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FTIR SPECTROSCOPY AS A NOVEL APPROACH TOWARDS PROVIDING EFFECT-BASED ENDPOINTS IN DUCKWEED TOXICITY TESTING

Running title: FTIR application in duckweed toxicity test

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Abstract: Traditional duckweed toxicity tests only measure plant growth inhibition as an endpoint, with limited effects-based data. This study aimed to investigate whether Fourier-transform infrared (FTIR) spectroscopy could enhance the duckweed (*Lemna minor* L.) toxicity test. Four chemicals (Cu, Cd, atrazine, and acetochlor) and four metals-containing industrial wastewater samples were tested. After exposure of duckweed to the chemicals, standard toxicity endpoints (frond number and chlorophyll content) were determined; the fronds were also interrogated using FTIR spectroscopy under optimized test conditions. Biochemical alterations associated with each treatment were assessed and further analyzed by multivariate analysis. The results showed that comparable ECx (x percent of effective concentration) values could be achieved based on FTIR spectroscopy in comparison to those based on traditional toxicity endpoints. Biochemical alterations associated with different doses of toxicant were mainly attributed to lipid, protein, nucleic acids and carbohydrate structural changes, which helped explain toxic mechanisms. With the help of multivariate analysis, separation of clusters related to different exposure doses could be achieved. This is the first study showing successful application of FTIR spectroscopy in standard duckweed toxicity tests with biochemical alterations as new endpoints.

Keywords: Biochemical fingerprint; Biospectroscopy; Duckweed; FTIR spectroscopy; Multivariate analysis; Toxicity test

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INTRODUCTION

In (eco-) toxicological research, toxic effects are determined based on estimation of various endpoints, a time-consuming aspect of many tests [1, 2]. Traditional toxicological endpoints often involve the examination of lethality or inhibition of the test organism, and physical characteristics [3-5]. Alterations in biomarkers, including lipid, protein, nucleic acids and carbohydrates, may also be determined as endpoints in toxicity tests [6, 7].

As a useful tool, infrared (IR) spectroscopy can provide detailed biochemical information, including lipids, proteins, DNA/RNA and carbohydrates of a biological sample [8]. Besides, alterations in secondary structure of proteins, protein phosphorylation could also be identified in the vibrations of functional groups of the biomaterials [9, 10]. So far, this tool has been used to diagnose disease, especially as an emerging, simple and nondestructive tool in cancer diagnosis [11-13]. Beyond that, IR spectroscopy could also be used in various fields such as forensic casework, biomes identification, and metabolomics stress response [14-18]. Even though the IR spectrum has been widely used in various biological materials like cells and tissues, comparative analysis between ATR and transmission was lacking [19].

Fourier-transform IR (FTIR) spectroscopy is emerging as a sensor-based tool for correlating the structure of biomolecules in different biological systems [20]. As different chemical bonds of biochemical samples absorb light in the mid-IR region (including 3000 to 2800 cm-1 and 1800 to 900 cm-1), the application of FTIR spectroscopy may interrogate the chemical structure of molecules in biochemical samples [15]. It generates a vibrational spectrum and the region of 1800-900 cm-1 is often known as a “biochemical fingerprint” [21, 22]. IR spectra generated from analysis as dataset can be considered in a multidimensional space and the dimensionality is best reduced

using sophisticated multivariate analysis techniques like principal component analysis (PCA) and linear discriminant analysis (LDA) that capture the most important variation [21, 23, 24]. Considering its advantages such as biochemical fingerprinting, sample nondestructive, and simple operation features, FTIR may be applied in ecotoxicity testing as a powerful tool.

The purpose of this study was to investigate whether FTIR spectroscopy can be applied in a standard eco-toxicity test using four test agents (Cu, Cd, atrazine and acetochlor as the representatives of metals and herbicides) and four industrial wastewater samples with duckweed used as test species. Following exposure of duckweed to the chemicals, standard toxicity endpoints (growth inhibition) were determined, while fronds were interrogated using FTIR spectroscopy under optimized test conditions. Biochemical alterations associated with each treatment were assessed and analyzed by multivariate analysis. These results may validate application of FTIR spectroscopy in the interpretation of toxic effects.

MATERIALS AND METHODS

Chemical agents. Four chemicals (Cu, Cd, atrazine, and acetochlor) were selected as test agents. CuSO4 and CdCl2 (purity >99%) were purchased from XILONG Chemical Co. (China), while atrazine and acetochlor (purity >99.9%) were obtained from Dr. Ehrenstorfer GmbH (Germany). Methanol and ethanol were HPLC grade and supported by Merck Corporation (Shanghai, China). Stock solutions of CuSO4, CdCl2, and acetochlor were prepared by dissolving the compounds in Milli-Q water at a concentration of 10000 mg/L, 10000 mg/L and 100 mg/L, respectively, whereas the stock solution of atrazine was pre-dissolved in methanol at 1000 mg/L. All other chemical reagents applied in this research were of HPLC or analytical grade.

