How *Chlorella sorokiniana* and its High Tolerance to Pb Might be a Potential Pb Biosorbent

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Abstract

The link between the acute toxicity of heavy metals on algae and the bioadsorption capacity of heavy metals by algae has seldom been reported. In the present study, an acute toxicity experiment was carried out to assess the toxic effects of Pb, Cu, and Cd for *Chlorella sorokiniana*, and the 96 h IC_{50} values were 0.249 mg/L, 0.485 mg/L, 46.108 mg/L, and 21.00 mg/L for Cu, Cd, Pb (total), and Pb (free ion), respectively, which implied that *Chlorella sorokiniana* showed high tolerance to Pb compared to Cu and Cd. Pb distribution analysis indicated that 73.40% to 98.15% of free Pb ions were accumulated on the algae cell wall to avoid further intracellular accumulation, resulting in irreversible metabolic disorders. Then the adsorption capacity of *Chlorella* was explored. It could be found that the Langmuir model (the R^2 were 0.988 and 0.962 for living and lifelss cells, respectively) was fit to explain the adsorption equilibrium data and the q_e calculated by this model were 1.54 and 2.97 mg /10^{10}cells for living and lifeless cells, respectively, which was consistent with the experimental result. In the competitive adsorption, *Chlorella* exhibited a greater affinity for Pb with the higher removal rate compared to Cu and Ni. Therefore, the renewable *Chlorella sorokiniana* and its dramatic resistance to Pb may serve as a potential biosorbent for Pb in the future.

Keywords: *Chlorella sorokiniana*, Pb, high tolerance, adsorption capacity

Introduction

With the dynamic development of industrialization and urbanization, the entrance of heavy metals from industrial discharge, domestic sewage, and atmospheric deposition into natural water, even at low concentrations, has induced a series of potential impacts on both aquatic ecosystems and human health [1]. Being an essential part of primary productivity, algae show high sensitivity to various heavy metals such as Pb [2]. Consequently, algae serve as a testing model to evaluate the quality of freshwater and estimate the toxicity of pollutants. By now, numerous studies have illustrated that most heavy metals, including Cd, Pb, Cu, and Ni, can interfere with the photosynthesis and enzymatic metabolism of algae, which results in growth inhibition and even death. Choudhary et al. found that Pb, Cu, and Zn could generate reactive oxygen species (ROS) and attack biomolecules within the cells, further leading to irreversible adverse effects on *Spirulina* [3]. The analogous toxic mechanism of Cu on the green

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algae *Chlamydomonas reinhardtii* was also discovered by Jiang et al. [4]. At sub-lethal concentrations, Ouyang et al. discovered that the addition of Cu at 5 μmol/L in algae culture vastly inhibited the growth of *Chlorella*, and similar inhibitory phenomenon were also observed in the same concentration for Cr [5]. Nevertheless, compared with other heavy metals such as Cd, Cu, As, and Sb, Pb with a higher IC_{50} to *Chlorococcum* seems to be less hazardous in aquatic systems [6]. Similarly, the algae growth inhibition test aimed at assessing the toxicity of Zn, Cu, Cd, and Pb demonstrated that Zn showed the most toxic effects and Pb had the least lethality for *Isochrysisgalbana* [7].

Many previous studies have concluded that one of the mechanisms responsible for heavy metal detoxification is that heavy metals are accumulated in algae in the form of chelate, i.e., the cell wall with abundant polysaccharides can dramatically adsorb a large proportion of heavy metals, preventing them from entering into the cell to cause further damage. Macfie and Welbourn found more than half of the total heavy metals aggregated on the cell surface of unicellular green algae (*Chlamydomonas reinhardtii*) in the exposure of Cd, Co, and Ni [8]. The research of Subramaniyam et al. has also indicated that the polysaccharides present in the cell walls of algae played a major role in the bioaccumulation of metal ions by algae [9]. Andrade et al. proved that a higher amount of polysaccharides of cell walls were synthesized by brown algae (*P. gymnospora*) living in heavy metal-contaminated waters than in normal conditions to avoid intracellular accumulation [10]. Therefore, it can be inferred that the difference of heavy metals in toxic effects on algae can be partly attributed to the adsorption ability of the cell wall on heavy metals. In addition, algae with a high tolerance to one kind of heavy metal may serve as the bioadsorbent to this metal.

