Aquatic Botany 200 (2025) 103888

Contents lists available at ScienceDirect

Aquatic Botany

journal homepage: www.elsevier.com/locate/aquabot

Nickel tolerance and phytoremediation potential of the aquatic plant *Lemna minuta* and the cyanobacterium *Trichormus variabilis* in monoculture and consortium

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ARTICLE INFO

Keywords:
Duckweeds
Cyanobacteria
Heavy metal
Nickel-contaminated freshwater
Phytotoxicity
Bioremediation

ABSTRACT

One of the main threats to aquatic conservation is heavy metal pollution, with nickel (Ni) among the most significant contaminants. The Ni tolerance and remediation potential of *Lemna minuta* (vascular plant) and *Trichormus variabilis* (cyanobacterium) in contaminated water were investigated. The phytotoxic effects of nickel sulfate ($NiSO_4 \cdot 7\,H_2O$; 6.47 mg/L) on these two species, were assessed after 7- and 14-days exposure by measuring morphological changes, growth (fresh weight) and key physiological parameters (chlorophyll, malondialdehyde, protein content and catalase activity). The ability of *L. minuta* and *T. variabilis* to remove Ni was compared in both monoculture (single species) and consortium (mixed species) by measuring the reduction in Ni concentration in the culture medium. Nickel exposure induced phytotoxic effects in both species, as shown by decreases in fresh weight, chlorophyll and protein content, and increases in malondialdehyde content and catalase activity. These effects were more pronounced in monocultures, particularly in *T. variabilis*, compared to consortia. *Lemna minuta* exhibited good Ni tolerance and remediation capacity, removing 75 % and 86 % of the metal from the solution after 7 and 14 days, respectively. *Trichormus variabilis* removed 36 % and 27 % of the Ni after the same exposure time. Consortia exhibited high Ni removal, reaching 80 % and 90 % after 7 and 14 days, but this was not statistically different to *L. minuta* monocultures. These results demonstrate the potential of *L. minuta* in the remediation of Ni-contaminated waters and suggest that consortia might enhance the tolerance and viability of both species under Ni-stress.

1. Introduction

One of the main threats to the conservation of aquatic ecosystems and their biodiversity is the chemical pollution that mostly derived from industrial wastewater (Kumaraswamy et al., 2020). Among the main chemical pollutants, heavy metals have garnered significant interest, especially from an ecotoxicological viewpoint, due to their intrinsic genotoxic nature in animals and plants (Sankhla et al., 2016; Ceschin et al., 2021). A heavy metal commonly found in the environment is nickel (Ni), as it constitutes 0.01 % of the Earth's crust and is present in many everyday objects, and metal alloys, in particular, aluminum, to improve antioxidant properties and resistance to high temperatures (Ezugwu et al., 1999).

Nickel's effects on plant growth are complex, ranging from an essential micronutrient to a harmful toxin, depending on its concentration in the growth medium and on the exposed plant species and its tolerance/sensitivity. As a micronutrient, nickel is crucial for normal growth, playing a vital role in numerous metabolic enzyme activities (e.g., urease, dehydrogenase, methyl reductase), nitrogen metabolism, iron uptake, and seed maturation. However, excessive nickel concentrations induce toxicity, reducing root and shoot growth, biomass accumulation, and often leading to chlorosis and necrosis. Nickel toxicity also induces oxidative stress, triggering the production of reactive oxygen species (ROS) that can inhibit photosynthesis and transpiration processes

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https://doi.org/10.1016/j.aquabot.2025.103888
Received 30 November 2024; Received in revised form 29 March 2025; Accepted 30 March 2025
Available online 4 April 2025

(Rizwan et al., 2017; Hassan et al., 2019).

For treating heavy metal contamination in freshwater, phytoremediation offers a promising and eco-friendly solution. This technique utilizes aquatic plants that not only exhibit tolerance towards heavy metal pollutants but also actively absorb and accumulate them (Ali et al., 2020). Among freshwater plants, species of the genus *Lemna* (duckweeds) have proved to be particularly efficient in the phytoremediation, able to grow whilst purifying organic- and nutrient-rich civil and agricultural wastewater (e.g., Ceschin et al., 2019; 2020; Zhou et al., 2023). These plants are also adept at treating industrial wastewater contaminated with inorganic pollutants, synthetic substances or heavy metals (e.g., Obek and Sasmaz 2011; Farid et al., 2022). Their remarkable tolerance and efficient bioaccumulation of pollutants enable them to remove large concentrations of contaminants. Because most *Lemna* species form freely floating mats on the surface of still or slow-flowing waters, their phytoremediation efforts are primarily focused on the upper water layers (Mkandawire and Dudel, 2007).

Several laboratory studies have shown that some *Lemna* species (*L. minor*, *L. gibba*) are efficient in the treatment of waters contaminated by heavy metals, such as cadmium (Cd), lead (Pb), silver (Ag) and nickel (Ni) (Axtell et al., 2003; Khan et al., 2020; Iannelli et al., 2022). Furthermore, evidence suggests that these species, when cultivated in consortia with other photosynthetic organisms, may exhibit enhanced heavy metal removal efficiency compared to monoculture systems. For example, the mercury (Hg) removal efficiency by *L. minor* and *Salvinia natans* (aquatic fern) was improved when present together rather than singularly (Sitarska et al., 2023).

In addition to duckweeds, many studies demonstrated the capacity of cyanobacteria to tolerate and bioaccumulate heavy metals in highly contaminated waters (Gismondi et al., 2016; Kulal et al., 2020). Since some cyanobacteria, such as the genus *Trichormus*, form filamentous populations that in slow-flowing waters mainly occupy the water column bottom, this suggests that any phytoremediation activity could be mainly localized here. Given the spatial distance between the growth habits of *Lemna* and *Trichormus*, and so, the likelihood they would not compete spatially, could indicate a potential accumulative phytoremediation potential when present together.

This study investigated, for the first time, the potential of the duckweed *Lemna minuta* and the cyanobacterium *Trichormus variabilis* for the remediation of Ni-contaminated freshwater. Selection of these two species was made by taking into consideration their biotechnological and remediation potential (Ceschin et al., 2019, 2020; Congestri et al., 2020; Alabiso et al., 2023), and their distinct spatial distribution within the water column. The hypothesis was that their combined use could potentially lead to a cumulative phytoremediation of contaminated waters throughout the water column. Therefore, the tolerance and remediation capacity of both species were assessed in monocultures (single species) and novel experimental consortia (mixed species) incubated under nickel stress and no-nickel stress by analyzing morphological, physiological and growth parameters of both species as well as water chemical and physical parameters.

