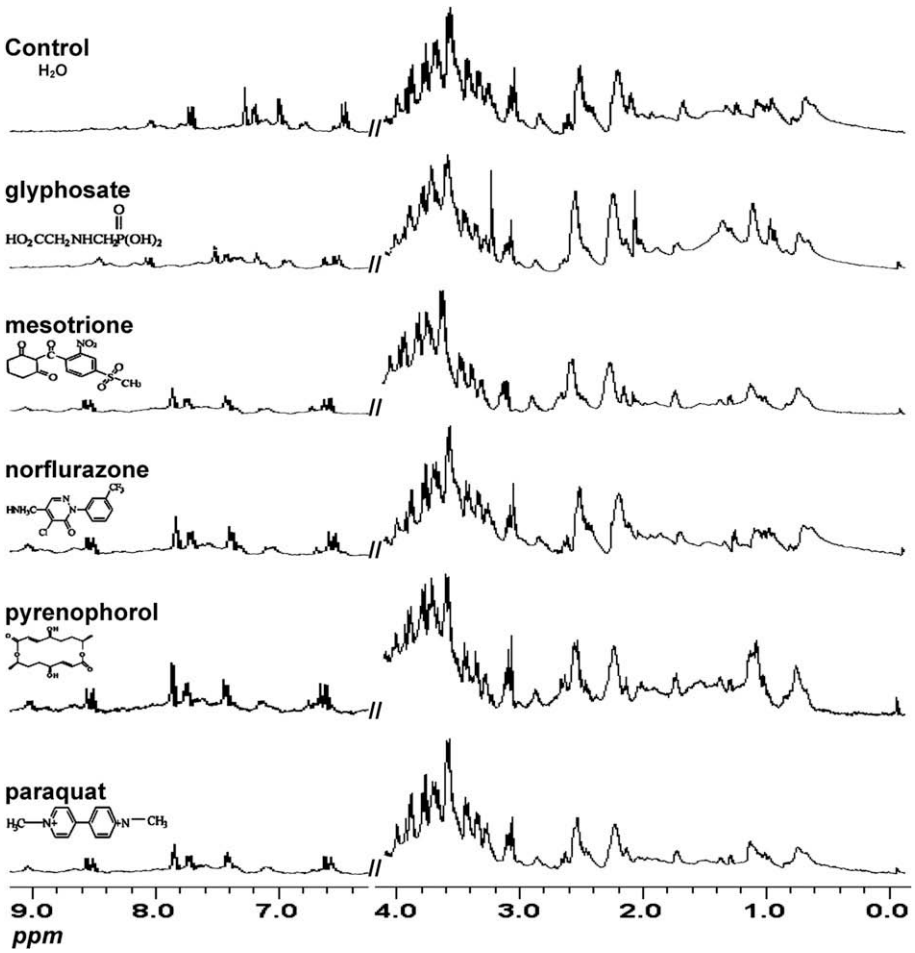

Fig. 1. ^{1}H nuclear magnetic resonance (NMR) spectra of *Lemna minor* crude extracts of untreated and treated colonies with phytotoxic substances before normalization. Spectra regions δ 4.10–6.50 were excluded from analysis.

the reproducibility of the applied protocol, experiments were repeated in weekly intervals. In total four experiments were performed with two replications for each phytotoxic compound in every experiment.

