Selenastrum Capricornutum biosorption in the binary system of Cadimum(II) and Copper(II)

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Introduction:

The literature and research are based on the using of *Selenastrum Capricornutum*, a microalga also known as *Raphidocelis subcapitata*, for the ability to extract heavy metals from a liquid phase. Different environmental variables and binary stress of two metal ions, Cu^{2+} and Cd^{2+}, are evaluated here to verify their influence on the microalgae. *Selenastrum Capricornatum* is commonly known as a bioindicator because of its comparatively higher growth rate, sensitivity to toxicants, and good reproducibility compared with other algae.

Heavy metal pollution is one of the most threatening environmental problems. The accumulation of heavy metal ions causes undesired effects on humans and other living creatures (1 Salar et al. 2019). Elevated levels of heavy metals can be traced from various chemical industries, such as fertilizer, petroleum, metallurgy and other inorganic chemical industries. The most commonly found metals of these industries are zinc, nickel, cadmium, lead, mercury, copper, chromium, uranium, thorium, etc. The conclusion can be made that the disposal of heavy metal waste is tightly related to the production process of human civilization.

Biosorption is considered to be the most effective in treating low concentrations(<20ppm) of cationic heavy metals in water (2Roy et al., 1993). The organisms immobilize, mobilize, or transform metals with extracellular precipitation

reactions (3 Corale L.1990). Studies stated dead, chemically treated biomass shown a similar process as ion exchange to remove the heavy metal in an aqueous system.

Microalgae exhibited great importance and advantages in this area. As the primary producer in aquatic environments, it has fast growth rates and hence produces a continuous supplement for the metal-removal process. Metals can also increase ROS (Reactive oxygen species) in algal cells (4 Alho,2019). The effect of heavy metal ions to change the proteins, DNA, and lipids will thereby create defense systems to minimize the damages. Shreds of evidence shows different algae have the ability to inherit this activation of defense mechanism. For example, in Chun-Di and Fu-Jun's experiment (5 2007), *Dunaliella tertiolecta* shows the highest growth rate under low concentration of Cd^{2+} ions stress.

The present work experiments heavy metal removal using *S. Cap*, specifically as Cadmium and Copper. The experiment is carried out in a semi-batch system to observe the kinetics of the concentration changes of heavy metal ions in the system over time. This microalga was chosen for its common presence in natural environments. An previous experiment from Yin's "*Effect of copper-cadmium combined stress on free radicals oxidative damage of Selenastrum capricornuium "(*6 2014) states that the algae have shown the potential of surviving under binary stress of Cd^{2+} -Cu^{2+} ions. However, most researches done on *S.Cap* are characterizing the toxicity of different containments and very few of them studied on the stress-training of algae and the limitation of metal removal from the system.

Therefore, the purpose of this research is to characterize the viability of *Selenastrum capricornutum*(*S.Cap*.) under the stress of the Cd^{2+} -Cu^{2+} system and analyze its effectiveness of removing both metal ions from solution. pH and hardness are also included in the study as an important influence of algae growth.

The results of this work show that the adsorption rate of Cd^{2+} and Cu^{2+} is deceased in a binary-stress system comparing to single Cd^{2+} stress and single Cu stress. The highest metal removal percentage decreases from 61.78% to 44.41% for Cd^{2+} and from 98.66% to 68.00% for Cu ions. The algae show the potential of removal during the 96 hr experiment period. The algae are acidic domesticated prior, but the removal rates are comparably lower in a more acidic solution(pH3.5) than a more neural solution (pH 6.5). The hardness of water seems to have a minor influence on the metal removal process.

Hypothesis/Objectives

The purpose of this research is to characterize the sustainability of *Selenastrum capricornutum* (*S.Cap*.) under the stress of the Cd^{2+} -Cu^{2+} system and analyze its effectivity of removing both metal ions. The microalgae are representative algae whose resistance to and uptake of metals can be extensible to other algae. The goal of this research is to use experimental and statistical method to analyze whether the algae is benefited from the antagonistic effect of Cd^{2+} -Cu^{2+} joint toxicity and increase the removal-ability under different pH, hardness and containment concentration.

Literature review:

This chapter presents a review of literature and research related to the use of microalgae, especially *Selenastrum Capricornutum*, for the viability of extracting heavy metals from wastewater. Research on metal ion adsorption mechanisms and binary stress of different metal toxicity are also discussed in detail. Cadmium(Cd^{2+}) and Copper(Cu^{2+}) are common contaminants in aqueous systems. Studies were done by Yin, Jiande, and Lei (7 2014), Tian, Yigang and Ruikang(8 2014), Qinlin and Biqin(10 1997), Expósito and Kumar(11 2017), Lays de Oliveira Gonçalves Alho(2019) all included a measurement of S.Cap uptaking Cu, Cd, and other heavy metal ions.

The following sections of the review will be first discussing the necessity of metal removal from aqueous systems by highlighting the background and the hazardous against humans and plants. The mechanism of metal uptaking by algae will be discussed in the next section in a general understanding. The third section explains the research actuality of binary stress upon microalgae and the interactions between different heavy metal ions, most about Cd^{2+} and Cu^{2+}, and their toxicity against algae's defense mechanism. The last section covers the introduction of the algae we used specifically.

1.Heavy metal:

According to EPA, over 2,800 cases of new chemicals are under reviewing during the last 4 years. (12) With the rapid development of industry and agriculture, more and more chemicals enter the natural environment. The damage to human health and the environment caused by these chemicals also goes along with the development history of modern society. Early as the 1956 outbreak of Minamata disease in Japan, people have realized that heavy metals in water can get into people's bodies and cause harm through the food chain. Heavy metals become one of the most serious environmental pollutants(13).