Accordingly, being a renewable natural biomass, algae with ample functional radicals, including amino, carboxyl, hydroxyl, and carbonyl, has attracted increasing attention in the removal of heavy metals from wastewater as a bioadsorbent [11-12]. Yalcin et al. found that dried marine brown macro algae *Cystoseirabarbata* showed the highest adsorption capacity for Pb with the highest calculated equilibrium adsorption quantity (q_e), compared to Cd and Ni [13]. Maznah et al. reported that the biosorption capacity of *Chlorella* for Cu and Zn were 33.4 and 28.5 mg/L, respectively [14]. Praveen et al. also indicated that algae *Kappaphycus alvarezii* provided high uptake for Pb, Cd, Cu, and Ni [15]. In addition, in the research of Zakhama et al., *Ulvalactuca* algae was proven to be a successful adsorbent in the removal of Ni, Cu, and Cd, and especially for Pb [16]. However, previous studies focused either on the heavy metal detoxification mechanism or on adsorption capacity, and there exists a lack of information about the relationship between these two parts. The objectives of the present work are to study the acute effects of Pb, Cd, and Cu on *Chlorella sorokiniana* to reveal the mechanism of algae with higher tolerance to Pb compared with other heavy metals, and to investigate the adsorption

properties and selectivity adsorption capacity of *Chlorella* on Pb.

Material and Methods

Algae Culture and Biomass Measurement

The algae *Chlorella sorokiniana* were cultured under a 16:8-h light/dark photoperiod at 25±1ºC. The pH of this medium is maintained at approximately 6.5. The algae cell density measured by the microscopic cell counter was closely correlated to optical density at 684 nm (OD_{684}), and a fine linear relationship was observed between the biomass and the optical density on *Chlorella*. Therefore, the cell counts were measured by a uv-vis spectrophotometer (UV-3100PC, MAPADA, China). To exclude possibility of the disturbance of heavy metals on the OD_{684}, the cell count was confirmed by the microscopic cell counter in each treatment.

Algae Growth Inhibition Test

Prior to the exposure experiment, algae were cultivated in the batch experiment through transferring and re-cultivating in culture medium once every four days to ensure that the experimental algae were in logarithmic phase [17]. The initial algae density was maintained at 2.13×10^6 in 80 mL.

The exposure concentrations of Pb, Cu, and Cd ranged from 0 to 80 mg/L , 0 to 0.64, and 0 to 1.25 mg/L, respectively. There were three replicates for each treatment. The culture vessels were shaken three times a day to keep the algae in suspension and to promote the exchange of CO_2. The biomass of *Chlorella* in each incubator was detected at 24, 48, 72, and 96 hours. The 96h-IC_{50} (half inhibitory concentration at 96h) of each heavy metal were calculated through the software SPSS 16.0. The free Pb ion in different treatments was calculated by Visual MINTEQ, due to those parts of Pb forming precipitation in the medium.

Pb Distribution

Algae in exponential phase were inoculated in the culture medium containing different concentrations of Pb from 2.5 to 25 mg/L. After four-day exposure, approximate solution taken from each sample was directly digested for total metal measurement. Meanwhile, an aliquot of 15 mL solution taken from each sample was centrifuged at 3,500 rpm for 10 min, and approximate supernatant containing water-soluble Pb was directly digested. The remaining biomass was washed with EDTA (4 mM) for 10 min twice to remove metal precipitations and metals adsorbed on the cell walls. After centrifuging, the supernatant containing EDTA-changeable Pb representing Pb free ion and precipitation on the cell wall was mixed. Subsequently, we collected the residual biomass containing intracellular Pb. All the samples

were digested with HNO_3 (65%) at 120ºC. The whole digestion liquid was properly diluted with deionized water and measured by an atomic absorption spectrophotometer (TAS-990, Persee, China). Three replicates were propelled in this section.

