Screening of trace metal elements for pollution tolerance of freshwater and marine microalgal strains: Overview and perspectives

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Abstract

Microalgae represent a putative solution to decontaminate metal polluted aquatic sites phycoremediation). Seven different freshwater and seawater microalgal strains (*Nannochloropsis* sp., *Dunaliella* sp., *Phaeodactylum* sp., *Chlorella* sp., *Isochrysis* sp., *Euglena* sp. and *Chlorogonium* sp.) were exposed to five metals (Cu, Zn, As (III), Fe and Ni) at three concentrations each, simulating highly polluted sites. The experiment was conducted for a week; the survival ability of each strain and the photosynthetic pigments content (chlorophyll a, b and carotenoids) were evaluated, together with the Optical Density of each culture, pH, growth rate and biomass. Results highlighted different resistance patterns towards metals characterizing each algal strain, and the tolerance of all the microalgal strains towards arsenite. For the first time, the metal resistance pattern of *Chlorogonium* sp. was evaluated. Finally, our *Euglena* sp. and *Dunaliella* sp. strains were considered among the most promising organisms for phycoremediation of freshwater and seawater polluted sites respectively.

1. Introduction

Trace metals are known to be the major environmental contaminants [39], thus representing a long-term threat for the environment and human health [76]. Toxic metal pollutants are introduced in

the environment from natural and anthropogenic sources; they can persist over time in environmental matrices [11], or can be accumulated in soils, water bodies or in the air, disrupting physiological functions in several biological systems [4]. In the last few years, the increasing need to decontaminate the aquatic and terrestrial environment from metals has led to the development of alternative solutions. Conventional physical and chemical methods are usually not usable at large scales and are often costly and not well accepted by the public opinion [37]. Many organisms are able to uptake and accumulate metals from the environment [56]. Plants [73], algae [50] and microorganisms, such as bacteria [e.g. [19]] and microalgae [52], have been largely studied in the last decades as a putative solution for new ecofriendly and low-cost techniques to decontaminate polluted sites [6]. Both soil phytoremediation by means of plants [10], and phycoremediation in aquatic environment by means of algae species [7,21] are strongly encouraged because they are highly applicable to large contaminated areas. In addition, they are less expensive, and they are also efficient for metal contaminants at low concentrations [1,35].

Microalgae are microscopic organisms that can be found both in marine and freshwater environments; usually the term includes both eukaryotic microorganisms and cyanobacteria (prokaryotes) [22]. Like plants they possess a photosynthetic mechanism, and for this reason they can be considered the world's largest group of primary producers of biomass. It is estimated that microalgae are responsible for at least 32% of global photosynthesis [65]. The removal of toxic metals from the environment by means of microalgae can be assessed through different mechanisms that depend on many factors, such as the algal species, the type of metal ions, the conditions of the solution in which phycoremediation takes place and the choice of a living or non-living algal biomass [25,50,71]. To this purpose, it is important to discriminate between biosorption, which is the binding of metals to cell surfaces, and bioaccumulation, which instead involves the active metal uptake at the intracellular level [86]. In the first case, the use of dead algal biomass is recommended since it reduces both the need to provide nutrients and toxic effects that pollutants may cause to the organisms [61]. In the second case, the process is active, and the algal biomass is alive and resistant to metals at

different concentrations. Bioaccumulation is more advantageous than biosorption since living algae maintain a constant number of individuals within their population and are active in metal uptake. On the other hand, the dead biomass used in biosorption reaches a maximum level of metal accumulation. Consequently, the costs for its removal, decontamination and possible reuse, must be considered in the entire process [e.g. [52,55]]. Microorganism tolerance to metals can be defined as the ability to survive metal toxicity by means of intrinsic properties and/or environmental modification of toxicity [42]. Intrinsic biochemical and structural properties that determine survival may include cell walls characteristics, metabolite excretion and the ability to convert the metal into an innocuous form, e.g. by binding or precipitation [20]. Despite the fact that metal recovery abilities of many algae (both micro and macro-algae) are well known and documented, an overall comparison between the removal performances and tolerance of different algae preserved in the same conditions has not yet been investigated in depth. Among environmental metal pollutants, Cu, Fe, Ni and Zn are, at very low concentrations, essential for life, because they play important roles in many metabolic processes of living cells [76]; conversely, As is one of the major environmental pollutants [14,41].

In this context, the aims of this study are the following: i) to test the tolerance of seven different microalgal strains from sea and freshwater towards a panel of five toxic metals - copper (Cu), iron (Fe), nickel (Ni), zinc (Zn) and the metalloid arsenic (As); ii) to apply for the first time three different concentrations, each one simulating very highly polluted events/environments (i.e. landfills and/or mining sites leachate, uncontrolled industrial spills); iii) to verify a prolonged exposure (seven days) to such metals respect to analogous studies [e.g. [44,53,85,69]]; iv) to identify the most promising strains to be used for putative phycoremediation perspectives. Macroscopic observation of cultures, pH values of treated tests, physiological parameters (chlorophyll a, chlorophyll b, total chlorophyll content, carotenoids, optical density, biomass and growth rates) have been measured to find the strains showing higher resistance patterns to the applied metals.

2. Materials and methods

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79 2.1. Microalgal strains and growth conditions

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81 A panel of seven microalgal strains, four from the sea and three from freshwater, was screened
82 for 1 week for five metals, each one tested at three different concentrations. The microalgal strains
83 employed in this work were *Dunaliella* sp., *Nannochloropsis* sp., *Isochrysis* sp. and *Phaeodactylum*
84 sp., as sea-water organisms, and *Chlorella* sp., *Chlorogonium* sp. and *Euglena* sp. from freshwater.
85 *Dunaliella* sp. and *Chlorogonium* sp. were kindly provided by the Dept. of Biology of the University
86 of Pisa, while all the other strains were available in the collections of the Dept. of Agriculture, Food
87 and Environment of the University of Pisa. All the microalgal strains were available in different
88 laboratory collections and preserved for several years in non-axenic, monostrain cultures (1 l volume
89 in pyrex flasks). Each monostrain culture was grown in sterile conditions, maintained in agitation and
90 kept in a specific growth medium (Table 1). Freshwater algae were grown in TAP medium [5]
91 modified in the nitrogen source: 15 g L^{-1} of ammonium chloride was substituted with 11.4 g L^{-1} of
92 ammonium nitrate, maintaining the same nitrogen concentration. Euglena sp. was grown in Cramer-
93 Myers medium [15], while marine algae were grown in F medium [27]. All the chemicals used for
94 media preparation were obtained from Sigma- Aldrich (Michigan, U.S.A.). All the microalgal cultures
95 were kept in the growth chamber, in the following conditions: 24/22 °C, 16/08 h day-night cycle and
96 a PPFD of 120 μmol photons m^{-1} s^{-1} from cool-white light lamps (Gavita Lep 330 Plasma fixtures,
97 Gavita Holland Light Emitting Plasma, Netherlands). Almost every 2 weeks, about 2/3 of old culture
98 was removed after overnight sedimentation and substituted with fresh medium; this periodic
99 maintenance was always performed under sterile bench, using sterile glass and bottles, and sterile
100 media. Moreover, each microalgae culture was periodically observed under optical microscopy
101 (Nikon TMS-F 301434, Japan) in order to monitor the general health status of the culture, and to
102 check the absence of possible eukaryotic contamination.

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104 2.2. Metal solutions, pre-culture, culture and experimental design

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106 Five different metals were chosen for the resistance pattern experiment: Cu, Zn, As (in the
107 form of the more toxic Arsenite), Fe and Ni. The concentrations to be used were established on the
108 basis of bacterial resistance patterns in [13], and by comparing metal concentration measured in some
109 of the most contaminated sites in the world [e.g. [70]]. Three different concentration levels were
110 tested for each metal, and indicated as “Low”, “Medium” and “High” (Table 2). Each concentration
111 was tested in triplicates, and three negative controls (the microalgal culture without any metal
112 solution) were established in every experiment.

