**The changes in *Lemna minor* metabolomic profile: a response to diclofenac incubation**

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**Abstract**

Metabolomics is an emerging approach that investigates the changes in the metabolome profile. In the present study, *Lemna minor —*considered as an experimental aquatic plant model— was incubated with 10 and 100 µM diclofenac (DCF) for 96 hours, respectively. Knowing that DCF is internationally often problematic in wastewater effluents and that it might affect particularly the metabolic profiles in aquatic plants, mainly the oxidoreductase, dehydrogenase, peroxidase, and glutathione reductase activities, here it was hypothesized (H) that in the common duckweed, DCF might increase the phenolic and flavonoids pathways, as an antioxidant response to this stress (H1). Also, it was expected DCF to alternate the physiological characteristics, especially the molecular interaction and biochemical properties, of *Lemna* (H2)*.* Metabolic changes were investigated with target and untargeted screening analysis using RPLC-HILIC-ESI-TOF-MS. Twelve amino acids were identified in all treatments, together with three organic acids (*p*-coumaric, cinnamic, and sinapic acids). In untargeted screening, the important metabolites to discriminate between different treatments were assigned to *Lemna* such as organic acids, lignin, sugars, amino acids, dipeptides, flavonoids, biflavonoids, fatty acids, among others. In resume, *Lemna* responded to both DCF concentrations, showing different stress patterns. A similar metabolic response had already been identified in other studies in exposing *Lemna* to other anthropogenic stressors (like pesticides).

**Keywords:** untargeted screening analysis, aquatic plants, anti-inflammatory compounds, RPLC-HILIC-ESI-TOF-MS, organic and amino acids, reducing potential

**Highlights: 85 characters per sentence with spaces**

1. The reducing contents increased due to diclofenac (DCF) incubation;
2. *Lemna´s* amino acid profile changed afterDCFincubation;
3. *Lemna´s* metabolic profile (by untargeted screening analysis) changed uponDCFincubation*.*

**Introduction**

Metabolomics is an approach for the overall investigation of metabolite variations in biological systems and is essential to characterize these profiles as the metabolites change significantly during biochemical reactions. Consequently, metabolic profiling can be used as a robust tool to discuss the metabolic response of plants regarding environmental disorders, such as xenobiotics, nutrient deficiency, high salinity, and temperature stress (Kralova et al., 2012). As known, primary (e.g. amino acids (AA)) and specialized (e.g. fatty acids, and flavonoids) metabolites reflect the plant's functional and physiological states of the cell and organism, respectively (Wu et al., 2020).

*Lemna minor* (commonly named duckweed) is the largest genus of the family *Lemna*ceae and can be found in tropical and subtropical countries*. Lemna* has an ecologically important role in the absorption of excess nutrients, heavy metals, and other contaminants (Chakrabarti et al., 2018). It is a free-floating aquatic plant thatis mainly used as fish and livestock feed as a source of various essential and non-essential AA, polyunsaturated fatty acids, β-carotene, and xanthophylls (Cao et al., 2018).

Plant metabolomics investigations have been formerly conducted with liquid chromatography (LC) and gas chromatography (GC) connected to the mass spectrometer (MS), respectively (Tugizimana et al., 2015; Kim J-Y et al., 2017). Moreover, a serial coupling of reversed-phase liquid chromatography (RPLC) and hydrophilic interaction liquid chromatography (HILIC) connected to a high-resolution mass spectrometer (HRMS) has been used to detect a wide range of polarities (from non-polar to high polar compounds; Bieber et al., 2017) in plant extracts in a single run (Greco et al., 2013; Wahman et al., 2019). However, metabolomics data require an analysis strategy that could recognize the changes between datasets. Thus, an untargeted screening strategy has been developed to assign such changes in metabolomics studies (Wahman et al., 2019). Using this strategy, the changes in plant metabolic profile could be investigated through two independent workflows: (1) the identification of the relevant metabolites, and (2) the statistical identification of the ‘unknown’ metabolomics indicators.

One prominent environmental problem is water pollution, especially with slow and/or non/transformed pollutants. Diclofenac (DCF) is a widely distributed non-steroidal anti-inflammatory drug that can be found in surface waters and is considered an environmental risk factor (Huber et al., 2012). Further, in 37 countries, including Germany, the average concentration of DCF is up to 1μg L-1 in surface water(Letzel et al., 2009; Fischer et al., 2020). The estimated removal efficacy for DCF using conventional wastewater treatment plants varies from 39% to 70% (Fischer et al., 2020). Furthermore, DCF’s photo-transformation products have high potential toxicity at concentration levels close to environmental concentrations (Schmitt et al., 2007). In 2019, Alkimin and co-authors (2019) reported that DCF incubation in *Lemna* caused a decrease in the content of photosynthetic pigments, relative fluorescence decay values of chlorophyll, and oxidoreductase and dehydrogenase activities. However, it led to increases in non-photochemical quenching, amount of reactive nitrogen and oxygen species in roots, lipid peroxidation, oxidized ascorbate and thiols, and glutathione-reductase activity (Alkimin et al., 2019). These findings induce concerns regarding the chronic exposure of plants in constructed wetlands.

