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Research Article

# Secondary metabolites production combined with lead bioremediation by *Halamphora* sp. marine diatom microalgae and their physiological response

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## Abstract

This study was designed to investigate the physiological and biochemical response of the diatom microalgae *Halamphora* sp. (SB1 MK575516.1) to the toxicity of lead (Pb) as well as its ability as phytoremediation. Four different concentrations of Pb (50, 100, 150, and 200 mg $L^{-1}$) were applied for 10 days. Fatty acid profile, mineral composition, secondary metabolite contents, and physiological responses have been determined in *Halamphora* biomass. We found that this metal was mainly removed by bio adsorption on cell surfaces and that *Halamphora* sp. could acclimatize upon long-term exposure to Pb stress. A decrease in the cell's number and size, polyunsaturated fatty acids as well as mineral content in *Halamphora* sp were observed under Pb stress. However, an increase in polyphenol, flavonoid, and carotenoid contents has been recorded at 100 mg Pb $L^{-1}$, with stimulation of the antioxidant capacity as measured by DPPH and ABTS radical scavenging activities. An increase in MDA, proline, and $H_2O_2$ levels were also observed. On the other hand, the deleterious effect of Pb resulting from the cellular oxidative state can be alleviated by the enzymatic system such as Superoxide dismutase (SOD), Glutathione peroxidase (GPx), and catalase (CAT). The present study indicates the ability of *Halamphora* sp. to remove heavy metals from the aquatic environment and produce antioxidant biomolecules.



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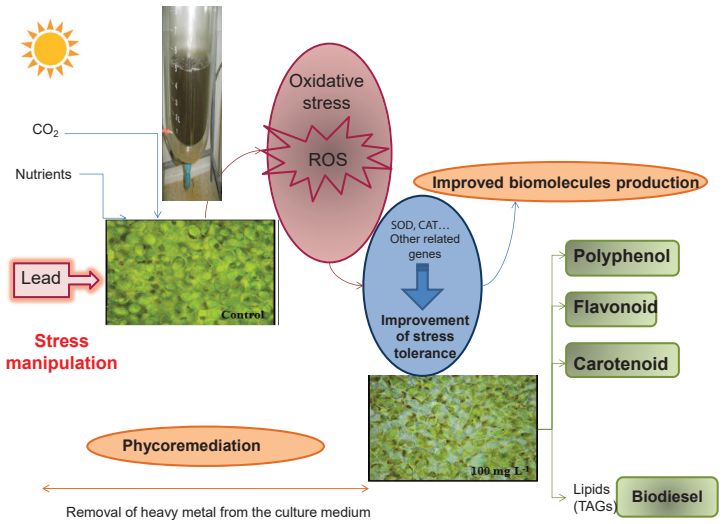


**Graphical abstract:**

## Abbreviations

ABTS: 2,2'-Azino-Bis (3-Ethylbenzothiazoline-6-Sulfonic Acid); Car: Carotenoids; CAT: Catalase; CE: Catechin Equivalent: Chl: Chlorophyll; DPPH; Diphenylpicrylhydrazyl: DW: Dry Weight; GAE: Acid Equivalent; GPx: Glutathione Peroxidase; MDA: Malondialdehyd; MUFA: Monounsaturated Fatty Acids; Pb: Lead; PUFA: Polyunsaturated Fatty Acids; ROS: Reactive Oxygen Species; SFA: Saturated Fatty Acids; SOD: Superoxide Dismutase

## Introduction

Among the most common heavy metals, lead (Pb) is involved in acute and/or chronic effects on organisms [1]. The concentration of this toxic metal occurs at ultra-trace concentrations in environments through natural processes such as weathering. Nevertheless, lead pollution has risen dangerously due to human activities [2]. Lead from anthropogenic sources can lead to concentrations above 10,000 ppm and may induce severe harm to aquatic life even at a low concentration of 10ppm [3]. This heavy metal can cause damage to the nervous system, kidneys, and disturbance of vitamin D metabolism, especially in children when the blood lead levels exceed 25µg/dL [4].

Microorganisms play an important role in the removal of various chemicals and physical pollutants from the environment. Therefore, bioremediation techniques are considered safe and sustainable methods to remove toxic substances from contaminated water. The bioremediation process uses different microbes such as bacteria, algae, fungi, and yeast to treat oil spills, contaminated soil, and contaminated water. Previous studies have well been reported on the approach of microalgae for the removal of various contaminants. In algae-based bioremediation, algae absorb nutrients like carbon, phosphate, and heavy metals from wastewater and produce new biomass, which is useful in the generation of bioenergy [5-8].

The algae have many characteristics that make them ideal candidates for the elimination and concentration of heavy metals, which include high tolerance to heavy metals, autotrophic and heterotrophic growth capacity, and genetic manipulation as well as the development of biofuels [9,10]. Several studies have shown that microalgae species have been used as biomarkers and bioindicators of water pollution and in determining the impact of toxic metals [11]. Recently, Al-Homaidan, Al-Ghanayem [12] demonstrated that the green algae *Enteromorpha* and/or *Cladophora* were used to estimate heavy metal concentrations in many parts of the world. In this regard, some species have already been used for the removal of heavy metals from contaminated water [13]. In recent years, the use of microalgae in the detoxification of wastewater has aroused great attention because of their key role in the fixation of carbon dioxide. In addition, *Amphora* biomass produced has great potential as feedstock for biofuel production [14-17]. These bioremediation capabilities of microalgae are useful for environmental sustainability [11]. In addition, recent investigations regarding microalgae were carried out to remediate domestic and industrial wastewater [7,8,18]. To our knowledge, literature pertaining to the remediation of toxic heavy metals by the diatom microalgae *Halamphora* sp. is relatively scarce. Moreover, the present survey for the first time has been conducted to remediate hypersaline waters.

The aim of this present study was to assess the phycoremediation potential of this wild microalga, *Halamphora* sp., by determining the bioaccumulation capacity and toxicity of lead to this microalga. The effect of lead on the biochemical and physiological responses of this alga was investigated.

## Materials and methods

### Strain and cultural conditions

*Halamphora* sp. SB1 MK575516.1 was isolated via micromanipulation and serial dilutions from the Sfax Solar Saltern pond with an average salinity of 107p.s.u. Isolated and purified microalgae were inoculated in 2L Erlenmeyer flasks



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containing 1000mL autoclaved F/2 culture medium, which was enriched with sodium silicate ($Na_2SiO_3$). The cultures were kept at 25°C under a 16h light/8h dark cycle provided by cool white fluorescence lights at 6000Lux irradiance and were fed by a $CO_2$ bubbling rate of 0.1vvm. The culture was then transferred to a 15L glass air bubble photobioreactor with a working volume of 10L.