The recipe of a modified Swedish Standard (SIS) medium [25] is given as follows: 75 mg/L MgSO4•7H2O, 85 mg/L NaNO3, 36 mg/L CaCl2•2H2O, 20 mg/L NaCO3, 13.4 mg/L KH2PO4,

1.0 mg/L H3BO3, 0.2 mg/L MnCl2•4H2O, 0.01 mg/L Na2MoO4•2H2O, 0.05 mg/L ZnSO4•7H2O, 0.005 mg/L CuSO4•5H2O, 0.01 mg/L Co(NO3)2•6H2O, 0.84 mg/L FeCl3•6H2O, and 1.4 mg/L Na2EDTA. All substances in the medium were prepared in Milli-Q water and the pH was adjusted to 6.5±0.2 by addition of NaOH or HCl solution.

Test species. Duckweed *Lemna minor* L. was used in the experiments. It was cultured for one month as a pre-culture in the laboratory in the SIS medium under the following conditions: 2000 lux; 25±1 °C; light cycle, 14 h : 10 h (day: night); humidity, 60%. The medium was replaced every two weeks.

Duckweed exposure experiments

Range finding test. Duckweed toxicity tests were conducted in a series of six well plates. Each well contained 10 mL of the SIS medium and 4 three-frond colonies with approximately the same size of *L. minor.* Six concentrations (0, 0.0001, 0.001, 0.01, 0.1, 1 or 10 mg/L) for each test compound were used in the range finding test, each treatment was replicated three times. Plants were maintained under the conditions shown in the above. The number of fronds in each well was counted every 48 h. After 96 h exposure to chemicals, plants were picked out for the analysis of biomass and chlorophyll content.

Test conditions determined. Test conditions including fixing methods and instrumental parameters were optimized using the following experiment.

The experiment was set up at the same exposure conditions for Cu as the test agent at a concentration of approximate EC50 value based on the range finding test results, and for the control without Cu. After exposure, the fronds of the control groups and Cu treatment groups were fixed with multiple methods for plant samples, including (A) 70% ethanol, (B) 0.9% NaCl, (C) 10% formalin in phosphate buffer saline (PBS), and (D) Conroy solution. After 12 h, the fixed samples

were tiled on the BaF2 glass slides, and dried in the desiccator for at least 24 h. Then the samples were interrogated using FTIR with the ATR mode and transmission mode.

Exposure to chemicals. Once the approximate EC50 value for each test agent was obtained, the exposure experiment could be set up at a range of exposure concentrations. The exposure concentrations of Cu, Cd and acetochlor were diluted with Milli-Q water while the exposure concentrations of atrazine were diluted with methanol from the stock solutions. Ten μL of each test agent were delivered in each well with 10 mL medium. Since the methanol solvent may affect the toxic effects [26], the accessory solvent of atrazine treatment group was dried before 10 mL medium was added in each well. Then pre-cultured four duckweed plants, each has three fronds, were selected for chemical exposure experiment. The number of fronds in each well was counted every 48 h. After 96 h exposure, several fronds were fixed with 10% (v/v) formalin and then stored at the room temperature until FTIR analysis.

The exposure concentrations were measured at intervals throughout the experiment by using atomic absorption spectrometry (Varian, USA) for Cu and Cd, and by using high performance liquid chromatography for atrazine and acetochlor on an Agilent system equipped with Eclipse XDB-C18 (5 μm, 4.6×150 mm, USA) column. As found in the Supporting Information (SI, Table S1), the nominal concentrations and measured concentrations of the agents showed no significant differences, except for copper due to the instrument systematic error.

Exposure to wastewater samples. Four wastewater samples were collected in a wastewater treatment plant of an electroplate factory in southern China, including A: the influent with a high concentration of copper, B: the effluent with the main component of copper, C: influent with a high concentration of chromium, and D: the effluent of mixed metals. All samples of wastewater were filtered through glass fiber filters (Whatman GF/F, 0.7 μm effective pore size, UK), and two-fold

diluted with the SIS medium (100%, 50%, 25%, 12.6%, 6.25%, and 3.125%). Then those solutions were used for duckweed toxicity tests with the same conditions as the four test agents. The metal concentrations were measured by atomic absorption spectrometry.

Traditional duckweed toxicity test endpoints

Following each exposure treatment, plant growth inhibition was calculated using the frond number and chlorophyll content. The fronds of duckweed were picked out carefully and put them on the blotting paper for several minutes, then weigh the all fronds of each treatment and put them to a 2 mL centrifuge tube with 2 mL 95% (v/v) ethanol for 48 h.

The supernatant was used for chlorophyll a (Ca), chlorophyll b (Cb) and Ca+b (Ca+Cb) estimation. Absorbance at 663 nm and 645 nm was measured using a microplate reader (BMG Labtech FLUOstar Omega), and the content of chlorophyll was obtained by a previous method [27]. The percent inhibition of plant growth compared with the control was calculated according to the protocol previously [25].

FTIR spectral measurement and analysis

Duckweed plants fixed with 10% (v/v) formalin were washed three times with PBS and water in sequence, then spread to BaF2 slides, and dried in the desiccator for at least 24 h. The prepared samples on BaF2 slides were interrogated using a Bruker Vector 70 FTIR spectrometer (Bruker Optics Ltd., Germany) equipped with a HYPERION microscope, which contained a liquid nitrogen cooled detector. The instrumental settings were optimized: transmission mode, 64 scans, and 8 cm-1 resolution. 25 spectra of each sample were acquired at different positions of the dried frond samples. Prior to starting the next slide, a background spectrum was taken for background noise subtraction.