113 The microalgae were collected from the original microalgal cultures by centrifugation (1000
114 g at room temperature for 10 min, Speedmaster 14R, Euro Clone, Milano, Italy), rinsed with sterile
115 distilled water and resuspended in fresh medium (modified TAP, F or Cramer-Myers medium) until
116 their Optical Densities (OD) achieved the values listed in Table 3 (T0), in a total volume of 1 l. This
117 experimental design resulted for 48 tests for each microalgal strain (5 metals×3 concentrations × 3
118 replicates, + 3 control). Every test was conducted on a total volume of 20 ml in sterile glass tubes (50
119 ml volume), under the same growth conditions in which microalgae cultures were kept during the
120 previous months/years. The glass tubes contained about 3/5 of air to guarantee appropriate gas
121 exchange. The metal solution was directly added to the microalgae culture (in sterile conditions)
122 without macroscopic change in the pH values.

123

124 2.3. Physiological parameters measured during the experiment

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126 The following parameters were measured for each algal culture: pH, culture Optical Density
127 (OD), photosynthetic pigments content and biomass expressed as g of dry weight L^{-1}. The pH was
128 measured by the sensors equipped in the Multiparameter AP-700 set (Eijkelkamp Soil & Water,
129 Giesbeek, The Netherlands). OD values for Euglena, Chlorogonium, Phaeodactylum and Isochrysis

were measured at 750 nm; *Dunaliella* [36] and *Nannochloropsis* [34] were measured at 680 nm, while *Chlorella* was measured at 530 nm [38] using a UV–vis spectrophotometer (UV-1800 Spectrophotometer, Shimadzu, Japan). The measured pigments were chlorophyll a (Chl-a), chlorophyll b (Chl-b, when present), total chlorophyll (Chl-Tot) and carotenoids. The photosynthetic pigments were extracted in 100% methanol (Sigma Aldrich, Michigan, U.S.A.). Samples (1 ml) were centrifuged at 1500 rpm for 5 min at 4 °C (Speedmaster 14R, Euro Clone, Milano, Italy). The supernatant was discharged, and the pellet was resuspended in 1 ml methanol (Sigma Aldrich, Michigan, U.S.A.), submitted to sonication (Branson 1210, Bransonic Ultrasonic Cleaner, U.S.A.) for 10 min, and kept in the dark at 4 °C for one night. Subsequently, a centrifugation at 12,000 rpm for 5 min was performed (Speedmaster 14R, Euro Clone, Milano, Italy) and the absorbance of the supernatant was spectrophotometrically analyzed (UV-1800 Spectrophotometer, Shimadzu, Japan) with regards to the blank at 665.2, 652.4 and 470.0 nm for Chla, Chl-b and carotenoids concentrations, according to the equations indicated by [46]. In addition, a primary observation and evaluation of the microalgal culture density and the color of each test was conducted at the end of the experiment and was compared with the control tests (C) (Table 5). Finally, at the end of the experiment, the dried weight was measured for each culture (both tests and control) after 1 week of oven drying at 60 °C. The biomass was g^{-1} in dried weight and used to compare the control and the tests in which the algal culture was macroscopically grown comparably to control. This was particularly true in the case of As treatments that moreover, were also the most important tests since this metalloid is the most toxic among all other metals. Unfortunately, the measures of the biomasses in presence of Fe, Zn, Ni and Cu, were partially invalidated by metal salts residues contained in the pellet after the 7-day oven drying (data not shown). This is presumably due to the fact that the amount of metal salts added to reach the tested concentrations was really high, while for As, the tested concentrations were the lowest, even if the toxicity level was the highest (Table 2).

2.4. Data interpretation and statistical analysis

Once the pigment concentrations were calculated, data were elaborated and expressed as % compared to the pigment content of C, representing the 100%. An increment in chlorophyll content compared to C was interpreted as a greater growth in the presence of the metal compared to the not-treated test [e.g. [24]]. An increase in carotenoid content coupled with a decrease in chlorophyll content represents an index of stress and sufferance of the microalgal cultures [e.g. [43]]. The weight ratio between Chl-a and Chl-b was calculated for microalgae having both pigments (*Chlorella* sp., *Chlorogonium* sp., *Euglena* sp. and *Dunaliella* sp.). This index is an indicator of the functional pigment equipment and light adaptation of the photosynthetic apparatus [45]. In addition, for all algal strains, the ratio between Chl-Tot and carotenoids was calculated as an indicator of the greenness, as explained in [47]. For every microalgae strain the calculated indices were submitted to the analysis of variance (ANOVA), performed using the PAST software v. 3.0 (PAleontological STatistic, [30]), in order to check whether the treatment had an effect on the overall health status of the cultures. When the obtained p value was significant ($p < 0.05$), the Tukey's pairwise post-hoc tests was applied to verify which values were statistically different from C, and, consequently, to verify which treatment mainly affected the population.

The growth rate of microalgal cultures was expressed as the ratio between the optical density variation from T0 and T1, and the experimental time expressed in d^{-1}: (ODT1 − ODT0)/d where ODT1-ODT0 are respectively the optical density of the culture after 1 week (T1) and the optical density measured at T0.

Growth rates were calculated for all strains in absence of treatments to better characterize the organisms from a physiological point of view; growth rates were also calculated for As treatments in comparison to C at T1. Results were plotted using the “ggplot2” and “ggpubr” packages of the R software [82], for boxplot construction and for statistical tests respectively. The same R packages and the same statistics were used to plot the biomass comparisons between controls and As tests for each algal strain.

3. Results and discussion

3.1. Growth of microalgal strains in the experimental conditions in absence of metals

In this work, both high concentrations and prolonged time were chosen to simulate a highly toxic environmental spill to be bioremediated in hypothetical conditions of real environmental pollution and/or accidental cause.

The calculated growth rates (Fig. 1) revealed significant differences among the strains in absence of treatment. The lower growth rates were attributed to *Phaeodactylum* sp., *Euglena* sp., followed by *Isochrysis* sp. and *Dunaliella* sp. *Chlorella* sp., and *Chlorogonium* sp. showed the highest growth rates, while *Nannochloropsis* sp. showed an intermediate growth rate. The OD values are frequently used as a rapid measurement of biomass in cultures of bacteria and other unicellular microorganisms [72,77]. In pigmented cells such as microalgae, the most recommended wavelength is outside the range of absorbance of the pigments (e.g. 750 nm) [26] or not interfering with them. For every microalgal strain, the C (T1) showed a higher pigment content after 1 week (Table 3) and a higher OD value compared to the T0 (Table 4). In all these cases, the observed differences in OD values were statistically significant ($p < .05$). Moreover, macroscopic observation of cultures revealed that the color of C was more intense than the T0 in almost all the experiment (Table 5).

All these parameters suggested that in absence of metals, each culture was able to grow in the experimental conditions (i.e. temperature, pH, medium, light and glass tube), which did not affect the overall growth of the organisms. Accordingly, the statistically significant increase in pigment content in all the strains, except for *Dunaliella* sp., confirmed this observation. Moreover, the ratios between Chl-a/Chl-b (when present) and between Chl-tot/carotenoids, did not differ significantly between T0 and C (Figs. 2 and 3). For these reasons, the observed lack of growth and decrease in pigment content

207 in some of the treated samples is reasonably related to the applied treatments (i.e. toxic metals and
208 concentrations).
209
210 3.2. pH changes during algal growth
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212 In all of the cases examined, the pH values increased during the seven day experiment in non-
213 treated samples (T0 and C) (Table 4). This increment is in agreement with literature; indeed, the pH
214 of microalgal cultures rises gradually during time due to the uptake of inorganic carbon [66].
215 Dissolved inorganic carbon is present in microalgal cultures in different forms - carbon dioxide,
216 carbonate, and bicarbonate- [57], and bicarbonates are the dominant form at pH levels compatible
217 with most algal cells growth (between 6.5 and 10) [16]. According to [12], in presence of the
218 equilibrium $H^+ + HCO_3^- \rightleftharpoons CO_2 + H_2O$, H^+, ions are used to convert HCO_3 – to CO_2 (the latter is
219 fixed during photosynthesis). OH^- is left in the cell, and this has to be neutralized by H^+ uptake from
220 the extracellular environment. Consequently, the reduction of H^+ in the culture medium occurs,
221 leading to an increased pH, which subsequently changes the equilibrium between different carbon
222 species. Notably, every time the microalgal strains demonstrated tolerance and growth (comparable
223 to C) in presence of the treatments, the pH values were in most cases similar to that of C. In all the
224 cases in which the pigment content did not exceed 20% in comparison to C, the pH values did not
225 increase, remaining similar to T0 values (Table 4). This explains the reduction of the photosynthetic
226 pigment content and, consequently, the lack of cell growth.
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228 3.3. Microalgal health status in presence of heavy metals: Macroscopical observation of cultures
229
230 The macroscopical observation of cultures and the comparison between C and treatments,
231 offered preliminary information on the general effect of the tested metal on the culture (Table 5). All
232 the control tests appeared as healthy growing microalgae cultures displaying intense pigmentation