In terms of physical and chemical properties of substances, metals are usually divided into heavy metals and light metals according to their densities. Metals with a density greater than 4g/cm^3 are usually included in the heavy metal groups, such as gold, silver, copper, lead, zinc, nickel, cobalt, chromium, mercury, cadmium and other 45 elements (14). In terms of environmental pollutants, Arsenic and Selenium are also included in this group for their similarity on chemical properties and toxicities. These heavy metals are not only unable to be naturally decomposed in the environment, but also easily be combined with other toxins in the water to form more toxic organic or inorganic substances.

1.1 Cadmium

Cadmium is a silvery-white shiny metal with a melting point of 320.9 ℃ and a boiling point of 765 ℃. Cadmium is slowly oxidized in humid air and loses its metallic luster. When heated, brown oxides are formed on the surface. At high temperature, cadmium can react with halogen or directly combine with sulfur to form cadmium sulfide. Cadmium oxides are also soluble in acids, but not in bases.

World reserves cadmium are estimated at 9 million tons (15 Shigang, 2010). In nature, it is mainly in the presence of Cadmium sulfide, but also a small amount in zinc ore, so it is also a by-product of zinc ore smelting. Cadmium alloys are widely used in low melting point alloys because of their high tensile strength and wear resistance. Nickel-Cadmium and Silver-Cadmium batteries are broadly used with the advantages of small size and large capacity. Cadmium has high oxidation potential and is also widely used as the electroplating protective film of iron, steel, and copper. Cadmium compounds are also used in the manufacture of pigments, phosphors, plastic stabilizers, etc. Cadmium compounds can also be used in the manufacture of pesticides, paints, fungicides, and pigments. We can make the statement that It is closely related to the modern industry.

Although it is not an essential element for many plants, Cadmium can easily penetrate the xylem of the root system and be absorbed. Its potential hazards to plants include: reducing chlorophyll content, thereby reducing photosynthesis, and interfering with carbon metabolism, and plant water and nutrient absorption. The

most important biochemical and toxicological properties of cadmium is its strong affinity for the mercapto groups of some compounds and its affinity for other side chains of proteins and phosphate functional groups. In-plant cells, cadmium can replace zinc and magnesium in many enzymes, resulting in denaturation of enzyme proteins and inhibition of their activities, thus affecting plant growth and development.

The air, water and food contaminated by cadmium are seriously harmful to human health. Cadmium, which is hard to metabolize, is highly concentrated in the body through food chains, water, smoking, and other pathways. Upon exposure to Cd, the liver is one of the susceptible organs with respect to the accumulation of toxicity (). Cadmium poisoning occurs when cadmium concentration reaches a certain level. It mainly damages the renal tubules and glomeruli, leading to proteinuria, amino acid urine and diabetes.

At the same time, as Cadmium ions replace Calcium ions in bone, Calcium deposition on bone is impeded, and bone collagen is also prevented from curing and maturation. It would, therefore, cause rickets. In the 1960s, a strange disease appeared in Toyama prefecture, Japan. The first clinical manifestation is back pain, knee pain and later throughout the body. Most of the patients were menopausal women and confined to the irrigated area of the Shintogawa river basin. When aching activity aggravates, pathological fracture happens more and causes serious deformity of skeletal. A few slight activities or even cough can cause a fracture. Patients had to lie in bed for a long term and cry painfully. The disease is then

named" *Itai-Itai disease* (it hurts-it hurts disease) ". Epidemiological investigations and experimental studies confirmed that " *Itai-Itai disease* " was caused by chronic Cadmium poisoning.

Reports proclaimed, at the end of the 2000s, approximately 45% of soils in the United Kingdom were polluted by Cd^{2+} ions from the nickel-cadmium batteries, pigments, etc. Cd^{2+} soil levels varied across different areas in the United States with a mean value of 0.32mg/Kg soil (16 Yingying et al. 2014). After all, Cadmium is one of the important challenges for humans in environmental researches.

1.2 Copper

Copper is a transition metal with a Burgundy color. It has a density of 8.92 g/cm^3, a melting point of 1083 ℃ and a boiling point of 2,567 ℃. Copper is one of the earliest metals discovered by human beings. It has good ductility, thermal conductivity, and electrical conductivity. It is the only metal that can be produced in large quantities naturally. Copper and its alloys have good corrosion resistance and are stable in dry air, but $Cu_2(OH)_2CO_3$ can be formed on the surface in moisty air. Copper and its alloys are used in electrical, light power, construction, defense industry and other fields. It is very relevant to human civilization.

Copper, as one of the essential metal nutrients, is the core of many oxidase activities (such as polyphenol oxidase, galactose oxidase, ascorbic acid oxidase, etc.).

Copper is involved in the receipt and transmission of electrons and plays an important role in the REDOX reaction in plants in the middle stage. It is also closely related to the formation of first-order protein and carbohydrate synthetic oil in the open air. Lack of copper will reduce the synthesis of plastocyanin and cytochrome oxidase, leading to growth inhibition and reduced photosynthesis and respiration (17). On the other hand, excessive copper can also cause negative effects, such as root deformity and chlorosis, or even the death of the whole plant.

With the development of human civilization, the demand for copper is increasing. These production activities will undoubtedly cause an abnormal rise of copper level in the local environment, resulting in copper pollution. Some water bodies have also been directly or indirectly affected, posing a serious threat to aquatic life and even causing the ecosystem to collapse.

Hongjing and his group studied the effect of copper on *Lemnan Spp* and found that increasing Cu2+ by 1μmol/L in the environment significantly inhibited the growth of *Lemnan Spp* (expressed in terms of fresh weight and dry weight). As the concentration of copper increased, the inhibiting effect on growth increased, the fresh weight and dry weight increased when above 8μmol/L and 2μmol/L respectively. The increase of copper concentration can significantly reduce chlorophyll content but has little effect on carotenoid content. It also affects the production of chlorophyll-a fluorescence, which means the surface photosynthetic organs have been damaged to some extent. At the same time, the level of the

copper content in plants also increased, indicating that Photosynthetic organ the lemna had some accumulative effect on copper(18).