2. Materials and methods

2.1. Biological material collection

Plants of *L. minuta* were collected in December 2022 from a wetland located at the Appia Antica Regional Park (Rome, Italy). These plants were transported to the laboratory within 30 minutes in a container filled with water from the collection site. In the laboratory, the plants were acclimatized for one week in glass tanks containing 10 L mineral water (Table S1), chemically similar to the water of the field water site. The strain of the heterocystous cyanobacterium *T. variabilis* (VRUC168) was isolated from phytobenthos of a Mediterranean coastal lagoon (Cabras Lagoon, Sardinia, Italy) and maintained in stock cultures using the standard Blue Green Medium (BG11.0). *Trichormus variabilis* was cultivated using an indoor column photobioreactor (8 L) at 18–20 °C, 80 μmol photons m^{-2} s^{-1} and 12:12 h light/dark cycle.

2.2. Experimental set-up

The experiment included Ni-treatments with and without *L. minuta* and *T. variabilis* (Ni-treatments) and Ni-free tests with organisms (controls), monitored at 0 (T_0), 7 (T_7), and 14 (T_{14}) days. Tests with organisms included monocultures (mono-species: Lm, Lm-Ni, Tv, Tv-Ni) and consortia (mixed-species: Mix, Mix-Ni). Three replicates of each test (Ni-treatments, controls), culture type (mono- and mixed) and time-point (0, 7, 14 days), were set up, for a total of 39 experimental units (Fig. S1). All units were incubated in a climate chamber under controlled conditions (25 °C, 100 μmol photons m^{-2} s^{-1} and 12:12 h light/dark cycle).

In detail, Ni-treatments were performed using 240 mL graduated glass beakers, each filled with 150 mL of a 6.47 mg/L nickel(II) sulfate heptahydrate ($NiSO_4 \cdot 7\,H_2O$) solution. The solution was prepared by dissolving nickel sulfate in the mineral water described in Table S1. Organism-free nickel treatments were also set-up to monitor Ni stability in solution. To minimize non-biological Ni adsorption (i.e. by beaker surfaces), all Ni-treatments beakers were pre-rinsed with a saturated nickel sulfate solution prepared in mineral water. This pre-treatment ensured saturation of potential Ni-binding sites on the glass surfaces before plant exposure. Therefore, nickel removal by the organisms was calculated as the difference between initial and post-exposure nickel concentrations in the treatments containing organisms. The initial Ni concentration of 6.47 mg/L, exceeding typical environmental levels, was selected for several reasons: comparability with previous nickel decontamination studies on *Lemna* plants (Axtell et al., 2003; Khan et al., 2020), relevance to nickel levels found in some industrial wastewaters (Ansari and Malik, 2010; Lokhande et al., 2011), and the need to elicit robust stress responses in the target organisms for more reliable analysis.

Simultaneously to Ni-treatments, control tests with organisms grown in 150 mL of Ni-free mineral water (Table S1) were established.

For each test with *L. minuta*, 5 g of *Lemna* fronds were added to form a monolayer covering the water surface, while 0.75 g of *T. variabilis* biomass was used in each cyanobacterial test. The different initial biomass amounts used for the two species target were chosen to ensure both species formed a monolayer that had a similar surface of interaction with the contaminant, therefore by ensuring a valid assessment of their removal potential in a biotechnological application as both single- than combined-cultures.

At T_0, T_7, and T_{14}, water samples of each experimental unit were analyzed for chemical and physical parameters, while plant and cyanobacterial samples were assessed for growth, morphology, physiology, and biochemical parameters.

2.3. Analyses of water chemical and physical parameters

In each test (Ni-treatment, control), water conductivity (μS/cm), pH, and dissolved oxygen saturation (%), were measured using specific probes (Hach-Lange 40 HQD). Water Ni concentration (mg/L) in each Ni-treatments was measured by spectrophotometric analyses (Hach Lange DR 3900) by using a cuvette-kit specific for nickel (LCK 337-Ni, Hach Lange) based on Dimethylglyoxime method, according to DIN 38406-E11 protocol standard (DIN 38406–11, 1991). Deionized water was used as a blank and standard curves prepared from 5 to 0.1 mg/L using a standard Ni solution (1000 mg/L $Ni(NO_3)_2$, NIST).

2.4. Biomass growth

Biomass (g, fresh weight) of *L. minuta* and *T. variabilis* was measured at each sampling time-point (T_0, T_7, T_{14}) to assess the effect of Ni exposure on their growth. Changes in biomass during the experiment were calculated for each test organism. *Lemna minuta* fronds were

collected, drained and partially dried on absorbent paper and then weighed (Falc electronic scale T500). Regarding *T. variabilis*, the fresh weight was recorded after collecting the biomass by natural settling in the glass beakers used for the experiments followed by centrifugation (4500 x *g*, 10 min) in pre-weighed 2-mL eppendorf tubes; the pellet fresh weights were measured using a precision balance (Gibertini E42S).

2.5. Morphological analyses

Morphological parameters, such as frond length (mm), width (mm), frond area (mm^2) and root length (mm), were measured on ten *L. minuta* fronds randomly selected from each *Lemna*-test, following Ceschin et al. (2016). All these parameters were analyzed using ImageJ program on digital photos taken with a stereoscope (Zeiss-Stemi 305).

2.6. Chlorophyll content determination

Total chlorophyll content in *L. minuta* and chlorophyll *a* in *T. variabilis* were determined following the spectrophotometric protocol of Huang et al. (2007) . Pre-weighted aliquots of *L. minuta* and of *T. variabilis* were soaked in 10 mL of 95 % (v/v) ethanol for three days, in a stoppered tube at room temperature in the dark. The samples were centrifuged at 3000 x *g* for 10 min, and the absorbance of the supernatant was measured at 663 and 645 nm (Perkin Elmer-Lambda 25 UV/Visible). Chlorophyll concentrations (mg/g FW) were calculated using the following equations (Huang et al., 2007):

Chl *a* = 12.72 A663 - 2.69 A645

Chl *b* = 22.90 A645 - 4.68 A663

Chl tot = Chl *a* + Chl *b*

where Chl *a*, Chl *b*, and Chl Tot represent the content of chlorophyll *a*, chlorophyll *b*, and total, respectively, and A663 and A645 are the absorbances at 663 and 645 nm, respectively. Results are expressed in mg of total Chl (Chl *a* + Chl *b*) per gram of plant tissue in fresh weight (mg/g FW) for *L. minuta* and in mg of Chl *a* gram of fresh weight for *T. variabilis*.