Knowing that DCF particularly affects the *Lemna* metabolic profiles (mainly the oxidoreductase, dehydrogenase, peroxidase, and glutathione reductase activities; Alkimin et al., 2019), here it was hypothesized (H1) that an increase of the reducing potential can be expected, as an antioxidant response to this stress. The reducing potential involves all compounds, which can reduce the Folin-Ciocalteu agent, such as phenolic compounds, ascorbic acid, dehydroascorbic acid, and reducing sugars (e.g. glucose and fructose; Rangel et al., 2013). They act as primary antioxidants or free radical scavengers. For this reason, it is reasonable to determine their total amount in the *Lemna* extracts.

Using duckweed as a potential phytoremediator (i.e., to clean the environment in long-term exposure), the plant’s survival mechanisms might be affected when continuously exposed to DCF. Therefore, an alteration in its physiological characteristics, especially the molecular interaction and biochemical properties, during exposure to the recalcitrant DCF is expected (H2).

This study uses a (target and untargeted) analytical workflow and to evaluate the changes in the metabolic profile of *Lemna* when incubated with two concentrations of DCF 10 and 00 µM. Moreover, an untargeted metabolomics analysis strategy was conducted to characterize the metabolic profile of *Lemna* and reveal the changes due to DCF incubation.

**2. Material and Methods**

2.1. Experimental trial and sample extraction

The experiments were performed with fronds of *Lemna minor* L., grown in aquaria under controlled conditions. Each, control (untreated *Lemna*), 10, and 100 µM DCF treatment was maintained for four days in Steinberg medium at 23 °C with a photoperiod of 8-16 h and an average light intensity of 43 μmol m-2 s-1, as described by Obermeier *et al*., (2015). After freeze-drying and grinding the *Lemna*, samples were extracted with 100% MeOH and 100% H2O, separately as mentioned in (Wahman et al., 2020). Detailed experimental setup and extraction method are provided in the supplementary material.

2.2. Total reducing potential

The total reducing potential was estimated by the Folin-Ciocalteu method (Singleton *et al*., 1999) as described in Wahman et al., 2020. The quantification of phenolics was based on the standard curve (with seven nominal concentrations) generated with the use of gallic acid, and expressed as gallic acid equivalent; details are mentioned in the supplementary material as well.

2.3. Instrumental analysis and quality controls

*Lemna* metabolic profile was obtained using a reversed-phase column Poroshell 120 EC-C18 (50.0 × 3.0 mm, 2.7 μm; Agilent Technologies, Waldbronn, Germany) coupled to a ZIC-HILIC column (150 × 2.1 mm, 5 μm, 200 Å; Merck Sequant, Umea, Sweden) with a T-piece (Upchurch, IDEX Europe GmbH, Erlangen, Germany), which was connected to the HILIC flow pump. The reversed-phase liquid chromatography (RPLC) mobile phase was 10 mM ammonium acetate in water: acetonitrile (90:10, v/v) (A) and 10 mM ammonium acetate in water: acetonitrile (10:90, v/v) (B). For the HILIC, acetonitrile (C) and water (D) were used as a mobile phase. The pH value of both was about 7. The gradient data was done as described by (Wahman et al., 2020). The injection volume was 10 µL injected in triplicates. The ion masses were detected with a ‘time-of-flight’ mass spectrometer (6230 TOF-MS; Agilent Technologies, Santa Clara, CA, USA), equipped with Jet Stream ESI interface in positive electrospray ionization mode with the following parameters: 325 ºC gas temperature, 10 L min-1 drying gas flow, 325 ºC sheath gas temperature, 7.5 L min-1 sheath gas flow, 45-psi nebulizer operating pressure, and 100 V fragmentor voltage. Ions were detected in positive ionization mode with a mass range of 50-2100 Dalton. Mass accuracy calibration was performed with a reference solution that consisted of 125 nM purine and 6.25 nM HP-921 MS tuning mix (Agilent Technologies, Waldbronn, Germany) in methanol/water (90/0, v/v). The resolution of the instrument was better than 10,000 at m/z 922 (using HP-921).

Three different standard mixtures were injected in triplicate during sample analysis at regular intervals (at the beginning, middle, and end of each batch) to confirm the analytical system's robustness. The mixtures consisted of M1 (kaempferol, rutin, and taxifolin), M2 (apigenin, resveratrol), and M3 (galangin, diclofenac, flavone, vitexin, and quercetin). All compounds were injected at a final concentration of 20 µM. The mass deviation, retention time (RT), standard deviation (SD), and relative standard deviation (RSD) were calculated and used to determine the stability, reproducibility, and accuracy of the LC system.

2.4. Metabolite’s identification

AA and organic acids standards were injected at a final concentration of 20 µM into RPLC-HILIC-ESI-TOF-MS. The mean monoisotopic mass and RT for each standard were calculated in Daltons and minutes, respectively. The compounds were identified when the absolute deviation in masses and RT were Δppm ≤ 10 Dalton and ΔRT ≤ 0.3 min., respectively. The AA in *Lemna* extractswere later confirmed using more differentiated QTOF analysis, see details in the supplementary material (Table S2).