2.4. ^{1}H NMR spectroscopy, data pre-processing and analysis

The ^{1}H NMR spectra of crude colony extracts were recorded on a Bruker DRX 500 NMR spectrometer equipped with a multi nuclear inverse broad band 5 mm probe with 1 s solvent presaturation (60 dB) and 256 transients of 32 K data points for each spectrum. The data were Fourier transformed, and the baseline and the phase were automatically corrected using MestReC software (version 461). The spectral regions δ 4.10–6.50 were removed to eliminate the effects of imperfect water suppression. Additionally, in order to reduce the variation between treatments, regions of the spectra were aligned prior to statistical analysis as proposed by Defernez and Colquhoun (2003) using the MestReC software. Spectra were overlapped and offsets of chemical shifts were corrected. Spectra were then exported to MS Excel® and prior to pattern recognition analysis they were normalized to the whole spectrum to remove any concentration effects. Multivariate data analyses were carried out using the SIMCA-P 10.5 (Umetrics, Umeå, Sweden) software. Data were Pareto scaled (1/xSD) and visualized by plotting the principal components scores, where each coordinate represents an individual treatment. The detection of differences in metabolic fingerprints was based on partial least squares-discriminant analysis (PLS-DA) and scaled PLS regression coefficients which unravel the most influential variables (chemical shifts) for the observed discrimination (Eriksson et al., 2001). Assignment of the shifts to the corresponding chemical groups was performed using several NMR spectra databases [ACD/C+H NMR Predictors and Database v.11.01, Advanced Chemistry Development, Inc. (ACD/Labs, Toronto, Canada), The Human Metabolome Database (http://www.hmdb.ca), and The Spectral Database for Organic Compounds SDBS (http://riodb01.ibase.aist.go.jp/sdbs/cgi-bin/direct_frame_top.cgi)].

In addition to PLS-DA, hierarchical cluster analysis (HCA) was applied to Pareto-scaled data in order to further examine the ability of the developed model to classify treatments according to the changes that were caused in the plant's metabolome using the SPSS software (v.11.0.0, SPSS Inc., Chicago, USA).

2.5. Performance statistics

The developed models were evaluated by the cumulative fraction of the total variation of the X's that could be predicted by the extracted components [$Q^2_{(cum)}$] and the fraction of the sum of squares of all X's (R^2X) and Y's (Y^2X) explained by the current component.

3. Results

Metabolic fingerprints of the Lemna colonies in the absence or in the presence of the phytotoxic compounds are depicted in the representative ^{1}H NMR spectra displayed in Fig. 1. At the time of harvesting, all treated Lemna colonies showed no visible symptoms of phytotoxicity.

For spectra analysis principal components analysis (PCA) was carried out on the whole dataset revealing one sample (mesotrione-treated) being outside the Hotelling T^2 95% confidence ellipse. This sample was excluded in order to avoid possible high leverage on the model. PCA of data after outlier removal revealed no samples being outside the Hotelling T^2 95% confidence ellipse and values of $R^2X = 0.73$ and $Q^2_{(cum)} = 0.52$ (seven principal components-*PCs*).

In order to examine the reproducibility of the applied protocol six PCA models were developed, one for each group of treatment. Analysis revealed tight classes for all groups with high values of explained variation [control ($R^2X = 0.83$, 3 *PCs*), glyphosate ($R^2X = 0.90$, 5 *PCs*), mesotrione ($R^2X = 0.87$, 3 *PCs*), pyrenophorol ($R^2X = 0.86$, 3 *PCs*), paraquat ($R^2X = 0.89$, 4 *PCs*), and norflurazon ($R^2X = 0.89$, 4 *PCs*)].

Initially, the hypothesis that performing metabolic fingerprinting differences between control and every other treatment group could be detected and classified was tested by applying PLS-DA to data sets, comparing each time the control group with another treatment group. Models were developed from training sets of observations of known membership class. PLS-DA t_1/t_2 score plots for all combinations are given in Fig. 2. Cross validation analysis revealed that the control group could be satisfactorily separated from the group treated with glyphosate [$Q^2_{(cum)} = 0.72$], mesotrione [$Q^2_{(cum)} = 0.74$], norflurazon [$Q^2_{(cum)} = 0.86$], paraquat [$Q^2_{(cum)} = 0.58$] or pyrenophorol [$Q^2_{(cum)} = 0.83$] (Table 1 and Fig. 2). The above estimates indicated that all substances applied at sub-lethal concentrations caused substantial metabolic changes in Lemna's metabolome detectable by examining ^{1}H NMR spectra of crude extracts. Multivariate analysis of spectra revealed that the differences in metabolic composition of the macrophyte after treatments were mainly due to differences in substances containing methylene, methine, hydroxy, amine, thiol, olefin, and aldimine groups (Table 1). Further examination on those areas could unravel biomarkers for toxicity attributable to the phytotoxin(s) being tested.

