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

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8

9 **Highlights-**

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

15

16 **Abstract-**

17 Eco-toxicological assays with species of economic interest such as *Diacronema lutheri* are
18 essential for industries that produce aquaculture feed, natural food additives and also in drug
19 developing industries. Our study involved the exposure of a single and combined toxicity of
20 arsenic (As V) to *D. lutheri* for the entire algal growth phase and highlighted that a combined
21 exposure of As V with other essential (Copper, Cu and Nickel, Ni) and non-essential
22 (Cadmium, Cd; Lead, Pb) trace metals reduced significantly the cell number, chlorophyll *a*
23 content, and also significantly increased the de-epoxidation ratio (DR) as a stress response
24 when compared to the single toxicity of As V. Arsenic, as one of the ubiquitous trace metal
25 and an active industrial effluent is reported to have an increased bio-concentration factor when
26 in mixture with other trace metals in this study. In the combined exposure, the concentration

27 of total As bio-accumulated by *D. lutheri* was higher than in the single exposure. Hence,
28 polluted areas with the prevalence of multiple contaminants along with the highly toxic trace
29 metals like As can impose a greater risk to the exposed organisms that may get further bio-
30 magnified in the food chain. Our study highlights the consequences and the response of *D.*
31 *lutheri* in terms of contamination from single and multiple trace metals in order to obtain a
32 safer biomass production for the growing need of natural derivatives.

33

34 **Keywords** – Arsenic, trace metals mixture, bioaccumulation, bioconcentration factor, de-
35 epoxidation ratio, chlorophyll *a*.

36

37 **1. Introduction**

38 Trace metals are persistent and have proved to be toxic to surrounding organisms
39 in all spheres of the ecosystem (Anandkumar et al., 2019; Rajaram et al., 2020; Das
40 et al., 2020a; Yang et al., 2020). The scenario post-industrialization has posed a
41 serious ultimatum to numerous species around the world with continuous exposure
42 to harmful substances that are often disposed of in coastal areas (Johnson et al.,
43 2003; Schwarzenbach et al., 2006; Webster et al., 2011). Several studies have
44 reported the effects of such contaminants on marine organisms and the
45 consequences on their growth, physiology, metabolism, morphology, etc.
46 (Baumann et al., 2009; Diaz et al., 2012; Facey et al., 2019; Mamun et al., 2019a;
47 Dayras et al., 2020). Some trace metals like copper (Cu), nickel (Ni), zinc (Zn) play
48 essential roles in the metabolism of the organism while others like arsenic (As),
49 cadmium (Cd), lead (Pb) have no such capacity (Zidour et al., 2019; Das et al.,
50 2020a). The essential trace metals are required at certain concentrations in the
51 organism which, when exceeded, can be toxic (Chen et al., 2018; Yang et al., 2020).

52 Amongst the non-essential trace metals, Arsenic is often considered as ubiquitous
53 in nature posing a high risk of toxicity through its exposure (Hak et al., 2020; Huang
54 et al., 2021). Further, As is considered as an extremely varied toxicant and a
55 predominant carcinogen that persists in the entire food chain of the ecosystem
56 existing in several organic and inorganic states (Sghaier et al., 2015; Mitra et al.,
57 2017; Mamun et al., 2019 a, b; Hussain et al., 2021). An inorganic state of As;
58 arsenate (As V) has been reported as the chief state that exists in an aerobic
59 ecosystem and often gets absorbed into the body surface of aquatic organisms
60 (Mitra et al., 2017; Mamun et al., 2019 a, b). In general, such toxic trace metals are
61 often present in a combined complexity with other metals and thus individual effect
62 on the organism depends on the bioavailability of each metal (Kadiene et al., 2019
63 a, b; Zidour et al., 2019; Das et al., 2020a). Laboratory-based studies of single
64 toxicity of a trace metal have however taken their place for few decades, but
65 comparisons of single and combined toxicity are still in the way of making a
66 profound understanding in eco-toxicology (Zidour et al., 2019; Das et al., 2020a).
67 Previous studies have focused on the single toxicity of As V on marine organisms
68 and its capacity of bio-accumulation and biotransformation whereas, a comparison
69 of varied pathways of exposure (single and combined toxicity) on the first trophic
70 level of the food chain has not been much explored (Upadhyay et al., 2018; Hak et
71 al., 2020; Huang et al., 2021). In addition, the industrial wastes that are usually
72 emitted into the adjacent water bodies, are often present as a cocktail of trace metals
73 and other organic pollutants that gets exposed to the inhabiting organisms, which
74 further substantiates the need of considering the effect of combined trace metal
75 and/or other contaminants in ecotoxicology. Therefore, experimental approaches

76 comparing culture conditions (control) with that of single toxicity and combined
77 toxicity of trace metals should be encouraged.

78 . To comprehend the effects of such contaminants, phytoplankton, being one of the
79 primary producers of the aquatic sphere played a major role as a bio-indicator in
80 assessing the threats prevailing in a particular environment and risk assessment
81 assays (Salama et al., 2019; Rajaram et al., 2020; Yang et al., 2020). The
82 bioaccumulation of trace metals in algae usually occurs by active or passive
83 transports of ions into the cell membranes that act as hydrocolloids, affecting its
84 growth by decelerating photosynthetic activity (Leal et al., 2016; Salama et al.,
85 2019). Trace metals can often cause oxidative stress and increase the cellular
86 reactive oxygen species (ROS) that can further cause alteration in enzymatic
87 activity, pigment contents including protein degradation (Cheloni and Slaveykova
88 et al., 2018; Salama et al., 2019). Apart from this, trace metals have also shown
89 adverse effects on the morphology, and the coherence of cell membranes
90 controlling the permeability of the cell wall of algal species (Mamun et al., 2019b;
91 Costa et al., 2019; Salama et al., 2019). Marine micro/macroalgae have shown
92 resilience to such toxicity and pathways to depurate these substances by
93 compartmentalizing the toxins in specific cellular cavities like vacuoles followed
94 by a release of the contaminants into the medium through efflux pump (Perales-
95 Vela et al., 2006; Juang and Chang et al., 2016; Salama et al., 2019). They have also
96 proved their adaptability to trace metals by the formation of metallothioneins and
97 phytochelatins (Navarrete et al., 2019, Costa et al., 2019). In general, algal species
98 are capable of bio-accumulating trace metals that often penetrate and get stored in
99 their tissues and also poses a threat of bio-magnification of contaminants to higher
100 trophic levels (Yin et al., 2011; Tao et al., 2012; Yang et al., 2020). Besides the risk