Nadia's researched the influence of Cu2+ to *Phragmites australis* and *Zea mays* in the range of 0.5μmol/L to 157μmol/L. It has shown Copper starts to suppress the root growth of *Phragmites australis* and *Zea mays* when the concentration reaches to 15.7 μmol/L and 78.7 μmol/L. Also as the result of copper toxicity, the concentration of N, P and K elements shown declining in the roots and sprouts of the plants(19).

Moreover, Morelli's Studies on the molecular mechanism of resistance to copper toxicity show the detection of Cu-plant complexin within one hour of treatment with Copper. (21) Catalase (CAT) and Superoxide dismutase (SOD)'s activation increased after a few hours too. The increment in the reference group is 200% and 40% respectively after 48 hours. The experiment determined CAT is the main enzyme that removes peroxides. Glutathione reductase activity decreased initially and then increased as well. This process means glutathione reductase and its polypeptides provided the resistance to copper-induced reactive oxygen damage. After all, at least three antioxidant enzymes increased activity to oppose to the oxidative stress from copper ions. (20)

Mechanisms of algae biosorption:

Algal technologies have been successfully used to treat wastewater for over 70 years. The first research using micro-bacteria and algae for wastewater treatment was established in the early 1950s (21. Sayed et al. 2014). From then on biologists and engineers considered it an important and useful technology: not only removing water-based pollutants previously unreachable for humans but also to produce high-quality biofuel as its byproduct.

The ability of algal absorption of nutrients (e.g., N and P) has been broadly understood by the public for a long time. Algal wastewater treatment has been used in industrial, municipal and agricultural field applications. Researches state a few kinds of algae can simultaneously remove more than 90 percent of nitrates and more than 50 percent of phosphorus as nutrients (22. Abdel-Raouf et al. 2012).

However, uncontrolled algae growth in natural systems due to excess amounts of N and P can be hazardous. It leads to water eutrophication, which threatens other living things with the shortage of oxygen and living space because of its strong ability to absorb nutrients and reproduction. The overgrowth of algae flooding in the top layer would not only consume most oxygen and suppress other organisms' growth, but the "death" of the ecosystem would also become the nutrient source and accelerate the blooming of algae. As things continue this way, the toxicity released from the dead algae would destroy the whole system.

Algae also show potential in absorbing heavy metal ions from water. As the primary producer in an aquatic system, algae are also the primary receptors of metal pollution. The biosorption process of the living organisms or derivatives thereof is the main source of ion adsorption. In combination with filtration and digestion process treatment, heavy metal ions can be selectively removed. This method of heavy metal treatment has the advantages of energy-saving, high treatment efficiency, easy separation and regeneration, and also high recovery of heavy metals (23.Changhao et al. 2016).

Math model

In order to get a better understanding of the viability of S.Cap. in industrial usage, I am here performing some simulation of the metal adsorption along with different reactor designs: Batch/semi-batch, CSTR, and PFR. This portion of the analysis is used to find an optimized conversion of the reactor with the same inlet stream under similar conditions.

Bioremoval usually involved a combination of active and passive transport mechanisms . The general understanding of the algae adsorption process can be divided into two stages: Biosorption and bioaccumulation. The biosorption process is acted on the cell wall of algae and it's a passive process, which indicates that even dead cells would have this ability; bioaccumulation, however, is related to the

metabolizing activities of the cell. Enzymes inside the algae would contribute more to transport the ions actively.

Langmuir and the Freundlich equations are considered to be the most satisfying models to start with. The general expression of Langmuir is:

$$C = \frac{K_l m}{a+m} \quad (1)$$

Where: C = specific metal uptake (mM adsorbed/mg adsorbent)
m = equilibrium concentration of adsorbate (mM/L)
Kl, n, a = constants (temperature dependent)

Langmuir model is the simplest model to describe the adsorption phenomena of one component. However, in this case, study, there are multiple species of chemical components involved. The Freundlich model is obtained by assuming that the surface fraction covered by the adsorbed solute has a logarithmic attenuation to the heat of adsorption.

Freundlich Isotherm:

$$C = K_f m^{\frac{1}{n}} \quad (2)$$

Kf, n = constants (temperature dependent)

The derivation of the Langmuir Adsorption requires that there is less than one complete monolayer on the surface occurred during the adsorption. In such this case all the surface is energetically uniform and has no interaction between the algae units and the Freundlich equation is a transform of it. They have been widely used to

characterize the uptake of heavy metals by illustrating the differences between algae species, especially the different morphological types of the same organism. On the other hand, the deficiencies are also readily noticed: both equations present the environmental factors and the “complex nature” of sorbent with the cell’s varied active sites.

Considering an ideal membrane system of metal uptake by algae. The model presented below is a refined work of Khummongkol(24.1982). It has been used to describe the uptake of different metals by algal cells not only singly but also in combination. It assumed the metal uptake is given in two stages: a rapid passive uptake on the surface of the cells and followed by a much slower active intracellular transportation.

