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Received: 26 September 2025

Accepted: 10 December 2025

Published online: 05 January 2026

Cite this article as: Mungunkhuyag K., Steingroewer J., Walther T. *et al.* Isolation and characterization of heavy metal tolerant microalgae from old mining areas of Saxony. *Sci Rep* (2025). <https://doi.org/10.1038/s41598-025-32393-0>

Khongorzul Mungunkhuyag, Juliane Steingroewer, Thomas Walther & Felix Krujatz

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Isolation and characterization of heavy metal tolerant microalgae from old mining areas of Saxony

Khongorzul Mungunkhuyag^{1,2}, Juliane Steingroewer¹, Thomas Walther¹, and Felix Krujatz^{3,*}

¹Chair of Bioprocess Engineering, Institute of Natural Materials Technology, Technische Universität Dresden, 01069, Germany

²Department of Biology, School of Arts and Sciences, National University of Mongolia, Ulan-Bator, 14200, Mongolia

³biotopa gGmbH, Radeberg, 01454, Germany

⁴Professorship Automatic Control and System Dynamics, Technische Universität Chemnitz, Reichenhainer Straße 70, 09126 Chemnitz, Germany

*corresponding author: felix.krujatz@etit.tu-chemnitz.de

ABSTRACT

Heavy metal contamination poses a major threat to ecosystems and human health, particularly in mining-impacted areas. Algal biosorption offers a promising, low-cost, and sustainable approach to mitigate this problem. In this study, five microalgal strains were isolated from abandoned mining sites and evaluated for their tolerance and ability to remove Cu, Cd, and Cr from aqueous solutions. Laboratory experiments were performed to assess heavy metal tolerance and biosorption efficiency under controlled conditions. The results demonstrated that isolates *Chlorella vulgaris* RG1-4 and *Tetradesmus obliquus* exhibited the highest tolerance and sorption capacity for Cu, with removal efficiencies of 100 mg/g and 89.73 mg/g, respectively. Isolate *Lobochlamys seignis* Ehr31-1 showed the highest tolerance and biosorption capacity for Cd, reaching 93.17 mg/g. These findings highlight the potential of locally adapted microalgal strains as effective biosorbents for remediating heavy metal-contaminated water sources.

Keywords

Microalgal isolates, phylogenetic analysis, heavy metal tolerance, heavy metal removal

Introduction

Heavy metals (HMs) and metalloids are elements with a density exceeding 5 g/cm³¹ that can be toxic to humans and other organisms, even at low concentrations in parts per billion (ppb). They can enter the environment through natural processes, such as soil erosion, forest fires, and volcanic eruptions, as well as through anthropogenic activities like mining, electroplating, and the use of pesticides, fertilizers, and certain consumer products². If contaminated areas are not remediated, HMs can

pollute surrounding water and soil, leading to human exposure via ingestion, inhalation, or dermal absorption³. Once inside the body, HMs can bind to biomolecules, disrupting biochemical pathways and impairing cellular functions. This can cause ion imbalances and generate reactive oxygen species (ROS), potentially harming various organs and increasing cancer risk^{2,4}. Among HMs, nickel (Ni), cadmium (Cd), arsenic (As), hexavalent chromium (Cr(VI)), uranium (Ur), and the fission product strontium (Sr) are classified as Group 1 by International Agency for Research on Cancer (IARC), meaning they are carcinogenic to humans⁵. Some of the toxic effects of HMs are detailed as follows:

When Cd enters cells, it primarily binds to sulfhydryl groups (-SH) in proteins, particularly amino acid cysteine, which can inhibit enzyme function and damage cellular structures like nucleus, endoplasmic reticulum, and mitochondria. This interference also affects DNA methylation, leading to epigenetic changes and potential health issues in humans, such as liver and kidney damage, bone mineralization loss (e.g., Itai-Itai disease), and cancer⁶. Copper is an essential element for biological functions, acting as a cofactor in various enzymes that are involved in energy production, iron metabolism, neurotransmitter synthesis, etc. Thus, adequate copper intake is crucial for human health⁷. However, excessive copper can cause liver and gastrointestinal damage⁸. In contrast, copper is more toxic to microorganisms and aquatic organisms^{9,10}. Its use in agriculture and as a food preservative should be careful. Otherwise, it can negatively impact the ecosystem by stunting the growth of aquatic organisms and altering the composition of microorganisms. Chromium exists in several oxidation states, with Cr(III) and Cr(VI) being the most stable. Of these, Cr(VI) is the most toxic. When Cr(VI) enters cells, it generates reactive oxygen species (ROS), which can damage macromolecules such as lipids, proteins, and nucleic acids. This can ultimately lead to allergic reactions, damage to sperm and the male reproductive system, and an increased risk of cancer¹¹.

Trace concentrations of HMs, measured in parts per billion (ppb), can significantly impact human health and the environment. Regulatory frameworks, such as those established by the World Health Organization (WHO), set stringent limits for HMs in drinking water: cadmium at 0.003 mg/L, arsenic at 0.01 mg/L, lead at 0.01 mg/L, chromium at 0.05 mg/L, and copper at 2 mg/L¹². Higher limits may apply to industrial effluents and irrigation water^{13,14}. HM concentrations near mining operations depend on soil exposure. For example, the Meca River in Spain, affected by acid mine drainage, showed arsenic at 0.13 mg/L, cadmium at 0.8 mg/L, copper at 9.2 mg/L, lead at 0.36 mg/L, and zinc at 4.09 mg/L¹⁵. In contrast, the Ib Valley coalfield in India reported lower levels of these metals¹⁶. Industrial processes also contribute to HM pollution. Wastewater from an electroplating factory contained copper concentrations up to 15560 mg/L, along with significant levels of nickel (245 mg/L) and zinc (505 mg/L). Additionally, wastewater from an industrial area in Delhi revealed lead at 326 mg/L and cadmium at 0.9 mg/L¹⁷.

HMs can be removed from water using various methods categorized as chemical, physical, and biological. Chemical and physical unit operations include adsorption, precipitation, membrane filtration, ion exchange, solvent extraction, electro-dialysis, neutralization, coagulation, and flocculation¹⁸. These methods often entail high costs for equipment and reagents, may not completely eliminate metals, and can cause secondary pollution requiring further treatment¹⁹. Their effectiveness

also decreases at low metal concentrations (10–100 mg/L). In contrast, biological treatment, or bioremediation, employs organisms such as plants, bacteria, yeast, fungi, and algae, showing effectiveness even at low concentrations²⁰. Bioremediation is cost-effective and environmentally friendly, although it can be time-consuming and may lack specificity in targeting various metal ions¹⁹.

Among them algae are a significant group of photosynthetic organisms. They can grow without organic carbon sources and are effective in fixing carbon, making them valuable for reducing greenhouse gas emissions. Microalgae, particularly, are versatile and can be cultivated in various systems²¹, demonstrating high tolerance to HMs. Algae exhibit higher HM biosorption capacities than conventional adsorbents, making them promising for environmental remediation. *Spirulina platensis* absorbs Cd at over 120 mg/g²², while *Chlorella vulgaris* absorbs 17.46 mg/g²³, compared to lignite's 40.46 mg/g²⁴. For Cu, *Callithamnion corymbosum* has an absorption capacity of 81.25 mg/g, and a biosorbent from agricultural waste shows 63.37 mg/g²⁵. *Chlorella vulgaris* ZBS1 absorbs 74.63 mg/g of Cr(VI)²⁶, while woody-activated carbon can absorb 241.6 mg/g²⁷.

Microalgae have developed different mechanisms to tolerate high concentrations of HM. The mechanisms are: adsorption outside the cell such as extracellular polymeric substance, cell wall, cell membrane, intracellular accumulation of metals to certain metalloproteins and other chelators, compartmentalization, changing metals into less toxic forms, and efflux transport²⁸.

While much research has focused on cultured algae, less attention has been given to algae from HM-contaminated environments. Saxony, with its 800-year mining history²⁹, may harbor microalgae that are well adapted to such conditions and exhibit high absorption capacities. This study aimed to isolate HM-tolerant microalgal species from mining areas, characterize them through molecular analysis, evaluate their tolerance and growth in response to copper (Cu), cadmium (Cd), and chromium (Cr(VI)), and assess their capability for HM removal. The reason why those HMs were studied is that at first, even small concentrations of those metals can be toxic to the environment and human health, second, the Cu and Cd concentration was higher in Roter Graben ditch³⁰.

Results and Discussion

Isolation and pre-screening of HM tolerant isolates

A total of 51 microalgae cultures were isolated from the collected samples, consisting of 44 green microalgae and 7 cyanobacterial strains. As shown in Table 1, most of the isolates exhibited tolerance to As(V) at 100 mg/L and Pb at 20 mg/L. However, Cd, Cu, and Cr(VI) were highly toxic, particularly Cr(VI), with few microalgae showing tolerance to these metals.