2.7. Malondialdehyde content determination

Malondialdehyde (MDA) content, a well-established marker of lipid peroxidation in oxidative stress studies (Morales and Munné-Bosch, 2019), was measured to assess plant responses to Ni exposure. Increased MDA levels indicate oxidative damage to cell membranes, serving as a reliable indicator of stress conditions in *L. minuta* and *T. variabilis*. Frozen plant samples from *L. minuta* and *T. variabilis* biomass were homogenized in a pre-chilled mortar and pestle with two volumes of ice-cold 0.1 % (w/v) trichloroacetic acid (TCA) and centrifuged for 15 min at 16,000 × *g*. An assay mixture containing 1 mL aliquot of supernatant and 2 mL of 0.5 % (w/v) thiobarbituric acid (TBA) in 20 % (w/v) TCA was heated to 95 °C for 30 min and then rapidly cooled in an ice bath. After centrifugation (16000 × *g* for 10 min at 4 °C), the supernatant absorbance was read at 532 nm, and values corresponding to non-specific absorption at 600 nm were subtracted. MDA concentration was calculated using the extinction coefficient (ε = 155 mM/cm).

2.8. Measurement of proteins content and catalase activity

To determine proteins and catalase activity, frozen samples from *L. minuta* and *T. variabilis* (0.05–0.1 g fresh weight) were extracted and homogenized using Tissue Lyser (Qiagen). Extraction buffer was 50 mM potassium phosphate (pH 7.0) containing 0.1 % ascorbic acid, 0.1 % Triton X-100, 1 mM EDTA and 1 % polyvinylpyrrolidone (PVPP). The samples were centrifuged (13,000 rpm) for 20 min at 4 °C and separated from cellular debris. The clear supernatant fraction was used to determine protein content and enzyme assay of catalase (CAT) activity. Protein concentration was quantified following Bradford (1976) using bovine serum albumin (BSA) as the standard. CAT (EC 1.11.1.6) activity was measured by following the consumption of hydrogen peroxide (ε = 39.4 mM^{-1} cm^{-1}) at 240 nm according to the protocol of Aebi (1984). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0) 10 mM of H_2O_2 and enzyme extract in a total volume of 1 mL. CAT activity was expressed as μmol of H_2O_2 mg^{-1}protein min^{-1}.

2.9. Statistical analyses

Statistical analyses were conducted in R (version 4.2.1). All results are presented as means ± standard deviations (SD) of three replicates. All data were checked for normality before analyses of variance (ANOVA). Differences between means were analyzed by Tukey's test following two-way ANOVA to evaluate the responses of the two test organisms (*L. minuta*, *T. variabilis*) in presence and absence of Ni, at different exposure time, in monocultures and consortia. All statistical tests were given in the p value categories: NS = no significant, * = $p < 0.05$, * * = $p < 0.01$, * ** = $p < 0.001$.

3. Results and discussion

3.1. Water chemical and physical parameters

Water conductivity remained similar over time (at T_7, T_{14}) across Ni-treatments and controls. Conversely, pH levels in the controls were significantly higher than those measured in the Ni-treatments, where they remained similar to the initial values (Fig. 1a). This could be due to the potentially toxic effect of nickel on exposed organisms, the stress of which would result in a lower production of photosynthetically active biomass compared to control tests (see 3.2). With less biomass available for photosynthesis, CO_2 uptake from the water would be reduced. This decreased CO_2 consumption would lead to a higher concentration of

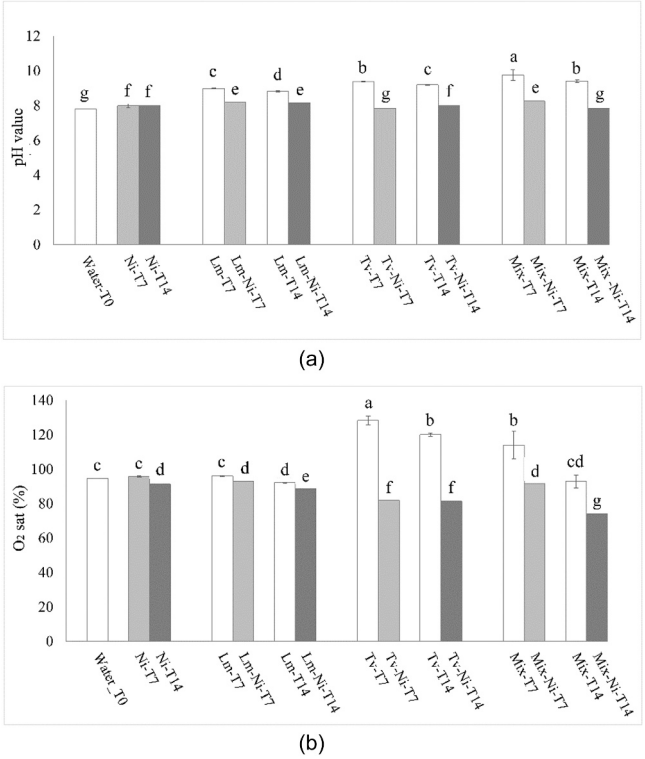

Fig. 1. Mean values of pH (a) and dissolved oxygen saturation (%) (b) measured in controls and Ni-treatments at different time-points (T_7, T_{14}). From left, Ni-treatments without organisms (Ni), *L. minuta*-tests in monoculture (Lm, Lm-Ni), *T. variabilis*-tests in monoculture (Tv, Tv-Ni), *Lemna-Trichormus* consortium (Mix, Mix-Ni). Different letters between values indicate a significant difference with $p < 0.05$ (n = 3).

carbonic acid (H_2CO_3), which dissociates into H^+ and bicarbonate (HCO_3^-), ultimately resulting in the observed decrease in pH (Lucas, 1983).