Adsorption of Pb with *Chlorella*

Adsorption Equibirium

For all absorption experiments, the cation in culture medium was substituted to chloride to avoid the formation of Pb precipitation. *Chlorella sorokiniana* were used as sorbent in the removal of Pb and the adsorption efficiency of living or dead algae cells was tested in this study. Fresh algae biomass were harvested by centrifuging at 3,500 rpm for 10 min and washed with sodium chloride solution (0.024%) three times. The collected algae were then re-dispersed in sodium chloride solution (0.024%) for Pb adsorption. The algae were heated at 55ºC for 45 min to produce dead algae cells. A 25 ml solution containing living or dead cells was exposed to different concentrations of lead chloride in 100 mL conical flasks. These flasks were shaken for four hours under 180 rpm at 25ºC. At 0 and 4 hours, an aliquot of 6 mL solution was filtered with 0.45 μm filter membrane, followed by the atomic absorption spectrophotometer (TAS-990, Persee, China) to measure the Pb concentration of filtrate. A control experiment in the absence of adsorbent was conducted to decide whether there exists any adsorption on container walls [18]. There were three replicates for each treatment.

Absorption capacity was evaluated by the following equation:

$$q_e = \frac{(c_o - c_e)V}{N} \tag{1}$$

…where C_o is the original metal concentration (mg/L), Ce is the equilibrium metal concentration (mg/L), V is the volume of the solution (L), N is the number of algae cells (10^{10}cells), and q_e is the quantity of Pb absorbed by per unit number of algae.

The removal rate was calculated through the expression:

$$R = \frac{c_o - c_e}{c_o} \tag{2}$$

…where P is the equilibrium adsorption ratio of the bioadsorbent (%).

Adsorption Isotherms

Langmuir and Freundlish models were used to study the equilibrium isotherms of adsorption. The Langmuir model assumes that the sorbent surface is homogeneous, on which the whole active sites are identical and have the same affinity for adsorbate, and a monolayer is formed when attaining equilibrium. The equation can be expressed as follows:

$$\frac{c_e}{q_e} = \frac{c_e}{q_m} + \frac{1}{K \cdot q_m} \tag{3}$$

… where q_m(mg/10^{10}cells) is the maximum adsorption capacity and K (L/mg) is Langmuir equilibrium constant associated with the affinity of the binding sites on adsorbent surface.

The Freundlish model predicts a reversible adsorption occurring on the heterogeneous surface and there is a consequently exponential distribution of binding sites. The linear form can be described as follows [19]:

$$\log q_e = \log K + \frac{1}{n} \cdot \log c_e \tag{4}$$

…where K ($mg^{1-1/n}$ $l^{1/n}$/10^{10}cells) and 1/n (dimensionless) are constants for a given sorbate and sorbent at a particular temperature, which are related to the relative sorption capacity and intensity, respectively.

Effect of pH

The pH dependence of *Chlorella* absorbability was explored through the sorption experiment in a pH range from 1.5 to 7.5, avoiding the hydroxide precipitation of Pb at pH value upon 8.50. NaOH or HCl was used for the pH adjustment and the actual pH was detected by a pH meter (LE438, Mettle Toledo, China). There were three replicates for each treatment.

Competitive Adsorption

Batch sorption experiments with three replicates were carried out in 250 mL conical flasks containing 50 mL experimental solution with dead algae cells prepared as above to study the metal adsorptive selectivity of *Chlorella*. The test was performed at the same initial concentrations of 25 ppm for each element, including Pb, Cu, and Ni. The entire flasks were shaken continuously for four hours to attain the equilibrium state. Subsequently, metal concentration was measured by ICP-MS (7700x, Agilent, USA).

