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Parametric Evaluation of Heavy Metal Removal from Iron and Steel Wastewater Using Chlorella vulgaris: Linking Operational Conditions to Biomass Composition and Treatment Performance

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ABSTRACT: The effects of pH, illumination duration, wastewater dilution, and carbon dioxide addition on heavy metal removal were investigated over seven days of application to real steel mill wastewater. The microalga was isolated from surface water samples obtained from Eflany Lake, which is a freshwater aquatic ecosystem located within the Karabuk Province in Turkey. The isolated strain showed 99% sequence identity to Chlorella vulgaris, confirming its assignment to this species. The molecular composition of biomass under different operating conditions using Fourier transform infrared spectroscopy (FTIR) revealed the biochemical interactions associated with metal concentration. During a period of seven days, the results showed that wastewater concentrations and treatment efficacy were inversely related. An 18-hour light period, pH 8, 30% wastewater concentration, and 500 mL/h CO_2 flow rate was identified as optimal, achieving the highest removal efficiency for metals (As: 45.0 %, Cd: 75.0%, Cr: 82.0%, Cu: 88.0%, Mn: 90.0%, Ni: 65.0%, Pb 94.0%, Fe: 85.0%). Under optimal conditions characterised by high elimination efficiency, an improved protein content was observed with strong Amide I/II extension. Furthermore, correlation of FTIR peak ratios with removal efficiency can produce predictive biomarkers.

1. INTRODUCTION

Iron and steel manufacture is one of the largest manufacturing industries in the World(Kabeyi & Olanrewaju, 2022). Steel production leads to approximately 10 to 15m^3 of process water and wastewater with dissolved and suspended pollutants and especially heavy metals such as iron (Fe), chromium (Cr), manganese (Mn), nickel (Ni), zinc (Zn), lead (Pb), cadmium (Cd), copper (Cu), arsenic (As)(European Commission, 2012; Kanamarlapudi et al., 2018). The discharge of untreated or inadequately treated wastewater from iron and steel plants into aquatic and nonaquatic environments is considered a serious environmental and health hazard. Chromium, particularly in its hexagonal form (Cr(VI)), is classified as a Group 1 carcinogen.(Sharma et al., 2022) Lead and cadmium accumulate in the food chain and cause neurological damage, kidney dysfunction, and reproductive problems(Balali-Mood et al., 2021). Excess manganese causes neurotoxicity and arsenic and its compounds increase the risk of cancer(A. Kumar et al., 2024).

Although traditional physical and chemical treatment methods, such as chemical precipitation, coagulation and flocculation, ion exchange, electrochemical treatment, and membrane separation are effective, they have many disadvantages, including high energy consumption, the creation of secondary chemical deposits that need further treatment, and are economically expensive for small and medium-sized industrial processes(Shofia et al., 2025).These limitations have motivated research aimed at biological treatment approaches which provide lower maintenance costs, low chemical usage, and the possibility of recovering valuable byproducts(Kuppan et al., 2024).

Microalgae, especially freshwater algae *Chlorella vulgaris*, have natural capacities to concentrate heavy metals from aqueous solutions by various mechanisms: bioaccumulation, biosorption, and extracellular precipitation through metabolite secretion.(Faruque et al., 2024). Algae-based treatment systems have multiple advantages . A high binding capacity characterizes these systems, as the cell walls of microalgae contain a variety of polysaccharides, proteins and lipids, in addition to functional groups (such as carboxyl, hydroxyl, amino acids, sulphates and phosphates) that interact with metal ions through coordination, complexation and chelation (Spain et al., 2021). They also allow the simultaneous removal of pollutants, allowing the simultaneous removal of more than one heavy metal pollution with the help of a single bioreactor (Aravind et al., 2023). In addition, this technology offers opportunities for biomass consumption: algae biomass is different from other conventional adsorbents, such as activated carbon, as it is renewable and biodegradable. It can be exploited by thermochemical conversion (pyrolysis, hydrothermal liquefaction) to produce biofuels or processed to extract pigments and other high-value components, thereby integrating with the principles of circular economy. However, it should be noted that heavy metal-filled biomass recovered from heavy metal treatment systems is not suitable for use in food, feed or nutraceuticals, and the final modes of use must be chosen according to the metal content of the harvested material (Munisamy Sambasivam et al., 2023).Microalgae cultivation also offers an environmental advantage because it consumes carbon dioxide (thereby reducing greenhouse gas emissions), produces oxygen, and requires minimal land area, especially with vertical algae cultivation technologies (Omokaro et al., 2025).

Chlorella vulgaris was selected for this study due to its rapid growth kinetics, exceptional biosorption capacity for multiple metals, and demonstrated efficacy in previous treatment applications, El-Sheekh et al (2012) used sewage wastewater to treat metals employing the species *Chlorella vulgaris*, whereas (Mohebi et al., 2024) utilized simulated industrial wastewater and obtained good results in the removal of heavy metals. However, biosorption efficiency is highly dependent on operational parameters, including pH, temperature, light intensity, light duration, nutrient composition, and wastewater characteristics(Kyratzopoulou et al., 2025).