2.5. Untargeted screening workflow

All samples were analyzed with RPLC-HILIC-ESI-TOF-MS. The data was processed with Agilent Profinder B.06.00 Software (Agilent Technologies, Santa Clara, CA, USA) to perform the peak picking. The main parameters were: ppm, which represented mass deviation (set for ±10 ppm), minimum/maximum chromatographic peak height (set with a filter of 1000 counts), and chromatographic signal-to-noise threshold (set to 3X the threshold setting of the MassHunter Data Acquisition Software). The complete workflow of data analysis with different parameters is provided in the supplementary material.

2.6. Data and Statistical analyses

The extracted data was then submitted to statistical analysis to investigate the changes in the *Lemna* metabolites profile after exposure to 10 and 100 µM DCF.

Statistical analyses were performed to assess differences in the total reducing potential, for the extracts (100% MeOH and 100% H2O) between the control and the 10 and 100 µM DCF treatments, using one-way ANOVA. The total reducing potential data were initially checked for normality (Shapiro-Wilk’s test) and homogeneity of variances (Brown-Forsythe’s test). The same data was transformed to try to ﬁt the assumptions for the analysis. When not possible, a Kruskal-Wallis test was applied. Additionally, Quantile-Quantile plots were also executed to verify the [distribution functions o](https://de.wikipedia.org/wiki/Verteilungsfunktion)f the statistical variables; data were also checked for outliers (ROUT alpha = 0.05). Dunn’s comparison test (non-parametric) was applied as post-hoc tests to assess differences between treatments for the 100% MeOH and 100% H2O extracts, individually. All statistical analyses were done using GraphPad Prism version 6.00.

The extracted compound groups were imported to Mass profile and Mass Profiler Professional Software (MPP, v.13.1.1) to start the untargeted data analysis workflow and the statistical analysis (Wahman et al., 2020); details are mentioned in the supplementary material. The obtained data set (from 100% MeOH and 100% H2O extracts) were used to assess the *Lemna* metabolic profile for all the treatments (control and incubated with 10 and 100 µM DCF, individually).

The MetaboAnalyst 4.0 software was used for the generation of Partial Least Square- Discriminant Analysis (PLS-DA) score plot, and the dendrogram using the metabolic profile of the treatments: control, incubated with 10 and 100 µM DCF.

The PLS-DA was used to discriminate the similarities and differences in the metabolites profile among the different treatments. The PLS-DA module was calculated between the metabolites data (X: variables) and the permuted treatments (Y: class labels) using the optimal number of components, which were determined by cross-validation for the model based on the original class assignment (Chong et al., 2019). The most important metabolites are based on the weighted coefficients of the PLS-DA model, which were used to discriminate between different treatment metabolic profiles. These coefficients are assigned to each variable to define each component, indicating the importance of each variable in PLS-DA. Importantly, each loading vector is associated to a particular component. Loading vectors are obtained so that the covariance between a linear combination of the variables from X (the X-component) and the factor of interest Y (the Y-component) is maximized. Pre-selection criteria of 50 were established for the coefficients (is a relative measure of variability that indicates the size of a standard deviation to its [mean](https://statisticsbyjim.com/glossary/mean/)) for each treatment (control, 10, and 100 µM DCF). About 76 compounds could not be identified via the in-house database due to the limited size of the database. These compounds without identify suggestions are listed in the supplementary material, Table S4.

**3. Results**

3.1 RPLC-HILIC-ESI-TOF-MS analysis

A wide polarity range of metabolites was extracted from *Lemna* samples by using two different solvents (100% MeOH and 100% H2O, individually), allowing untargeted metabolomics analysis. The %RSD of the RT of the quality control ranged between 0.1 and 0.3%, showing a very high chromatographic robustness. Further, the mass deviation was less than 5 ppm. The results are summarized in Table S1. Subsequently, the RTs and masses were assigned to perform the untargeted analysis of the different treatments *Lemna* underwent in this study. The *Lemna* metabolic fingerprint showed low %RSD values (< 2%) indicating the robustness and the reproducibility of the method.

The RT-massplots displayed different patterns (i.e., dispersion of features according to polarity, Fig. S1). For both extracts, plots are divided into two parts: 1) the polar part (from 0-15 min. that is eluted from the HILIC phase material), and 2) the non-polar fraction (from 15 min until the end of the run, that is eluted from the RP column). Using the 100% H2O extract, significantly higher amount molecules (2232) could be separated in the HILIC phase than in the 100% MeOH extract (1965). However, in the RPLC phase, more molecules were separated and detected in the 100% MeOH (1704) than in the 100% H2O extract (986).

For the comprehensible investigation of different treatments, a scatter plot between control and incubated samples (10 and 100 µM DCF) was drawn according to the average intensity of different molecules, respectively (see Fig. S2).

The scatter plot shows a change in duckweed metabolites intensities and metabolomics upon 10 and 100 µM DCF exposure (Fig. S2). For example, looking into the part I and V of this scatter plot, some compounds are absent in the control samples and other new ones are present in the treated ones; detailed information can be seen in Table S3.