Nr.	Isolate	As(V)	Pb	As(III)	Cu	Cd	Cr(VI)
1	RG1-4	100	83	100	4	17	0.0
2	RG1-5						
3	RG5-5						
4	Ehr11-1						
5	Ehr13-1						
6	Ehr13-2						
7	Ehr15-3						
8	Ehr15-6						
9	Ehr21-15						
10	Ehr23-2						
11	Ehr23-5						
12	Ehr23-9						
13	Ehr23-11						
14	Ehr25-3						
15	Ehr25-7						
16	Ehr25-8						
17	Ehr25-9						
18	Ehr31-1	83	100	55	3	95	3
19	Ehr31-2						
20	Ehr33-1						
21	Ehr33-6	100	100	0.0	93	18	0.0
22	Ehr33-7						
23	Ehr33-9	87	82	31	75	4	0.0
24	Ehr35-1						
25	Ehr35-3						
26	Ehr35-5						
27	Ehr35-6						
28	Ehr35-7						
29	Ehr35-9						
30	Ehr35-14						
31	Zin11-2						
32	Zin15-3						
33	Zin21-1						
34	Zin21-2						
35	Zin21-3						
36	Zin21-6						
37	Zin23-1						
38	Zin23-2						
39	Zin23-5						
40	Zin23-6						
41	Zin25-2						
42	Zin25-6						
43	Zin25-7						
44	Zin25-8						
45	Ehr15-5	(++)	(+++)	(+/-)	(++)	(+)	(+++)
46	Ehr23-8	(+/-)	(+)	(-)	(-)	(-)	(+)
47	Ehr33-3	(+/-)	(++)	(-)	(++)	(+)	(++)
48	Ehr33-8	(+/-)	(++)	(-)	(-)	(-)	(++)
49	Zin13-3	(++)	(+)	(-)	(+)	(+/-)	(++)
50	Zin13-5	(++)	(+++)	(+)	(+)	(+)	(++)
51	Zin15-5	(+)	(++)	(+/-)	(++)	(+)	(++)
52	<i>C. vulgaris</i>	75.1	69.4	0.8	2.5	0.0	2.8
53	<i>L. maxima</i>	100	72	61	0	0	65

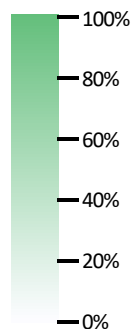


Table 1. Algal tolerance to heavy metals. For green algae, the growth in the media containing heavy metals were compared to growth of the isolates in the normal medium (control) presented as percentage and the colour scale is given beside the table. For cyanobacteria, the growth was compared to its control and the coloration was measured by naked eye. The meaning of the marks: (+++) – growth is same as control, (++) - growth is slightly lower than control, (+) – growth is low, (+/-) – no growth, (-) – discoloration. *C. vulgaris* (*Chlorella vulgaris* SAG211-11b) and *L. maxima* (*Limnospira maxima* CCALA27) are reference strains for comparison.

The strain Ehr31-1 was most resistant to Cd, while Ehr33-6 and Ehr33-9 showed the highest resistance to Cu. The isolate RG1-4 was notably resistant to both As(III), As(V), and Pb, with moderate tolerance to Cd, making it the most resilient green microalgae from Roter Graben.

Additionally, cyanobacterial strain Ehr15-5 was resistant to Cr(VI), maintaining growth at 15 mg/L. These five isolates were selected for further analysis and their microscopic images presented in Fig. 1 and their morphological descriptions presented in Table 2.



Fig. 1. Phase contrast microscopic images (above) and agar medium culture pictures (below) of five selected isolates.

Morphological and molecular identification of selected isolates

Molecularly identified species and their morphological descriptions are given in Table 2.

Isolate	Genes used for identification and their sequence GenBank accession no.	Identified species	Morphology
RG1-4 ¹	Combination of <i>rbcL</i> (PV805455), <i>tufA</i> gene (PV805456)	<i>Chlorella vulgaris</i> Beijerinck 1890	Cells are spherical, 2.9 to 6.4 µm in diameter, with cup-shaped chloroplasts that have one pyrenoid.
Ehr31-1 ²	rRNA genes (PV823513)	<i>Lobochlamys segnis</i> (H. Ettl) Pröschold, B. Marin, U. W. Schlösser & Melkonian 2001	The cells are ellipsoid, measuring 10-12 µm by 6-8 µm. Each contains a large, rough-surfaced, cup-shaped chloroplast with a single pyrenoid near the base. The nucleus is slightly anterior. Asexual reproduction produces four zoospores, each of which can

			divide into two, resulting in up to eight zoospores.
Ehr33-6 ²	ITS (PV823514)	<i>Chlamydomonas ulvaensis</i> R. A. Lewin 1957	This organism resembles <i>Chlamydomonas ulvaensis</i> morphologically, with a broad, ovoid cell size of 5.4 to 8.5 µm by 3.6 to 7.3 µm. The chloroplast does not extend to the flagella base, the nucleus is central, and the pyrenoid is slightly lateral. It lacks papillae and reproduces asexually, forming two to four zoospores.
Ehr33-9 ²	Combination of <i>rbcL</i> (PV805459), <i>tufA</i> gene (PV805458)	<i>Tetrademus obliquus</i> (Turpin) M. J. Wynne 2016	Cells are spindle-shaped and can be single, in pairs, or in groups of four, sometimes forming aggregates. Single cells measure 4.4 to 9.4 µm in length and 2 to 5.9 µm in width, while groups of four measure 8.1 to 14.5 µm by 5.4 to 10.0 µm. The chloroplast typically covers the entire cell and contains one pyrenoid, but in some single cells, it is on one side.
Ehr15-5 ³	16S rRNA (PV822044)	<i>Pseudanabaena catenata</i> Lauterborn 1915	Filamentous blue-green algae lack a sheath or mucilaginous layer. Active cultures show individual cells averaging 3.0 ± 0.7 µm in length and 1.5 ± 0.1 µm in width. The cylindrical cells typically have rounded ends and are longer than wide, but immediately after division, they are of similar length and width. Hyaline bridges connect the cells, and division occurs perpendicular to the filament's long axis.

¹ isolated from Roter Graben

² isolated from Ehrenfriedersdorf – sampling point 3

³ isolated from Ehrenfriedersdorf – sampling point 1

Table 2. Identification and morphology of selected isolated species.

Isolates RG1-4 and Ehr33-9 successfully amplified the 18S rRNA gene, ITS gene, plastidial *rbcL* and *tufA* gene using the selected primer pairs. However, the highly conserved 18S rRNA gene does not

resolve species-level differentiation, as seen with isolate Ehr33-9, which shares similarity with other *Scenedesmus* species. In contrast, the combination of the plastidial *rbcL* gene and *tufA* gene provides improved resolution for species differentiation and these two genetic barcodes were recommended³¹.

Isolate RG1-4

After sequencing, 1004 nt length of *rbcL* gene (GenBank accession no. PV805455) and 598 nt length of *tufA* gene (PV805456) were obtained. These sequences were compared to the other sequences in the GenBank in the NCBI database using BLAST. The percentage of identity were 99.80% and 100.00% (*Chlorella vulgaris* UTEX259 (MK948102.1)) for *rbcL* and *tufA* genes respectively. Then, the sequences from same strain from each result were combined and aligned. The Maximum Likelihood (ML) tree was then inferred from the alignment with nucleotide substitution model GTR+I+G. The isolate RG1-4 is more similar to *C. vulgaris* than other species and it belongs to the same clade where other *C. vulgaris* strains belong. Due to cell morphology, percentage of identity and the ML tree inferred below this isolate is further identified as *C. vulgaris* Beijerinck 1890 (Supplementary Figure S1).

Isolate Ehr31-1

For the isolate Ehr31-1, *rbcL* gene (PV805457) and SSU rRNA genes (PV823513) were sequenced but few gene sequences with high similarity to *rbcL* gene were found in GenBank and the query cover was low. Therefore, small subunit ribosomal ribonucleic acid (SSU rRNA) genes (18S rRNA, ITS, partial 23S rRNA) were used for the phylogenetic analysis of this isolate (Supplementary Figure S2). The length of 2488 nt sequence SSU genes were compared by BLAST. The SSU rRNA genes were most similar (98.96%) to *Lobochlamys segnis* strain NH-29 (MT735192.1). In the ML tree, isolate Ehr31-1 is in the clade which other branches are species *Lobochlamys segnis*, (H. Ettl) Pröschold, B. Marin, U. W. Schlösser & Melkonian 2001 and is closely related to genus *Chlamydomonas*.

Isolate Ehr33-6

From this isolate, only the ITS sequence (PV823514) could be successfully amplified. According to NCBI BLAST result, the ITS sequence was most similar to *Chlamydomonas inflexa* CCAP 11/42 (FR865584.1) (99.16%) and then to *Chlamydomonas ulvaensis* SAG2.72 (AJ749612.1) (99.07%). However, these two sequences were 100% identical to each other. In the Genbank, there were only few sequences of these two species were available. From the phylogenetic tree inferred (Supplementary Figure S3), isolate Ehr33-6 belong to also the same clade of *Chlamydomonas pulvinata* and *Chlamydomonas* sp. CCAP 11/166, CCAP 11/154, and CCAP 11/158. They were 98.32-98.48% similar to isolate Ehr33-6.

The genus *Chlamydomonas* is divided into nine species groups based on traits such as chloroplast shape, pyrenoid count, and their positioning. *Chlamydomonas ulvaensis* belongs to the *Chlamydomonas* group, characterized by a cup-shaped chloroplast and one lateral pyrenoid. In contrast, *Chlamydomonas inflexa* is categorized in the *Chlorogoniella* group, which has a chloroplast located on one side of the cell, also with one lateral pyrenoid³². The isolate Ehr33-6 exhibits a large, cup-

shaped chloroplast and one lateral pyrenoid, indicating that it belongs to the *Chlamydomonas* group. Following the morphological characters in the identification key, it aligns with *Chlamydomonas ulvaensis* R. A. Lewin 1957.

Isolate Ehr33-9

After sequencing, 1009 nt length of *rbcL* gene (PV805459) and 599 nt length of *tufA* gene (PV805458) were obtained. The best substitution model that is calculated by JModeltest software was TIM2+I+G (Akaike information criterion (AIC) was 19985.34). However, there are only several nucleotide substitution models available in the RAxML software. One of them is GTR+I+G, which was the third best choice (AIC was 19987.41). Therefore, GTR+I+G substitution model was used to infer ML tree. This merged sequence was 99.5-100% similar to *Tetradismus obliquus* strains. Moreover, isolate Ehr33-9 belongs to *Tetradismus obliquus* clade in the ML tree inferred (Supplementary Figure S4). Therefore, the isolate Ehr33-9 would be regarded as *Tetradismus obliquus* (Turpin) M. J. Wynne 2016.