Despite the growing body of literature on microalgal bioremediation, three critical gaps remain unaddressed. First, the vast majority of published studies on *Chlorella vulgaris* have employed synthetic single-metal or dual-metal solutions prepared in distilled water, rather than authentic industrial effluents. Real steel mill wastewater presents a fundamentally different challenge, containing eight or more co-occurring metals at varying concentrations, together with suspended solids, oils, and competing ions that modify biosorption kinetics in ways that synthetic systems cannot replicate. To our knowledge, fewer than five peer-reviewed studies have evaluated Chlorella-based treatment using actual steel mill effluent, and none have simultaneously examined pH, photoperiod, dilution, and CO_2 enrichment as interacting variables in such a matrix. Second, while individual operational parameters have been optimized in isolation in previous work, the interactive effects among pH, light duration, wastewater concentration, and inorganic carbon supply on both removal efficiency and biomass biochemical composition remain poorly characterized. The present study evaluates these parameters through a cumulative sequential one-factor-at-a-time (OFAT) design capturing the stepwise optimization profile for steel industry applications. This integrated multi-parameter approach is essential for designing scalable systems but has not been systematically investigated for steel industry applications. Third, most prior studies using commercially sourced or laboratory-maintained algal strains have not considered the adaptive advantage of locally isolated strains. An indigenous isolate from the same geographic region as the industrial facility may exhibit superior tolerance to site-specific metal combinations due to prior environmental exposure. The present study addresses all three gaps by: (i) testing a locally isolated *Chlorella vulgaris* strain from Eflany Lake against authentic Karabuk steel mill wastewater; (ii) conducting one factor at a time evaluation covering pH, photoperiod, dilution ratio, and CO_2 supplementation simultaneously; and (iii) linking operational outcomes to biomass biochemical composition through FTIR spectroscopy to establish mechanistic evidence for treatment performance.

Evaluating the effectiveness of microalgae in treating wastewater from the steel and iron industries. Assessing the effects of pH, illumination duration, wastewater dilution, and carbon dioxide addition on heavy metal removal over seven days of application to real steel plant wastewater.

Identify the optimal operating conditions that ensure maximum heavy metal removal efficiency. Characterising the change in the molecular composition of biomass under different operating conditions using Fourier transform infrared spectroscopy (FTIR); and establishing the relationship between operating parameters, biomass chemical composition, and treatment outcomes. Providing quantitative information and operational guidelines suitable for transfer of technology to pilot and commercial industrial applications.

2. MATERIALS AND METHODS

2.1 Source of wastewater and its Characteristics

Steel and iron wastewater was collected from the wastewater outlet of the integrated iron and steel production facility. Samples were collected in pre-cleaned, acid-washed polyethylene containers and transported to the laboratory at 4°C within four hours of collection. The physicochemical characterisation of the raw wastewater, including heavy metal concentrations measured by inductively coupled plasma optical emission spectrometry (ICP-OES, Agilent 720ES, USA), was performed upon receipt and the results are reported in Section 3 (Results and Discussion) as part of the baseline characterisation of the influent wastewater.

2.2 Culture and Inoculation of Microorganisms

The microalgal was isolated from surface water samples obtained from Eflani Lake (41.42680946504968 ° N, 33.0098904373218 ° E), which is a freshwater aquatic ecosystem located within the Karabuk Province in Turkey. Water samples (1 L) were brought to the laboratory at 4 C ° and microalgae were enriched cultures on B G11 medium under ambient illumination in the laboratory. Serial subculture was done to get unialgal cultures(serial dilution-enrichment cultivation). The isolated microalgal strain was then gradually acclimated in steel mill wastewater using stepwise exposure and the same dilution ratios as used for the experimental design (10%, 20%, 30%, 40% v/v). Each acclimation step was continued for 7-10 days before increasing the wastewater fraction to the next fraction (L. Lin et al., 2007).

The molecular identification of the dominant green microalga was carried out by PCR amplification and sequencing of the nuclear 18S rRNA gene region(Mastercycler Pro thermal cycler Eppendorf, Hamburg, Germany). Genomic DNA was extracted from harvested biomass following standard protocols and the 18S rDNA region was amplified using a set of universal algal primers targeting the 18S rRNA-ITS1-5.8S rRNA region following previously described protocols for Chlorella species (Khaw et al., 2020; Rasoul-Amini et al., 2009). PCR products were subjected to Sanger sequencing. For all subsequent cultivation experiments, *Chlorella vulgaris* was kept in BG11 medium under normal laboratory conditions: temperature 20-24 °C, white LED light: 120 µmol photons m^{-2} s^{-1}, automatic 12-hour light-dark cycles. Pre-cultured inocula (20 day grown cultures) with fresh BG11, Count was 10^7 cell/mL (Taikhao & Phunpruch, 2025).

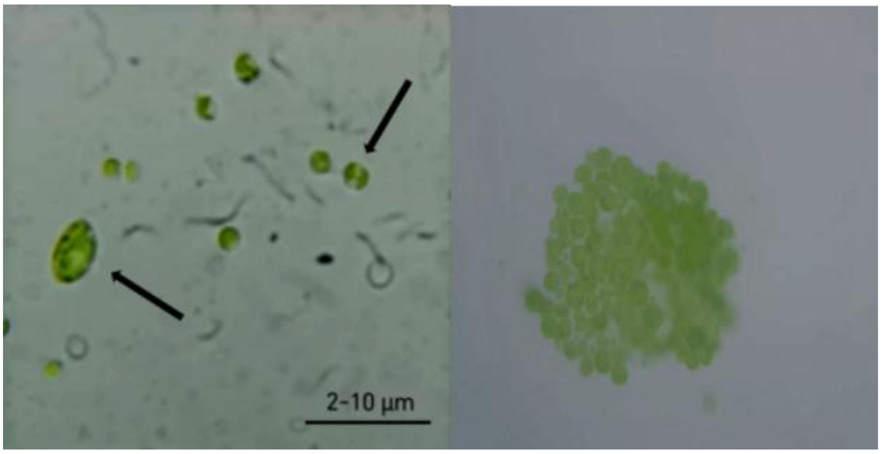

Figure 1. Microscopic image (100× objective) of the isolated *Chlorella vulgaris* strain collected from Lake Eflany, Karabuk Province, Turkey