In a second step, the validity and the ability of the developed model to discriminate all the groups of treatments were assessed performing PLS-DA for selected datasets. Since results of PLS-DA can become tricky comparing simultaneously more than three groups of treatments (Eriksson et al., 2001), PLS-DA was applied for all possible combinations of treatments in groups of three. PLS-DA t_1/t_2 score plots for all combinations are given in Fig. 3 and results from cross validation in Table 2. It was evident that all substances applied at sub-lethal concentrations caused substantial metabolic changes in *L. minor's* metabolome detectable by examining ^{1}H NMR spectra of crude plant extracts.

HCA also revealed the high discriminative ability of the developed model. A satisfactory classification could be achieved for all groups of treatments (Fig. 4) with the exception of control and paraquat groups, which is in agreement with results of PLS-DA (Table 1 and Fig. 2). Out of the 47 treatments that were simultaneously analyzed, only one treatment of control group got incorrectly classified into the paraquat group which means that 97.9% of the treatments could be correctly classified.

In order to further correlate the changes in the metabolome of the plant caused by the natural phytotoxin pyrenophorol to those of the phytotoxic compounds with known mode-of-action, PLS-DA was applied comparing the group of pyrenophorol treatment to the rest groups of treatments, one at time. The predictive abilities of the pyrenophorol–control ($Q^2_{(cum)} = 0.83$, 2*PCs*), pyrenophorol–mesotrione ($Q^2_{(cum)} = 0.91$, 3*PCs*), and pyrenophorol–norflurazon ($Q^2_{(cum)} = 0.91$, 2*PCs*) models were higher than that of the pyrenophorol–glyphosate model ($Q^2_{(cum)} = 0.81$, 3*PCs*). These results in combination with results of HCA (Fig. 4) indicate that pyrenophorol causes metabolic changes in *L. minor* in a pattern more similar to that of glyphosate than the other phytotoxic compounds being tested.

4. Discussion

The automatization of the assignment of ^{1}H NMR chemical shifts in complex mixtures to the corresponding compounds is a challenging task for high-throughput metabolomics and metabonomics studies. Nevertheless, although ^{1}H NMR spectroscopy lacks

