Biosorption by green alga *Chlorella vulgaris* for decontamination of Pb(II) from wasters at near benign concentration

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Abstract

Lead ion, i.e., Pb(II), is one of the most toxic heavy metal ions (HMI) that constitutes a significant threat to aquatic ecosystems and human health. It is non-biodegradable and, therefore, could build up in human bodies to a harmful level via biomagnification and bioaccumulation, even when it exists at a very low level in the environment. Its presence in the environment is mainly due to human activities such as mining, burning fossil fuels, and industrial operations, which contaminate natural water sources through pathways such as precipitation of dust in the contaminated atmosphere, runoffs of contaminated soils, and discharge of contaminated wastewater. Corrosion of pipelines may also cause leakage of heavy metals into tap water. The levels of HMI, including Pb(II), in contaminated natural waterbodies are mostly at near-benign concentrations (10-100ppb), which is harmful but very difficult to decontaminate with conventional methods. This study investigated the potential of utilizing growing green alga *Chlorella vulgaris* in removing Pb(II) from water sources contaminated with near-benign concentrations (10-100ppb). The growth and behavior of *C. vulgaris* cells under Pb(II) exposure were examined. It was first established that the biosorption of Pb(II) by growing *C. vulgaris* in culture containing near-benign level Pb(II) was very similar to that of non-growing cells with short contact time. Then, biosorption tests were carried out with non-growing cells to analyze the effects of various factors such as pH, contact time, biomass concentration, and Pb(II) concentration. Results indicate that *C. vulgaris* exhibits promising biosorption abilities, with efficient removal of Pb(II) attributed mainly to surface adsorption mechanisms. PH played an important role in biosorption, with increasing pH enhancing biosorption in the tested range of pH 2.0- pH 7.0. The rapid adsorption kinetics with the plateau achieved within 5 minutes contact

time and the desorption of over 85.7% of lead from cells using EDTA buffer support the hypothesis that the biosorption of Pb(II) at near-benign levels is surface adsorption, which has been well reported for the biosorption of Pb(II) and other HMI by microalgae at high-level biosorption tests. Results of the study show that using *C. vulgaris* could effectively decontaminate Pb(II) contaminated water to meet drinking water standards within a well-defined operation window.

Key words: bioremediation, Lead pollution, near-benign level contamination, biosorption, microalgae

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Chapter 1. Introduction

Lead has been considered to be one of the most toxic heavy metal [1]. It is non-biodegradable and, therefore, could accumulate in human bodies through consuming contaminated water, agricultural products grown in tainted soil, as well as fishery products from polluted water[2]. Furthermore, lead could reach humans at dangerous levels through biomagnification moving up along the aquatic food web even when its level in an aquatic system is very low. Consequently, health authorities around the globe have set safe standards for lead to very low levels. For instance, the drinking water standards of lead content are set to 10 and 15 μg/L (i.e., ppb) by the World Health Organization (WHO) [12] and the Environmental Protection Agency (EPA) of the USA [13], respectively. Decontaminating waters contaminated by lead ions not vastly above these standards, i.e., the near-benign levels, is very challenging using conventional separation technologies. In this thesis, the near-benign level of lead is rather arbitrarily defined as 10 – 100 ppb Pb.

Lead is one of the most toxic heavy metals, which poses a significant risk due to unreversible build-up within the body. It may disturb the functions of almost every organ in the human body, primarily accumulating in human bones, brain, kidneys, and muscles [3]. It is considered to be both carcinogenic (Group 2B) and a neurotoxin to humans [6] and has been a threat to human health worldwide. For instance, according to an EPA report [4], over 250,000 children in the USA experienced measurable IQ losses from drinking lead-contaminated water.

With the increase of urban and industrial activities of humans, leakage of heavy metal ions (HMI), including lead, into water systems has increased tremendously in the past century[5].

Wastes containing HMI from can be discharged into the atmosphere, soil, and water[6], and all of them may eventually end up in the aquatic ecosystem by a variety of different pathways. These pathways include precipitation of dust from contaminated atmosphere and storm runoffs from contaminated soils. High levels of Pb ions have also been reported from service lines of tap waters due to the corrosion of pipelines. In Addis Ababa, Ethiopia, water showed elevated lead levels at an average of 62.6 ppb [9][10], from the twelfth century to the 1930s. The United Kingdom faced significant lead levels in drinking water, mainly derived from mining wastewater, with 10% of homes in England and 33% in Scotland consuming water with lead levels above 50 µg/L[11]. In Glasgow, Scotland, where the water was plumbosolvent, approximately 48.7% in 1981 exceeded 10 µg/L [4]. Prior to the 1986 US Pb ban, brass/bronze alloys also contributed to long-term exposure to lead leakage in drinking water [7]. For instance, the average lead concentration in water consumed over a one-week sampling period ranged from 1.1–30.7 ppb, with a median level of 4.8 ppb in Canada [8]. According to the WHO Water Development Report 2023, there are still 26% of the global population still suffers from unsafe drinking water services[14]. Consequently, more and more attention has been directed at the treatment of heavy metal-contaminated drinking water sources. In 2018, EPA declared a "war on lead" plan to address the water lead issue while acknowledging "there is no safe level of lead exposure".

Numerous separation technologies have been developed for the decontamination of heavy metal ions (HMI), including Pb (II). Some of the most typical ones include chemical precipitation, electro-precipitation, reverse osmosis membrane separation, nanofiltration, ion exchange, and adsorption. While the research and application of these technologies have been around for

decades, they all share two common downsides, i.e., high costs and incompleteness in removing HMI from contaminated waters when the Pb level is low [15]. Indeed, until recently, most studies on HMI decontamination using conventional technologies have been focused on wastewaters containing HMI contaminants at 1000 ppb or higher concentration, even though these levels are magnitudes higher than the drinking water standards set by the health authorities of different jurisdictions around the globe.

Decontamination of HMI using biosorption with living organisms as biosorbents presents a promising alternative [16], levering the self-propagation of living organisms. The contaminated natural waterbodies such as rivers and lakes are typically characterized by low contaminant levels usually in the near-benign range, vast volumes, and the capacity to sustain the growth of natural biosorbents, including photosynthetic organisms and heterotrophic microorganisms. Among these organisms, microalgae, with their high biomass productivity, rapid growth, and environmental adaptability, have shown particular effectiveness [17]. Of particular relevance, green alga *Chlorella vulgaris* is one of the most common microalgae species commercially grown nowadays, which is also widely found in the aquatic ecosystem. Numerous studies have been carried out to investigate its biosorption for the removal of organic compound[18] and HMI [17][19] including Pb ions[20][21], which has established it as an excellent biosorbent. However, to our best knowledge, all the previous studies have been carried out at Pb concentrations above 1000 ppb, i.e., beyond the near-benign range.

In this study, we explored the potential of *C. vulgaris* for bioremediation of water contaminated by Pb(II) at the near-benign concentration range by assessing the growth of *C. vulgaris* in medium containing near-benign level Pb(II), investigating the mechanism of Pb(II) biosorption,

studying adsorption kinetics, as well as the effects of different biosorption conditions on biosorption capacity, Pb(II) removal% and residual lead concentration. According to the experimental data, an operational window was established defining the boundaries of required biomass concentration for the treatment of lead contaminated water of given lead concentration to achieve the residual lead concentration that is lower than the drinking water standard, i.e., 15 ppb, per WHO. To achieve these objectives, the study was structured into three phases as follows:

1. Confirmation of biosorption ability and cell growth of *C. vulgaris* at near-benign Pb(II) levels;
2. Evaluation of the effects of a few key parameters on the effectiveness of Pb(II) biosorption using *C. vulgaris*: pH, contact time, concentration of lead ions, and concentration of biomass. This phase aimed to assess how variations in these parameters influenced the efficacy of Pb(II) ion uptake by *C. vulgaris* and make comparisons to the drinking water standard.
3. Analysis of biosorption mechanisms: In the 3rd phase, the mechanisms underlying the binding of Pb(II) ions to *C. vulgaris* cells were examined. This involved analyzing surface functional groups present on the algal cells and conducting kinetic studies to understand the Pb(II) ion and cell interactions. By elucidating these binding mechanisms, the study aimed to provide insights into the underlying processes driving the biosorption of Pb(II) ions by *C. vulgaris*.

The thesis is composed of five parts. Chapter 1 serves as a brief introduction, providing an overview of the thesis. Chapter 2 presents a literature review, focusing on the toxicity of heavy metal ions (HMI), in particular lead, and biosorption of HMI by microalgae, in particular *C. vulgaris*, for decontamination of contaminated waters. In Chapter 3, a detailed description of the laboratory protocol is provided, including the preparation of *C. vulgaris* culture, biosorption of

Pb(II) by growing and non-growing cells, and analytic methods. Chapter 4 presents the experimental data and discussions of these results, including near-benign concentration lead toxicity assessment, the influence of different parameters, including pH, lead concentration, and biomass concentration on the biosorption of lead, and exploration of the mechanism of binding through determination of binding on the cell surface. Finally, Chapter 5 offers a summary of the conclusions derived from the study results, along with recommendations on potential future research.