2.3 Experimental Design

The experiments were conducted sequentially using a one-factor-at-a-time (OFAT) methodology to evaluate how four specific parameters affect the result: pH, photoperiod, wastewater dilution, and CO_2 supplementation(Table 1).The initial trials focused on pH adjustment while maintaining other variables at a fixed photoperiod of 12 hours, following the recommendations of Ratomski & Hawrot-Paw (2021). The initial wastewater concentration was set at a 20% dilution to mitigate acute metal toxicity, in accordance with the guidelines of Mubashar et al. (2020). To ensure nutrients, the wastewater was mixed with BG11 medium, including 10^7CFU/mL of Chlorella vulgaris. A low initial CO_2 flow rate (100 mL/h) was applied, based on principles of low-flow aeration commonly used in microalgal studies (Chiu et al., 2008). This baseline control condition effectively integrated a photoperiod of 12 hours, a 20% dilution of wastewater and a starting point for gas aeration.The experiments began with the assessment of pH levels (9, 8, 7, 6, 5), while maintaining other parameters in these baseline conditions. The pH was adjusted with HCl or NaOH and checked by a pH meter. Once the optimal pH value is determined, it replaces the baseline value at the next stage and evaluates wastewater dilution in the test amounts of 10%, 20%, 30%, and 40%. This cumulative optimization process has been repeated step by step for the photoperiod (6, 12, 18, and 24 hours) and finally for CO_2 supplementation. The CO_2 supplement tests were 0, 100, 500 and 1000 mL/h. High-purity carbon dioxide gases (99% concentration) are added by blowing in humidified gases, and a glass flow sensor (Rotameter) is used to control the required flow rate through continuous ventilation. At this stage, the pH was monitored and periodically adjusted because it was found that the introduction of carbon dioxide had a negative impact on the pH level, so each parameter was rigorously evaluated according to the previously optimized conditions. Each specific condition was tested three times, requiring a total of 51 bioreactors. The heavy metal levels were monitored daily for 7 days by ICP-OES, resulting in 2,856 measurements. During this time, samples were collected daily to avoid daily changes in metabolism.

Table 1. Experimental design parameters and levels tested in one factor at a time study

Parameter	Levels Tested	Notation	Unit
pH	5, 6, 7, 8, 9	pH	dimensionless
Illumination Duration	6, 12, 18, 24	h	hours
Wastewater Dilution	10, 20, 30, 40	% effluent	% v/v
CO_2 Supplementation	0, 100, 500, 1000	ml/h	ml/h

2.4 Cultivation Protocol and Sampling Protocol

Cultivation was performed in 600-mL borosilicate glass cylindrical reactors at 20-24 +- 1 °C. Illumination was granted by white light-emitting diode (LED) panels (60 W/m2 light intensity) with electronic timers for controlling 6, 12, 18 and 24 h photoperiod. Each reactor was inoculated with 500 ml of prepared wastewater medium containing 2 ml of *Chlorella vulgaris* pre-culture (about 10^7 cells/mL inoculum density of preculture in the reactor) ,The experiments were conducted at pH 8, except for those specifically designed to investigate the effect of varying pH,Mixing and aeration were carried out using air supplemented with concentrated carbon dioxide, whereas at 0% carbon dioxide concentration, only air was used at a pumping rate of 500 mL/min. Figure. 2

Figure 2. Reactor used for algae growth

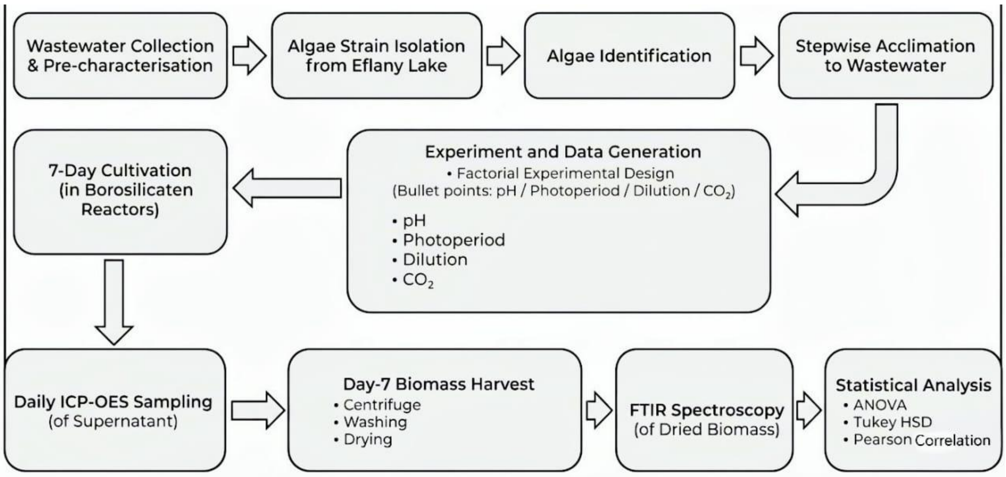

Figure 3. Experimental Process Flowchart

A process flowchart is required here, illustrating the following sequential steps: Wastewater collection and pre-characterisation → Strain isolation from Eflany Lake → Molecular identification → Stepwise acclimation to wastewater → one factor at a time experimental design (pH / photoperiod / dilution / CO_2) → 7-day cultivation in borosilicate reactors → Daily ICP-OES sampling of supernatant → Day-7 biomass harvest (centrifugation, washing, drying) → FTIR spectroscopy of dried biomass → Statistical analysis (ANOVA, Tukey HSD, Pearson correlation) As shown in Figure 3.

Heavy metal levels were measured on days 1, 2, 3, 4, 5, 6 and 7 on the same days each day to minimise circadian variation in cellular metabolism. At each time point, a 25 ml aliquot of culture was sampled, centrifuged (4000rpm for 10 min) and the supernatant was filtered (0.22 µm syringe filters, Millipore) into preacid-washed sample vials (10% HNO3). The samples were weighed and acidified to pH 2 concentrated HNO3 and stored at +4 ^{o}C prior to ICP-OES analysis.(Sasmaz Kislioglu et al., 2025).

Cumulative removal efficiency (%) has been calculated as:

$$\text{Removal Efficiency \%} = \frac{C_{\text{initial}} - C_{\text{day 7}}}{C_{\text{initial}}} \times 100$$

where C"initial" is the concentration of metal on day 1 and C "day 7" is the concentration after 7 days of treatment (H.-C. Lin et al., 2024).

