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# Single toxicity of arsenic and combined trace metal exposure to a microalga of ecological and commercial interest: *Diacronema lutheri*

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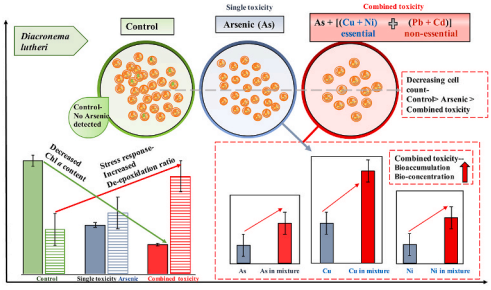
## HIGHLIGHTS

- Cell count significantly reduced in trace metal mixture than Arsenic (As V).
- Total As reduced in the single exposure of As V than in mixture on day 20.
- Bioaccumulation and bio-concentration factor of As V increased in mixed trace metal.
- Chl *a* content was lowest in mixed trace metal when compared to As V treatment.
- De-epoxidation ratio in the mixed trace metal was higher than in As V alone.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Eco-toxicological assays with species of economic interest such as *Diacronema lutheri* are essential for industries that produce aquaculture feed, natural food additives and also in drug developing industries. Our study involved the exposure of a single and combined toxicity of arsenic (As V) to *D. lutheri* for the entire algal growth phase and highlighted that a combined exposure of As V with other essential (Copper, Cu; Nickel, Ni) and non-essential (Cadmium, Cd; Lead, Pb) trace metals reduced significantly the cell number, chlorophyll *a* content, and also significantly increased the de-epoxidation ratio (DR) as a stress response when compared to the single toxicity of As V. Arsenic, as one of the ubiquitous trace metal and an active industrial effluent is reported to have an increased bio-concentration factor when in mixture with other trace metals in this study. In the combined exposure, the concentration of total As bio-accumulated by *D. lutheri* was higher than in the single exposure. Hence, polluted areas with the prevalence of multiple contaminants along with the highly toxic trace metals like As can impose a greater risk to the exposed organisms that may get further bio-magnified in the food chain. Our study highlights the consequences and the response of *D. lutheri* in terms of contamination from single and multiple trace metals in order to obtain a safer biomass production for the growing need of natural derivatives.

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## 1. Introduction

Trace metals are persistent and have proved to be toxic to surrounding organisms in all spheres of the ecosystem (Anandkumar et al., 2019; Rajaram et al., 2020; Das et al., 2020a; Yang et al., 2020). The scenario post-industrialization has posed a serious ultimatum to numerous species around the world with continuous exposure to harmful substances that are often disposed of in coastal areas (Johnson et al., 2003; Schwarzenbach et al., 2006; Webster et al., 2011). Several studies have reported the effects of such contaminants on marine organisms and the consequences on their growth, physiology, metabolism, morphology, etc. (Baumann et al., 2009; Diaz et al., 2012; Facey et al., 2019; Mamun et al., 2019a; Dayras et al., 2020). Some trace metals like copper (Cu), nickel (Ni), zinc (Zn) play essential roles in the metabolism of the organism while others like arsenic (As), cadmium (Cd), lead (Pb) have no such capacity (Zidour et al., 2019; Das et al., 2020a). The essential trace metals are required at certain concentrations in the organism which, when exceeded, can be toxic (Chen et al., 2018; Yang et al., 2020). Amongst the non-essential trace metals, Arsenic is often considered as ubiquitous in nature posing a high risk of toxicity through its exposure (Hak et al., 2020; Huang et al., 2021). Further, As is considered as an extremely varied toxicant and a predominant carcinogen that persists in the entire food chain of the ecosystem existing in several organic and inorganic states (Sghaier et al., 2015; Mitra et al., 2017; Mamun et al., 2019a,b; Hussain et al., 2021). An inorganic state of As; arsenate (As V) has been reported as the chief state that exists in an aerobic ecosystem and often gets absorbed into the body surface of aquatic organisms (Mitra et al., 2017; Mamun et al., 2019a,b). In general, such toxic trace metals are often present in a combined complexity with other metals and thus individual effect on the organism depends on the bioavailability of each metal (Kadiene et al., 2019a,b; Zidour et al., 2019; Das et al., 2020a). Laboratory-based studies of single toxicity of a trace metal have however taken their place for few decades, but comparisons of single and combined toxicity are still in the way of making a profound understanding in eco-toxicology (Zidour et al., 2019; Das et al., 2020a). Previous studies have focused on the single toxicity of As V on marine organisms and its capacity of bio-accumulation and biotransformation whereas, a comparison of varied pathways of exposure (single and combined toxicity) on the first trophic level of the food chain has not been much explored (Upadhyay et al., 2018; Hak et al., 2020; Huang et al., 2021). In addition, the industrial wastes that are usually emitted into the adjacent water bodies, are often present as a cocktail of trace metals and other organic pollutants that gets exposed to the inhabiting organisms, which further substantiates the need of considering the effect of combined trace metal and/or other contaminants in ecotoxicology (Das et al., 2020b). Therefore, experimental approaches comparing culture conditions (control) with that of single toxicity and combined toxicity of trace metals should be encouraged.