Data preprocessing and multivariate analysis

Raw infrared spectra obtained from interrogated samples were analyzed using the irootlab toolbox (http://irootlab.googlecode.com/) [28] running on Matlab r2010a. Each spectrum was cut at the "biochemical fingerprint" region (1800-900 cm-1) and CH stretching region (3000-2800 cm-1), followed by rubber-band baseline correction, and normalization to the Amide I peak (~1650 cm-1) in biochemical fingerprint region and to the max in the CH stretching region (~2920 cm-1) [21]. Following these preprocessing, PCA-LDA was applied to each dataset separately and allowed to identify biochemical alterations that segregate treated groups from each other [15]. PCA was applied to the spectral dataset to reduce the dimensions of the datasets [29]; while LDA was applied to discriminate treated groups [15, 30].

In scores plots, nearness in the first LDA factor (LD1) between samples indicates the similarity of toxic effects, while distance means difference [15]. Dose-response curves were derived based on the distance between each treatment mean and the control mean in the LD1 space.

Statistical analysis

Data on duckweed growth inhibition were presented as mean ± standard deviation (SD) in each treatment, and analyzed for statistical differences by analysis of variance (ANOVA) in order to understand the difference of each treatment. All ANOVA tests were achieved in GraphPad Prism 4 (GraphPad Software, USA). Dose-response curves were fitted with the logistic model. Pearson correlation analysis was achieved by using SAS 9.1 software (SAS Institute, UCLA).

RESULTS AND DISCUSSION

Optimization of sample processing method and instrumental conditions

Before duckweed toxicity test, test conditions were optimized, mainly for the fixative and FTIR instrumental conditions. A typical IR spectrum of the fronds of duckweed derived from the FTIR spectroscopy is shown in Figure S1. The datasets generated from the FTIR analysis are

complex and multidimensional. No obvious differences between different treatment groups could be readily observed throughout the selected spectral CH stretching region (3000-2800 cm-1) and biochemical fingerprint region (1800-900 cm-1). Given the large numbers of spectra generated, computational analyses including PCA-LDA were thus applied to discriminate treatment groups and distinguish correlative biomarkers contributing to variance.

Four fixing solutions (A, 70% ethanol; B, 0.9% NaCl; C, 10% formalin in PBS; and, D, Conroy solution) and two instrumental modes (ATR mode *vs.* transmission mode) were compared to find an optimized test method for application in the toxicity characterization. Since the ATR mode is mainly applied to homogeneous samples, it primarily expresses the surface information of the tissues if the sample is thick. In fact, the IR can penetrate the fronds of the duckweed, so the transmission mode would better reflect the toxic effects inside plant cells than the ATR mode. The cluster vectors plots demonstrated this difference (Figure S2).

Among the four fixing solutions, the cluster for the solution B (0.9% NaCl) segregated away from those for the other three solutions in both control group and Cu treated group under the ATR and transmission modes (Figure S2). As the cluster vector plots showed in Figure S2 (bottom), the duckweed fronds treated with this solution showed biochemical alterations at lipid and protein structures during drying process. It was also observed that Conroy solution (D) might induce the alteration of lipid and protein. The solution A (70% ethanol) showed few variations in the Cu-treated group, but it induced several changes in C-H stretching region in the control group because of its lipid soluble nature. In comparison to the other three solutions, the solution C (10% formalin in PBS) induced fewer alterations in both ATR mode and transmission mode for the control group and chemical-treated group.

In fact, both ethanol and formaldehyde are common fixatives with different reactions [31].

As its molecular structure is similar to water, ethanol competes for protein hydrogen bonds with water to replace the water molecules in the organization; and the protein precipitates in the isoelectric point by reducing the protein bound electric constant, and blocks the combination of antibodies-epitope [32]. Besides, alcohols can also dissolve the lipid materials, and have weak penetration as forming a layer of membrane protein, which may block the fixed liquid penetrating into, and cause the intermediate organization poorly fixed [33, 34]. So ethanol sometimes is not a good fixative for some biological samples. In contrast, formaldehyde is the most common fixative for retaining the tissue and cell protein targets as it depends on the formation of cross-linked protein-protein and protein containing methylene (CH2-)-nucleic acids [35]. Thus the solution C (10% formalin in PBS) was selected as fixative for FTIR analysis of fronds under the transmission mode.

Effects of duckweed exposure to chemicals

Following exposure of duckweed to the two metals (Cu and Cd) and two pesticides (atrazine and acetochlor), the dose-response curves were obtained for each chemical based on traditional toxicity endpoints (Figure S3) and FTIR spectroscopy (Figure 1). General observations of the toxic effects on duckweed following exposure to the four chemicals are given in Table S2, while distinct FTIR peak assignments at different exposure concentrations are presented in Table S3. Various effective concentrations (ECx: EC1, EC10, and EC50) for the four chemicals were calculated based on the endpoints of plant growth rate inhibition, total chlorophyll inhibition at 96h, alterations of biochemical fingerprint region (1800-900 cm-1) and C-H stretching vibration region (3000-2800 cm-1) along LD1 space (Table 1). When compared to the traditional endpoints, generally comparable results were achieved from FTIR spectroscopy. The EC50 values for Cu, Cd, atrazine and acetochlor based on PCA-LDA results of biochemical fingerprint region were 0.198, 0.673,

0.0302, and 0.0028 mg/L whereas those EC50 of duckweed growth inhibition (I96h) were 0.157, 1.27, 0.0405, and 0.00410 mg/L respectively (Table 1). Overall, statistically significant correlations existed between the phenotypic and the biochemical alterations (Table S5). Specifically, significant correlations have been found among the growth inhibition rate, total chlorophyll and biochemical alterations for the metal groups and herbicide group ($R2 > 0.9$, $\rho < 0.05$). This suggests the applicability of FTIR spectroscopy in duckweed toxicity test, and availability of additional biochemical alteration information.