2.5 Biomass Harvesting and FTIR Analysis

On the seventh day, the cultures were centrifuged (4000 rpm for 10 minutes), and the biomass was washed three times with deionised water to remove residual mineral salts and dissolved substances. The mixture was then oven-dried at 80 °C for 24 hours. The biomass was ground into a fine powder using a mortar and pestle (Al-Homaidan et al., 2015). A total of 17 samples, one sample for each level of the variables, were prepared and processed accordingly.

The Fourier transform infrared (FTIR) spectra of solids were read using a Nicolet 6700 FTIR spectrometer (Thermo Scientific) with KPR in the 4000-400 cm^{-1} range with a resolution of 4 cm-1, with particular emphasis on the wavenumber Range of proteins, carbohydrates, and Lipids, as presented in Table 2. The spectral data were processed using OMNIC software with automatic compensation for atmospheric effects and baseline correction.

Table 2. Peak heights and peak area integrals were extracted for key characteristics of key biochemical classes and major bands:

Component	Functional Group / Feature	Wavenumber Range (cm^{-1})
Lipids	C-H Stretching	2920, 2850 cm^{-1}
	Ester C=O	1740 cm-1
Proteins	Amide I (C=O stretch)	1650-1680 cm^{-1}
	Amide II (N-H bend)	1540-1560 cm^{-1}
Carbohydrates	C-O stretching	1200-1000 cm^{-1}
	Ring C-O bending	900-700 cm^{-1}
Structural features	o-H stretch (including water)	3400 cm^{-1}
	p-o stretch (phospholipids)	1200-900 cm^{-1}

A reference FTIR spectrum of standard *Chlorella vulgaris* (cultured under optimal growth conditions in nitrogen-replete BG11, with an 18-hour period photoperiod, pH 8 and no heavy metal stress) was included for variation comparisons for all samples analysed.

2.6 Statistical Analysis

Statistical differences between treatment conditions were assessed using ANOVA followed by Tukey's test at a significance level of $\alpha = 0.05$, using SPSS software (version 26, IBM, USA). Analysis of variance (ANOVA) was performed and normality and homogeneity of variance were verified. Pearson correlation analysis was performed to assess the relationship between the intensity of the FTIR peaks and metal removal efficiency.

3. RESULTS AND DISCUSSION

3.1 Isolation and Characterization of the Microalgal Strain

Sequence identity was verified by comparison with reference sequences from the National Center for Biotechnology Information database (NCBI; GenBank) using the BLAST software (Canelli et al., 2021). The isolated strain showed 99% sequence identity to *Chlorella vulgaris*, confirming its assignment to this species.

The morphological characteristics further supported this identification; the unicellular, round, green cells, 2-10 µm in diameter (Figure 1), containing starch-filled sacs or thylakoid membranes was identical to published standard descriptions of C. vulgaris (Coronado-Reyes et al., 2022).

3.2 Characterisation of Raw Steel Mill Wastewater

Table 3. Heavy Metal Concentrations in Raw Wastewater

Metal	Concentration (mg/L)
Iron (Fe)	10.12
Nickel (Ni)	3.5187
Copper (Cu)	1.9973

Manganese (Mn)	1.2627
Chromium (Cr)	0.9333
Arsenic (As)	0.514
Lead (Pb)	0.4684
Cadmium (Cd)	0.0514

The raw undiluted wastewater collected from the Karabuk iron and steel facility was characterised by ICP-OES (Agilent 720ES, USA) upon receipt. The measured heavy metal concentrations were (Table 3): As (0.514 mg/L), Cd (0.0514 mg/L), Cr (0.9333 mg/L), Cu (1.9973 mg/L), Mn (1.2627 mg/L), Ni (3.5187 mg/L), Pb (0.4684 mg/L), and Fe (10.12 mg/L). Iron was the dominant metal by concentration, consistent with the iron-intensive nature of blast furnace and electric arc furnace operations at this facility. Although arsenic and cadmium are present in lower absolute concentrations, they are still dangerous in this amount if they reach a drinking water source. Nickel and copper concentrations exceeded typical discharge limits for industrial effluents in (Çevre ve Orman Bakanlığı, 2004), confirming the need for effective treatment prior to any release to receiving water bodies. These baseline values served as the reference concentrations (C_0) for calculating removal efficiencies in all subsequent experimental conditions.

3.3 pH-Dependent Metal Removal and Surface Charge Modulation

The pH value is one of the most critical factors in the treatment of heavy metals using algae, as it governs the surface charge properties and the ionisation of functional groups(Ajayan et al., 2012; Zeraatkar et al., 2016). These parameters directly influence the biosorption process. Five pH levels (5, 6, 7, 8, and 9) were evaluated, producing different results as presented in Table 4 .

Table 4. Effect of pH on heavy metal removal efficiency (%) by *Chlorella vulgaris* from steel mill wastewater

pH	As	Cd	Cr	Cu	Mn	Ni	Pb	Fe
5	27.0±1.2	45.0±1.8	49.2±2.0	52.8±1.9	54.0±2.1	39.0±1.5	56.4±2.2	51.0±1.8
6	33.8±1.4	56.3±2.1	61.5±2.3	66.0±2.2	67.5±2.4	48.8±1.9	70.5±2.5	63.8±2.2
7	40.5±1.6	67.5±2.4	73.8±2.6	79.2±2.7	81.0±2.8	58.5±2.2	84.6±2.9	76.5±2.6
8	45.0±1.5	75.0±2.5	82.0±2.7	88.0±2.8	90.0±2.9	65.0±2.3	94.0±3.0	85.0±2.8
9	42.8±1.7	71.3±2.6	77.9±2.8	83.6±2.9	85.5±3.0	61.8±2.4	89.3±3.1	80.8±2.7