101 of biomagnification, studies have also highlighted the ability of micro/macroalgal
102 species for bioremediation, reporting high removal efficiency of trace metals,
103 secondary pollutants and hydrophobic compounds by some algal species like
104 *Spirulina*, *Chlorella*, *Cladophora* and many others (Mahdavi et al., 2012; Jasrotia
105 et al., 2014; Yang et al., 2015; Gao et al., 2016). Hence, studies on the effect of
106 highly toxic trace metals like As and its combined toxicity with other trace metals
107 can further benefit our understanding on the removal efficiency of such
108 contaminants by microalgae near anthropogenic quarters. *Diacronema lutheri*
109 (Droop) Bendif & Véron, 2011 is one such ecologically and economically important
110 microalgae that is widely cultivated worldwide. Indeed, this microalga constitutes
111 loads of phytosterols and is also extensively used as a feed in the aquaculture
112 industry (Ahmed et al., 2015; Bernaerts et al., 2018; Huang et al., 2020). Such
113 phytosterol rich organic sources are the natural alternatives that are often used as
114 additives in food items for their ability to lower the levels of low-density lipoprotein
115 (LDL) cholesterol and also beholds anti-inflammatory, anti-cancerous and anti-
116 oxidative properties (Kim et al., 2008; Carmona et al., 2010; Ahmed et al., 2015).
117 Therefore, toxicological assays implementing toxicity from varied sources and
118 pathways involving economical species with medicinal and therapeutic benefits like
119 *D. lutheri* should be encouraged. Out of the few studies conducted on the eco-
120 toxicological aspect involving *D. lutheri*, some of them reported that when *D.*
121 *lutheri* was exposed to dissolve organic contaminant originating from nearby
122 industrial catchment areas in Scotland, showed significant inhibition of growth rate
123 in nearly all the stations for higher concentrations of pesticides; that was useful in
124 assessing the risk of that particular habitat (Emelogu et al., 2013; 2014). Therefore,
125 the objective of our study was to monitor the effect of As V which is predominant

126 and shows high adsorption ability, as a single source of toxicant and a combined
127 mixture of essential (Cu; Ni) and non-essential (Cd; Pb) trace metals to comprehend
128 the bioaccumulation and bio-concentration of these metals when detected in a
129 mixture. The further objective was to analyze the effect of the bioaccumulation of
130 trace metals on the growth curve and the pigment contents of *D. lutheri* comparing
131 it with the controlled culture conditions. The tangential aim of this study was to
132 highlight the risks of bioaccumulation and bio-concentration of trace metals to
133 optimize the growth and production of this economically beneficial algal species
134 when used in medicines and the aquaculture industry as a feed for higher trophic
135 communities. *Diacronema lutheri* is used as an aquaculture feed in our laboratory
136 regularly for years, and the amount of trace metal used in this study corresponds to
137 the lethal concentration of the bio-indicator copepod *Eurytemora affinis* (Kwok et
138 al., 2015, Souissi and Souissi, 2021), with regards to enhancing our understanding
139 on the consequences in case of a trace metal contamination in our culture systems
140 emerging through the algal feed source. Such eco-toxicological assays with *D.*
141 *lutheri* are scarce and can help to better understand the mechanism of environmental
142 toxicity and the cocktail effect of contaminants in addition to the benefits in the
143 commercial industries to improve the quality and quantity of biomasses that can be
144 safely used as a natural alternative to drugs.

145

146 **2. Material and methods**

147 **2.1 Algal species- *Diacronema lutheri***

148 *Diacronema lutheri* from the Roscoff Culture Collection (RCC-1537), France was
149 cultivated in artificial seawater (osmosed water with artificial salt, Coral salt-pro)
150 in round bottom flasks of 6 L and was maintained in an incubator with controlled

151 physical parameters (12:12h light: dark in 18°C, salinity 33) (Arias et al., 2017;
152 Gnouma et al., 2017, Dayras et al., 2020). The formulation of the culture was
153 ensured by adding an adequate supply of nutrition through the Conway medium in
154 every culture flask by following the same composition mentioned in Tlili et al.,
155 2016.

156

157 **2.2 Exposure to trace metals: single and combined toxicity**

158 To understand the variability in growth, pigment concentration, and ability to bio-
159 accumulate trace metals by *D. lutheri*, two different treatments with trace metal
160 stress along with control were designed in triplicates. The trace metal stress applied
161 to the algae was single toxicity of As V and a mixture of trace metals (As V + Cd +
162 Cu + Ni + Pb). The metal concentrations used in this study corresponds to the 10%
163 lethal concentration (LC50%) of each trace metal when exposed to the invertebrate
164 aquatic model *Eurytemora affinis*, a bio-indicator, calanoid copepod which is often
165 fed with *D. lutheri* in the mass cultures of our laboratory (Zidour et al., 2019;
166 Kadiene et al., 2019b; Das et al., 2020a, Dayras et al., 2020). The LC 50% (96
167 hours) for Cd, Ni, Cu, and Pb when exposed to *E. affinis* was used according to our
168 previously reported studies (Cd - 108.5 $\mu\text{g.L}^{-1}$, Cu - 33.5 $\mu\text{g.L}^{-1}$, Ni - 125.5 $\mu\text{g.L}^{-1}$,
169 Pb - 413.5 $\mu\text{g.L}^{-1}$) (Zidour et al., 2019; Das et al., 2020a). The lethal concentration
170 used for As V (96 hours, 449.78 $\mu\text{g.L}^{-1}$) was determined in our laboratory following
171 the same protocol as reported in Tlili et al., 2016 (supplementary data, Table 1S).
172 The exposure for all the treatments was executed in 6 L sterilized flasks using
173 artificial seawater, under controlled light and temperature conditions along with
174 constant re-suspension for a symmetrical availability of nutrients, light, and
175 temperature for all the algal cells (Tlili et al., 2016; Arias et al., 2017; Gnouma et

176 al., 2017, Dayras et al., 2020). The exposure started by adding sterilized algal
177 culture at the log phase along with the Conway medium and vitamin (vitamin B1,
178 vitamin B12) to the experimental flasks to anticipate a successive exponential
179 growth. The composition of Conway medium and vitamin was the same as reported
180 in Arias et al., 2017 and Tlili et al., 2016. The entire growth curve of *D. lutheri* with
181 daily cell count was accounted for in our study from day 1 (T1) until day 23 (T23).

182

183 **2.3 Cell counting: Daily growth monitoring**

184 Cell counting was done every day from T1 to T23 for monitoring the daily growth
185 of the algal cells in each treatment and replicate by using an inverted microscope at
186 100-fold magnification (Olympus, IX 71, Tokyo, Japan) attached to a high storage
187 capacity computer to preserve all photographs of the fields observed under
188 microscope for quantification of cell numbers (Guillard and Sieracki 2005; Arias et
189 al., 2017). A volume of 2 mL sample from each treatment was collected at a
190 particular time daily in Eppendorf tubes and was fixed with 5% lugol solution in
191 triplicates and then stored at 4°C before quantification (Iwasawa et al., 2009; Arias
192 et al., 2017). A haemocytometer (Malassez cell slide, 0.1 mm depth, Japan) was
193 used for observing the algal cells under the microscope and each field was
194 photographed for counting the number of cells (Gnouma et al., 2017, Dayras et al.,
195 2020). A software named ImageJ (<https://imagej.nih.gov/ij/>) was later used for
196 counting the number of cells in each field from each sample and replicates.