3.2. Total reducing potential

Considering the extracts independently (Fig. 1), the increasing pattern for the 100% MeOH extracts was observed, as control ˂10 µM DCF˂ 100 µM DCF while for the 100% H2O extracts, *Lemna* control, and the one incubated with 10 µM DCF samples presented approximately the same gallic acid equivalent content (ca. 730 µg GAE/g DW). Furthermore, the reducing potential increased significantly (about 1.8-fold) in samples incubated with 100 µM DCF (Fig. 1A).



**Fig. 1:**Totalreducing potential expressed as gallic acid equivalent (µg GAE/ g of dry weight (DW)) extracted from 100% MeOH (black) and 100% H2O (grey) and represented by treatments (control, 10 and 100 µM DCF); lower-case (100% MeOH) and upper-case (100% H2O) letters represent statistical differences between DCF treatments and control group (one-way ANOVA followed by the Dunn’s multiple comparisons test); (n=9) ± SD.

3.3 Metabolites identification

The changes in *Lemna* metabolic profile were investigated with reference standards to follow the changes in the intensities after 10 and 100 µM DCF. Several AA (serine, tyrosine, aspartic acid, glutamic acid, proline, histidine, phenylalanine, tryptophan, valine, alanine, isoleucine, leucine, and proline) were identified in duckweed samples extracted with 100% MeOH. The pattern of each AA was compared in the different samples incubated with 10 and 100 µM DCF against control and expressed as a relative percentage against control in 100% MeOH extracts (Table 1 and Fig. 2A).

**Table 1:** List of amino acids (AA) and organic acids detected in the different *Lemna* treatments (control, 10 and 100 µM DCF) from 100% MeOH extract; details such as name, the monoisotopic mean mass, and the corresponding mean RT, the absolute deviation in mass (Δppm), and RT (ΔRT), the intensities (inten.) of the standards and the treated samples are represented.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Name | Standard | Control | 10 µM DCF | 100 µM DCF |
| Mean Monoisotopic Mass(Da) | MeanRT(min) | Mean Monoisotopic Mass(Da) | Δppm | MeanRT(min) | ΔRT | Inten. | Mean Monoisotopic Mass(Da) | Δppm | MeanRT(min) | ΔRT | Inten. | Mean Monoisotopic Mass(Da) | Δppm | MeanRT(min) | ΔRT | Inten. |
| Serine | 105.0428 | 13.44 | 105.0433 | 4.13 | 13.45 | 0.02 | 13.74 | 105.0433 | 4.76 | 13.49 | 0.05 | 1.45 | — | — | — | — | — |
| Tyrosine | 181.0730 | 11.96 | 181.0726 | 2.49 | 11.94 | 0.02 | 5.67 | 181.0725 | 1.75 | 11.96 | 0.001 | 8.54 | — | — | — | — | — |
| Aspartic acid | 133.0375 | 12.71 | 133.0369 | 4.76 | 12.88 | 0.17 | 5.41 | 133.0375 | 0 | 12.83 | 0.12 | 12.93 | 133.0376 | 0.5 | 12.93 | 0.04 | 3.16 |
| Glutamic acid | 147.0529 | 12.63 | 147.0522 | 2.04 | 12.75 | 0.04 | 3.00 | 147.0520 | 1.81 | 12.73 | 0.02 | 25.90 | 147.0529 | 0 | 12.77 | 0.04 | 2.60 |
| Proline | 115.0636 | 12.17 | 115.0633 | 2.35 | 12.03 | 0.14 | 1.76 | 115.0632 | 3.48 | 12.40 | -0.24 | 3.33 | 115.0634 | -0.32 | 12.36 | -0.19 | 31.75 |
| Histidine | 155.0687 | 15.35 | 155.0696 | 6.02 | 14.65 | 0.7 | 9.42 | 155.0692 | 3.44 | 15.05 | 0.3 | 12.83 | 155.0704 | 3.44 | 15.23 | 0.11 | 3.11 |
| Phenylalanine | 165.0787 | 10.71 | 165.0786 | 2.30 | 10.70 | 0.01 | 0.16 | 165.0783 | 4.32 | 10.70 | 0.01 | 3.68 | 165.0799 | 5.77 | 10.67 | 0.04 | 7.27 |
| Tryptophan | 204.0899 | 11.45 | 204.0900 | 0.82 | 11.43 | 0.03 | 0.84 | 204.0898 | 0.33 | 11.43 | 0.02 | 0 | 204.0908 | 4.25 | 11.46 | 0.01 | 3.46 |
| Valine | 117.079 | 11.64 | 117.0790 | 0.28 | 11.60 | 0.03 | 3.52 | 117.0791 | 0.85 | 11.62 | 0.02 | 4.60 | 117.0796 | 5.12 | 11.71 | 0.07 | 3.68 |
| Alanine | 89.0477 | 12.84 | 89.0478 | 1.12 | 13.11 | 0.27 | 0.89 | 89.0475 | 2.25 | 12.99 | 0.15 | 12.46 | 89.0479 | 1.87 | 12.89 | 0.06 | 1.823 |
| Isoleucine | 131.0944 | 10.82 | 131.0949 | 4.07 | 10.87 | 0.04 | 12 | 131.0947 | 2.8 | 10.83 | 0.01 | 13.33 | 131.0947 | 2.8 | 10.67 | 0.16 | 13.12 |
| Leucine | 131.0941 | 10.79 | 131.0943 | 1.78 | 10.98 | 0.19 | 3.07 | 131.0947 | 4.83 | 10.68 | 0.11 | 9.94 | 131.0947 | 4.83 | 10.67 | 0.12 | 10.43 |
| Cinnamic acid\* | 148.0519 | 10.61 | 148.0521 | 1.35 | 10.6 | 0.01 | 4.55 | 148.0520 | 0.90 | 10.52 | 0.09 | 5.15 | 148.0523 | 2.70 | 10.50 | 0.11 | 3.08 |
| *p*-coumaric acid | 164.0468 | 7.72 | 164.0479 | 6.50 | 7.75 | 0.03 | 2.00 | 164.0472 | 2.64 | 7.80 | 0.05 | 1.00 | — | — | — | — | — |
| Sinapic acid | 224.0683 | 11.95 | 224.0677 | 2.83 | 12.14 | 0.19 | 2.69 | 224.0679 | 1.79 | 12.17 | 0.22 | 6.87 | — | — | — | — | — |
| *The symbol (—) means below the LOD**\*Identified only in the 100% H*2*O extract* |