Dissolved oxygen saturation was significantly reduced in all Ni-treatments with organisms (Lm-Ni, Tv-Ni, Mix-Ni) compared to their relative controls (Lm, Tv, Mix) (Fig. 1b). This reduction likely reflects the altered balance between photosynthesis and respiration. Both *L. minuta* and *T. variabilis* showed signs of Ni toxicity with a reduced photosynthetic capacity, indicated by a lower chlorophyll concentration, and a subsequent decrease in oxygen released in the aqueous medium. This oxygen decrease was considerably more pronounced in *Trichormus*- rather than *Lemna*-tests. Compared to their respective controls, in *Lemna* (Lm-Ni), *Trichormus* (Tv-Ni) and consortium (Mix-Ni) treatments were recorded reductions in dissolved oxygen saturation of 3.0 % and 3.8 %, 36 % and 32 % and 19.5 % and 21 %, at T_7 and T_{14}, respectively. These results show that the dissolved oxygen reduction in Tv-Ni was more evident than in Lm-Ni-treatments, likely due to a greater senescence of cyanobacterial filaments caused by Ni-induced stress. Indeed, this greater reduction in Tv-Ni treatments is probably due to the combination effect of a reduction in photosynthetic biomass, photosynthesis in general, and an increase in the microorganism respiration during the decomposition of dead cyanobacterial cell material, which consumes additional oxygen. This could also explain the lower reduction in dissolved oxygen in *Lemna*-treatments, where the fronds floating on the water surface were in direct contact with atmospheric oxygen, unlike the *Trichormus* filaments, which, being at the bottom of the water column, were further from this oxygen source. These results also underline that the effect of the mixed species on the dissolved oxygen was conservative with the resultant saturation lying between the values of both the single species.

3.2. *Lemna minuta and Trichormus variabilis growth*

In Ni-free conditions, *T. variabilis* monocultures and consortia with *L. minuta* exhibited a significant ($p < 0.001$; Table S2) increase in biomass content compared to *L. minuta*. This increase was particularly pronounced in *T. variabilis*, with biomass rising by 63 % at T_7 and 68 % at T_{14}, whereas *L. minuta* showed increases of only 23 % and 20 %, respectively. Conversely, in Ni-treatments, the biomass of both species decreased when compared with their respective controls at each time-point, although this response was more evident in *T. variabilis* (Fig. 2). In particular, *L. minuta* showed biomass reductions of 22.5 % (6.15 vs 4.76 g) and 20 % (6.00 vs 4.80 g) (Fig. 2a), while *T. variabilis* biomass was reduced by 39 % (1.22 vs 0.75 g) and 45 % (1.26 vs 0.69 g) (Fig. 2b). The evident reduction in biomass was a strong indicator of Ni phytotoxicity, in accordance with previous studies that showed similar values for other *Lemna* species (Xylander et al., 1993; Khellaf and Zerdaoui, 2010) and cyanobacteria (Kumar et al., 2019; Kaamoush et al., 2022) depending on the exposed concentrations. For example, Khellaf and Zerdaoui (2010) showed a decrease in *L. gibba* growth of 50 % in water with 0.75 mg/L Ni and a complete inhibition at 1 mg/L Ni, while Kaamoush et al. (2022) found a 50 % reduction in growth of the cyanobacterium *Spirulina platensis* at 2 mg/L Ni.

In Mix-Ni treatments, the biomass production of both organisms, but especially *Trichormus*, was lower compared to the controls. In the absence of Ni, there was a significant increase in biomass during the experiment. In Mix-Ni-treatments, compared to those Ni-free, the biomass of *L. minuta* and *T. variabilis* was significantly reduced by 3 % and 16 % at T_7, and 23 and 50 % at T_{14}, respectively (Fig. 2). However, *Trichormus* exhibited greater Ni tolerance in consortia with biomass reductions of 16 %, compared to monocultures values of 39 % at T_7 (Fig. 2b). This could be associated with the ability of *L. minuta* to remove Ni from aqueous medium and effectively alleviating Ni toxicity to *T. variabilis*.

3.3. *Nickel effect on morphological parameters*

There were no significant differences ($p > 0.05$) in root length and width and area of *Lemna* fronds among controls and treatments (both mono- and mixed). However, there was a significant difference in the length of *Lemna* frond in consortium when exposed to Ni, with reductions of 8.6 % and 7.4 % at T_7 and T_{14}, respectively.

In *Trichormus*-tests, macro-observations of the cyanobacterial populations revealed that, in Ni-free tests, they showed an even distribution at the bottom of the beakers, whereas in Ni-treatments, they aggregated at the center of the bottom, forming compact three-dimensional disk-like structures (Fig. S2). This arrangement was also observed for the same strain when included in a microbial consortium used to reclaim household wastewater (Alabiso et al., 2023). In Ni-treatment, the medium was more turbid than in the control, possibly due to the lysing of *T. variabilis* cells, resulting in the release of exopolymeric substances into medium in response to the Ni stress (Congestri et al., 2020). When in consortium, *T. variabilis* did not form aggregates in response to Ni and was more evenly distributed on the bottom of the beaker.

3.4. *Nickel toxic effect on physiological parameters*

3.4.1. *Chlorophyll content*

Lemna minuta and *T. variabilis* exhibited significant reductions in total chlorophyll ($p < 0.001$) and chlorophyll *a* content ($p < 0.01$),