Treatment of duckweed with the chemicals showed increased alterations of biomolecules with chemical exposure concentrations (Figure 2; Table S2). For Cu, the main alterations associated with the EC50 dose included vibrations related with glycogen (~1000 cm-1), Amide I (~1600 cm-1), Amide II (~1540 cm-1), amino acid residues (~1405 cm-1) and protein phosphorylation (~950 cm-1) (Figure 2). Comparing low-dose and high-dose exposures, discriminating loadings throughout the biochemical fingerprint region were observed. The observed toxic effects were associated with alterations to lipids (~1740 cm-1), nucleic acids (~1080 cm-1) at high doses, and carbohydrate (~1140 cm-1) and Amide III (~1260 cm-1) at low-doses, respectively. The results from this study showed that Cu could affect the protein in organisms through combining with protein molecules and amino acids. As an essential element, Cu can be incorporated into various enzymes that perform essential metabolic functions [36]. Copper can also exist in oxidized, or reduced forms in living cells; and it acts as catalyst in the production of reactive oxygen species, which can cause oxidative damage and induce adverse effects [37-39]. In addition, Cu can also result in pernicious effect on chlorophyll, so the chlorophyll may be more sensitive than the frond number and the biomass [40].

Cadmium showed different biochemical alterations to Cu in duckweed (Figure 2). The biochemical alterations induced by Cd were associated with Amide II (~1550 cm-1), Amide III

(~1315 cm-1), lipid (~1740 cm-1), and nucleic acids (~1080 cm-1). The low-dose treatments did not cause significant alterations at coefficient in cluster vectors. However, the high dose treatments brought out discriminating loadings at Amide I (~1650 cm-1), lipid (~1740 cm-1), amino acids residues (~1400 cm-1), nucleic acids (~1080 cm-1) and glycogen (~1050 cm-1). Cd may induce lipid peroxidation and change antioxidant system [41]. Cd is easy to combine with –OH, -NH2 and –SH groups in protein [42, 43]. It can cause the conformation changes of membrane proteins, thus affecting membrane lipid fluidity and changing the function of membrane protein and liquidity, leading to the damage of membrane lipid [44]. Cd can also replace the function of Zn in the body, damage Zn-containing enzyme function, which has a relationship with the respiratory and other physiological processes, finally inhibit the growth of plants and lead to death [45].

For atrazine, the main biochemical alterations in duckweed of the low-dose treatments were related to Amide I (~1650 cm-1), which is concerned with the secondary structure of protein, and carbohydrate (~1000 cm-1). At the doses above a toxic unit, changes occurred at protein and lipid, and became more obvious with the increasing doses. Besides, the alterations associated with nucleic acids (~1080 cm-1) and carbohydrate (~1150 cm-1) were also observed in duckweed. Atrazine mainly destroys the photosynthesis, and it can also disturb the plant hormone and iron balance, leading to the collapse of the metabolism and thus affecting the RNA, enzyme and protein synthesis [46].

For acetochlor, the biochemical alterations at low-doses were associated with protein secondary structure (~1650 cm-1), Amide II (~1540 cm-1), carbohydrate (~1180 cm-1) and glycogen (~1030 cm-1). While at high doses, the biochemical alterations showed distinguishing wavenumbers associated with Amide II (~1535 cm-1), Amide I (~1650 cm-1), asymmetric phosphate (~1226 cm-1), carbohydrate (~1030 cm-1) and protein phosphorylation (~995 cm-1). The

toxic mechanism for acetochlor in plants is mainly through inhibition of cell growth, making the plants stop growing by blocking the protein synthesis [47].

The two-dimension scores plots of biochemical region (1800-900 cm-1) showed different segregation patterns from the control and their wavenumber basis (Figure 3; Figures S4 and S5). The results from this study clearly showed that different chemicals induced diverse alterations at the same toxic dose due to their different toxic mechanisms. Thus, the bio-spectroscopy not only showed the toxic effects in duckweed induced by chemicals at varying concentrations, but also explained specific biochemical alterations.

FTIR spectroscopy application to wastewater toxicity test

FTIR spectroscopy was also applied to the toxicity tests of four industrial wastewater samples. The wastewater samples A and C were influents, while samples B and C were effluents. The sample A contained mainly Cu (110 mg/L) and several other metals (Al, Fe, Cr and Zn) at 2 to 10 mg/L, while the sample B from the same wastewater treatment system contained only a low concentration of Cu (0.1 mg/L). The sample C mainly contained Cr (128 mg/L), whereas the sample D contained various metals (Ni, Cu and Zn) at concentrations of 3 to 5 mg/L. Growth inhibition was observed for the two influent samples even after dilution to 3.13%; but for the two effluent samples, growth inhibition was found only in 100% of the sample B and 100% and 50% of the sample C.