## Heavy metal exposure

Exponentially grown cells used for different treatments were harvested and cultured in 2000 mL flasks with 1000mL of F/2 medium with final Pb concentrations of 0, 50, 100, 150 and 200mg$L^{-1}$. Pb solutions were prepared by dissolving analytical-grade $PbCl_2$ in sterilized F/2 medium at the desired concentrations just before the experiment. During the whole experiment, all cultures were kept under the same previous conditions.

Biomass was separated from the culture medium by sedimentation and lyophilized at -70°C.

## Determination of fixed lead

Fixed lead was evaluated using a modified method of Stauber and Florence [19] described by Folgar and Torres [20].

Total lead removed: total lead removed in cells was measured by filtration of 25mL aliquots from each culture through two superposed 1.2-µm Millipore filters. The lead concentrations were determined in both filters and the lower filter was used as blank.

Lead removed intracellularly: Intracellular lead was determined in the following way.

The aliquots of 25mL of each culture were centrifuged at 4500rpm for 15min and the pellet was resuspended for 20min in 25mL of 0.02M EDTA dissolved in an F/2 medium. EDTA's function is to eliminate the lead adsorbed onto the cell surface, thus allowing only the intracellular lead fraction to be measured. Then, the culture was centrifuged for 15min at 4500rpm and the pellet was washed twice with seawater and centrifuged again at 4500rpm for 15min.

**Bioadsorbed lead:** The quantification of adsorbed lead onto the cell surface was calculated by subtracting the intracellular lead concentration from the total lead removed (adsorbed lead = total lead - intracellular lead).

**Measurement of lead level:** Each one of the filters from the determination of total lead removed and the pellets from the determination of lead removed intracellularly were separately digested in a mixture of 5mL of 1M $HNO_3$ and 0.2mL of HCl (37 %). Digested samples were brought to a final volume of 25mL with distilled water. Pb presenting the samples was measured by atomic absorbance spectroscopy (205 AAS; USA).

## Growth

The cell density in the cultures was measured using a hemocytometer. Ten µL of Lugol's solution was added to 50µL *Halamphora* culture to immobilize and stain the cells. Growth inhibition was calculated according to the following equation:

$$\% I_r = \frac{\mu_c - \mu_r}{\mu_c} \times 100 \quad (1)$$

Where $\% I_r$ is the percent inhibition in average specific growth rate, $\mu_c$ is the average specific growth rate (µ) in the control group, and $\mu_r$ is the average specific growth rate for the treatment replicate.

## Mineral composition analysis

Analyses of sodium ($Na^+$), potassium ($K^+$), calcium ($Ca^{2+}$), magnesium ($Mg^{2+}$), Copper ($Cu^{2+}$), and Zinc ($Zn^{2+}$) minerals content in *Halamphora* sp. were performed using the inductively coupled plasma optical emission spectrophotometer (ICP-OES) (Model4300 DV, Perkin Elmer, Shelton, CT, USA).

## Determination of pigments contents

Carotenoid content in the algal cultures was determined spectrophotometrically according to the method described by Lichtenthaler and Buschmann [21].

Extraction of polyphenols was executed as reported by Gouvinhas, Santos [22], with minor modifications. Briefly, samples (0.1g) were mixed with 1.5mL of methanol/distilled water (80:20 v/v), vortexed, and incubated in the dark for 20min under agitation at 40°C. Subsequently, the mixture was centrifuged for 2min at 6000rpm. The supernatants were stored at 4°C.

Total phenol content in *Halamphora* extracts of different groups was estimated by the Folin–Ciocalteu method [23]. The absorbance of the mixture was recorded at 760nm after incubation for 30min in the dark. Gallic acid was used as a reference standard, and the content of total phenolics was expressed as milligram gallic acid equivalent (mg GAE $g^{-1}$ DW).

The total flavonoids content of microalga extracts was determined according to the modified method of [24]. Absorbance was measured at 510nm against the blank. Total flavonoid content was expressed as milligram catechin equivalent (mg CE $g^{-1}$ DW).

## Antioxidant activity

***Diphenylpicrylhydrazyl (DPPH) free radical scavenging assay:*** The measurement of the DPPH radical scavenging activity was carried out as described by Blois [25], with a slight modification. The diluted phenolic extracts (0.06–1mg $mL^{-1}$) were reacted with 1mL of DPPH radical solution 0.1mM in ethanol, and 450µL of 50mM Tris–HCl buffer (pH 7.4) was added. The solution was incubated for 30min at room temperature, and the reduction of DPPH free radicals was measured by reading the absorbance at 517nm. The control solution was prepared by mixing ethanol and DPPH radical solution. The scavenging activity percentage was determined according to the following equation:



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$$\text{Activity}(\%) = \left[\frac{\left(A_{\text{control}} - A_{\text{test}}\right)}{A_{\text{control}}}\right] \times 100 \quad (2)$$

Where $A_{control}$ is the absorbance of ethanolic DPPH solution and $A_{test}$ is the absorbance of samples. The antioxidant activity of each extract was expressed as $IC_{50}$ values, defined as the concentration of the extract required to inhibit 50% of radical.

### ABTS radical scavenging activity

This assay, based on the ability of substances to scavenge 2, 2'- azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) radical cation, was performed according to Katalinic, Modun [26] with minor modifications. Briefly, the ABTS radical cation ($ABTS^{\cdot+}$) was prepared by mixing 7mM ABTS stock solution with 2.45mM potassium persulfate and incubating the mixture for 12–16h before use. The $ABTS^{\cdot+}$ solution was diluted with phosphate-buffered saline (PBS; pH 7.4) until absorbance of 0.70 ± 0.02 at 734nm. The photometric assay was conducted on 2 mL of diluted $ABTS^{\cdot+}$ solution and 100µL of tested extract, or Trolox standard. The reaction mixture was incubated for 30min at 30°C. The decrease in absorbance was recorded at 734nm. All measurements were performed in triplicate. ABTS radical scavenging activity was expressed as µmol Trolox equivalent (µM TEq $g^{-1}$ DW).