197

198 **2.4 Analysis for trace metal concentration – ICP-MS**

199 The analysis for trace metals was executed with an inductive coupled plasma
200 quadrupole mass spectrometer (ICP-MS) (ICP-MS 7900, Agilent Technologies,

201 United States) equipped with a standard introduction system [borosilicate Micro
202 Mist concentric nebulizer (0.4 mL min^{-1}), quartz double pass spray chamber cooled
203 at 2°C , quartz torch (2.5 mm ID) and nickel cones. The algal samples were
204 collected at every phase of the entire growth curve of *D. lutheri*. The sub-sampling
205 intervals for trace metal analysis were day 7 (T7, exponential phase), day 14 (T14,
206 early stationary phase), day 17 (T17, late stationary phase), and day 20 (T20, lysis
207 phase). The algal samples collected at the mentioned intervals were filtered on
208 sterile membrane filters of $0.45 \mu\text{m}$ from all the conditions and were dried for
209 further pre-treatment. The pre-treatment of the filters comprised of acid digestion,
210 where nitric acid and hydrochloric acid were mixed in the ratio of 1:3 and added to
211 the Teflon tube containing the filter. These tubes were then put in a water bath of
212 100°C for at least 4 hours placed inside a laminar flow cabinet. After the digestion,
213 a final volume of 5 ml was adapted in each Teflon tube with ultrapure double
214 distilled water (Milli Q). The samples were then transferred to sterilized Falcon
215 tubes and were analyzed for trace metal concentration under ICP-MS. The trace
216 metal concentration remaining in the residual seawater after filtering the algal
217 biomass was also considered by collecting the filtrate (15 mL) directly into
218 sterilized Falcon tubes and fixing it with ultrapure nitric acid ($20 \mu\text{L}$). These tubes
219 were kept in dark until further analysis under ICP-MS without any need of pre-
220 treatment (Ouddane et al., 1990; Zidour et al., 2019; Kadiene et al., 2019a; Das et
221 al., 2020a). In the entire process of trace metal analysis, all the quality control
222 measures were implied by maintaining the optimum quality for all the standard
223 solutions, and for trace metal identification a reference of multi-elemental standard
224 was utilized (Ouddane et al., 1990). Certified Reference Materials (CRM) PACS-3
225 and HISS-1 was used to validate the trace metal concentrations and the

226 mineralisation procedure constituted of $\leq 10\%$ total analytical errors. The bio-
227 concentration factor (BCF) of each trace metal in *D. lutheri* was calculated from the
228 concentration of trace metal bio-accumulated in algae and the residual trace metal
229 in the medium for each condition (Das et al., 2020a).

230

231 **2.5 Analysis for pigment concentration – HPLC**

232 Samples for pigment analysis were collected at all phases of the algal growth curve
233 of *D. lutheri*, in the same manner as for trace metals, on day 7, day 14, day 17, and
234 day 20. After filtration through GF/F Whatman filters, they were stored at -20°C in
235 the dark until extraction. Pigment extraction was performed by grinding the filters
236 with methanol and 4 drops ($30\mu\text{L}$) of methylene chloride, avoiding exposure to
237 light. The extracts were further concentrated after filtration on
238 polytetrafluoroethylene membrane filter of $0.45\ \mu\text{m}$ by dry-evaporation under a
239 nitrogen stream. Desalination of the extract was accomplished with a mixture
240 (50:50 v/v) of distilled water and methylene chloride. Phase separation was
241 followed to obtain the organic portion from each sample, which was then dry-
242 evaporated under nitrogen gas. Each sample was dissolved in $40\ \mu\text{L}$ of methanol
243 before injection, from which a volume of $20\ \mu\text{L}$ was injected in the high-
244 performance liquid chromatography instrument (Shimadzu, Nexera XR; C18
245 reverse-phase column, Allure, Restek). The separation was performed using a
246 solvent delivery profile adapted from Arsalane et al. (1994). Along with the
247 photosynthetic pigments, the photoprotective pigments were also studied to denote
248 any possible stress response that triggered in the algal cells when exposed to trace
249 metals from single and combined toxicity. The xanthophyll cycle has often proved
250 to be a sensitive parameter to indicate metal stress and its role in the photoprotection

251 process underneath high-light has been reported in previous studies (Bertrand et al.,
252 2001; Brunet et al., 2014; Yan et al., 2017). The reversible reaction between
253 diadinoxanthin and diatoxanthin in the xanthophyll cycle often referred to as the
254 de-epoxidation ratio (DR) was calculated by the following equation (1) (Brunet et
255 al., 2014; Yan et al., 2017):

256

$$\text{Eq. (1)..... DR} = \frac{\text{Diatoxanthin}}{\text{Diadinoxanthin} + \text{Diatoxanthin}}$$

258

259 **2.6 Statistical analysis**

260 The daily cell count, chlorophyll *a* concentration, de-epoxidation ratio (DR),
261 bioaccumulation of trace metals, bio-concentration factor in each treatment
262 including the control were compared by using analysis of variance (ANOVA) at
263 $p < 0.05$, after meeting all the requirements of normality and homogeneity of
264 variance. To test the normality and the homogeneity a Shapiro Wilk normality test
265 and a Levene's Test for Homogeneity of Variance were carried out respectively. To
266 compare the significance in each condition Tukey multiple comparisons of means
267 (95% family-wise confidence level) was applied to the data. All the analyses were
268 accomplished by using R studio (4.0.4).