The results from FTIR spectroscopy analysis showed clear segregation of the influent samples (A and C) from the effluent samples (B and D) and the control (Figure 4). This suggests significant differences in bio-molecular alterations induced by different wastewaters. Along LD1, the major difference was induced by the influent sample C with a high Cr concentration, while at LD2, it was obvious for the influent sample A with a high concentration of Cu. Cluster separation was not obvious between the control and effluents. Anyway, there were clear segregation between

the influents and effluents, indicating large differences in toxicity.

Biochemical alterations observed in duckweed following exposure to the Cu-containing influent A were found in second structures associated with Amide I (~1670 and ~1640 cm-1), DNA (~1225 cm-1), and protein phosphorylation (~980 cm-1) (Figure 4). The effluent B caused the major alterations in Amide I (~1640 cm-1), and minor changes in carbohydrate (~1000 cm-1), nucleic acids (~1080 cm-1), and Amide II (~1520 cm-1). The Cr-induced alterations for the influent C were noted mainly in the Amide II (~1580 cm-1), followed by Amide III (~1320 cm-1), asymmetric phosphate stretching vibration in DNA (~1225 cm-1). The changes in duckweed from exposure to the effluent D were found mainly in Amide III (~1310 cm-1), followed by Amide I (~1680 cm-1), Amide II (~1570 cm-1) and nucleic acids (~1080 cm-1). Biochemical alterations in duckweed induced by different contaminants could be used as a biomarker at the bio-macromolecular level for characterizing their biological mechanisms of toxicity.

In summary, the results indicate that FTIR spectroscopy can be applied in duckweed toxicity test and biochemical alterations can be used as new endpoints. Comparable results of ECx values were achieved based on the FTIR spectroscopy when compared to the traditional endpoints. FTIR spectroscopy showed its capability of monitoring the effects in biochemical molecules such as protein, lipid, DNA /RNA and carbohydrate due to exposure of duckweed to the chemicals at different levels.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

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Data Availability—Data, associated metadata, and calculation tools are available on request from the corresponding author (guang-guo.ying@gig.ac.cn; guangguo.ying@gmail.com).

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Figure 1. Dose-response curves showing effects of duckweed exposure to various doses of Cu, Cd, atrazine and acetochlor (96 h) based on FTIR spectroscopy. PCA-LDA scores plots and resultant cluster vectors were derived from triplicate experiments (n=75 spectra per chemical treatment). Panels in the left column represent IR alterations concentrated in biochemical fingerprint region (1800-900 cm-1); those in the right column were concentrated in lipid region (3000-2800 cm-1). Each symbol indicates one independent experiment containing an average of 25 separated IR spectra per slide.

Figure 2. Clusters vectors peaks plots indicating the wavenumber basis for segregation following treatment of fronds with the chemicals. Each treatment is compared to the control. The size of the symbol in the plot is proportional to the height of the corresponding peaks, which are relative to the extent of biochemical alterations compared to the control. The black horizontal line represents the control. The hint line represents a typical infrared spectrum of the biochemical fingerprint region (1800-900 cm-1). Note: because the treated concentrations of metals were the same, so the fisrt two figures shared a legend; so as the pesticides did.

Figure 3. PCA-LDA score plots showing effects of the treatment of fronds with the chemicals at a toxic unit (EC50). In the scores plots in two dimensions (90% confidence ellipsoids) of biochemical region (1800-900 cm-1) (above), clusters vectors peaks plots indicate the wavenumber basis for segregation following treatment of fronds. The size of the symbol in the plot (bottom) is proportional to the extent of biochemical alteration compared to the vehicle control. The hint line represents a typical IR spectrum of the biochemical fingerprint region (1800 - 900 cm-1).

Figure 4. PCA-LDA scores plots of biochemical region (1800-900cm-1) (above) of duckweed following exposure to the wastewaters from a plating plant. A: the influent with a high

concentration of copper, B: the effluent with a low concentration of copper, C: influent with a high concentration of chromium, D: the effluent with several metals at low concentrations. Confidence ellipsoids (90%) are drawn assuming a normal distribution in 3-D scatters plots. Clusters vectors peaks plots indicate the wavenumber basis for segregation following treatment of fronds. The size of the symbol in the plot (bottom) is proportional to the extent of biochemical alteration compared to the vehicle control.

Table 1 The EC_x values of different chemicals

EC_x (mg/L)	NOEC (EC_1)				LOEC (EC_{10})				EC_{50}			
	I_{96h}[a]	CI[b]	P1[c]	P2[d]	I_{96h}[a]	CI[b]	P1[c]	P2[d]	I_{96h}[a]	CI[b]	P1[c]	P2[d]
Cu	0.0058	0.0018	0.0006	0.0004	0.0256	0.0117	0.008	0.0092	0.157	0.114	0.198	0.418
Cd	0.0861	0.0049	0.0415	0.0165	0.289	0.0345	0.145	0.103	1.27	0.379	0.673	0.969
Atrazine	0.003	0.0025	0.0001	0.0003	0.0097	0.0129	0.0013	0.0059	0.0405	0.0959	0.0302	0.256
Acetochlor	0.0001	n	n	n	0.0005	0.0003	0.0001	0.0002	0.00410	0.0039	0.0028	0.003

[a] Growth rate inhibition at 96h;

[b] Total chlorophyll inhibition;

[c] PCA-LDA results of biochemical fingerprint region (1800-900 cm^{-1});

[d] PCA-LDA results of C-H stretching vibrations (lipid region 3000-2800 cm^{-1});

n The data below 0.0001.