### Fatty acids profiling

Fatty Acid Methyl Esters (FAME) were prepared from the lipid extract by transesterification using a direct transmethylation method [15]. Then, the FAMEs were extracted with hexane and analyzed quantitatively by GC-MS (HP 5975B inert MSD) equipped with FID and HP-5MS capillary column (30m length; 0.25mm i.d.; 0.25mm film thickness). The carrier gas (He) was used at a 1mL$min^{-1}$ flow rate. The oven temperature program was 1 min at 100°C ramped from 100 to 250°C at 4°C$min^{-1}$ and 10min at 260°C. The split ratio was 1:100. Components identification was assigned by matching their mass spectra with Wiley and NIST library data.

### Antioxidant enzymes biomarkers

The algal pellets were suspended in phosphate buffer solution (PBS, pH 7.4). Cells were physically disrupted by three cycles of freezing (-80°C). Broken cells were centrifuged at 10,000rpm for 5 min at 4°C, and the supernatants were used for the quantification of antioxidant status.

Catalase (CAT) activity was determined spectrophotometrically according to Radi, and Torrens [27]. The absorbance was measured at 240 nm and the results were expressed as µmol $H_2O_2$ $min^{-1}$ $mg^{-1}$ of protein.

Glutathione Peroxidase (GPx) activity was estimated according to Vega-López, and Ayala-López [28]. The absorbance was measured at 340 nm and the results were expressed as µmol GSH $min^{-1}mg^{-1}$ of protein.

Superoxide Dismutase (SOD) activity was determined according to the method of Cano-Europa, López-Galindo [29] by measuring its ability to inhibit the photoreduction of nitrobluetetrazolium (NBT). SOD activity was expressed as units (U) $mg^{-1}$ protein.

### Determination of oxidative stress biomarkers

Proline was extracted using 3% sulphosalicylic acid and estimated using L-proline as a standard as described by Bates, and Waldren [30].

For the determination of $H_2O_2$ content in microalga, cells were centrifuged at 10,000rpm for 10 min and the pellet was homogenized in 0.1% w/v TCA solution. The homogenate was centrifuged at 10,000rpm for 10min. 0.5ml supernatant was mixed with 0.5mL of 10mM phosphate buffer (pH 7.0) and 1mL of 1MKI. Absorbance was recorded at 390nm. $H_2O_2$ content was expressed as µmoL $H_2O_2$ $g^{-1}$ fresh weight [31].

For the determination of lipid peroxidation, microalgal cells were harvested by centrifugation at 10,000rpm for 10min. The cell pellet was homogenized in 2mL of 80:20 (v/v) ethanol: water. The homogenate was centrifuged at 10,000rpm for 10min. 1mL supernatant was mixed with 1 mL of 0.65% TBA prepared in 20% TCA solution containing 0.01% BHT and heated at 95°C for 25min. After cooling at room temperature, the mixture was centrifuged at 10,000 rpm for 10min. Absorbances of supernatants were read at 450nm, 532nm, and 600nm. MDA content was calculated using the following equation [32]:

MDA (µmol $g^{-1}$ fresh weight) = $[6.45 \times (OD_{532} - OD_{600})] - [0.56 \times OD_{450}]$/ fresh weight (g) (5).

### Statistical analysis

Statistics were performed on SPSS software (version 20). Data were expressed as the mean ± standard deviation and analyzed by one-way ANOVA. The significance level was determined ($p<0.05$) and a significant difference was separated using Duncan's Multiple Range Test (DMRT).

## Results

### Phycoremediation potential

The content of total lead, adsorbed lead, and intracellular lead removed by the *Halamphora* sp. cells were observed in the least different lead concentrations (50, 100, 150 and 200mg $L^{-1}$). The total removed lead by the cells increased proportionally as much as the concentration of Pb in the culture media increased (Figure 1). The maximum removal of Pb occurred for a concentration of 100mg $L^{-1}$ recording 27.64%. However, the Pb removal efficiency decreased by increasing the initial concentration of Pb to 150 and 200mg $L^{-1}$ and reached 24.65 and 20.65%, respectively.

The determination of the Pb bio adsorbed to the cells surface of *Halamphora* sp. is given in Figure 1. For the lowest Pb concentration used (50mg$L^{-1}$), bio adsorbed Pb represents 99.21% of the total Pb removed. Also, for the highest Pb concentration (200mg$L^{-1}$), the bio adsorbed fraction represents 97.36% of the total Pb removed.



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Figure 1 also represents the quantity of Lead removed intracellularly by the cells of *Halamphora* sp. Pb ion removal by *Halamphora* sp. biomass depends largely on the initial concentration of the metal ions in the solution phase. For the lower concentration of 50mg $PbL^{-1}$, *Halamphora* sp. was capable to remove 0.04±0.01pg $cell^{-1}$ which corresponds to 6.89% of the total removed Pb. The metal accumulation into *Halamphora* sp. cell increased at the higher concentration (200mg$L^{-1}$) and reached 0.07±0.02 pg $cell^{-1}$, which represents 7.95% of the total removed Lead.

## Effects of lead on *Halamphora* growth

The impact of Pb concentrations on *Halamphora* sp. growth was studied during 10 days of culture. The result revealed that the cell growth was significantly influenced by lead after 2 days of metal exposure (Figure 2a). In fact, Figure 2a shows that the number of cells of *Halamphora* sp. decreased proportionally with increasing the concentration of Pb in the microalgae medium. Cultures treated with the highest concentration of Pb (200mg $L^{-1}$) showed the most marked phytotoxic effect of heavy metals on the growth of *Halamphora* sp. until the last day of culture. In this condition, the number of cells decreased by 49% as compared to the control group. This might be due to the degradation of indole acetic acid, the hormone which stimulates growth and multiplication, induced by the presence of Pb in the culture medium. At a Pb concentration of 50mg$L^{-1}$, only a slight inhibition of algal growth occurred.