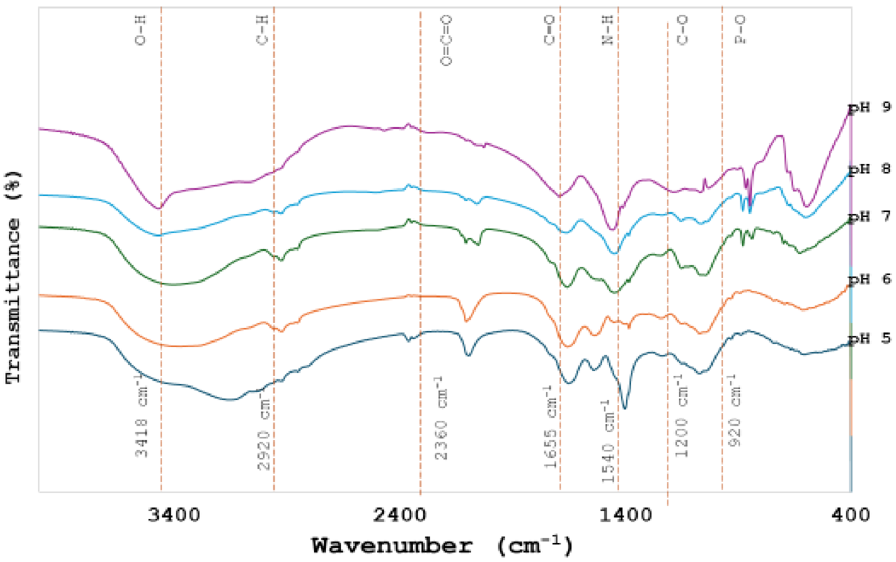

Figure 4. FTIR spectra of algal biomass at Ph5,6,7,8,9 levels showing the influence of protonation/deprotonation on functional groups responsible for metal binding

According to the data in Table 4, pH 8 was identified as the optimal condition, achieving removal efficiencies ranging from a minimum of 45% for arsenic to a maximum of 94% for lead, with favourable results for other metals as well,These findings are consistent with previous studies(S. D. Kumar et al., 2013; Rahmatina et al., 2025). At lower acidic conditions (pH 5–6), the removal efficiency decreased significantly, particularly at pH 5. This reduction is attributed to the diminished surface charge caused by extensive protonation, which weakens the electrostatic attraction between metal ions and binding sites. Furthermore, the high proton concentration competes with metal ions for active binding sites, The observed reduction in arsenic removal efficiency is primarily ascribed to the electrostatic repulsion between it and *Chlorella vulgaris*, thereby limiting the biosorption process(Alharbi et al., 2023).

At pH 9, the removal efficiency decreased slightly (approximately 3%), as reported by (Bilal et al., 2018), who noted that strong alkalinity can damage the cell membrane. This damage leads to the deprotonation of amino groups, thereby increasing electrostatic repulsion and reducing binding affinity. The neutral medium (pH 7) produced lower results compared to pH 8, indicating that mild alkalinity is required for effective heavy metal removal(Lapeñas et al., 2023).

FTIR analysis provided partial evidence to support these findings (Figure 4). At pH 8, the biomass exhibited strong absorption at 1400 cm^{-1}, corresponding to COO^- stretching, and at 3200–3600 cm^{-1}, indicative of hydroxyl stretching. These signals confirm the abundance of functional groups involved in metal adsorption. On the contrary, at pH 5, the intensity of carboxyl and protein bands (1650 and 1540 cm^{-1}) decreased, consistent with the observations of (Alharbi et al., 2023).

3.4 Concentration of wastewater: Balancing Removal Efficiency with Treatment Capacity

To achieve a balance between volume processing capacity and removal efficiency, four different concentrations (10%, 20%, 30%, 40%) were evaluated. The dilution is done using deionised water in the optimal conditions of other parameters (pH 8, 12-hour exposure to light, 100 mL/hour). The results are presented in Table 5.

Table 5. Effect of wastewater concentration on metal removal efficiency (%) by *Chlorella vulgaris*

Concentration	As	Cd	Cr	Cu	Mn	Ni	Pb	Fe
10%	47.3±1.6	78.8±2.5	86.1±2.7	92.4±2.9	94.5±3.0	68.3±2.4	98.7±3.1	89.3±2.8
20%	45.9±1.5	76.5±2.6	83.6±2.8	89.8±2.9	91.8±3.0	66.3±2.4	95.9±3.1	86.7±2.8
30%	45.0±1.5	75.0±2.5	82.0±2.7	88.0±2.8	90.0±2.9	65.0±2.3	94.0±3.0	85.0±2.8
40%	38.3±2.2	63.8±3.3	69.7±3.5	74.8±3.6	76.5±3.7	55.3±2.9	79.9±3.8	72.3±3.4

Note the increase in standard deviations at 40% concentration, reflecting heterogeneous responses to cellular stress. ANOVA: $F_{3,8} > 89.3$, $p<0.001$ *for all metals.*

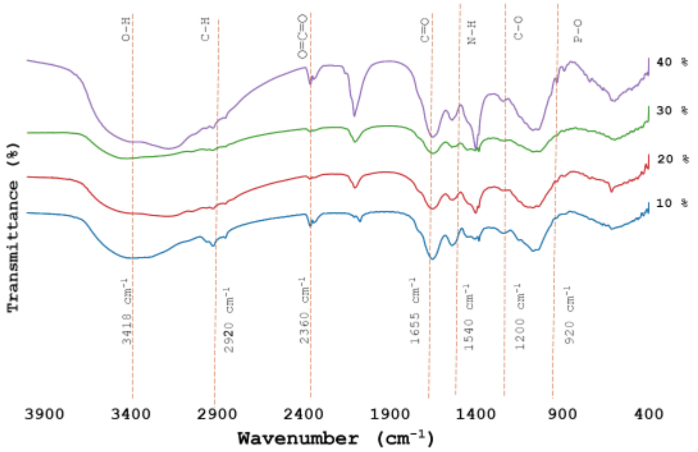

Figure 5. FTIR spectra of algal biomass treated with 10% 20% 30%40 wastewater concentrations showing functional-group variations associated with metal binding

The results obtained over seven days show that the removal efficiency decreased as the initial wastewater concentration increased, consistent with previous studies (Expósito et al., 2021). The 10% concentrations achieved the best performance (As 47.3 %,Cd 78%, Cr 86.1 %,Cu 92.4 %,Mn 94.5%, Ni 68.3 %, Pb 98.7 %, Fe 89.3 %) and were comparable to 20 and 30 %. Therefore, 30% dilution was selected for economic feasibility, since it was able to treat three times the volume of wastewater, compared to 10%, while the efficiency of removal decreased only by 2-4 percent. On the contrary, 40% concentration showed significant efficiency reductions (15–25%) and data variability (standard deviations increased from 2.3–3.3 to 2.9–3.8), indicating a previous low tolerance of algae to high metal concentrations (Agoun & Avci, 2025).