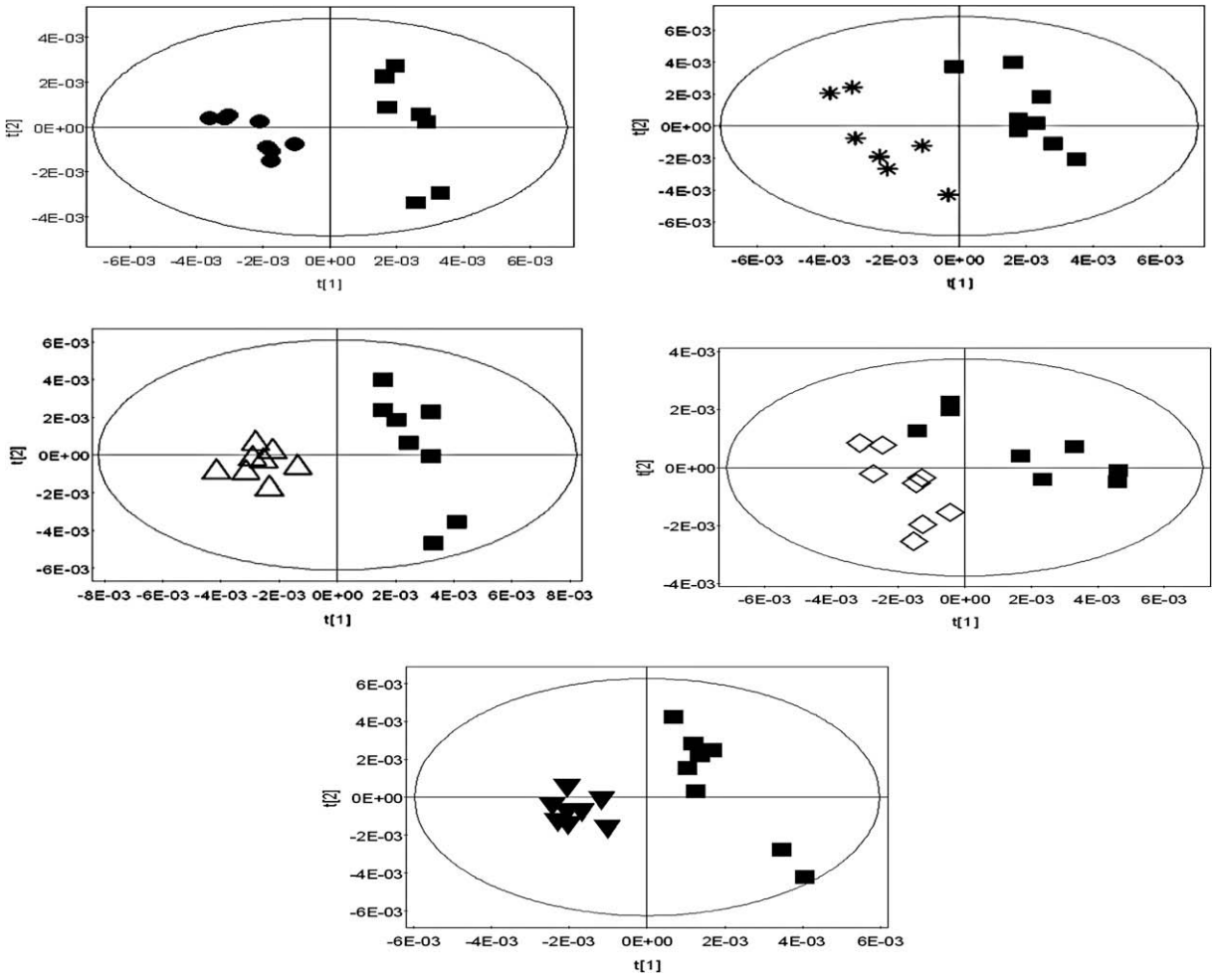

Fig. 2. Partial least squares-discriminant analysis (PLS-DA) t_1/t_2 score plots based on the observations of control group (■) plotted versus groups of observations of glyphosate (●), mesotrione (∗), norflurazon (▼), paraquat (◇), and pyrenophorol (Δ). The ellipse represents the Hotelling T^2 with 95% confidence.

Table 1
Cross validation parameters of paired treatment groups performing partial least squares-discriminant analysis (PLS-DA) with corresponding ^{1}H nuclear magnetic resonance (NMR) spectra regions responsible for the discrimination.

Treatment groups	Parameters[a]				Shift ranges, δ (ppm)	Chemical groups
	PCs	R^2X	R^2Y	$Q^2_{(cum)}$		
Control-glyphosate	2	0.45	0.91	0.72	2.14–2.15, 2.28–2.31, 2.58–2.60, 3.11–3.12, 3.62–3.63	Methyl, methylene, methine, hydroxy, amine, thiol, olefin
Control-mesotrione	2	0.41	0.92	0.74	0.85–0.88, 1.23–1.25, 1.47–1.48, 1.57–1.60, 3.11–3.12, 3.26–3.27, 7.15	Methyl, methylene, methine, hydroxy, amine, thiol, olefin, aldimine
Control-norflurazon	3	0.58	0.96	0.86	1.07–1.12, 1.21–1.24, 2.25–2.29, 2.33–2.38, 2.56–2.59, 3.46–3.48	Methyl, methylene, methine, hydroxy, amine, thiol
Control-paraquat	4	0.81	0.94	0.58	1.07–1.13, 1.22–1.25, 2.12–2.17, 2.54–2.55, 2.61–2.68, 3.12, 3.16, 3.28–3.32, 3.35–3.36, 6.99–7.00	Methyl, methylene, methine, hydroxy, amine, olefin, aldimine
Control-pyrenophorol	2	0.51	0.94	0.83	1.17–1.19, 3.60–3.62	Methyl methylene, methine, hydroxy, amine, thiol