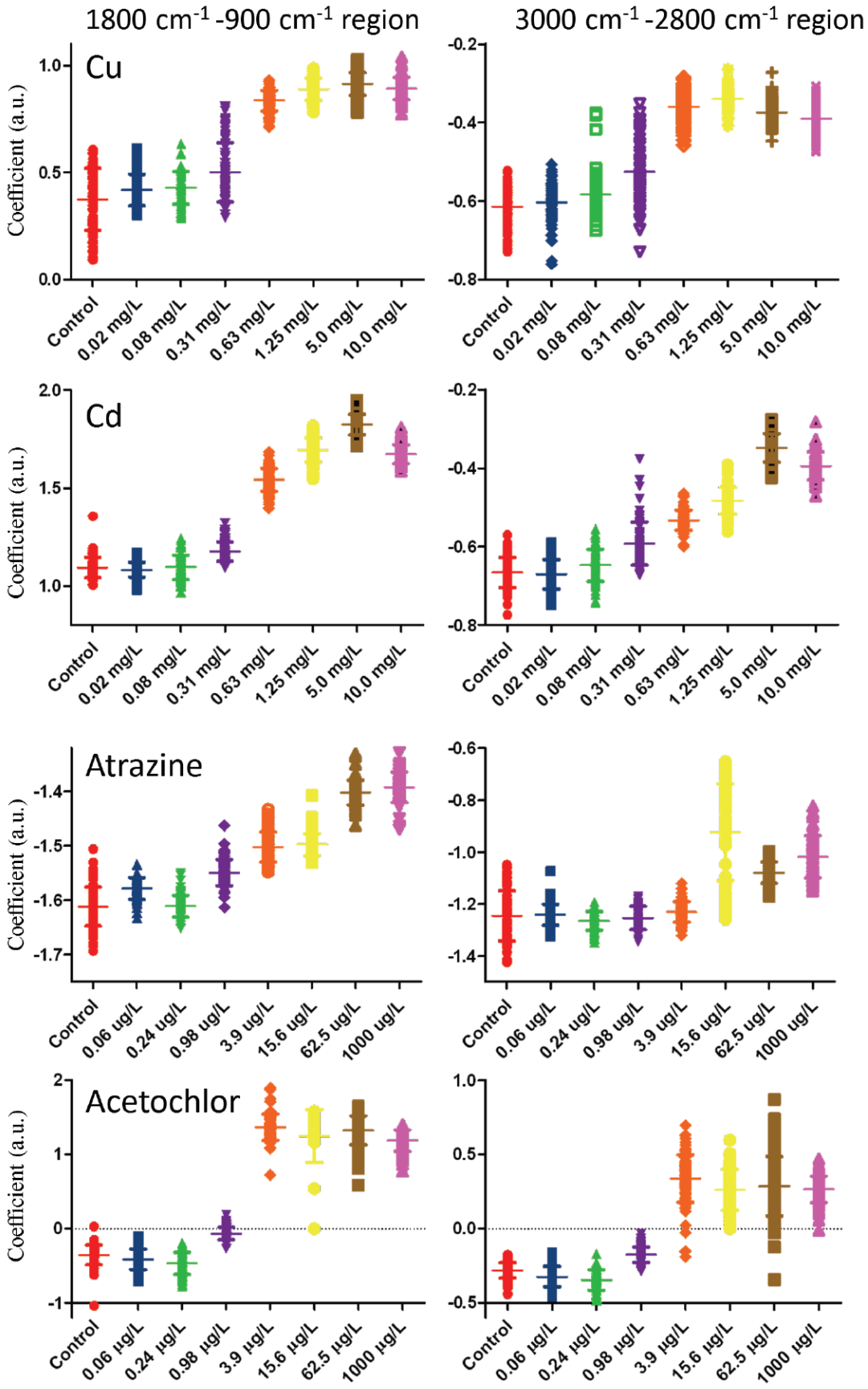

Figure 1