To comprehend the effects of such contaminants, phytoplankton, being one of the primary producers of the aquatic sphere played a major role as a bio-indicator in assessing the threats prevailing in a particular environment and risk assessment assays (Salama et al., 2019; Kwaansa-Ansah et al., 2019; Rajaram et al., 2020; Yang et al., 2020). The bioaccumulation of trace metals in algae usually occurs by active or passive transports of ions into the cell membranes that act as hydrocolloids, affecting its growth by decelerating photosynthetic activity (Leal et al., 2016; Salama et al., 2019). Trace metals can often cause oxidative stress and increase the cellular reactive oxygen species (ROS) that can further cause alteration in enzymatic activity, pigment contents including protein degradation (Cheloni and Slaveykova et al., 2018; Salama et al., 2019). Apart from this, trace metals have also shown adverse effects on the morphology, and the coherence of cell membranes controlling the permeability of the cell wall of algal species (Mamun et al., 2019b; Costa et al., 2019; Salama et al., 2019). Marine micro/-macroalgae have shown resilience to such toxicity and pathways to depurate these substances by compartmentalizing the toxins in specific cellular cavities like vacuoles followed by a release of the contaminants into the medium through efflux pump (Perales-Vela et al., 2006; Juang and Chang et al., 2016; Salama et al., 2019). They have also proved their adaptability to trace metals by the formation of metalothioneins and phytochelatins (Navarrete et al., 2019; Costa et al., 2019). In general, algal species are capable of bio-accumulating trace metals that often penetrate and get stored in their tissues and also poses a threat of bio-magnification of contaminants to higher trophic levels (Yin et al., 2011; Tao et al., 2012; Yang et al., 2020). Besides the risk of bio-magnification, studies have also highlighted the ability of micro/-macroalgal species for bioremediation, reporting high removal efficiency of trace metals, secondary pollutants and hydrophobic compounds by some algal species like *Spirulina*, *Chlorella*, *Cladophora* and many others (Mahdavi et al., 2012; Jasrotia et al., 2014; Yang et al., 2015; Gao et al., 2016). Hence, studies on the effect of highly toxic trace metals like As and its combined toxicity with other trace metals can further benefit our understanding on the removal efficiency of such contaminants by microalgae near anthropogenic quarters. *Diacronema lutheri* (Droop) Bendif and Véron (2011) is one such ecologically and economically important microalgae that is widely cultivated worldwide. Indeed, this microalga constitutes loads of phytosterols and is also extensively used as a feed in the aquaculture industry (Ahmed et al., 2015; Bernaerts et al., 2018; Huang et al., 2020). Such phytosterol rich organic sources are the natural alternatives that are often used as additives in food items for their ability to lower the levels of low-density lipoprotein (LDL) cholesterol and also beholds anti-inflammatory, anti-cancerous and anti-oxidative properties (Kim et al., 2008; Carmona et al., 2010; Ahmed et al., 2015). Therefore, toxicological assays implementing toxicity from varied sources and pathways involving economical species with medicinal and therapeutic benefits like *D. lutheri* should be encouraged. Out of the few studies conducted on the eco-toxicological aspect involving *D. lutheri*, some of them reported that when *D. lutheri* was exposed to dissolve organic contaminant originating from nearby industrial catchment areas in Scotland, showed significant inhibition of growth rate in nearly all the stations for higher concentrations of pesticides; that was useful in assessing the risk of that particular habitat (Emelogu et al., 2013, 2014). Therefore, the objective of our study was to monitor the effect of As V which is predominant and shows high adsorption ability, as a single source of toxicant and a combined mixture of essential (Cu; Ni) and non-essential (Cd; Pb) trace metals to comprehend the bioaccumulation and bio-concentration of these metals when detected in a mixture. The further objective was to analyze the effect of the bioaccumulation of trace metals on the growth curve and the pigment contents of *D. lutheri* comparing it with the controlled culture conditions. The tangential aim of this study was to highlight the risks of bioaccumulation and bio-concentration of trace metals to optimize the growth and production of this economically beneficial algal species when used in medicines and the aquaculture industry as a feed for higher trophic communities. *Diacronema lutheri* is used as an aquaculture feed in our laboratory regularly for years, and the amount of trace metal used in this study corresponds to the lethal concentration of the bio-indicator copepod *Eurytemora affinis* (Kwok et al., 2015; Souissi and Souissi, 2021), with regards to enhancing our understanding on the consequences in case of a trace metal contamination in our culture systems emerging through the algal feed source. Such eco-toxicological assays with *D. lutheri* are scarce and can help to better understand the mechanism of environmental toxicity and the cocktail effect of contaminants in addition to the benefits in the commercial industries to improve the quality and quantity of biomasses that can be safely used as a natural alternative to drugs.

## 2. Material and methods

### 2.1. Algal species- Diacronema lutheri

*Diacronema lutheri* from the Roscoff Culture Collection (RCC-1537),

France was cultivated in artificial seawater (osmosed water with artificial salt, Coral salt-pro) in round bottom flasks of 6 L and was maintained in an incubator with controlled physical parameters (12:12 h light: dark in 18 °C, salinity 33) (Arias et al., 2017; Gnouma et al., 2017; Dayras et al., 2020). The formulation of the culture was ensured by adding an adequate supply of nutrition through the Conway medium in every culture flask by following the same composition mentioned in Tlili et al. (2016).

### 2.2. Exposure to trace metals: single and combined toxicity

To understand the variability in growth, pigment concentration, and ability to bio-accumulate trace metals by *D. lutheri*, two different treatments with trace metal stress along with control were designed in triplicates. The trace metal stress applied to the algae was single toxicity of As V and a mixture of trace metals (As V + Cd + Cu + Ni + Pb). The metal concentrations used in this study corresponds to the 10% lethal concentration (LC50%) of each trace metal when exposed to the invertebrate aquatic model *Eurytemora affinis*, a bio-indicator, calanoid copepod which is often fed with *D. lutheri* in the mass cultures of our laboratory (Zidour et al., 2019; Kadiene et al., 2019b; Das et al., 2020a, Dayras et al., 2020). The LC 50% (96 h) for Cd, Ni, Cu, and Pb when exposed to *E. affinis* was used according to our previously reported studies (Cd - 108.5 $\mu g.L^{-1}$, Cu - 33.5 $\mu g.L^{-1}$, Ni - 125.5 $\mu g.L^{-1}$, Pb - 413.5 $\mu g.L^{-1}$) (Zidour et al., 2019; Das et al., 2020a). The lethal concentration used for As V (96 h, 449.78 $\mu g.L^{-1}$) was determined in our laboratory following the same protocol as reported in Tlili et al. (2016) (supplementary data, Table 1S). The exposure for all the treatments was executed in 6 L sterilized flasks using artificial seawater, under controlled light and temperature conditions along with constant re-suspension for a symmetrical availability of nutrients, light, and temperature for all the algal cells (Tlili et al., 2016; Arias et al., 2017; Gnouma et al., 2017; Dayras et al., 2020). The exposure started by adding sterilized algal culture at the log phase along with the Conway medium and vitamin (vitamin B1, vitamin B12) to the experimental flasks to anticipate a successive exponential growth. The composition of Conway medium and vitamin was the same as reported in Arias et al. (2017) and Tlili et al. (2016). The entire growth curve of *D. lutheri* with daily cell count was accounted for in our study from day 1 (T1) until day 23 (T23).

### 2.3. Cell counting: daily growth monitoring

Cell counting was done every day from T1 to T23 for monitoring the daily growth of the algal cells in each treatment and replicate by using an inverted microscope at 100-fold magnification (Olympus, I×71, Tokyo, Japan) attached to a high storage capacity computer to preserve all photographs of the fields observed under microscope for quantification of cell numbers (Guillard and Sieracki, 2005; Arias et al., 2017). A volume of 2 mL sample from each treatment was collected at a particular time daily in Eppendorf tubes and was fixed with 5% lugol solution in triplicates and then stored at 4 °C before quantification (Iwasawa et al., 2009; Arias et al., 2017). A haemocytometer (Malassez cell slide, 0.1 mm depth, Japan) was used for observing the algal cells under the microscope and each field was photographed for counting the number of cells (Gnouma et al., 2017; Dayras et al., 2020). A software named ImageJ (https://imagej.nih.gov/ij/) was later used for counting the number of cells in each field from each sample and replicates.