[a] *PCs*, principal components; PLS-DA, partial least squares-discriminant analysis; $Q^2_{(cum)}$, the cumulative fraction of the total variation of the *X*'s that can be predicted by the extracted components; R^2X and R^2Y, fraction of the sum of squares of all *X*'s and *Y*'s, respectively, explained by the current component.

the sensitivity of mass spectrometry it is a platform of high potential for metabolomics studies due to its reproducibility and minimum requirements for sample preparation. Thus, the final decision for the selection of the analytical platform for metabolomics studies depends on the aim of the study and the available instrumentation (Lindon and Nicholson, 2008).

This work represents the first step for the development of a validated model for the assessment of ecotoxicological risk through ^{1}H NMR metabolic fingerprinting using duckweed as the model organism. The major task was the discrimination and classification of metabolic changes caused in plant's metabolome by xenobiotics without the identification of the compounds contributing to such separation to be a primary task.

The development and standardization of the method was based on the use of herbicidal compounds with a known and selective mode of action at the subcellular level causing specific changes in plant's metabonome. Furthermore, the selected substances all have high hydrophilicity which facilitates the preparation of test

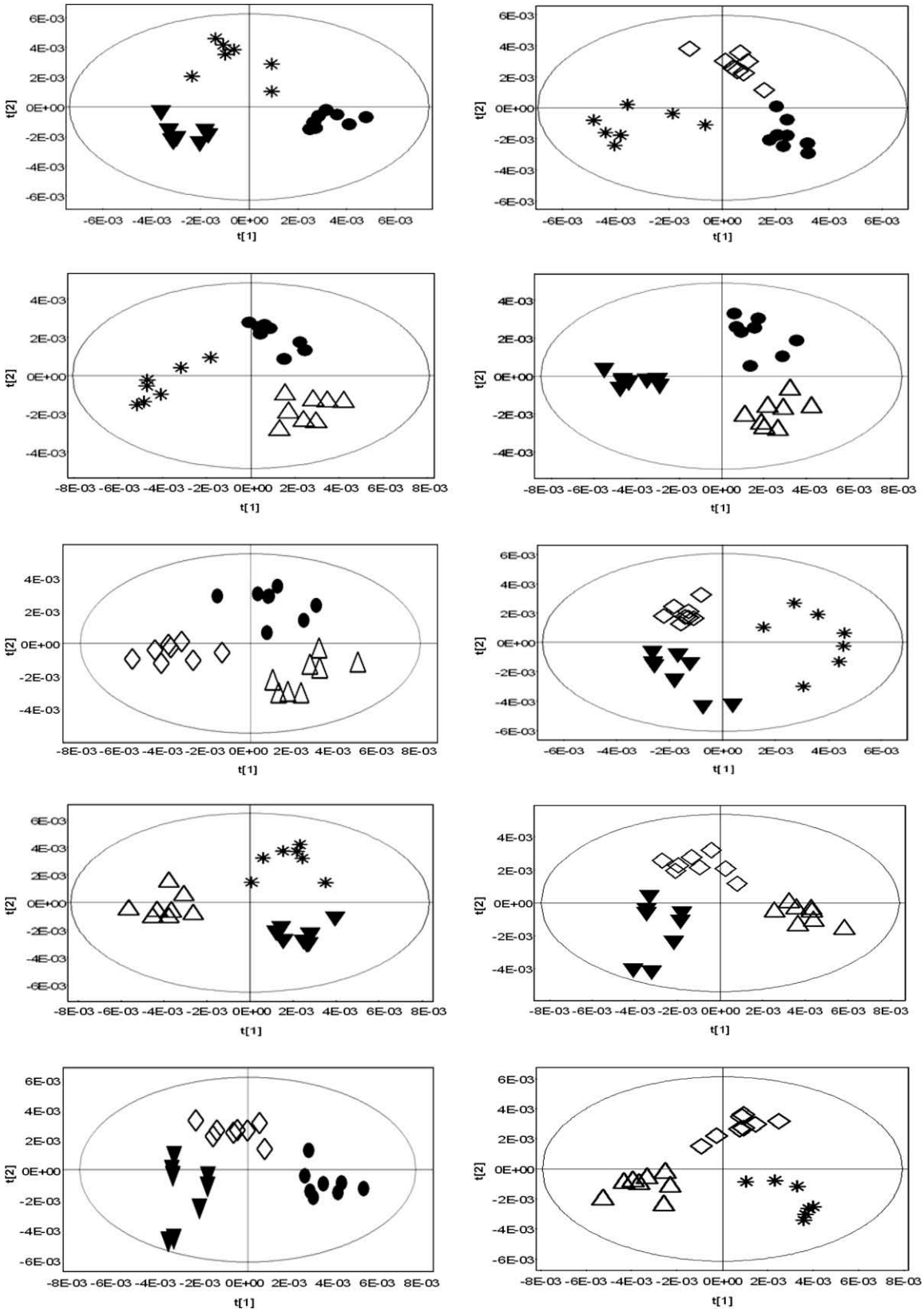

Fig. 3. Partial least squares-discriminant analysis (PLS-DA) t_1/t_2 score plots for all possible combinations of observation groups per three. The ellipse represents the Hotelling T^2 with 95% confidence [glyphosate (●), mesotrione (∗), norflurazon (▼), paraquat (◇), and pyrenophorol (Δ)].

