Phenotypic changes in microalgae at acidic pH mediate their tolerance to higher concentrations of transition metals

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ABSTRACT

Acclimatory phenotypic response is a common phenomenon in microalgae, particularly during heavy metal stress. It is not clear so far whether acclimating to one abiotic stressor can alleviate the stress imposed by another abiotic factor. The intent of the present study was to demonstrate the implication of acidic pH in effecting phenotypic changes that facilitate microalgal tolerance to biologically excess concentrations of heavy metals. Two microalgal strains, Desmodesmus sp. MAS1 and Heterochlorella sp. MAS3, were exposed to biologically excess concentrations of Cu (0.50 and 1.0 mg L$^{-1}$), Fe (5 and 10 mg L$^{-1}$), Mn (5 and 10 mg L$^{-1}$) and Zn (2, 5 and 10 mg L$^{-1}$) supplemented to the culture medium at pH 3.5 and 6.7. Chlorophyll autofluorescence and biochemical fingerprinting using FTIR-spectroscopy were used to assess the microalgal strains for phenotypic changes that mediate tolerance to metals. Both the strains responded to acidic pH by effecting differential changes in bio-chemicals such as carbohydrates, proteins, and lipids. Both the microalgal strains, when acclimated to low pH of 3.5, exhibited an increase in protein (>2-fold) and lipid (>1.5-fold). Strain MAS1 grown at pH 3.5 showed a reduction (1.5-fold) in carbohydrates while strain MAS3 exhibited a 17-fold increase in carbohydrates as compared to their growth at pH 6.7. However, lower levels of biologically excess concentrations of the selected transition metals at pH 6.7 unveiled positive or no effect on physiology and biochemistry in microalgal strains, whereas growth with higher metal concentrations at this pH resulted in decreased chlorophyll content. Although the bioavailability of free-metal ions is higher at pH 3.5, as revealed by Visual MINTEQ model, no adverse effect was observed on chlorophyll content in cells grown at pH 3.5 than at pH 6.7. Furthermore, increasing concentrations of Fe, Mn and Zn significantly upregulated the carbohydrate metabolism, but not protein and lipid synthesis, in both strains at pH 3.5 as compared to their growth at pH 6.7. Overall, the impact of pH 3.5 on growth response suggested that acclimation of microalgal strains to acidic pH alleviates metal toxicity by triggering physiological and biochemical changes in microalgae for their survival.

Introduction

The widespread occurrence of heavy metals (HMs), in excess concentrations, in the environment is mostly due to intensive industrial and mining activities. In the extreme habitats like acid mine drainages (AMDs), the HMs that occur commonly are copper (Cu), cadmium (Cd), zinc (Zn), manganese (Mn), arsenic (As), iron (Fe), aluminum (Al), nickel (Ni), lead (Pb), and chromium (Cr) (Venkateswarlu et al., 2016; Abinandan et al., 2018). Especially, Cu, Zn, Mn, and Fe are among the seven essential transitional metals, only in trace quantities or biologically relevant concentrations, involved in several metabolic and cellular functions in phytoplankton (Bataller and Capareda, 2018). For instance, organelles such as chloroplasts and mitochondria use Cu, Zn, Mn, and Fe for photosynthetic and metabolic functioning of microalgal cells (Merchant et al., 2006). As active non-protein cofactors for stabilizing protein structures, these metals also catalyze certain enzymatic reactions in microalgae (Hansch and Mendel, 2009). Concentrations of HMs that are deficient (sub-optimal) or in excess (detrimental) tend to alter the levels...
of macromolecules such as carbohydrates, proteins, and lipids due to changes in metabolic pathways (Hsieh et al., 2013; Höhner et al., 2013).

The impact of stress, imposed by excess concentrations of those HMs that serve as micronutrients, on the production of carbohydrates, proteins and lipids in microalgae has been widely investigated at neutral pH conditions (Battah et al., 2015; Rocha et al., 2016; Kona et al., 2017; Liu et al., 2017; Palma et al., 2017). Excess Cu⁺⁺ concentrations reduced the cell density of Selenastrum gracile, and increased protein and lipid synthesis (Rocha et al., 2016). Addition of Cu, Zn or Mn at various concentrations to a synthetic seawage increased the accumulation of lipids in Chlorella sp. (Liu et al., 2017). The detrimental effects of transition metals are not only governed by the concentration but also on their bioavailability that is expected to be higher at low pH (Wren and Stephenson, 1991). Nevertheless, the toxicity of any metal toward microalgae is higher in acidic pH rather than neutral pH and was related to the predominant occurrence of free and hydrated metal ions (Wilde et al., 2006; Abinandan et al., 2019a). Krishnamurit et al. (2004) demonstrated that metal organic complex such as Cd-citrate and Cd-dissolved organic matter were better tolerated and exerted toxicity to a soil alga, Chlorococcum sp. (Degens et al., 2016). In a study on a microalgae, Dunaliella salina, exposed to a representative sample of acid mine waters (Abinandan et al., 2018). However, a detailed information on phenotypic changes that occur in microalgae to cope with biologically excess concentrations of the essential metals, Cu, Fe, Mn, and Zn (Table S1) at pH 3.5 and 6.7 (control) to provide a final cell density of 50 × 10⁶ cells mL⁻¹ (measured using Hemocytometer) and incubated for 16 days under constant illumination as described earlier (Abinandan et al., 2019a, 2019b). The selection of metal concentrations (Cu at 0.5 and 1.0 mg L⁻¹, Fe and Mn at 5 and 10 mg L⁻¹, and Zn at 2.5 and 10 mg L⁻¹) was based on our earlier observations on toxicity of these metals to both the strains of microalgae (Abinandan et al., 2019b).