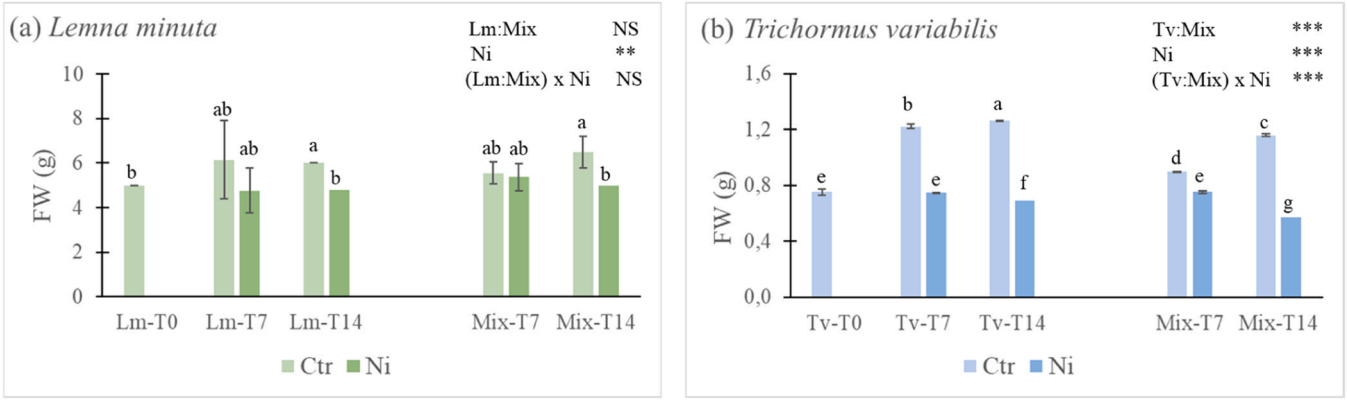

Fig. 2. Biomass (Fresh weight-FW, g) of *L. minuta* (a) and *T. variabilis* (b) in monoculture (Lm, Tv) and consortium (Mix), measured at T_0, T_7 and T_{14} in controls (Ctr, Ni-free tests) and treatments with nickel (Ni). Mean values ± standard deviations (SD) with different letters indicate a significant difference with $p < 0.05$. Asterisks indicate significant differences between single organism and treatments (*** = 0.001; ** = 0.01; * = 0.05; NS = no significant) as determined by two-way ANOVA analysis (organism:Mix, Lm or Tv with consortium Mix; [Ni] Lm or Tv in presence of Nickel; [(organism:Mix) x Ni] interaction between Lm or Tv with consortium Mix and Ni) (n = 3).

respectively (Table S2), in response to Ni-exposure, although the severity of the effect varied with exposure time (Fig. 3). In monocultures, at T_7, chlorophyll content was significantly reduced in *L. minuta* (25 %) (Fig. 3a), but less so in *T. variabilis* (5 %) (Fig. 3b). However, at T_{14}, a further decrease was observed both in *L. minuta* (53 %) and *T. variabilis* (35 %). This reduction in chlorophyll content aligns with previous studies demonstrating similar effects of heavy metals on *Lemna* species (Jayasri and Suthindhiran, 2017; Al-Nabhan, 2022) and cyanobacteria (Deniz et al., 2011).

In consortia, *L. minuta* appeared to tolerate Ni exposure better than *T. variabilis*, particularly at T_{14} (Fig. 3). At T_7, *L. minuta* exhibited a 28 % reduction, while *T. variabilis* showed a less severe reduction of 12 %. This difference became more pronounced at T_{14}, with reductions of 35 % and 50 % in *L. minuta* and *T. variabilis*, respectively. The lower reduction of chlorophyll content in *L. minuta* when combined with *T. variabilis* compared to its monoculture suggests that *L. minuta* benefited more from the consortium compared to *T. variabilis*. Previous studies on monocultures of other duckweeds (*Lemna minor*, *Spirodela polyrhiza*) (Appenroth et al., 2010; Van Dyck et al., 2023) and green algae (*Chlorella vulgaris*, *Desmodesmus* sp.) (Rugnini et al., 2017), which had been exposed to heavy metals, reported similar effects on chlorophyll content. Consistent with the chlorophyll content measurements, some chlorosis was observed in *L. minuta* fronds during the experiment. This chlorosis was more evident at T_{14} in monocultures exposed to Ni (Lm-Ni), while it appeared mitigated in Mix-Ni treatments (Mix-Ni) (Fig. S3).

3.4.2. MDA content

When exposed to Ni, the MDA content of *L. minuta* and *T. variabilis* significantly increased ($p < 0.001$; Table S2) in both monocultures and consortia compared to controls. This suggests that exposure to Ni induced a state of high oxidative stress in both organisms. Both species exhibited significant increases in MDA content at T_7 and T_{14} (Fig. 4). Compared to the respective controls, the MDA content in *L. minuta* samples increased by 43 % at T_7 and 89 % at T_{14}, while a greater increase of MDA was recorded in *T. variabilis* samples with 91 % at T_7 and 102 % at T_{14}. The observed increase in MDA, a well-established biomarker of oxidative stress (Morales and Munné-Bosch, 2019), concurs with results emerging from previous research on plants exposed to various heavy metals, including cadmium, silver and Ni (Rizwan et al., 2017; Xue et al., 2018; Iannelli et al., 2022).

Even in consortia exposed to Ni, *L. minuta* and *T. variabilis* showed increased MDA content (Fig. 4). However, *L. minuta* exhibited significantly higher MDA values, with increases of 55 % and 45 % at T_7 and T_{14}, respectively, compared to control. In contrast, *T. variabilis* displayed significantly higher MDA values, reaching 126 % at T_7 and 113 % at T_{14}, compared to control. Interestingly, MDA content of *Lemna* in the combined cultures (Mix-Ni) at T_{14} was significantly lower (approximately 45 %) when compared to the monocultures (Lm-Ni) at the same time. The reduced oxidative stress in *L. minuta* in the consortium following Ni exposure suggests a beneficial interaction with *T. variabilis*.

3.4.3. Soluble protein content

Ni exposure significantly decreased soluble protein content in *L. minuta* and *T. variabilis* monocultures compared to their respective controls ($p < 0.001$; Table S2) at both T_7 and T_{14} (Fig. 5). Specifically, protein content declined by 32 % and 53 % for *L. minuta* and by 51 % and 65 % for *T. variabilis* at T_7 and T_{14}, respectively. Previous studies document similar reductions in some terrestrial cultivated plants, such as *Brassica juncea* and *Helianthus annus*, exposed to high Ni concentrations (100 and 60 mg/L, respectively) (Sharma et al., 2008; Ashraf et al., 2011). Decrease of soluble protein content in *L. minor* was found as a stress response to other heavy metals, like cadmium (Cd) and copper (Cu) (Hou et al., 2007). In contrast to monoculture tests, *L. minuta*-*T. variabilis* consortium offered a clear advantage in terms of protein content. Protein content in *L. minuta* within consortium was significantly higher than within Mix-Ni-free, increasing by 18 % at T_7 and 20 % at T_{14} (Fig. 5a). Protein content in *T. variabilis* combined with *L. minuta* showed a slightly lower reduction compared to monocultures at both T_7 and T_{14}, with no significant difference from Mix-Ni-free (Fig. 5b). This suggests that the co-occurrence of these two species mitigates the negative effects of Ni contamination on protein production in both organisms.

