Performance of chlorophyll \textit{a} fluorescence parameters in \textit{Lemna minor} under heavy metal stress induced by various concentration of copper

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The objective of the present investigation was to understand the efficacy of chlorophyll fluorescence analysis and to identify the specific photosynthetic parameters for early and rapid detection of Cu-induced HM-stress in plants. Aquatic angiosperm \textit{Lemna minor} was exposed to various concentrations (0–40 µM) of Cu. We observed that the $F_v/F_m$ (Efficiency of the water-splitting complex on the donor side of PSII), quantum yield for electron transport, and quantum yield of primary photochemistry were decreased however, dissipated quantum yield was increased with Cu concentration. \textit{ABS/CSM}, \textit{TRo/CSM}, \textit{ETo/CSM} and maximum quantum yield were displayed the dose–response relationship under Cu stress. Performance indexes were increased initially due to the beneficial effects of Cu at lower concentration while decreased significantly ($p \leq 0.05$) at highest concentration of Cu. The outcomes of the present research revealed that the ChlF analysis is very sensitive tool that can be used to determine the toxicity of heavy metals in plants.

In nature, plants are continually exposed to abiotic and biotic stresses. Heavy metals (HMs) like Hg, Cu, Pb, Zn, Ni, Co, Mn, As, etc. have been accumulating in soils for a long time due to anthropogenic activity such as the use of chemical fertiliser, sewage, industrial and smelting wastes. HMs are non-biodegradable elements that cannot be eliminated from the environment through natural processes. Some of them are said to be immobile, that unable to move from the site where they have accumulated, others are referred to as mobile because they can be taken up by the root system of plants via diffusion, endocytosis, or metal transporters. However, some metals, such as zinc, copper (Cu), nickel, etc. are important micronutrients that must be absorbed in small amounts as cofactors for several enzymes. Besides these, some HMs found in pesticides such as Cd, Pb, Hg etc. have no beneficial properties and become harmful when their concentration exceeds a threshold limit. These HMs may or may not be essential for the proper growth of plants but accumulate in the plants (as a natural accumulator) from soil and water. Metal accumulation rates and plant tolerance vary from species to species. Some of the HMs become more toxic than others causing chlorosis, stunted growth, root browning, and mortality are some of the apparent indicators of HM toxicity in plants.

Cu (Cu) is an important element in plants that serves several functions at the physiological and molecular levels. However, the excessive levels of it might constitute a risk to the survival of plants. Cu is a key component of plastocyanin and cytochrome oxidase that are essential for photosynthesis and respiration which have a crucial function in plant carbon assimilation and ATP generation. Cu-stressed plants exhibit a variety of visible symptoms, including chlorosis, stunted development, ion leakage and reduced root growth. Excessive levels of Cu in plants can lead to oxidative stress that causes severe damage to membranes and macromolecules, as well as having a negative impact on many metabolic pathways. Neelima and Reddy investigated the effects of Cu in \textit{Solanum melongena} seeds and revealed that excess Cu reduces germination, seedling length, and root number. All these consequences are extremely harmful to the plant.

Chlorophyll \textit{a} fluorescence (ChlF) is a commonly used method to detect plant stress conditions in plant research, frequently in association with other morphological, chemical, and physiological variables. Chl \textit{a} fluorescence (ChlF) is the natural phenomenon describing the dissipation and heat radiation or re-emission of
the portion of absorbed energy which is not utilised to drive photosynthesis\textsuperscript{23, 41–46}. ChlF measurement provides information about changes in photosynthetic efficiency and heat dissipation\textsuperscript{47, 48}. It is an incredibly simple, non-invasive, extremely sensitive, rapid, and accurate method and providing a quantitative assessment of oxygenic photosynthesis\textsuperscript{49}. Plants exposed to HM ions disrupt photosynthesis as a result of a single or cumulative event of HM interaction with protein which increase the rate of ROS generation and which replaces essential kations in protein active centers\textsuperscript{50}.

Some HM ions, for example, Cu, Hg, Cd, Zn, or Ni can replace the core Mg ion in chlorophyll molecules, resulting in chlorophyll-metal complexes and a reduction in PSII quantum efficiency\textsuperscript{49–51}. Apart from evaluating specific parameters, of which the F\textsubscript{v}/F\textsubscript{o} and F\textsubscript{v}/F\textsubscript{m} are the most well-known and extensively utilised, the interpretation of double normalised curves using the JIP test is becoming increasingly popular in environmental research practices\textsuperscript{42}. Plots are formed using data collected at a high sampling rate within a second of the dark-adapted leaf being exposed to light, as the independent variable on a logarithmic timeline. On such plots, inflection points (J–I–P) are noticed when the recorded fluorescence increases which provide the foundation for inferences regarding the photosynthetic apparatus’ structure and function. the O–I–P transient is prime source of observed variations in the efficiency of the chlorophyll antenna in capturing light energy and transfer to plastoquinone Q\textsubscript{A} (the electron acceptor) is the only limitation of photochemical conversion in PSII\textsuperscript{52, 53}. Even though, ChlF there are years of in-depth expertise, valid interpretations of ChlF data still require more research\textsuperscript{54}. ChlF measurement has become a simple, effective, and dependable technique for outdoor environmental research to improve knowledge and current technology\textsuperscript{42, 43, 45, 55–59}.