FTIR analysis revealed the biochemical interactions associated with metal concentration(Figure 5). With 40% of the 2920 cm^1, the expansion shows an increase in lipid content, suggesting that algae use lipid accumulation as a mechanism for membrane repair and energy storage, not for growth and metal binding (Alwaleed et al., 2025; Yang et al., 2025). Meanwhile, the amide peak decreases, indicating a decrease in protein content, while the carbohydrate peak decreases, further indicating a reduction in metal removal efficiency by 40% (Expósito et al., 2021).

3.5 Photoperiod Optimisation: Energy Requirements for Active Metal Sequestration

Chlorella vulgaris, as an obligatory photoautotrope, requires light energy to activate mechanisms for the absorption and accumulation of heavy metals. Accordingly, four light systems (6, 12, 18 and 24 hours of daily illumination) have been evaluated using white LED lamps that provide 60 W/m2 of illumination (120 µmol photons m^{-2} s^{-1}), while other parameters have been maintained at their optimal levels (pH 8, 30% wastewater, 100 mL/h CO_2). The following results are presented in Table 6.

Table 6. Effect of the photoperiod on the metal removal efficiency (%) of *Chlorella vulgaris*

Photoperiod	As	Cd	Cr	Cu	Mn	Ni	Pb	Fe
6 h	24.8±1.3	41.3±1.7	45.1±1.3	48.4±1.2	49.5±2.0	35.8±1.5	51.7±2.1	46.8±1.9
12 h	36.0±1.5	60.0±2.2	65.6±2.4	70.4±2.5	72.0±2.6	52.0±2.0	75.2±2.7	68.0±2.4
18 h	45.0±1.5	75.0±2.5	82.0±2.7	88.0±2.0	90.0±2.9	65.0±2.1	94.0±3.0	85.0±2.8
24 h	44.1±1.8	73.5±2.6	80.4±2.8	86.2±2.9	88.2±3.0	63.7±2.4	92.1±3.1	83.3±2.8

The optimal photoperiod (18 h) is highlighted. Continuous illumination (24 h) showed modest photoinhibition effects (2-3% reduction). ANOVA: $F_{3,8}$ > 156.4, p<0.001 for all metals.

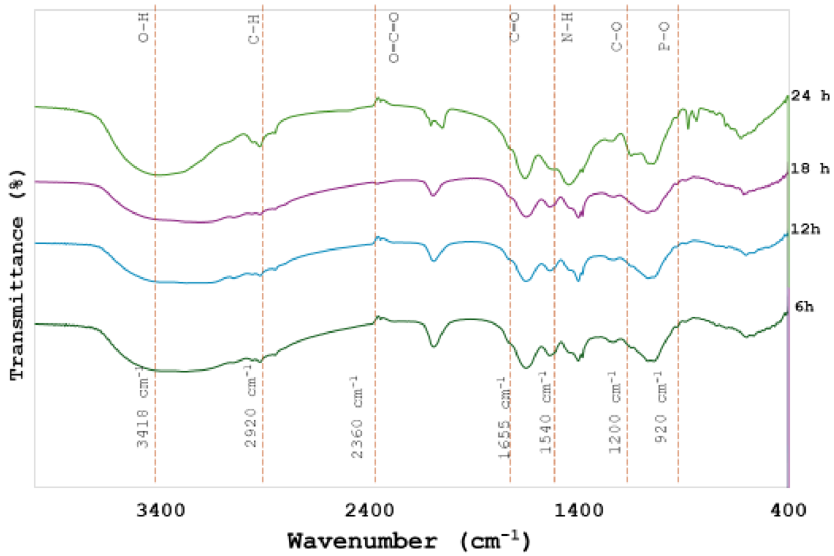

Figure 6. FTIR spectra of algal biomass exposed to 6,12,18,24 h light-exposure durations, showing changes in major functional groups associated with metabolic activity and metal-binding capacity

An 18-hour light period was identified as optimal, achieving the highest removal efficiency for metals (As: 45.0 %, Cd: 75.0%, Cr: 82.0%, Cu: 88.0%, Mn: 90.0%, Ni: 65.0 %, Pb: 94.0%, Fe: 85.0 %). However, 6 hours of daily lighting produced the lowest removal efficiency, with reductions of 45–50% compared to the 18 hour regime. This decrease is due to insufficient light energy for ATP production, which provides the cell energy needed for metal absorption(Cheng et al., 2015; Suresh Kumar et al., 2015).This explains the superiority of 18 hours over 12 hours of illumination, despite relatively small differences in removal efficiency, as shown in Table 4. At 24 hours of continuous illumination, a slight reduction in efficiency was observed, probably due to the impairment of the photosynthetic device known as photoinhibition. This phenomenon is due to excessive exposure to light without a black recovery period(Nishiyama & Murata, 2014; Wu et al., 2015). Algae generally require dark phases to perform essential processes such as cell respiration for ATP regeneration, as well as dark reactions to repair protons and build carbon skeletons(Zones et al., 2015).

3.6 CO_2 Enrichment: Biomass Enhancement and Carbon Sequestration Synergies

The availability of carbon dioxide constitutes a fundamental constraint on algal growth; therefore, we evaluated the addition of different CO_2 flow rates (0, 100, 500, and 1000 mL/h), while maintaining the other operating factors at their optimal levels (pH 8, 30% wastewater, and 18 h light exposure). The corresponding outcomes are summarized in Table 7.