Aliquots (50 μL) of microalgal cultures growing at logarithmic phase (10 days at pH 3.5 and 7 days at pH 6.7), were withdrawn, the cells were harvested by centrifugation (8000 × g, 5 min), and the pellet was washed with deionized water. Cell suspensions (100 μL) in deionized water were transferred into a black 96-well microplate (Nunc, Thermofisher, USA). The chlorophyll autofluorescence in microalgae were measured (560–620 nm, Excitation 440 nm, Emission 680 nm) and expressed in terms of relative fluorescence units (RFUs), as described earlier (Abinandan et al., 2020a). Chemical speciation, in terms of free ion concentration, of metals supplemented at varying levels (Table S1) to the modified BBM at pH 3.5 and 6.7 (control) was determined using Visual MINTEQ (V 3.1) at 25°C.

### ATR-FTIR spectroscopy

Microalgal strains were grown in presence of heavy metals for 10 days at pH 3.5 and 7 days at 6.7, and 2 ml aliquots, in triplicates, were withdrawn and centrifuged at 8000 × g for 10 min to obtain microalgal pellets. The biomass was washed twice with sterilized modified BBM, and the cells were resuspended in the same medium. The aqueous samples were then placed on a horizontal plane ATR (HATR) trough prism made of ZnSe crystal (Reflective index 2.4) for IR measurements (PIKE PN: 022-2010-45). A trapezoid-shaped specific accessory of dimensions 80 × 10 × 4 mm (length, width, and thickness) with 45° internal critical reflection angle and multiple reflections up to 10 times, was incorporated into the HATR base assembly (PIKE PN: 022-1213). The microalgal samples, placed onto ZnSe trough, were held tightly by a pressing accessory (PIKE PN: 022-3052) using a HATR pressure clamp (PIKE PN: 022-3050) to ensure maximum proper contact of the sample. A working matrix without microalgal sample was included to obtain a background spectrum to negate the effects of BBM peaks that might interfere with the spectra of microalgal biomass. All the spectra were scanned in Agilent Technologies Cary 660 FTIR spectrometer by placing HATR accessory inside the sample compartment. Spectral scans were obtained in the mid-IR energy range (4000-400 cm⁻¹) taking into consideration the spectral cut-off for ZnSe prism with a spectral resolution of 8 cm⁻¹ and by co-averaging 16 scans for each sample (a total of 90 samples that include all the treatments of both the cultures) using Agilent IR Resolutions Pro software. The deuterated triglycerine sulfate

### Materials and methods

**Microalgal strains and growth in presence of transition metals**

Two acid-tolerant microalgae, Desmodesmus sp. MAS1 and Heterochlorella sp. MAS3, (Fig. S1) were initially maintained in modified Bold’s basal medium (BBM) with low phosphate (1/10th) at pH 3.5 and sub-cultured twice with an interval of 10 days to ensure acclimatization to acidic pH. In an earlier study, we observed restoration of homeostasis in the microalgal strains after an extended lag phase of 10 days at acidic pH (Abinandan et al., 2019a). However, the microalgal strains started growing rapidly with reduced lag phase after repeated subculturing at low pH, indicating the acclimation of cells to the acidic environment (Abinandan et al., 2019b). Exponentially growing cultures were used to inoculate modified BBM (30 mL) contained in 100 mL Erlenmeyer flask, in triplicates, supplemented with varying concentrations of Cu, Fe, Mn and Zn (Table S1) at pH 3.5 and 6.7 (control) to provide a final cell density of 50 × 10⁶ cells mL⁻¹ (measured using Hemocytometer) and incubated for 16 days under constant illumination as described earlier (Abinandan et al., 2019a, 2019b). The purpose of including low phosphate in modified BBM was to avoid phosphate-metal complexation and maximize metal bioavailability. The cells grown at pH 3.5 attained a stationary phase at 16th day as demonstrated in our earlier studies (Abinandan et al., 2019a, 2019b). The selection of metal concentrations (Cu at 0.5 and 1.0 mg L⁻¹, Fe and Mn at 5 and 10 mg L⁻¹, and Zn at 2.5 and 10 mg L⁻¹) was based on our earlier observations on toxicity of these metals to both the strains of microalgae (Abinandan et al., 2019b).

**Microalgal strains**

Recent studies (Abinandan et al., 2019a) established a clear mechanistic understanding of pH-induced toxicological bioavailability of metal on microalgae is lacking. Francois et al. (2007) observed an increase in metal flux in Chlamydomonas reinhardtii due to pH changes that caused conformational changes in surface transport protein. Thus, pH could be an important factor that determines the bioavailability of metals in microalgae at biologically excess concentrations. A perusal of the literature indicates that microalgae exhibit phenotypic response through changes in physiological and metabolic signature molecules such as carbohydrates, proteins, and lipids for their growth in any environmental cues (Charles et al., 2019; Abinandan et al., 2020a; Perera et al., 2021a,b,c)). Metals at higher concentrations that are biologically in excess may trigger certain undesirable effects on the growth of microalgae (Abinandan et al., 2018). However, a detailed information on phenotypic changes that occur in microalgae to cope with biologically excess concentrations of transition metals at acidic pH is lacking.