HM pollution is becoming more prevalent in the environment, demanding rapid and effective solutions for metal remediation. The use of metal-accumulating plants for remediation has recently given rise to a new technology known as phytoremediation\textsuperscript{60}. An ideal hyperaccumulator plant species must meet two requirements for this technology to be viable are HM tolerance and accumulation. Consequently, a better knowledge of the metal tolerance mechanism(s) is critical for the development of effective phytoremediation techniques\textsuperscript{64}. The chlorophyll a fluorescence has long been used to measure the effects of environmental stress on plants, because they provide a quick approach to determine injury in the absence of visual signs\textsuperscript{62, 63}. Therefore, the ability to identify the toxic effects before any morphological symptoms can be seen makes phytoremediation an extremely effective method for identifying metal-tolerant plants.

Duckweeds have high potential to grow under HM stress because of their potential to bioremediate HMs through either by rhizofiltration or phytotransformation. Therefore, besides use in bioremediations, duckweeds serve a rich source of essential HMs such Cu and Zn for improving feed efficiency of animals\textsuperscript{64}.

Chlorophyll (Chl) a fluorescence signals have become one of the potent indicators for early detection of HMs in soil and aquatic bodies\textsuperscript{25, 65, 66}. In the present study, we used the chlorophyll (Chl) a fluorescence transient to investigate the effects of HM stress induced by various concentration of Cu in \textit{L. minor} plants grown in a nutrient medium.

Materials and methods

\textbf{Plant material and growth condition.} \textit{L. minor} plants were collected from the region of Ayad river located at Udaipur, India (24° 35’ 14.97” N, 73° 42’ 14.97’’ E) (As per the Biological Diversity act, 2002 of National Biodiversity Authority of India, the Indian researchers neither require prior approval nor need to give prior intimation to SBB for obtaining biological resource for conducting research\textsuperscript{67}). The plant was identified by Dr. Vineet Soni based on the morphological characteristics (oval shaped fronds, 2–5 fronds remained together, presence of three nerves in each frond and cylindrical root sheath with two lateral wings). The collected fronds (stock culture) were maintained in plastic (PVC) aquariums in Jacob culture media as per the OECD guideline of 2002\textsuperscript{68}. The stock culture and Cu treated plants were kept in controlled conditions at 150–230 µmol/m\textsuperscript{2}/s (PAR) by using white fluorescent light, 14:10 h light: dark cycle, and 25/20 °C day/night temperature. This medium consisted of the following: Stock solution (A): Ca (NO\textsubscript{3})\textsubscript{2}, 60.0 g/L, Stock solution (B): MgSO\textsubscript{4}·7H\textsubscript{2}O, 102.0 g/L, KNO\textsubscript{3}, 100.0 g/L; KH\textsubscript{2}PO\textsubscript{4}, 14.0 g/L, Stock solution (C): H\textsubscript{3}BO\textsubscript{4}, 0.300 g/L; MnCl\textsubscript{2}·4H\textsubscript{2}O, 0.3145 g/L; ZnSO\textsubscript{4}·7H\textsubscript{2}O, 0.0356 g/L; Na\textsubscript{2}MoO\textsubscript{4}·2H\textsubscript{2}O, 0.0118 g/L, Stock solution (D): CuSO\textsubscript{4}·5H\textsubscript{2}O, 0.0125 g/L; FeEDTA (Ethylenediaminetetraacetate acid), 1.8520 g/L. The stock culture (stock culture) were maintained in plastic (PVC) aquariums in Jacob culture media as per the OECD guideline of 2002\textsuperscript{68}. The stock culture and Cu treated plants were kept in controlled conditions at 150–230 µmol/m\textsuperscript{2}/s (PAR) by using white fluorescent light, 14:10 h light: dark cycle, and 25/20 °C day/night temperature. This medium consisted of the following: Stock solution (A): Ca (NO\textsubscript{3})\textsubscript{2}, 60.0 g/L, Stock solution (B): MgSO\textsubscript{4}·7H\textsubscript{2}O, 102.0 g/L, KNO\textsubscript{3}, 100.0 g/L; KH\textsubscript{2}PO\textsubscript{4}, 14.0 g/L, Stock solution (C): H\textsubscript{3}BO\textsubscript{4}, 0.300 g/L; MnCl\textsubscript{2}·4H\textsubscript{2}O, 0.3145 g/L; ZnSO\textsubscript{4}·7H\textsubscript{2}O, 0.0356 g/L; Na\textsubscript{2}MoO\textsubscript{4}·2H\textsubscript{2}O, 0.0118 g/L, Stock solution (D): CuSO\textsubscript{4}·5H\textsubscript{2}O, 0.0125 g/L; FeEDTA (Ethylenediaminetetraacetate acid), 1.8520 g/L. The stock solutions were kept in a refrigerator and growth media prepared by adding 10 mL of each stock to 1 L of distilled water and then adjusting the pH 6.0 using NaOH or HCl\textsuperscript{69}.