Table 7. Effect of CO_2 supplementation on metal removal efficiency (%) by *Chlorella vulgaris*

CO_2 Level	As	Cd	Cr	Cu	Mn	Ni	Pb	Fe
0 mL/h	29.3±1.4	48.8±1.9	53.3±2.1	57.2±2.0	58.5±2.2	42.3±1.7	61.1±2.3	55.3±2.1
100 mL/h	38.3±1.5	63.8±2.3	69.7±2.5	74.8±2.6	76.5±2.7	55.3±2.1	79.9±2.8	72.3±2.5
500 mL/h	45.0±1.5	75.0±2.5	82.0±2.7	88.0±2.8	90.0±2.9	65.0±2.3	94.0±3.2	85.0±2.0
1000 mL/h	45.5±1.9	75.8±2.5	82.8±2.7	88.9±2.8	90.9±3.0	65.7±2.3	94.9±3.0	85.9±2.8

Optimal CO_2 concentration (500 mL/h**) is highlighted. Saturation evident at 1000** mL/h **(<1% additional improvement). ANOVA: $F_{3,8} > 243.7$, $p<0.001$.**

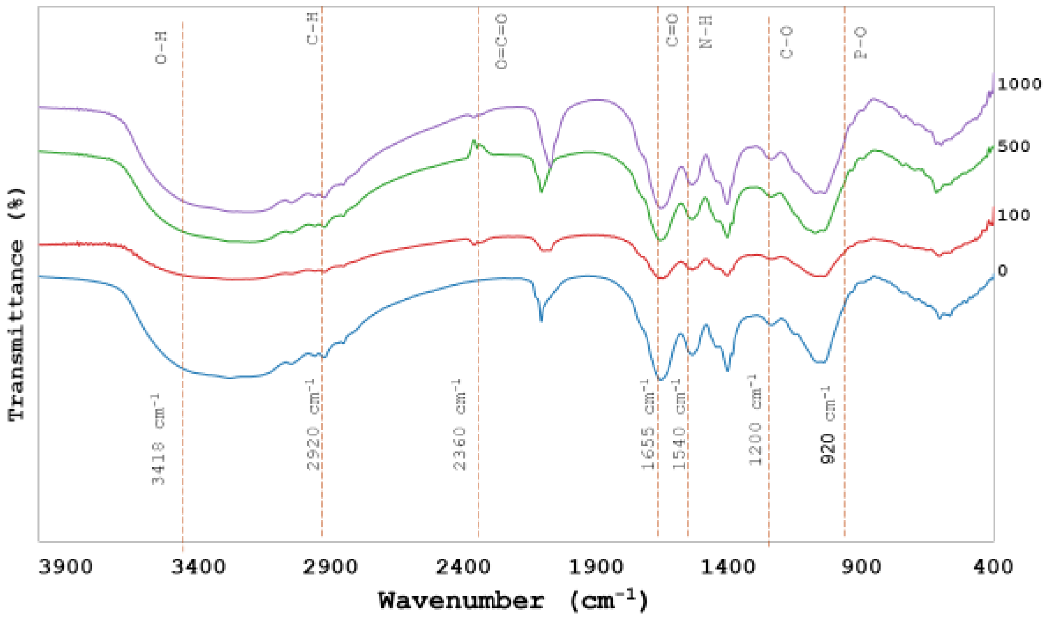

Figure 7 . FTIR spectra of algal biomass cultured under 0,100,500,1000 mL/h exposure durations and CO_2 levels, showing functional-group changes associated with metabolic activity and biochemical response to environmental conditions

The addition of carbon dioxide resulted in a significant improvement in the efficiency of heavy metal removal. On the other hand, the absence of additional CO_2 (air/ambient control) resulted in a significant decrease in efficiency of removal by approximately 30–40% compared to 500 and 1000 mL/h CO_2 treatments. The flow rate of 1000 mL/h CO_2 achieved the highest efficiency of removal; however, the improvement was not significantly different from that of treatment of 500 mL/h. Consequently, the level of 500 mL/h was considered the most appropriate from an economic point of view. However, when CO_2 is available from an environmentally-relevant source, such as recycled emissions from steel industry flue gases, the level of 1000 mL/h can be used. The enhanced removal efficiency observed at CO_2 concentrations of 500–1000 mL/h can be attributed to an increase in the saturation of the enzyme ribulose1,5 bisphosphate carboxylase/oxygenase (RuBisCO), the key enzyme responsible for carbon fixation during photosynthesis. Higher CO_2 availability promotes greater photosynthetic activity and biomass accumulation, thereby increasing the number of available metal-binding sites (Kao et al., 2012; Raven et al., 2014). In addition, FTIR analysis(Figure 7) revealed an increase in the peak of amide and an increased intensity of functional groups such as COO and P=O, together with a strong enhancement of the C–O absorption bands within the region 1000–1200 cm^1 that provides abundant ligands for metal biosorption (Gonçalves et al., 2016).