### 2.4. Analysis for trace metal concentration – ICP-MS

The analysis for trace metals was executed with an inductive coupled plasma quadrupole mass spectrometer (ICP-MS) (ICP-MS 7900, Agilent Technologies, United States) equipped with a standard introduction system [borosilicate Micro Mist concentric nebulizer (0.4 mL $min^{-1}$), quartz double pass spray chamber cooled at 2 °C, quartz torch (2.5 mm ID) and nickel cones. The algal samples were collected at every phase of the entire growth curve of *D. lutheri*. The sub-sampling intervals for trace metal analysis were day 7 (T7, exponential phase), day 14 (T14, early stationary phase), day 17 (T17, late stationary phase), and day 20 (T20, lysis phase). The algal samples collected at the mentioned intervals were filtered on sterile membrane filters of 0.45 μm from all the conditions and were dried for further pre-treatment. The pre-treatment of the filters comprised of acid digestion, where nitric acid and hydrochloric acid were mixed in the ratio of 1:3 and added to the Teflon tube containing the filter. These tubes were then put in a water bath of 100 °C for at least 4 h placed inside a laminar flow cabinet. After the digestion, a final volume of 5 mL was adapted in each Teflon tube with ultrapure double distilled water (Milli Q). The samples were then transferred to sterilized Falcon tubes and were analyzed for trace metal concentration under ICP-MS. The trace metal concentration remaining in the residual seawater after filtering the algal biomass was also considered by collecting the filtrate (15 mL) directly into sterilized Falcon tubes and fixing it with ultrapure nitric acid (20 μL). These tubes were kept in dark until further analysis under ICP-MS without any need of pre-treatment (Ouddane et al., 1990; Zidour et al., 2019; Kadiene et al., 2019a; Das et al., 2020a). In the entire process of trace metal analysis, all the quality control measures were implied by maintaining the optimum quality for all the standard solutions, and for trace metal identification a reference of multi-elemental standard was utilized (Ouddane et al., 1990). Certified Reference Materials (CRM) PACS-3 and HISS-1 was used to validate the trace metal concentrations and the mineralisation procedure constituted of ≤10% total analytical errors. The bio-concentration factor (BCF) of each trace metal in *D. lutheri* was calculated from the concentration of trace metal bio-accumulated in algae and the residual trace metal in the medium for each condition (Das et al., 2020a).

### 2.5. Analysis for pigment concentration – HPLC

Samples for pigment analysis were collected at all phases of the algal growth curve of *D. lutheri*, in the same manner as for trace metals, on day 7, day 14, day 17, and day 20. After filtration through GF/F Whatman filters, they were stored at −20 °C in the dark until extraction. Pigment extraction was performed by grinding the filters with methanol and 4 drops (30 μL) of methylene chloride, avoiding exposure to light. The extracts were further concentrated after filtration on polytetrafluoroethylene membrane filter of 0.45 μm by dry-evaporation under a nitrogen stream. Desalination of the extract was accomplished with a mixture (50:50 v/v) of distilled water and methylene chloride. Phase separation was followed to obtain the organic portion from each sample, which was then dry-evaporated under nitrogen gas. Each sample was dissolved in 40 μL of methanol before injection, from which a volume of 20 μL was injected in the high-performance liquid chromatography instrument (Shimadzu, Nexera XR; C18 reverse-phase column, Allure, Restek). The separation was performed using a solvent delivery profile adapted from Arsalane et al. (1994). Along with the photosynthetic pigments, the photoprotective pigments were also studied to denote any possible stress response that triggered in the algal cells when exposed to trace metals from single and combined toxicity. The xanthophyll cycle has often proved to be a sensitive parameter to indicate metal stress and its role in the photoprotection process underneath high-light has been reported in previous studies (Bertrand et al., 2001; Brunet et al., 2014; Yan et al., 2017). The reversible reaction between diadinoxanthin and diatoxanthin in the xanthophyll cycle often referred to as the de-epoxidation ratio (DR) was calculated by the following equation (1) (Brunet et al., 2014; Yan et al., 2017):

$$DR = \frac{Diatoxanthin}{Diadinoxanthin + Diatoxanthin} \quad (1)$$

### 2.6. Statistical analysis

The daily cell count, chlorophyll *a* concentration, de-epoxidation ratio (DR), bioaccumulation of trace metals, bio-concentration factor in each treatment including the control were compared by using analysis of variance (ANOVA) at $p < 0.05$, after meeting all the requirements of normality and homogeneity of variance. To test the normality and the homogeneity a Shapiro Wilk normality test and a Levene's Test for Homogeneity of Variance were carried out respectively. To compare the significance in each condition Tukey multiple comparisons of means (95% family–wise confidence level) was applied to the data. All the analyses were accomplished by using R studio (4.0.4).

## 3. Results

### 3.1. Daily growth

Fig. 1 corresponds to the growth curve of *D. lutheri* from T1 to T23 (day 1- day 23), in each treatment, namely control, As V (single exposure) and mixed trace metals (combined exposure/ mixture). The phases of algal growth curve are well distinguished in the control treatment with the highest concentration of cells reaching on day 15 with $28 \times 10^6$ cells $mL^{-1}$. The cell density in control increased from T7 to T15 and then reached a stationary phase from T16 to T18, followed by the lysis phase or death phase with decreasing number of cells until T23. The trace metal exposed treatments (As V and combined) showed significantly lower concentration of cells than control from T7 until T23. After 7 days of continuous exposure the combined trace metal mixture showed statistically the least concentration of cells when compared to As V exposure and control. The single exposure of As V and the trace metal mixture differed significantly only at T7 and thereafter from T16 to T23. However, the cell count in As V was significantly lower than control, but higher than combined exposure. In both the As V and combined trace metal exposure, the phases of algal growth were not well defined and showed high variability especially during the early to late stationary phase. The lysis phase for combined exposure started at T16 showing significantly lower no. of cells from As V exposure until T23. The single exposure to As V however showed higher no. of cells than the combined trace metal mainly in the exponential and lysis phase. The sub-lethal concentrations of single trace metal exposure (As V) and combined trace metal exposure (As + Cd + Cu + Ni + Pb) could reduce the biomass significantly throughout the algal growth curve.

### 3.2. Pigment concentrations

#### 3.2.1. Chlorophyll a concentration

Fig. 2 shows the chlorophyll *a* (chl *a*) concentration in each treatment at specific intervals in the entire growth curve of *D. lutheri*. The control treatment at each interval had significantly higher amount of chl *a* content than the trace metal exposed treatments. The highest chl *a* content was observed on day 14 (T14), when the pigment value increased significantly compared to the exponential phase at T7 in case of control and As V exposure, but however, the pigment content did not significantly increase in the combined trace metal treatment. In comparison between the trace metal exposed treatments, As V exposure showed higher chl *a* concentration at each interval of growth phases, although not significantly. In both the trace metal exposed treatments, the chl a content decreased significantly at T20 gradually from T14. The chl *a* content and the cell count of *D. lutheri* at specific intervals of the growth curve in all the treatments including control showed a significant positive correlation, with a p-value $<0.01$, marking the coherence of both the parameters in this study.