The relative amount of tyrosine increased in *Lemna* 100% MeOH incubated with 10 µM DCF, while it decreased under the measurable limit in 100 µM DCF incubation. The relative amount of serine decreased in *Lemna* incubated with 10 µM DCF and fell below the LOD for the 100 µM DCF.

Further, aspartic acid, glutamic acid, histidine, valine, and alanine patterns increased in *Lemna* 100% MeOH incubated with 10 µM DCF and decreased in *Lemna* incubated with 100 µM DCF. For phenylalanine, proline, leucine, and isoleucine levels increased in both incubated samples. They were directly proportional to DCF concentration. However, tryptophan decreased in *Lemna* incubated with 10 µM DCF and increased in the 100 µM incubated sample.



**Fig. 2:** Relative intensities (%) of different metabolites identified in 10 and 100 µM DCF treatments against the control; A) amino acids (AA); B) organic acids; the asterisk symbol (\*) means intensities below the LOD.

Regarding organic acids, *p*-coumaric and sinapic acids were identified in all the samples extracted in 100% MeOH. In 10 µM DCF, these compounds presented opposite pattern; i.e., *p*-coumaric acid decreased while the sinapic acid increased. For 100 µM DCF, both were below the LOD. For cinnamic acid, it was increased and decreased in the 10 µM DCF and 100 µM DCF in 100% H2O extract, respectively, as shown in Table 1 and displayed in Fig. 2B.



**Fig.3:** Schematic figure of the investigated amino acids biosynthesis pathways in *Lemna* exposed to DCF; the green and blue colors represent the *Lemna* incubated with 10 µM DCF and 100 µM DCF, respectively.

3.4 Alterations in *Lemna* metabolic profile

The present study was built on the LC-MS metabolomics analysis to investigate the metabolic profile of *Lemna* changes due to incubation with 10 and 100 µM DCF. After assigning an accurate mass, 1177 metabolites were selected and used to differentiate between control, and 10 and 100 µM DCF treatments, which are represented in separated clusters in the PLS-DA plot (Fig. 4).



**Fig. 4** PLS-DA Scores plot of *Lemna* control (red) and 10 and 100 µM DCF treatments (green and blue, respectively) between the first and second components using both 100% MeOH and 100% H2O extracts. The explained variances are shown in brackets (95% confidence level); circles represent control, triangles represent 10 µM DCF, and diamonds represent 100 µM DCF treatment samples (n=6).

The first and second significant PLS components explained 14.0% and 8.4% of the total variance, respectively. The separation was observed clearly between treatments being more evident for the 100 µM DCF incubation. Within treatments, a high variation was observed among replicates due to the two different types of extraction (100% H2O and 100% MeOH) and biological variability.

The same data was plotted in a dendrogram of hierarchical cluster analysis for detailed visualization to illustrate the correlation between different extracts and treatments (Fig. S3). The hierarchical cluster analysis demonstrates the individual extracts behind the differentiations between the treatments.

The contribution of metabolites to the discrimination between the *Lemna* control and incubated with DCF metabolic profiles is based on the coefficients of the PLS-DA, which were given in (Table 2). A total of 108 compounds were selected, through the coefficients of PLS-DA, as important metabolites in the discrimination between the different treatments. 30% of them were detected as organic acids, lignin, sugars, AA, dipeptide, flavonoids, bioflavonoids, fatty acids, and miscellaneous (Table 2).

The concentrations (intensities) according to the Metaboanalyst software varied among treatments:

* gallic acid and truxillic acid, N-alanyl-alanine (dipeptide), Luteolin-8-C-beta-D-glucopyranoside (flavonoid), propelargonidin and n-pentadecanoic acid (fatty acids), 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, decreased due to incubation with DCF;
* syringaresinol (lignan), D-hexose and ribose (sugars), myricetin, vicenin-1 and pentamethoxyflavone (flavonoids), 1-pentadecane carboxylic acid and stearamide (fatty acids), and 2,4-dihydroxy-1,4-benzoxazine-one (DIBOA) increased in the 10 µM DCF treatment;
* 3-heptadecyl-5-methoxy phenol, 3-ketosphingosine, 5,8,11,14-eicosatetraenoic acid, oleamide, C14 fatty acids, and undecenoic acid derivatives (all fatty acids) increased in 100 µM DCF mainly to control.