Fourier Transform Infrared Spectroscopy (FTIR) Studies

The raw algae and the metal-loaded algae were collect by centrifuge and dried naturally, followed by FTIR spectrometer (FTIR-6700, Madison, USA) to record the Fourier transform infrared spectra (FTIR).

Results and Discussion

The toxic effects of Cu, Cd, and Pb on the growth of *Chlorella* are shown in Fig. 1. The metal concentration dependence of growth inhibition could be evidently observed from Fig. 1, and there was an obvious decline in algae biomass with increasing levels of heavy metal concentrations. The 96h IC_{50} values of Cu, Cd, total Pb, and free ion of Pb were 0.249 mg/L (0.163~0.386 mg/L, 95% CI), 0.485 mg/L (0.429~0.538 mg/L, 95% CI), 46.108 mg/L (38.971~56.060 mg/L, 95% CI), and 21.003 mg/L (17.528~25.912 mg/L, 95% CI), respectively, illustrating that *Chlorellasorokiniana* was most sensitive to Cu, followed by Cd and Pb. Similar results have also been observed in previous studies. Carfagna et al. found that *Chlorella* showed relatively high tolerance to Pb while appearing to be more sensitive to Cd [20]. A toxicity assessment of Cd, Pb, Co, Ni, and Zn by Lin et al. indicated that Pb was the least lethal on *Chlorella* with the highest IC_{50} value among the five kinds of heavy metals [21]. Similarly, high 96h IC_{50} values of Pb for *Chlorella* were reported in the research of Zhang et al., where the 96h IC_{50} values for *Chlorella vulgaris* and *Chlorella protothecoides* were 79.20 mg/Land 73.44 mg/L, respectively [22]. The higher IC_{50} values in the research by Zhang et al. than in the present study could be attributed to the culture condition, initial algae cell density, and species difference [22]. However, Debelius et al. have reported that the 72h IC_{50} value of Pb for *Nannochloropsisgaditana*, i.galbana, Rhodomonassalina and *Tetraselmischuii* were 0.740±0.020 mg/L, 1.340±0.020 mg/L, 0.900±0.090 mg/L, and 2.640±0.200 mg/L, respectively – apparently lower than the IC50 values for *Chlorella in the study of Zhang* and the present study [23]. *Jiang et al. also found that Chlamydomonas reinhardtiihad had greater tolerance*
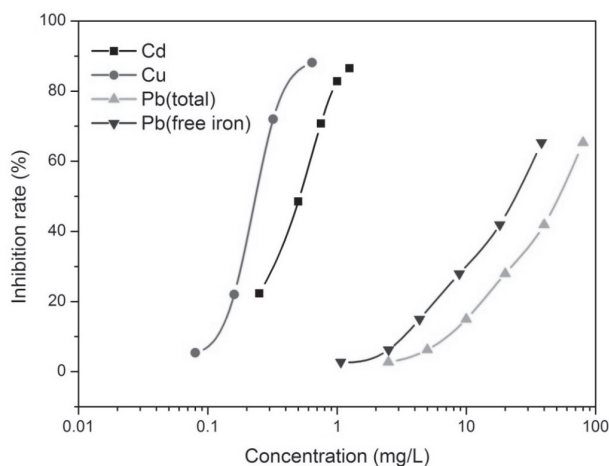

Fig. 1. The inhibitory effect of Cu, Cd, and Pb for *Chlorella sorokiniana*.
This growth inhibition test was carried out with initial cell density of $0.213*10^{10}$ cells/L. The Pb(free ion) concentrations were calculated on the basis of Pb(total) using Visual MINTEQ software.
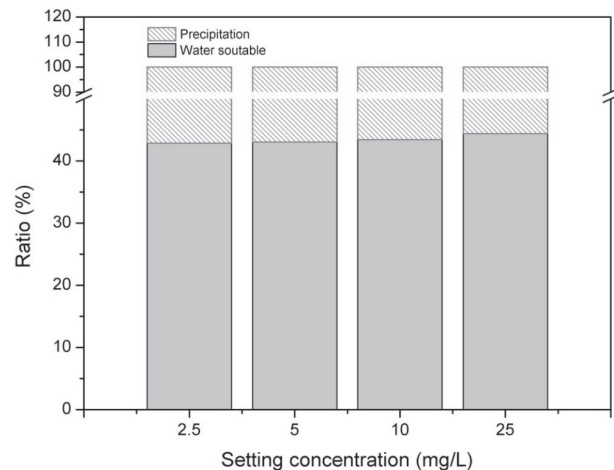