269

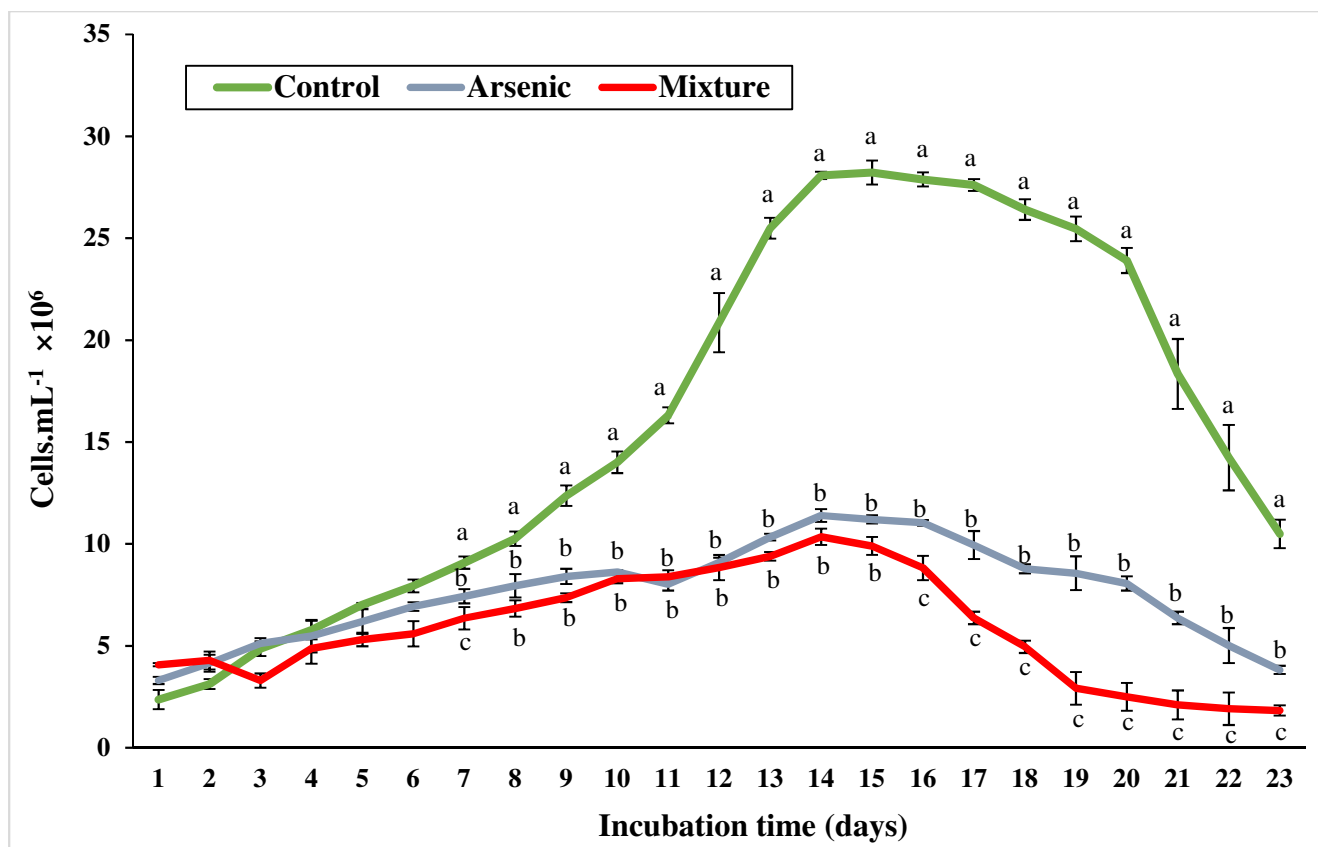
270 **3. Results**

271 **3.1 Daily growth**

272 Fig. 1 corresponds to the growth curve of *D. lutheri* from T1 to T23 (day 1- day
273 23), in each treatment, namely control, As V (single exposure) and mixed trace
274 metals (combined exposure). The phases of algal growth curve are well

275 distinguished in the control treatment with the highest concentration of cells
276 reaching on day 15 with 28×10^6 cells mL⁻¹. The cell density in control increased
277 from T7 to T15 and then reached a stationary phase from T16 to T18, followed by
278 the lysis phase or death phase with decreasing number of cells until T23. The trace
279 metal exposed treatments (As V and combined) showed significantly lower
280 concentration of cells than control from T7 until T23. After 7 days of continuous
281 exposure the combined trace metal mixture showed statistically the least
282 concentration of cells when compared to As V exposure and control. The single
283 exposure of As V and the trace metal mixture differed significantly only at T7 and
284 thereafter from T16 to T23. However, the cell count in As V was significantly lower
285 than control, but higher than combined exposure. In both the As V and combined
286 trace metal exposure, the phases of algal growth were not well defined and showed
287 high variability especially during the early to late stationary phase. The lysis phase
288 for combined exposure started at T16 showing significantly lower no. of cells from
289 As V exposure until T23. The single exposure to As V however showed higher no.
290 of cells than the combined trace metal mainly in the exponential and lysis phase.
291 The sub-lethal concentrations of single trace metal exposure (As V) and combined
292 trace metal exposure (As + Cd + Cu + Ni + Pb) could reduce the biomass
293 significantly throughout the algal growth curve.

294



295

296 **Fig. 1** Growth curves of control and trace metal exposed treatment from day 1 (T1) until day
 297 23 (T23). The letters (a, b, c) signify statistical differences between each treatment, at $p < 0.05$.
 298 The error bar shows the standard deviation between the three replicates in each treatment.

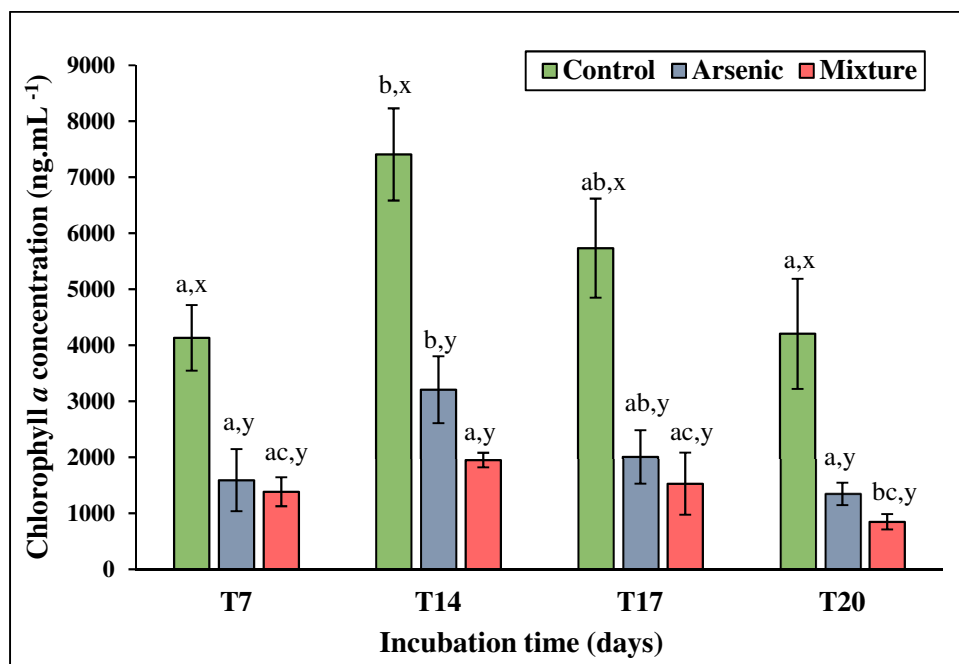
299

300 3.2 Pigment concentrations

301 3.2.1 Chlorophyll *a* concentration

302 Fig. 2 shows the chlorophyll *a* (chl *a*) concentration in each treatment at specific
 303 intervals in the entire growth curve of *D. lutheri*. The control treatment at each
 304 interval had significantly higher amount of chl *a* content than the trace metal
 305 exposed treatments. The highest chl *a* content was observed on day 14 (T14),
 306 when the pigment value increased significantly compared to the exponential
 307 phase at T7 in case of control and As V exposure, but however, the pigment
 308 content did not significantly increase in the combined trace metal treatment. In

309 comparison between the trace metal exposed treatments, As V exposure showed
 310 higher chl *a* concentration at each interval of growth phases, although not
 311 significantly. In both the trace metal exposed treatments, the chl *a* content
 312 decreased significantly at T20 gradually from T14. The chl *a* content and the
 313 cell count of *D. lutheri* at specific intervals of the growth curve in all the
 314 treatments including control showed a significant positive correlation, with a *p*-
 315 value <0.01, marking the coherence of both the parameters in this study.