Isolate Ehr15-5

The partial 16S rRNA gene (PV822044) of isolate Ehr15-5 was polymerised and sequenced. This sequence was most similar to two different strains', 99.64-99.93% similar to *Pseudanabaena catenata*'s 16S rRNA gene and 99.78-99.86% similar to *Pseudanabaena limnetica*'s 16S rRNA gene. In the ML tree, *Synechococcus elongatus* PCC 630's sequence was used as an outgroup. The best substitution model that is calculated by JModeltest software was TIM1+I+G (AIC was 7232.75). However, the third best substitution model, GTR+I+G (AIC was 7235.13) was utilized, which was available in the RAxML software and the ML tree is shown in Supplementary Figure S5. Compared to the descriptions of these two similar species in book³³, Ehr15-5 is distinctly constricted between two adjacent cells in the filament (Fig. 1), which resembles *Pseudanabaena catenata* more closely. Furthermore, the average cell width of Ehr15-5 is $1.5 \pm 0.1 \mu\text{m}$ (with observed filaments reaching up to $1.8 \mu\text{m}$ wide), which is slightly wider than *Pseudanabaena limnetica*, which has a width range of 1.2 to $1.5 \mu\text{m}$. With regards of these two differences, based on the analyses, it was determined that the isolate Ehr15-5 is now considered *Pseudanabaena catenata* Lauterborn 1915.

T. obliquus, *P. catenata*, and *Chlamydomonas* genus species were commonly found in Saxony. However, the "List of Algae Species in Saxony" from the State Office for Environment, Agriculture, and Geology did not include *L. segnis* or *C. ulvaensis*, and *C. vulgaris* had little evidence of presence between 1897 and 1989³⁴. In the brown coal and lignite post-mining areas near Sokolov (Czech Republic) and Cottbus (Germany), *C. vulgaris* appeared in 25-50% of certain locations, alongside frequent occurrences of *Chlamydomonas* microalgae³⁵. *C. vulgaris*, *T. obliquus*, and *C. reinhardtii* are well-studied organisms known for their rapid growth and ability to tolerate and uptake HMs^{28,36}.

HM tolerance of selected isolates

Microalgal growth was evaluated at HM concentrations of 0 to 10 mg/L, with some cultures exposed to up to 20 mg/L. Measurements were taken using optical density at 750 nm (OD₇₅₀). As the metal

concentrations increased, microalgal growth was inhibited compared to the control group (refer to Supplementary Figure S6). However, at lower metal concentrations, such as 0.5 mg/L of Cu and up to 2 mg/L of Cd for *C. vulgaris* RG1-4, growth was slightly enhanced compared to the control. *Asterarcys quadricellulare* and *Chlorococcum minutum* also showed increased growth at 0.4 mg/L of CdCl₂, but growth declined at higher concentrations³⁷. Among the tested microalgae, *C. vulgaris* RG1-4, *C. ulvaensis* Ehr33-6, and *T. obliquus* Ehr33-9 displayed the highest tolerance to copper, even at concentrations up to 20 mg/L. In contrast, *P. catenata* Ehr15-5, a cyanobacterium, died after 4 days at a concentration of 0.5 mg/L of copper. The microalga most tolerant to cadmium was *L. segnis* Ehr31-1; minimal adverse effects on growth were observed, even when cadmium concentrations were elevated to 20 mg/L. However, Cr(VI) proved to be highly toxic to most microalgal species, with the cyanobacterium *P. catenata* Ehr15-5 showing the greatest tolerance.

From the growth curves, the half-maximal effective concentration (EC₅₀) was determined (fitted curves are represented in the Supplementary Figure S7), and the findings were compared with values reported in previous studies (Fig. 2). Among the isolates tested for Cu tolerance, three exhibited the highest levels: *C. vulgaris* RG1-4, *C. ulvaensis* Ehr33-6, and *T. obliquus* Ehr33-9, with EC₅₀ values of 22.13 ± 2.07 mg/L, 20.11 ± 0.32 mg/L, and 20.88 ± 0.52 mg/L, respectively. These values are significantly higher than the average EC₅₀ value of 4.53 mg/L found in the literature (Supplementary Table S8). In contrast, the diatom isolate *Navicula permitis* demonstrated the highest EC₅₀ value at 29.41 mg/L under similar conditions³⁸. Notably, the same species of *C. vulgaris* and *T. obliquus* exhibited much lower tolerance to Cu, with EC₅₀ values of only 0.1 mg/L³⁹ and 5.84 mg/L⁴⁰, respectively. And in another study, *T. obliquus* had an even lower EC₅₀ value of 0.05 mg/L⁴¹. Other copper-tolerant microalgae included *Scenedesmus quadricauda* (CPCC 158), *Chlamydomonas reinhardtii* (CPCC 243), and *Dunaliella salina*, which had EC₅₀ values of 19.97 mg/L, 23.30 mg/L⁴², and 18.14 mg/L⁴³, respectively.

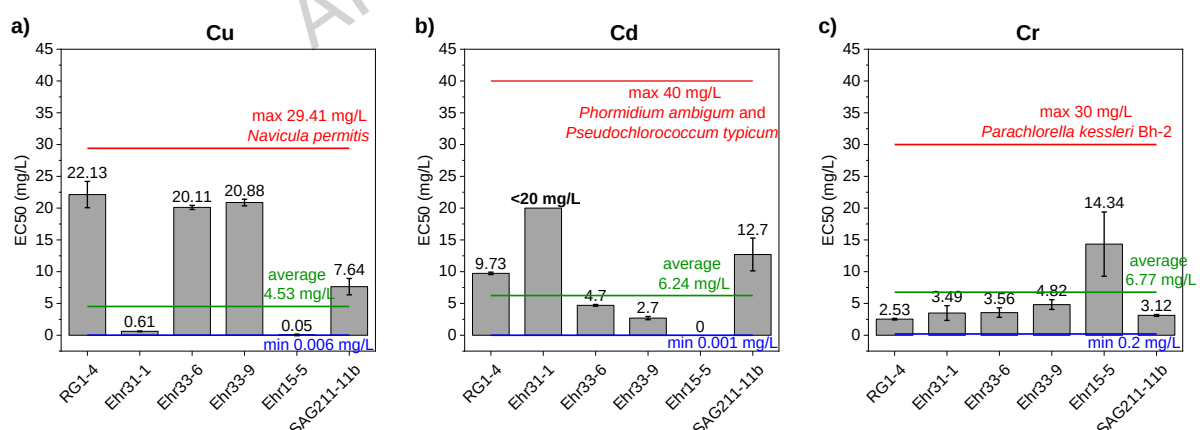


Fig. 2. Comparison of EC₅₀ values for metal tolerance among isolates: a) Cu, b) Cd, and c) Cr(VI). The maximum, average, and minimum EC₅₀ values found in literature are included. Here, SAG211-11b serves as the reference strain. The growth rate inhibition experiments were done in three biological replicates (n=3) and the EC₅₀ values were calculated by fitting the sigmoidal curve of Hill1 function

Two isolates demonstrated higher tolerance to Cd than the average EC₅₀ value of 6.24 mg/L reported in the literature (Fig. 2). The isolate *L. segnis* Ehr31-1 showed no growth inhibition at a Cd concentration of 20 mg/L, displaying only a 1.33% reduction in growth compared to the control after 96 hours. This indicates that the isolate *L. segnis* Ehr31-1 can tolerate Cd concentrations exceeding 20 mg/L. The other tolerant isolate, *C. vulgaris* RG1-4, had an EC₅₀ value of 9.73 ± 0.19 mg/L, which is lower than the reference strain *C. vulgaris* SAG211-11b, which exhibited an EC₅₀ of 12.70 ± 2.58 mg/L. One study reported that two strains of cyanobacteria could tolerate 40 mg/L of Cd⁴⁴, but previously reported EC₅₀ values were lower than the current results (Supplementary Table S9).

None of the isolated and reference green microalgae showed tolerance to high chromium concentrations, except for strains *C. ulvaensis* Ehr33-6 and *T. obliquus* Ehr33-9, which grew at 2 mg/L without significant negative effects. The cyanobacterium *P. catenata* Ehr15-5 displayed the highest tolerance, experiencing only minor effects at up to 10 mg/L, with an EC₅₀ of 14.34 ± 5.06 mg/L. However, it could not tolerate Cu or Cd at 0.5 mg/L, leading to cell death and bleaching. Conversely, strain *C. vulgaris* RG1-4 was the least tolerant, with an EC₅₀ of 2.53 ± 0.12 mg/L. Most literature reported lower EC₅₀ values (Supplementary Table S10). However, *Craticula subminuscula* able to tolerate Cr(VI) up to EC₅₀ of 15.12 mg/L⁴⁵ and *Phaeodactylum tricornutum* up to EC₅₀ of 15.38 mg/L⁴⁶; both species are diatoms. Furthermore, *Parachlorella kessleri* Bh-2 was identified in the literature as the most tolerant microalgae, with an EC₅₀ of 30 mg/L⁴⁷. Cr(VI) can be particularly toxic as it easily penetrates and disrupts cell membranes⁴⁸.