233 and marked turbidity. When Fe was present in the solution, especially at a higher concentration, the
234 culture was red and the precipitate was reddish. In few cases the microalgal growth was so high as to
235 dominate the reddish pigment of the iron solution with a greenish color, especially at low
236 concentrations (i.e. *Dunaliella* sp., *Chlorogonium* sp., *Chlorella* sp., and *Euglena* sp.). For this reason,
237 the OD measures of the Fe-treated cultures were not used for growth rate calculations since the values
238 did not only refer to cell density, but also to the different color of the solution compared to C. All the
239 tests performed in presence of arsenite looked similar to their C, and sometimes the color intensity
240 appeared to be even higher. This was true for all the strains with the only exception of *Phaeodactylum*
241 sp. (As-Med and As-High) and *Chlorella* sp. (As-High). In presence of Zn, all the microalgal cultures
242 turned into a white colored culture in which a white precipitate (reasonably death or broken cells and
243 salts) was observed, with the only exceptions of *Dunaliella* sp. (Zn-Low) and *Euglena* sp. (all tests).
244 Similar results were observed in presence of Ni and Cu, despite for Cu, since the initial solution
245 looked light blue colored, the final experiments looked light blue as well and not white. For these
246 reasons and similarly to Fe, the O.D. was not used for the calculation of growth rates, as in the cases
247 of Ni, Cu and Zn.

248 Overall, Chl-a content is often used as a quantitative measure of phytoplankton in water bodies
249 [32]. Accordingly, the increase in pigment content (mainly Chl-a) measured in each experiment after
250 one week treatment is an indicator of the increased growth, while decreased values of chlorophylls
251 stands for a partial (or total) inhibition of microalgal growth [24]. In parallel, carotenoid content was
252 used in this context to evaluate the stress status of each microalgal strain since it has been
253 demonstrated that carotenogenesis in green microalgae occurs under stress response [e.g. [8,43]].

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255 3.4. Effects of arsenite on microalgal strains

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In this experiment, all the strains were able to survive and, in most cases, grow in presence of at least one toxic metal. Interestingly, all the strains could tolerate arsenite, even at the highest concentration

(Fig. 4). For this reason, the growth rates and the biomass of each organism treated with As were also calculated and statistically compared with C (Figs. 5 and 6 respectively). Microalgae are able to undergo different processes to reduce arsenic toxicity in the environment (for a review, see [79]). Even though it is well known that the synthesis of phytochelatins represents a defense mechanism of *P. tricornutum* against arsenate toxicity [58], very little is known about arsenite toxicity responses.

In any case, our results revealed that after a seven day exposure at low As concentration (0.5 mM, corresponding to about 37 mg L^{-1} As), the amount of pigments detected was less than the 20% compared to C (Fig. 4). This result might suggest that some cells can survive in presence of this toxic metal. Interestingly, while decrease in growth rate is statistically significant when As exposure occurs (Fig. 5), the biomass does not seem to be affected by the treatment (Fig. 6). This evidence suggests that the remaining cells increase in biomass. Further investigation will highlight possible minimal inhibitory arsenite concentrations for our *Phaeodactylum* sp. strain, and possible defense mechanisms. The growth of *Euglena* sp. observed in As-Low, As-Med and As-High tests (respectively corresponding to 37, 75 and 187 mg L^{-1}), is in accordance with previously published results, demonstrating the tolerance of *Euglena gracilis* to As (III) up to 200 mg L^{-1} in a eight day experiment [54]. The fact that in our *Euglena* sp. at all the tested concentration the pigment amount was about 150% (and even more) compared to C, might suggest the presence of a possible mechanism leading to a growth promoting effect due to the presence of As in the medium. Additionally, it is remarkable that health indices revealed a comparable situation than in C, in presence of As for *Euglena* sp. (Figs. 2 and 3), as well as the growth rate (Fig. 5) and the biomass (Fig. 6).

Very little is known about *Nannochloropsis* sp. tolerance to arsenic species. Sun et al. [74] evaluated the growth conditions of *Nannochloropsis* sp. and the microstructures of microalgal cells

283 with arsenic (III) adsorption, using arsenic concentrations of 10, 20, 30, 50, and 100 mg L^{-1}. Results
284 highlighted that *Nannochloropsis* sp. was able to grow until 50 mg L^{-1} of As (III). Our results
285 highlighted that our *Nannochloropsis* sp. strain was able to grow in presence of greater concentrations
286 of As (III) up to 2.5 mM corresponding to 187.3 mg L^{-1} As (Figs. 4). At all tested concentrations, the
287 index Chl-Tot/carotenoids revealed that the health status of As treatments was comparable to that of
288 C (Fig. 4), as well as growth rate (Fig. 5) and biomass, the latter being even significantly higher than
289 C at As-High concentrations (Fig. 6). At the same time, our Isochrysis sp. strain was tolerant to all the
290 As (III)

291 tested concentrations as well (from 0.5 to 2.5 mM, corresponding to 37–187 mgL^{-1}), and the
292 health status (Fig. 3), the growth rate (Fig. 5) and the biomass (Fig. 6) of the treated cultures were
293 comparable to C. However, a progressive reduction in photosynthetic pigment content (up to 70% at
294 As-High) compared to C was observed (Fig. 4) against comparable pH values (Table 4). Despite the
295 limited information on the effect of arsenic on the growth of *Isochrysis*, the study of Sanders and
296 Vermersch [68] demonstrated that *I. galbana* was very sensitive to arsenate, with a concentration of
297 0.4 pg/cell necessary to suppress 50% of the cells. This is the first report showing an arsenite
298 resistance pattern for Isochrysis, and, opening new perspectives for possible applications in
299 phycoremediation of As (III) polluted marine areas.

300 The behavior of *Chlorella* sp. with arsenite has also been widely investigated; Ohki et al., [60]
301 showed the survival abilities of this organism at concentrations in the 0–100 μg mL^{-1} range, which is
302 comparable to our results (Fig. 4). Recently, in the study of Saavedra et al. [67], *Chlorella* sp. uptake
303 of arsenate at a concentration of 11.7 mg L^{-1} was evaluated, and the growth was not affected by the
304 presence of As in the concentrations tested. In this work, we demonstrated the tolerance of *Chlorella*
305 sp. to As (III) up to 1 mM (corresponding to 75 mg L^{-1}), and health indices confirmed a good health
306 status in these conditions (Figs. 2 and 3). Moreover, the statistically significant decrease in Chl-a/Chl-
307 b ratio suggests that the As-High concentration leads to a sufferance of the algal strain and inhibits

308 growth. This result is also enforced by the significant decrease in growth rate due to As-High (Fig. 5)
309 even if the biomass does not reflect this significance, but just confirms the trend (Fig. 6).

310 *Dunaliella* sp. was investigated for its ability to accumulate arsenic [75,83]; in presence of
311 arsenic, both As (III) and As (IV), ranging from 1 to 100 mg L^{-1}, Takimura et al. [75] described a
312 reduction mechanism of arsenite into arsenate. Yamaoka and colleagues [84] observed *Dunaliella* sp.
313 growing in a medium containing 100 mg L^{-1} of arsenite in a 7-day culture. More recently, a non-
314 axenic culture of *Dunaliella* sp. was examined for its resistance and detoxification performances in
315 arsenite up to of 0.5 mM [80]. Our experiment demonstrates a high tolerance of As (III) up to 2.5 mM
316 (187.3 mg L^{-1}) as well as a general optimum health status, analogous to the C test (Fig. 2). In these
317 cases, the pH values correspond to C (Table 4). Interestingly, despite the fact that the biomass remains
318 comparable to that of C (Fig. 6), a statistically significant increase in the growth rate was observed
319 (Fig. 5).