Recently, we isolated two acid-tolerant microalgal strains, Desmodesmus sp. MAS1 and Heterochlorella sp. MAS3, from soils and lake waters having neutral pH conditions (Abinandan et al., 2019a), that have great potential in removing HMs and yielding good amounts of biodiesel when grown at pH 3.5 (Abinandan et al., 2019b, 2019c; Abinandan et al., 2020b). These strains also exhibited physiological and metabolome changes to survive in samples collected from such an extreme environment as AMD (Abinandan et al., 2020b). The present novel study was therefore aimed at providing a better insight into the phenotypic changes and verifying the metabolic trade-off in Desmodesmus sp. MAS1 and Heterochlorella sp. MAS3 when grown in presence of biologically excess concentrations of the essential metals, Cu, Fe, Mn, and Zn, at an acidic pH of 3.5 by comparing with those grown at pH 6.7. The physiological and metabolic signature molecules investigated in the microalgal strains included chlorophyll, carbohydrates, proteins, and lipids. Here, we used attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy (Qiao et al., 2017), a robust technique in obtaining relevant IR spectra than the conventional mode of measurements (Gurbano et al., 2016), to determine the phenotypic changes imposed by biologically excess concentrations HMs in the selected microalgal strains.
(DTGS) detector of the spectrophotometer was air-cooled at room temperature for optimizing the sensitivity of the measurements. Since all the spectral data were processed through chemometrics datasets, no ATR corrections were performed. Areas of biochemical fingerprints or peaks of interest were calculated based on the sum of total peak areas (900–1200, 1244–1697 and 1744, 2850–2947 cm⁻¹) across the treatments, measured in triplicates, and the change (in fold) was calculated using the formula:

\[
\text{Relative fold change}(+) = \frac{(T_p - T_p^c)}{T_p^c}
\]

where, \(T_p^c\) is the total peak area of control (pH 6.7) and \(T_p\) is the total peak area of treatment (pH 3.5). Similarly, the data of spectral peak area obtained in respective treatments were normalized to control (organisms grown in the same pH without the addition of metals).

**Spectral processing and multivariate analysis**

The spectra obtained were processed following spectroscopy skin in SIMCA 15 software (Umetrics, Sweden). The datasets exported in .csv format from Agilent Resolution’s Pro software were averaged and assigned with primary variable Id (wavenumber label), primary observation Id (overall sample label), secondary observation Id (treatment label), and X (frequency) and Y (attained absorbance value) variables before importing into SIMCA 2017 dataset. Spectral filters were applied under control filters tab to obtain secondary derivative spectra. The principal component analysis (PCA) was performed through PCA tab by auto fitting option for the second derivative spectra to account for the most variation in the data sets since the raw spectroscopic data contains numerous peaks that make the interpretation difficult (Dao et al., 2017). The autofit option in SIMCA 2017 will extract many components based on significant values obtained from raw spectra. Second derivative spectra were obtained through processing of raw data based on standard normal variate algorithm and applied to remove the baseline and to replace maxima bands in raw spectra to minima for elucidating better differences (Gurbanov et al., 2018). Score plots were used to visualize clustering of the data and variation among them. The important macromolecules in microalgal cells, displayed at wave numbers 900–3000 cm⁻¹ (Table 1), were only considered for data analysis. To improve the prediction error of the data acquired from spectral filterings such as derivatives, the orthogonal partial least square (OPLS) regression was employed in the analysis since OPLS distinguishes the variations in another way compared to the partial least square (PLS) even though both tend to possess the same predictive properties. However, PLS considers only components 1 and 2, while OPLS relies on both the components along with an orthogonal component and multiple data that incorporate large data sets (Gorzsiá et al., 2011; Easmin et al., 2017). Both the X (absorbance) and Y (wave number) variables for the dataset were selected and processed for OPLS regression under the OPLS tab of spectroscopy skin, and all the data were transformed to avoid error and center-scaled to derive the prediction data set.

**Statistical analysis**

The standard deviations for the experimental data means (\(n = 3\)) were calculated following Graphpad Prism V.8 software, and the statistical significance (\(P \leq 0.05\)) was determined following Duncan’s multiple range test using IBM Statistical Product and Service Solutions (SPSS) software version 24 (Abinandan et al., 2020a).

**Results**

**pH-Dependent phenotypic changes in microalgal strains MAS1 and MAS3**

A clear difference on the effect of pH was evident in the microalgal strains wherein the maxima spectral bands corresponding to the metabolic shifts received negative scores in the second derivative spectra (Fig. 1a). At pH 6.7, strain MAS1 exhibited a positive score for both PC1 and PC2 whereas strain MAS3 showed negative scores that were closely

![FTIR spectra](image_url)

**Table 1**

| Wave number (cm⁻¹) | Metabolites as per spectral assignments | Strain MAS1 (pH 6.7) | Strain MAS3 (pH 3.5) | Change (fold) |
|-------------------|----------------------------------------|----------------------|----------------------|---------------|
| 900-1200          | Carbohydrates                          | 2.78 ± 0.12a         | 1.77 ± 0.01b         | ↓1.50         |
| 1244–1697         | Proteins                               | 2.09 ± 0.03a         | 5.96 ± 0.22b         | ↑12.80        |
| 1744, 2850-2947   | Lipids                                 | 0.82 ± 0.03a         | 0.2 ± 0.01b          | ↓4.10         |

Change (fold) in metabolites was calculated based on relative increase/decrease in pH 3.5 as compared to pH 6.7. ↑” and “↓” represent upregulation and downregulation, respectively. Means (\(n = 3\)) for a microalgal strain sharing the same letter are not significantly different at (\(P \leq 0.05\)) according to Tukey’s test.
clustered and the maximum variation in PC1 and PC2 was 62.5 and 33.1%, respectively (Fig. 1b). Interestingly, strain MAS3 gave a positive score when grown at pH 3.5 whereas strain MAS1 showed a negative score at acidic pH. Relative changes during metabolic shifts in such biomolecules as carbohydrates, proteins, and lipids in response to different pH are presented in Table 1. Strain MAS1 exhibited 1.5-fold change in metabolites as carbohydrates, proteins, and lipids in response to score at acidic pH. Relative changes during metabolic shifts in such score when grown at pH 3.5 whereas strain MAS1 showed a negative stretch, respectively.