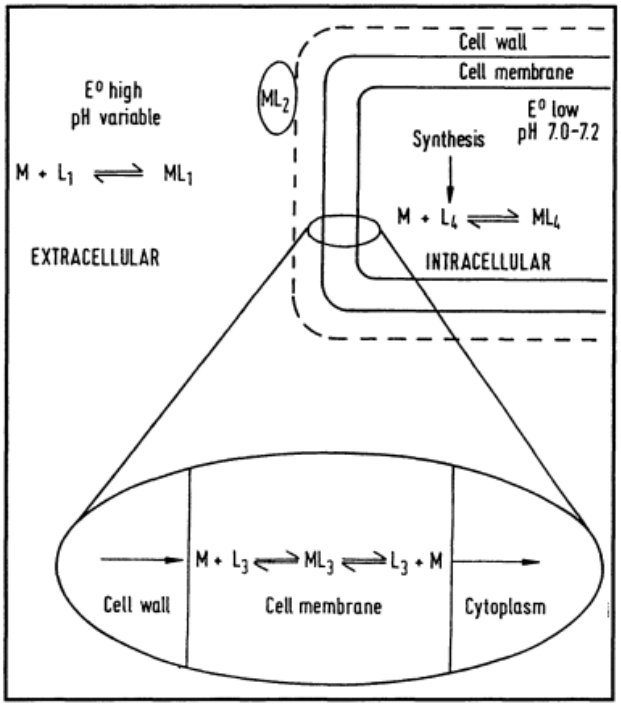


Fig.1 general model of cell membrane adsorption

As a numerical model, here are some assumptions suggested by Ian G. Prince(25.1998):

1) The metal in solution at the cell surface, m (mM metal/L), is in equilibrium with the metal adsorbed on the cell, C1(mM metal/mg cell dry wt). A linear relationship is assumed.

$$m = KC1 \text{(K=adsorption constant)} \tag{3}$$

2) The metal ion adsorbed on the cell surface traverses the cell membrane through the agency of carrier molecules present on both sides of the membrane. The mechanism is presented in an enzyme-substrate coupling scheme:

$$C_1 + E \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} CE \underset{k_{-2}}{\overset{k_2}{\leftrightarrow}} C_2 + E \tag{4}$$

Where:

C1 is the extracellular metal concentration and C2 is the intracellular one.

E is the carrier

CE is the metal-carrier complex

k1, k2,k-1,k-2 are the chemical reaction rate constants.

3) The diffusion of the CE through the membrane is rapid and the concentrations of E and CE are negligible compared with C1 and C2

4) Pseudo Steady-state, no accumulation of the metal -carrier complex and

$$\frac{d(CE)}{dt} = 0 \tag{5}$$

5) The carrier content of a cell is essentially constant, and so the total carrier concentration in the system, Et,

$$\mathrm{Et} = [\mathrm{E}] + [\mathrm{CE}] \tag{6}$$

6) One mole of carrier reacts with one mole of metal ion to produce one mole of CE complex

$$\frac{d(x[C_2])}{dt} = xR_1\{[C_1] - R_2[C_2]\} \tag{7}$$

where the left-hand side denotes the rate of intracellular transport;

x = the biomass concentration

The metal mass balance gives:

$$A = m + x\{[C_1] + [C_2]\} \tag{8}$$

Therefore, with the substituting of Equation 3 and 8 into Equation 7,

$$\frac{d(x[C_2])}{dt} = \frac{xR_1}{K+x}\{A - [C_2](x + R_2(K+x))\} \tag{9}$$

To simplify the scenario, even though the hardness of water is included during the measurement and they are presented with the concentration of Mg^{2+} or Al^{3+} ions in the sample, the analysis only covers their effect on the Cd^{2+} and Cu^{2+} metal adsorption rather than quantification. Therefore, the system is still considered to be

binary. The empirical extension of the Freundlich model of the binary mixture can be given as follows:

$$C_1 = \frac{a_1 C_{1,eq}^{b_1^0 + b_{11}}}{C_{1,eq}^{b_{11}} + a_{12} C_{2,eq}^{b_{12}}} \quad (10)$$

$$C_2 = \frac{a_2 C_{2,eq}^{b_2^0 + b_{22}}}{C_{2,eq}^{b_{22}} + a_{21} C_{1,eq}^{b_{21}}} \quad (11)$$

The basic concepts of Joint toxicity

In the actual production and living environment, a variety of chemical pollutants often exist at the same time, acting together on the organism. Organisms exposed to a complex mix of pollutants produce biological effects that are different from those produced by any chemical contamination individually. Therefore, we refer to the comprehensive toxicity effect combined by two or more chemical pollutants as joint toxicity. As concluded by Shigang, four major types of joint toxicity are commonly categorized in the study of chemical pollutants: Synergistic effect, Additive effect, Independent effect, and Antagonistic effect.

A synergistic effect is defined as the effect caused when exposure to two or more chemicals at one time results greater than the sum of the effects of the individual chemicals. The common understanding is that one of the toxic components can be promoting the body absorbing other toxic components, degradation blocking, accumulation increasing, slow excretion or producing high toxic metabolites, so as to increase the toxicity of the mixture. For example, a combination of carbon tetrachloride and ethanol, ozone and sulfuric acid aerosols will occur with a synergistic effect. Using the inhibition rate as the observation index, the independent inhibition rate of the two poisons is respectively defined as M1 and M2, then Mtotal $>$ M1 + M2.

An additive effect is defined as the effect caused when exposure to two or more chemicals, usually with similar chemical structure or properties, the toxicity of the

mixture would be equal to the summary of each individual. Similar toxicants normally effective on the same organism with a similar reaction mechanism, therefore, the result of the joint will be presented as an additive effect. For example, a combination of Acrylonitrile and acetonitrile will occur with an additive effect. Using the inhibition rate as the observation index, the independent inhibition rate of the two poisons is respectively defined as M1 and M2, then Mtotal = M1 + M2.

If the toxic components have a difference in the invasion approach, effective organism and mechanism, the effect of the process are called independent effect. Because the results of the biological reaction differ from each other, the overall toxicity is lower than the addictive effect but higher than the individual chemical. For example, a combination of phenobarbital and xylene will be affected independently. The inhibition rate of the two poisons cannot be added directly.

Last, of all, the antagonistic effect described the phenomenon of the overall toxicity is less than the sum of the individual toxins. In another way, the composition of one toxin suppressed the effect of another one by increasing decomposed speed, reducing reactions or producing fewer toxic compounds. In some bio-toxicity tests, dichloromethane and ethanol, selenium and mercury, selenium and cadmium or nitrite and cyanide can be considered with this effect. Using the inhibition rate as the observation index, the independent inhibition rate of the two poisons is respectively defined as M1 and M2, then Mtotal < M1 + M2.