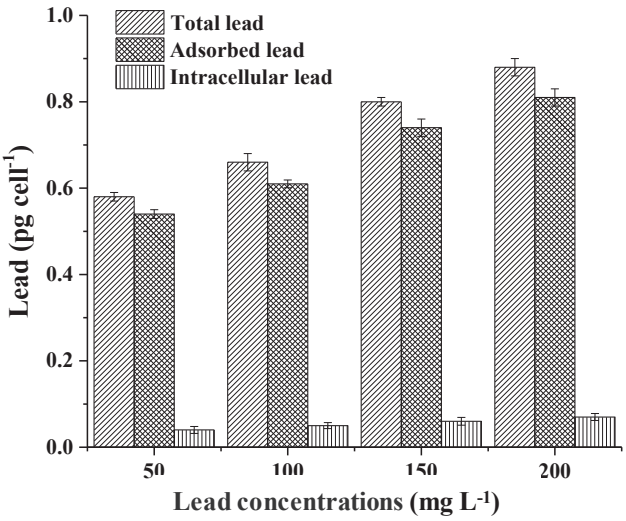


**Figure 1:** Total lead, adsorbed lead, and intracellular lead were removed by *Halamphora* sp. cells grown in an F/2 medium supplemented with different lead concentrations ( 50, 100, 150, and 200mg$L^{-1}$). Data presented are the average of three replicates ± standard error (SE).

The toxicity symptoms were also observed in heavy metal-treated cultures of *Halamphora* sp. (Figure 2b). The morphological structure included partial disorganization of the cell wall, increasing in crystalline inclusions, and a reduction in cellular size at different lead concentrations.

## Effects of lead on the mineral composition of *Halamphora* sp.

The changes in the concentration of mineral composition in the control and experimental group are presented in Table 1. Results of the ANOVA test showed that the mineral element contents of different groups treated with lead were significantly lower ($p< 0.05$) than that of the control group. Lower mineral contents were found in cells grown under different concentrations of Pb which could be a consequence of the growth inhibition during the response to metal stress.

## Effects of lead exposure on *Halamphora sp.* fatty acid profiles

Fatty acids composition of *Halamphora* sp., exposed to Pb stress, was analyzed by extraction and transesterification of the lipids and analysis of the fatty acid methyl ester by GC-MS. *Halamphora* sp. accumulated fatty acids in the range of C14 to C20. Under control conditions, the major fatty acid components were C14:0, C16:0, C16:2, and C16:3 (Table 2). In addition, the levels of saturated fatty acids (SFA) were significantly lower, while the levels of polyunsaturated fatty acids (PUFA), including C16:2, C16:3, C20:4, and C20:5 were the highest compared to stressful conditions.

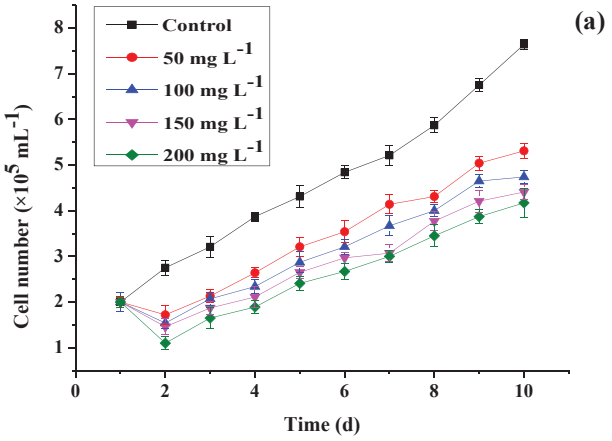


**Figure 2:** Effects of lead exposure on (a) Growth kinetics of *Halamphora* sp. depending on the lead exposure (mg$L^{-1}$) and (b) Cell morphology of *Halamphora* sp. exposed to lead during 10 days of culture. Values are the mean of three replicates ± standard error (SE).



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**Table 1:** Effect of lead concentrations on the mineral composition of *Halamphora* sp. Biomass.

| Treatments (mg $L^{-1}$) | Na | K | Ca | Mg | Fe | Zn |
|---|---|---|---|---|---|---|
| 0 | 1.12 ± 0.05$^{e}$ | 0.48 ± 0.08$^{e}$ | 0.58 ± 0.07$^{e}$ | 0.74 ± 0.04$^{d}$ | 0.008 ± 0.001 | 0.008 ± 0.002 |
| 50 | 1.08 ± 0.07$^{d}$ | 0.46 ± 0.04$^{de}$ | 0.56 ± 0.03$^{de}$ | 0.72 ± 0.08$^{de}$ | 0.006 ± 0.001 | 0.005 ± 0.001 |
| 100 | 1.04 ± 0.08$^{c}$ | 0.43 ± 0.02$^{c}$ | 0.54 ± 0.05$^{c}$ | 0.69 ± 0.12$^{c}$ | ND | ND |
| 150 | 1.02 ± 0.11$^{b}$ | 0.39 ± 0.11$^{b}$ | 0.52 ± 0.07$^{b}$ | 0.67 ± 0.02$^{b}$ | ND | ND |
| 200 | 0.98 ± 0.06$^{a}$ | 0.36 ± 0.04$^{a}$ | 0.49 ± 0.07$^{a}$ | 0.64 ± 0.03$^{a}$ | ND | ND |

Different superscript letters within column indicate significant differences at ($p< 0.05$)

In this study, by the report to control culture, *Halamphora* sp. fatty acids profile showed an increase of SFA from 36.89% to 60.64% at 100mg$L^{-1}$Pb and then decreased to reach 16.64% at 200mg $L^{-1}$Pb (Table 2). As shown in table 2, Pb addition induced saturation of fatty acids by decreasing PUFA percentage and increasing SFA and monounsaturated fatty acids (MUFA) in *Halamphora* sp. cultures. At 150mgPb$L^{-1}$, the proportions of C16:0 and C16:1 were highest, while those of C20:4 and C20:5 were lower.

## Effects of lead stress on secondary metabolites of *Halamphora sp.*

Table 3 represents the effects of lead exposure on *Halamphora* sp. secondary metabolites and pigments. Results showed a pronounced increase in polyphenols content in *Halamphora* sp. at low Pb concentrations of 50 and 100 mg $L^{-1}$ (1.58 ± 0.04 and 2.06 ± 0.07mg GAE $g^{-1}$ DW respectively) compared to untreated cells (1.38 ± 0.03mg GAE $g^{-1}$ DW) (Table 3). This means that moderate metal concentrations resulted in higher phenolic contents in *Halamphora* sp. culture. However, the amounts of phenolic contents per cell decreased at high concentrations of 150 mg $L^{-1}$ and 200mg $L^{-1}$.

In our study, the flavonoid content varied similarly to the phenolic content increasing from 0.29mg Catechin Equivalent (CE) $g^{-1}$ DW in untreated cells to 0.43mg CE $g^{-1}$ DW in treated cells at 100mgPb $L^{-1}$ and then decreased to 0.25mgCE$g^{-1}$ DW at 200mgPb $L^{-1}$ (Table 3).