Fig. 2. The ratio of water-soluble Pb and Pb precipitation in culture medium.
The water-soluble Pb was calculated on the basis of Pb(total) using Visual MINTEQ software. The content of water-soluble Pb mainly consisted of the Pb free ion.

for Cu with the highest IC_{50}, followed by Cd and Pb [24]. Consequently, this distinction of metal tolerance might be attributable to the algae species, and the present results indicated that *Chlorella sorokiniana* seem to possess a higher tolerance to Pb compared with other algae.

Pb Accumulation and Distribution in *Chlorella*

Pb accumulation and distribution in *Chlorella* was quantified on the basis of growth inhibition bioassay to identify if the Pb distribution responsible for the explanation of high resistance of *Chlorella* for Pb. Fig. 2 shows that Pb in the culture medium consisted of two fractions: the precipitation form of Pb and water-soluble Pb, almost in the form of a free ion, which accounted for 42.88% and 44.41% of the total Pb, respectively. The free ion activity model (FIAM) has illustrated that the metal toxicity effect was vastly controlled by the free metal ion concentration rather than the total concentration [25-26]. The Pb distribution in *Chlorella* showed that a relatively large proportion of Pb (free ion) was present in the EDTA-changeable (free ion) section, whereas a small fraction was accumulated within the algae cell (Fig. 3). The ratios of EDTA-changeable Pb (free ion)/total Pb (free ion) under 2.5 mg/L, 5 mg/L, 10 mg/L, and 25 mg/L of Pb were 73.4%, 78.1%, 84.0%, and 98.2%, respectively. Meanwhile, the ratio was increased according to the increasing levels of Pb concentrations. This high extracellular accumulation and climbing tendency has proven that the cell wall as the foremost barrier can immensely adsorb the majority of Pb and prohibit the further intracellular aggregation to cause serious metabolic disorders. Few studies have reported the inherent correlation between heavy metal distribution and metal detoxification in algae, while the protection mechanism of the cell wall for algae has been verified. Macfie and Welbourn have demonstrated that the
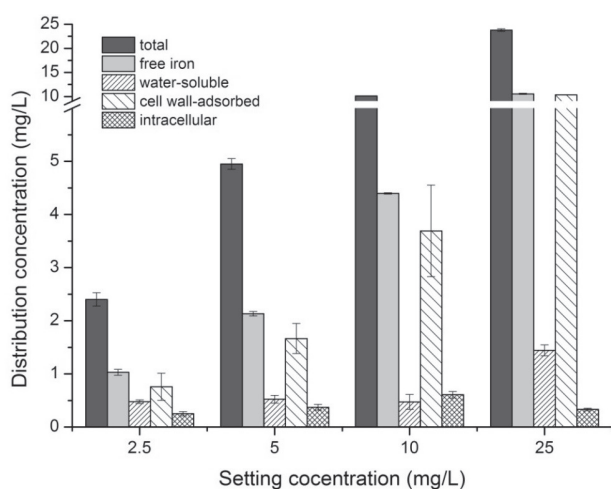
Fig. 3. Pb distribution in *Chlorella sorokiniana* after four-day exposure.
The Pb free ion adsorbed on the cell wall was calculated by the following equation: cell wall adsorbed = EDTA-changeable – precipitation. The setting Pb total concentrations were 2.5, 5.0, 10, and 25 mg/L, and the actual total concentrations measured by AAS were 2.40±0.13 mg/L, 4.95±0.10 mg/L, 10.12±0.03 mg/L, and 23.79±0.26 mg/L.