3.7 Biomass Composition and Treatment Efficiency

The relationship between the biochemical composition of biomass and the performance of metal removal was examined through Fourier transform infrared spectroscopy (FTIR) in this study (Figure 4, 5, 6 and 7). It is important to note that FTIR peak intensities provide semi-quantitative, not absolute, compositional information; accordingly, the following discussion refers to relative trends in spectral features rather than precise quantitative measurements of biomass composition. Under different treatment conditions, changes in the relative intensity of spectral peaks corresponding to lipids (C–H stretching at 2920/2850 cm^{-1}), proteins (Amide I at 1650 cm^{-1}, Amide II at 1540 cm^{-1}) and carbohydrates (C–O stretching at 1000–1200 cm^{-1}) suggested differential involvement of these cellular components in the metal binding process(Kyratzopoulou et al., 2025). Under optimal conditions characterised by high elimination efficiency, an improved protein content was observed with strong Amide I/II extension. This is due to the presence of proteins rich in metal-coordinating amino acids, such as histidine, cysteine, and glutamate(Kyratzopoulou et al., 2025) . Furthermore, changes in the phosphate peaks (900–1200 cm-1) suggest metal interactions with phospholipids and nucleic acids, and the expansion of the hydroxyl vibration region H (3400 cm-1) suggests metal interactions with free hydroxyl groups (Kyratzopoulou et al., 2025; Liu et al., 2022). In contrast, under stress conditions (40% wastewater concentration, a short photoperiod of 6 hours, and no CO_2), the following spectral patterns were observed: a decrease in the protein peaks reflecting cell damage and protein degradation in response to toxicity(Expósito et al., 2021); changes in the lipid profiles showing membrane damage or protection transformation; and a reduction in the carbohydrate content due to cell energy consumption to resist stress(Expósito et al., 2021).

3.8 Correlation Between FTIR Signatures and Metal Removal Efficiency

To identify which functional groups are involved in metal binding, all important spectral peak assignments were recorded and classified before and after metal exposure (Table 8). These include the O–H extension of about 3400 cm^1, which refers to hydroxy group in polysaccharides; the N–H and C–H extension of 2920–2850 cm^1 from lipid acyl chains; the C=O extension of 1650–1680 cm1 for proteins; and the N–H extension of 1540–1560 cm^1 for proteins. Other important signals: asymmetric COO stretching close to 1400 cm^1 marks carboxylate groups; P=O stretching close to 1230 cm1 relates to phospholipids or nucleic acids; and C–O stretching from 1000–1200 cm^1 highlights carbohydrates or polysaccharides. After exposing the samples to metals, consistent shifts were observed, usually 5 to 15 cm^1 in the direction of lower wave numbers. These changes show that metal ions interact directly with these functional groups, corresponding to (Liu et al., 2022). FTIR, however, provides structural, qualitative hints rather than solid quantitative evidence. To gain a deeper mechanical understanding, methods such as X-ray photoelectron spectroscopy (XPS) or acid-treated biomass analysis with ICP-MS, and I suggest that these methods be included in future work.

Pearson correlation analysis across all experimental conditions revealed strong quantitative relationships between FTIR peak intensities and metal removal efficiency. Carboxyl groups (COO^- at 1400 cm^{-1}) showed the strongest correlations ($r = 0.88$–0.93, $p<0.001$ for all metals), confirming their pivotal role in metal binding. Protein content (Amide I at 1650 cm^{-1}) exhibited very strong positive correlations ($r = 0.84$–0.91, $p<0.001$), reflecting the importance of amino acid side chains in metal coordination. Phosphate groups (P=O at 1230 cm^{-1}) also displayed strong correlations (r = 0.79–0.85, p<0.001), while carbohydrates showed moderate positive correlations (r = 0.73–0.78, p<0.01).

Importantly, lipid content showed negative correlations (r = –0.40 to –0.48, p<0.05), confirming its value as a stress indicator inversely associated with treatment efficiency. These relationships enable FTIR-based quality monitoring: biomass that met spectral criteria (Amide I > 70% of the observed maximum, COO^- > 75%, Amide I/Lipid ratio > 2.0) consistently achieved >85% removal of priority metals (Pb, Mn, Cu, Fe).

Table 8. Correlation coefficients (r) between FTIR peak intensities and metal removal

FTIR Peak (Functional Group)	Pb	Mn	Cu	Cd	Fe
Amide I (1650 cm^{-1}) Proteins	r = 0.89***	r = 0.91***	r = 0.87***	r = 0.84***	r = 0.86***
COO^- (1400 cm^{-1}) Carboxyl groups	r = 0.93***	r = 0.90***	r = 0.92***	r = 0.88***	r = 0.89***
P=O (1230 cm^{-1}) Phosphate groups	r = 0.82***	r = 0.85***	r = 0.80***	r = 0.79***	r = 0.81***
C-O (1000-1200 cm^{-1}) Carbohydrates	r = 0.76***	r = 0.78***	r = 0.75***	r = 0.73**	r = 0.77***
C-H (2920 cm^{-1}) Lipids	r = -0.45*	r = -0.48*	r = -0.42*	r = -0.40	r = -0.44*
O-H (3400 cm^{-1}) Hydroxyl/Polysaccharides	r = 0.71**	r = 0.74**	r = 0.68**	r = 0.66**	r = 0.70**

**** p<0.001, ** p<0.01, * p<0.05. Strong positive correlations (r>0.8, highlighted) indicate critical functional groups for metal binding. Negative lipid correlation reflects stress-induced accumulation under suboptimal conditions.*

CONCLUSIONS

This paper demonstrates that Chlorella vulgaris offers an effective and sustainable heavy metal removal system to remove the toxic heavy metal content in steel mill wastewater. Most heavy metals achieved maximum 7-day cumulative removal efficiencies of 69–91%, with lead removal exceeding 90%. pH 8 was identified as optimal, providing conditions for both surface biosorption and physicochemical co-precipitation of metal hydroxides. Maximisation of photosynthesis at an 18-hour photoperiod favoured growth and cellular homeostasis. Optimized dilution of 30 percent of wastewater provided a feasible engineering balance, and CO_2 supplementation of 500 mL/h increased metal removal up to 2.8 times compared to the ambient control.However, it should be noted that FTIR evidence is semiquantitative and structural. The confirmation of the accumulation of metals in harvested biomass by ICP-OES or ICP-MS analysis is strongly recommended as a critical next step to verify the biosorption mechanism. Concerning biomass exploitation, processed metal-laden biomass is not suitable for use as feed or food supplement due to the high concentrations of potentially toxic metals accumulated during treatment. The appropriate end-use of this biomass includes thermal chemistry conversion through pyrolysis and coprocessing in metal recovery plants. Before commercial use, pilot-scale validation and rigorous technological and economic analysis are required.

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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.

Availability of Data and Materials:

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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