Carotenoid and chlorophyll contents were also affected by Pb treatment (Table 3). A significant increase in carotenoid content was observed at 50 and 100mg Pb $L^{-1}$ compared to control microalgae. An increase in carotenoid content in treated cells appears to be in response to the metal detoxification mechanism. On the other hand, the higher concentration of Pb (150 and 200mg Pb $L^{-1}$) decreased carotenoid content when compared to control microalgae (Table 3). In contrast, *Halamphora* sp. showed significant reductions in chlorophyll *a* and *b* with the increased metal concentrations ($p<0.05$; Table 3).

## Effects of lead stress on the antioxidant potential of *Halamphora sp.*

The antioxidant capacity of the phenolic extract of *Halamphora* sp. was estimated through the DPPH and ABTS scavenging tests (Tables 3,4). For the DPPH, the lowest $IC_{50}$ (0.11mgm$L^{-1}$) was obtained for the highest concentration of

**Table 2:** Fatty acid (FA) profiles of *Halamphora sp.* grown under different concentrations of lead.

| | Treatments (mg $L^{-1}$) | | | | |
|---|---|---|---|---|---|
| FA (%) | 0 | 50 | 100 | 150 | 200 |
| C14:0 | 16.95 | 36.70 | 23.87 | 13.71 | 2.98 |
| C16:0 | 11.02 | 16.50 | 28.59 | 32.75 | 12.40 |
| C18:0 | 7.36 | 6.21 | 6.26 | 4.67 | 0.94 |
| C24:0 | 1.56 | 2.44 | 1.92 | 1.37 | 0.31 |
| SFA | 36.89 | 61.85 | 60.64 | 52.49 | 16.64 |
| C16:1 | 9.15 | 9.99 | 11.58 | 16.60 | 5.56 |
| C18:1 | 1.84 | 3.77 | 11.92 | 17.78 | 74.00 |
| MUFA | 10.99 | 13.76 | 23.51 | 34.37 | 79.56 |
| C16:2 | 16.76 | 6.83 | 1.28 | 2.12 | 1.43 |
| C16:3 | 19.83 | 9.64 | 7.51 | 6.20 | 1.61 |
| C20:4 | 6.09 | 1.28 | 1.81 | 0.84 | 0.16 |
| C20:5 | 9.45 | 6.64 | 5.25 | 3.97 | 0.61 |
| PUFA | 52.12 | 24.38 | 15.85 | 13.14 | 3.80 |

**Table 3:** Effect of lead concentrations on secondary metabolites and pigments levels in *Halamphora* sp.

| Treatments (mg $L^{-1}$) | Polyphenols (mg GAE $g^{-1}$ DW) | Flavonoids (mg CE $g^{-1}$ DW) | Car (µg $mL^{-1}$) | Chl *a* (µg $mL^{-1}$) | Chl *b* (µg $mL^{-1}$) |
|---|---|---|---|---|---|
| 0 | 1.38 ± 0.03 | 0.29 ± 0.02 | 1.33± 0.01 | 7.95±0.1 | 4.31±0.39 |
| 50 | 1.58 ± 0.04 | 0.31 ± 0.03 | 1.46± 0.04 | 7.34±0.2 | 4.17±0.05 |
| 100 | 2.06 ± 0.07 | 0.43 ± 0.02 | 1.51± 0.01 | 6.24±0.27 | 3.64±0.14 |
| 150 | 1.67 ± 0.03 | 0.34 ± 0.04 | 1.25± 0.02 | 5.67±0.06 | 2.78±0.03 |
| 200 | 1.17 ± 0.02 | 0.25 ± 0.01 | 1.07± 0.05 | 4.85±0.4 | 2.21±0.03 |

Data are expressed as the mean of three replicates ± standard error (SE). GAE: Gallic Acid Equivalent, CE: Catechin Equivalent, DW: Dry Weight.

**Table 4:** Effect of lead intoxication on antioxidant activities of *Halamphora* sp. Extracts.

| Treatments (mg $L^{-1}$) | $IC_{50}$ (mg $mL^{-1}$) | | ABTS radical scavenging activity (µM TEq $g^{-1}$ DW) |
|---|---|---|---|
| | DPPH activity | BHT | |
| 0 | 0.32 ± 0.04 | 0.17± 0.04 | 0.54 ±0.04 |
| 50 | 0.27 ±0.03 | 0.17± 0.03 | 0.58 ±0.05 |
| 100 | 0.41 ±0.02 | 0.17± 0.02 | 0.75 ±0.05 |
| 150 | 0.25 ±0.01 | 0.17± 0.04 | 0.61 ±0.03 |
| 200 | 0.11 ±0.03 | 0.17± 0.03 | 0.20 ±0.01 |

TEq: Trolox Equivalent. Data presented are the average of three replicates ± standard error (SE).

200mgPb$L^{-1}$, while the highest $IC_{50}$ was 0.41mgm$L^{-1}$ which was recorded for the mild Pb concentration (100mg$L^{-1}$). The ABTS scavenging capacity of *Halamphora* sp. extracts increased



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with the Pb concentration, ranging from 0.54 µM Trolox equivalent (TEq) $g^{-1}$ DW for the control to 0.75µM TEq$g^{-1}$ DW at 100mg$PbL^{-1}$. Then, this parameter decreased significantly and reached 0.20mMTEq $g^{-1}$ DW at 200mg$PbL^{-1}$.

Results showed the relative antioxidant efficiency of the diatom extracts against the DPPH radical. Antioxidants suppressed the absorbance at 515 nm on a time scale dependent on the antioxidant activity of the extracts.

The extracts derived from the diatom exposed to lead showed the highest radical scavenging activities $IC_{50}$ of 0.41mg$mL^{-1}$ at 100mg$L^{-1}$ of lead exposure compared to the extract derived from the control (0.32mg$mL^{-1}$). The different measurements showed that *Halamphora* sp. has an antioxidant capacity that rises with the increase in lead in the medium.

### Effects of lead exposition on antioxidant enzymes and oxidative stress markers in *Halamphora sp.* cells

This is the first study to investigate the toxic effect of lead on the diatom alga *Halamphora* sp. The impact of different concentrations of Pb on the activities of the antioxidant enzymes SOD, GPx, and CAT of *Halamphora* are shown in Figure3. The result revealed that the activities of the antioxidant enzymes involved in antioxidant defense were negatively correlated with the concentrations of Pb (Figure 3).