wall-less *Chlorophyceae* was more sensitive to Cd, Co, Cu, and Ni than the normal algae cell [8]. Perales-Vela et al. predicted that extracellular polymers in cell walls, such as carbonhydrates, played an eventful role on metal adsorption to prevent the indiscriminate entrance of metal ions into the cell [27]. Kadukova also reported that surface sorption was the main metal detoxification mechanism for *Parachlorella Kessleei* [28]. The analogous defense mechanism has also been found in plants, in which more cell wall polysaccharides were likely to gather a larger amount of toxic metals and isolate them from reactive sites in the protoplast [12, 29-30]. It can be inferred that the large proportion of Pb absorbed on the cell wall was a remarkable detoxification pattern of *Chlorella*, and the higher sensitivity of *Chlorella* to Cd and Cu indicated the lower adsorption efficiency of cell wall on those heavy metals. Taken together, this implied that the *Chlorella* may be applied as a potential adsorbent in Pb wastewater treatment.

Adsorption Properties of *Chlorella*

Effect of pH

The Pb ions removal rate was 6.97% at pH of 1.56, while it has been raised to 91.61% at pH of 7.59. In addition, there is a prominent increase of the adsorption ratio rising from 24.81 to 79.61%, when the pH ranged from 4.48 to 6.03. A similar phenomenon in which adsorption of metal irons increased with gradually increasing pH was also been reported in by Alhomaidan et al. [31]. In acid conditions, the majority of reactive groups on the cell surface were protonated, which means that the metal biding sites were occupied by protons, resulting in a

decline in metal adsorption by algae [32]. With increasing pH, the cell wall of biomass is gradually negatively charged, which implies that the functional groups of the cell wall have a greater attraction for positive metal ions. According to the FTIR analysis in Fig. 4, the strong peak at 3,278 cm^{-1} was due to the hydroxyl stretching of carboxylic groups and the stretching of amino [33]. The strong bands at 1,644 and 1,530 cm^{-1} can be assigned to the vibration of amide I andamide II from protein fraction, and the slight peak at 1,238 cm^{-1} was the stretching band of phosphate [34]. Compared with raw and Pb-loaded biomass, a general decline in absorbency can be observed after adsorption, which implies that there were interactions between Pb ions and the functional groups, including hydroxyl, amino, and phosphate. Previous studies have proven that the cell wall ligands, including amino, hydroxyl, and phosphate at low pH can be protonated and restrict the approach of metal ions because of repulsive force, and the groups would have greater affinity for metal ions under higher alkalinity conditions [35]. Accordingly, the following adsorption experiments were performed at pH 6.7.

Equilibrium Adsorption Capacity

The adsorption capacity of *Chlorella sorokiniana* for Pb was evaluated with equilibrium sorption capacity (q_e) and removal rate (R) as shown in Table 1, and it can be discovered that both living and lifeless algae cells have great Pb binding ability. In batch experiments, q_e increased with the raising initial Pb concentration (C_o), probably due to the border collisions between biomass and the metal ions [36]. However, there was a progressive saturation of functional sites for metal binding with continuously increasing C_o. As a result, q_e maintained at the same values when the C_o exceeded approximate 2.6 mg/L. Contrary to q_e, a downtrend of removal rate (R) was observed with increasing C_o, indicating that Pb adsorption with *Chlorella* was a passive process [37]. Compared to living cells, lifeless algae biomass showed a higher affinity for Pb
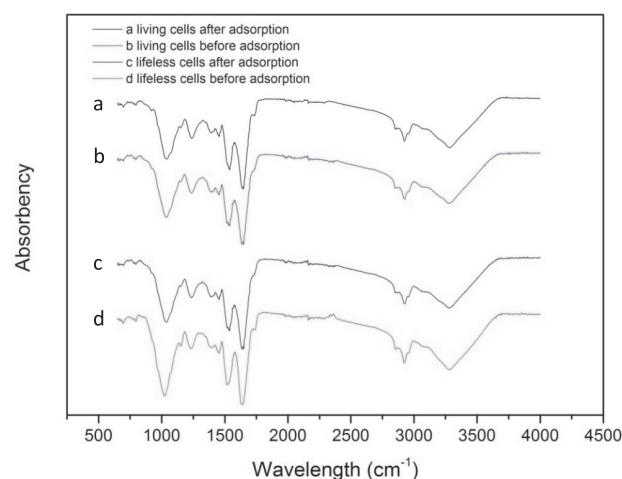