#### 3.2.2. De-epoxidation ratio

Fig. 3 shows the de-epoxidation ratio for all the treatments at each incubation time throughout the growth curve of *D. lutheri*. A significantly higher de-epoxidation ratio was observed for the trace metal combined treatment at the linear phases (T14, T17) and the lysis phase (T20). At the exponential phase, the DR was not significantly different between the treatments control, As V exposure and combined toxicity of

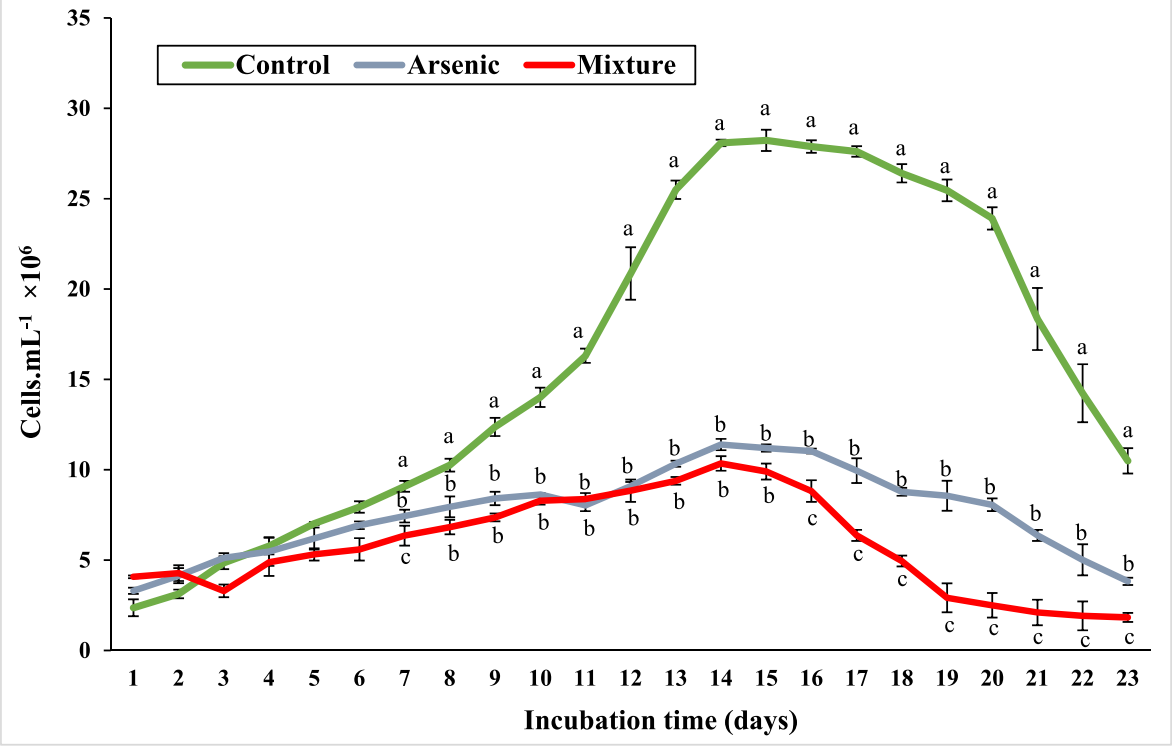


**Fig. 1.** Growth curves of control and trace metal exposed treatment from day 1 (T1) until day 23 (T23). The letters (a, b, c) signify statistical differences between each treatment, at $p < 0.05$. The error bar shows the standard deviation between the three replicates in each treatment.

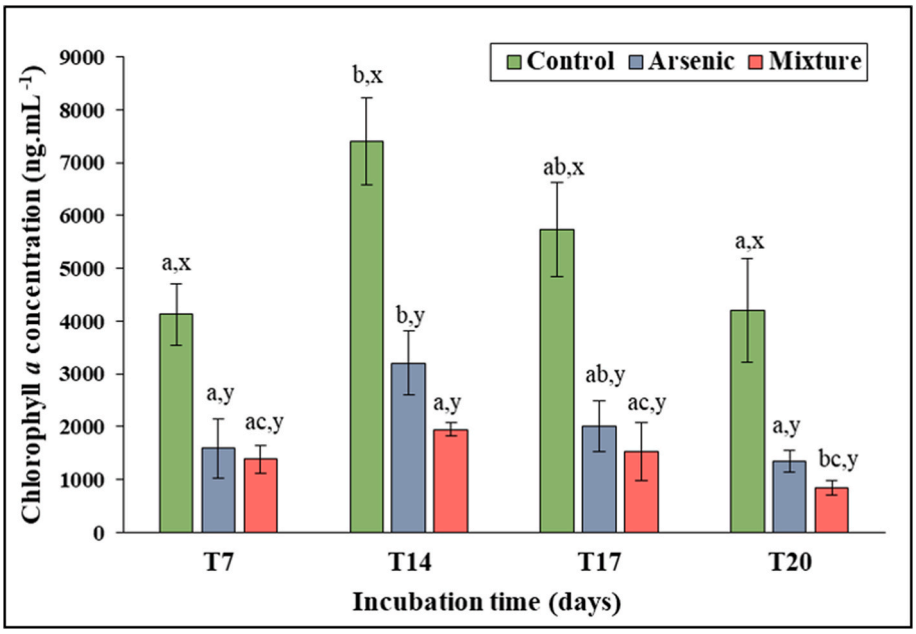


**Fig. 2.** Chlorophyll *a* concentration for each treatment at all phases of growth in *D. lutheri*. Letters a, b, c shows significant differences between incubation times; x, y shows significant differences between treatments, at $p < 0.05$. The error bar shows the standard deviation between the three replicates in each treatment.