The highest Pb concentration (200mg$L^{-1}$) significantly decreased SOD, GPx, and CAT activities in *Halamphora* sp. by 78.51%, 51.68%, and 63% ($p<0.01$), respectively, compared to the control (Figure 3a, b, c).

Our result indicated an increase in the oxidative damage markers induced by Pb ions in *Halamphora* sp. where, a considerable increase in lipid peroxidation (MDA), proline, and $H_2O_2$ levels was recorded. This increase was dose-dependent. After 10 days of exposure, the MDA content was significantly enhanced ($p<0.05$) by about 3.4-folds by the high dose of Pb (200mg$L^{-1}$) in *Halamphora* sp. Similar to the present study, Dahmen-BenMoussa, Athmouni [13] found an increase in lipid peroxidation level with the increase of nickel in the growth medium from 0 to 500mg$L^{-1}$.

Similarly, $H_2O_2$ content was also extremely increased with increasing Pb concentration (Figure 3f), indicating that metal concentration was dependent on free radical production in the culture medium. The low concentration (50mg$L^{-1}$) increased the $H_2O_2$ level from 2.42µmol$g^{-1}$ FW to 6.34µmol$g^{-1}$ FW ($p<0.05$) and the high concentration (200mg$L^{-1}$) continued to increase it to 26.78µmol$g^{-1}$FW. Similarly, Piotrowska-Niczyporuk, and Bajguz [2] reported increases in $H_2O_2$ amount in *Acutodesmus obliquus* exposed to different concentrations of lead (0-500µM).

Similarly, at the high concentration proline content was elevated from 15µmol$g^{-1}$ FW to 34µmol$g^{-1}$ FW ($p<0.05$). On the other hand, the maximum proline content was observed at the mild concentration of Pb, *i.e.*, 47.45µmol$g^{-1}$ FW (Figure 3e).

Generally, the production of proline increases under stress produced by some metals, since these molecules contribute to the antioxidant response against reactive oxygen species (ROS).

## Discussion

During the exposition period, algae treated with (200mg $PbL^{-1}$) contained the highest concentration of lead (0.88pg $cell^{-1}$). This is often in agreement with the earlier study on *Acutodesmus obliquus* [2]. The tolerant diatom *Halamphora* sp. showed high efficiency of heavy metal biosorption. This may be due to their smaller size and larger cell wall surface area [10]. Therefore, even small differences in the organization and composition of the cell wall can greatly influence the tolerance of a particular metal to the different species of microalgae, as well as the level of tolerance toward metals. Microalgae-based technology is highly recommended for being applied for the biotreatment of heavy metals [33].

The mechanism of microalgal bioremediation of lead may occur in two ways i.e. biosorption process (a part of the lead removed by the cells is adsorbed on the cellular surface) and bioaccumulation process (another part is accumulated intracellularly). The results of this study agree well with the previous observation of Singh, et al. [34], who find that no more considerable increase in metal sorption is observed by a tandem increase of metal ions concentration. Uptake of Pb ions in algae occurs via the $Ca^{2+}$ pathway. Since Pb can imitate Ca, a reasonable explanation for the increased absorption of $Pb^{2+}$ could be that $Pb^{2+}$ could block the $Ca^{2+}$-dependent $Pb^{2+}$ efflux system [35]. This second phase is known as the absorption of intracellular ions. It consists of the active sorption of heavy metal ions within the cytoplasm of algae cells and depends on cell metabolism (Shivaji and V L Dronamaraju 2019). It's also been confirmed that the absorption of intracellular ions has a great contribution to the biosorption and detoxification of heavy metal ions [36].

A higher lead concentration was found to be present as loosely bound to the frustules instead of accumulating intracellularly, which suggests an efficient mechanism of metal-exclusion that binds the metal in the extracellular environment and prevents the entrance of lead into the cell. In our assay, the diatom *Halamphora* sp. leads to high adsorption removal capacity due to the rigidity, the high area surface, and therefore the strong binding affinity to heavy metals. There is some evidence to prove that sulfhydryl and amide groups of enzymes involve in binding Pb, altering their configuration and diminishing their activities [1]. Low molecular proteins such as phytochelatins, may also participate in binding toxic metals [2]. Algae uptake metals both passively and actively; some metals such as strontium (Sr) and Pb may be passively adsorbed by charged polysaccharides in the cell wall, and intracellular matrix [11], while others (eg Zn, Cd) are actively absorbed against a large intracellular concentration of gradients [37].

Our data indicate that the *Halamphora* sp. strain examined can tolerate the toxicity of Pb even at higher concentrations. Other algae such as *Scenedesmus quadricauda*, *Phormidium ambiguum*, and *Chlorella* sp. (3,38) are also able to tolerate Pb concentration up to 100mg $L^{-1}$. It was determined that the toxic effect of Pb on growth and some key metabolic processes in *Halamphora* sp. cells is caused by its intracellular accumulation.