Marking's "Method for Assessing Additive Toxicity of Chemical Mixture" proposed an additive index (AI) for the measurement of joint toxicity(1977)(32). The equation is shown below:

$$S = \frac{A_m}{A_i} + \frac{B_m}{B_i} + \cdots \quad (12)$$

Where S is the biological activity of mixed poisons, A and B are the experimented toxin, I and m stand for individual chemical's half effective value and the overall half effective value. When $S \leqslant 1$, AI =(1/S) -1; when $S \geqslant 1$, AI =1-S. If AI >0, then the effect of the chemicals is synergistic effect; if AI = 0, it is additive effect; if AI < 0, the result is the antagonistic effect.

Researches on Joint toxicity:

Fucheng's(33) group studied the effects of cadmium and mercury combined pollution on lipid peroxidation and antioxidant enzyme activity in *Ceratophyllum demersum L*'s cell membrane. The results showed that SOD activity increased significantly and reached the maximum value under the treatment of low concentration cadmium and mercury (2mg/L Cd^{2+}, 05mg/L Hg^{2+}). With the increase of concentration, sod activity decreased slowly and was lower than the control value at 2mg/l Hg^{2+} and 10mg/L Cd^{2+}.

Xunan(34 2002) studied the toxic effect of mercury, cadmium and compound pollution on duckweed leaves. Through result analysis, the lethal concentration of Hg on duckweed leaves was between 5-7mg/L; the lethal concentration of Cd^{2+} is 2.5-5mg/L while the lethal concentration of the mixture is at 1-2.5mg/L Hg + 1 -2.5 mg/L Cd^{2+}. The result has shown the mixture has a synergistic effect on antioxidant enzyme system activity, proline, and soluble protein content but the antagonistic effect on the Chlorophyll concentration.

In O.P shukla's research (35 2007) on the combined toxicity of cadmium and chromium to *Bacopa Monnier L*, the results showed that the soluble protein content decreased with the increase of the combined concentration of chromium and cadmium. The accumulation effect of chromium was greater than that of cadmium. In combined contamination, the accumulation of cadmium was greater than that of chromium. It is speculated that in two non-essential elements of metal chains and solutions, one metal may promote the absorption of the other metal, thereby increasing its toxicity to plants. However, when essential and non-essential metals interact together, the absorption of essential elements will increase and its toxicity to the environment will be reduced.

More importantly, as directly related to this thesis, Yin's research on *S.Cap* analyzed the joint effect of Cd^{2+} and Cu^{2+} ions, too. The result has shown the AI reading of *S.Cap* is -1.63, which exhibits a typical reading of antagonistic effect. With the increase of toxicity concentration, malondialdehyde increased, showing a significant concentration-effect relationship. The soluble proteins generally showed

a tendency of rising and then falling. The mechanism of antagonism of Cu^{2+} and Cd^{2+} in microalgae may also be related to the inhibition of lipid peroxidation of heavy metals due to antagonism.

***Selenastrum capricornutum*:**

Selenastrum capricornutum has crescent-shaped or sickle-shaped cells that are longer than broad and strongly curved or twisted. The genus can form non-mucilaginous colonies from clusters of 4, 8, or 16 cells that are not intertwined and are instead arranged with their convex sides facing each other. (36. Wiki "Archived copy". Archived from the original on 12 December 2011. Retrieved 9 February 2012.) This species is quite sensitive to the presence of toxic substances and has the advantage of commonly presenting. The algal is the most frequently used bioindicator in the ecotoxicological area for its high growth rate and sensitivity to toxicants comparing with other algae or bacteria.

However, despite its popularity in the biotoxicity area, the knowledge of its cell biology is limited. The patterns of nuclear and cytoplasmic division in remain unclear. Machado and Soare's visualization research found that it is proliferated by forming four daughter cells after nuclear divisions. The autosports released directly through the rupture of the parental cells without forming colonies. But at the same time, the process of achieving cytokinesis in the cells before autospore released is still unknown.

Researchers also pointed out that PbCl would promote the growth of *Selenastrum capricornutum* until the concentration reaches 38.5mg/L (Dong et al. 1997). Also, observed under a 96h joint toxic effect experiment, scientists suggested that there

exists the resistance mechanism of this alga against the stress of the heavy metal (Yin et al. 2014).

Method:

Materials:

Algae: *Selenastrum capricornutum*

Selenastrum capricornutum(S.Cap.) is produced and purchased from "Carolina Base "and further cultivated for the experimental use. The microalgal is cultivated with distilled water in an aquarium with no water current. 500 ml modified Bold's Basal Medium solution was added into the system every 2 weeks. The aquarium is shaken back and forth once every day until well-mixing. 1600lx light is provided 12 hours a day from the top of the aquarium.

Water: Distilled water is produced from the lab directly

Chemicals: $Cd(NO_3)_2$, $CuSO_4$, Bold's Basal Medium*, HNO_3

Equipment: 20-1/4" x 10-1/2" x 12-9/16" aquarium, 2 1L Erlenmeyer flask, 68 50mL beaker, Polypropylene tubes, pH meter, light meter, weight scale, heat stove, centrifuge

General procedures:

All experiments were carried out in the same lab with constant temperature and humidity. All containers were rinsed with 0.1 mM/L nitric acid before using to prevent glassware from adsorbing metal ions and minimize the contamination.

* See Appendix A

Algae would be exposed to different concentrations of the single stress of Cd^{2+} ions, complex stress of Cd^{2+} -Cu^{2+} composition with different controlled elements of pH, hardness and length of illuminance. After a 96hr period of the adsorption process, the algal sample would be collected and separated from water by centrifuge.