Change (fold) in metabolites was calculated based on relative increase/decrease in pH 3.5 as compared to pH 6.7. However, strain MAS3 grown at pH 3.5 showed a 17-fold increase in neutral pH. Such a differential response to pH among the selected strains MAS1 and MAS3 could be due to significant increase in ribose rings in RNA and polysaccharides at 946, 995 and 1078 cm\(^{-1}\) in the spectrum (Table 2). In strains MAS1 and MAS3 grown at pH 3.5, the increase in protein band frequency at 1244–1697 cm\(^{-1}\) comprising amide regions (I-III) was 2.8- and 10-fold, respectively (Table 2). Such a specific increase in protein could be due to symmetric stretching of C=O for amide I as evident at 1648 cm\(^{-1}\). In addition, a 2.7-fold increase in lipid accumulation was observed at pH 3.5 in strain MAS3 while it was a significant 4.1-fold decrease in strain MAS1. Although microalgae, in general, respond to environmental stress through increased lipid accumulation, it was evident only in strain MAS3. The critical spectral bands that represent microalgal lipids and fatty acids in the selected strains were found at 1744, 2850, 2889 and 2947 cm\(^{-1}\) that are characteristics of fatty acid esters, triglycerides, and methylene groups of lipids (Table 2). Interestingly, the peak area for both the strains was higher in the region 1744 cm\(^{-1}\), indicating the accumulation of triglycerides at pH 3.5 that at neutral pH.

Impact of transition metals at two pH regimes on chlorophyll synthesis

Therefore, chlorophyll in terms of RFUs was employed as an indicator in the present study to assess the impact of excess concentrations of transition metals on microalgae at pH 3.5. Thus, the increase in chlorophyll of strain MAS1 exposed to lower concentrations of Cu, Fe and Mn at pH 6.7 was 7, 17 and 27%, respectively, while the percent increase with Fe, Mn, and Zn at pH 3.5 was 12, 3.3 and 36, respectively. Clearly, Cu at pH 3.5 and Zn at pH 6.7 was toxic to strain MAS1, as there was a decrease in chlorophyll by 38 and 15%, respectively as compared to its growth in modified BBM alone. On the other hand, growth of strain MAS3 increased by 30–64% in presence of lower levels of transition metals at pH 6.7. Even at pH 3.5, chlorophyll was not affected when this strain was grown in modified BBM supplemented with the transition metals although bioavailability of free metal ions could be higher at pH 3.5 than 6.7 (Abinandan et al., 2019b). Thus, the reduction in chlorophyll of strain MAS1 was 10, 8 and 43% for Cu, Fe and Zn, respectively, while 16% decline in the growth pigment was evident with Cu in strain MAS3 (Fig. 2). Both the strains selected in the present study accumulated metals intracellularly, implying that pH might have induced changes in metabolism in response to excess concentrations of transition metals.

Metabolic shifts in microalgal strains in response to transition metals

In terms of biochemical response, concentrations of Cu (0.50 and 1.0 mg L\(^{-1}\)) supplemented to BBM significantly affected carbohydrate synthesis in both the strains grown at pH 6.7 (Fig. 3a). Nevertheless, both

### Table 2

| Wave number (cm\(^{-1}\)) | Strain MAS1 pH 6.7 | Strain MAS1 pH 3.5 | Strain MAS3 pH 6.7 | Strain MAS3 pH 3.5 | Vibrational frequency | Region |
|---------------------------|-------------------|-------------------|-------------------|-------------------|----------------------|--------|
| 906 | + | – | – | – | trans – C-H out-of-plane | Ribose-phosphate main chain vibration involving 2′-OH group of ribose rings in RNA |
| 946 | – | † | † | † | ν (C-H) | RNA |
| 995 | – | – | † | † | ν (C-H) | CH\(_2\)OH groups of polysaccharides of glycosidic bonds inside groups |
| 1029 | † | † | † | † | ν (C=O); δ (CH\(_3\)) | Carbohydrates |
| 1078 | † | † | † | † | ν (C-O-C); ν (CH\(_2\)) | Carbohydrates |
| 1140 | † | – | – | – | ν\(_{as}\) (CO-O-C) | Group of glycerol and nucleic acids (DNA and RNA) |
| 1165 | – | – | † | – | ν\(_{as}\) (C-O-C) | Esters |
| 1244 | † | † | † | † | ν\(_{as}\) (C-O-C) | Amide III: β-sheet |
| 1315 | – | – | † | † | ν\(_{as}\) (C-O-C) | Amide II: α-helix |
| 1339 | † | † | † | + | ν (CH\(_2\)) | α-CH\(_2\) groups in polyethylene chains |
| 1370 | – | † | † | † | δ\(_s\) (CH\(_3\)) | Cholesterol and fatty acid radicals |
| 1396 | – | † | † | † | δ\(_s\) (CH\(_3\)) δ (CH\(_2\)) | Lipids and proteins |
| 1421 | † | † | † | † | δ\(_s\) (CH\(_3\)) | Di-substituted cis-olefins |
| 1457 | † | † | † | † | δ\(_s\) (CH\(_3\)) | α-CH\(_2\) groups in polyethylene chains |
| 1469 | † | † | † | † | δ\(_s\) (CH\(_3\)) | α-CH\(_2\) groups in polyethylene chains |
| 1520 | † | † | † | † | ν (C-N) δ (N-H) | Amide II from proteins |
| 1540 | † | † | † | † | ν (C-N) δ (N-H) | Amide II from proteins |
| 1557 | † | † | † | † | ν (C-N) δ (N-H) | Amide II from proteins |
| 1626 | † | † | † | † | ν (C-O) ν (C-C) | α-Helix and antiparallel β-sheet of amide II |
| 1648 | † | † | † | † | ν (C-C) | Antiparallel β-sheet of amide I of carbohydrate and aromatic regions |
| 1683 | – | – | † | † | ν (C-O) | α-Helix and antiparallel β-sheet of amide II |
| 1697 | † | † | † | † | ν (C-C) | Di-substituted cis-olefins and α-helix of amide I |
| 1734 | † | † | † | † | ν (C-O) | β-Sheet of amide I |
| 2850 | † | † | † | † | ν\(_{as}\) (C-H) | Amide I proteins |
| 2889 | † | † | † | † | ν\(_{as}\) (C-H) | Fatty acid esters and triglycerides |
| 2947 | † | † | † | † | ν\(_{as}\) (C-H) | –CH\(_2\) groups of lipids |
| 2947 | † | † | † | † | ν\(_{as}\) (C-H) | –CH\(_2\) groups of lipids |