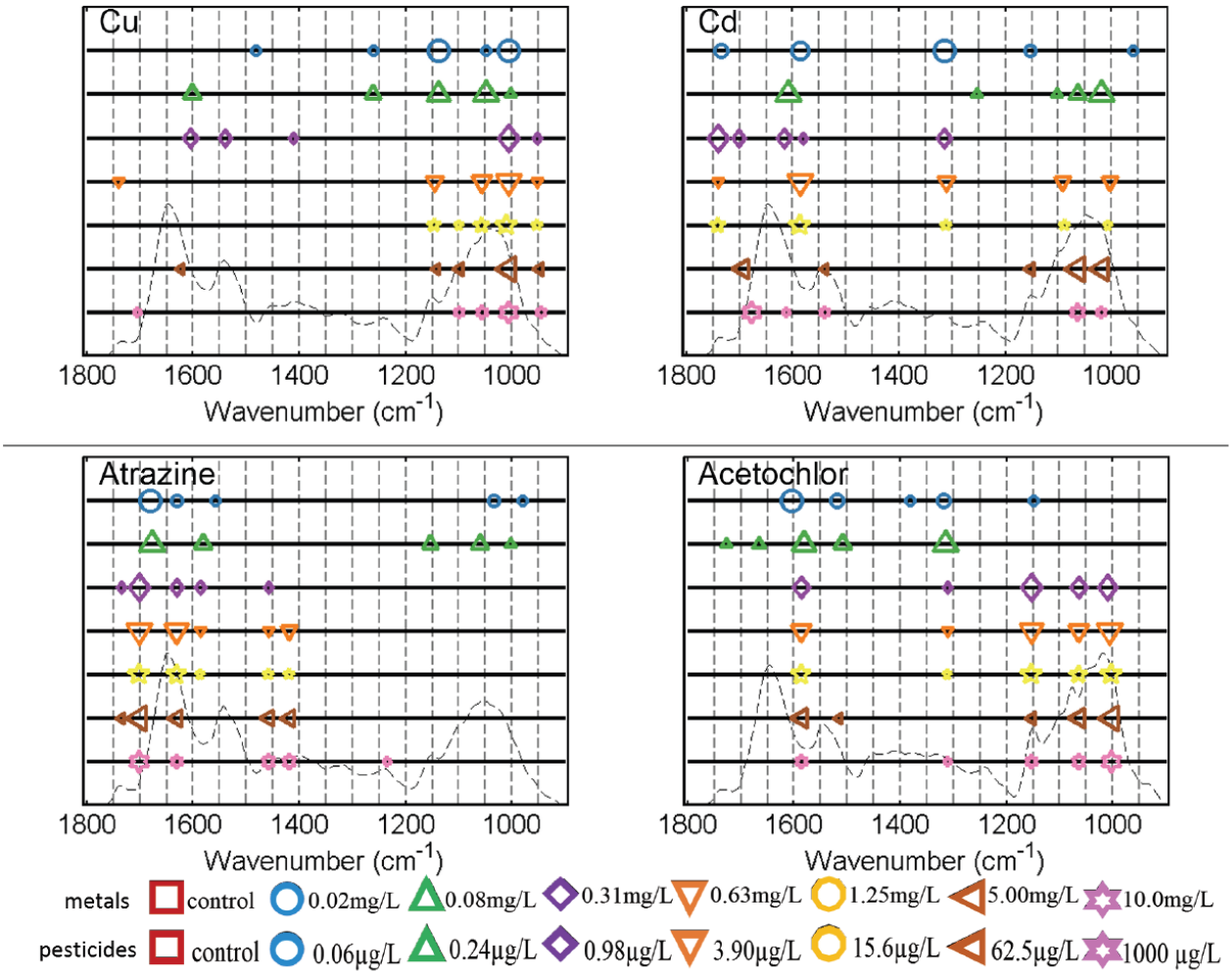

Figure 2

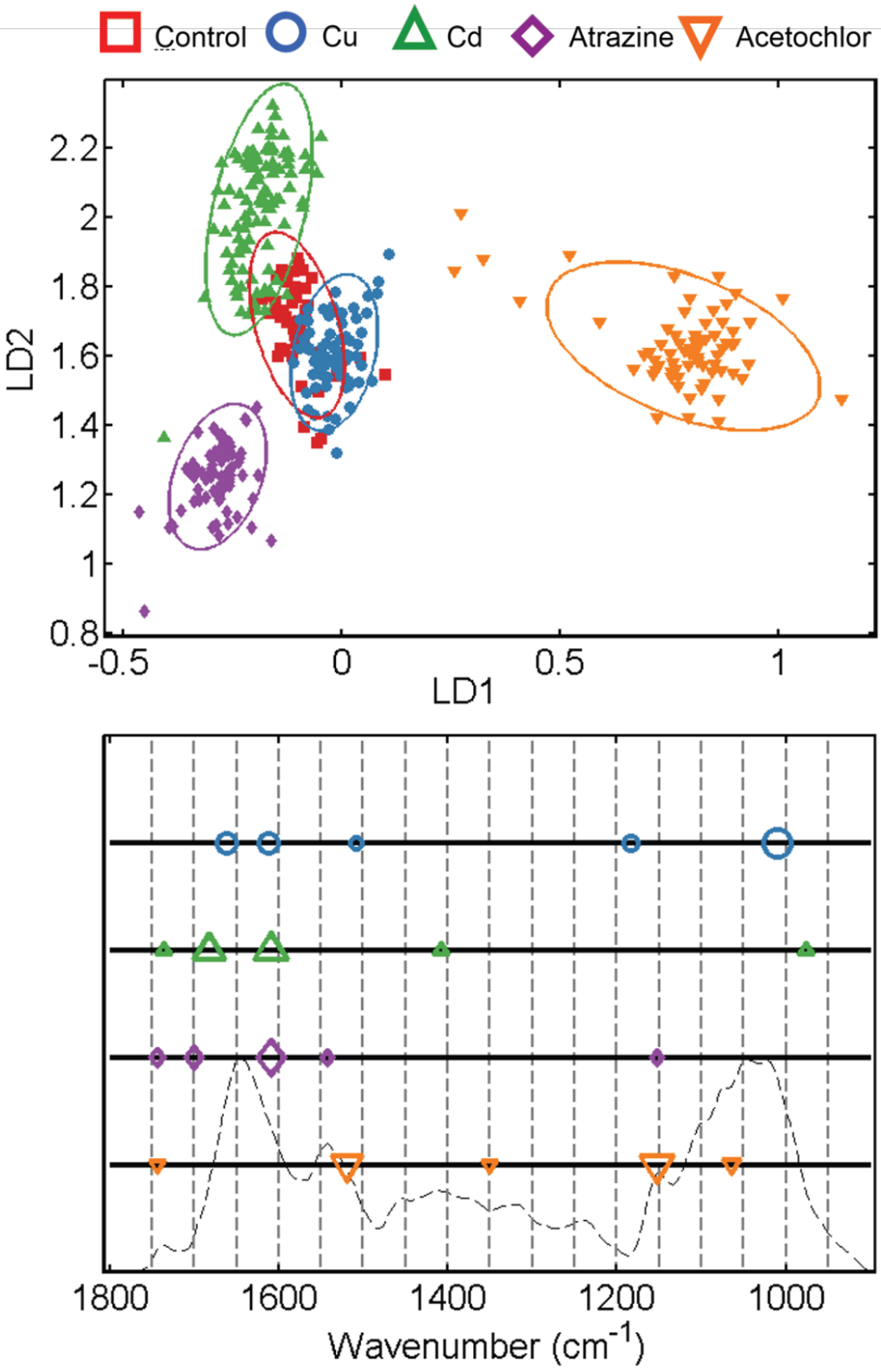