\textbf{Cu exposure.} For the ChlF experiment ~ 30 two or three-fronded, healthy plants (300 mg) were taken from stock culture and transferred to glass bottle containing 250 mL of growth medium without EDTA and exposed to various concentrations of CuSO\textsubscript{4}·5H\textsubscript{2}O (Sigma Aldrich, C8027, ≥ 98%) (0, 10, 20, 30, and 40 µM) for 24 h. The metal exposure experiments were performed according to procedure described by Teissere and Guy using EDTA free growth medium since it is a chelating agent and alter the metal adsorption process in plants (can increase the bioavailability of metal)\textsuperscript{70}. Control plants were grown under both EDTA and Cu free growth medium. The experiment glass bottles were placed in a controlled environment as described above.

\textbf{Chlorophyll a fluorescence transient.} ChlF was measured using a plant efficiency analyser (Handy PEA fluorimeter, Hansatech instruments Ltd. England). Before measurement fronds were dark-adapted for 50–60 min at 26 °C. Thereafter, ChlF signals were analysed with the Biolyzer v.3.0.6 software (developed by Laboratory of Bioenergetics, University of Geneva, Switzerland). The experiments were done in six replicates and repeated three times to ensure the results. JIP-test method has been developed by which several selected phenomenological and biophysical parameters quantifying the PSI and PSII behaviors are calculated. Several parameters can be derived from the polyphasic ChlF rise (OJIP curve) that provide information about photo-
synthetic fluxes. Abbreviations, formulas, and definitions of the JIP-test parameters used in the current study are presented in Table 1.

### Principal component analysis (PCA), grid correlation matrix and heat map.

The relations between the selected JIP-test parameters were tested by Principal Component Analysis. ChlF parameter was selected for the PCA analysis to classify the variables that show the maximal fluctuations. Dimension 2 (PC 2) described the maximum of the variability which accounted for 79.15% and dimension 1 (PC 1) accounted for 18.17%, respectively. The positive and negative correlation between the parameters also shows the variation of the parameters in the respective principal components (dimensions) (Table 2). The correlation between all ChlF parameters investigated in this paper were analysed through grid correlation matrix by using Python software which expressed between + 1 and − 1 with colour code. The calculated JIP parameters were also presented by the heat map, through normalizing them between 1 and 100 by using a color code green to red.

### Statistical analysis.

Statistical analysis was performed using analysis of variance (ANOVA), followed by a Tukey HSD test ($p = 0.05$) using XLSTAT 2020. Only significant values ($p \leq 0.05$) of measurements are presented in figures. The heat map was prepared by normalizing the values and bringing them all to a range between 1 and 100 to provide an unbiased color code. Three color code combination of red for high (100%), yellow for medium (50%), and green for the lowest value (1%) was used to represent the heat map. The MS excel and CorelDraw software were used for calculation and designing of the heatmap. In addition, a principal component analysis (PCA) was conducted by eigenvalue decomposition of a data correlation matrix using OriginPro 2016. PCA was applied to find the patterns of the fluorescence parameter and variations in the experimental data. The 48-h lethal dose (LD50 and LD90) was determine by Probit Analysis using SPSS (22.0). Comparision of mortility ratios between experimental and control groups in the deferent concentrations was performed with Chi-square testing.

### Results

Cu stress significantly altered the growth and productivity of L. minor through the modulation of the photosynthetic process. In the present studies, impacts of Cu-induced HM stress on ChlF kinetics, specific energy fluxes, phenomenological energy fluxes, and performance indexes were studied in L. minor.

### ChlF rise.

ChlF rise of L. minor was measured after 24 h of Cu treatment and a typical OJIP induction curve was displayed when plotted on the logarithm time scale (Fig. 1). With increasing the Cu concentration, the fluorescence yield at various intermediary steps, such as J, I, and P was reduced. In control plants, two intermediate peaks $F_1$ (chlorophyll fluorescence at 2 ms) and $F_2$ (chlorophyll fluorescence at 300 ms) were formed between $F_0$ and $F_m$. ChlF increased continuously from initial ($F_{0}$) to maximal ($F_m$) fluorescence intensity in L. minor growing under control conditions. HM induced reduction in PSII photochemistry and electron transport activity were severe at the highest concentration of Cu.
### Basic parameters calculated from the extracted data

| Parameter | Description | Formula |
|-----------|-------------|---------|
| \( F_{O} \) | Fluoence when all PSII RCs are open (\( \geq \) to the minimal reliable recorded fluorescence) | \( F_{O} \) |
| \( T_{M} = t_{F_{M}} \) | Time (in ms) to reach maximal fluorescence \( F_{M} \) | \( T_{M} = t_{F_{M}} \) |
| \( F_{M} = F_{O} \) | Maximal fluorescence, when all PSII RCs are closed (= \( F_{O} \) when the actinic light intensity is above 500 \( \mu \text{mol} \text{photon} \text{m}^{-2} \text{s}^{-1} \) and provided that all RCs are active as \( Q_{A} \)-reducing) | \( F_{M} = F_{O} \) |
| \( F_{V} = F_{O} - F_{O} \) | Maximal variable fluorescence | \( F_{V} = F_{O} - F_{O} \) |
| \( S_{M} = \text{Area}/(F_{M} - F_{O}) = \text{Area}/F_{V} \) | Normalised Area to \( F_{V} \) | \( S_{M} = \text{Area}/(F_{M} - F_{O}) = \text{Area}/F_{V} \) |
| \( N = S_{M} \times (M_{F}/V_{F}) \) | Turnover number (expresses how many times \( Q_{A} \) is reduced in the time interval from 0 to \( F_{O} \)) | \( N = S_{M} \times (M_{F}/V_{F}) \) |
| \( V_{J} = (F_{J} - F_{O})/(F_{M} - F_{O}) \) | Relative variable fluorescence at \( t = 2 \text{ ms} \) | \( V_{J} = (F_{J} - F_{O})/(F_{M} - F_{O}) \) |
| \( V_{O} = (F_{O} - F_{M})/(F_{M} - F_{O}) \) | Relative variable fluorescence at \( t = 30 \text{ ms} \) | \( V_{O} = (F_{O} - F_{M})/(F_{M} - F_{O}) \) |