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Chapter 2. Literature review

2.1 Heavy Metals Materials

Heavy metals (HM) constitute a group of chemical elements known for their persistence in the environment and their detrimental effects on living organisms. They can be categorized into three groups [1] i.e.,: toxic metals (e.g., Hg, Cr, Pb, Zn, Cu, Ni, Cd, As, Co, Sn), precious metals (e.g., Pd, Pt, Ag, Au, Ru), and radionuclides (e.g., U, Th, Ra, Am). Some heavy metals, like zinc (Zn), copper (Cu), manganese (Mn), nickel (Ni), and cobalt (Co), are essential micronutrients necessary for cell growth. Others, such as cadmium (Cd), lead (Pb), and mercury (Hg), have no known biological functions [2]. All heavy metal elements could constitute environmental pollution when they exist in air, water, and soil above a certain threshold. Since they are not biologically degraded, bioaccumulation would happen in organisms having long-term exposure to contaminated environments. Furthermore, biomagnification would happen moving up along the food web in a contaminated ecosystem that would amplify the level of their threats tremendously. Sitting at the very top of the food web, humans could uptake health-threatening levels of HM even when they exist in the environment at trace amounts in the range of several to dozens of parts per billion parts (ppb). Consequently, the World Health Organization (WHO) and the health authorities in different nations have set strict regulations on the discharge of wastewater and drinking water standards concerning HM [3].

2.1.1 Health Impacts of Lead Exposure

Lead is one of the most toxic heavy metals, which poses a significant risk due to its capacity of unreversible build-up within the body. It may disturb the functions of almost every organ in the human body [4]. It is considered carcinogenic (Group 2B) to humans [5] and also a neurotoxin [6]. The half-life of Pb in the brain is 2 to 3 years, whereas in the blood, it is 30 days[7]. Encephalopathy is a condition where there is a progressive deterioration in the parts of the human brain, major manifestations of encephalopathy include headache, dullness, poor attention span, memory loss, and hallucinations within a few weeks of exposure. Studies have shown that a significant average IQ drop of 1.56 and 3.58 can be found in children with blood lead levels of 20-50 ppb (μg/L) and 50-100 ppb [8][9]and WHO concluded that for every 100 ppb increase in blood lead levels, IQ decreases by 1–5 points. Another survey study also shows that the mean blood lead level was 18-19 ppb among children and also found that children had neural development, linguistic and processing problems, decrement in memory power, and difficulty in comprehension of visuospatial skills [10]. Accumulation of lead may also result in abnormal bone matrix development in childhood and inhibits the synthesis of hemoglobin. Lead acts as a calcium analog in formation of bone[11], thus it is easily absorbed in people and effect any bioactivities utilize calcium, zinc, and iron. Pregnant women with low calcium, iron or zinc levels are prone to the effects of lead accumulation and pass on the adsorbed lead to her infant [12].

2.1.2 Bioaccumulation, biomagnification, and human exposure

Unlike many other contaminants, HM, like lead, is not biodegradable. Consequently, they tend to accumulate progressively over time through a process called bioaccumulation, increasing the potential for toxicity. Furthermore, in an aquatic system, the content of unbiodegradable toxins, including HM, in aquatic plants and animals may increase dramatically, moving up along the ladders of the food web. This process is referred to as bioaccumulation. The combined effects of bioaccumulation and biomagnification make it possible for unbiodegradable toxins to reach humans through the consumption of fishery products originating from waters containing very low concentrations of such toxins.

Atmospheric dust, automobile exhaust, polluted food, and contaminated drinking water are some of the key pathways for human exposure[13–15]. Drinking water with defection in purification and pipeline corrosion, and food in the canning industry are two of major sources of daily Pb intake due to their necessity and reachability [16].

Mining and fossil fuels result in lead exhaust releases into the atmosphere, which are recycled back down into the soil by rain and snow [17]. Disposal of lead-containing waste products, especially waste batteries, may also cause lead leakage, and natural water bodies will accumulate heavy metal ions (MHI) through atmosphere precipitation, soil runoffs and surface waters. Lead and other toxic HMI are not only harmful to humans. Once these are released into natural water bodies, they may disrupt the self-purification processes of aquatic systems and steadily accumulate, causing harm to microorganisms, aquatic plants, and animals[7]. This persistence and indestructibility of heavy metals have far-reaching environmental, public health, and economic impacts[14].

Research conducted in Pakistan in 2014 [18] suggested that local Pb ion uptake by humans are 65% from food, 20% from water, and 15% from air. In 2000-2004, Washington DC experienced a water lead (Pb) crisis when the disinfectants were switched from free chlorine to chloramine, causing the average drinking water lead contamination to rise up to 79 ppb. The crisis resulted in 4 times higher blood lead levels for children aged 1.3 years or less [19]. Pb service lines prior to the 1986 US Pb ban and brass/bronze alloys also contributed to the prolonged exposure to lead in drinking water in some regions due to lead leakage via corrosion [20]. Historically, humans utilized containers made from lead and consumed traditional medicines made with lead due to a lack of knowledge of the poisonous of this heavy metal[21].

The toxicity of lead and other HMI, in combination with bioaccumulation and biomagnification, makes them a realistic threat to human health even at extremely low contamination levels and health authorities around the globe have set the safe levels of them accordingly. For instance, the US Environmental Protection Agency (EPA) [22] and the World Health Organization (WHO) [3] have defined the maximum levels of Pb in drinking water to be 15 and 10 ppb (μg/L), respectively, to restrict human daily exposure to lead.

2.2 Heavy metal ion (HMI) decontamination methods

The existing treatment approaches for water contaminated with HMI involve various physicochemical mechanisms, including chemical precipitation, filtration, adsorption, and membrane separation.[23] These methods have traditionally been employed to eliminate toxic metals from wastewater and are widely used in water treatment plants. However, all these methods have their inherent limitations, including common issues such as low efficiency, incomplete metal removal, and the generation of toxic sludge (or other metal-containing waste

products) necessitating costly disposal procedures, driving the overall costs of treatment to high levels [24]. Studies also show that conventional techniques can not completely remove HMI, leaving residual HMI in treated waters at greatly lowered levels but nonetheless still higher than the strict standards set out by health authorities [25]. These drawbacks constrain the practical application of these conventional methods, particularly for the treatment of contaminated natural waterbodies, which are characterized by monstrous volumes and concentrations that are extremely low for removal by concentration approaches but nonetheless harmful to affected humans and environments. The key advantages and disadvantages of conventional techniques for metal removal are outlined in Table 1 [23].

Table 1 Comparison of HMI decontamination methods Reference:[26][27]

Method	**Advantage**	**Disadvantage**
Ion exchange	High effectiveness Possibility of metal recovery	Sensitivity to presence of particles High maintenance and operation costs
Membrane filtration	Low solid waste generation; Low chemical consumption	Membrane fouling; High maintenance and operation costs
Reverse osmosis	Pure effluent generation	High-pressure requirement
Chemical precipitation	No metal selectivity	A large amount of metal-containing sludge generated
Electrochemical treatment	No chemical requirements Tolerance to suspended solids	Expensive for high-concentration treatment; Requires floc filtration
Adsorption	High capacity; Fast kinetics	Adsorbent-dependent performance
Bioremediation	High efficiency; High selectivity [28]	Special environment conditions for growth; may require pretreatment

2.3. Microalgae for decontamination of HMI-contaminated waters

The bioremediation process has been established as a promising alternative way of treating HMI-contaminated wastewater and natural water bodies. It has been extensively studied for the last twenty years. It has also started to slowly apply in industry as a supplement to conventional methods, with benefits such as reducing the requirement for chemicals, lowering operating costs, and enhancing efficiency at low levels of contamination. [29] Various types of readily available and inexpensive biomass for metal removal, including microalgae, bacteria, fungi, and agricultural plant wastes, have been investigated.

2.3.1 Mechanisms of heavy metal removal using microalgae

Generally, the bioremediation of microalgae involves two mechanisms: cell surface adsorption and intracellular bioaccumulation. Cell-surface adsorption (extracellular) usually involves the binding of positive HMI onto negatively charged functional groups, which are abundant on the cell surface and are given the name biosorption. [30] The ion interaction usually takes place between the HM cations and the cell surface presence of hydroxyl (OH^-), carboxyl (-COOH), phosphate (-PO4), and sulfonic ($-SO_2H$) [13]. These interactions could be simply ion exchange or chelating. These processes are rapid and can take place in both living and dead cells as long as the cell surface fragments are not damaged.

The other mechanism is the internalization of the HMI (intracellular) when the cell completely engulfs the HMI. This process is known as bioaccumulation. This is a process that requires ATP and specialized ion channels. Research suggests that P-type ATPases are involved in the simulation of Pb(II), Cd(II), Cu(II), Co(II), and Ag(I) crossing the cell membrane [31].

Bioaccumulation is much slower than biosorption and will occur only with living cells that are active metabolically.

The first bioremediation process occurs in both living and nonliving cells since cell fragments also carry out negative charge on the membrane, whereas the second one is a much slower and weaker process and takes place only in living ones. Notably, research also suggests that the detoxification activity results in the binding of metal ions to proteins and may result in the precipitation of the HMI complex [25].

2.3.2 Toxicity of HMI to microalgae

It's worth noting that the accumulation of heavy metals doesn't consistently correlate with their toxicity, due to different algal species exhibiting varying reactions to different heavy metals, as well as different surface charges leading to different adsorption capacity on the cell surface (which is the first barrier before algae are exposed to heavy metal). After metal ions cross the cell membrane, the toxicity of heavy metals to algae stems from their capacity to generate high levels of reactive oxygen species (ROS). Heavy metals can induce oxidative damage by elevating cellular ROS levels directly [32] and by diminishing cellular antioxidant capacity[33] . Algal cells may suffer impairments in various biological functions, such as lipid peroxidation, protein oxidation, and nucleic acid damage.