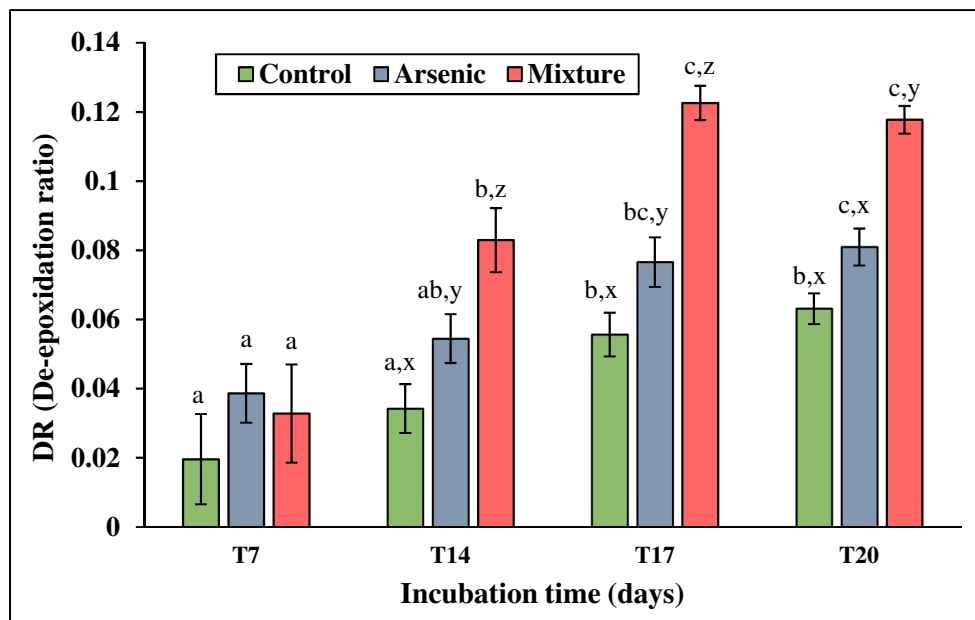
316
 317 **Fig. 2.** Chlorophyll *a* concentration for each treatment at all phases of growth in *D. lutheri*.
 318 Letters a, b, c shows significant differences between incubation times; x, y shows significant
 319 differences between treatments, at *p* < 0.05. The error bar shows the standard deviation between
 320 the three replicates in each treatment.

321

322 3.2.2 De-epoxidation ratio

323 Fig. 3 shows the de-epoxidation ratio for all the treatments at each incubation
 324 time throughout the growth curve of *D. lutheri*. A significantly higher de-

325 epoxidation ratio was observed for the trace metal combined treatment at the
 326 linear phases (T14, T17) and the lysis phase (T20). At the exponential phase,
 327 the DR was not significantly different between the treatments control, As V
 328 exposure and combined toxicity of trace metals (As V+ Cd + Cu + Ni + Pb). In
 329 the single exposure of As V treatment, the de-epoxidation state was significantly
 330 higher than in control in the linear phases (T14, T17) but not at the exponential
 331 and the lysis phase. In comparison between the trace metal exposed treatments,
 332 mixed trace metal showed significantly higher DR than the As V exposed
 333 treatment at all the stages, except the exponential phase (T7).

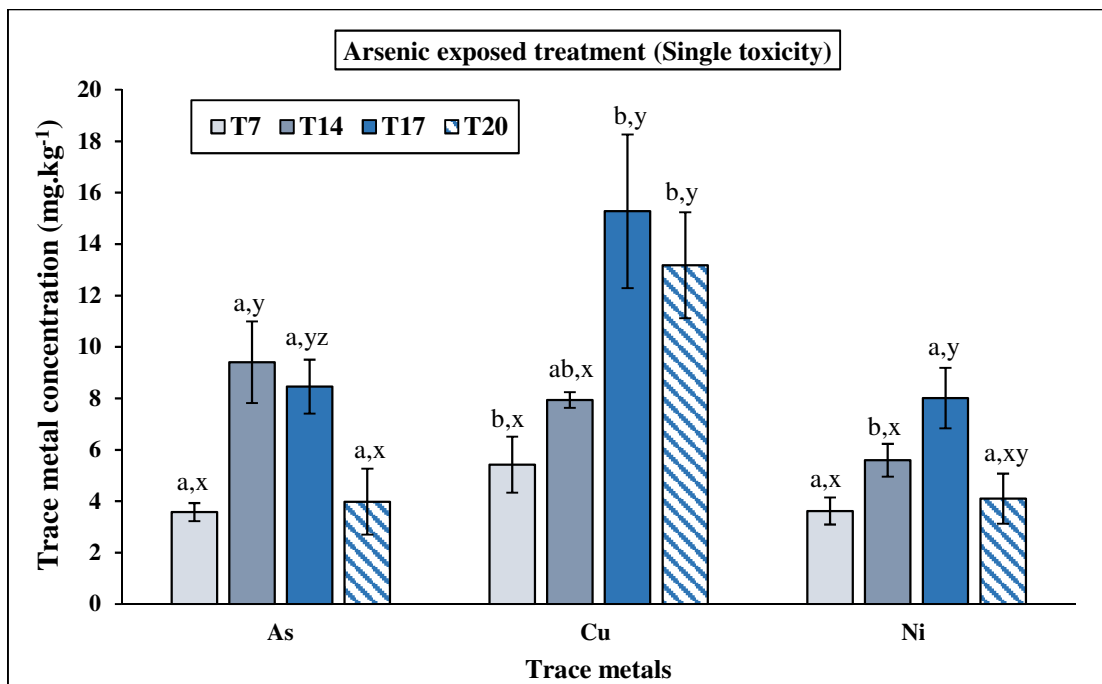


334
 335 **Fig. 3.** De-epoxidation (DR) state for each treatment at all phases of growth in *D. lutheri*.
 336 Letters a, b, c shows significant differences between incubation times; x, y, z shows significant
 337 differences between treatments, at $p < 0.05$. The error bar shows the standard deviation between
 338 the three replicates in each treatment.