### Biophysical parameters derived from the basic parameters

#### Deexcitation rate constants of PSII antenna

- \( k_{0} = (ABS) \times k_{F} \times (1/F_{O}) \)
- \( k_{0} = (ABS) \times k_{F} \times (1/F_{O} - 1/F_{O}) = k_{0} \times (F_{O}/F_{O}) \)

#### Specific energy fluxes (per RC: QA-reducing PSII reaction centre), in \( \text{ms}^{-1} \)

- \( \text{ABS/RC} = M_{A} \times (1/V_{J}) \times (1/\phi_{P}) \)
- \( \text{TR}_{O}/\text{RC} = M_{A} \times (1/V_{J}) \)
- \( \text{ET}_{O}/\text{RC} = M_{A} \times (1/V_{J}) \times (1-V_{J}) \)
- \( \text{DI}_{O}/\text{RC} = \text{ABS/RC} - \text{TR}_{O}/\text{RC} \)

#### Phenomenological energy fluxes (per CS: QA-reducing PSII cross section), in \( \text{ms}^{-1} \)

- \( \text{TR}_{O}/\text{CS}_{O} = (F_{O}/F_{O}) \times \text{ABS/CS}_{O} \)
- \( \text{ET}_{O}/\text{CS}_{O} = (F_{O}/F_{O}) \times (1-V_{J}) \times \text{ABS/CS}_{O} \)
- \( \text{DI}_{O}/\text{CS}_{O} = \text{ABS/CS}_{O} - \text{TR}_{O}/\text{CS}_{O} \)
- \( \text{ABS/CS}_{O} = \text{Fo} \)

#### Quantum yields and efficiencies

- \( \phi_{D} = \text{TR}_{O}/\text{ABS} = [1 - (F_{O}/F_{O})] \quad \text{Maximum quantum yield for primary photochemistry} \)
- \( \phi_{E} = \text{ET}_{O}/\text{ABS} = [1 - (F_{O}/F_{O})] \times (1-V_{J}) \quad \text{Quantum yield for electron transport (ET)} \)
- \( \phi_{E} = \text{ET}_{O}/\text{TR}_{O} = (1-V_{J}) \quad \text{Efficiency/probability that an electron moves further than \( Q_{A} \)} \)
- \( \phi_{D} = F_{O}/F_{O} \quad \text{Quantum yield (at } t = 0 \text{) of energy dissipation} \)

#### Performance indexes

- \( \Pi_{\text{ABS}} = \frac{(F_{O}/F_{O})}{M_{A}/V_{J}} \times \frac{F_{O}}{F_{O}} \times \frac{1-V_{J}}{V_{J}} \quad \text{Performance index for energy conservation from photons absorbed by PSII until the reduction of intersystem electron acceptor} \)
- \( \Pi_{\text{CS}} = \frac{\text{ABS}/(F_{O}/F_{O}) \times \frac{F_{O}}{F_{O}} \times \frac{1-V_{J}}{V_{J}}}{\text{TR}_{O}/\text{ABS}} \quad \text{Performance index on cross section basis} \)

### Abbreviations, formulas, and definitions of the JIP-test parameters.

#### Biophysical parameters derived by the ‘JIP-test’ equations

- \( F_{O} \) and \( F_{M} \): The minimal fluorescence intensity \( (F_{O}) \) and the maximum fluorescence intensity \( (F_{M}) \) both are decreased with increasing the Cu concentration (Fig. 2A). Fluorescence intensity recorded at 50 µs is denoted as \( F_{O} \) and at this time the all primary quinone acceptor \( (Q_{A}) \) is in the open (oxidized) state.

- The primary maximum yield of photochemistry of PS I \( (F_{O}/F_{O}) \) are related with photosynthetic efficiency of plant and increased value of \( F_{O}/F_{O} \) indicates proper functioning of PSII. The \( F_{O}/F_{O} \) ratio (ratio between the rate constants of photochemical and nonphotochemical deactivation of excited Chl molecules) for \( L. \ minor \) plants decreased gradually at 10 µM (94.86% of control) and 30 µM (89.44% of control) concentration of Cu (Fig. 2A). Further, a significant decline in \( F_{O}/F_{O} \) ratio (43.58% of control) was recorded at a high level (40 µM) of Cu exposure as a result of a significant decrease in \( F_{O} \) (41.00% of control) as shown in Figs. 2A and 3.

- The relative variable fluorescence at 2 ms \( (J \text{ step}) \) is denoted as \( V_{J} \) which is the measure of the fraction of primary quinone electron acceptor of PSII in its reduced state \( [Q_{A} - Q_{A \text{ reduced}}] \). At the lower level of HM stress, a slight reduction in the value of \( V_{J} \) was observed but with increasing the metal concentration to a high level the \( V_{J} \) value was increased to 224.74% of control (Figs. 2A, 3).