ν, ν\(_{as}\) and ν\(_{s}\): Vibrational stretching, symmetric vibrational stretching, and asymmetric vibrational stretching, respectively.

δ, δ\(_s\), and δ\(_{as}\): Plane bending vibration, symmetric deformation, asymmetric deformation (bend), and plane scissor bending vibration, respectively.

ν\(_{s}\): Out of plane deformation.

Vongsvirut et al. (2013); Kardas et al. (2014); Kalmadia et al. (2015).
the concentrations of Cu at near neutral pH inhibited protein synthesis in strain MAS1, but significantly increased protein accumulation (2.3 to 2.7-fold) in strain MAS3. The lipid content only in strain MAS1 increased by 1.2 to 1.7-fold at pH 6.7. In fact, proteins containing amide I and amide II increased (>100%) in MAS3, whereas in MAS1 the enhancement in lipids that resolved at 1744 and 2850 cm\(^{-1}\) was 30–40 and 100–200%, respectively (Table S2). Since higher concentrations of Cu were algalidial at acidic pH (Abinandan et al., 2019a), only 0.5 mg L\(^{-1}\) of Cu was used at pH 3.5 in the present study. Thus, the reduction in carbohydrates was 30% in both the strains, while lipids accounted for 50 and 66% decline in MAS1 and MAS3, respectively (Fig. 3b). Interestingly, strain MAS1 grown at pH 3.5 showed significant increase in proteins containing amide III (24.6%), amide II (18.1%) and amide I (14.3%) (Table S3).

Both the levels of Fe used in the present study significantly affected the overall synthesis of carbohydrates, while proteins and lipid were slightly reduced in strain MAS1 at pH 6.7 (Fig. 3c). On the contrary, strain MAS3 showed significant increase in all biomolecule synthesis especially with a pronounced increase of protein accumulation (>300%) through all the amide bands with metal concentrations (Fig. 2, Table S4). The strain MAS1 exhibited a significant decrease in all the biomolecules at higher Fe concentration at pH 3.5 (Fig. 3d). Although Fe significantly affected proteins and lipids in strain MAS3, carbohydrates accumulation was quite significant. Thus, in strain MAS3 the significant band that corresponds to carbohydrates at 1078 cm\(^{-1}\) in cultures of strain MAS3 increased by >140% (Table S5).

In all, growth of strain MAS3 in presence of Mn led to enhanced accumulation of carbohydrates, proteins, and lipids, especially at lower levels of the metal (Fig. 4a). Among the biomolecules, the significant increase was >400% for lipids followed by protein (>200%) as evidenced by the vibrational stretching of amide I band and symmetric and asymmetric vibrational stretching of C–H from methylene group of lipids (Table S6). Particularly, the vibrational stretching at the region 1697 cm\(^{-1}\) represents amide I proteins with an increase up to 15% while the frequency at 2947 cm\(^{-1}\) increased to 78.95% along with a new frequency at 2984 cm\(^{-1}\), indicating that Mn favors for lipid accumulation than proteins (Fig. 4b). Two redox tyrosine residues are involved in membrane-protein complexes of PSII that are dominated by symmetrical vibrational stretch observed between 1620–1720 cm\(^{-1}\), clearly supporting the effect of Mn in strain MAS1 (Berthomieu and Hienerwadel, 2005). The carbohydrate region at 1078 cm\(^{-1}\) with vibrational stretching of C–O–C and C–OH exhibited 189% increase in MAS3 (Table S7).