The digestion using nitric acid would then determine the percentage of heavy metal adsorbed. Once an optimal combination of the condition is chosen, the removal capacities would then be analyzed.

Statistical methods are used to evaluate which controlled element was most affected by the adsorption rate.

Algae Cultivation and Acquisition:

Selenastrum capricornutum(S.Cap.) is purchased from "Carolina Base" (Item #152520) and further cultivated for the experimental use. Cd^{2+}, Cu^{2+} ions are obtained from CuSO4 and Cd(NO3)2 diluted in distilled water. The growth medium is a modified Bold's Basal Medium which will be described in the appendix.

Algae will be cultivated in a 19.5in x 15in x 10in aquarium for the experiment. The room temperature will be 25 ℃ and a light source around 1600lx measured at the surface of the liquid will be provided 12hr/day. The tank will be shaken back and forward several times until it's well mixed once a day and medium will be added bi-weekly.

During the experiment, a considerable number of anions such as OH^-, Cl^-, NO_3^- and SO_4^{2-} were also presented in the environment. There are researches stated that the toxic effects of anions on algal chl a chl b and total chlorophyll contents increased at higher concentration. Due to self-protective mechanism of algae cells, the number of starch granules and electron-dense deposits will be increased followed by the destruction of cell walls and membrane. However, the paper by Xia also mentioned that this effect "alleviated at lower concentrations over time" (37 Xia 2018). Therefore, considering about the experiment length and the concentrations of these anions were initially the same in all of the samples, the assumption is made that there would not be effect of anions on Cd^{2+} and Cu^{2+} adsorption.

Algae Acidic domestication

The pH change is often encountered in the process of heavy metal adsorption by microalgae. For example, when the heavy metal enters the reaction tank, the carbon dioxide and nitrogen or sulfur oxides introduced together pass directly into the culture solution, which will lead to a sharp decline in pH value, and then inhibit the growth of microalgae, resulting in poor persistence of microalgae. Generally speaking, people can reduce the harmful influence on the growth of microalgae by adding alkaline substances to neutralize the acid or using pH feedback control and regulation to solve the acidification problem in the reaction tank. However, the application of these methods not only increased the cost but also could not fundamentally solve the damage of the acidic environment to the growth of microalgae.

Fei-Fei's article (38) provided acidic domestication might be a solution. Improving the S.Cap. 's ability to adapt to an acid environment can increase the feasibility of heavy metal adsorption in the industrial size. In terms of this experiment, the acid-tolerant microalgae can also express the adsorption ability of protease to heavy metals under different acid-base conditions with the same concentration remain the same despite the effect of algal death.

The reason to induce this method in the experiment is to ensure the number of living algae remain stable with at a more acidic environment. Growing half of the

algae in another 19.5in x 15in x 10in aquarium. Algae in this tank will be also provided the same amount of Bold's Basal Medium at the same rate. Light intensity was 8000lux and the temperature was 25℃ 800ml. Triplicated 800ml Algae solution would be moved into 1L glass bottles. With other environment elements remain the same, diluted nitric acid would be inducted into the samples to adjust the pH to acidity. The pH gradients for domestication are 5, 4.5, 4 and 3.5. Algae would be cultivated under each gradient for 24hr and the pH will be monitor and adjust to the current gradient every 2 hours. Two control groups will be used to compare at the end of the domestication.

The domestication is successful, the algae are directly used for the Cu^{2+} -Cd^{2+} complex stress experiment.

Cadmium

After cultivating, *S.Cap*. is inoculated into 15 150ml-beakers with a final volume of 100ml and then cultured under the same illuminance. Three stages of measurement are done in order to determine the different characteristics of *S.Cap* with the addition of $Cd(NO_3)_2$.

In the first stage, add $Cd(NO_3)_2$ into the samples until the final cation concentration reaches 0.02mg/l, 0.1mg/l, and 0.4 mg/l as the experiment group. Each sample will run in triplicate. The experiment will also include 3 control samples: samples only with distilled water, samples only with microalgae and samples only with 0.1mg/l $Cd(NO_3)_2$ solution each run in duplicate. Samples would be settled under the same condition as the cultivating tank and be shaken twice a day.

With the preliminary result presenting, the ability of *S.Cap* adsorbing Cadmium is verified. Therefore, Cadmium concentration is increased based on Yin's result.

Add $Cd(NO_3)_2$ into the samples to let the final cation concentration reaches 2mg/l, 4mg/l and 8mg/l as the experiment group. Each sample will run in triplicate. The experiment will also include 3 control samples: samples only with distilled water, samples only with microalgae and samples only with 4mg/l $Cd(NO_3)_2$ solution each run in duplicate. Samples would be settled under the same condition as the cultivating tank and be shaken twice a day.

Last of all, to verify the possibility of algae domestication under Cadmium stress, another batch containing 2 L of *S.Cap*. with the same concentration are treated with 1mg/l $Cd(NO_3)_2$ solution to reach a final concentration at 0.1mg/l. The domestication process will take 24 hours. The solution will then be treated as previous steps. Add $Cd(NO_3)_2$ into the samples to let the final cation concentration reaches 2mg/l, 4mg/l and 8mg/l as the experiment group. Each sample will run in triplicate. The experiment will also include 3 control samples: samples only with distilled water, samples only with microalgae and samples only with 4mg/l $Cd(NO_3)_2$ solution each run in duplicate. Samples would be settled under the same condition as the cultivating tank and be shaken twice a day.

The concentration of the heavy metal would be measured by the Atomic absorption spectrometer (AA) with graphite furnace at the following time schemes: 0.5 hr, 1.5 hr, 3.0 hr, 6.0 hr, 18.0 hr, and 36.0 hr. Samples will be diluted to meet the measurement range of AA. The concentration of Cd^{2+} remains in the sample would be recorded to analyze the absorbing rate of *S.Cap.*. After 36hr, the algae sample would be separated and digested to prove the percentage of missing heavy metals was taken up by the microalgae.