2.4 Chlorella vulgaris for decontamination of HMI

C. vulgaris is a photosynthetic microorganism belonging to the *Chlorellaceae* family, which was discovered by the Dutch researcher Willen Beijrenick in 1890.[34] It is a unicellular green alga, having spherical cells with a diameter of 2 to 10 micrometers. A mother cell would divide to produce four daughter cells, resulting in a very high rate of growth. Rapid growth, easy and flexible terms of culture, and resistance to unfavorable factors are some advantages that make these microalgae appropriate for various applications in the food industry, aquaculture, cosmetics, pharmaceutical, wastewater treatment, and biofuel production. It is also a popular food additive in Japan. Research has proved the high remediation effect on heavy metal ions in wastewater. It has been a very successful module in the field of heavy metal contact. The study on tolerance of *C. vulgaris* on metallic ions started in 1965[35] and the first attempt of utilizing *C. vulgaris* for bioremediation was in 1984 [36]. Since then, *C. vulgaris* has been a popular choice for bioremediation.

A more recent study reported the toxicity of in short- and long-term Pb(II) exposure on *C. vulgaris* and *Chlorella protothecoides*[37]. Results showed that Pb(II) of 50-80 mg L^{-1} could significantly inhibit the growth and chlorophyll synthesis of both algae in almost all the treatments and dose-response relationships could be clearly observed. The EC50 of short-term exposure was in the range of 24-120 h, corresponding to a Pb(II) range of 67.73-172.45 mg L^{-1}. Whereas the long-term exposure had EC50 of 7-28 days for Pb(II) concentration of 50.41-63.91 mg L^{-1}. Not surprisingly, the long-term exposure demonstrated a much stronger toxicity to both algae. It was reported that the activities of superoxide dismutase (SOD) and catalase (CAT) activities of both algae after exposed to medium to high level of Pb(II) were significantly promoted, and their response might be more susceptible in short-term exposure. Both SOD and CAT are important defense mechanisms of cells against harms from strong oxidants such as

reactive oxygen species (ROS). Similarly, Rahman and Sathasivam [66] found that high level of HMI induced the secretion of antioxidant enzymes, including SOD, glutathione peroxidase (GPX) and ascorbate peroxidase (APX)[38] These data suggest that the toxicity of HMI, including Pb(II), could be related to the promotion of oxidants such as ROS in cultures. Numerous mechanisms have been proposed for reducing HM toxicity in organisms. For instance, studies suggest that algal tolerance to HM is highly dependent upon the defense response against possible oxidative damages.

2.5 Factors affecting the biosorption of HMI on Microalgae

Understanding the factors that influence the biosorption of heavy metals by microalgae is crucial for optimizing bioremediation processes. In this extensive section, we explore various factors in detail.

2.5.1 Conditioning of microalgae for HMI decontamination

Microalgal biomasses of different forms have been investigated for the decontamination of HMI, including Pb(II). Molazadeh et al. [39] studied Pb(II) adsorption from contaminated water by dried *Chaetoceros sp*. and *Chlorella sp.* at different adsorbent dosages (0.2, 0.5, 1.5 and 2 g/L), pH (3, 4, 5, 6, 7, 8), temperatures (20, 25, 30, 35, 40 °C), contact times (30, 60, 90, 180, 360 minutes), particle sizes (20, 40, 60, 100, 140 mesh) and Pb concentrations (20, 40, 60 mg/L). The study reported a Pb(II) removal efficiency of 78% with *Chlorella sp.*, which was significantly higher than the 68% removal obtained with *Chaetoceros sp.* Sun et al. [40] studies the HMI

adsorption by the dry powers of cyanobacterium *Spirulina platensis*. The maximum amount of Pb^{2+} biosorption was reported as 253 mg/g.

Bayramoğlu et al. [41] evaluated the capacities of immobilized microalga *Chlamydomonas reinhardtii* to remove Hg(II), Cd(II), and Pb(II) ions from aqueous solutions using bare Ca-alginate bead as a control system. Effects of pH, temperature, initial concentration of metal ions, and biosorbent dosages on the adsorption of Hg(II), Cd(II), and Pb(II) ions were studied. Adsorption capacities of 384.4, 88.6, and 116.8 mg/g were reported for the adsorption of Pb(II), Hg(II), and Cd(II) ions at an initial HMI concentration of 200 mg/L. It should be mentioned the adsorption capacities of alginate beads were 285.7, 31.4, and 38.9 mg/L for Pb(II), Hg(II), and Cd(II), respectively.

Das et al. [42] investigated the suitability of the residual biomass of green algae *Phormidium sp.*, a microalgal strain meant for biodiesel production to remove lead (Pb2+) ions from aqueous solution in both batch-type stirred system and a semi-batch-packed bed adsorber. The biosorption equilibrium was established in 40 min. The maximum removal efficiency of Pb^{2+} on *Phormidium* sp. at equilibrium was reported to be 92.2% at pH 5.0, with an initial Pb2+ concentration of 10 mg/L and an adsorbent dosage of 4 g/L.

Giarikose et al. [43] compared the capacities of *Neochloris minuta* and *Neochloris alveolaris* grown in nitrogen-rich (+N) and nitrogen-depleted (-N) media for biosportion of five HMI, i.e., Pb^{2+}, Cd^{2+}, Zn^{2+}, Cu^{2+}, and Ni^{2+}. When comparing the two types of algae, *N. alveolaris* grown in either +N or -N media had higher adsorption capacities (q_{max}) for all five metals than *N. minuta*. In both algae, nitrogen depletion (-N) caused an increase in the q_{max} values for Zn^{2+} and Cu^{2+}. Additionally, the max of *N. minute* for Cd^{2+}, Zn^{2+}, Cu^{2+}, and Ni^{2+} increased by nitrogen depletion, demonstrating that the treatment can be applied to improve the biosorption capacity of

a particular alga for multiple heavy metals. The biosorption capacity of these algae for heavy metals was also discussed in terms of their biomass compositions and the type of hard or soft metal acid according to the Pearson theory of Hard-Soft Acid-Bases (HSAB).

Our group[44] compared the biosorption of two HMI ions, i.e., Pb(II) and Cd(II) by three different forms of the biomass of *C. vulgaris*, i.e., wet, dried, and residual biomass after extraction of lipids. The Pb(II) biosorption capacities of residual, wet, and dried biomass were 262.29, 114.85, and 82.81 mg/g, respectively, and that of Cd(II) were 55.13, 33.21, and 21.41 mg/g, respectively. The biosorption capacities of the residual biomass for Pb(II) and Cd(II) were 3.18 and 2.58-fold of that of dried biomass. The vastly larger adsorption capacities of residual biomass in comparison to that of dried biomass were tentatively explained by a hypothesis arguing that the internal wall surface, which was made accessible for the residual biomass, has a similar or larger adsorption capacity than the outer surface of the cell wall.

Table 2 lists a few examples of algal biomass processed into different forms, including dried algal biomass, residual algal biomass, and immobilized microalgae, for the adsorption of Pb(II). While the removal efficiency ranges between 67% and 92.2%, it is worth noting that all the experiments were carried out in the Pb(II) concentration range of 10-500 ppm, which was magnitude above the near-benign level of contamination.

Table 2 Comparison of different microalgal species for decontamination of Pb(II) at 25 ºC

Microalga	**Biomass**	**pH**	**Pb(II) (ppm)**	**Time (min)**	**Removal %**	**Ref.**
Chaetoceros sp.	Dry	6	20	180	60	[39]
Chlorella sp.		6	20	180	78	[39]
Chlamydomonas reinhardtii	Ca-alginate Immobilized	6	500	120	-	[41]
Phormidium sp.	Residual	5	10	40	92.2	[42]
Spirulina platensis	Dry	5	50	240	69	[40]
Neochloris oleoabundans	Wet, dry, residual	6.5	50	30	90	[44]
Neochloris alveolaris	Dry	5	50	60	87	[43]
Neochloris minuta	Dry	5	50	60	67	[43]

2.5.2 Effects of Biomass Concentration

The amount of HM removed by microalgae from aqueous solution is greatly affected by biomass concentration, as it increases the presence of negatively charged groups on the surface[45]. However, the removal of HM metals is not linear proportional to the concentration, since the amount of Pb(II) adsorption per unit mass of cells would decrease as the biomass concentration increases. This is expected since for a given Pb(II) concentration, the amount of Pb(II) available per unit mass of algal cells would decrease with the increase of biomass concentration. This may be further explained by the partial aggregation of biomass that reduces the effective surface area available for sorption as well as by a decrease of the average distance between available adsorption sites, which makes it hard for lead ions to bind [46][47].

2.5.3 Biosorption pH

pH levels play a pivotal role in heavy metal biosorption by microalgae. [46][48][49]

In principle, pH affects the deprotonation of the functional groups on the cell surface, which are negatively charged when the suspension pH is above their corresponding pKa values. Since the composition of surface functional groups varies among algal species, pH has different impacts on the adsorption Pb(II) and other HMI by different algal species [46] [50]. At low pH, functional groups are associated with protons, i.e., H^+ ions, and the binding of metal ions is therefore very weak. As pH increases, those functional groups become deprotonated and, therefore, negatively charged, allowing the binding of HMI ions. However, it should be noted that, with the increase of pH, Pb(II) will start forming insoluble $Pb(OH)_2$ when the solution pH is 8 or above. [51] [52] Therefore, both acidic and basic pH should be avoided in this set of experiments.