Lower concentration of Zn (5 mg L\(^{-1}\)) exhibited upregulation of carbohydrates, proteins, and lipids while 10 mg L\(^{-1}\) Zn enhanced only lipids (Fig. 4c). Although Zn has been shown to be toxic by competing for metal-binding sites in other proteins which cause oxidative stress (Malasarn et al., 2013), the bands in spectral regions at 1029, 1744 and 2889 cm\(^{-1}\) that represent carbohydrates and lipids significantly increased even at higher concentrations of Zn in strain MAS1 (Table S6). Overall, the biomolecules in strain MAS1 exposed to even higher concentrations of Zn were upregulated with a significant increase in protein up to 130%. Especially, the band intensities of amide III and amide II proteins as well as those in 1744 and 2947 cm\(^{-1}\) related to lipids were >150% higher. Growth of strain MAS1 in presence of the selected concentrations of Zn at pH 3.5 resulted in significant increase in carbohydrates (90%) and proteins (22%) (Fig. 4d). Contrary to the response of strain MAS1, the biomolecules such as carbohydrates and lipids tested in strain MAS3 decreased when grown in presence of Zn. In fact, the increase in the protein region was predominated by amide III by >200%.

Discussion
Phenotypic changes that occur in microalgae through alterations in physiology and biochemistry are a common phenomenon in response to environmental perturbations (Perera et al., 2021). Environmental stress imposed by acid pH causes disruption in homeostasis that leads to an extension in lag phase required for restoration of homeostasis through changes in the metabolism during acclimation (Borowitzka, 2018). We reported earlier an extended lag phase (>10 days) in microalgae grown at acidic pH. Also, microalgae exhibited heavy metal tolerance upon exposure to various concentrations (Abinandan et al., 2019a). In fact, biochemical responses in microalgal cells are the distinct metabolic fingerprints, which are a prerequisite to monitor immediate changes under environmental stresses (Abinandan et al., 2019b). Therefore, initially a comparison has been made of the shifts, in terms of FTIR spectral peak areas, of important metabolic indicators in the selected microalgal strains, Desmodesmus sp. MAS1 and Heterochlorella sp. MAS3, grown at pH 6.7 and 3.5. The predominant occurrence of bands related to biomolecules such as carbohydrates (900–1200 cm\(^{-1}\)), proteins (1100–1396 and 1300–1650 cm\(^{-1}\)) and lipids (1740 and 2650–2960 cm\(^{-1}\)) observed in the present study (Table 1) corroborates with the data reported in several studies (Vongsavivut et al., 2013; Kardas et al., 2014; Kalmodia et al., 2015). PCA revealed that both the microalgal strains were clearly separated into various clusters when grown at pH 6.7 and 3.5, indicating differential metabolism that supports the growth (Fig. 1). For instance, microalgae make a shift in the metabolism of carbon uptake through passive diffusion rather than bicarbonate uptake at low pH that results in alteration in metabolic activity (Abinandan et al., 2019a). However, during the acclimation process, carbohydrate, protein, and lipid accumulation was modified in cells growing at acidic pH compared to pH 6.5. Buayam et al. (2019) observed a significant decrease in synthesis of polysaccharides and amino acids in Desmodesmus sp. exposed to pH 4.0. Similarly, Khalil et al. (2009) reported that a microalga, Chlorella ellipsoidea, accumulated 142.5% protein at pH 4.0 as compared to its growth at pH 7.5. Lipids and fatty acids play a significant role in cellular metabolism, energy storage and membrane
activities (Bi et al., 2014). Osundeko et al. (2014) also observed distinct metabolic fingerprints, in terms of significant reduction in carbohydrate metabolism and increased lipid at 1740 cm$^{-1}$ in acclimated cultures of microalgae that tolerated oxidative stress. Transition metals such as Cu, Fe, Mn, and Zn play an important role in photosynthetic and respiratory electron transport across thylakoid and inner membrane system of mitochondria, respectively (Vigani et al., 2019). In fact, the pigment chlorophyll is considered as an important indicator of microalgal growth, particularly in heavy metal-exposed cells (Stoiber et al., 2010; Charles et al., 2019). In addition, Kruskopf and Flynn (2006) demonstrated that in vivo chlorophyll fluorescence poorly correlate to phytoplankton biomass determination but is a reliable indicator in assessing physiological stress. Thus, in vivo analysis of chlorophyll fluorescence is a commonly used approach to understand the physiological status in microalgae (Mallick and Mohn, 2003). Here, the term ‘excess concentrations’ is referred to such higher concentrations of metals that do not exhibit pronounced undesirable effects on growth of microalgae particularly at pH 3.5 wherein the bioavailability of metals is expected to be greater (Abinandan et al., 2019b). Thus, the two higher levels (up to 20 mg L$^{-1}$) of the transition metals selected (Table S1) at pH 3.5 served as the concentrations in excess that might enforce acclimation strategies in the selected microalgae. In general, both the microalgal strains grew well in modified BBM supplemented with additional concentrations of transition metals at lower levels (Fig. 2). Higher concentrations of Cu, Fe and Zn affected chlorophyll synthesis at pH 6.7 in both the microalgae. Kalinowska and Skowronska (2010) also observed a decrease in chlorophyll of Stichococcus minor, isolated from Cu-contaminated soil, exposed to 5 μM Cu at pH 6.7. Subramaniyam et al. (2016) reported that ferrous sulphate was more toxic than ferric chloride to Chlorella sp., Chlamydomonas sp. and Chlorococcum sp., which indicated that Fe speciation other than free ions, could have reduced chlorophyll. According to Hamed et al. (2017), Zn in excess concentration may replace magnesium in chlorophyll molecules thereby disrupting photosynthesis. Francois et al. (2007) observed that metal uptake by microalgae was reduced with a decrease in pH of the medium, suggesting a non-competitive inhibition as the proton binding sites differ from the metal complexing sites. Metabolic shifts, in terms of changes in biochemicals such as carbohydrates, proteins and lipids, under the influence of the selected transition metals in microalgal strains MAS1 and MAS3 grown at pH 3.5 and 6.5 were investigated following ATR-FTR spectroscopy. In all cases, the data on biochemical changes imposed by heavy metals in both the microalgal strains grown at pH 3.5 were compared with those obtained in microalgal strains cultured at pH 6.7 (controls). Cu is an important co-factor involved in various intracellular enzymatic functions and redox reactions favoring cellular respiration besides promoting Fe uptake (Bossuyt and Janssen, 2005). However, Cu affects microalgal growth directly under the influence of abiotic stress (Johnson et al., 2007). The differences in metabolic shifts during interactions with metals observed in strains MAS1 and MAS3 appear to be species-specific. Based on amide I and amide II signatures as well as carbohydrates, Charles et al. (2019) reported that microalgal cells, acclimated to higher Cu concentrations, were highly distinct when compared with non-acclimated cells. Excepting proteins in strain MAS1, all the biomolecules were significantly inhibited in both the strains at this concentration (0.5 mg L$^{-1}$) of Cu (Fig. 3b). Wilde et al. (2006) reported that in microalgal cells Cu can either be detoxified or cause adverse effect. Olsson et al. (2015) reported that higher concentrations of Cu upregulated several transcripts in an
acidophilic microalga, *Chlamydomonas acidophila*. Under replete conditions, Cu content in plastocyanin and Cytochrome c oxidase increased with an increase in growth (Kropat et al., 2015). Fe, with a predominant portion (80%) located in chloroplasts encompassing various groups of Fe-containing proteins, is a vital redox metal ion involved in essential cellular functions (Terauchi et al., 2010).