**Table 2**. The relevant coefficients of compounds, produced by *Lemna,* indicate the effect of DCF incubation. The range of concentrations (intensity range: low to high) is represented by a designated color of the bar.



|  |  |
| --- | --- |
| **Compound name** | **Coefficients**  |
| **mean** | **control** | **10 µM DCF** | **100 µM DCF** |
| **Organic acids** |  |  |  |  |
| Gallic acid | 34.44 | 50.18 | 41.02 | 12.13 |
| Hydroxymethyl furfural | 55.57 | 43.52 | 40.95 | 82.25 |
| Nicotinic acid | 80.85 | 98.57 | 80.18 | 63.81 |
| Truxillic acid | 68.88 | 100.00 | 76.91 | 29.72 |
| **Lignan** |  |  |  |  |
| Syringaresinol  | 26.99 | 12.03 | 16.01 | 52.95 |
| Symplocosin | 45.59 | 39.18 | 34.92 | 62.66 |
| **Miscellaneous** |  |  |  |  |
| 1,2,3,4-Tetrahydro-β-carboline-3-carboxylic acid | 52.53 | 73.70 | 58.07 | 25.83 |
| N,N-Dimethyltryptamine | 47.08 | 47.80 | 41.05 | 52.39 |
| 5-Hydroxytryptamine | 25.27 | 8.70 | 13.06 | 54.04 |
| 2,4-Dihydroxy-1,4-benzoxanzin-34H-one (DIBOA) | 49.94 | 46.62 | 42.31 | 60.90 |
| **Sugars** |  |  |  |  |
| D-Hexose | 47.52 | 36.57 | 35.42 | 70.58 |
| Ribose | 53.29 | 47.10 | 42.01 | 70.75 |
| **Amino acids** |  |  |  |  |
| Glutamine | 30.10 | 8.59 | 13.22 | 68.48 |
| Histidine | 78.13 | 74.23 | 65.63 | 94.53 |
| **Dipeptide** |  |  |  |  |
| N-Alanyl-alanine | 41.34 | 69.82 | 52.59 | 1.62 |
| **Flavanoids** |  |  |  |  |
| Luteolin-8-C-beta-D-glucopyranoside | 50.64 | 73.15 | 57.61 | 21.15 |
| Myricetin | 40.05 | 65.73 | 47.56 | 6.86 |
| Vicenin 1 | 35.54 | 56.51 | 42.76 | 7.34 |
| Pentamethoxyflavone | 38.40 | 19.09 | 21.61 | 74.51 |
| **Biflavanoids** |  |  |  |  |
| Propelargonidin | 28.27 | 14.80 | 17.97 | 52.04 |
| Sciadopitysin  | 26.78 | 12.71 | 16.31 | 51.32 |
| **Fatty acids** |  |  |  |  |
| Propylamylcarbinol | 31.03 | 51.78 | 39.72 | 1.60 |
| 1-Monopalmitoylglycerol | 30.40 | 14.68 | 18.90 | 57.63 |
| 1-Pentadecanecarboxylic acid | 50.77 | 74.25 | 55.27 | 22.79 |
| 3-Heptadecyl-5-methoxyphenol | 33.27 | 14.95 | 18.71 | 66.15 |
| *(continued)* |

|  |  |
| --- | --- |
| **Compound name** | **Coefficient**  |
| **mean** | **control** | **10 µM DCF** | **100 µM DCF** |
| 3-ketosphingosine\* | 41.76 | 50.91 | 39.88 | 34.47 |
| 5,8,11,14-Eicosatetraenoicacid | 46.44 | 45.32 | 40.31 | 53.69 |
| Oleamide\* | 53.18 | 75.28 | 56.70 | 27.55 |
| n-Pentadecanoic acid | 42.19 | 53.87 | 43.11 | 29.59 |
| Stearamide\* | 34.71 | 50.99 | 38.11 | 15.03 |
| Undecenoic acid derviatives\* | 42.84 | 57.21 | 46.14 | 25.18 |
| C14 fatty acid  | 51.26 | 71.98 | 54.30 | 27.49 |

**4. Discussion**

In the last decades, metabolomics has been expanded especially in plant science. This approach has been used to illustrate the stress response, characterization of biomonitors, and identifying the influential metabolites (Kumar et al., 2017). The present study investigated the potential alteration in *Lemna* metabolic profile after incubation with DCF (10 and 100 µM) over four days based on the fact that a lower concentration of DCF (20 µg L-1) can already act as a signal, and might affect the circadian expression of reactive oxygen species response selected genes in lettuce (Bigott et al., 2021).

The extraction was performed with 100% MeOH and 100% H2O to study the metabolic profile due to incubation. The large difference in the polarity ensured the extraction of a wide range of metabolites. Further, the LC-system consisted of a two-column setup in serial coupling which allowed separation of a wide range of different metabolites, from very-polar to non-polar ones (Wahman et al., 2019; Wahman et al., 2020) in positive ionization mode, which is sufficient for untargeted screening strategy to obtain a global overview of the differences and similarities between samples, as suggested by De Vos and coworkers (2012).