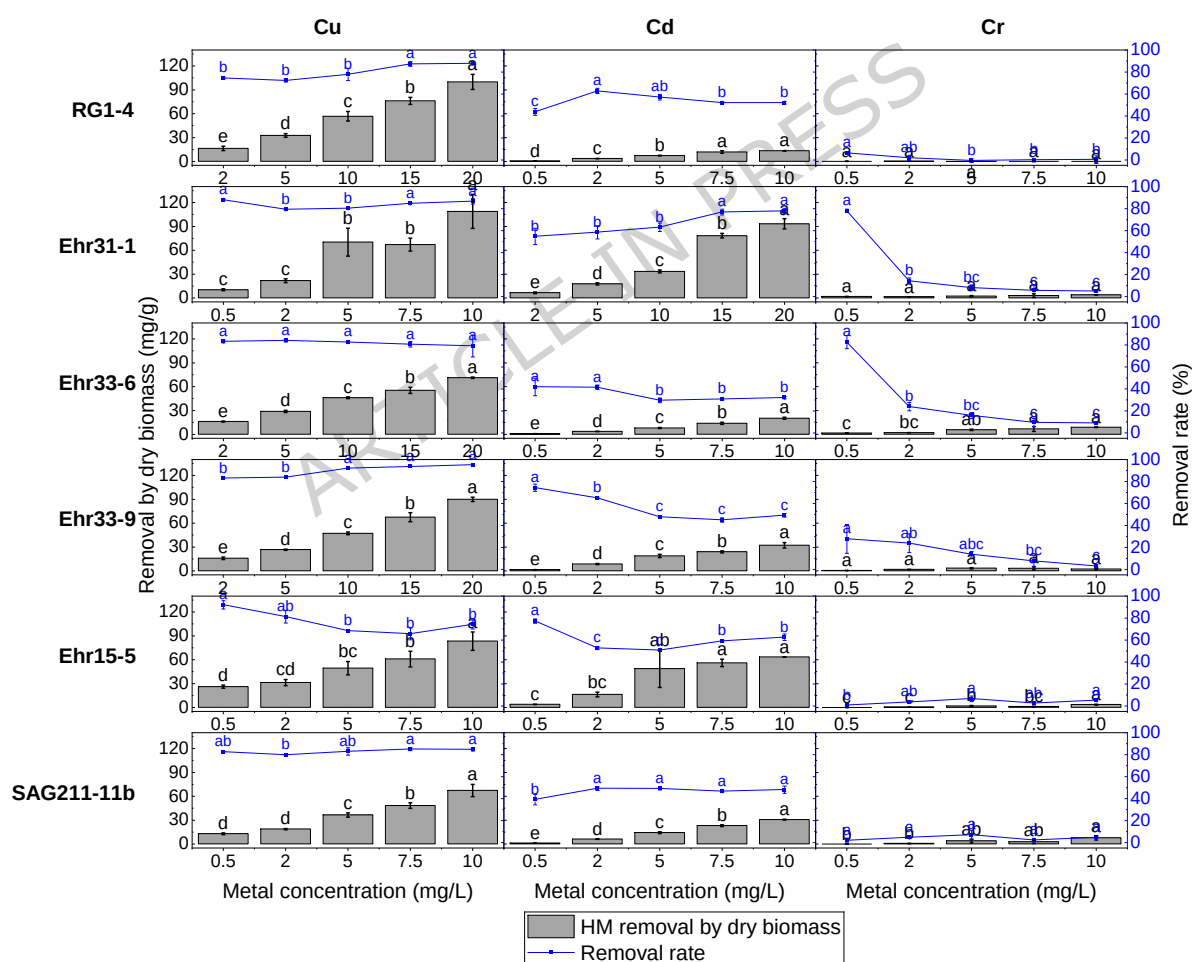
Turning metals into less toxic forms is the one of the strategy of algae to alleviate the HM toxicity. One of the possible reason that cyanobacterium *P. catenata* Ehr15-5 was able to tolerate Cr(VI) is that it may have reduced Cr(VI) to Cr(III) using its own chromium reductase similarly to *Spirulina* sp.⁴⁹. Lee et al. found that live *C. vulgaris* was more effective at reducing Cr(VI) than dead cells, while its chromium reductase activity was lower than that of other bacteria⁵⁰, and Cr(III) is less toxic than Cr(VI) as it is less soluble and more difficult to penetrate cell membranes⁵¹.

HM removal of selected isolates

The extent of HM absorption by microalgal cells was measured in two ways: as a percentage of the total HM removed by the microalgal culture and as the amount of HM absorbed by the dry biomass (see Fig. 3). The raw data from the ICP-MS measurements, including heavy metal (HM) concentrations, dry biomass, and the calculated removal rates along with removal efficiency based on dry biomass, are presented in Supplementary Tables S1 to S6. The results of the overall one-way ANOVA test can be found in Supplementary Table S7. While the microalgae generally tolerated HMs well, their uptake typically increased with higher metal concentrations. Notably, isolate *P. catenata* Ehr15-5 demonstrated good tolerance to hexavalent chromium but exhibited the lowest uptake of this metal. Similarly, isolates *C. vulgaris* RG1-4 and the reference strain *C. vulgaris* SAG211-11b also showed poor uptake performance (as indicated in the third column of Fig. 3).

The removal rate of Cu was highest in *T. obliquus* Ehr33-9 at $95.26 \pm 0.46\%$ and second highest in *C. vulgaris* RG1-4 at $87.92 \pm 1.45\%$, both measured at a concentration of 20 mg/L of copper. The dry

mass uptake for *T. obliquus* Ehr33-9 was 89.73 ± 2.77 mg/g, while *C. vulgaris* RG1-4 exhibited a higher uptake of 100.00 ± 9.52 mg/g. Their growth inhibition rates were $48.21 \pm 3.39\%$ and $40.05 \pm 5.03\%$, respectively. These values were significantly higher than the uptakes reported for fresh biomass, which were 69.41 mg/g for *T. obliquus*⁵², 9.89 mg/g for *C. vulgaris*⁵³, and 15.89 mg/g for frozen *C. vulgaris*⁵⁴. These experiments were conducted under optimal conditions, including appropriate pH, temperature, metal concentration, and algal dosage. When the copper concentration was 10 mg/L, the isolate *L. segnis* Ehr31-1 showed the highest absorption, with 108.85 ± 21.45 mg/g in terms of dry mass uptake. However, the growth of *L. segnis* Ehr31-1 was nearly completely inhibited (almost 100%) starting from a concentration of 2 mg/L of copper (see Supplementary Figure S6). At low concentrations, copper is an essential trace element for cells, playing crucial roles in photosynthesis, cellular respiration, the formation of superoxide dismutase with anti-ROS activity, and in the transport of iron across membranes⁵⁵. However, at high concentrations, copper can damage the membranes of organisms like *Chlorella sorokiniana* and *Scenedesmus acuminatus*, resulting in increased antioxidant activity⁵⁶.



284

Fig. 3. Heavy metal removal after 96 hours of cultivation. The left axis shows the removal rate per dry biomass of microalgae (mg/g), while the right axis displays the percentage of heavy metal removal (%) relative to the initial concentration. The x-axis represents heavy metal concentration. The experiments were done in three replicates (n=3). Different letters above bars indicate statistically

significant differences among HM concentrations ($p < 0.5$, one-way ANOVA with post-hoc Tukey's test).

The cadmium removal rate was highest for *L. segnis* Ehr31-1, achieving $77.86 \pm 2.40\%$ at a cadmium concentration of 20 mg/L, with a cadmium removal per dry biomass of 93.17 ± 6.39 mg/g. Importantly, the growth of this isolate was inhibited by only 1.33% compared to the control. The experiment was carried out with cadmium concentrations ranging up to 20 mg/L, but it seems that this isolate can tolerate higher concentrations and absorb more cadmium than those levels. When comparing cadmium removal per dry weight, *L. segnis* Ehr31-1 outperformed other similar species. For instance, the fresh biomass of *Chlamydomonas reinhardtii* had a removal rate of 42.6 mg/g⁵⁷, while the dried biomass of *Chlamydomonas* sp. recorded 44.75 mg/g⁵⁸. *Chlamydomonas reinhardtii* immobilized in alginate beads demonstrated a higher rate of 79.7 mg/g⁵⁹. Additionally, *P. catenata* Ehr15-5 was tested with 10 mg/L cadmium, resulting in a removal rate of $62.56 \pm 2.96\%$ and a dry weight absorption of 63.51 ± 0.22 mg/g of cadmium. This microalga also showed a strong capacity for copper absorption, as illustrated in Fig. 3. However, its growth was significantly inhibited by $92.26 \pm 5.80\%$ at 0.5 mg/L copper and completely inhibited at 0.5 mg/L cadmium (as shown in Supplementary Figure S6). Fig. 4 demonstrated that its chlorophyll content was nearly absent, indicating negative impact on the cell physiology. For other species, *C. vulgaris* RG1-4 and *T. obliquus* Ehr33-9 removed $52.15 \pm 1.12\%$ and $49.31 \pm 1.68\%$ of cadmium, respectively, with dry biomass absorption rates of 13.32 ± 0.37 mg/g and 32.15 ± 3.35 mg/g. Their growth was inhibited by over 50% at 10 mg/L cadmium. However, these absorption capacities were still lower than those of *C. vulgaris*, which had a freeze-stored biomass absorption of 15.89 mg/g⁵⁴ and a dried biomass absorption of 85.3 mg/g⁶⁰. *T. obliquus* had a freeze-dried biomass absorption of 68.6 mg/g⁶¹, while *D. pleiomorphus* (ACOI 561) also recorded a dried biomass absorption of 85.3 mg/g⁶². In a study by Nourbala Tafti et al., the addition of Fe₃O₄ nanoparticles to *C. vulgaris* and *T. obliquus* significantly enhanced their cadmium uptake capacity, reaching 280.8 mg/g and 331.4 mg/g, respectively⁶³.