Aung *et al.* (2013) [45] showed that pH 6 at a temperature of 30°C on the absorption of lead metal ions by *C. vulgaris*, with an initial concentration of 0.1 M, had the highest removal efficiency, reaching 99.4% while another species, i.e., *Spirogyra sp.*, showed a maximum amount of adsorption at pH 5 [46].

In the case of utilizing live cells, it also influences the growth and behavior of the algae. For *C. vulgaris*, the suitable pH is within the range of 6-8 [53].

2.5.4 Temperature

Temperature plays an important role in adsorption. For example, Aksu *et al.* (2002) [54] have utilized *Chlorella vulgaris* and reported an increase of Nickel(II) sorption capacity with the increase of temperature from 15 to 45 °C and contact time of 60 min, but conflicted with the result of Lau *et al.* (1999) [55] which they suggested a decrease of metal uptake with the temperature range of 15-40 °C and a contact time of 3h, where the maximum is achieved at 15 °C, suggesting the metal surface interaction as an exothermic process. This difference in result could be because of the effect of contact time and growth conditions. On the other hand, different algal species also behave very differently, Gupta *et al.* (2008) [46] suggested a negative correlation between temperature and Cd(II) using *Oedogonium sp*., on other species of algae. There are also research results suggesting some HMI adsorption is fully independent of temperature within the tested temperature range [49][48].

2.5.5_Competition of other metal ions

In real-life practice, contaminated wastewater or natural waterbodies contain more than one metal ion. For example, significant levels of Zn^{2+}, Cr^{2+}, Cu^{2+}, Cd^{2+}, Pb^{2+} and Fe^{2+} were found in the groundwater of Hong Kong[56] . Biosorption of targeted HM ion can be significantly affected by the presence of another metal ion in solution, adsorption isotherm analysis of a Cu^{2+}/Ni^{2+} adsorption on *C. vulgaris* experiment done by Lau *et al.* (1999) [55] suggested that it is due to competition of adsorption sites. Gu & Lan [57] investigated the biosorption of *N. oleoabundans* (wet biomass)on five strategically selected bivalence HMI, i.e., Pb(II), Hg(II), Zn(II), Cd(II) and Cu(II). By introducing the concept of impact factor, which was defined as the ratio of the electronegativity and radius of an ion, it was demonstrated that the adsorption

capacity of *N. oleoabundans* biomass to the tested bivalence HMI was proportional to the electronegativity and inversely proportional to the radius of the HMI. Furthermore, Gu & Lan [58] reported that univalent metal ions such as Na^+ and K^+ had much larger affinity and adsorption capacity than bivalent metal ions such as Pb(II), Cd(II), and Zn(II) for biosorption onto wet *N. oleoabundans* biomass. Consequently, high concentration of univalent metals could substantially reduce the adsorption of bivalent HMI.

2.5.6 Desorption:

Based on the cell surface structure with negative charges, the metal adsorbed on the surface can be desorbed under appropriate conditions or a desorbing solution. There are two widely used methods of separating heavy metal ion from the cell surface, one is lowering the pH of the solution the other one is to wash by desorbing reagent e.g. EDTA solution. As we mentioned in the pH section, the metal ions with positive charge were attached to the cell surface; lowering the pH causes protonation of the negatively charged functional groups on the cell surface and causes displacement of the attached ion back into the solution. Research has reported that the highest efficiency of desorption can be found using HNO_3 [57] and HCl [58], but the acid treatment damages the cell surface and causes a decrease in metal biosorption ability after the treatment. In comparison, cells treated with EDTA solution don't seem to lose their function during the desorption process as well as show a similar desorption ability compared to decreasing pH [59] . EDTA, as a strong chelating agent, forms a soluble chelate-metal complex and extracts HM ions from the cell surface [60]. Utilizing 0.1 M EDTA Solution for desorption of heavy metal can be found in many research[61][62][63]. In fact, EDTA solution is a medication used in the

treatment of heavy metal toxicity as the binding of metal to EDTA is extremely strong and prevents further toxic effects [60].

2.6 Other applications of C. vulgaris.

Due to *C. vulgaris* 's fast growth rate and easy control conditions, Extensive research has shown that *C. vulgaris* is effective in removing inorganic materials from wastewater, including nitrogen, phosphorus, and ammonia.[64]. Studies have shown for a 2 days test time *C. vulgaris* is capable of removing 86% inorganic N and 70% inorganic P[65].

Apart from its use in heavy metal treatment, *C. vulgaris* finds applications in various fields due to its rapid growth and easily controllable growth conditions. Additionally, beyond bioremediation, *C. vulgaris* is a widely utilized algae species in biodiesel and biogas production, as well as in the production of supplementary food and animal feed.

2.7 Knowledge Gaps

Lead ion pollution in drinking water sources poses a significant threat due to its high toxicity and bioaccumulation. Prevention remains the primary approach to mitigate lead poisoning, as there is no effective treatments for lead poisoning. Various species of microalgae have been utilized as promising candidates for wastewater treatment. Microalgae as a bioremediation tool have been demonstrated to have great potential in comparison to conventional approaches due to their fast growth rate and the potential to treat large volumes of water, such as contaminated rivers and lakes, at relatively low cost, leveraging its self-propagating capacity. Furthermore, numerous studies have been carried out to understand the mechanisms of surface binding based on negatively charged functional groups and intercellular accumulation, and we have garnered

knowledge on manipulating different factors to facilitate the biosorption of different HMI, including Pb(II) by a few microalgal species in different processed forms. However, most of these studies were carried out at HMI levels that are magnitudes higher than the drinking water standards issued by different health authorities around the globe.

Therefore, understanding the behaviors, influencing factors, toxicity, and adsorption potentials of microalgae, such as commercially important *C. vulgaris*, under near benign concentrations of lead ions, is warranted.

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Chapter 3. Methodologies

3.1 Algal culture and medium

3.1.1 microalgal strain and media

The microalga, green alga *C. vulgaris* (UTEX 2714), was purchased from the UTEX Culture Collection of Algae at the University of Texas, Austin. Modified Bold's Basal Medium (MBM) was used throughout the study for the cultivation of *C. vulgaris* cells. One liter of MBM consisted of the following nutrients: 0.175g KH_2PO_4, 0.025g $CaCl_2•2H_2O$, 0.075g $MgSO_4•7H_2O$, 0.25g $NaNO_3$, 0.075g K_2HPO_4, 0.025g NaCl, 0.01g $Na_2EDTA•2H_2O$, 0.0062g KOH, 0.00498g $FeSO_4•7H_2O$, 0.0115g H_3BO_3, and 1 ml/L of Trace Metal Solution were added as well, 1L of Trace Metal Solution containing 2.86g H_3BO_3, 1.81g $MnCl_2•4H_2O$, 0.222g $ZnSO_4•7H_2O$, 0.390g $Na_2MoO_4•2H_2O$, 0.079g $CuSO_4•5H_2O$, 0.0494g $Co(NO_3)_2•6H_2O$. The pH of the medium was adjusted by 0.1 N H_2SO_4 and 0.1 N NaOH to 6.8 or as indicated in the text. The medium was sterilized at 121 °C for 15 minutes before inoculation.

3.1.2 Preparation of seed culture:

To prepare a new seed culture, tips full of *C. vulgaris* existing in seed culture gel were transferred to a sterile 500 mL cultivation bottle containing 250 mL MBM. It was then incubated in a box illuminated with white LED light strips going through a 12h light-12 h dark cycle at 25 °C. The light was initially given by 1000 lux (with Aluminum foil cover) for the starter culture and stabilized at 15000 lux with the growth of the culture. The flask was shaken gently with a magnetic stirrer.

3.1.3 Cultivation of C. vulgaris

For *C. vulgaris* culture, 600 mL fresh MBM was added to 1 L cultivation bottles and autoclaved at 121 ^{o}C for 15 min with the bottle head. The sterile bottle was then cooled to room temperature and inoculated with 50 mL seed culture before incubating under the same conditions of the seed culture. The culture in a bottle was agitated with a magnetic stir and aerated with CO_2-enriched air at a flowrate of 0.5 vvm, which was first passed through a bottle of deionized water for moisturization and then filtered through a 0.47 μm microfilter. The CO_2 fraction in the aeration stream was adjusted to control culture pH at the setting value as indicated in the text.

3.2. Pb(II) biosorption by growing cells

For Pb(II) biosorption by and toxicity to growing cells, the seed culture was inoculated to sterile MBM medium to a starting optical density of 0.1 OD_{700}. An appropriate volume of stock PbCl2 solution was added right after the inoculation of the seed culture to an initial lead concentration as specified in the text. Biomass concentration and lead adsorption were measured daily.

3.3 Pb(II) biosorption kinetic

In the experiments of Pb(II) biosorption kinetic, *C. vulgaris* biomass in culture was harvested by centrifugation at 6000 rmp (4193 RCF) for 5 min. The pellets were resuspended in sterile MBM to establish a biomass concentration as specified in the text. Then, appropriate volume of stock $PbCl_2$ solution was added to a pre-determined lead concentration. The mixture was then thoroughly mixed by vertexing for 30-60 s. Sampling was conducted at intervals of 5 minutes, 15 minutes, 30 minutes and 60 minutes for measuring of Pb(II) biosorption. To note that in this operation condition, 5 minute is the minimum contact time that could measure.