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321 3.5. Phisiological conditions of treated samples: Cu, Zn, Ni and Fe

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323 *Phaeodactylum* sp. was the most sensitive to the tested metal showing an almost complete
324 growth inhibition in all the experiments, except for As-Low (see previous paragraph), Fe-Low and
325 Zn-Low concentrations, with<20% of the pigments compared to C. In these cases, *Phaeodactylum*
326 sp. did not show any significant difference in the index values in any treatment (Fig. 4). According to
327 literature, *Phaeodactylum* spp. is one of the most sensitive marine microalgae to copper [44,81].
328 Moreover, as in higher plants, the excess in Cu or Zn leads to a reduction of the biomass production
329 in diatoms [51]. However, Horvatic & Persic [33] observed a tolerance of Zn (up to IC 50 of 140 mg
330 L^{-1}), and of Ni (up to IC 50 7 mg L^{-1}) in *P. tricornutum*. Compared to the metal concentrations tested
331 in the present work (5–15 mM Zn corresponding to about 326–980 mg L^{-1}, and 2.5–10 mM Ni
332 corresponding to about 147–586 mg L^{-1}), the aforementioned toxic concentrations of Zn and Ni for
333 *P. tricornutum* are lower.

In *Nannochloropsis* sp. exposed to Ni (all concentration), about 20% Chl-a content was measured compared to C with pH values ranging from 7.7 to 7.8 (Table 4). In all other cases, the growth inhibition was observed since pigment content was almost zero in every experiment, and the pH values were lower than the control. *Nannochloropsis* sp. did not show any significant difference in the index values in any treatment (Fig. 3). This situation was similar in *Isochrysis* sp., showing a comparable pattern of pigment content; almost a 20% Chl-a was measured in Ni as well (all concentrations) with pH values ranging from 7.5 to 7.7. *Isochrysis* sp. show a statistically significant increase in the index value for Ni-Low treatment respect to C (Fig. 3).

In *Dunaliella* sp., both Chl-a and carotenoid content drastically increased in Zn (all concentrations, corresponding to 326, 653 and 980 mg $^{-1}$ of Zn) and Fe-Low compared to C. In the first case, the pH ranged between 6 and 6.5, while in Fe-Low it was 4.9. All other experiments showed a really low pigment content compared to C (< 20%), and in all cases, the pH values were lower than C. Interestingly, statistics confirmed a significance in the higher value of Chl-a/Chl-b index in Zn-Low concentration compared to C (Fig. 3). *Dunaliella* sp. was the only tested strain that showed a tolerance and growth ability in presence of Zn, which was even higher than the C test. Studies on the uptake of Cu and Zn in *Dunaliella* sp. revealed that in presence of Zn 50 mg L^{-1}, the growth of this organism is not inhibited and the amount of Chl-a detected in the culture medium is comparable to the one of the control test in a 4-day experiment [62]. More recently, Kutlu and Mutlu [40] highlighted a high tendency of *Dunaliella* sp. to accumulate Zn and Cu, when exposed to the mentioned metal at concentrations ranging between 10 and 800 μmol L−1. Our results revealed that our strain is more tolerant to higher Zn concentration compared to similar studies.

Einicker-Lamas and colleagues [17] reported the IC 50 for Zn of 0.88 mM in *Euglena* sp. and observed that cell division seems to be impaired in metal-exposed cells in a three-day experiment. In our experiment, *Euglena* sp. showed tolerance of Zn at all the tested concentrations up to 15 mM, but the amount of pigments measured was in all cases about half that of the C test (Fig. 4). In the same work conducted by Einicker-Lamas and colleagues [17], *Euglena* sp. cells displayed an IC 50 for Cu

of 0.22 mM, and the cell division was impaired even at the minimal $CuCl_2$ concentration (0.02 mM), thus confirming the toxicity of Cu at high concentrations that we observed in our study. In addition, the decrease in Chl-a/Chl-b index for Cu with a statistical significance, confirms the intolerance of the microalgae to this metal as well. Nevertheless, *Euglena* sp. is the only freshwater strain analyzed in this screening that is able to tolerate the Zn concentrations tested, and, for this reason, it is one of the candidates for remediation in Zn polluted environmental matrices.

Although iron uptake is strictly required for phytoplankton for the development of the photosynthetic apparatus, its excess might generate oxidative stress, as an increase in the steady state concentration of O_2 radical intermediates occurs [9], causing severe damage to membranes, proteins, and DNA [29]. *Chlorella vulgaris* under laboratory conditions showed an increase in biomass when iron was added, up to 90 μM. Results also showed that increase in iron availability (500 μM iron) was responsible for a substantial increase in the content of lipid radicals of the membranes [18]. Our *Chlorella* sp. strain also showed a tolerance of Fe up to 2.5 mM (about 140 mg L^{-1}), which is a greater concentration if compared to the mentioned study. Moreover, it showed about a 20% increase in pigment content compared to C (120% measured values, Fig. 2). Interestingly, the lower pH values correspond to the lower pigment amount of the experiments (Table 4). Additionally, the Chl-a/Chl-b index significantly decreased against the control test in Fe-Med and Fe-High treatments (Fig. 2) while the Chl-Tot/carotenoid index (Fig. 3) only significantly increased in Fe-Low treatment. Tolerance to Fe-Low occurred also in our *Euglena* sp. and *Dunaliella* sp. Additionally, for this last strain, the statistics confirmed a significant increase in Chla/Chl-b ratio in Fe-Low (Fig. 2). Consequently, these three strains might be implicated for iron remediation in contaminated water bodies.

Interestingly, *Dunaliella* sp. and *Euglena* sp. can grow moderately (up to about 50% compared to C) on Ni. *Euglena* sp. has already been investigated for its ability to tolerate and accumulate Ni; indeed, in the work by Garcia-Garcia et al., [23], *Euglena gracilis* showed maximal nickel accumulation when exposed to 1 mM Ni for 24 h. In addition, accumulated nickel was observed to be partially released after 72 h. Our work confirmed the tolerance of our strain to nickel, even if the

386 health index (Chl-a/Chl-b) showed a statistically significant decrease compared to C in all tested
387 concentrations.
388
389 3.6. Metal tolerance pattern in *Chorogonium* sp.
390
391 One of the novelties of this study is the analysis of the metal tolerance pattern of
392 *Chlorogonium* sp., since this aspect, relative to this microalgal genus, has never been investigated in
393 detail. Indeed, the only available information refers to the changes in glutathione and polyamine
394 content, in *Chlorogonium elongatum* exposed to mercury [2]. In *Chlorogonium* sp., an increase in
395 Chl-a and Chl-b content was observed in Fe-Low and Fe-Med treatments (Fig. 2); in these cases, the
396 pH values of the culture were not significantly different from the C samples (Table 4). At As-Low
397 concentration, we observed a 50% growth compared to C. The presence of As-Med and As-High
398 concentrations did not result in the total absence of growth since pigments have been measured, even
399 if in lower amounts; in these cases, the pH values ranged from 7.4 (high) to 9.8 (low). In parallel, the
400 growth rate significantly decreased at all the concentrations (Fig. 5), while the biomass was
401 significantly lower in As-Med and As-High treatments (Fig. 6). All other metals caused a sort of
402 growth inhibition since the pigment content in all cases was about 0–10% compared to C. In all these
403 cases, the pH was lower than C. In *Chlorogonium* sp., the Chl-a/Chl-b index is statistically lower
404 compared to C in all the treatments, with the exception of As tests. To the best of our knowledge, this
405 is the first report revealing a tolerance of *Chlorogonium* sp. to arsenite, and an increment of
406 chlorophyll and carotenoid production in the presence of Fe 2.5 and 5 mM. This last aspect might be
407 interpreted in light of the Chl-tot/carotenoid index (Fig. 4); indeed, the index in Fe-Low experiment
408 in *Chlorogonium* sp. is significantly higher compared to C. This might be due to the fact that in these
409 tests, the measured Chl-Tot content was higher than the C test; since the increase in chlorophyll
410 content might be directly related to the increase of the number of cells in the microalgal population,