339 3.3 Bioaccumulation of trace metals

340 3.3.1 Single exposure – Arsenic

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



352 **Fig. 4.** Bio-accumulation of trace metals from the single toxicity exposed treatment of arsenic.
 353 Letters a, b shows significant differences between trace metals; x, y, z shows significant
 354 differences between incubation times within a single trace metal, at $p < 0.05$. The error bar
 355 shows the standard deviation between the three replicates in each treatment.
 356

357 **3.3.2 Combined exposure of trace metals (As + Cd+ Cu+ Ni+ Pb)**

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

360 The highest bioaccumulation was by Pb at T17 followed by Cu > Ni > As > Cd.

361 All the exposed trace metals showed highest bioaccumulation at T17 correlating
362 positively with the increase in cells concentration in the linear phase. However,

363 at the lysis phase (T20) each combined metal showed a decrease in trace metal
364 concentrations, out of which Cu and Pb showed a significant decrease. This

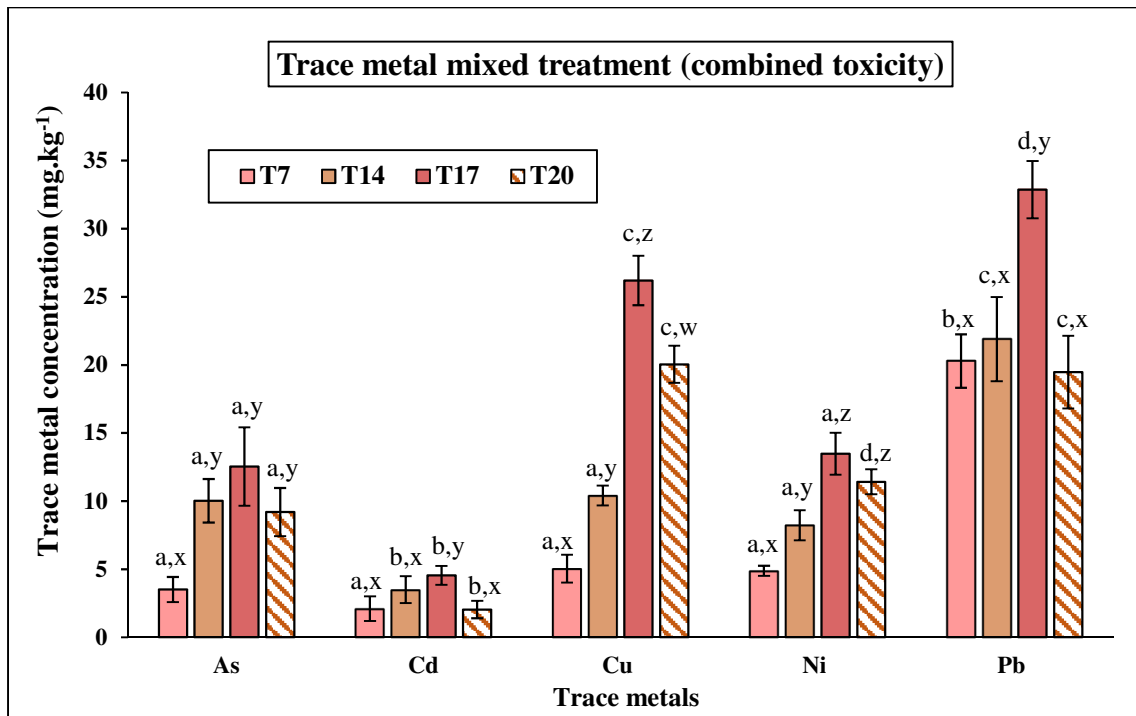
365 finding was in contrast to the single exposure of As V treatment (Fig. 4) in

366 which Cu did not show any significant reduction at T20 given the fact that this
367 metal has essential metabolic functions. Inversely, when As V was exposed

368 through a single exposure (Fig. 4) it showed a significant decrease in
369 bioaccumulation at T20 but in combination with other trace metals no such

370 reduction was noted (Fig. 5). The total As concentration in the single exposure
371 was highest at T14 (Fig. 4), but when combined with other trace metals, the

372 highest bioaccumulation was reached at T17 also denoting higher values of total
373 As when in mixture with other trace metals.



374

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

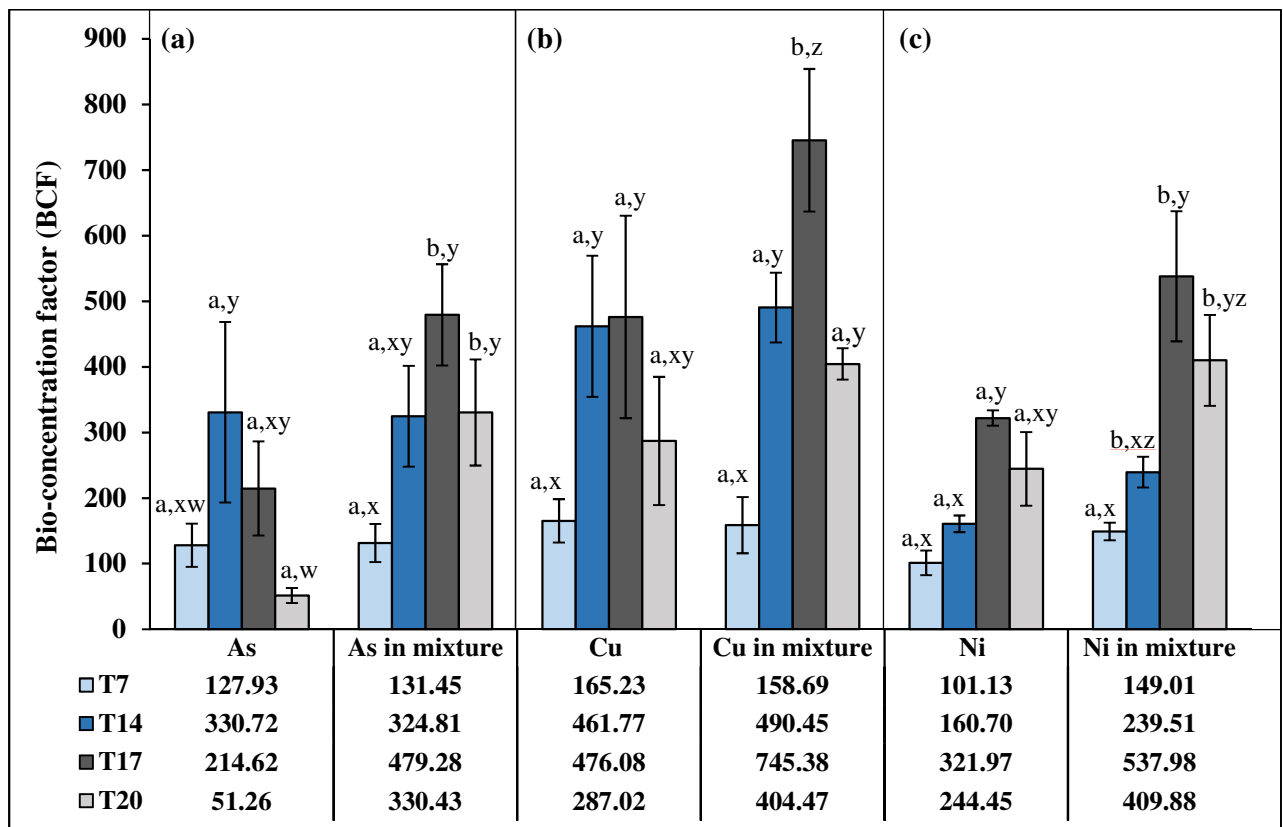
379

3.3.3 Bio-concentration (BCF) of trace metals

380

In the single exposure to As V treatment, only Cu and Ni were detected (without
 381 any addition) marking their essential role in algal metabolism. However, no Cd
 382 and Pb was detected in this treatment. Hence, Fig. 6a shows a comparison
 383 between the BCF of As exposures (single and combined with other trace
 384 metals), Fig. 6b, shows Cu exposure (naturally present and combined with other
 385 trace metals) and Fig. 6c, shows Ni exposure (naturally present and combined
 386 with other trace metals). In As V single exposure, the highest BCF was reached
 387 at T14, increasing significantly from T7 and further reducing significantly at
 388 T20. On the other hand, when As V was combined with other trace metals, the
 389 highest BCF was noted at T17, significantly increasing from T7 but showing no