3.4 Lead biosorption by non-growing cells

Total lead biosorption: A sample of 50 mL cell culture in biosorption by growing cells or suspension in biosorption by non-growing cells was centrifuged at 6000 RPM (4193 RCF) for 10 min and the pellet was dried overnight at 60°C in a vacuum oven. Then, the dried cells were digested overnight using 1 mL concentrated HNO_3 solution (~68%). The digestate was then centrifuged at 14500 RPM (14100 RCF) (MiniSpin Plus, Eppendorf). The supernatant was then subjected to analysis using Flame Atomic Absorption Spectrometry (FAAS), utilizing 10 mA Lead lamp (iCE 3000 FAAS, Thermo Scientific). The total lead biosorption q_e (µg/g) by biomass at equilibrium was calculated from C_0 (µg/L), which was the concentration of Pb(II) in the digested sample subjected to FAAS analysis and the biomass concentration of the 50 mL culture sample in adsorption using growing cells or the cell suspension in adsorption using nongrowing cells (x, g/L):

$$q_e = \frac{C_0}{50x} \quad (1)$$

The residual Pb(II) concentration in the supernatant after adsorption, i.e., Ce (µg/L), was calculated by mass balance using the following equation:

$$C_e = C_i - x^* q_e \quad (2)$$

3.5. Analytics Methods

3.5.1 Measurement of Biomass Concentration

The biomass concentration within the culture was assessed through optical density measurements using a spectrophotometer (Genesys 10S UV-Vis) set to 700 nm. Samples were obtained and

subsequently diluted several times using deionized (DI) water. Afterward, the samples underwent centrifugation at 6000 RPM (4193 RCF) utilizing the Allegra V-15 Centrifuge by BECKMAN COULTER. The resultant samples were then subjected to overnight drying at 60°C. To establish the relationship between optical density (OD) and biomass, a standard curve was created by the equation: $x = K \times OD_{700}$, where x is biomass concentration (g DCW/L) and K the conversion factor. For this study, the K value determined according to a pre-established standard chart was 0.375 g/L.

3.5.2 Extracellular lead adsorption and intracellular lead uptake by cells

The extracellular lead adsorption of cells was measured by washing off the surface-bound Pb^{2+} ion without affecting the intracellular lead[1]. Briefly, cells of 50 mL culture in biosorption of growing cells or suspended in biosorption by non-growing cells were harvested by centrifugation, and then 1 ml of 0.02 M EDTA solution was added to the pellets. The mixture was vortexed and then incubated at room temperature for 5 minutes. The supernatant was collected by centrifugation and then subjected to FAAS assessment to determine the content of lead adsorbed on the surface of the cells. The Extracellular lead adsorption and intracellular lead uptake were calculated using the following equations:

3.5.3 Chlorophyll content of cells

The algae cells were collected by centrifuging 1 mL cell culture at 14500 RPM (14100 RCF) for 5 min. The pellets of biomass were collected and then mixed with 1 mL of 95% methanol in the dark at 4 ◦C for 24 h. Then the mixture was centrifuged again at 14500 RPM (14100 RCF) for 5min. The absorbance of the supernatant was measured at 647 and 664.5 nm wavelengths using

95% methanol as the blank. The following formula was used for calculating chlorophyll contents [2]:

$$\text{Chlorophyll a} = [16.72 \times OD_{665.2}] - [9.16 \times OD_{652.4}] \quad (3)$$

$$\text{Chlorophyll b} = [34.09 \times OD_{652.4}] - [9.16 \times OD_{665.2}] \quad (4)$$

3.5.4. FTIR spectroscopy

The Cary 630 FTIR spectrophotometer was used to analyze the cell surface. Samples with or without exposure to lead were dried overnight at 60 ^{o}C to form round, thin flakes for FTIR analysis.

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Chapter 4. Results and Discussions

4.1 Effect of pH on cell growth and lead removal

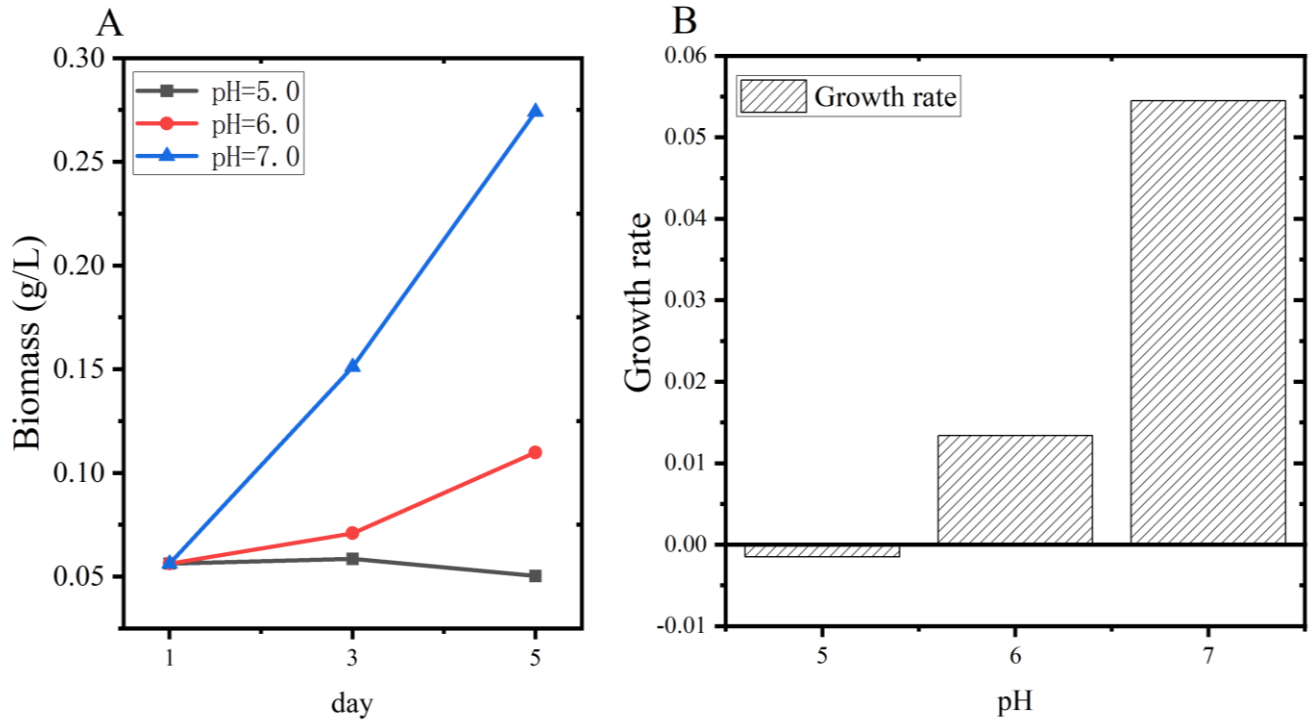

Figure 1 Effects of culture pH on cell growth of C. vulgaris: biomass concentration time course profile (A) and average cell growth rate (B).

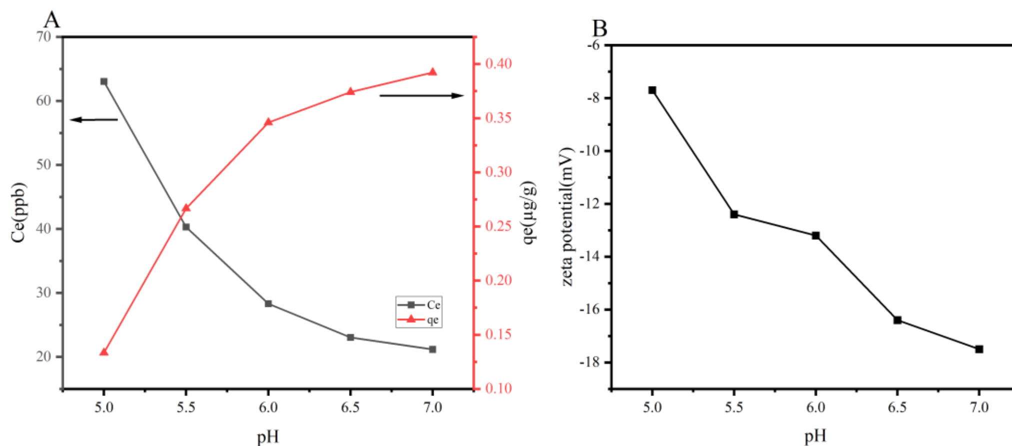

Figure 2. Effect of buffer pH on equilibrium lead adsorption (q_e) and residual lead concentration in supernatant (C_e) (A) and zeta potential of cells (B) in adsorption tests of 30 minutes contact time. The mixture contained 100 ppb Pb(II) and biomass 0.3 g/L.

The role of pH in the growth of *C. vulgaris* and its binding of lead is critical, as it influences various aspects of the adsorption process. The adsorption pH exerts profound effects on the deprotonation of functional groups and, therefore the surface charges involved in the adsorption process[1].

The growth profiles of *Chlorella vulgaris* incubated for 5 days at culture pH of 5 to 7 are shown in Figure 1. It can be seen that the growth of *C. vulgaris* experienced inhibition when the medium pH was 6 or lower. Based on previous studies [2], the controlled pH of 7.5-8.0 is the most suited, resulting in the highest growth rate; inhibition of cell growth was observed at pH 6.9 or below and 8.3 or above [3].

As shown in Figure 2A, the residual Pb(II) concentration in supernatant (C_e) decreased and the equilibrium lead adsorption (q_e) increased with the increase of adsorption pH in the range of 5.0 - 7.0. As shown in Figure 2B, the zeta potential of cells changed from -7.9 to -17.9 mv when cell suspension pH increased in the same range of 5.0-7.0, which can be attributed to the

deprotonation of functional groups on cell surface. At high buffer pH, proton concentration is low and more functional groups would be deprotonated, leading to more negatively charged functional groups interacting with metal ions such as Pb(II).