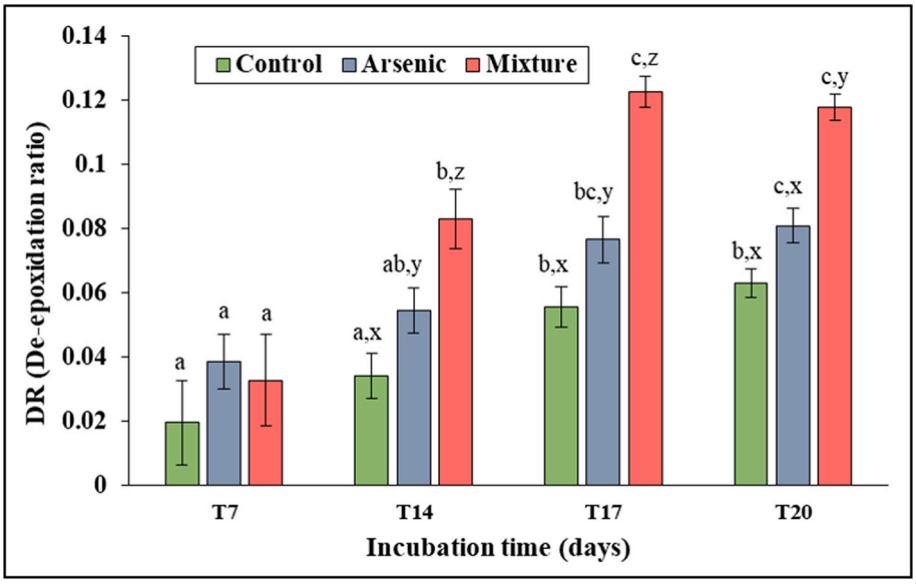


**Fig. 3.** De-epoxidation ratio (DR) for each treatment at all phases of growth in *D lutheri*. Letters a, b, c shows significant differences between incubation times; x, y, z shows significant differences between treatments, at $p < 0.05$. The error bar shows the standard deviation between the three replicates in each treatment.

trace metals (As V + Cd + Cu + Ni + Pb). In the single exposure of As V treatment, the de-epoxidation ratio was significantly higher than in control in the linear phases (T14, T17) but not at the exponential and the lysis phase. In comparison between the trace metal exposed treatments, mixed trace metal showed significantly higher DR than the As V exposed treatment at all the stages, except the exponential phase (T7).

### 3.3. Bioaccumulation of trace metals

#### 3.3.1. Single exposure – arsenic

In the single exposure of As V, the essential trace metals (Cu and Ni) were also detected in *D. lutheri* at the specific intervals (T7, T14, T17 and T20) as shown in Fig. 4. The bioaccumulation of total As measured at each interval showed significantly higher value at T14 when compared to the exponential phase (T7) and lysis phase (T20). Moreover, at T20 we observed a significant decrease in the total As concentration bio-accumulated in *D. lutheri*. In this treatment no additional Cu and/or Ni was added in the exposure beaker but was detected in the trace metal analysis as they play essential roles in algal metabolism. The highest bioaccumulation of Cu and Ni was found at T17 increasing significantly from T7. However, at T20 both Cu and Ni did not show any significant decrease of trace metal concentration compared to T17.

#### 3.3.2. Combined exposure of trace metals (As V + Cd + Cu + Ni + Pb)

Fig. 5 shows the bioaccumulation of all the trace metals combined in one exposure at specific incubation times in the entire growth curve of *D. lutheri*.

The highest bioaccumulation was by Pb at T17 followed by Cu > Ni

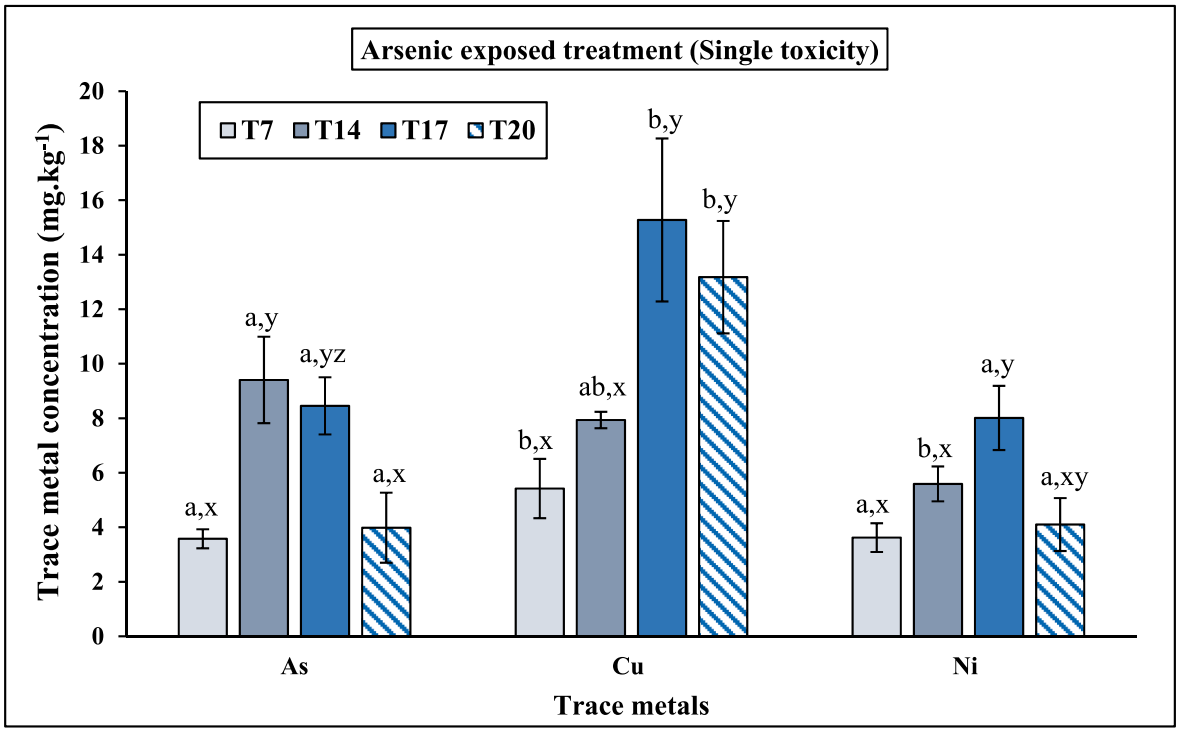


**Fig. 4.** Bioaccumulation of trace metals from the single toxicity exposed treatment of arsenic. Letters a, b shows significant differences between trace metals; x, y, z shows significant differences between incubation times within a single trace metal, at $p < 0.05$. The error bar shows the standard deviation between the three replicates in each treatment.