solutions in water avoiding the use of organic solvents. Several physiological processes are involved in the phytotoxic activity of the compounds, including oxidative stress through the production of reactive oxygen species (ROS) [paraquat-photosystem I (PSI) electron diverter], inhibition of amino acid synthesis [glyphosate-inhibitor of 5-enolpyruvylshikimate-3-phosphate (EPSP) syn-

Table 2
Cross validation parameters comparing treatments in groups of three performing partial least squares-discriminant analysis (PLS-DA). Acronym definitions are given in Table 1.

Treatment groups	Parameters			
	PCs	R^2X	R^2Y	$Q^2_{(cum)}$
Glyphosate–mesotrione–norflurazon	3	0.51	0.94	0.82
Glyphosate–mesotrione–paraquat	3	0.49	0.89	0.75
Glyphosate–mesotrione–pyrenophorol	3	0.54	0.91	0.71
Mesotrione–norflurazon–paraquat	5	0.65	0.94	0.83
Mesotrione–norflurazon–pyrenophorol	3	0.59	0.93	0.84
Glyphosate–norflurazon––pyrenophorol	3	0.57	0.91	0.80
Norflurazon–pyrenophorol–paraquat	3	0.68	0.92	0.83
Mesotrione–pyrenophorol–paraquat	3	0.56	0.91	0.80
Glyphosate–pyrenophorol–paraquat	3	0.54	0.87	0.67
Glyphosate–norflurazon–paraquat	4	0.61	0.91	0.76

thase], inhibition of photosynthetic pigment biosynthesis [mesotrione-inhibitor of 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD) and norflurazon-inhibitor of phytoene desaturase (PDS)]. Pyrenophorol was included in the tests because it is a natural phytotoxin which does not share a common mode of action with any of the above mentioned herbicides (Aliferis and Chrysayi-Tokousbalides, 2006).