- Complimentary Area \( (S_{M}) \) is also an important parameter that is directly proportional to the number of reduc-tion and oxidation of one \( Q_{A} \) molecule during the fast OJIP transient or number of electrons passing through the electron transport chain. The turnover number \( (N) \) is represented as the number of times \( Q_{A} \) becomes reduced and re-oxidized another time, till the \( F_{M} \) (Maximum fluorescence intensity) is reached. At severe Cu stress the increased value of turnover number \( (N) \) value was recorded (145.40% of control) which was also represented by PSI cyclic electron transport as photoprotection (Fig. 2A). The increased values of \( S_{M} \) in \( L. \ minor \)
under Cu exposure (131.40% of control at moderate HM treatment) displayed the reduced electron transport between these photosystems.

Quantum yield. The quantum yield of primary photochemistry $F_{\text{v}}/F_{\text{m}}$ ($\phi_{\text{Po}}$), which reflects the overall photosynthetic potential of active PSII reaction centers, was not affected by Cu-induced HM stress in plants. However, a slight decline in $F_{\text{v}}/F_{\text{m}}$ was recorded at 40 µM Cu (Fig. 2C). A similar trend was observed in ET/ABS ($\phi_{\text{Eo}}$) (Figs. 2C, 3). The lowest values of $\phi_{\text{Eo}}$, approximately half of control, were recorded when $L. \text{minor}$ was subjected to 40 µM Cu. In contrast, DI/ABS ($\phi_{\text{Do}}$) remained almost the same until the exposure of 30 µM Cu and thereafter enhanced about two folds of the control level in plants grown in media containing 40 µM Cu (Figs. 2C, 3).

Specific energy flux (membrane model). The specific energy fluxes such as absorption flux per reaction center (ABS/RC), trapped energy flux per reaction center (TRo/RC), electron transport flux per reaction center (ETO/RC), and dissipated energy flux per reaction center (DIO/RC) were analyzed to determine the photosynthetic performance of active PS II reaction centers of $L. \text{minor}$ subjected to various concentrations of Cu (Figs. 3, 4). Up to 30 µM Cu, no significant variations in absorption flux per reaction center (ABS/RC) was recorded while at 40 µM, a remarkable enhancement in absorption potential of active reaction centers was recorded (162.83% of control) (Figs. 3, 4). A similar trend in TRo/RC was observed as shown in the heatmap (Fig. 3). TRo/RC remained almost constant up to 30 µM Cu and thereafter increased with increasing the severity of Cu-induced HM stress (129.68% of control). In contrast, no significant changes in electron transport flux per reaction center (ETO/RC) were recorded up to 20 µM Cu concentration while decreased at severe Cu stress (73.58% of control). On the contrary, the DIO/RC remained constant up to 30 µM Cu and then increased about three folds with the progression of Cu concentration (297.67% of control) as shown in Fig. 2B. The effects of Cu-induced HM stress on the specific energy fluxes (absorption flux per reaction center, trapped energy flux per reaction center, electron transport flux per reaction center, and dissipated energy flux per reaction center) are presented diagrammatically through thylakoid membrane models (Fig. 4). It is of interest to investigate if HM stress changes the ratio among antenna light-harvesting complex (ABS) and active PSII reaction centers (RC). According to the leaf pipeline model in severe Cu stress, there is more active RC and the higher value of specific energy flux (ABS/RC, TRo/RC, and DIO/RC) shows the increased ability of RC to the reduction of plastoquinone (Fig. 4).

Phenomenological energy flux (leaf model). Phenomenological energy fluxes mean absorption flux per cross-section (ABS/CS), trapped energy flux per cross-section (TRo/CS), electron transport flux per cross-section (ETO/CS), and dissipated energy flux per cross-section (DIO/CS) significantly modulated by Cu-induced HM stress in $L. \text{minor}$. Absorption flux per cross-section (ABS/CS) did not alter with increasing concentrations of Cu up to 30 µM (Fig. 5). The lowest values of ABS/CS, TRo/CS, and ETO/CS were noticed at the highest concentration of Cu (92.09%, 73.34% and 41.61% of control respectively). Absorption potential per cross-section
significantly declined when plants were treated with 30 µM Cu for 48 h. TRO/CSM reduced remarkably up to 30 µM Cu and thereafter declined up to 50% with increasing concentration of Cu (Fig. 5).

Electron transport efficiency of plants was found tolerant to Cu-induced HM stress. A high concentration of Cu (40 µM) extremely reduced the electron transfer system in thylakoid membranes (Fig. 5). DIO/CSM was increased significantly ($p \leq 0.05$) at 30 µM Cu treatment and after that decreased slightly.

KP and KN. De-excitation rate constants for nonphotochemical reaction ($K_N$) increased under Cu stress and at severe stress conditions $K_N$ value approaches 198.46% of the control (Figs. 2C, 3). While de-excitation rate constants for photochemical reaction ($K_P$) lowered slightly (86.48% of control) at 40 µM Cu concentration.