411 we can suggest that the presence of Fe in the medium might favor “algal bloom” with a similar
412 mechanism observed for diatoms in oceans [78].
413
414 **4. Conclusions**
415
416 In this study, we screened seven microalgal strains in order evaluate which one(s) would
417 survive, grow and potentially decontaminate wastewater. Results helped highlight the potential of
418 each strain to survive in toxic metal environments and opened new perspectives for their full-scale
419 application directly in environmentally contaminated matrices. All the freshwater strains were able to
420 survive and grow in arsenite up to 2.5 mM, and in presence of iron up to 10 mM. *Euglena* sp. was the
421 only freshwater strain able to tolerate all of the tested metals at all the tested concentrations. Among
422 marine strains, *Dunaliella* sp. was the one showing the best tolerance against zinc (up to 15 mM).
423 Additional investigation will be necessary to measure the uptake rates of each algae strain, and the
424 decontamination rates of polluted water. Finally, future investigation will be directed both towards
425 the quantification of metal removal from the medium, and towards the description of metal tolerance
426 mechanisms; this will help evaluate the best candidates for in situ phycoremediation experiments.
427
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435
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639

Organism sp.	Phylum	Marine/freshwater	Main pigments	References (pigments)	Growth medium	Growth medium pH
Dunaliella sp.	Chlorophyta	Marine	Chl-a; Chl-b; Carotenoids	[64]	F medium	7.03
Phaeodactylum sp.	Heterokonta (Stramenopiles)	Marine	Chl-a; Chl-c; Fucoxanavoid; Diadinoxanthin; β-carotene	[31,49,63]	F medium	7.03
Nannochloropsis sp.	Heterokonta (Stramenopiles)	Marine	Chl-a	[48]	F medium	7.03
Isochrysis sp.	Haptophyta	Marine	Chl-a; Chl-c; Fucoxanthin	[59]	F medium	7.03
Chlorogonium sp.	Chlorophyta	Freshwater	Chl-a; Chl-b; Carotenoids	[3]	Modified TAP medium	7.54
Euglena sp.	Euglenozoa	Freshwater	Chl-a; Chl-b; Carotenoids	[70]	Cramer & Myers medium	6.97
Chlorella sp.	Chlorophyta	Freshwater	Chl-a; Chl-b; Carotenoids	[28]	Modified TAP medium	7.54

Table 1

Microalgal strains used in the screening for metal pollutants. The main characteristics are listed for each one. Chl-a: chlorophyll a; Chl-b: chlorophyll b; Chl-c: chlorophyll c.

Metal	Salt	pH of the stock solution	Concentration Stock solution (mM)	Low final concentration	Medium final concentration	High final concentration
Cu	$CuCl_2 \times 2H_2O$	5.07	500	2.5 mM	5 mM	10 mM
Zn	$ZnSO_4 \times 7H_2O$	5.6	500	5 mM	10 mM	15 mM
As (III)	$NaAsO_2$	9.98	100	0.5 mM	1 mM	2.5 mM
Fe	$Fe(SO_4) \times 7H_2O$	2.84	500	2.5 mM	5 mM	10 mM
Ni	$NiSO_4 \times 6H_2O$	5.95	500	2.5 mM	5 mM	10 mM

Table 2

List of metals used in the experiment. Three concentrations were applied for each metal and are listed in the table as “Low”, “Medium” and “High” concentration. The pH of every stock solution is indicated.

	Day 1 – Start of experiment T0				Day 7–1 week experiment T1			
Organism sp.	OD	pH	Total Chl (mg/ml fresh liquid culture)	Carotenoids (mg/ml fresh liquid culture)	OD	pH	Total Chl (mg/ml fresh liquid culture)	Carotenoids (mg/ml fresh liquid culture)
Euglena sp.	OD_{750} = 0.304 ± 0.02	7	6.64 ± 1.56	1.79 ± 0.54	OD_{750} = 0.696 ± 0.12	7.67	15.86 ± 5.29	4.23 ± 1.10
Dunaliella sp.	OD_{680} = 0.639 ± 0.009	8.31	13.29 ± 3.37	0.92 ± 0.08	OD_{680} = 1.23 ± 0.003	9.39	17.17 ± 1.12	0.77 ± 0.18
Chlorella sp.	OD_{530} = 0.326 ± 0.00	8	3.04 ± 0.15	0.58 ± 0.01	OD_{530} = 1.37 ± 0.16	11.48	12.94 ± 1.73	2.64 ± 0.41
Nannochloropsis sp.	OD_{680} = 0.362 ± 0.00	6.8	1.04 ± 0.16	0.31 ± 0.04	OD_{680} = 1.07 ± 0.12	9.43	3.30 ± 0.24	0.79 ± 0.06
Chlorogonium sp.	OD_{750} = 0.851 ± 0.08	7.8	12.88 ± 5.02	2.47 ± 0.88	OD_{750} = 1.901 ± 0.07	10.45	36.59 ± 4.54	5.91 ± 0.49
Phaeodactylum sp.	OD_{750} = 0.360 ± 0.01	8.5	2.42 ± 0.25	1.12 ± 0.08	OD_{750} = 0.699 ± 0.12	9.495	4.45 ± 0.78	1.92 ± 0.39
Isochrysis sp.	OD_{750} = 0.405 ± 0.00	8.13	1.81 ± 0.15	0.54 ± 0.03	OD_{750} = 0.912 ± 0.02	9.5	4.35 ± 0.31	1.41 ± 0.03

Table 3

Comparison of Optical Densities, pH and pigment content of each microalgal strain at the beginning of the experiment (T0) and T1 (1 week) grown in experimental conditions without treatment. Values are given as the average value of the replicates (n = 3); standard errors are indicated as±n.

	C	As_Low	As_Med	As_High	Cu_Low	Cu_Med	Cu_High	Fe_Low	Fe_Med	Fe_High	Ni_Low	Ni_Med	Ni_High	Zn_Low	Zn_Med	Zn_High
Chlorella sp.	11.5	11.5	11.4	8.4	7.4	6.5	5.9	9.9	7.1	5.1	8.3	8.1	7.8	7.6	7.4	7.3
Chorogonium sp.	10.2	9.8	8.7	7.4	6.3	5.7	5.4	9.9	8.6	4.5	7.4	7.3	7.0	7.2	6.9	6.8
Euglena sp.	7.7	7.6	7.7	8.0	7.6	6.9	5.1	6.7	6.7	4.7	7.0	6.9	6.6	7.0	5.4	4.5
Dunaliella sp.	9.4	9.2	9.2	9.2	5.2	5.1	5.8	4.9	3.8	3.6	8.3	8.1	7.8	6.5	6.5	6.0
Isochrysis sp.	9.5	9.5	9.5	9.4	5.8	5.5	5.2	5.9	4.0	3.9	7.5	7.7	7.6	6.7	5.3	5.7
Nannochloropsis sp.	9.4	9.2	9.4	9.4	5.9	5.4	5.1	4.3	4.0	4.8	7.8	7.7	7.7	7.2	6.9	6.7
Phaeodactylum sp.	9.5	8.2	8.2	8.2	7.0	5.5	5.2	5.3	3.9	3.6	7.4	7.6	7.5	7.0	6.8	6.7

Table 4

pH values of the tests after 1 week treatment (T1).