390 significant reduction of trace metal concentration at T20. When both exposures
 391 (single and combined) were compared, a significant difference in BCF of total
 392 As at T17 and T20 was noted. The Cu detected in the single exposure of As V
 393 showed significantly higher BCF at T14 and T17 than T7 thereafter reducing at
 394 T20 although not significantly. Further, the comparison between the naturally
 395 detected Cu and the Cu added with other trace metals showed, a significantly
 396 higher BCF of Cu at T17 in mixture along with a significant reduction at T20.
 397 On the other hand, Ni when detected in the single exposure of As V showed
 398 highest BCF at T17 increasing significantly from T7 and T14 but lacking any
 399 reduction of trace metal concentration at T20. The comparison between Ni
 400 naturally detected and when in mixture with other trace metals showed
 401 significantly higher BCF in mixed trace metal exposure at T14, T17 and at T20
 402 without any significant decrease in both the cases.



403

404 **Fig. 6 (a).** Comparison of the bio-concentration factor (BCF) of total As in single toxicity and
405 in mixture, where, w, x, y, z denotes the significant differences between the incubation time in
406 a single treatment and letters a, b denotes the significant differences between total As single
407 and mixture treatment on a single day of exposure.

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

414 **4. Discussion**

415 **4.1 Cell growth of *Diacronema lutheri***

416 Previous studies have reported that exposure to trace metals along with other
417 contaminants such as organic pollutants and/or microplastics affected the growth
418 rate of many algal species, in general (Emelogu et al., 2014; Chia et al., 2015;
419 Bellingeri et al., 2019, Mamun et al., 2019 a, b; Navarrete et al., 2019; Wilson et
420 al., 2019; Huang et al., 2021). However, these studies had varied exposure strategies
421 ranging from combined toxicity of trace metals, microplastics, and organic
422 pollutants, etc. coupled with several nutrient limiting exposures (Chia et al., 2014,
423 2015; Navarrete et al, 2019; Huang et al., 2021). A recent study on single exposure
424 of As and combined exposure of As and Cu on three algal species showed a lower
425 growth rate of the algal cells in the combined exposure after 10 days (Huang et al.,
426 2021). In this study, a similar observation was noted, where the algal cell density in
427 the combined exposure of the essential (Cu, Ni) and non-essential (As, Cd, Pb) trace
428 metals varied significantly after 7 days of exposure from the single toxicity of As

429 V and control. Furthermore, similar to our observation, other studies also reported
430 the significant inhibition in the growth rate after the exponential stage and described
431 it as a combined effect of the toxicity from trace metal and the nutrient limiting
432 condition mainly at the end of each algal growth curve (Serra et al., 2010; Monteiro
433 et al. 2011; Chia et al., 2013, 2014, 2015). Huang et al., 2021 also observed a time-
434 dependent effect on the declining growth rate of the algal species irrespective of the
435 concentration of As used in their study. In comparison between single toxicity and
436 combined toxicity, previous studies on varied organisms have shown a decrease in
437 the growth rate when exposed to a combination of trace metals, microplastics, and
438 nano-plastics (Yan et al., 2015; Tang et al., 2015; Kim et al., 2017; Barboza et al.,
439 2018; Huang et al., 2021). Synergistic toxicity of Cd, Cu, Pb, and Zn significantly
440 affected the enzyme activities and chlorophyll *a* content in an estuarine plant
441 (*Avicennia marina*) in comparison to their exposures, that posed no significant
442 effects (Mac Farlane and Burchett, 2002; Yan et al., 2015). In addition to our study,
443 a combination of trace metals like As, Cd, Cu has been reported to damage the
444 membranes of cells disrupting the permeability and its capacity to uptake nutrients
445 because of the trace metals binding to the sulfhydryl groups and preventing several
446 enzymatic activities that are crucial for the metabolism, growth, physiological
447 conditions and morphological characteristics (Chattopadhyay et al., 2002; Zheng et
448 al., 2008; Hu and Zhou, 2010; Chia et al., 2014, Huang et al., 2021).

449

450 **4.2 Pigment concentrations of *D. lutheri***

451 *Diacronema lutheri* is a haptophyte (pavlovophycean) consisting of flagellum at the
452 hind-site with two flagella of unequal length. They contain pigments like
453 fucoxanthin, carotenoids, type A pigments that includes chl *a*, *c1*, *c2*,
454 chlorophyllides, pheophytine *a*, diadinoxanthin, diatoxanthin and β -carotene (Van

455 Lenning et al., 2003; Bendif et al., 2011; Kim et al., 2021). The pigment chl *a* has
456 been an indicator to the trace metal stress in previous studies, which reported a
457 decrease in its content when algal cells are often impaired by the trace metals, and
458 as a result affecting its ability to assimilate CO₂, hindering the process of
459 photosynthesis and lowering the biomass in acute and chronic exposures (Qian et
460 al., 2009; Chia et al., 2013; Liu et al., 2014; Li et al., 2018). Additionally, in a recent
461 study, exposure to both the forms of arsenic (As III and As V) could induce a
462 significant decrease in the chl *a* and *b* content in an aquatic plant, *Vallisneria natans*
463 (Li et al., 2018). Similar to the previous findings, our study has shown a significant
464 decrease in chl *a* content of *D. lutheri* in the trace metal exposed treatments when
465 compared to control conditions. Studies have also reported similar observations on
466 varied trace metals when exposed to large terrestrial flora, where soluble forms of
467 trace metals having a high affinity towards the sulfhydryl group bind and affects
468 the pigment contents and enzyme activities retarding the growth of the organism
469 (Tomar et al., 2000; Ali et al., 2002; Li et al., 2018). However, when related to the
470 number of cells, algal species like *Chlorella vulgaris*, *Scenedesmus obliquus*, some
471 cyanobacterial and periphyton communities have shown higher contents of chl *a*
472 per cell, along with high carbohydrate and lipid biomolecules in response to trace
473 metal stress and limitation of nutrient (Soldo and Behra, 2000; Omar 2002, Okmen
474 et al., 2011; Chia et al., 2015, Mamun et al., 2019a). Although a significant decrease
475 in chl *a* content from control to trace metal exposed treatments was observed in our
476 study, a comparison between the single exposure and combined exposure to trace
477 metal showed no significant differences. On the other hand, the comparison of cell
478 density in the single and combined trace metal exposures showed significant
479 differences in the exponential phase and at the stationary phase. Such observations