The scatter plot (Fig S2) showed an alteration in the metabolic profile as expected; hence, some compounds disappeared and others appeared after DCF incubation; these results show that plants were capable of dealing with DCF, even at higher concentrations. Duckweed increased the production of stress-defensive compounds such as fatty acids, saturated carboxylic acids, and flavonoids (Vladimirova and Georgiyants, 2014), as we expected (H1), which was already mentioned in other studies using duckweed. For example, in 2012, Forni and coworkers revealed that *Lemna* has higher production of phenols during the first three days of treatment with sodium dodecyl sulfate (an anionic surfactant) with a higher concentration (up to 50 mg L-1) than when exposed for seven days to a lower dosage (25 mg L-1), even if the phenol content was lower than the control for the last one. Further, in 2013, Varga and co-authors showed that the total phenolic content of *Lemna* changed significantly when exposed, for 24 hours, to Hg in comparison to other metals (Cd or Cr) at concentrations ranging from 0.02 to 20 mg L-1. Furthermore, in 2020, Kostopoulou and coworkers reported that *Lemna* increases the production of aromatic amino acids (AAAs) after incubation with drugs such as glyphosate, metribuzin, and their mixture.

In the same way, the AA profile also changed. In plants, serine biosynthesis proceeds by different pathways. As known, plants use the phosphorylated pathway in response to an infection, and to environmental and abiotic stresses (Igamberdiev and Kleczkowski, 2018). In this pathway, plants produce glutamate, which, in turn, synthesizes proline. In plants, intracellular proline levels have been found to increase by more than 100-fold during stress, as observed in 100 µM DCF treatment. Rhodes and co-authors (1986), suggested that proline increased after exposure of *Lemna* to methionine sulfoximine. Later, it was been proven that the concentration of proline increased because the glutamate pool served as its precursor (Delauney and Verma, 1993). Also, the glutamic acid concentration decreased in *Lemna* incubated with metribuzin, glyphosate, and their mixtures for 72 hours (Kostopoulou et al., 2020); this may explain the decrease in the concentration of glutamic acid in our data for 100 µM DCF treatment.

Tryptophan, tyrosine, and phenylalanineare the AAAs, which are required for protein biosynthesis in all living cells. In plants, AAAs serve as precursors of a wide variety of plant natural products that play crucial roles in plant growth, development, reproduction, defense, and environmental responses such as alkaloids, phytoalexins, and indole glucosinolates as well as numerous phenolic compounds (Maeda and Dudareva, 2012). The increase in phenylalanine pattern was observed when incubated with DCF, which was accompanied by an increase of cinnamic acid, supporting once again H1. Cinnamic acid is the first compound in the phenylpropanoid pathway that begins with the deamination of phenylalanine (Maeda and Dudareva, 2012). In light of the current data, it seems that the phenylpropanoid pathway is induced, which protects *Lemna* from oxidative stress against DCF, increasing the patterns of myricetin, syringaresinol, pentamethoxyflavone, and vicenin 1. Phenylpropanoids also increased to protect *Lemna* against reactive oxygen species (ROS) (Buchanan et al., 2015). The biflavonoids pattern decreased in both treatments reaching lower levels for the highest concentration (100 µM DCF treatment). The flavonoids content also decreased, when *Lemna gibba* was exposed to several environmental challenges (stressors); according to Akhtar and co-authors (2010), this might be a result of the promotion of the photosynthetic electron transport chain reduction, causing flavonoid reduction. Moreover, *p*-coumaric and sinapic acid decreased for the 100 µM treatment, which can be related to the alternative usage of NADPH to degrade DCF instead of producing these organic acids (Huber et al., 2012); the same profile was also reported by Kostopoulou et al., (2020).

It has been known that the utilization of glutamate decreases the intensity of tryptophan since it uses glutamine in its biosynthesis (Delauney and Verma, 1993). The same was observed in the current study with *Lemna* incubated with 10 µM DCF. Interestingly, the opposite behavior was observed for the plants incubated with 100 µM DCF, which might be due to over-expression of tryptophan synthase β gene-like like it happened for plants treated with Cd (Sanjaya et al., 2008). Higher DIBOA levels were observed for 10 µM DCF incubation. As we know, DIBOA is synthesized by tryptophan synthase α that catalyzes its formation from indole (as a precursor) instead of tryptophan (Frey et al., 1997; Buchanan et al., 2015).

Aspartic acid increased in *Lemna* incubated with 10 µM DCF, which might be due to an increase of isoleucine and valine synthesis. Rhodes and coworkers (1986), expected an increase of isoleucine due to protein degradation, in *Lemna* exposed to methionine sulfoximine, for 24 hours. However, a recent study indicated that the increase is due to aspartic acid catabolism, such as in a study where *Arabidopsis* was subjected to bacterial infection (Yang and Ludewig, 2014). However, for the 100 µM DCF incubation, the opposite was observed for aspartic acid. This might be a response to an abiotic stress (in this case, the high concentration of DCF) which aspartic acid can be used to provide energy to the tricarboxylic acid cycle (TCA), as suggested by (Galili, 2011).