Therefore, to maximize the efficiency of adsorption experiments, taking into account the effects of pH on the cell growth and Pb(II) adsorption, and avoid any precipitation of lead ions due to an alkaline environment, a pH level of 7.0 was selected and maintained to ensure optimal conditions for the growth, biological activities, and adsorption capacity of *Chlorella vulgaris* [4].

4.2 Measurement of lead's effect to C. vulgaris

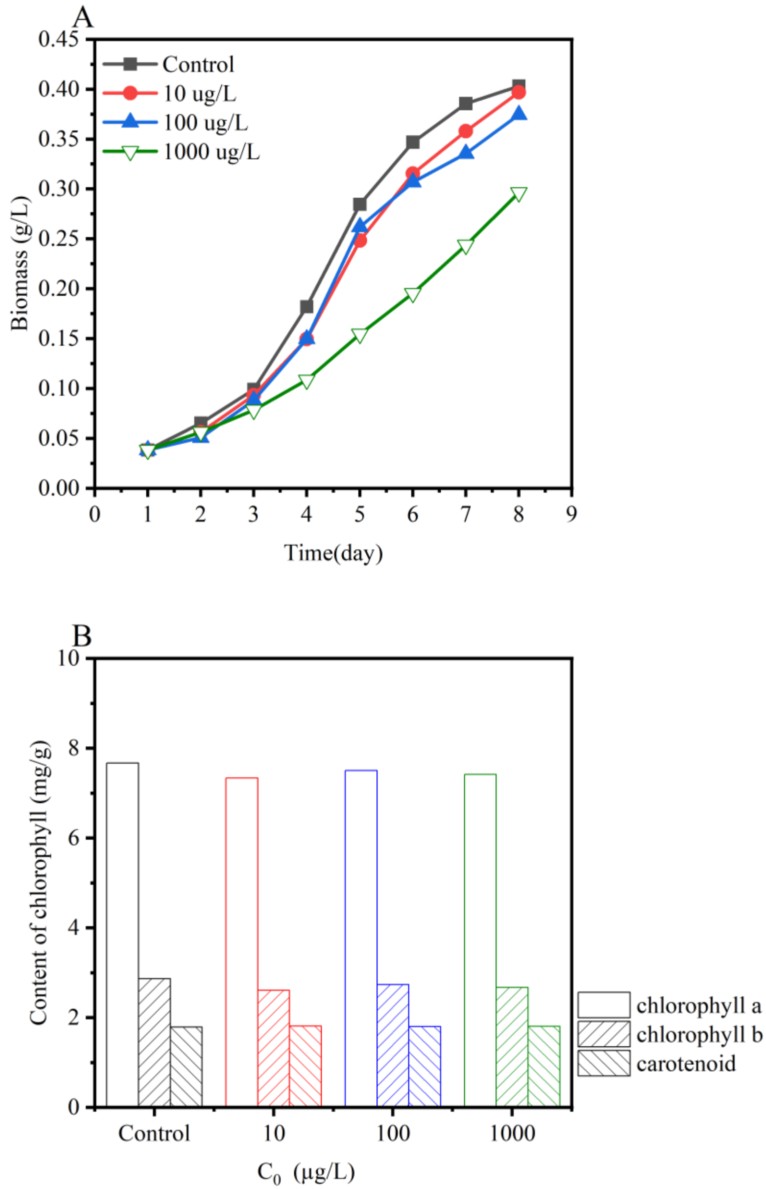

Figure 3 Biomass concentration profile (A) and chlorophyll cell content profile (B) of C. vulgaris at the end of 8-day cultivation with a varied initial concentration of Pb(II) in the medium at pH 7.0.

As shown in Figure 3a, the growth of *C. vulgaris* in cultures containing up to 100 ppb of Pb(II) only showed slight inhibition to cell growth while cultures containing 1000 ppb of Pb(II) exhibited substantial growth inhibition. This observation suggests that Pb(II) concentrations at the near-benign level would only have a moderate impact on *C. vulgaris* under the tested conditions. Different microalgal species have very different tolerance to lead. For instance, Debelius et al. [5] studied the toxicity of lead to five different marine microalgae, i.e., *Tetraselmis chuii, Rhodomonas salina, Chaetoceros sp., Isochrysis galbana (T-iso)* and *Nannochloropsis gaditana*, and reported half maximal effective concentration (ranging from 0.5 µM (105 ppb) for *Chaetoceros sp.* to 13 µM (2640 ppb) for *T. chuii* [5]. EC50 is the concentration of a toxin that would cause the specific growth rate of microalgae to reduce by 50%, which has been widely used to characterize the toxicity of toxins to algal cells. As for *C. vulgaris,* an earlier study reported no inhibition to cell growth at 1000 pb Pb(II) and significant inhibition at 5000 ppb or above[6]. In another study, Bajguz and Godlewska-Zylkiewicz [7] observed a negligible inhibitory effect of lead at a concentration of 1 µM (approximately 207 ppb) after 48 hours of incubation and significant inhibition at 10-100 µM. However, all these concentrations mentioned above are much higher than the near-benign concentration (10-100ppb) used in the experiment.

The levels of photosynthetic pigments in microalgal cells have been widely used as an indicator of various types of stress and senescence. For instance, Bajguz and Godlewska-Zylkiewicz [7] reported a negligible impact on the chlorophyll contents of C. vulgaris of lead at a concentration of 1 µM (207 ppb) and a significant reduction of chlorophyll cellular contents at 10 (2070 ppb)

and 100 μM (20700 ppb) Pb(II). The results are compatible with our observation that there are no detectable impacts on the chlorophyll content of *C. vulgaris* at Pb(II) of 1000 ppb or below.

In summary, the results presented in this section demonstrate that Pb(II) concentrations up to 100 ppb, i.e., in the near-benign range, do not significantly hinder biomass growth in *C. vulgaris*, and photosynthetic activities remain stable. Considering the almost negligible inhibitory effect of near-benign levels of lead exposure on the growth and behavior of *C. vulgaris*, it can be concluded that the presence of lead ions does not affect *C. vulgaris* cells at this level. The tolerance of *C. vulgaris* to Pb(II) at near-benign levels makes it potentially effective for bioremediation of lead-contaminated waters. Utilizing growing cells can take advantage of the self-propagation of microalgae for treating vast volumes of contaminated natural water bodies such as lakes, rivers, or bays.

4.3 Mechanism of lead biosorption at near-benign concentration: extracellular surface adsorption or intracellular uptake?

FTIR spectra of *C. vulgaris* cells exposed to 80 ppb Pb(II) for 30 min at room temperature are shown in Figure 4. Blueshift of the peak at 1340 cm^{-1}, which corresponds to P=O bond, indicates the likely involvement of phosphoryl functional groups in Pb(II) adsorption. Phosphoryl groups could be found in hybrid biomacromolecules such as phospholipids and phosphoproteins, which are abundant on cell wall [1]. Furthermore, redshift was observed with peaks corresponding to the C=O in amide group and C=O bond in carboxyl group, as well as the C-O-C bonds. A common feature of these functional groups is that they all possess oxygen atoms, which offers negative or partial negative charges due to the significantly larger electronegativity of oxygen

(3.44) in comparison to other atoms, i.e., phosphorus (2.19) and carbon (2.55). This negative charge abundance would facilitate the binding of Pb(II) to the *C. vulgaris* cell surface[8].

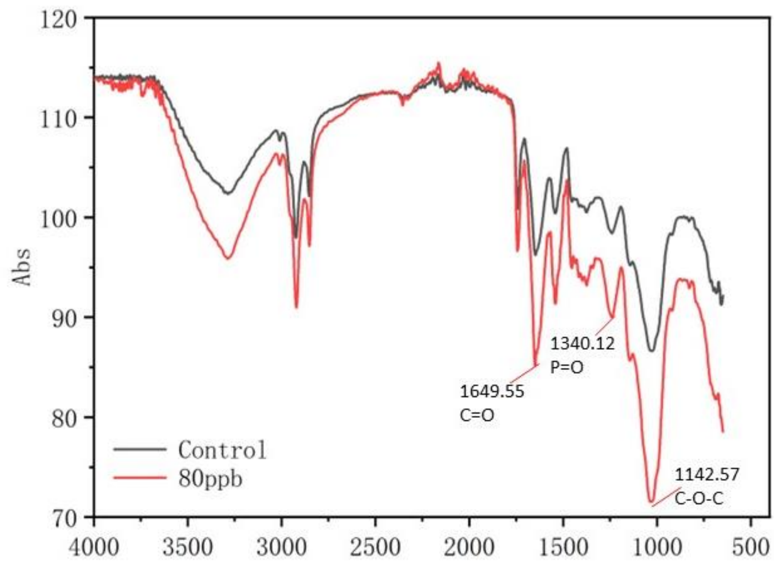

Figure 4 FTIR spectra of C. vulgaris cells and cells treated with 80 ppb Pb(II) solution

Table 3 FTIR absorbance redshift of bonds that might be involved caused by Pb(II) adsorption [8]

Cells	Cells loaded with Pb (CM^{-1})	Redshift (CM^{-1})	Bond	Ref.
1146.3	1142.57	3.73	C-O-C	[8]
1340.14	1346.67	-6.53	P=O	[9]
1649.55	1647.68	1.87	C=O (Amide)	[10]
1744.61	1743.68	0.93	C=O (carboxyl)	[10]