The highest Cr(VI) removal was observed in *C. ulvaensis* Ehr33-6 and *L. segnis* Ehr31-1, with absorption rates of $82.65 \pm 5.99\%$ and $78.00 \pm 1.52\%$, respectively, at a concentration of 0.5 mg/L. However, as the concentration of Cr(VI) increased, their absorption capacities decreased, and their specific growth rates were inhibited by about 30% at this concentration. At a Cr concentration of 10 mg/L, the absorption capacities were measured at 8.92 ± 0.32 mg/g for *C. ulvaensis* Ehr33-6 and 3.94 ± 0.18 mg/g for *L. segnis* Ehr31-1. These values are relatively low compared to other studies; for instance, dried *Chlamydomonas* sp. was reported to absorb 152 mg/g Cr(VI)⁶⁴, dried *C. vulgaris* absorbed 14.78 mg/g, and *Scenedesmus acutus* absorbed 20.08 mg/g⁶⁵. The fresh biomass of *Chlamydomonas reinhardtii* showed an absorption capacity of 18.2 mg/g, which increased to 25.6 mg/g after drying⁶⁶. *P. catenata* Ehr15-5 showed some tolerance to chromium but absorbed very little. Foster et al. noted that irradiation increased extracellular polysaccharide production⁶⁷, possibly hindering Cr(VI) uptake due to the negatively charged exopolysaccharides secreted by it⁶⁸.

No microalgae were found to effectively absorb all three HMs. However, the highest uptake of HMs was observed in *C. vulgaris* RG1-4 and *T. obliquus* Ehr33-9 for copper, with relatively less growth

inhibition. For cadmium, *L. segnis* Ehr31-1 exhibited the best absorption. Therefore, these isolates can potentially be used for bioremediation of copper and cadmium. Additionally, hexavalent chromium was best absorbed by *L. segnis* Ehr31-1 and *C. ulvaensis* Ehr33-6 at a concentration of 0.5 mg/L. While *P. catenata* Ehr15-5 showed the highest tolerance to Cr(VI), it absorbed Cr(VI) less effectively than the other isolates. It is evident that the tolerance and absorption of these HMs varied among different microalgae species.

Extracellular polymeric substances (EPS) are secreted by algal cells and consist of polysaccharides, proteins, DNA, humic substances, and inorganic ions. They surround algal cells and serve a defensive role⁶⁹. Studies have shown that among the components of EPS isolated from sludge, Cu and Cd are more effectively adsorbed by proteins. The amide I and amide II groups of proteins, along with the carbonyl (C=O) and aromatic (C=C) stretching of humic acids, contribute to the sorption of these metals⁷⁰. Shi et al. studied the changes in EPS from *Chlorella vulgaris* in response to Cu exposure. They found that the secretion of protein-like substances increased in loosely bound (soluble) EPS when exposed to Cu. Interestingly, at low Cu concentrations, the amount of polysaccharides in EPS increased. However, as Cu concentrations increased, the amount of polysaccharides decreased while the protein content in loosely bound EPS steadily increased. Under normal conditions, proteins are primarily located in tightly bound EPS⁷¹. Additionally, when three different groups of algae, green alga *Uronema confervicolum*, the cyanobacteria *Phormidium autumnale*, and the diatom *Nitzschia palea*—were exposed to Cu and Zn, the protein content in their EPS increased at high concentrations of these metals⁷². This can be the one of the possible explanations of why *C. vulgaris* RG1-4 and *T. obliquus* Ehr33-9 exhibited greater tolerance to high concentrations of Cu.

L. segnis Ehr31-1, in comparison to RG1-4 and Ehr33-9, formed mucoid colonies on agar medium (Fig. 1). It also generated a slimy biomass that adhered to the walls of old cultures in liquid medium. This phenomenon may occur because the oxygen produced by photosynthesis cannot escape, leading to the formation of bubbles on solid medium due to the secretion of strong polysaccharides. The primary component of the outer capsule or biomass of *L. segnis* SAG 1.79 is chlamyhyaluronic acid, and its production increases under stress conditions⁷³. In the second column of Fig. 1, four closely located cells can be seen within a single capsule. Recently, Visconte et al. studied *L. segnis* SAG 1.79 and found that under stress conditions, a capsular polysaccharide (CPS) similar to hyaluronan was produced in large quantities. This CPS exhibited a highly branched random coil structure and demonstrated reducing power, antioxidant activity, and thickening properties, with the capability of secreting up to 1 g/L of EPS under light stress conditions⁷⁴. Hyaluronan is typically produced by vertebrates and a few pathogenic bacteria; it is also produced in the *Chlorella* genus of microalgae when infected by phycodnaviruses⁷⁵. Due to these differences, the capsule of *L. segnis* Ehr31-1 may have absorbed a significant amount of Cd to protect the cells from Cd toxicity.

Chlorophyll a content in dry biomass

When measuring growth through optical density, some cultures exhibited a visual decrease in green colour, yet their optical density values suggested good growth. Chl *a* is a crucial photosynthetic

pigment found in all organisms that perform oxygenic photosynthesis. Besides measuring optical density, Chl *a* can serve as an additional indicator of HM impacts on cell physiology. Fig. 4 demonstrates the correlation between varying concentrations of HMs and the alteration of Chl *a* levels within the dry biomass. Chl *a* concentration in dry weight was measured at the end of cultivation, specifically for treatments with Cu, Cd, and Cr(VI) at a concentration of 10 mg/L. Despite some microalgal cultures showing higher optical density than controls at low HM concentrations (see Supplementary Figure S6), Chl *a* content in dry biomass was consistently lower than controls when exposed to HMs. Notably, isolates Ehr33-6 and Ehr33-9 significantly reduced Chl *a* content even at low Cu and Cr(VI) levels. While Cd had minimal impact on strain Ehr31-1 growth, Chl *a* content slightly decreased as Cd concentration increased.

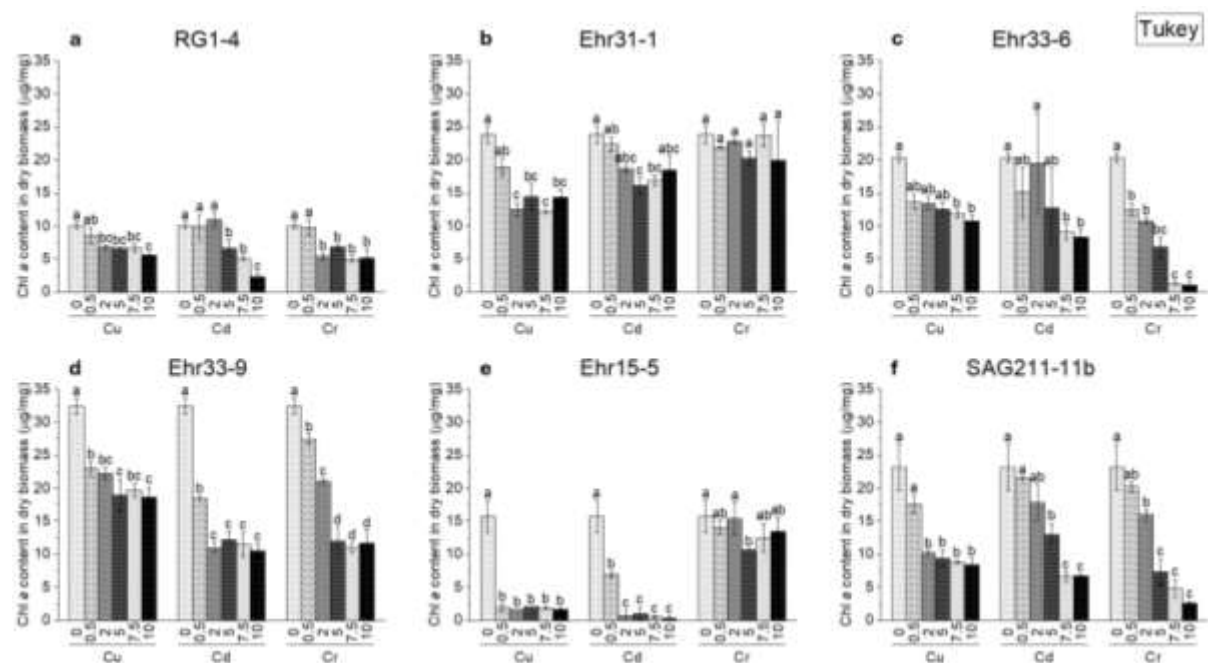


Fig. 4. Chlorophyll *a* content in dry biomass treated with different concentration of heavy metals. Here, a. RG1-4, b. Ehr31-1, c. Ehr33-6, d. Ehr33-9, e. Ehr15-5, f. *C. vulgaris* SAG211-11b – reference strain. The heavy metals and their nominal concentrations are given on the x-axis. The experiments were done in three replicates (n=3). Different letters above bars indicate statistically significant differences among HM concentrations (p < 0.05, one-way ANOVA with post-hoc Tukey's test).

The changes in Chl *a* content observed in *C. vulgaris* RG1-4, *P. catenata* Ehr15-5, and *C. vulgaris* SAG211-11b were consistent with the changes noted in optical density. However, in *L. segnis* Ehr31-1, Cr(VI) reduced the optical density without significantly impacting the Chl *a* content in the dry biomass. This suggests that while the cells did not grow well, they remained intact despite the lack of biomass growth.