3.4.4. Catalase antioxidant enzyme activity

Exposure to Ni significantly enhanced catalase activity (CAT) in both species in monocultures and consortia compared to controls ($p < 0.001$; Table S2) (Fig. 6). In *L. minuta* monoculture, CAT activity increased 1.7- and 2.8-fold at T_7 and T_{14}, respectively (Fig. 6a). In contrast, *T. variabilis* monoculture exhibited a larger CAT response, increasing 3.4-fold at T_7 and 7.0-fold at T_{14} when exposed to Ni (Fig. 6b). During Ni exposure both *L. minuta* and *T. variabilis* in the consortia exhibited increased CAT activity compared to their respective controls. *Lemna minuta* CAT activity increased 1.2- and 2.0-fold at T_7 and T_{14}, respectively. Higher CAT activities in *T. variabilis* were measured showing a 2.7- and 6.6-fold increase at T_7 and T_{14}, respectively. However, it is noteworthy that in the consortia, and more evidently in *L. minuta*, CAT activity was significantly lower compared to the respective monocultures (Fig. 6a).

The observed increase in CAT activity likely represents an enzymatic response to manage the production of reactive oxygen species (ROS) generated by the organisms under Ni exposure, especially H_2O_2. It is known that exposure to Ni induces oxidative stress, leading to ROS production. Plants and algae, in response to ROS, increase the

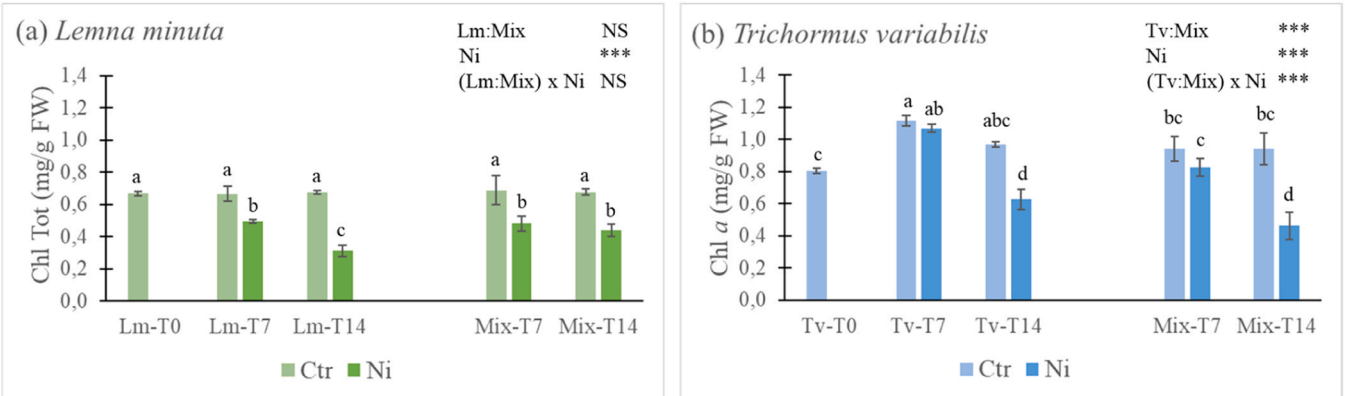

Fig. 3. Content of total chlorophyll (Chl Tot) in *L. minuta* (a) and chlorophyll *a* in *T. variabilis* (b) in monoculture (Lm, Tv) and consortium (Mix), measured at T_0, T_7 and T_{14} in controls (Ctr, Ni-free tests) and treatments with nickel (Ni). Mean values ± standard deviations (SD) with different letters indicate a significant difference with $p < 0.05$ ($n = 3$). For explanation of asterisks see caption of Fig. 1.

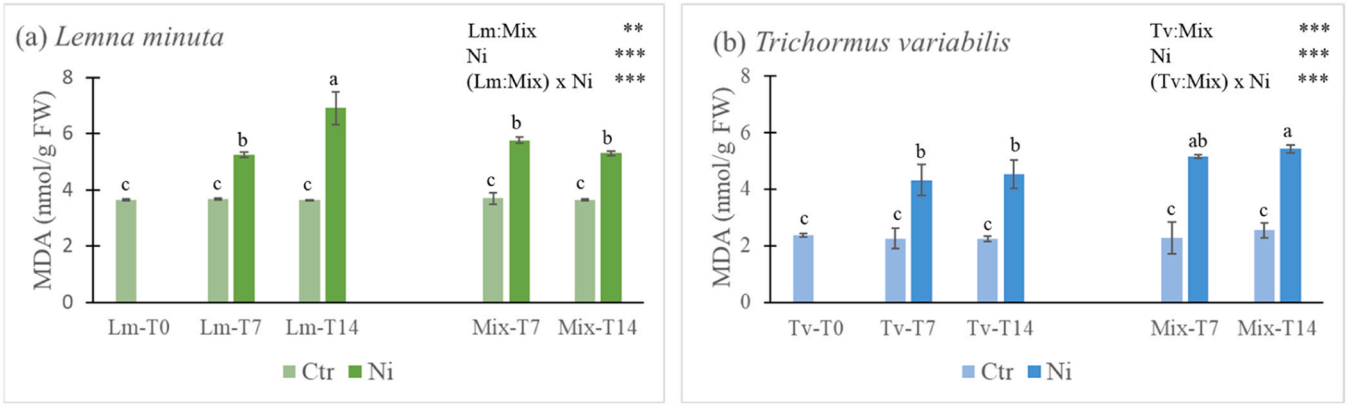

Fig. 4. Content of malondialdehyde (MDA) in *L. minuta* (a) and *T. variabilis* (b) in monoculture (Lm, Tv) and consortium (Mix), measured at T_0, T_7 and T_{14} in controls (Ctr, Ni-free tests) and treatments with nickel (Ni). Mean values ± standard deviations (SD) with different letters indicate a significant difference with $p < 0.05$ ($n = 3$). For explanation of asterisks see caption of Fig. 1.