		Dunaliella sp.	*Phaeodactylum* sp.	*Nannochloropsis* sp.	*Isochrysis* sp.	*Chlorogonium* sp.	*Euglena* sp.	*Chlorella* sp.
	Control	Green, HG	Brown, HG	Green, HG	Green, HG	Green, HG	Green, HG	Green, HG
Fe	Low	Red and greenish solution. HG	Brownish solution, due to the presence of Iron combined to the survival of some cells	Red solution, DB	Red solution, DB	Green, HG	Green, HG	Red and greenish solution, HG
	Med	Deep red solution with precipitate						Deep red solution with precipitate
	High					Deep red solution with precipitate		
As	Low	Greener than Control, HG	Light brown, survival of some organisms	Green, HG	Green, HG	Green, HG	Green, HG	Greener than Control, HG
	Med		White solution, DB					Green, HG
	High					Green, less than Low and Med, HG		White solution, DB
Zn	Low	Green, HG	Light brown, survival of some organisms	White solution, DB	White solution, DB	White solution, DB	Green, HG	White solution, DB
	Med	Green, HG						
	High	Less green than control, growth	White solution, DB					
Ni	Low	Less green than control, growth	White solution, DB	Light green solution, survival of some cells	White solution, DB	White solution, DB	Green, HG	White solution, DB
	Med	White solution, probably DB						
	High			White solution, DB				
Cu	Low	Aggregates, blue flocks, probably due to the color of Cu solution, DB	White solution, probably DB	Light blue solution, probably due to the presence of Cu, DB	Light blue solution, probably due to the presence of Cu, DB	Light blue solution, probably due to the presence of Cu, DB	Light blue solution, probably due to the presence of Cu, DB	Light blue solution, due to the presence of Cu, DB
	Med							
	High							

Table 5

Results of the observation and description of the appearance of each algal culture after 1-week experiment. HG: Healthy Growth; DB: Death Biomass.

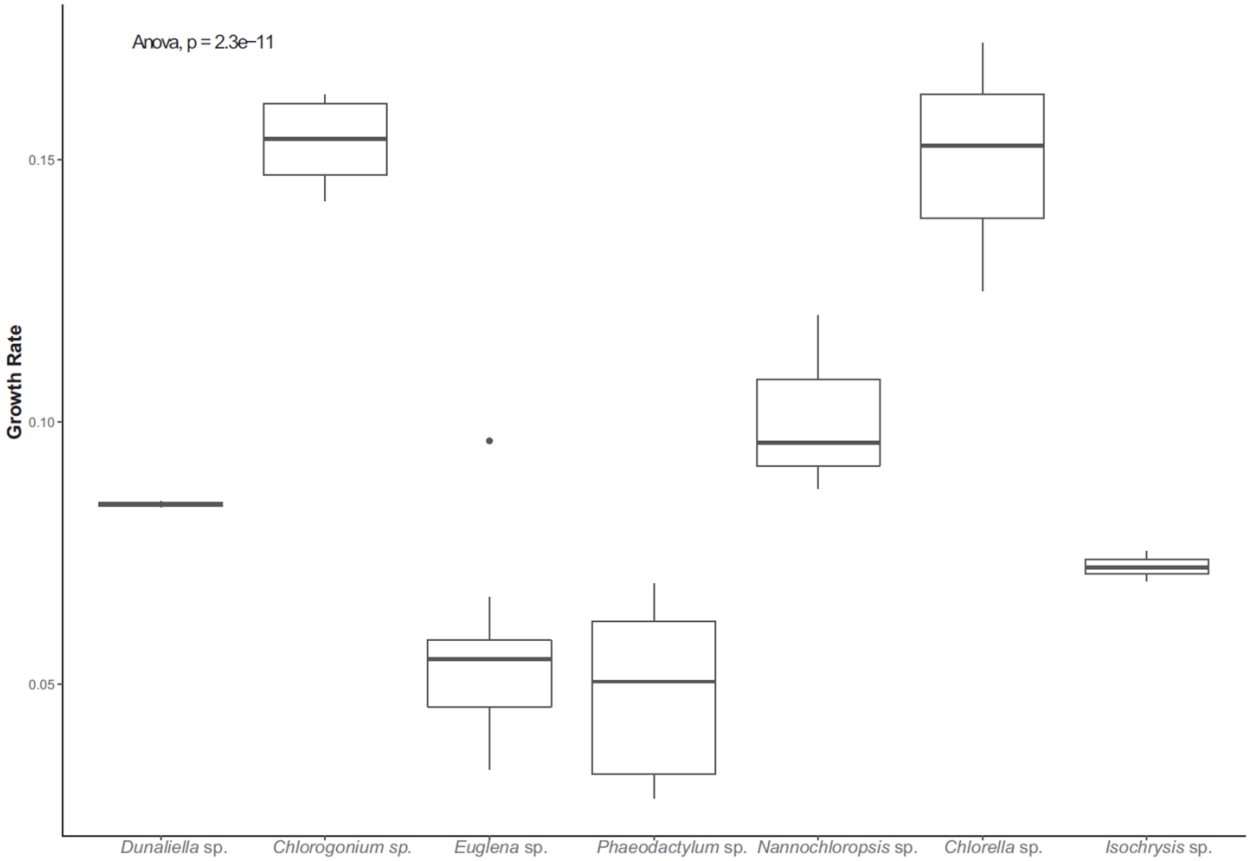


Figure 1

Growth rate of the microalgal strains in absence of treatments, during a seven day-experiment.