In another study involving *Scenedesmus quadricauda*, it was found that Chl *a* content decreased as the concentration of Cr(VI) increased from 5 ppm to 10 ppm⁷⁶. Additionally, increases in Cu and Cd concentrations above 0.014 mg/L and 0.04 mg/L, respectively, also decreased the Chl content⁷⁷. For the microalgae *Asterarcys quadricellulare* and *Chlorococcum minutum*, treated with CdCl₂ at a

concentration of 0.4 mg/L, both cell growth and total chlorophyll content increased compared to the control (TAP medium). However, as cadmium concentration rose, both cell growth and total chlorophyll content decreased³⁷. This trend was also observed in *C. reinhardtii*, *C. luteoviridis*, and *P. kessleri*⁷⁸, as well as in various aquatic plants⁷⁹ and bryophytes⁸⁰. Nevertheless, a study by Ye et al. demonstrated that when *C. reinhardtii* was exposed to half the EC50 concentration of Cu for 96 hours, the chlorophyll content was higher than that of the control group⁸¹.

The decrease in Chl *a* content may be linked to various cellular changes induced by HMs. These changes may include elevated levels of ROS, metal accumulation in chloroplasts, disruption of membrane ion balance, damage to thylakoid membranes, reduced photosynthetic activity, and alterations in gene expression resulting from these damages^{36,82}. For instance, in *C. reinhardtii*, exposure to cadmium for a short period led to a decrease in the expression of the enzyme ferredoxin NADP⁺ reductase, which was associated with a reduction in chlorophyll content. Furthermore, prolonged exposure to cadmium significantly reduced the expression of enzymes involved in the synthesis of chlorophyll and carotenoids⁸³. Similarly, in the brown macroalga *Ectocarpus siliculosus*, short-term copper treatment resulted in the repression of enzymes such as chlorophyll synthase, Mg chelatase, and protoporphyrinogen oxidase, all of which play roles in the steps of chlorophyll synthesis⁸⁴. In these studies, protective protein expression was also up-regulated. The decrease in chlorophyll may be attributed to the allocation of more cellular resources and energy toward protective activities, resulting in fewer resources and energy available for other normal functions.

Future research directions

The isolates *C. vulgaris* (RG1-4) and *T. obliquus* (Ehr33-9) were able to absorb higher amounts of copper (Cu), while *L. segnis* (Ehr31-1) demonstrated a greater capacity to absorb cadmium (Cd). Therefore, these isolates can be utilized for treating wastewater containing Cu and Cd as the primary pollutants. The main sources of Cd pollution include nickel-cadmium (Ni-Cd) batteries and the electroplating industries⁸⁵. In contrast, higher concentrations of Cu are typically found in wastewater from copper mining⁸⁶.

To further enhance the HM biosorption capacity of these isolates, several studies could be conducted. Potential approaches might include chemically treating the biomass, supplementing the growth medium to boost the production of EPS, providing additional sulphur and phosphate to strengthen the cells' biosorption, and genetically engineering the cells to increase metallothionein production, enhance EPS production, or even display metal-binding proteins on their surfaces^{87–89}. Kalita and Baruah found that treating *Leptolyngbya* sp. GUEco 1015 with 0.1 mM CaCl₂ and 0.3 mM HCl reinforced the microalgae's filament surface, enabling better Cu absorption⁹⁰. Similarly, adding more sulphate to the medium of *Chlamydomonas moewusii* resulted in increased production thiol compounds, thereby enhancing Cd tolerance⁹¹. By supplementing medium with additional phosphorus, the microalgae can produce more polyphosphate bodies, leading to increased sequestration of Cd and Zn in species like *Chlamydomonas reinhardtii*⁹² and *Tetradismus obliquus*⁹³. Additionally, genetically engineering *Chlamydomonas reinhardtii* to display metalloreulatory protein (MerR) allowed it to uptake five times more Hg than the wild type⁹⁴.

When developing these isolates for field applications, three key considerations must be taken into account. First, it's essential to understand the kinetic and isotherm behavior of the metal adsorption by these isolates. Adsorption typically occurs rapidly within the first 20 to 30 minutes of contact with metals, after which the rate slows until equilibrium is reached. This information can help determine the optimal duration for using these isolates to treat metal contaminants. Understanding the isotherm will provide insights into the maximum adsorption capacity and the binding characteristics of the isolates^{52,53,95}. Second, it is crucial to test these isolates under various conditions, including changes in pH, temperature, salinity, and the presence of co-existing metal ions, to evaluate their effects on metal binding rates and capacities^{88,96}. Third, if these isolates are used in the field, they will need to be harvested. Given that these isolates are single-celled organisms with a diameter ranging from 4 μm to 12 μm , harvesting can be challenging and costly. To overcome this obstacle, methods such as immobilization⁹⁷ using alginate⁹⁸, water-hyacinth derived pellets⁹⁹, or loofah sponge¹⁰⁰ can be employed. Additionally, microalgae can be magnetized by using Fe_3O_4 nanoparticles, enabling easy harvesting with magnets⁶³.

Conclusion

Since the increase in industrialization worldwide, heavy metals have been released into the environment due to human activities. Their presence poses a significant threat to living organisms and can lead to biomagnification in the food chain, which in turn presents a high risk to human health. One environmentally friendly and cost-effective method to reduce heavy metal pollution is the use of microalgae. In a search for suitable microalgae in old mining areas of Saxony, Germany, Cu-absorbing strains of *Chlorella vulgaris* (RG1-4) and *Tetradesmus obliquus* (Ehr33-9), along with a Cd-absorbing strain of *Lobochlamys segetis* (Ehr31-1), were successfully isolated. These strains demonstrate a relatively high capacity to absorb Cu and Cd compared to other strains, making them promising candidates for treating wastewater primarily contaminated with these heavy metals. Future research should focus on understanding the kinetics and isotherms of metal uptake, as well as how factors such as pH, temperature, and contact time influence their ability to absorb metals. Additionally, exploring the possibility of immobilizing these microalgae for practical applications could enhance their effectiveness in pollution mitigation.

Methods

Sampling locations

Samples were taken from three locations, Roter Graben near Freiberg (50°56'24.1"N 13°22'20.1"E), three other locations near Ehrenfriedersdorf (around 50°38'36.0"N 12°58'59.6"E) and Visitor's Mine Zinnwald (50°74'11.6"N, 13°76'35.9"E) from Erzgebirge (Ore Mountains) in south-eastern Germany (Saxony). Sampling locations are shown on the map (Fig. 5). The map was created using ArcGIS Desktop version 10.8 software (Environmental Systems Research Institute, Inc. (Esri))¹⁰¹. Sample

collection was conducted in accordance with local regulations, and no special permission was required.

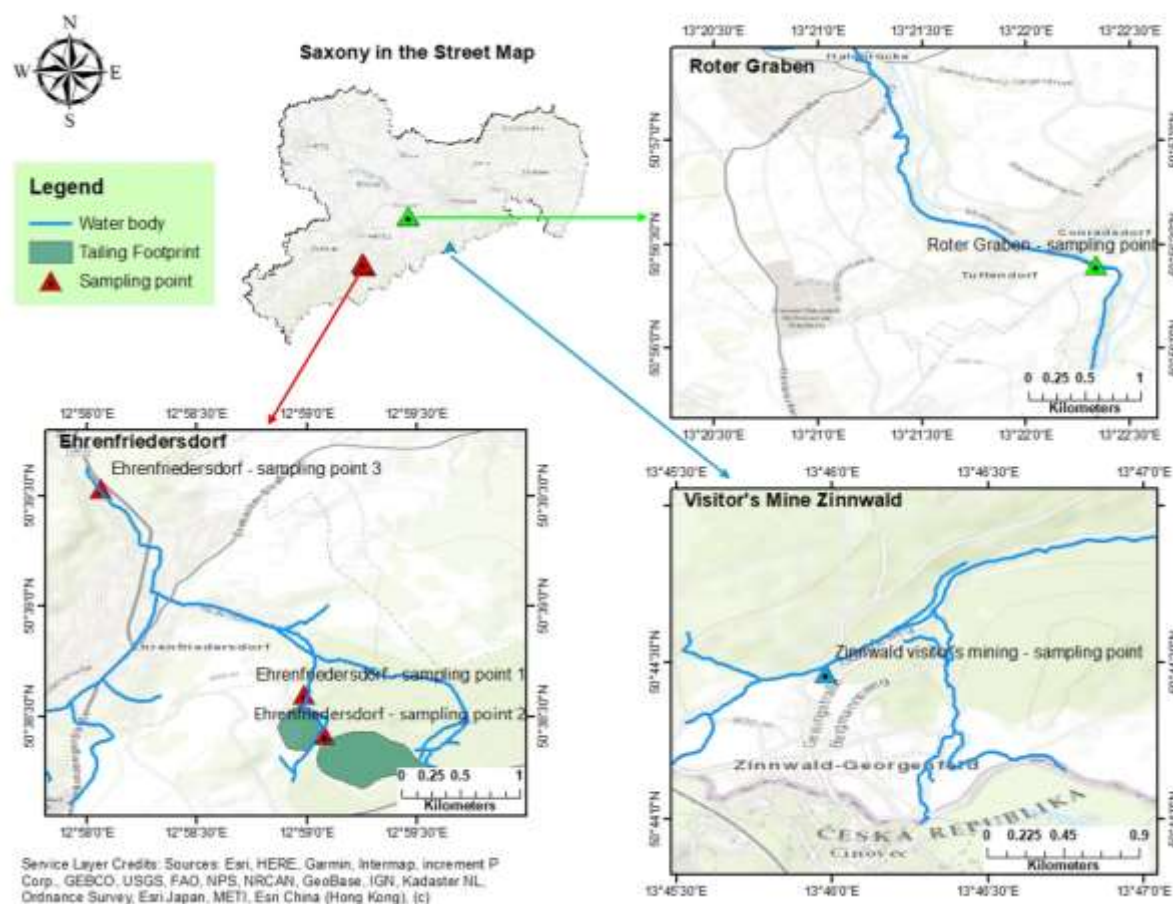


Fig. 5. Sampling locations in Erzgebirge, Saxony. The basemap utilized is the World Street Map, and the credits for the basemap are indicated at the bottom left¹⁰². The water body data was taken from the River Basin Districts and Catchment Boundaries in Germany by Esri Deutschland (© Bundesamt für Naturschutz (BfN) 2023)¹⁰³.