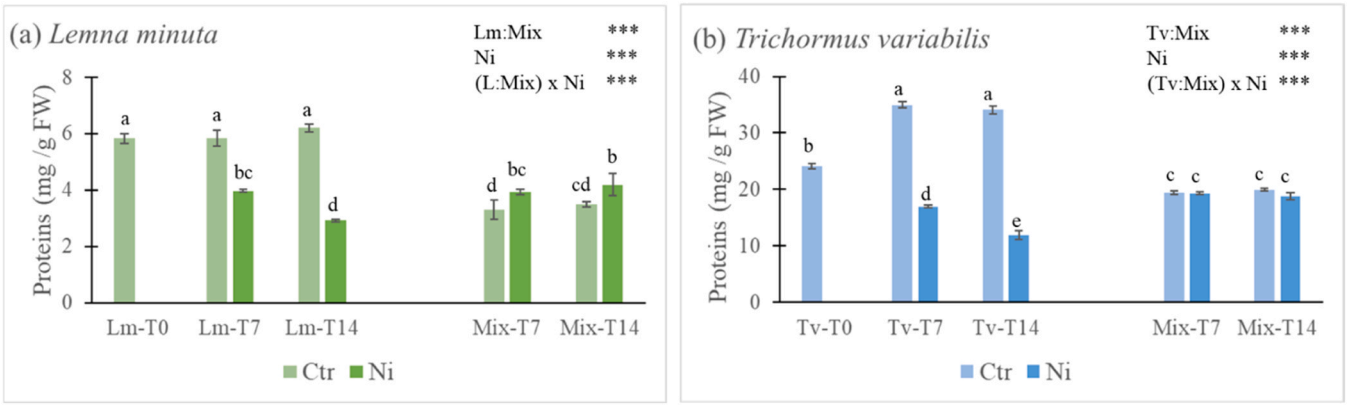

Fig. 5. Proteins content in *L. minuta* (a) and *T. variabilis* (b) in monoculture (Lm, Tv) and consortium (Mix), measured at T_0, T_7 and T_{14} in controls (Ctr, Ni-free tests) and treatments with nickel (Ni). Mean values ± standard deviations (SD) with different letters indicate a significant difference with $p < 0.05$ ($n = 3$). For explanation of asterisks see caption of Fig. 1.

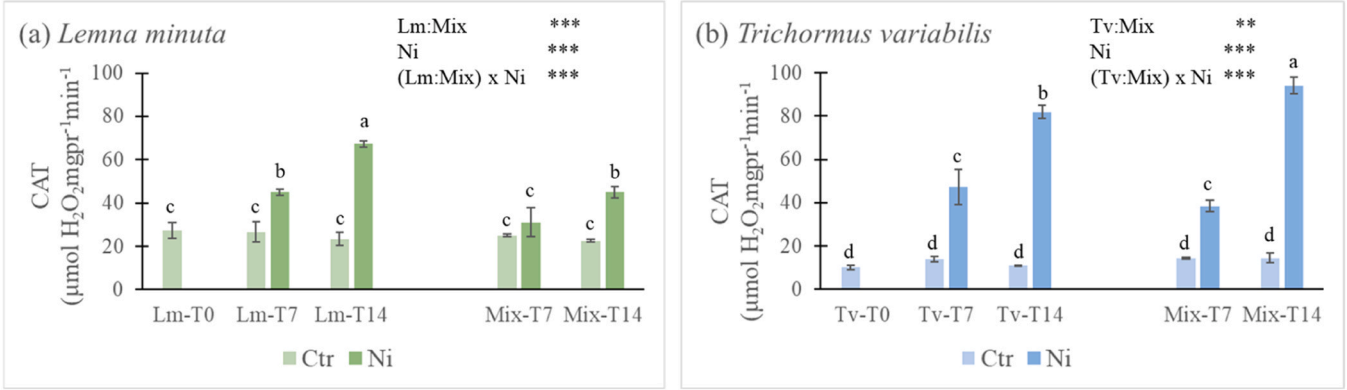

Fig. 6. Activity of catalase (CAT) in *L. minuta* (a) and *T. variabilis* (b) in monoculture (Lm, Tv) and consortium (Mix), measured at T_0, T_7 and T_{14} in controls (Ctr, Ni-free tests) and treatments with nickel (Ni). Mean values ± standard deviations (SD) with different letters indicate a significant difference with $p < 0.05$ ($n = 3$). For explanation of asterisks see caption of Fig. 1.

production of antioxidant enzymes. The amount of these enzymes is often proportional to the level of oxidative stress and therefore to the degree of contamination. Similar increases in CAT activity due to Ni exposure were also documented in other plants, including maize (*Zea mays*) (Ghasemi et al., 2013) and sweet potato (*Ipomoea batatas*) (Kumar et al., 2022). Yilmaz and Parlak (2011) found that *L. gibba* exhibited catalase activity increase in the presence of Ni. In some cyanobacteria, such as *Microcystis aeruginosa* (Martínez-Ruiz and Martínez-Jerónimo, 2016), and in two *Anabaena* species (Prajapati et al., 2018), catalase activity increases in response to nickel concentration was also recorded.

3.5. Biological removal of nickel

Nickel concentration decreased significantly ($p < 0.001$) in all

Table 1
Nickel concentration mean values (mg/L) and removal percentage of Ni (%) by monocultures of *L. minuta* (Lm-Ni), *T. variabilis* (Tv-Ni) and consortia (Mix-Ni) at T_7 and T_{14} (p values for difference in concentration with *** = 0.001).

	Test	Ni (T_0) (mg/L)	Ni ± SD (mg/L)	p value	Ni solution (%)	Ni removed (%)
T_7	Ni	6.47	6.530 ± 0.187	-	99.1	-
	Lm-Ni	6.47	1.594 ± 0.189	* **	24.6	75.4
	Tv-Ni	6.47	4.102 ± 0.221	* **	63.4	36.6
	Mix-Ni	6.47	1.311 ± 0.486	* **	20.3	79.7
T_{14}	Ni	6.47	6.400 ± 0.181	-	98.9	-
	Lm-Ni	6.47	0.886 ± 0.347	* **	13.7	86.3
	Tv-Ni	6.47	4.695 ± 0.211	* **	72.6	27.4
	Mix-Ni	6.47	0.703 ± 0.013	* **	10.9	89.1

treatments containing *L. minuta*, *T. variabilis* or their consortium compared to the initial concentration (T_0) (Table 1). Conversely, Ni concentration remained almost constant in all Ni-treatments without organisms throughout the entire experimentation (Fig. 7). Ni bioremoval activity by the organisms is the likely cause of the significant decrease in Ni concentrations observed in all Ni-treatments. Beaker pretreatment with a saturated Ni solution was crucial to eliminate abiotic Ni removal via adsorption to the beaker walls. This ensured that any observed decrease in Ni concentration could be attributed solely to the organism activity.