Results from PLS-DA and HCA indicated that *Lemna* spp. metabonomics could be exploited for ecotoxicological studies. The method has the advantages of the robustness and sensitivity as compared to the already applied methods for the estimation of the toxicity of xenobiotics on *Lemna* species, allowing the in-depth examination of their effects on the metabolome of the plant. Treatments could be satisfactorily grouped according to the metabolic changes in the macrophyte, which means that the model can be further developed with the inclusion of other potential pollutants to aquatic environments. Furthermore, although the pyrenophorol group of treatments could be strongly discriminated from the rest groups (Tables 1 and 2, Figs. 2–4), it seems that the toxicological effect of this phytotoxin in *L. minor* resembles that of glyphosate, an inhibitor of EPSP synthase.

The effects of the applied substances on *Lemna* spp. could be examined by the developed protocol offering a complementary tool to the already existing methods. Furthermore, it would be considered as a method applicable to biochemistry-driven research on the development of novel ecotoxicological biomarkers useful in studies on pollution of rural and urban environments and on risk assessment of xenobiotics.

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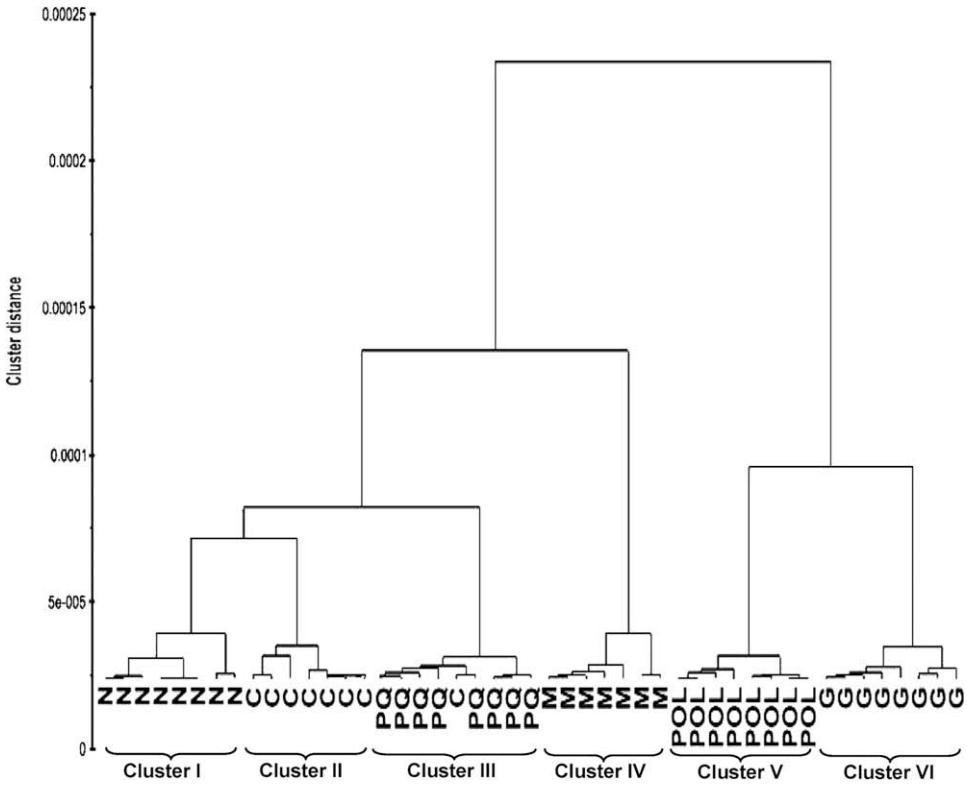

Fig. 4. Hierarchical tree diagram illustrating the hierarchical cluster analysis of the pre-processed data collected by ^{1}H NMR using the Ward's method [control (C), glyphosate (G), mesotrione (M), norflurazon (N), paraquat (PQ), and pyrenophorol (POL)].

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