Performance index. Overall effects of Cu-induced HM stress on various photosynthetic parameters are presented in the form of a radar plot (Fig. 2). To analyze the effects of Cu-induced HM stress on overall photosynthesis performance, PIABS and PICS were determined in L. minor exposed to various intensities of Cu stress. Cu stress led to a significant effect on the performance index on absorption basis (PIABS) and performance index of PS II and PS I (PICS) in L. minor. PIABS and PICS continuously increased with increasing concentration of Cu up to 30 µM, and then declined sharply with the progression of Cu-induced HM stress. The lowest performance index on absorption basis (PIABS) and performance index of PS II and PS I (PICS) were recorded in plants cultivated on media containing 40 µM Cu (Figs. 2C, 3).

Figure 2. Radar plots (A–C) showing various technical fluorescence parameters. Each line represents the average of 6 measurements per treatment. Asterisk denote the significance at $p \leq 0.05$ level.
The PCA results displayed that Dim 1 and Dim 2 represented 97.32% of the variation in the ChlF parameter under Cu induced HM stress in *L. minor* (Fig. 6). The loadings for ABS/CSO, ETo/RC, TRo/CSO, ETo/CSO, Fv/Fm, PICS and PIABS are in quadrate I and IV while, TRo/RC, ABS/RC, DIO/RC, DIO/CSO and FO/FM are accounted in quadrate II and III. All treatments, except 40 µM, are located in quadrate I and IV. The loading arrow of 40 µM is longer than others in all quadrates. Thus, the higher concentration of Cu was significantly affecting the major JIP parameters located in quadrate II and III. The mild Cu stress up to 20 µM was less toxic as compared to severe stress and plants performed better which was described by performance index parameters in quadrate IV (Fig. 6).

In Table 3, values of the lethal dese responsible for 50% mortality (LD50) and 90% mortality (LD90), calculated by probit analysis with 95% probability level, are given. The LD50 and LD90 values of Cu was 25.70 µM and 87.80 µM respectively.

**Discussion**

Many studies on the plant’s physiological changes under various HM stress have been reported. These studies indicated that plants have developed a series of mechanisms to protect themselves from these adverse environmental threats. ChlF analyses have been shown to detect complex biochemical alteration in photosynthetic
apparatus in a vast range of plant species, including both terrestrial and aquatic\(^8\). The present investigation shows the Cu induced changes in various fluorescence parameters of photosystem II in duckweed \textit{L. minor}.

**ChlF rise.** Excess energy enhanced the utilisation capacity of plants in extreme environments, after which photoprotective systems quenched the extra Chl radical, and the extra energy was dissipated as heat\(^79, 81, 82\). The reduction in ChlF from PSII is being used to quantify these mechanisms, which are jointly referred to as non-photochemical quenching (NPQ)\(^41, 83, 84\). The J-I and I-P, and the J step still appeared at the severe Cu stress (40 µM), indicating the tolerance capacity of the plant\(^85\). The O-J is a photochemical phase and J-I-P is a thermal phase, these are two characteristics of OJIP transient and presented three various reduction processes of the electron transport chain\(^52, 74, 86, 87\). The photochemical phase (O-J) is mainly light-dependent and comprises

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**Figure 5.** Energy pipeline leaf model of phenomenological fluxes (per cross section, CS) in \textit{L. minor} fronds when subjected to various concentration of Cu, (A); control, (B); 10.0 µM, (C); 20.0 µM, (D); 30.0 µM and (E); 40.0 µM.

**Figure 6.** The principal component analysis with four Cu treatment conditions. The PCA is based on the chlorophyll fluorescence data. Arrows represent the Chlorophyll \textit{a} fluorescence parameter on the corresponding dimensions (PC 1 and PC2), where PC 2 expressed most of the variability in the data.
information regarding antenna size and connection between PSII reaction centers. Further, the reduction in remaining ETC is denoted by the thermal phase (J-I-P) rise.

Biophysical parameters derived by the 'JIP-test' equations. The values of minimal fluorescence intensity are an important parameter and can provide insight in the irreversible damage of PSII, associated with light-harvesting complex II (LHCII) and hindering the electron transfer on the reduced side of PSII. Because of conformational changes in the D1 protein under Cu stress, which cause changes in the characteristics of PSII electron acceptors, decreasing \( F_M \) under HM stress may be related to less efficient PSII activity.

In determining the maximum primary yield of photochemistry, \( F_v/F_o \) is a parameter that accounts for simultaneous variations in \( F_M \) and \( F_o \). The decreased values of \( F_v/F_o \) in fronds under Cu stress show the alteration in the electron transport rate to the primary electron acceptors from PSII and a reduction in the number and size of the reaction center. Martinazzo et al. and Janka et al. also reported the environmental stress-induced decrease in the \( F_v/F_o \) ratio in different plant species. The increased level of relative variable fluorescence under Cu treatment indicates that the electron transfer at the donor side of PSII was affected. The affected \( F_v/F_o \) can be due to the modified unquenchable fluorescence (\( F_o \)) that altered the energy relay from antenna complex to reaction center.