Fig. 4. The FTIR spectra of living and lifeless algae biomass.

Table 1. The capacity of *Chlorella sorokiniana* for Pb adsorption.

Alage	C_o	C_e	q_e	R
	mg/L	mg/L	mg/10^{10}cells	%
Living cells	0.916	0.440	0.643	51.90
	1.321	0.757	0.763	42.67
	1.772	1.047	0.981	40.92
	2.292	1.474	1.106	35.66
	2.653	1.822	1.125	31.31
	3.083	2.208	1.185	28.38
Lifeless cells	0.892	0.226	0.902	74.67
	1.332	0.365	1.310	72.63
	1.785	0.462	1.791	74.10
	2.249	0.759	2.018	66.25
	2.673	1.027	2.229	61.58
	3.068	1.440	2.204	53.06

The adsorption experiment was carried out at ambient pH 6.7 with initial algae density of 0.739*10^{10} cells/L.

ions with the higher removal ratio and q_e, as the removal efficiency raised from 28.38 to 53.06% at an initial concentration about 3.0 mg/L, which was also observed in another algae species of *Chlamydomonas* [1].

Besides the reactive groups analyzed above, it can be seen from Fig. 4 that the intense peak at 2,925 cm^{-1} was caused by stretching CH belonging to the carbon chain, and the band at 1,035 cm^{-1} could be assigned mostly to the vibration of carbon-carbon bond and carbon-oxygen bond [34, 37]. For both living and lifeless cells, a general decrease at 3,278 cm^{-1} can be observed in the Pb-loaded biomass, indicating that there was an interaction between the Pb ions and both amino and hydroxyl groups through the electron lone pairs. Furthermore, absorbency declining at peaks of 1,644 and 1,530 cm^{-1} has suggested a combination between metal ions and amide. Also, the peak of 1,035 cm^{-1}, corresponding to the stretching of carbon-carbon bond and carbon-oxygen bond, was slightly reduced, which provides further information in heavy metal sorption. These results were in agreement with the data obtained by other authors [11, 37-38]. The decreasing amplitude among different functional groups after metal sorption was roughly similar and this has suggested that both of the groups played a significant role on metal biding. Meanwhile, in comparison with the spectrum of raw biomass (a and c) in Fig. 4, incremental absorbency was observed at the peak of amino, amide I, amide II, and carbon-carbon and carbon-oxygen bonds, implying that more functional groups with metal binding sites were exposed after thermal treatment. Consequently, this may explain that the greater adsorption capacity of lifeless cells than the living biomass was observed.