Figure 3

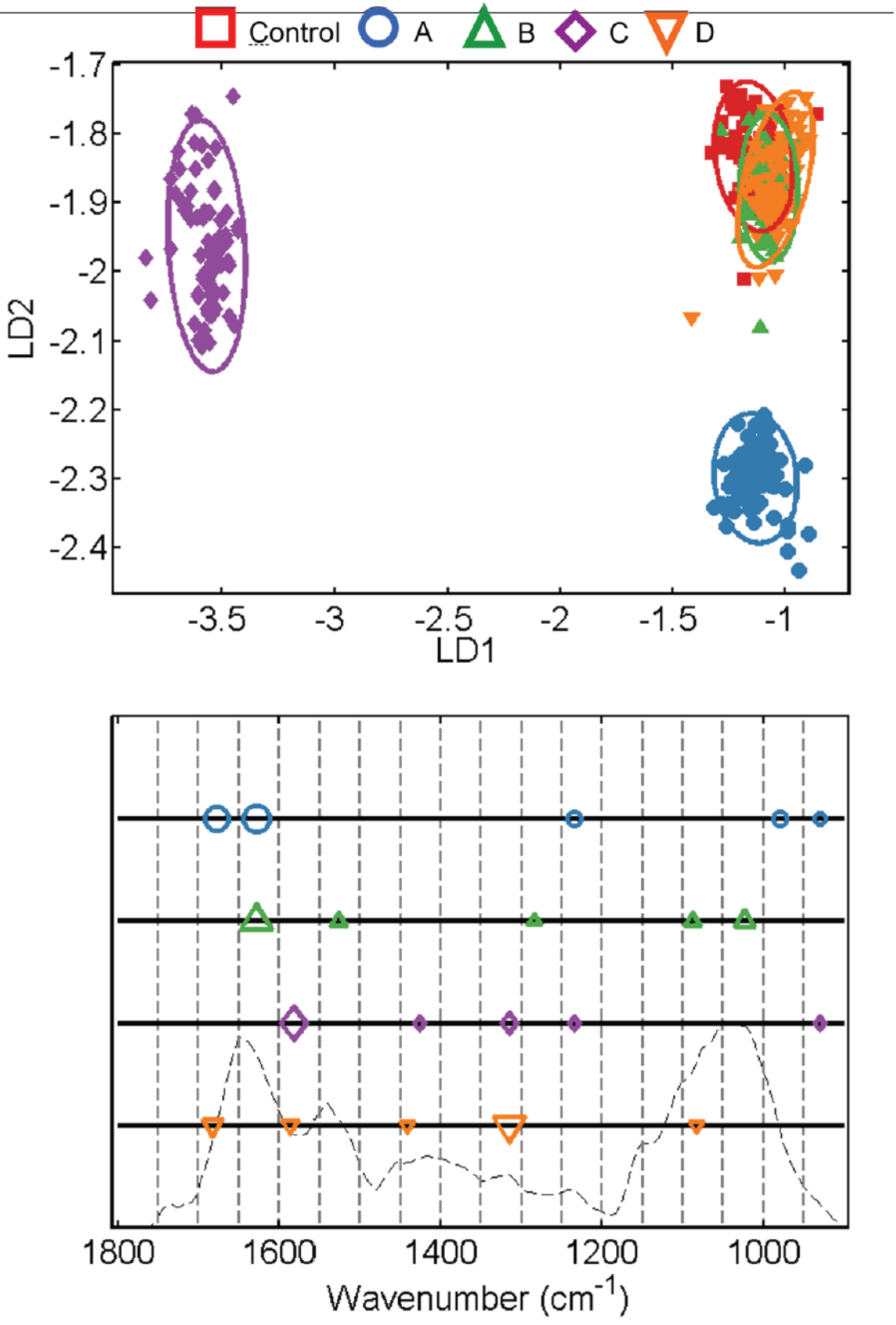

Figure 4