480 where the cell growth was retarded and yet a higher content of chl *a* per cell was
481 observed in toxic exposures have been illustrated in previous studies as a probable
482 mechanism of increasing the chl *a* content followed by the synthesis of sugars,
483 carbohydrates, and other biomolecules to compensate the declined growth of the
484 algal cells (Okmen et al., 2011; Chia et al., 2013, 2015, Tang et al., 2015). Chia et
485 al. (2013; 2015) further explained this strategy as a stress response observed in the
486 algae *Chlorella vulgaris* when exposed to Cd in several nutrient limiting conditions.
487 Our study reports similar observation in the case of combined toxicity of trace
488 metals that included Cu and Ni (essential trace metals) in sub-lethal concentrations
489 which presumably induced such stress responses as reported by previous studies
490 (Davaranah et al., 2015; Wang et al., 2015; Ashraf et al., 2016; Hang et al., 2016;
491 Huang et al., 2021). However, conclusively the synergistic effect of As, Cd, and Pb
492 (non-essential elements) led to a reduced content of chl *a* when compared to
493 individual exposure of As V, however not significantly. Chlorophyll *a* was however
494 the most abundant pigment found in *D. lutheri* followed by fucoxanthin that ranged
495 from 1950 ng.mL⁻¹ to 2100 ng.mL⁻¹ in control cells, 600 ng.mL⁻¹ to 400 ng.mL⁻¹
496 in single exposure of As and the lowest concentration found in the combined trace
497 metal treatment ranging from 350 ng.mL⁻¹ to 200 ng.mL⁻¹; all measured at the
498 exponential phase. In addition, considerable amount of pheophytine *a* was observed
499 with control showing the highest value of 550 ng.mL⁻¹ on an average, followed by
500 single exposure of As (258 ng.mL⁻¹ on an average), and mixture of trace metals
501 (137 ng.mL⁻¹ on an average) all measured at the exponential phase. The de-
502 epoxidation ratio (DR), in previous studies, have been reported as a good indicator
503 of the photo-oxidative stress response when high-light or any form of toxic
504 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., 2018, 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 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).

521

522 **4.3 Bioaccumulation of trace metals in *D. lutheri***

523 Trace metal accumulation by algae has been reported to cause considerable
524 damages in cell structure, growth rate, pigment contents, enzyme activities, and
525 several other metabolic functions in previous studies (Zhang et al., 2019; Mamun
526 et al., 2019 a, b; Hussain et al., 2021; Huang et al., 2021). Studies considered the
527 trace metal accumulated in the biomass/algal mass and also the residual trace metal
528 concentration in the culture medium to emphasize the importance of studying bio-
529 concentration factors (Baumann et al., 2009; Huang et al., 2021). Our study has
530 collectively considered both the parameters to highlight the harmful effects caused

531 by the toxic exposures of As V and combined trace metals (As + Cd + Cu + Ni+
532 Pb). In this study the bioaccumulation of all the trace metals in the combined metal
533 exposed treatment increased significantly in the linear phase (Day 17), and the order
534 of the bioaccumulation was $Pb > Cu > Ni > As > Cd$. This observation was in
535 accordance with some previous studies that also observed Cu and Pb have the
536 highest and Cd to have a lower concentration of bioaccumulation in varied species
537 of macroalgae like *Ulva intestinalis*, *Cladophora rupestris*, *Chondrus crispus*, and
538 microalgae like *Amphora coffaeiformis*, *Dunaliella salina*, *Euglena* sp. and many
539 others (Yu and Wang, 2004; Baumann et al., 2009, Chiellini et al., 2020; Elleuch et
540 al., 2021). In a recent investigation on phytoplankton blooms, the authors reported
541 that Cu and Pb has a greater potential to be bioavailable from their particulate forms
542 and thereby can get rapidly bio-accumulated by phytoplankton posing higher risks
543 for bio-magnification whereas trace metals like Cd, Chromium (Cr), Cobalt (Co)
544 has shown lower particle reactivity (Cabrita et al., 2020; IAEA report, 2004).
545 Mamun et al. (2019a), in a study based on the toxicity of As V, reported a rapid
546 accumulation of As by the macroalgae *Sargassum horneri* that resulted in
547 significant differences of residual total As in the medium throughout the exposure
548 period of 7 days. This observation was similar to our study on microalgae, as we
549 observed the total As accumulation to significantly increase from T7 to T14 in the
550 single exposure thereafter showing a trend of significant reduction at T20 (Mamun
551 et al., 2019 a, b; Huang et al., 2021; Hussain et al., 2021). However, in the combined
552 exposure of As V and other trace metals, the total As accumulation was highest at
553 T17 increasing significantly from T7 with an increasing period of exposure but
554 lacking any significant decline in the trace metal concentration at the lysis phase
555 (T20). Although literature with such comparison is deficient, a recent study on the

556 single toxicity of As V and combined As V and Cu toxicity reported a similar
557 observation (Huang et al., 2021). In addition to some previous studies, they reported
558 the sharp increase in the total arsenic content of the algal species and towards the
559 lysis phase a lower concentration in both the treatments (single and combined) but
560 reporting a higher absorption rate and bioaccumulation of As from the combined
561 exposure (Zheng et al., 2008; Dong et al., 2014; Wang et al., 2015; Hussain et al.,
562 2021; Huang et al., 2021). In our study, we found a similar trend of higher
563 bioaccumulation of total As in the case of combined exposure but, the absorption
564 rate was slower in contrast to the above study, perhaps because of the comparative
565 use of lower concentration of all the trace metal depicting an environmentally
566 realistic condition. Moreover, the speciation of As throughout the exposure period
567 in Huang et al., (2021), showed that As V can have enhanced detoxification when
568 Cu was added and can be bio-transformed to a less toxic methylated state of As.
569 However, in the current study, the combined exposure showed no significant
570 reduction of trace metal at the lysis phase, perhaps because total As concentration
571 was taken into account and the analysis for the speciation of the As species was
572 lacking, which can be considered in the future. The present study followed the
573 growth phases in batch mode comparing single toxicity of As V and combined
574 toxicity in environmentally realistic conditions to comprehend the mechanisms
575 developed by microalgae and possibly denote certain stress indicators of trace metal
576 toxicity when exposed through varied pathways. Along with the As speciation, a
577 detailed comprehension of how As can physiologically affect the photosynthetic
578 machinery of *D. lutheri* can be considered in the future.

579

580 **5. Conclusion**

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

600

601

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614

615

616

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