Adsorption Isotherm

In this section, both the Freundlich and Langmuir models were applied to equilibrium data to study the sorption isotherms as shown in Fig. 5. The regression coefficient R^2 (0.959 and 0.863 by Freundlich model, 0.988 and 0.962 by Langmuir model for living and lifeless cells, respectively) has suggested that the Langmuir model was more fitful for describing the sorption process. Therefore, it can be inferred that the surface of algae biomass was homogeneous and a monolayer of Pb ions would be formed when attaining equilibrium. The maximum adsorption capacity values (q_m) calculated by the Langmuir model were 1.54 and 2.97 mg/10^{10}cells for living and lifeless cells, respectively, which was consistent with the experimental result. On the other hand, the Langmuir equilibrium constant K (L/mg) related to the affinity of the binding sites for Pb ions was 2.41 L/mg and 1.52 L/mg for lifeless biomass and living cells, respectively [39]. Consequently, compared to living cells, the higher q_m and K also illustrated a greater Pb adsorption capacity for lifeless cells.
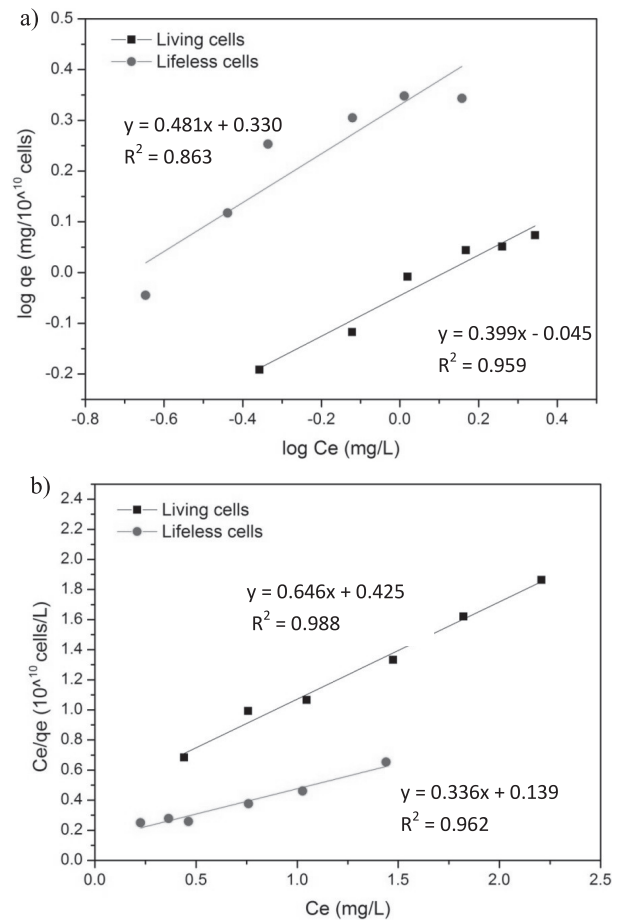

Fig. 5. The equilibrium adsorption fitting by a) Langmuir models and b) Freundlish models.

Competitive Adsorption

A competitive adsorption experiment was carried out to study the selective capacity of metal adsorption by *Chlorella*. The results showed that the removal rates were 17.86%, 13.64%, and 10.53% for Pb, Cu, and Ni, respectively. Meanwhile, in the mixture system, the q_e for Pb was 1.36 mg/10^{10}cells – lower than that in the single Pb system, which meant that the Pb, Cu, and Ni ions were competing for the same binding sites of the cell wall [1]. However, *Chlorella* showed the highest removal rate and highest affinity for Pb ions compared to other heavy metals, which confirmed our prediction that the *Chlorella* may serve as a potential bioadsorbent in Pb wastewater treatment. Sulaymon et al. reported that larger ionic radius may result in greater adsorption efficiency [40]. The ionic radius of three metals was decreased in the following order: Pb>Cu>Ni [41]. The ionic radius of Pb may be closer to the space of metal binding sites in the cell wall, which may explain the selectivity of Pb.

Conclusions

A toxicity assessment of Pb, Cu, and Cd showed that *Chlorella sorokiniana* had a rather high tolerance to Pb compared with Cu and Cd. Pb distribution in algae indicated that the majority of free Pb ions were adsorbed on the reactive sites of the cell wall, which might be one of the most important detoxification mechanisms for *Chlorella sorokiniana* in exposure to Pb. The adsorption experiment of *Chlorella sorokiniana* suggests that this algae biomass showed high q_e and removal rate in both single and competitive adsorption systems. Therefore, the algae with a high affinity for Pb may serve as an efficient Pb adsorbent.

Acknowledgements

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