The Erzgebirge has several metals that are mined from the 12th to the 20th century. The silver ore was discovered first, and it was mined continuously from 1168 to 1968. Between 1460 and 1560, the Erzgebirge was the most renowned silver producer in Europe. The second metal that was extracted from the region was a tin. Then the region became an important uranium producer in the world at the end of 19th century. During the 800 years of mining, the landscape of Erzgebirge had shaped a lot. Therefore, the Erzgebirge region was registered as a UNESCO World Heritage Site in 2019^{29,101}.

Isolation and selection of HM tolerant microalgae

Isolation of pure microalgal culture

To enrich the microalgae from the samples, they were cultured in both solid media containing 1.5% agar and liquid media: BG11, Bold's Basal Medium (BBM), Spirulina medium¹⁰², and Z8 medium^{103–105}. Microalgal colonies were collected with a fine needle and streak-plated onto new solid media for

isolation. However, repeated streak-plating was often insufficient to eliminate bacteria from most of the isolates. Consequently, the microalgae were treated with various antibiotics.

Single antibiotics were insufficient to eliminate various types of bacteria and fungi, so multiple antibiotics were tested for their effectiveness without harming microalgal growth. These included Penicillin (25 – 1000 µg/mL), Streptomycin (10 – 250 µg/mL), Gentamicin (10 – 250 µg/mL), Kanamycin (10 – 250 µg/mL), Chloramphenicol (5 – 100 µg/mL), and Cycloheximide (5 – 100 µg/mL). After selecting up to three antibiotics at the appropriate concentration, they were applied sequentially for 24 hours each.

The antibiotics mentioned above function by inhibiting cell wall formation and protein synthesis and they work well on actively growing cells. Especially, antibiotics that inhibit the cell wall formation kill bacteria that are only actively dividing. Therefore a small amount of nutrients should be added to enhance bacterial growth¹⁰². A sterile nutrient solution (SNS) was added (2 mL of SNS solution into 100 mL medium), consisting of 2.5% sucrose, 0.5% Difco yeast extract, and 0.5% Difco Bacto-Peptide¹⁰⁶. After each 24-hour treatment, most of the bacteria were reduced, but not eliminated. Thus, from each treatment, 20 µL samples were streaked onto suitable solid media. The colonies were then grown in liquid media, and their purity was examined by culturing them in BII, BIV, and BV media containing organic sources¹⁰⁷. The presence of bacteria was assessed under a microscope (Axioskop-2 MOT, Carl Zeiss, Oberkochen, Germany) to confirm their absence.

Selection of microalgal cultures that are tolerant to high concentrations of HMs

The isolated strains' and as references *Chlorella vulgaris* SAG 211-11b, and *Limnospira maxima* CCALA 27's tolerance to high concentration of heavy HM(oid)s: As(III) – 100mg/L (NaAsO₂), As(V) – 100mg/L (Na₂HAsO₄), Cd – 10 mg/L (Cd(NO₃)₂ · 4H₂O), Cr(VI) – 15 mg/L (K₂Cr₂O₇), Cu – 8 mg/L (CuSO₄ · 5H₂O), and Pb – 20 mg/L (Pb(NO₃)₂) were determined. The concentrations used were based on research articles assessing microalgal tolerance to HMs¹⁰⁸ and the culture media BBM and Z8 were prepared without EDTA due to its chelating property of metals^{109,110}. The experiment was conducted in a 24-well plate with a working volume of 1 mL (Nunc™ cell culture multi-dishes, treated, Thermo Fisher Scientific, Waltham, MA, USA). Microalgal growth was measured as OD₇₅₀ (optical density at 750 nm wavelength of light) daily by a plate reader (Infinite® 200 PRO, TECAN, Männedorf, Switzerland)¹¹¹. Microalgae were grown for 4 days in the same culture cabinet. The experiment was done in 3 replicates. For green microalgae, the average of 3 wells' optical density which containing normal medium, and microalgae after 4 days was considered as 100% of growth. The growth rates of other wells were then compared to this percentage. Cyanobacteria were observed for growth in a medium containing HM, comparing it to the control, with observations made visually.

Morphological and molecular identification

Morphological identification

The morphology of selected isolates was observed under Axioskop-2 MOT microscope. The morphological features of each isolates were compared to identification keys^{32,33,112}. Morphological identification in higher levels of taxonomy helped to choose the right primer pairs for further molecular identification.

Genomic DNA isolation

Genomic DNA from the cultures was isolated using Chelex-100^{113,114}. When cells are treated with Chelex-100 at 100°C, they are disrupted, releasing the DNA into the media. Chelex-100 acts as a chelating agent, binding magnesium ions, which are cofactors for deoxyribonucleases. This binding protects the released DNA from degradation. From the fresh liquid culture, 3 mL samples were taken and centrifuged using refrigerated centrifuge (Sigma 3-30KHS, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 14000×g. The supernatant was discarded, and 200 µL of a 5% Chelex-100 solution (pH 8.0, in 1X TE buffer) was added to the pellet in a 1.5 mL Eppendorf tube. Because Chelex-100 tends to precipitate easily, the solution was thoroughly mixed using a magnetic stirrer while pipetting. The mixture of fresh biomass and Chelex-100 was then vortexed and heated in a water bath at 100°C for 15 min with an additional vortex at the 8-minute mark. Following this, the tubes were centrifuged at 12000×g for 90 s at room temperature. The supernatant was transferred to a new tube, avoiding any Chelex resin. The DNA concentration and purity were measured using a Infinite® 200 PRO microplate reader. Finally, the extracted DNA was stored at -20°C.

Polymerase Chain Reaction (PCR) and sequencing

For molecular identification SSU genes (18S rRNA, ITS, partial 23S rRNA), ITS gene, and plastidial *rbcL* and *tufA* genes were used for identification of green microalgal isolates and 16S rRNA gene used for cyanobacterial isolate. The primer pairs used for PCR (polymerase chain reaction) are given in Table 3.

Target gene	Primer	Sequence	Product length, nt	Reference
<i>rbcL</i>	RcbLZ-F	5'-CAACCAGGTGTTCCASCTGAAG-3'	≈1323	31
	RcbLZ-R	5'-CTAAAGCTGGCATGTGCCATAC-3'		
<i>tufA</i>	tufA-SF	5'-TGGATGGTGCWATTYTWG-3'	789	31
	tufA-SR	5'-GGTTTTGCWAAAACCATWCCACG-3'		
ITS	ITS-AF	5'-CGTTTCCGTAGGTGAACCTGC-3'	≈700	115

	ITS-BR	5'-CATATGCTTAAGTTCAGCGGGT-3'		
SSU (18S rRNA, ITS)	EAF3 – Fw	5'-TCGACAATCTGGTTGATCCTGCCAG-3'	≈2400	116
	ITS055R - Rv	5'-CTCCTTGGTCCGTGTTTCAAGACGGG-3'		
16S rRNA	27F - fw	5'-AGAGTTTGATCCTGGCTCAG-3'	≈1480	117
	16C - rv	5'-AAGGAGGTGATCCAGCCGCA-3'		118

Table 3. Detailed information about primers.

PCR (Polymerase Chain Reaction) mix: 5x Phusion HF or GC buffer 4 µL, 10 mM dNTPs 0.4 µL, 10 µM forward primer 1 µL, 10 µM reverse primer 1 µL, Phusion DNA polymerase 0.2 µL (New England Biolabs GmbH), template DNA 1 µL, ddH₂O until the volume reaches 20 µL. The PCR was conducted in thermocycler (peqSTAR 96X, PEQLAB Biotechnologie GmbH, Erlangen, Germany) and the PCR condition was: Initial denaturation at 98°C for 30s, 35 cycles of denaturation at 98°C for 10s, annealing at appropriate temperature calculated by Tm Calculator v.1.16.6¹¹⁹ for each primer pairs, extension at 72°C for 1 min and final extension at 72°C for 10 min. The PCR products were kept at -20°C.

PCR products were cleaned by Monarch® PCR & DNA Cleanup Kit T1030 (New England Biolabs GmbH) – to obtain the PCR produced 16S rRNA and 18S rRNA genes. The gene sequences were sequenced by Sanger sequencing method in GENEWIZ (Azenta Life Sciences, Leipzig, Germany). And sequences were submitted to GenBank, then the Accession numbers are given in the results.

Data analysis and phylogenetic tree

Sequences were manually edited using the software BioEdit v7.2 software¹²⁰. Then, NCBI BLASTn (Basic Local Alignment Search Tool) tool¹²¹ was used to align isolates' sequences with other sequences in GenBank. Then some of the sequences from this alignment were used for further phylogenetic analysis. The alignment is done by Clustal Omega software¹²². Model of nucleotides substitution selected by AIC method by jModelTest v2.1.10 software¹²³. The Maximum Likelihood tree inferred on this alignment and nucleotide substitution model by RAxML v8.2 software¹²⁴ and illustrated by FigTree v1.4.4 software¹²⁵.