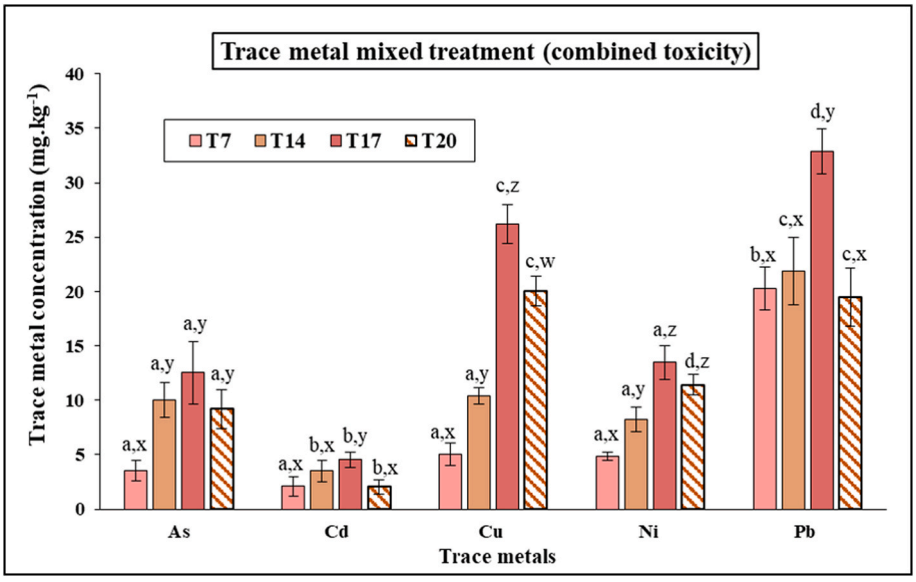


**Fig. 5.** Bioaccumulation of trace metals from mixture treatment. Letters a, b, c, d shows significant differences between trace metals; w, x, y, z shows significant differences between incubation times within a single trace metal, at $p < 0.05$. The error bar shows the standard deviation between the three replicates in each treatment.

> As > Cd. All the exposed trace metals showed highest bio-accumulation at T17 correlating positively with the increase in cells concentration in the linear phase. However, at the lysis phase (T20) each combined metal showed a decrease in trace metal concentrations, out of which Cu and Pb showed a significant decrease. This finding was in contrast to the single exposure of As V treatment (Fig. 4) in which Cu did not show any significant reduction at T20 given the fact that this metal has essential metabolic functions. Inversely, when As V was exposed through a single exposure (Fig. 4) it showed a significant decrease in bioaccumulation at T20 but in combination with other trace metals no such reduction was noted (Fig. 5). The total As concentration in the single exposure was highest at T14 (Fig. 4), but when combined with other trace metals, the highest bioaccumulation was reached at T17 also denoting higher values of total As when in mixture with other trace metals.

### 3.3.3. *Bio-concentration (BCF) of trace metals*

In the single exposure to As V treatment, only Cu and Ni were

detected (without any addition) marking their essential role in algal metabolism. However, no Cd and Pb was detected in this treatment. Hence, Fig. 6a shows a comparison between the BCF of As exposures (single and combined with other trace metals), Fig. 6b, shows Cu exposure (naturally present and combined with other trace metals) and Fig. 6c, shows Ni exposure (naturally present and combined with other trace metals). In As V single exposure, the highest BCF was reached at T14, increasing significantly from T7 and further reducing significantly at T20. On the other hand, when As V was combined with other trace metals, the highest BCF was noted at T17, significantly increasing from T7 but showing no significant reduction of trace metal concentration at T20. When both exposures (single and combined) were compared, a significant difference in BCF of total As at T17 and T20 was noted. The Cu detected in the single exposure of As V showed significantly higher BCF at T14 and T17 than T7 thereafter reducing at T20 although not significantly. Further, the comparison between the naturally detected Cu and the Cu added with other trace metals showed, a significantly higher BCF of Cu at T17 in mixture along with a significant reduction at T20. On the other hand, Ni when detected in the single exposure of As V showed highest BCF at T17 increasing significantly from T7 and T14 but lacking any reduction of trace metal concentration at T20. The comparison between Ni naturally detected and when in mixture with other trace metals showed significantly higher BCF in mixed trace metal exposure at T14, T17 and at T20 without any significant decrease in both the cases.

## 4. Discussion

### 4.1. Cell growth of *Diacronema lutheri*

Previous studies have reported that exposure to trace metals along with other contaminants such as organic pollutants and/or microplastics affected the growth rate of many algal species, in general (Emelogu et al., 2014; Chia et al., 2015; Bellingeri et al., 2019, Mamun et al., 2019a,b; Navarrete et al., 2019; Wilson et al., 2019; Huang et al., 2021). However, these studies had varied exposure strategies ranging from combined toxicity of trace metals, microplastics, and organic pollutants, etc. coupled with several nutrient limiting exposures (Chia et al., 2014, 2015; Navarrete et al., 2019; Huang et al., 2021). A recent study on single exposure of As and combined exposure of As and Cu on three algal species showed a lower growth rate of the algal cells in the combined exposure after 10 days (Huang et al., 2021). In this study, a similar observation was noted, where the algal cell density in the combined exposure of the essential (Cu, Ni) and non-essential (As, Cd, Pb) trace metals varied significantly after 7 days of exposure from the single toxicity of As V and control. Furthermore, similar to our observation, other studies also reported the significant inhibition in the growth rate after the exponential stage and described it as a combined effect of the toxicity from trace metal and the nutrient limiting condition mainly at the end of each algal growth curve (Serra et al., 2010; Monteiro et al., 2011; Chia et al., 2013, 2014, 2015). Huang et al. (2021) also observed a time-dependent effect on the declining growth rate of the algal species irrespective of the concentration of As used in their study. In comparison between single toxicity and combined toxicity, previous studies on varied organisms have shown a decrease in the growth rate when exposed to a combination of trace metals, microplastics, and nano-plastics (Yan et al., 2015; Tang et al., 2015; Kim et al., 2017; Barboza et al., 2018; Huang et al., 2021). Synergistic toxicity of Cd, Cu, Pb, and Zn significantly affected the enzyme activities and chlorophyll *a* content in an estuarine plant (*Avicennia marina*) in comparison to their exposures, that posed no significant effects (Mac Farlane and Burchett, 2002; Yan et al., 2015). In addition to our study, a combination of trace metals like As, Cd, Cu has been reported to damage the membranes of