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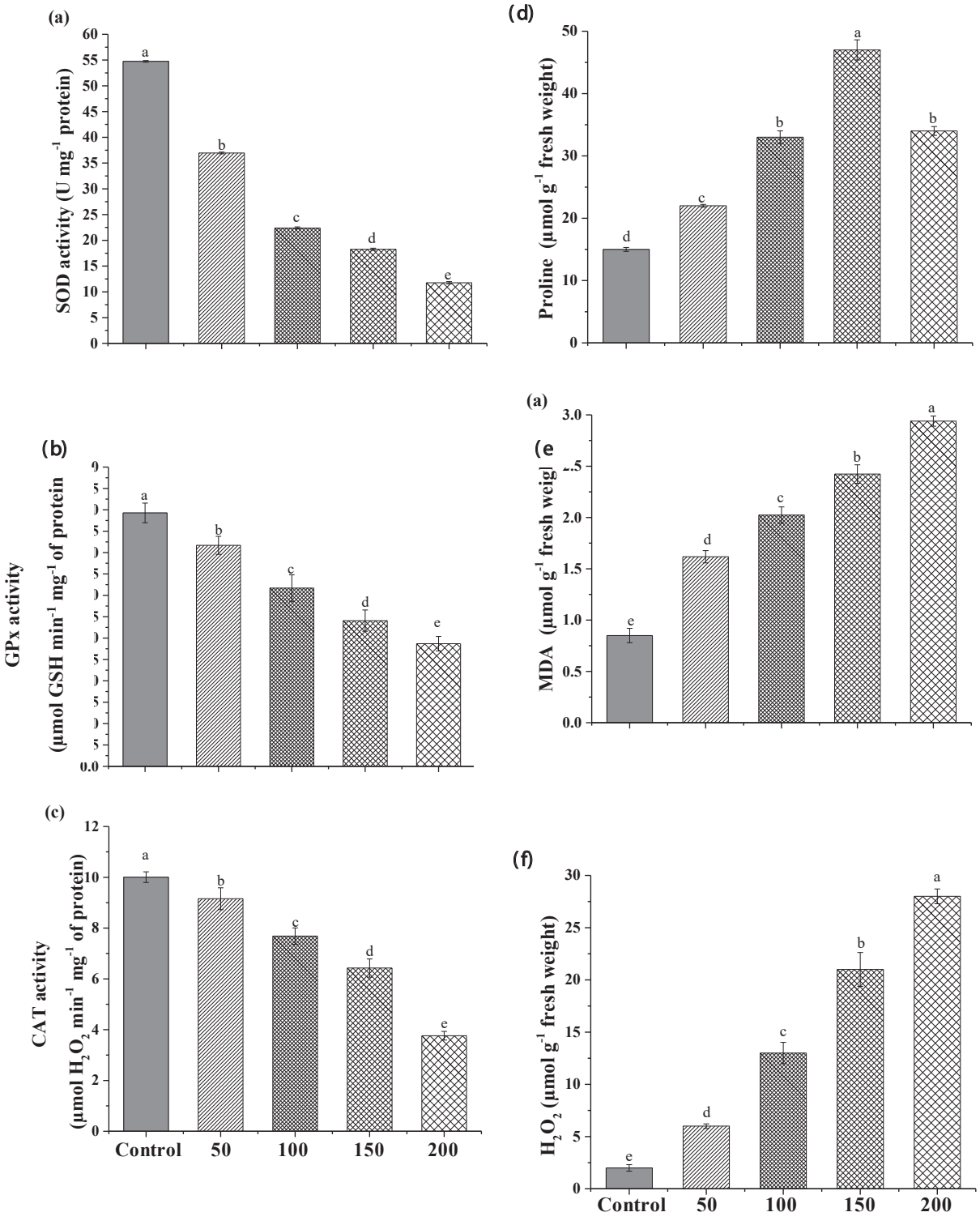


**Figure 3:** Effect of lead on Superoxide dismutase (SOD) (a), glutathion peroxidase (GPx) (b) and catalase activities, Proline (d), MDA (e), and $H_2O_2$ (f) in the diatom alga *Halamphora* sp. grown under different lead concentrations. Data presented are the average of three replicates ± standard error (SE). Different letters within the bar indicate significant differences at $p<0.05$.

Once in the cell, the lead induces the synthesis of class II phytochelatins and metallothionein which can enhance the detoxification effect [3]. Growth inhibition in microalgae is linked to the number of heavy metal ions bound to the algal cell surface, in some cases, and also to the quantity of intracellular heavy metal ions depending on their chemical nature Piotrowska-Niczyporuk, Bajguz [2,39]. The suppression of growth by lead was oberved in *Chlorella vulgaris* as reported by Piotrowska-Niczyporuk, Bajguz [2], Teoh and Wong [3], Bajguz [40], Piotrowska-Niczyporuk, Bajguz [41].

The changes in morphological structure confirm the effect



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of this metal on the growth of *Halamphora* sp. A study with the diatom *Amphora coffeaeformis* reported that heavy metals such as copper and cadmium, at all concentrations, cause fewer branched filaments with the presence of many small vacuoles in the cells [42]. Similarly, Piotrowska-Niczyporuk, Bajguz [2,43] also observed a decrease in the cell size of the green microalga *Acutodesmus obliquus,* which may be associated with the toxic effect of Pb on the cell wall structure and the leakage of ions or cell components through the cytoplasmic membrane thus generating necrosis and irreversible cell damage. Recently, it has been demonstrated that Pb intoxication induces cellular death and oxidative stress, DNA damage, activation of caspase-3, and externalization of phosphatidylserine [1].

Lower mineral contents were found in cells cultured in the presence of Pb, which could be a consequence of reduced growth during the metallic stress response. Yedjou, Tchounwou [1] reported that one of the major mechanisms by which lead exerts its toxic effect is through biochemical processes that include lead's ability to inhibit the action of calcium and to interact with proteins [44]. Within the cell, Pb may also compete with metallic cations for binding sites or alter the transport of essential metallic cations such as calcium [36].

The fatty acids profile of *Halamphora* sp., exposed to Pb stress showed a higher SFA and MUFA contents and a lower PUFA (C20:4 and C20:5) content in comparison with the control culture. Eicosapentaenoic acid (EPA) in control cells can be used as a nutraceutical or pharmacological agent for the prevention of cardiovascular and inflammatory diseases [45]. Moreover, EPA is the most valuable FA found in microalgae with a high content [46], which makes them suitable for aquaculture feeding [17,47]. Metal stress can be applied to alter the composition of fatty acids in microalgal cells to produce biodiesel [10].

A previous study reported that exposure of *Amphora subtropica* to nickel stress at 300mg $L^{-1}$ led to an increase in C16:0, C18:0, and C18:1 lipid globules and a decrease in C18:2, C20:4, and C20:5 thylakoid membrane lipids (Dahmen-Ben Moussa, 2017). These changes in fatty acid composition resulted in a reduced degree of unsaturation of the total fatty acid pool. High levels of SFA and MUFA combined with low levels of PUFA are essential for biodiesel [48]. MUFAs are better than PUFAs for the cetane number and iodine value while SFA is favorable for combustion and oxidative stability–related biodiesel properties [48]. Finally, the coupling of heavy metal treatment and the production of biodiesel by microalgae makes it possible to combat eutrophication and industrial pollution linked to the production of renewable energies [18,35,36,49]. Nanda and Jaiswal [10] have also compared and reported similar findings for the use of microalgae in Pb bioremediation of contaminated wastewater in conjunction with biofuel production.

Polyphenols represent a group of chemical compounds arising from a common intermediate, phenylalanine, or a close precursor, shikimic acid [50]. Polyphenols can protect cells from oxidative stress by different mechanisms; they can chelate transition metal ions, directly scavenge molecular species of active oxygen can inhibit lipid peroxidation by trapping the lipid alkoxyl radicals [50].