Samples are diluted to meet the measurement range of AA. A standard curve is adjusted at the beginning and the end of the measurement.

Copper:

After cultivating, *S.Cap*. will be inoculated into 15 150ml-beakers with a final volume of 100ml and then cultured under the same illuminance. Add CuSO4 into the samples to let the final cation concentration reaches 0.1mg/l, 0.2mg/l, and 0.4 mg/l as the experiment group. Each sample will run in triplicate. The experiment will also include 3 control samples: samples only with distilled water, samples only with microalgae and samples only with 0.1mg/l $CuSO_4$ solution each run in duplicate. Samples would be settled under the same condition as the cultivating tank and be shaken twice a day.

The concentration of the heavy metal would be measured by the Atomic absorption spectrometer (AA) with graphite furnace at the following time schemes: 0.5 hr, 1.5 hr, 3.0 hr, 6.0 hr, 18.0 hr, and 36.0 hr. The concentration of Cd^{2+} remains in the sample would be recorded to analyze the absorbing rate of *S.Cap*. . After 36hr, the algae sample would be separated and digested to prove the percentage of missing heavy metals was taken up by the microalgae.

Samples are diluted to meet the measurement range of AA. The standard curve is adjusted at the beginning and the end of the measurement.

Cu(mg/l)	Algae		reps	Cd(mg/l)	Algae		reps
0	0	Control	2	0	0	Control	2
0	1	Control	2	0	1	Control	2
0.4	0	Control	2	0.4	0	Control	2
0.1	1		3	0.02	1		3
0.2	1		3	0.1	1		3
0.4	1		3	0.54	1		3

Table 1: The design of Cu stress experiment and Cd stress experiment

Complex

Process the same method of the experiment as the previous one. As the observation made by Yin's experiment, the toxic effect of copper ions is 22 times stronger than Cd^{2+}. On the other hand, with the jointing of Cu, antagonism is also presented to strengthen the capacity of microalgae surviving under toxic conditions. Therefore, although the concentration of Cu^{2+} is minor compared to other experiment elements, it is necessary to attach attention to and identify the existence of this phenomenon in the experiment. Cu^{2+} ions would be prepared as $CuSO_4$ solution and the concentration in each sample would be 0 mg/L, 0.2 mg/L, and 0.40mg/L. At the same time, the concentration of Cd^{2+} ion is designed to be 0 mg/L, 5 mg/L and 10mg/L due to the accomplishment of the first stage result. The other factors designed are pH at 6.5 and 8.5; water hardness at 0 and 100mg/L; light time length at 24hr/day and 12hr/day. Algae would be sampled into 10 duplicated reference groups and 16 triplicated experimental groups. A reduced design matrix is exhibited as below:

Cu(mg/l)	Cd(mg/l)	Algae	pH	Hardness(mg/l)	Light time(hr/day)	reps	
0	0	0	6.5	30	24	Control	2
0	0	0	6.5	100	24	Control	2
0	0	0	8.5	30	24	Control	2
0	0	0	8.5	100	24	Control	2
0.2	0	0	6.5	30	24	Control	2
0.2	0	0	8.5	100	12	Control	2
0	5	0	6.5	30	12	Control	2
0	5	0	8.5	100	12	Control	2
0.2	5	0	6.5	30	12	Control	2
0.2	5	0	8.5	100	12	Control	2
0.2	0	1	6.5	30	24		3
0.2	0	1	8.5	30	24		3
0.2	0	1	6.5	100	24		3
0.2	0	1	8.5	100	24		3
0	5	1	6.5	30	12		3
0	5	1	8.5	30	12		3
0	5	1	6.5	100	12		3
0	5	1	8.5	100	12		3
0.2	5	1	6.5	30	24		3
0.2	5	1	8.5	30	24		3
0.2	5	1	6.5	100	24		3
0.2	5	1	8.5	100	24		3
0.4	10	1	6.5	30	12		3
0.4	10	1	8.5	30	12		3
0.4	10	1	6.5	100	12		3
0.4	10	1	8.5	100	12		3

Table 2: The design of Cd-Cu stress experiment

Measure the change of concentration as the previous stage, notice with the addition of copper ions, each sample needed to be run under the different sets of spectrometers twice. The sampling scheme is at 1 hr, 5hr, 9hr, 20hr, 44hr, and 96hr.

The experiment process of this stage is much complicated than the previous ones. Due to the capacity and measuring the speed of the AA spectrometer, each measurement would take about an hour to be done. Therefore, the adjustment of the concentration calibration curve will be done at the beginning of each measurement and after measuring every ten samples.

Hardness modification:

The hardness of water is usually identified by the amount of dissolved calcium and magnesium in the water. In this experiment, the only the amount of calcium is counted as a simplification. Calcium carbonate was added into the sample beakers to present different levels of hardness: 30mg/L Ca^{2+} as “soft” water and 100mg/L Ca^{2+} as “hard” water.

Digestion

Since the algal used for this research has a simple structure and the concentration of Cd^{2+} and Cu^{2+} are both assumed to be presented at a high level(>0.1mg/L), a standard method using nitric acid for digestion is applied for the experiments.

Apparatus:

1) Hot plate, acid-washed 125-mL Erlenmeyer flasks followed by washing with DI water

2) Volumetric flasks, 100-mL.

3) Watch glasses, ribbed and unribbed.

Reagent: HNO_3

Procedure:

Transfer a 100-ml well-mixed algae sample into the centrifuge, remove the top clear layers as much as possible. Add in distilled water to 100ml and repeat the process 2 more times. Preserve the sample in 0.2% HNO_3 to the volume of 100ml again. In a hood, add 5 ml concentrated HNO_3. Add boiling chips into the flask and bring it to a slow-boiling and evaporation on a hot plate. Reduce the amount of liquid to the lowest volume as possible before precipitation. Continue adding concentrated HNO_3 if necessary, until the sample is shown as a light-colored and clear solution. Make sure the sample is not dried during the digestion.