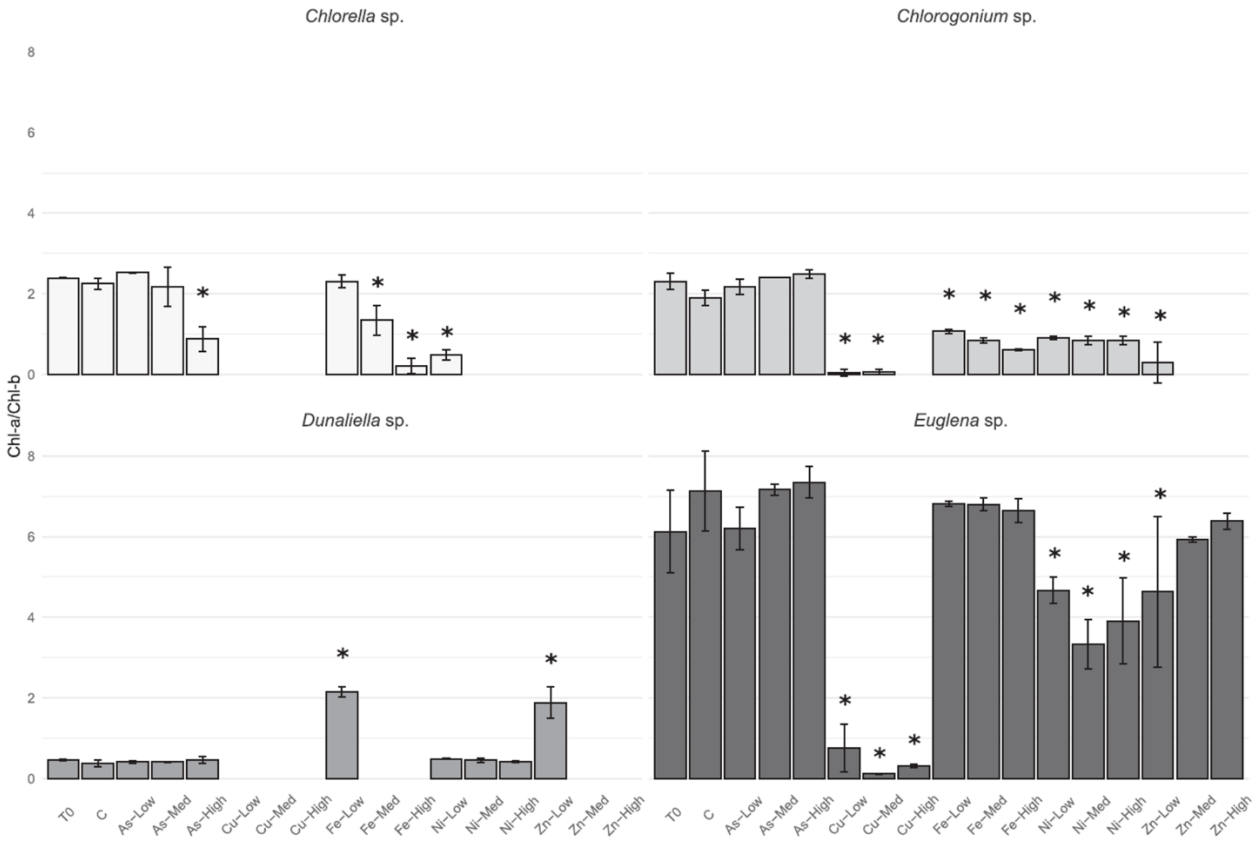
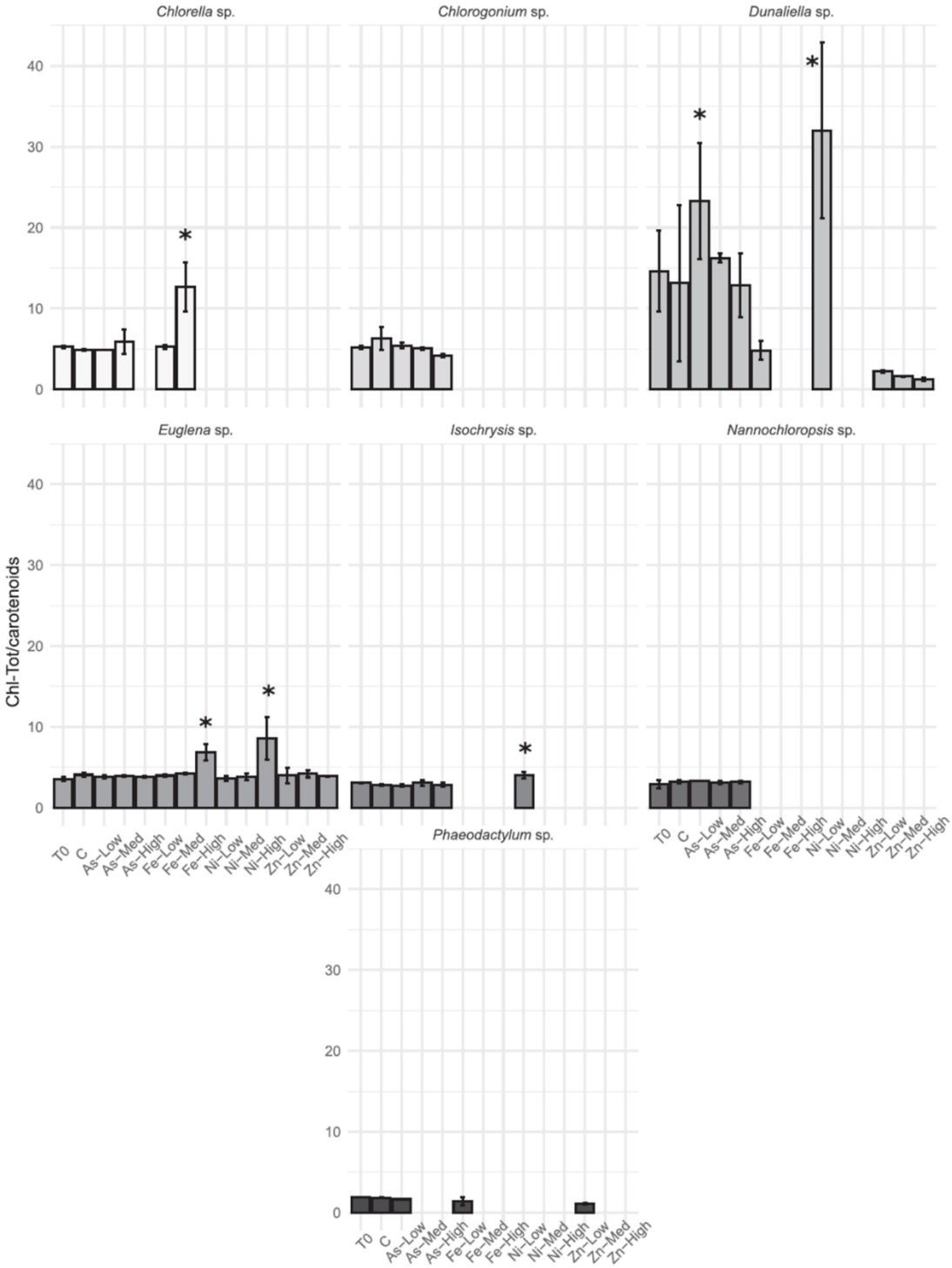


Figure 2

Chl-a/Chl-b index calculated from pigment content data of the algal strains. Asterisks indicate statistically significant differences between control (C) and treatments. Error bars refer to the standard error calculated on the replicate measures (n=3). Missing values stand for tests in which no pigment was present after 1-week experiment.



689

690 **Figure 3**

691 Chl-Tot/carotenoids index calculated from pigment content data of the algal strains. Asterisks indicate
692 statistically significant differences between control (C) and treatments. Error bars refer to the standard
693 error calculated on the replicate measures (n = 3). Missing values stand for tests in which no pigment
694 was present after 1-week experiment.

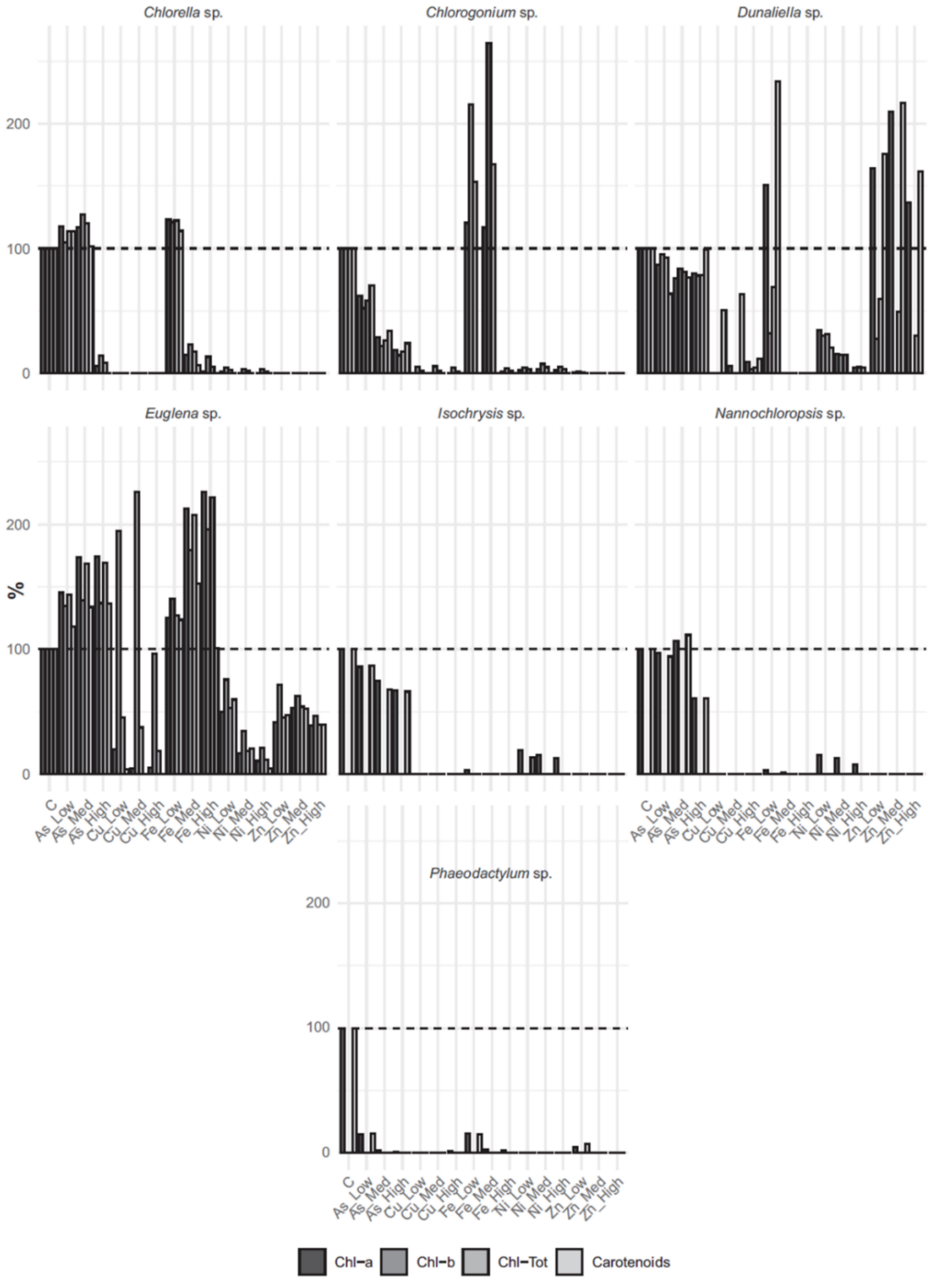


Figure 4

Pigment contents expressed as % compared to the pigment content of control test (C) in the algal strains. The 100% pigment content of C is represented by the dashed line.