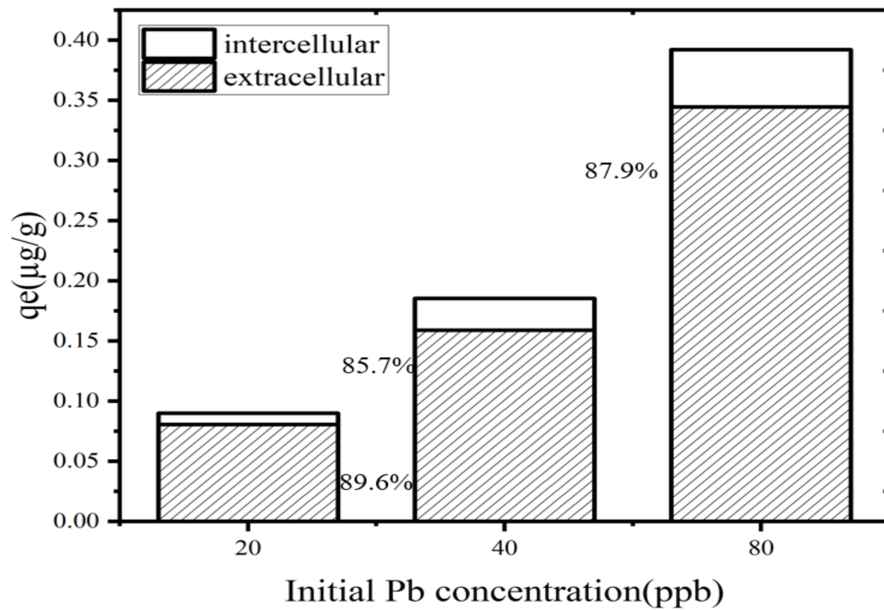

Figure 5 Extracellular and intracellular Pb(II) after exposure of cells to 80 ppb Pb(II) for 30 min

An EDTA washing experiment was conducted to determine the extent of lead adsorption on the surface. According to Expósito et al., 2021, the Pb(II) exhibits a strong interaction with EDTA

by chelating, making EDTA an efficient agent capable of washing out lead from cell surface while leaving intercellular lead unaffected [11]. Following 30 min of lead exposure and a 5-min EDTA washing period, both dissolved lead from EDTA supernatant and intracellular metal from cell pellet were measured and the results are shown in Figure 5. Approximately 90% of the adsorbed lead was effectively removed through EDTA incubating. Additionally, intracellular lead adsorption increased with the rise in lead concentration. These data indicate that the biosorption of Pb(II) at near-benign levels was primarily achieved through surface adsorption due to the excess amount of biomass. The FTIR data, which suggests the occurrence of ion-dipole interactions between the lead metal ion and the negatively charged functional groups on cell surface, are in accordance to this hypothesis.

4.4 Biosorption of Pb(II) by growing cells and nongrowing cells: similar behaviors

Figure 6 compares the lead adsorption of growing cells vs non-growing cells at different biomass concentration in the range of 0.03-0.335 g/L with an initial Pb(II) concentration of 80 ppb and adsorption/culture of pH 7.0. For adsorption by growing cells, the seed culture was transferred to a fresh medium containing 80 ppb Pb(II) to an initial biomass concentration (0.05 g/L), and the culture pH was maintained at pH 7.0. The biomass concentration and residual Pb(II) concentration were measured daily. On the other hand, the tests with nongrowing cells were carried out by mixing appropriate volumes of stock Pb(II) stock solution, pre-harvested fresh cells, and deionized water to 80 ppb Pb(II) and certain biomass concentration and incubating for 30 min before the residual Pb(II) in supernatant was measured. As shown in Figure 6, all the three examined parameters, i.e., Pb(II) removal (%), lead adsorption (q, mg/g), and residential

lead concentration (*C*, mg/L) showed similar trends of dependency on biomass concentrations for both growing cells and nongrowing cells. The data of Figure 6a indicates that both growing and nongrowing cells exhibit a linear increase in the percentage of lead removal with increasing biomass concentration. The similar slope K values of the linear regression fit suggest that regardless of whether the culture is actively growing or not, the trend of lead removal percentage increase remained consistent. Continuing to Figure 6b, the adsorption of lead on the unit mass of biomass decreased sharply with the increase of biomass concentration of both growing cells and nongrowing cells at the low biomass concentration range and then continued to decrease gradually at the relatively high biomass concentration range. Similarly, the residual lead concentration had similar trends of decreasing with the increase of biomass regardless of the means of biomass concentration increase, either by cell growth, i.e., self-propagation, or by addition of nongrowing cells. The fact that adsorption of Pb(II) by growing and nongrowing cells demonstrates similar dependency on biomass concentration has both practical and theoretical significance. From a practical perspective, we can rest assured that we can use data from adsorption by nongrowing cells, which is much easier to control and tremendously more time efficient than adsorption by growing cells, to replace tests using growing cells. Meanwhile, adsorption by growing cells is of more practical significance in the application scenario of treating vast volumes of contaminated lakes and rivers, taking advantage of the fast self-propagation of algal cells. Theoretically, these similar trends imply that the adsorption mechanisms, or at least the primary adsorption mechanisms, are not dependent on cell growth, confirming the aforementioned hypothesis that surface adsorption is the primary mechanism for the biosorption of lead ions by algal cells.

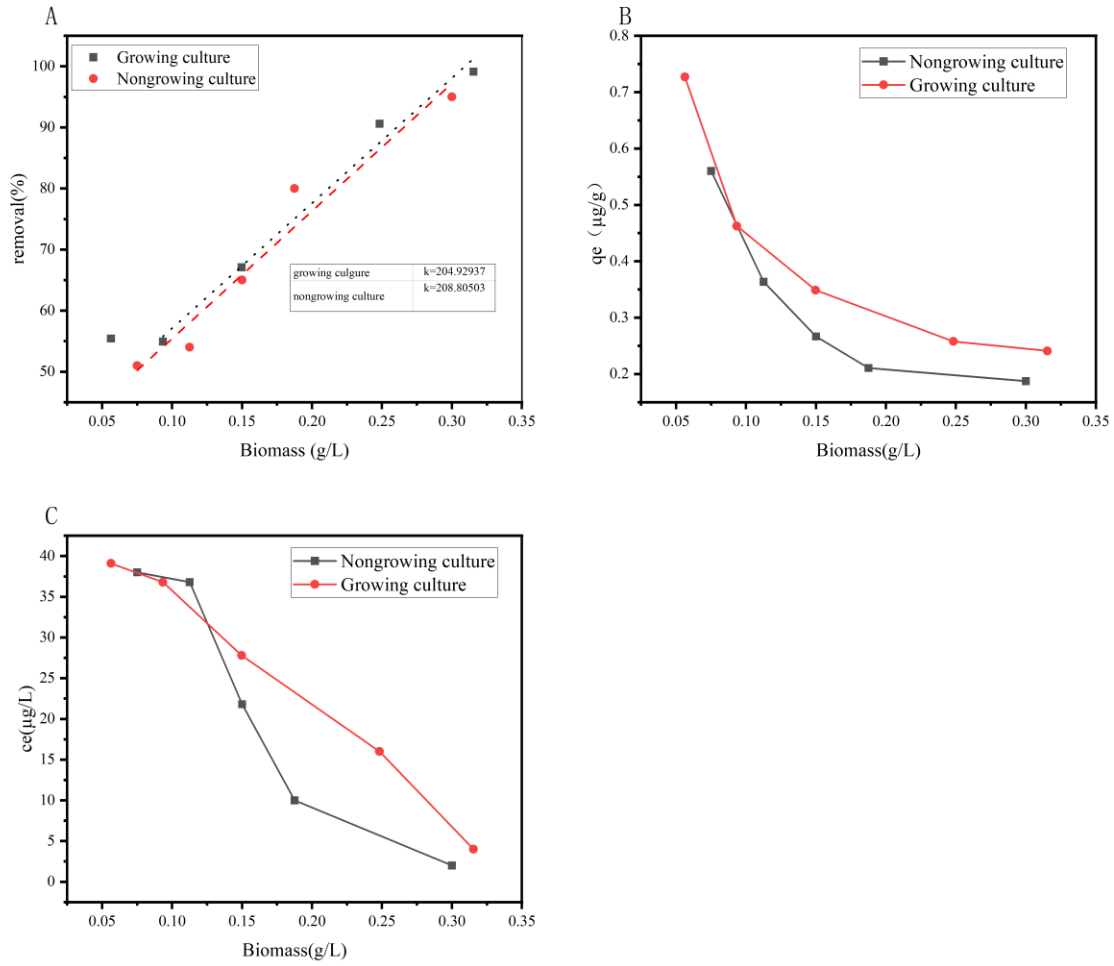

Figure 6 Dependance of Pb(II) removal (A), residual Pb(II) in supernant (B), and Pb(II) adsorption on unit biomass (C) on biomass concentration at pH 7.0 and 80 ppb initial Pb(II) concentration: comparision between growing and nongrowing cells.