One plausible reason that accounts for less accumulation of carbohydrates in strain MAS1 grown in presence of biologically excess concentrations of Fe could be the preferential requirement of chelated Fe for intracellular functions (Rizwan et al., 2017). Dao et al. (2017) reported that microalgae respond to stress environment through modifying carbohydrate or lipid molecules rather than protein synthesis which may indicate that growth at excess concentrations of Fe do not impose stress in strains MAS3 and MAS1. The reduction in protein content could be due to the availability of free ions of Fe at acidic pH (Abinandan et al., 2019b). Sutak et al. (2012) found that, at higher Fe concentrations, uptake of cell wall-bonded Fe in marine microalgae increased only when grown in Fe-deprived medium. According to Ismaiel et al. (2018), protein-rich cyanobacterium, *Arthrospira platensis*, exposed to higher Fe concentrations showed protein reduction under abiotic stress, thus corroborating with the results of the present study. Furthermore, the lipids in the microalgal strain grown in presence of excess Fe concentrations at low pH were significantly reduced (Fig. 3d). Urzica et al. (2013) observed adjustment in the metabolic pathway of lipids in a microalga, *Chlamydomonas* sp., as result of Fe starvation, indicating that iron deficiency can cause changes in distribution and fatty acids of lipid content.

Yet another micronutrient, Mn, is actively involved in PSII of photosynthetic apparatus as Mn-cluster in microalgae (Yruela, 2013). Carbohydrates, proteins, and lipids were significantly downregulated in strain MAS1 grown in excess concentrations of Mn at pH 6.7 (Fig. 4a). However, the data on enhanced chlorophyll accumulation in MAS1 indicated that increased levels of Mn did not affect the photosystem (Fig. 2). On the contrary, Liu et al. (2017) reported that excess concentrations of Mn inhibited growth of *Chlorella* sp. by imposing ionic stress. Nevertheless, higher doses of Mn did not affect the expression of Mn superoxide dismutase gene in a green microalga, *Closterium ehrenbergii*, which suggested that Mn functioned as a cofactor than a toxicant (Wang and Ki, 2020). In fact, amide I band corresponds to β-sheet of protein secondary structure that contains ribulose-bis-phosphate carboxylase and histone proteins in chloroplasts of microalgae (Heraud et al., 2005). In addition, the higher intensity of symmetric and asymmetric stretching of lipids correspond to the synthesis of shorter chain lipids in membrane at higher Mn concentrations (Kardas et al., 2014). Interestingly, Mn concentration supported the accumulation of proteins and lipids, but not carbohydrates, in strain MAS1 grown at pH 3.5 (Fig. 4b). Liu et al. (2017) demonstrated that *Chlorella* sp. exposed to Mn concentrations significantly increased total lipids, particularly triacylglycerol. Kardas et al. (2014) reported that region at 1083 cm⁻¹ was significant in bacteria because of their exposure to cobalt that brings about changes in backbone of nucleic acids in positive response towards metabolism or epigenetic modifications. Although Zn is an essential nutrient because of its role in catalysis and in protein stabilization, its presence in excess concentrations is deleterious (Malasarn et al., 2013). Both Zn and Mn deficiencies cause hyper-accumulation of triacylglycerol in microalgae (*Hsieh et al., 2013; Malasarn et al., 2013*); however, we observed an increase in lipid accumulation in both the strains under the influence of higher concentrations at pH 6.7. The observed lipid accumulation at lower concentration of Zn in strain MAS1 could be due to metal stress (Hamed et al., 2017). The FTIR peak
The increased toxicity of metal to microalgal cells depends upon the speciation and bioavailability (Lopes et al., 1999). In growth medium, metal chelates with other ions such as EDTA and phosphates leading to the decreased metal availability (Abinandan et al., 2019b). However, there exists limited information on the effects of pH on metal toxicity at biologically excess levels toward organisms. In this study, we therefore investigated the relative response of microalgae to biologically excess metal concentrations at pH 3.5 and 6.7. The impact of metals on microalgal growth, in terms of chlorophyll, suggests that lower levels of excess concentrations significantly increased the growth at pH 6.7, and there was a 50% reduction in growth at higher concentrations (Fig. 2). Virtually, the chlorophyll content of microalgal strains exposed to both the selected concentrations of metals at pH 3.5 was nearly the same. This observation could be substantiated with the metal speciation in modified BBM alone which revealed no difference in free ion concentration of Fe, Mn, and Zn at pH 6.7 and 3.5 at both the levels included (Table 3). Also, these predicted data revealed that the free ion concentration in modified BBM alone (control) was higher at pH 3.5 than at 6.7, and the free ion concentrations associated with the levels of metals in excess were significantly higher at pH 6.7 as compared to pH 3.5. Acidophilic microalgae commonly exist in extreme environments such as AMDs that contain dissolved heavy metals in surplus amounts because they develop tolerance mechanisms through several physiological and metabolic adaptations (Souza-Egipsy et al., 2011; Abinandan et al., 2018). Thus, the present observations clearly indicate that the microalgal strains were acclimated to higher free ion concentrations of metals even while growing in modified BBM, and the toxic effect of metals at higher concentrations was masked at acidic pH by mediating the required phenotypic changes for their survival and growth.