The results showed that *L. minuta* and *T. variabilis* were able to remove Ni from the solution. Based on the amounts of biomass used for the two species, *L. minuta* and *T. variabilis* in monoculture (Lm-Ni, Tv-Ni) removed a mean of 74.4 % and 36.6 % of the initial Ni concentration by T_7, respectively, and 86.3 % and 27.4 % at T_{14} (Table 1). The removal capacity shown by *L. minuta* is consistent with results emerging from some studies on other *Lemna* species. Axtell et al. (2003) demonstrated a high Ni removal rate (82 %) from Pb- and Ni-contaminated water by *L. minor* in 72 h. Similarly, Khan et al. (2020) found *L. minor* exhibited a greater removal capacity for Ni compared to lead and cadmium in multi-metal contaminated waters.

For comparing *L. minuta* and *T. variabilis* in terms of nickel removal efficiency, it would have been necessary to evaluate this efficiency as a function of the exposed biomass unit, considering that generally the removal efficiency increases with the amount of depurating biomass (Obek and Sasmaz, 2011; Bokhari et al., 2019). However, this study did not aim to directly compare the two species, but rather to assess their ability to bioremove Ni either as single or combined species, in order to establish a functional system (i.e. consortium) applicable in a biotechnological context for nickel remediation. To achieve this, the initial different biomass amounts of the two species were not standardized based on weight but rather on absorbing surface area, ensuring that both species formed a monolayer of comparable coverage. This approach was

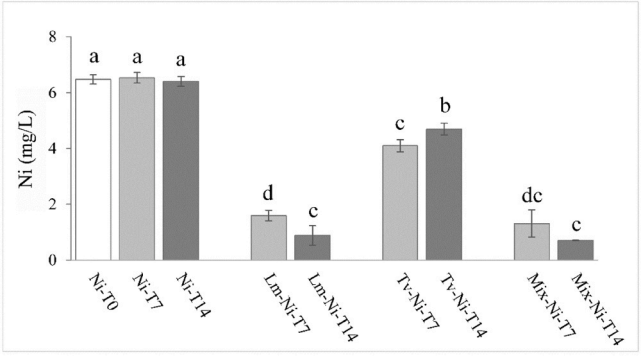

Fig. 7. Nickel (Ni) concentration measured in Ni-treatments without and with organisms at different time-points (T_0, T_7, T_{14}). From left, control tests with only mineral water and nickel (Ni), tests with monoculture of *L. minuta* (Lm-Ni) and *T. variabilis* (Tv-Ni) and consortium (Mix-Ni). Mean values with different letters indicate a significant difference with $p < 0.05$ ($n = 3$).

essential to create experimental conditions that would allow an effective evaluation of their combined performance in a potential large-scale bioremediation system.

Despite the biomass difference, *L. minuta* generally exhibited greater Ni tolerance than *T. variabilis* throughout the experiment. Its continued Ni removal beyond 7 days, coupled with its growth and positive physiological responses, supports this conclusion. Conversely, *T. variabilis* showed clear signs of stress from T_7 onward, forming compact, circular colonies on the beaker bottom. Furthermore, *T. variabilis* appeared to cease Ni removal as the experiment progressed; Ni concentrations even began to rise again. This suggests that *T. variabilis* may have released previously absorbed Ni back into the solution. Indeed, cellular degradation in response to stress is a known phenomenon in cyanobacteria and it would likely include the release of cellular contents (Congestri et al., 2020), including bioremoved heavy metal.

Mix-Ni-treatments achieved 79.7 % and 89.1 % Ni removal at T_7 and T_{14}, respectively, that is an improvement of 4.3 % and 2.8 % Ni removal compared to *L. minuta* monocultures at the same time-points. However, these differences were not statistically significant ($p > 0.05$). Therefore, regarding Ni removal, *Lemna-Trichormus* consortium offered no significant advantage over *Lemna*-monocultures.

4. Conclusions

This study is the first investigation on the phytoremediation potential of *L. minuta* and *T. variabilis* incubated as both single and combined species in water highly contaminated with nickel.

The results showed that the presence of *L. minuta* was essential for achieving higher Ni removal efficiency, exhibiting *L. minuta* monocultures and consortia 86.3 % and 89.1 % Ni removal, respectively. Ni removal by *L. minuta* continued beyond 7 days, unlike *T. variabilis*, which began to release Ni after this period. The Ni removal efficiency of the *L. minuta-T. variabilis* consortium was not significantly different from that of *L. minuta* monocultures, and this suggests that the presence of *T. variabilis* was not a strong contributing factor. Despite the lack of significant improvement in Ni removal, the benefit of using the consortium lies in the enhanced viability and tolerance of the combined species compared to the monocultures. Physiological data recorded in Mix-Ni-treatments support this. Higher total chlorophyl and protein content, lower malondialdehyde and catalase levels, and reduced chlorosis in *L. minuta*, as well as an inconspicuous cell aggregation in *T. variabilis*, suggest that the consortium is able to create more favorable growth conditions for both species. In addition, this consortium approach may offer a more sustainable strategy for long-term remediation projects by promoting the health and efficacy of the remediating organisms. However, it should be noted that, while *Lemna*-based phytoremediation offers several advantages, its application is linked to the habit of these plants to grow floating on the water surface. Thus, their effectiveness is largely confined to the upper water column, limiting their use to shallow aquatic environments, whether in constructed phytoremediation systems or natural water bodies.

Future research could focus on the optimization of the experimented consortia by combining *L. minuta* with other cyanobacteria exhibiting

greater tolerance to the target contaminant. Furthermore, it would be useful to investigate on the key mechanisms driving the interspecific synergies that could occur within these new consortia.

CRediT authorship contribution statement

Carioti Valeria: Writing–review & editing, Writing–original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Savio Saverio**: Writing–review & editing, Resources, Investigation, Data curation, Conceptualization. **Fabriani Marco**: Investigation, Data curation. **Ellwood Neil T.W.**: Writing–review & editing, Investigation, Formal analysis, Data curation. **Gemin Luca**: Writing–review & editing, Investigation, Data curation. **Congestri Roberta**: Writing–review & editing, Validation, Supervision, Resources. **Iannelli Maria Adelaide**: Writing–review & editing, Visualization, Validation, Supervision, Resources, Investigation, Formal analysis, Data curation. **Ceschin Simona**: Writing–review & editing, Writing–original draft, Visualization, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization.

Funding sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of Competing Interest

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

Acknowledgements

The Grant of Excellence Departments 2023–2027, Miur (Art. 1, Commi 314–337 Legge 232/2016), is gratefully acknowledged. The authors thank E. Pelella, E. Manganello, B. Luzzi for their support during the experiment.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aquabot.2025.103888.

Data availability

Data will be made available on request.

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