Toxicity test of selected isolates in different concentrations of HM

The experimental setup

The selected microalgae were grown in medium with different concentrations of HM, Cu (0.5 – 10 mg/L or 2 – 20 mg/L) (CuSO₄ · 5H₂O), Cd (0.5 – 10 mg/L or 2 – 20 mg/L) (Cd(NO₃)₂ · 4H₂O), Cr(VI) (0.5 – 10 mg/L) (K₂Cr₂O₇) and their growth was observed. The test was conducted for 96 hours, during which the growth conditions were maintained at 25°C with shaking at 150 rpm. The light/dark

cycle was 14/10 hours with a light intensity ranging from 46 to 65 $\mu\text{mol}/(\text{m}^2\text{s})$. A total of 50 mL of culture was prepared in a 100 mL conical flask using a chelator (EDTA) - free medium: BBM for green microalgae and Z8 medium for cyanobacteria. In the HM tolerance test, microalgae growth was measured daily using optical density at a wavelength of 750 nm (OD_{750}). In addition, chlorophyll *a* content, dry weight, and pH levels were assessed at both the beginning and the end of the test. Furthermore, 10 mL of filtered samples were collected at the experimental endpoint for subsequent elemental analysis and stored at -20°C . The experiment was done in three biological replicates.

Inoculum preparation

Inoculum cultures were prepared by growing green microalgae in a bubble column and a cyanobacterium in a CellDEG system (CellDEG GmbH, Berlin, Germany). The bubble column utilized mixed red and blue LED light at intensities of 50 to 100 $\mu\text{mol}/(\text{m}^2\text{s})$, starting lower and gradually increasing as the culture turned green. Mixing was achieved with a magnetic stirrer at approximately 150 rpm, and aeration was provided via a sparger with 0.45 μm filtered air. Cultures were incubated in a sterile environment for 5 to 6 days. Cyanobacteria in the CellDEG system were illuminated with white LED light at 20 to 30 $\mu\text{mol}/(\text{m}^2\text{s})$. They were maintained in 150 mL vessels at 125 rpm, aerated with 3% CO_2 for 7 days. Cultures in the exponential growth phase were then used for HM tolerance experiments.

Inoculum preparation for the HM resistance assay commenced with an initial concentration of $\text{OD}_{750} = 0.1$. The inoculum was separated from the culture medium by centrifugation at 5000 rpm for 10 minutes using a centrifuge (Megafuge 16R, Thermo Fisher Scientific, Osterode am Harz, Germany) and washed twice with sterile distilled water (dH_2O).

Growth measurement

Each day, a 1 mL sample was collected and thoroughly mixed with a pipette if necessary. The sample was then analysed for absorbance at 750 nm using a UV-VIS spectrophotometer (GENESYS™ 180, Thermo Fisher Scientific, Waltham, MA, USA) referenced as OD_{750} . Blanks were prepared using the medium without a chelator, as well as the same medium with each concentrations of HMs. A growth curve was generated from these measurements, and growth inhibition (GI) was calculated using specific equations¹²⁶:

$$K_{HM} = \frac{\ln \text{OD}_{t2} - \ln \text{OD}_{t1}}{t_2 - t_1} \quad (1)$$

$$K_C = \frac{\ln \text{OD}_{Ct2} - \ln \text{OD}_{Ct1}}{t_2 - t_1} \quad (2)$$

$$GI = \frac{K_{HM}}{K_C} \times 100\% \quad (3)$$

Where:

OD_{t1} – optical density at day 0

600 OD_{t_2} – optical density at day 4

601 $t_2 - t_1$ – cultivation period (4 days)

602 K_{HM} – specific growth rate when microalgae treated with HM

603 K_C - specific growth rate when microalgae grown in the control

604 GI – growth inhibition

605 The growth inhibition for each concentration of HM is plotted and the EC50 value was calculated by
606 fitting sigmoidal curve by Hill1 function on OriginPro 2025 software¹²⁷. This function's sigmoidal curve
607 equation is given below.

$$y = a + \frac{b - a}{1 + \left(\frac{k}{x}\right)^n} \quad (4)$$

608 Where:

609 a – minimum asymptote/growth inhibition when HM concentration is 0 mg/L, indicating that the algae
610 were cultured in the control medium. Therefore, this value has been manually set to 0%.

611 b - maximum asymptote/growth inhibition when HM concentration is high, indicating complete growth
612 suppression. Therefore, this value has been manually set to 100%.

613 k – the HM concentration at which 50% of the microalgal population are expected to show growth
614 inhibition (e.g. EC50 value)

615 n – the slope at the steepest part of the curve (Hill slope)

616 ***Chlorophyll a measurement***

617 Chl a content was measured at the start and end of the experiment and expressed as Chl a content in
618 dry biomass. It was measured when isolates were treated with HMs up to 10 mg/L concentration.
619 Pigment extraction followed the method in reference¹²⁸. A 1 mL of the sample was transferred to a 2
620 mL tube and centrifuged at 14000 g for 10 minutes using a Megafuge 16R centrifuge. After discarding
621 the supernatant, the pellet was resuspended in 1 mL DMSO, vortexed, and incubated at 70°C and
622 700 rpm for 10 minutes in a Thermomixer (Thermomixer Comfort 5355, Eppendorf SE, Hamburg,
623 Germany). Following incubation, the mixture was centrifuged again at 14000 g for 10 minutes, and the
624 supernatant was transferred to a new 2 mL tube. The pellet underwent the same treatment with
625 DMSO, and the resulting supernatant was combined with the previous one. Then the absorption of
626 this 2× diluted solution measured at wavelengths of 649 nm, 665 nm (664 nm for cyanobacteria), and
627 750 nm using a spectrophotometer. The Chl a content of green microalgae was calculated using
628 Equation 5¹²⁹, while the Chl a content of cyanobacteria was calculated using Equation 6¹³⁰:

$$C_a (\mu g mL^{-1}) = 12.19A_{665nm} - 3.45A_{649nm} \quad (5)$$

$$C_a(\mu g mL^{-1}) = 11.4063 \times A_{664} \quad (6)$$

Dry weight measurement

Dry weight was measured for inoculum and at the end of the experiment. A 20 mL of the culture was filtered using filtered using glass fibre filters (ROTILABO®, Type CR261, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) with a vacuum pump. After drying at 60°C for 24 hours, the filtered material was cooled in a desiccator for 40 minutes, and then the dry weight was measured.

HM removal measurement

At the end of the experiment (after 96 h), the 10 mL of the remaining filtered solutions were acidified with 500 µl concentrated HNO₃ (~65%) and stored at -20°C for further analysis of HM concentrations. Samples' metal concentration were analysed by an inductively coupled plasma mass spectrometer (iCAP™ RQ ICP-MS, Thermo Fisher Scientific Inc., Waltham, MA, USA) at Institute of Resource Ecology, Helmholtz Zentrum Dresden Rossendorf.

The details of ICP-MS calibration procedures were: Scandium (Sc) and rhodium (Rh) were used as internal standards. The linearity of the calibration curve (R² values) was 1.000 for all elements. The limits of detection were 0.01 µg/L, 0.001 µg/L, and 0.068 µg/L for Cu, Cd, and Cr respectively. The quantification limits were 0.25 µg/L, 0.1 µg/L, and 0.1 µg/L for Cu, Cd, and Cr respectively. The data on recovery or reproducibility were 1.28%, 1.40%, and 1.42% for Cu, Cd, and Cr respectively.

HM removal was expressed in two ways, removal by dry biomass (mg/g) and removal rate (%). The HM concentrations for the calculation were the ICP-MS measured concentrations. And they were calculated accordingly:

$$RB(mg/g) = \frac{C_{HM}(before) - C_{HM}(after)}{DW} \quad (6)$$

$$RR(\%) = \frac{C_{HM}(before) - C_{HM}(after)}{C_{HM}(before)} \times 100\% \quad (7)$$

Where:

RB(mg/g) - removal by dry biomass

C_{HM}(before) – HM concentration at the beginning of the treatment, mg/L

C_{HM}(after) – HM concentration at the end of the treatment, mg/L

DW – dry weight of microalgal biomass per 1 L culture

RR(%) – removal rate

Statistical analysis

Experiments were conducted with three biological replicates (n=3). Statistical analysis was conducted using the statistical package of OriginPro 2025 software¹²⁷. One-way ANOVA with Tukey's post hoc

test was used to evaluate treatment significance at a 5% level (p-value). Data are presented as mean $\pm$ standard deviation (mean $\pm$ S.D.).

Writing, editing, and proofreading

Grammarly and ChatGPT were used only to produce error-free English text with clear and coherent composition. All analyses, interpretations, and scientific content were developed and verified by the authors.

Acknowledgements

We would like to express our gratitude to Mr. Philipp Büttner from the recomine alliance project for facilitating the collection of microalgal samples from mining areas. We also appreciate our colleagues Rustiana Yuliasni, Yob Ihadjadene, and Richard Bleisch for their assistance in sample collection and their valuable advice on the methodology.

Funding

The work was made possible with the support of a scholarship from the German Academic Exchange Service (DAAD, grant-ID 5758837).

Data availability

All sequences obtained for the identification of algal isolates have been deposited in a public sequence repository [GenBank – INSDC] under the following accession numbers: *Chlorella vulgaris* RG1-4 *rbcL* (PV805455.1), *tufA* (PV805456.1); *Lobochlamys seignis* Ehr31-1 rRNA genes (PV823513.1); *Chlamydomonas ulvaensis* Ehr33-6 ITS (PV823514.1); *Tetradismus obliquus* Ehr33-9 *rbcL* (PV805459.1), *tufA* (PV805458.1); *Pseudanabaena catenata* Ehr15-5 16S rRNA (PV822044.1). Data will be made available on request.

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Author contributions

K.M. conceived the study with guidance from F.K. K.M. performed the experiments, collected and analysed the data. K.M. drafted the manuscript. F.K. assisted with experimental design, material preparation, and manuscript editing. F.K., J.S., and T.W. revised and provided critical feedback. F.K., J.S., and T.W. also assisted with funding administration and provided support with scholarship documentation. All authors reviewed and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at ...

Correspondence and request for materials should be addressed to F.K.