According to PCA analysis the quantum yield was positively correlated with the electron transport per cross-section while negatively correlated with \( FO/F_M \) located in the opposite direction of the PCA loading axis. Table 3. The acute 48-h LD50 values of Cu and their confidence limits in L. minor according to Logit analysis. *Logarithm base = 10.*

Table 3.

| Probability | 95% Confidence limits for Cu dose | 95% Confidence limits for log(Cu dose)* |
|-------------|----------------------------------|----------------------------------------|
|             | Estimate | Lower bound | Upper bound | Estimate | Lower bound | Upper bound |
| 0.010       | 2.763    | 0.422       | 5.580       | 0.441    | −0.375      | 0.747       |
| 0.020       | 3.588    | 0.685       | 6.693       | 0.555    | −0.165      | 0.826       |
| 0.030       | 4.235    | 0.931       | 7.516       | 0.627    | −0.031      | 0.876       |
| 0.040       | 4.798    | 1.172       | 8.204       | 0.681    | 0.069       | 0.914       |
| 0.050       | 5.310    | 1.414       | 8.812       | 0.725    | 0.150       | 0.945       |
| 0.060       | 5.789    | 1.658       | 9.368       | 0.763    | 0.220       | 0.972       |
| 0.070       | 6.244    | 1.906       | 9.886       | 0.795    | 0.280       | 0.995       |
| 0.080       | 6.682    | 2.159       | 10.376      | 0.825    | 0.334       | 1.016       |
| 0.090       | 7.107    | 2.417       | 10.845      | 0.852    | 0.383       | 1.035       |
| 0.100       | 7.522    | 2.682       | 11.297      | 0.879    | 0.429       | 1.053       |
| 0.150       | 9.515    | 4.115       | 13.413      | 0.978    | 0.614       | 1.128       |
| 0.200       | 11.469   | 5.756       | 15.441      | 1.060    | 0.760       | 1.189       |
| 0.250       | 13.462   | 7.637       | 17.517      | 1.129    | 0.883       | 1.243       |
| 0.300       | 15.545   | 9.774       | 19.759      | 1.192    | 0.990       | 1.296       |
| 0.350       | 17.762   | 12.164      | 22.310      | 1.249    | 1.085       | 1.348       |
| 0.400       | 20.158   | 14.767      | 25.378      | 1.304    | 1.169       | 1.404       |
| 0.450       | 22.783   | 17.506      | 29.257      | 1.358    | 1.243       | 1.466       |
| 0.500       | 25.700   | 20.295      | 34.319      | 1.410    | 1.307       | 1.536       |
| 0.550       | 28.890   | 23.102      | 40.998      | 1.462    | 1.364       | 1.613       |
| 0.600       | 32.765   | 25.971      | 49.837      | 1.515    | 1.414       | 1.698       |
| 0.650       | 37.184   | 29.004      | 61.631      | 1.570    | 1.462       | 1.790       |
| 0.700       | 42.487   | 32.338      | 77.676      | 1.628    | 1.510       | 1.890       |
| 0.750       | 49.062   | 36.167      | 100.261     | 1.691    | 1.558       | 2.001       |
| 0.800       | 57.588   | 40.792      | 133.789     | 1.760    | 1.611       | 2.126       |
| 0.850       | 69.413   | 46.768      | 187.937     | 1.841    | 1.670       | 2.274       |
| 0.900       | 87.800   | 55.358      | 289.186     | 1.943    | 1.743       | 2.461       |
| 0.910       | 92.928   | 57.637      | 321.035     | 1.968    | 1.761       | 2.507       |
| 0.920       | 98.838   | 60.211      | 359.670     | 1.995    | 1.780       | 2.556       |
| 0.930       | 105.771  | 63.165      | 407.596     | 2.024    | 1.800       | 2.610       |
| 0.940       | 114.091  | 66.626      | 468.782     | 2.057    | 1.824       | 2.671       |
| 0.950       | 124.381  | 70.792      | 549.948     | 2.095    | 1.850       | 2.740       |
| 0.960       | 137.663  | 76.006      | 663.573     | 2.139    | 1.881       | 2.822       |
| 0.970       | 155.951  | 82.924      | 836.132     | 2.193    | 1.919       | 2.922       |
| 0.980       | 184.076  | 93.072      | 1137.301    | 2.285    | 1.969       | 3.056       |
| 0.990       | 239.053  | 111.575     | 1848.088    | 2.378    | 2.048       | 3.267       |
plot, which was also confirmed by the Correlation matrix (Fig. 7). Another possibility of reduced maximum primary yield under Cu stress can be the substitution of central atom of chlorophyll molecule, Mg by Cu. This substitution can hinders photosynthetic light-harvesting in the affected chlorophyll molecules.

The "JIP test" of fluorescence transient in photosynthetic organisms, subjected to abiotic stress revealed a marked decrease in $\phi_{P_{0}}$96. The slight reduction in $\phi_{P_{0}}$ might be due to a decrease in PSII photochemical efficiency resulting from Cu stress (in most higher plants having usually a value in the range of 0.78–0.8497). In the light condition, a reduction in the maximum quantum yield of PSII ($\phi_{P_{0}}$) shows that HM stress inhibits the redox reaction following $Q_{A}$ and causes a delay in electron transport between $Q_{A}^{-}$ and $Q_{B}$. These parameters are very important and provide relevant information on electron transport activity at the PSII acceptor sites. The present finding suggested that Cu treatment reduces the electron transport at the PSII acceptor site in L. minor.