The alanine pattern fluctuated significantly between *Lemna* incubated with 10 µM and 100 µM DCF. The same was observed in plant response to hypoxic stress, which was accompanied by higher rates of glycolysis and ethanol fermentation causing fast depletion of sugar stores and carbon stress (Limami et al., 2008). Consequently, in 10 µM DCF incubation, hexoses such as glucose, galactose, mannose, raffinose, and ribose, potentially increased due to higher energy demand to tolerate the stress effect of DCF incubation through exhaustion of sugar stores. Also, it has been reported that non-soluble sugars have osmoprotectant and antioxidant activities (Sivaram et al., 2019). However, in 100 µM DCF incubation, their concentrations decreased which might be due to the fluctuation of sugars due to mechanisms that are affected by changes in the genotype (e.g., INV, SuSy, ATB2 bZIP, and α-amylase gene) and stress factors (Rosa et al., 2009).

Similar patterns were also reported for *Lemna* exposed to pesticides (Kostopoulou et al., 2020). In that experiment, aspartic acid, isoleucine, and valine concentrations increased after 72 hours of exposure, while for alanine, the scenario was not consistent, changing among pesticide treatments.

The untargeted screening can assign important variables without reference standards. In the current study, control samples presented higher concentrations of gallic, nicotinic, and n-pentadecanoic acids than samples incubated with 100 µM DCF or 10 µM DCF.

Gallic acid plays an important role in plant defense against stress conditions. This finding is illustrated by the increase in phenylalanine and the concomitant decrease in *p*-coumaric, sinapic, and truxillic acids in DCF incubated samples. Gallic acid has two pathways either through phenylalanine or 5-dehydroshikimic acid. It seems that *Lemna* preferred the formation of phenylalanine → protocatechuic acid → gallic acid through the β-oxidative pathway when incubated with DCF. This pathway was observed in the mutant strain of *Neurospora crassa* that blocks the conversion of 5-dehydroshikimic acid into shikimic acid leading to a strict production of gallic acid from protocatechuic acid (Dewick and Haslam, 1969).

The syringaresinol that is biosynthesized from sinapic alcohol by peroxidase enzymes (Habib et al., 2018), increased in 10 µM DCF treatment; this is an expected result of activation of peroxidase and oxidase enzymes in *Lemna* by DCF (Alkimin et al., 2019).

Moreover, the sinapic acidintensity increased together with syringaresinol, showing that 10 µM DCF evoked the phenylpropanoid pathway as expected (H1 and H2). Regarding 100 µM DCF treatment, the saturated and unsaturated fatty acids, and sphingosine concentrations increased to protect the *Lemna* against the ROS because of stress degradation, which are produced in the presence of DCF and/or its transformation products under a high dose of DCF (Alkimin et al., 2019). Therefore, the n-pentadecanoic acid concentration also increased.

Saturated and unsaturated fatty acids induce broad-spectrum resistance against infections in the plant, such as *Pseudomonas syringae* in tomatoes (Lim et al., 2017). Some of them (stearic, oleic, and palmitic acids) increased in *Lemna* incubated with glyphosate, and glyphosate metribuzin mixture for 72 hrs (Kostopoulou et al., 2020); this might explain the increase in reducing potential in the 100 µM DCF treatment, because of unsaturated fatty acid formation/accumulation.

**5. Conclusions**

Metabolomics analysis of *Lemna* incubated with 10 and 100 µM DCF was performed using RPLC-HILIC-ESI-TOF-MS. The spectral and statistical results showed changes in the metabolic profile of *Lemna* due to DCF incubation after 4 days. The PLS-DA analysis identified the significant differences between the control, samples treated with 10 and 100 µM DCF, respectively. The AA and organic acids exhibited changes in their intensities as a response to DCF incubation, mostly for the highest concentration being related to stress defense mechanisms. However, it could not explain the causality, in some points, as for the organic acids. The untargeted strategy enabled the investigation of changes in *Lemna* metabolic profile such as organic acids, lignin, sugars, AA, dipeptides, flavonoids, bioflavonoids, fatty acids, and some others more. Hence, untargeted metabolomics has a fundamental function in determining the metabolic changes in plants due to xenobiotics exposure. These results provided insights into untargeted metabolomics as they serve as a workflow to monitor the changes in *Lemna* metabolic profile that can be extended to target quantitative studies. In conclusion, *Lemna* responded differently to both treatments, showing that concentrations have a great impact on the metabolic profile of this aquatic plant, as it was also observed with other anthropogenic stressors (like pesticides). Such a difference in reactions might influence the efficiency of phytoremediation or productivity of aquatic species.

**6. CRediT author statement**

**R. Wahman:** conceptualization, methodology, validation, formal analysis, investigation, writing-original draft, visualization; **C. Cruzeiro:** conceptualization, formal analysis, writing-review and editing, visualization, supervision; **J. Grassmann:** writing-review and editing, supervision; **P. Schröder:** conceptualization, methodology, resources, writing-review, and editing, supervision; **T. Letzel:** conceptualization, methodology, resources, writing-review and editing, funding acquisition, supervision.

**7. Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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