The increase in the flavonoid content is explained by the fact that these molecules contribute to protecting the cells against the environmental pollution caused by the presence of lead in the medium at moderate concentrations. However, flavonoid content decreased, when higher concentrations of Pb were applied, as a result of the inhibitory effect of Pb on phenol synthesis and the cell damage affected by Pb at high concentrations [1].

The inhibition of pigment content observed in our experiment may be related to the sensitivity of the total carotenoid pigment towards the heavy metal ions at high concentrations. Some earlier studies have reported decreased carotenoid content, when higher concentrations of lead were used, as a consequence of the poisoning of the intracellular enzyme systems [51,52].

A wide range of valuable by-products (such as bioethanol and biodiesel), valuable nutrients, and bioactive compounds can be extracted from the produced biomass [35]. In this work, appropriate Pb concentrations (≤100mg Pb $L^{-1}$) have enhanced phenolic, flavonoid, and carotenoid production. On the cost side, integrated algal-based wastewater treatment and bioactive compounds production can not only efficiently remove potentially hazardous contaminations such as toxic metal pollutants but also reduce the inputs and costs of algal biomass production [53].

In this research, the phenolic extract of *Halamphora* sp. showed a moderate antioxidant capacity. These results are consistent with the findings of many research groups that have reported a positive correlation between total phenolic content and antioxidant activity [54]. We suggest that the increase of non-enzymatic antioxidants, represented by phenolic compounds (including flavonoids) and the carotenoids, is responsible for the increase in the antioxidant capacity in *Halamphora* sp. Phenolic compounds from plants can act as antioxidants by chelating metal ions [55]. Consequently, plants exposed to heavy metals accumulate high amounts of phenolic compounds allowing their protection against cellular damage [56]. Similar to the present study, Belghith, Athmouni [57] reported that as Cd exposure increased up to 25mg$L^{-1}$, phenolic contents of green alga *Dunaliella salina* increased. An increase in phenolic compound content has been reported also, in *Phaeodactylum tricornutum* exposed to high doses of copper (Cu(II)) [58]. These compounds help to minimize stress on the cell, which reduces the metabolic effort needed to absorb the metal [58]. Previous research has shown that flavonoids, a class of phenolic metabolites, have significant antioxidant and chelating properties [50].

As for chlorophyll content, showed decreasing values of both chlorophyll *a* and *b* for all Pb concentrations exposure. The increased Pb concentrations exposure may increase the inhibitory stress towards the photosynthesis of cells due to the destruction of chlorophyll pigments essential for photosynthesis. Hee, Shing [59] have supported the detrimental effects of Pb on the growth and chlorophyll content of *Chlorella Vulgaris*. They had reported a decrease in chlorophyll pigments per cell after an exposure of 32mg$L^{-1}$ of Pb. When microalgae



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are exposed to Pb, the oxidative potential of metal ions causes the inhibition of key enzymes that play a crucial role in the $CO_2$ fixation cycle. Such a decrease in the activity of these enzymes reduces the synthesis of ATP in the fixation of $CO_2$ and the production of ATP and NADPH which therefore affects the rate of photosynthesis [3].

Several studies indicated that the heavy metal exposition affected the antioxidant enzymes activities implicated in the scavenging of ROS [13]. Batsalova, Teneva [60] found that microalgae are sensitive to exposure to lead due to its toxicological effect. The results of the present study are consistent with the observations of Piotrowska-Niczyporuk, Bajguz [2] who reported that lead produced ROS and the elevated concentrations of these species suppressed the antioxidant response of SOD, CAT, and GPx in *Acutodesmus obliquus* culture treated with 500µM Pb. Many researchers found that Pb exposure significantly decreases the antioxidant status in the green alga *Cladophora* [61]. For instance, Cao, Shi [62] reported a decrease in SOD activity in the green alga *Cladophora*, with the increase of Pb concentrations in the growth medium from 0 to 200mg $L^{-1}$. Additionally, Huang, Lai [61] revealed that lead exposure induced oxidative stress in *Phanerochaete chrysosporium* by decreasing the catalase activity. Decreased expression of antioxidative enzymes may be due to a corresponding increase in oxidative damage. In our present study, the possible reason for suppressed activities of SOD, CAT, and GPx enzymes might be due to the down-regulation of gene expression of these enzymes. Another possible reason for decreased activity of antioxidant defense system might be due to brassinosteroids signaling (BRs). It has been reported that BRs enhanced the level of the antioxidant system (SOD, CAT and GPx) under heavy metals stress conditions. However, Bajguz [40] found that Pb exposure decreased BRs level in the green algae *Chlorella vulgaris.*

Microalgae can annul any effects associated with the overproduction of ROS by enzymatic and non-enzymatic antioxidants. Proline is an important non-enzymatic biomolecule produced by microalgae to prevent stress [63]. Accumulation of proline by cells under metal stress is an adaptive strategy to reverse oxidative stress injury via scavenging free radicals, maintaining osmotic balance, regulating cellular redox potential, and protecting membranes, enzymes, and ribosomes [64]. Overall, high lipid peroxidation level and damage to the membrane structure suggest that the induced antioxidant enzymes may not have been able to maintain ROS below the toxic level. Previous studies assumed that the protective response of algae cells against heavy metal ions strongly depends on their resistance to oxidative damage [65,66]. In addition, the presence of excessive toxicity of heavy metal ions could lead to denaturation of the protein structure, replacing essential elements, or damaging the oxidative balance of living algae. The intensity of stress on algae cells depends on the protein and oxidized lipid content of these cells [67,68]. This stress tolerance was attributed to the high antioxidant defense system of *Halamphora* sp. and its biodegradation capacity.

## Conclusion

In a conclusion, the data obtained in the current study demonstrate that the strain of *Halamphora* sp. studied was capable of tolerating high lead concentrations of at least 200mg L-1. Lead inhibited the *Halamphora* sp. cells' growth, decreased the antioxidant status, and enhanced oxidative stress at high concentrations. Pb treatment affected the content of polyphenols, flavonoids, and pigments in *Halamphora* sp. as well as its fatty acids profile. Given the potential of *Halamphora* sp. in bioremediation, these metabolites most probably contribute to the use of *Halamphora* sp. to remove heavy metals and may help it to survive in Pb-contaminated environments.

### Credit authorship contribution statement

IDB and SB designed and performed the experiments, analyzed the data, and wrote the paper. KA participated in the design and execution of the experiments. DB and HA critically reviewed the manuscript. All authors read and approved the final manuscript.

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