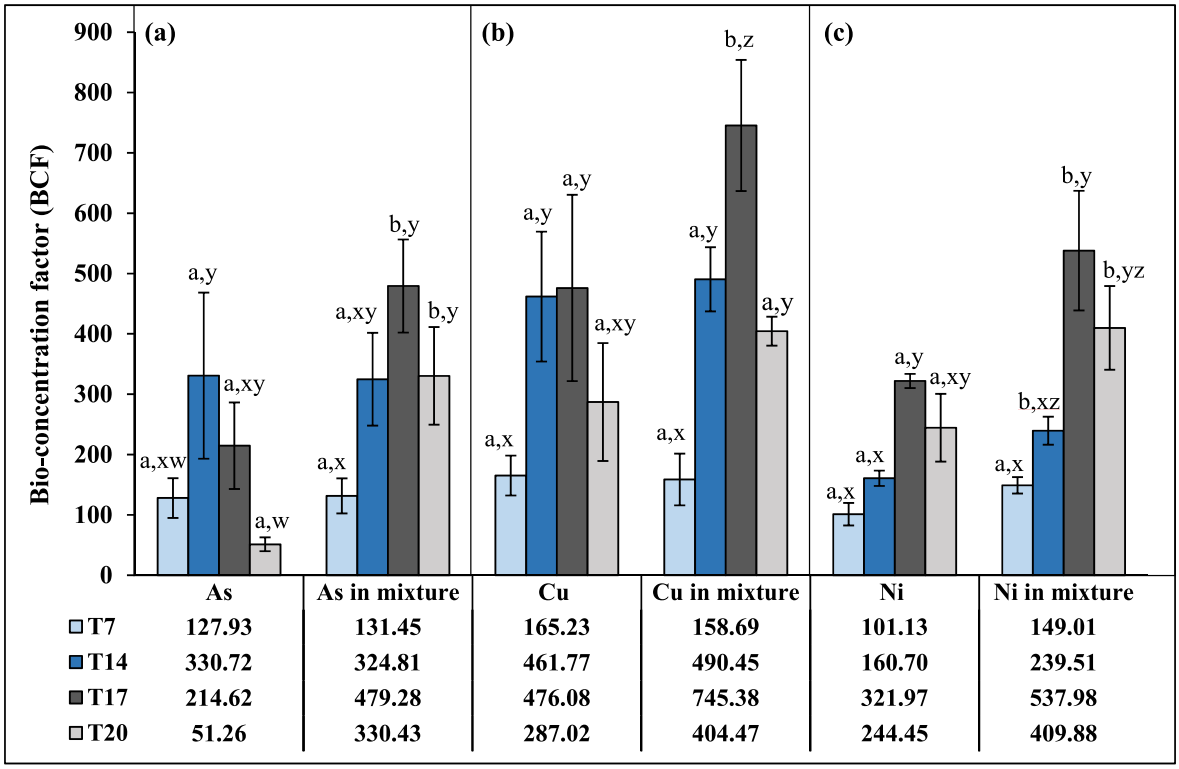


**Fig. 6.** (a). Comparison of the bio-concentration factor (BCF) of total As in single toxicity and in mixture, where, w, x, y, z denotes the significant differences between the incubation time in a single treatment and letters a, b denotes the significant differences between total As single and mixture treatment on a single day of exposure. **(b and c).** Comparison of the bio-concentration factor of Cu and Ni detected in the single exposure of As V (due to their essential roles) and in trace metal mixture, where x, y, z denotes the significant differences between the incubation times in a single treatment and letters a, b denotes the significant differences between Cu single and mixture treatment and Ni single and mixture treatment on each day of exposure at $p < 0.05$. The error bar shows the standard deviation between the three replicates in each treatment.

cells disrupting the permeability and its capacity to uptake nutrients because of the trace metals binding to the sulfhydryl groups and preventing several enzymatic activities that are crucial for the metabolism, growth, physiological conditions and morphological characteristics (Chattopadhyay et al., 2002; Zheng et al., 2008; Hu and Zhou, 2010; Chia et al., 2014; Huang et al., 2021).

### 4.2. Pigment concentrations of D. lutheri

*Diacronema lutheri* is a haptophyte (pavlovophycean) consisting of flagellum at the hind-site with two flagella of unequal length. They contain pigments like fucoxanthin, carotenoids, type A pigments that includes chl a, c1, c2, chlorophyllides, pheophytine a, diadinoxanthin, diatoxanthin and β-carotene (Van Lenning et al., 2003; Bendif et al., 2011; Kim et al., 2021). The pigment chl *a* has been an indicator to the trace metal stress in previous studies, which reported a decrease in its content when algal cells are often impaired by the trace metals, and as a result affecting its ability to assimilate $CO_2$, hindering the process of photosynthesis and lowering the biomass in acute and chronic exposures (Qian et al., 2009; Chia et al., 2013; Liu et al., 2014; Li et al., 2018). Additionally, in a recent study, exposure to both the forms of arsenic (As III and As V) could induce a significant decrease in the chl *a* and *b* content in an aquatic plant, *Vallisneria natans* (Li et al., 2018). Similar to the previous findings, our study has shown a significant decrease in chl *a* content of *D. lutheri* in the trace metal exposed treatments when compared to control conditions. Studies have also reported similar observations on varied trace metals when exposed to large terrestrial flora, where soluble forms of trace metals having a high affinity towards the sulfhydryl group bind and affects the pigment contents and enzyme activities retarding the growth of the organism (Tomar et al., 2000; Ali et al., 2002; Li et al., 2018). However, when related to the number of cells, algal species like *Chlorella vulgaris*, *Scenedesmus obliquus*, some cyanobacterial and periphyton communities have shown higher contents of chl *a* per cell, along with high carbohydrate and lipid biomolecules in response to trace metal stress and limitation of nutrient (Soldo and Behra, 2000; Omar 2002; Okmen et al., 2011; Chia et al., 2015; Mamun et al., 2019a). Although a significant decrease in chl *a* content from control to trace metal exposed treatments was observed in our study, a comparison between the single exposure and combined exposure to trace metal showed no significant differences. On the other hand, the comparison of cell density in the single and combined trace metal exposures showed significant differences in the exponential phase and at the stationary phase. Such observations where the cell growth was retarded and yet a higher content of chl *a* per cell was observed in toxic exposures have been illustrated in previous studies as a probable mechanism of increasing the chl *a* content followed by the synthesis of sugars, carbohydrates, and other biomolecules to compensate the declined growth of the algal cells (Okmen et al., 2011; Chia et al., 2013, 2015; Tang et al., 2015). Chia et al. (2013, 2015) further explained this strategy as a stress response observed in the algae *Chlorella vulgaris* when exposed to Cd in several nutrient limiting conditions. Our study reports similar observation in the case of combined toxicity of trace metals that included Cu and Ni (essential trace metals) in sub-lethal concentrations which presumably induced such stress responses as reported by previous studies (Davarpanah et al., 2015; Wang et al., 2015; Ashraf et al., 2016; Hang et al., 2016; Huang et al., 2021). However, conclusively the synergistic effect of As, Cd, and Pb (non-essential elements) led to a reduced content of chl *a* when compared to individual exposure of As V, however not significantly. Chlorophyll *a* was however the most abundant pigment found in *D. lutheri* followed by fucoxanthin that ranged from 1950 ng $mL^{-1}$ to 2100 ng $mL^{-1}$ in control cells, 600 ng $mL^{-1}$ to 400 ng $mL^{-1}$ in single exposure of As and the lowest concentration found in the combined trace metal treatment ranging from 350 ng $mL^{-1}$ to 200 ng $mL^{-1}$; all measured at the exponential phase. In addition, considerable amount of pheophytine *a* was observed with control showing the highest value of 550 ng $mL^{-1}$ on an average, followed by single exposure of As (258 ng $mL^{-1}$ on an average), and mixture of trace metals (137 ng $mL^{-1}$ on an average) all measured at the exponential phase. The de-epoxidation ratio (DR), in previous studies, have been reported as a good indicator of the photo-oxidative stress response when high-light or any form of toxic substances are encountered by the algal cells (Eskling et al., 1997; Bertrand et al., 2001; Ait Ali et al., 2008; Yan et al., 2017; Cabrita et al., 2018; Zsiros et al., 2020). Zsiros et al. (2020), showed that *Chlorella variabilis*, exhibited a higher DR value when the algal cells were exposed to the trace metal Chromium (Cr). Our study showed a similar finding, where significantly higher DR was observed in case of trace metal exposed treatments, consistently at all phases excepting the exponential phase (first 7 days of exposure) where algal cells perhaps could combat the toxic effects of trace metals with sufficient nutrient availability and increasing enzymatic activity induced by the toxic exposure (Okmen et al., 2011; Chia et al., 2013, 2015; Das et al., 2019; Mamun et al., 2019a). Although studies on the comparison of single and combined exposure of trace metals and its effect on the DR values are rare, in general, trace metal stress on organisms have shown to regulate the DR, through non-photochemical quenching and dissipation of excess energy in order to protect the cell membranes from reactive oxygen species, indicating varied oxidative stress responses induced by sublethal concentrations of environmental toxicants, which was also observed in the present study (Demmig-Adams et al., 2014; Yan et al., 2017; Bethmann et al., 2019; Zsiros et al., 2020).