4.5 Biosorption Kinetics

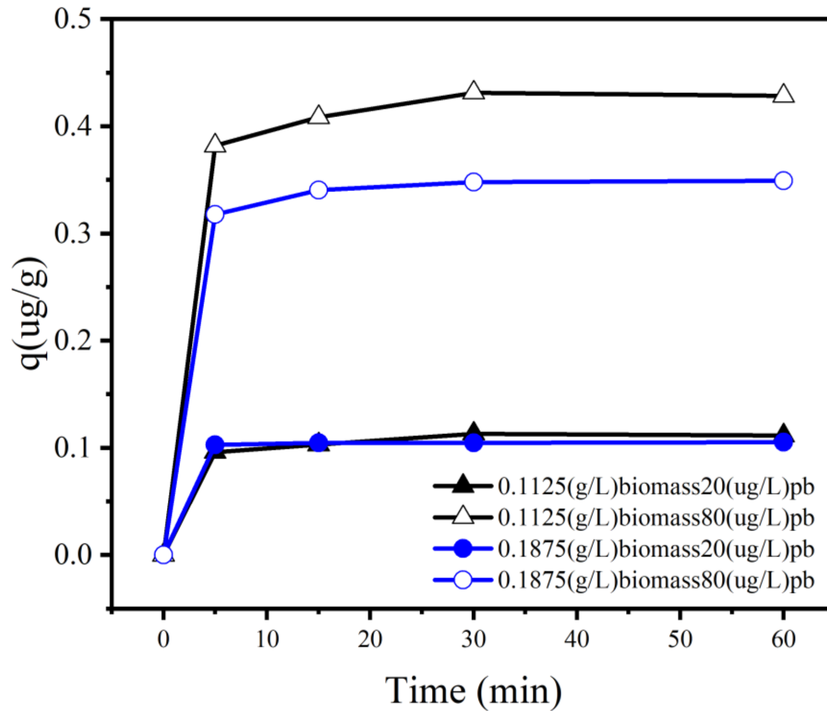

Figure 7 Biosorption kinetics of Pb(II) by nongrowing cells at different biomass concentrations and initial Pb(II) concentrations: residual Pb(II) concentration in supernatant

Figure 7 shows the results of the kinetics of Pb(II) adsorption at two different combinations of initial lead concentration and biomass concentration. Data were collected at 5, 15, 30, and 60 min of incubation. the adsorption q_e plateaued within 5 min for all the test runs, which was the shortest incubation time we could manage since 5 minutes were required for sample transfer and centrifugation. In other words, the adsorption plateaued in less than 5 minutes.

The fast adsorption kinetics of lead ions support the hypothesis that the biosorption of lead ions by algal cells is predominantly achieved by surface adsorption since the uptake of Pb(II) needs positive transportation across the phospholipid bilayer of the cytoplasmic membrane, which is a slow process requiring ATP and specialized ion channels.

Furthermore, as shown in Figure 7, the kinetics and the plateaued values of Pb(II), i.e., q_e, were very similar when compared at the same buffer Pb(II) concentration of 20 ppb. The q_e with 0.1125 g/L biomass concentration (0.45 μg/g) was substantially higher than that with 0.1875 g/L biomass concentration when the buffer Pb(II) concentration was 80 ppb. In the meantime, when compared at the same biomass concentration, the q_e with 80 ppb Pb(II) was always significantly higher than that with 20 ppb Pb(II). These observations can be explained by the fact that the testes were carried out at extremely low Pb(II) levels, i.e., 20-80 ppb. At a Pb(II) concentration of 20 ppb, the binding sites, i.e., the functional groups on the algal cell surface, would be overwhelming to the available Pb(II) ions even at the relatively low biomass concentration of 0.1125 g/L. Therefore, elevating biomass concentration to 0.1875 g/L did not impact the adsorption kinetics and q_e significantly. In other words, the Pb(II) adsorption was independent of biomass concentration while dependent on Pb(II) at such systems of extremely low Pb(II) concentration and relatively high biomass concentration.

4.6 Operation Window

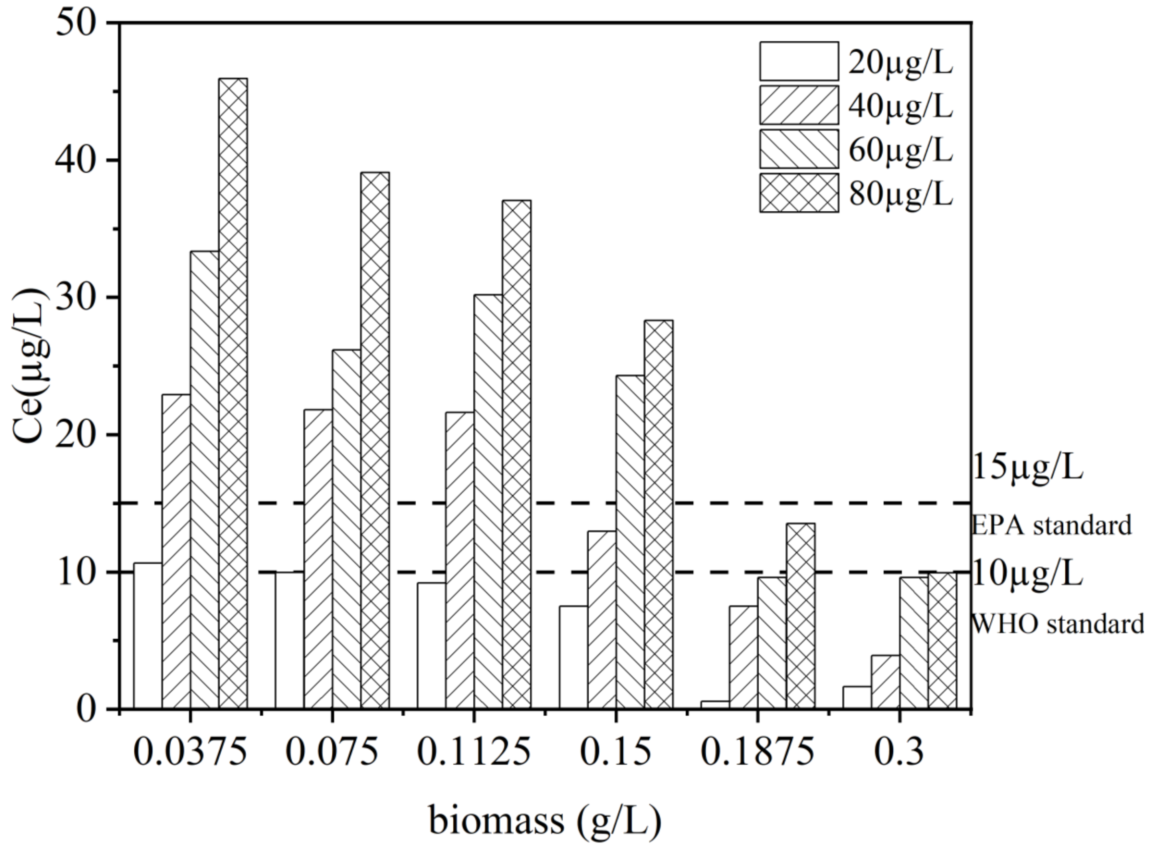

Figure 8 Operation window of residual Pb(II) concentration after 1 hour of adsorption under different biomass and initial Pb(II) concentration.

The similarity in adsorption mechanisms between the two scenarios suggests that the data generated by the adsorption of lead with nongrowing cells, which is time-saving and easy to control, could be representative of lead adsorption by growing cells, which has more application potentials in restoring contaminated natural waterbodies such as lakes and rivers, which is

typically contaminated by lead or/and other heavy metal ions in the near-benign range, leveraging the fast self-propagation of microalgae. Therefore, adsorption data from different initial lead and biomass concentrations with a 30-minute contact time with nongrowing cells were gathered to establish an operation window for lead removal. Two benchmarks were used, i.e., the EPA drinking water standard of 15 ppb lead and the WHO standard of 10 ppb. As shown in Figure 6, Pb(II) concentration is the key to the decontamination of lead ions from the solution, and a higher level of lead contamination would require a larger biomass concentration to ensure the treated water reaches the standard. For example, when the contaminating level is 20 ppb lead, then a biomass concentration of 0.0375 g/L or above would be sufficient to reduce the residual lead concentration to below 15 ppb, but 0.075 g/L biomass would be required to drive the lead concentration to below 10 ppb. In the meantime, a waterbody contaminated by 80 ppb lead would require biomass concentrations of 0.1875 and 0.30 g/L to reach the standard of 15 and 10 ppb lead, respectively.

Chapter 5. Conclusions and Future Works

This study demonstrates that *C. vulgaris* living cells offers a promising and potentially effective solution for reducing Pb(II) concentrations from waters contaminated by near-benign levels of Pb(II) to below drinking water standards. Our findings suggest that the efficiency of lead removal increases proportionally with the concentration of cell biomass, indicating a promising operational window for implementation. Notably, the insensitivity of lead toxicity at near-benign concentrations enables living *C. vulgaris* cells to grow normally during long-term exposure and adsorption process. Moreover, the adsorption kinetic results revealed comparable biosorption behaviors between growing and non-growing cells, with surface adsorption identified by the FTIR and EDTA washing experiment as the predominant mechanism facilitated by negatively charged functional groups on the cell surface. We also observed a significant influence of adsorption pH on both cell growth and adsorption efficacy.

This environmentally friendly and sustainable approach holds promising results for reducing lead contamination in water sources within the near-benign levels. Further exploration into the potential of other algal species, such as *N. oleoabundans*, and filamentous species such as cyanobacterium *S. platensis* is warranted to broaden our understanding of algal-based bioremediation processes. By continuing to investigate microalgae bioremediation methods, the advance of efficient and eco-friendly solutions for water pollution caused by heavy metal contaminants like lead can be done.

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