Similarly, FTIR data on microalgal response to excess metal concentrations at pH 3.5 indicated clear metabolic shifts exhibiting new and enhanced vibrational stretching at specific frequencies (see Section 3.1). Overall, Fe, Mn and Zn predominantly influenced lipid and carbohydrate synthesis in both the strains at pH 3.5. Thus, the results indicate that microalgal strains grown only at acid pH mediated metabolic changes for a better tolerance to metal exposure. Wilde et al. (2006) reported that Cu and Zn were toxic to Chlorella sp. especially at increasing pH from 5.5 to 8.0 and provided a protective effect of H⁺ ions at low pH by reducing the competition of metal ions onto the surface. Bossuyt and Janssen (2005) observed that acclimation of a microalga, Pseudokirchneriella subcapitata, to Cu regulated Cu concentrations to maintain homeostasis. Very recently, we reported that the microalgal strains MAS1 and MAS3, adapted to acidic pH by growing for > 100 generations at pH 3.5, exhibited significant physiological and metabolic alterations when exposed to acid mine drainage samples (Abinandan et al., 2020a). In line with our present observations, distinct metabolic signatures were reported by Charles et al. (2019) in non-acclimated Chlamydomonas reinhardtii when exposed to different ionic stress. Another advantage of acclimation to metal concentrations is the alleviation of detrimental effects induced by the deficiency of other metals such as Fe as reported in Synechocystis sp. (Salomon and Keren, 2015). Overall, our data clearly indicate that acclimation of microalgal cultures to acid pH resulted in significant increase in metabolic activity that potentially masked the toxicity of metals in biologically excess concentrations.

Conclusions

Metals in biologically excess concentrations, especially at acidic pH, are known for their deleterious effects on microalgal growth. In this study, we demonstrated the response of microalgal strains MAS1 and

| Metal | Free ion (%) | pH 6.7 Control | Lower level | Higher level | pH 3.5 Control | Lower level | Higher level |
|-------|--------------|----------------|-------------|-------------|----------------|-------------|-------------|
| Cu    | ND (0.02)    | ND (0.5)       | ND (1.0)    | ND (0.02)   | ND (0.5)       | ND (1.0)    | ND (1.0)    |
| Fe    | 0.47 (1.0)   | 31.69 (5)      | 39.4 (10)   | 16.32 (1.0) | 34.29 (5)      | 40.85 (10)  |
| Mn    | 1.19 (0.50)  | 42.62 (5)      | 48.33 (5)   | 25.82 (0.50)| 44.75 (5)      | 49.81 (10)  |
| Zn    | – (0.10)     | 27.53 (2)      | 37.51 (5)   | 0.20 (0.10) | 28.17 (5)      | 38.36 (10)  |

Values in parentheses are the concentrations of metals supplemented to modified BBM (mg L⁻¹).

ND – Not detected because of Cu complexation with EDTA.

Modified BBM with stipulated concentrations of transition metals.

MAS3 to excess metal concentrations at pH 3.5 as compared to pH 6.7. Both the strains showed a positive response, in terms of carbohydrates, proteins and lipids, especially at lower metal concentrations than higher levels at pH 6.7. The selected microalga tolerated excess concentrations of metals at pH 3.5 and grew relatively well by mediating distinct changes in metabolic signatures as revealed by ATR-FTIR spectroscopy. While strain MAS1 exhibited higher accumulation of lipids, strain MAS3 expressed higher amounts of carbohydrates and proteins under excess concentrations of metals at pH 3.5, which is contrary to the long-held notion of metal toxicity at acidic pH. Such a response could be ascribed to the masked effect of enhanced metal availability at acidic pH resulting in phenotypic changes in microalgae towards excess metal concentrations. Overall, it is apparent from our present study that the strains MAS1 and MAS3 acclimate to pH 3.5 and tolerate and grow well in presence of excess concentrations of transition metals. Our present study demonstrates an interesting finding that acclimation of microalgae to stress mediated by certain abiotic factors such as acidic pH can alleviate the stress imposed by other factors like heavy metals through tolerance mechanism. However, further investigations involving other microalgal strains and abiotic stresses are needed to arrive at such a generalization.

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

Not applicable

Code availability (software application or custom code)

Not applicable

Declaration of Competing Interest

The authors declare no conflicts of interest.
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Supplementary materials

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