### 4.3. Bioaccumulation of trace metals in D. lutheri

Trace metal accumulation by algae has been reported to cause considerable damages in cell structure, growth rate, pigment contents, enzyme activities, and several other metabolic functions in previous studies (Zhang et al., 2019; Mamun et al., 2019a,b; Hussain et al., 2021; Huang et al., 2021). Studies considered the trace metal accumulated in the biomass/algal mass and also the residual trace metal concentration in the culture medium to emphasize the importance of studying bio-concentration factors (Baumann et al., 2009; Huang et al., 2021). Our study has collectively considered both the parameters to highlight the harmful effects caused by the toxic exposures of As V and combined trace metals (As V + Cd + Cu + Ni + Pb). In this study the bioaccumulation of all the trace metals in the combined metal exposed treatment increased significantly in the linear phase (Day 17), and the order of the bioaccumulation was Pb > Cu > Ni > As > Cd. This observation was in accordance with some previous studies that also observed Cu and Pb to have the highest and Cd to have a lower concentration of bioaccumulation in varied species of macroalgae like *Ulva intestinalis*, *Cladophora rupestris*, *Chondrus crispus*, and microalgae like *Amphora coffaeiformis*, *Dunaliella salina*, *Euglena* sp. and many others (Yu and Wang, 2004; Baumann et al., 2009, Chiellini et al., 2020; Elleuch et al., 2021). In a recent investigation on phytoplankton blooms, the authors reported that Cu and Pb has a greater potential to be bioavailable from their particulate forms and thereby can get rapidly bio-accumulated by phytoplankton posing higher risks for bio-magnification whereas trace metals like Cd, Chromium (Cr), Cobalt (Co) has shown lower particle reactivity (Cabrita et al., 2020). Mamun et al. (2019a), in a study based on the toxicity of As V, reported a rapid accumulation of As by the macroalgae *Sargassum horneri* that resulted in significant differences of residual total As in the medium throughout the exposure period of 7 days. This observation was similar to our study on microalgae, as we observed the total As accumulation to significantly increase from T7 to T14 in the single exposure thereafter showing a trend of significant reduction at T20 (Mamun et al., 2019a,b; Huang et al., 2021; Hussain et al., 2021). However, in the combined exposure of As V and other trace metals, the total As accumulation was highest at T17 increasing significantly from T7 with an increasing period of exposure but lacking any significant decline in the trace metal concentration at the lysis phase (T20). Although literature with such comparison is deficient, a recent study on the single toxicity of As V and

combined As V and Cu toxicity reported a similar observation (Huang et al., 2021). In addition to some previous studies, they reported the sharp increase in the total arsenic content of the algal species and towards the lysis phase a lower concentration in both the treatments (single and combined) but reporting a higher absorption rate and bioaccumulation of As from the combined exposure (Zheng et al., 2008; Dong et al., 2014; Wang et al., 2015; Hussain et al., 2021; Huang et al., 2021). In our study, we found a similar trend of higher bioaccumulation of total As in the case of combined exposure but, the absorption rate was slower in contrast to the above study, perhaps because of the comparative use of lower concentration of all the trace metal depicting an environmentally realistic condition. Moreover, the speciation of As throughout the exposure period in Huang et al. (2021), showed that As V can have enhanced detoxification when Cu was added and can be bio-transformed to a less toxic methylated state of As. However, in the current study, the combined exposure showed no significant reduction of trace metal at the lysis phase, perhaps because total As concentration was taken into account and the analysis for the speciation of the As species was lacking, which can be considered in the future. The present study followed the growth phases in batch mode comparing single toxicity of As V and combined toxicity in environmentally realistic conditions to comprehend the mechanisms developed by microalgae and possibly denote certain stress indicators of trace metal toxicity when exposed through varied pathways. Along with the As speciation, a detailed comprehension of how As can physiologically affect the photosynthetic machinery of *D. lutheri* can be considered in the future.

## 5. Conclusion

In this study, we report that the effects caused by exposure of As V to *D. lutheri* can lead to varied responses when microalgae are exposed to single toxicity or when combined with other trace metals in a mixture (both essential and non-essential). The cell concentration in the combined trace metal exposure was significantly lower than in single exposure of As V after the stationary phase. The bioaccumulation and its bio-concentration factor of total As increased in *D. lutheri* when exposed along with a mixture of other trace metals. Our study also highlighted the loss of chl *a* pigment and the significantly higher value of DR as a stress response when As V is mixed with other trace metals. Hence, in aquatic areas where the trace metals are prevalent, the consequences of the contamination can be adverse depending on the exposure to multiple toxicants along with As V. Such studies can help in bio-remediation given the higher bio-concentration factor in the mixture for As, and also can reveal the risks of bio-magnification into higher trophic organisms. Eco-toxicological assays using *D. lutheri* are extremely rare and such observations can shed light on the resistance and/or vulnerability of this economically beneficial microalga species to enhance its production for safe and healthy therapeutic products. Detailed speciation of As in single toxicity and a mixture when exposed to *D. lutheri* or other economical algal species awaits, which can further improve the production and understanding of the varied responses for human benefits.

## Credit author statement

**Shagnika Das**: Performed the experiment and drafted the manuscript. **Francois Gevaert**: Analyzed the pigment concentrations and helped in reviewing the manuscript. **Baghdad Ouddane**: Performed heavy metals analyses and quality control. **Gwendoline Duong**: Formal analysis in ICP-MS for pigments. **Sami Souissi:** Conceived the project and supervised the MS drafting. All co-authors: read and commented on the Manuscript.

## Declaration of competing interest

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

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2021.132949.

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