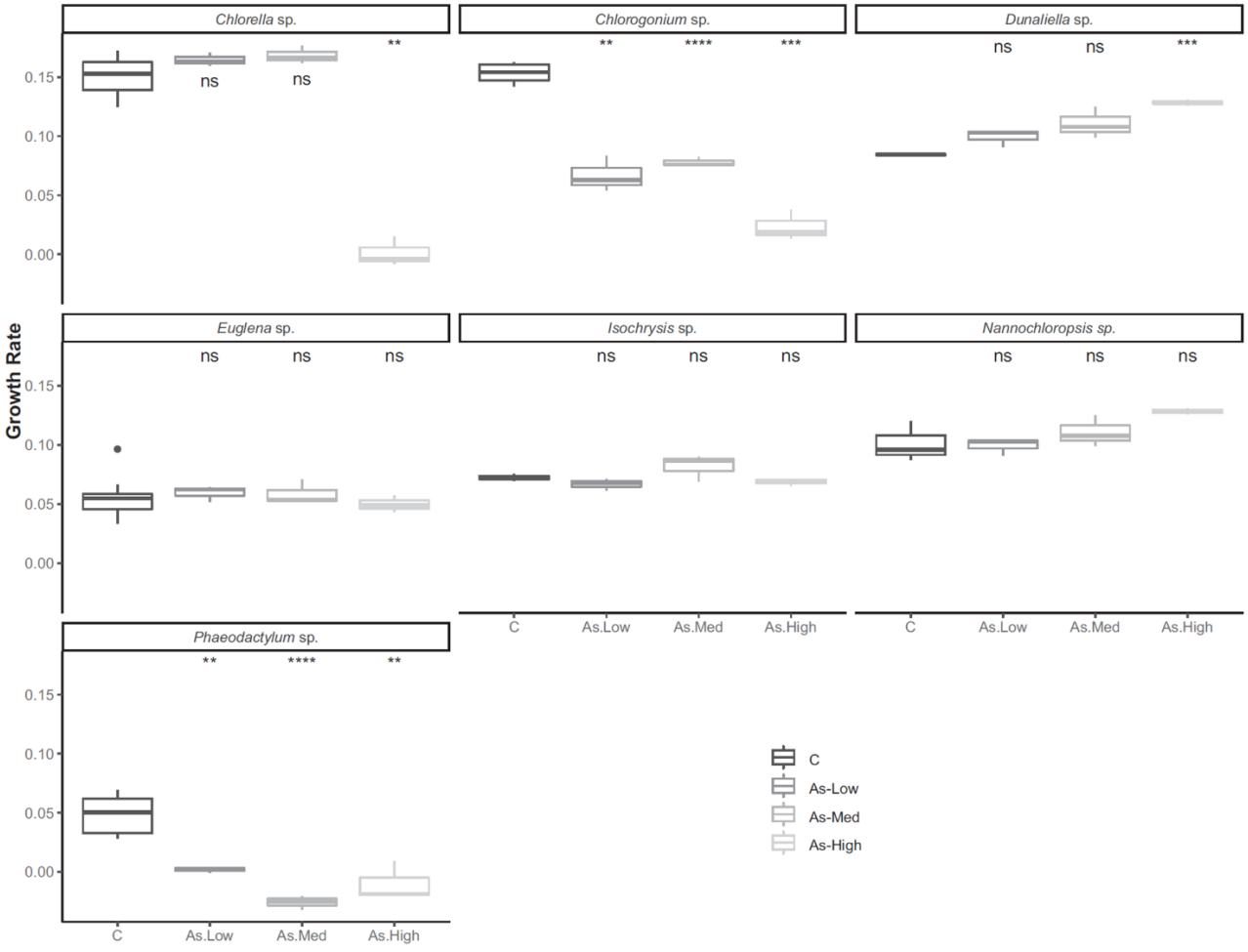


Figure 5

Growth rates of As treated cultures in comparison to C not-treated cultures. t-test has been applied. Significant differences are indicated with asterisks. Not significant differences are indicated as n.s.

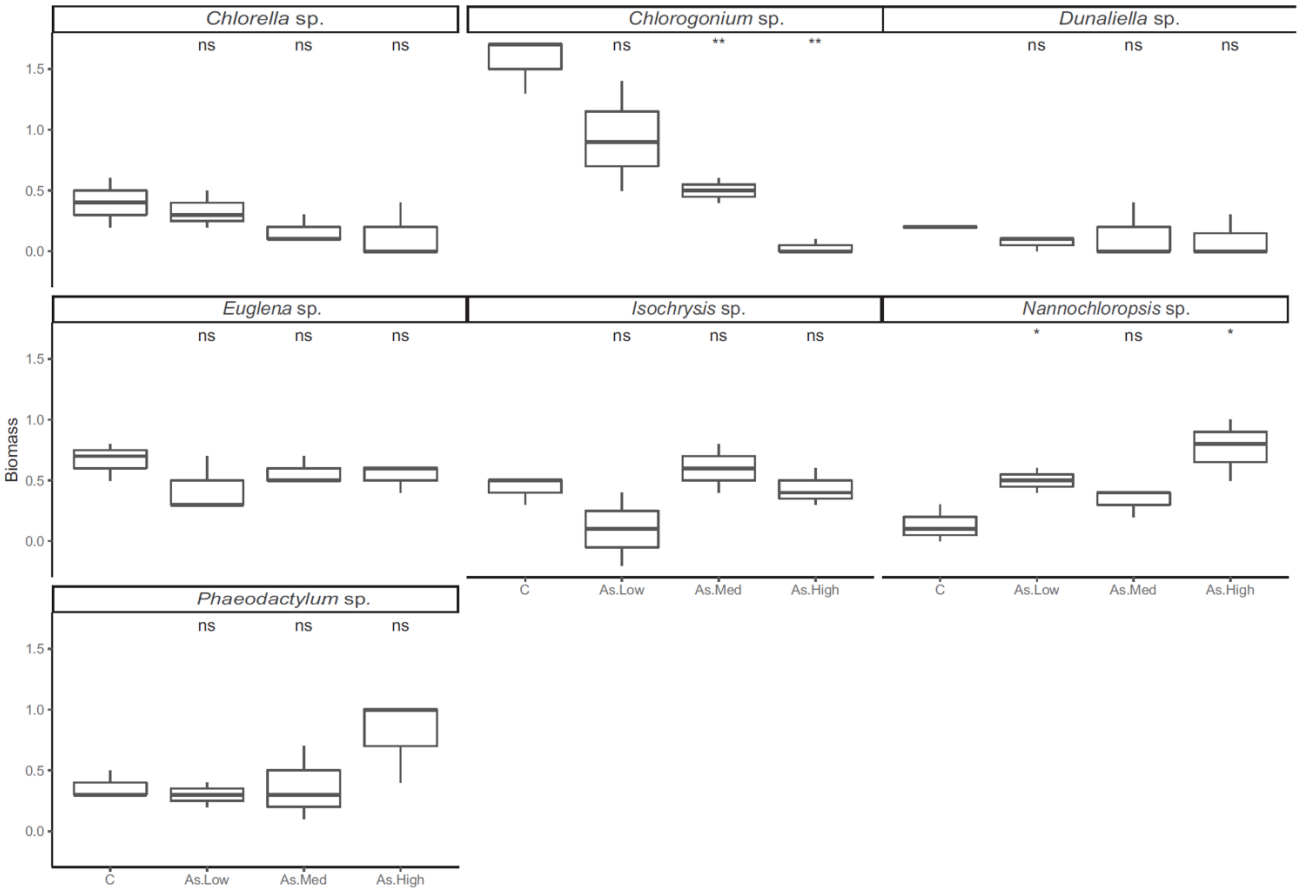


Figure 6

Biomass expressed as g L^{-1} calculated for controls and As-treated microalgal cultures at T1. Significant differences are indicated with asterisks. Not significant differences are indicated as n.s.