Energy pipeline models (membrane and leaf model), presented in Figs. 4 and 5, have displayed that various sites in PSII are sensitive to several environmental stresses98–100. Based on present results, $TR_{O}/CS_{M}$ and $ET_{O}/CS_{M}$ decreased with increasing the Cu concentration because active RCs are converted into inactive or closed (dark circle in model) RCs consequently decreasing the trapping efficiency and electron transport from PSII74, 81, 101. PCA biplot shown $ET_{O}/CS_{M}$, $DL_{O}/CS_{M}$ are positively correlated, which is also observed by grid correlation matrix (Figs. 6, 7). The ABS/RC is determined by taking the total amount of photons absorbed by Chl molecules throughout all RCs by the total number of active RCs58. The ratio of active/inactive RCs influences it, and as the number of active centers rose, the ABS/RC ratio reduced. $TRO/RC$ is the maximum rate at which an exciton is captured by the RC, resulting in a decrease in $Q_{A}$. An increase in this ratio indicates that all the $Q_{A}$ has been reduced83. Reduction in $ET_{O}/RC$ describes that the re-oxidation of reduced $Q_{A}$ through electron transport in an

Figure 7. Grid correlation matrix shows the correlation between all calculated chlorophyll a fluorescence parameter with color code.
active RC is decreased because a greater number of the active RC are available, hence it only reflects the activity of active RCs. Figure 4 demonstrates a reduction in per active RC electron transport but an overall increase in electron transport. The ratio of total dissipation of un-trapped excitation energy from all RCs to the number of active RCs is defined as DL/Rc. Dissipation arises as heat, fluorescence, and energy transfer to other systems and the ratio of active/inactive RCs also have an impact. Due to the effective utilisation of energy by the active RCs, the ratio of total dissipation to the number of active RCs (DL/Rc) is not very impacted.

The Fv/Fm ratio = (Fm − Fo)/Fm is an important JIP parameter that represents the conversion efficiency of primary light energy in the PS II reaction center and is used as a stress indicator in a large number of photosynthetic studies. However, since it is dependent on Fv and Fm fluorescence levels, this quantitative parameter is not usually sensitive enough to detect alteration across samples. Srivastava et al. employed the performance index (PI), a novel, more responsive, and significant parameter to measure photosynthetic efficiency under stress. The performance index, PI, is derived using three (or four) components based on reaction center density, trapping efficiency, and electron transport efficiency, in the same way as a Goldman equation. As a result, if any of these components is affected by stress, the effect will be visible in the performance index, which has a higher sensitivity. Performance index (PIabs) is calculated on an energy absorption basis while the performance index on cross-section (Plcs) is obtained by multiplying the performance index on absorption basis (PIabs) by the phenomenological energy flux, ABS/CS = Fo (or Fm): and the value of Plabs and Pcs significantly lowered in a plant grown under Cu stress (Fig. 2C). Plabs are decreased due to reduced activity of the RC so the overall activity of the RC is decreased based on results in this study and statistical models (PCA and Correlation matrix) of some of the important JIP parameters such as Phenomenological energy flux (ABS/CSm, TRm/CSm and ETm/CSm), maximum quantum yield (qFm), Performance index per absorbance (Plabs) and per cross-section (Plcs) are displayed the dose–response relationship under Cu stress. Probit analysis is usually used in toxicology to determine the relative toxicity of chemicals to living organisms based on results in this study and statistical models (PCA and Correlation matrix) and the effect will be visible in the performance index, which has a higher sensitivity. Performance index (PIabs) is calculated on an energy absorption basis while the performance index on cross-section (Plcs) is obtained by multiplying the performance index on absorption basis (PIabs) by the phenomenological energy flux, ABS/CS = Fo (or Fm):

**Conclusion**

In the present study, the efficacy of ChlF kinetics in the detection of Cu-induced HM stress was analysed in L. minor. Treatment of lower concentration of Cu (0.0–20.0 µM) had mild negative effect on photosynthesis. As the Cu is an essential micronutrient and plays a vital role in many biochemical processes, hence under moderate metal concentration the L. minor performed normally without any deleterious effect. A typical OJIP curve was obtained which shows that the plant efficiently used the solar energy for photosynthesis which is expressed in the term of increased active reaction center and performance index. In contrast, at higher Cu concentration (30.0–40.0 µM), the OJIP curve has been flattened due to a reduction in electron transport towards PSI (P700), and a major portion of absorbed energy was dissipated in the form of heat because of an increased number of the inactive reaction center. Conclusively, phenomenological energy flux (ABS/CSm, TRm/CSm and ETm/CSm), maximum quantum yield (qFm), performance indexes (Plabs and Plcs) are powerful indicators of HM stress in plants and can be used for rapid detection of HM-induced water pollutant. Additionally, the key OJIP parameters screened in this paper could be a good tool for the rapid detection of primary mode of action of HM on the photosynthetic apparatus in L. minor.

**Data availability**

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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**Author contributions**

H.S. conceived the original screening and research plans; V.S. supervised the experiments; H.S.; D.K. and V.S. performed the experiments; H.S. designed the experiments and analyzed the data; H.S. conceived the project and wrote the article with contributions of all the authors; V.S. supervised the experiments; H.S.; D.K. and V.S. performed the experiments; H.S. designed the experiments and analyzed the data; H.S. conceived the project and wrote the article with contributions of all the authors; V.S. supervised and completed the writing and agrees to serve as the author responsible for contact and ensures communication.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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