Wash down the flask and the glass cover with DI water, filter the sample in case of cell skeleton remain. Transfer the filtrate to a 100-ml flask with water. Cool down, dilute to mark and mix thoroughly. The filtrate should be ready for the AA spectrometer to determine the metal concentration.

Statistical Analysis:

The results are analyzed using F test. The test is done using the method of least squares to fit general linear models. ANOVA table and other statistical results are generated using SAS® University Edition.

Result and discussion

Algae were cultured and experimented during the year of 2018 to 2019. The average biomass concentration after cultivation is about 2.0 g/l. Due to the sensitivity of the measurements, the data collected shown unclear steady-state values. Fortunately, the overall adsorption is reasonable, and the percentage of metal recovered from digestion exceeds 85%.

Cd^{2+}:

For the first stage of the experiment, all three samples showed a significant reduction of the Cd^{2+} amount remains in the solution. The adsorption appears to be better modeled by the Langmuir isotherm. It is also speculated that this is reasonable because an algae adsorption reaction is typical related to the concentration of a solute on the surface of an adsorbent. However, the final value of the concentration remaining in the solute does not seem to converge to the same level, nor a similar ratio of reducing. The group of 20μg/l and 100 μg/l showed similar efficiencies of Cd^{2+} adsorption, which are 40.63% and 41.34%. However, the third group of 400 μg/l Cd^{2+} exhibited enhanced adsorption of about 93.70% of metal ions are removed. These values are also confirmed by the digestion process,

which showed recoverey of 83.45%, 99.62%, and 96.3%.

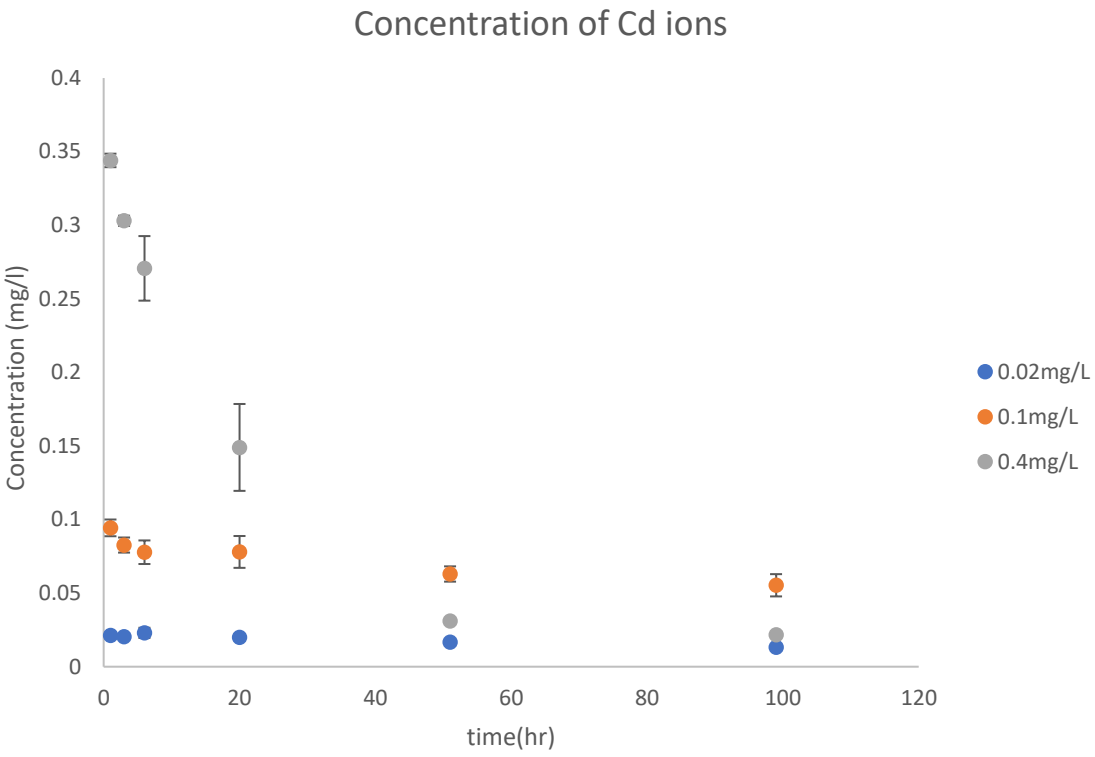


Fig 2. Average Cd^{2+} concentration in the solvent

According to Yin, the EC50 value of Cd^{2+} is around 16.02mg/l which indicates the algae growth rate is suppressed to 50% at this level. Therefore, at a concentration of Cadmium is around 8mg/l, the suppress rate is less than 33.8% in the first three days of growth and show a bounce after. This highest concentration is chosen with consideration based on this basis.

For the second stage, concentration reduction seems to be less significant than the previous stage with a higher initial concentration. However, the reaction rate is reported to be at a constant rate from the first 10 hours result. The concentration of

Cadmium is reduced to 1.2mg/l, 2.3mg/l and 4.8mg/l for 2mg/l, 4mg/l and 8mg/l group. The reaction rate slowed down after 10 hours and completely stopped after 48 hours. The macroscopic evidence has shown algae are congregating at the bottom of the sample beaker.

In Yin's essay, he pointed out that under the stress of heavy metal, photosynthetic pigments inside this type of microalga didn't start declining within the first 24hr under 0.5 and 1.0 TU (toxic units, this unit value is defined by his own group, which contains about 2.92mg/L Cd^{2+} ions and is much higher than my design). He proclaimed this may be caused by the automatic defense mechanism within the microalga cells, the amount of heavy metal ions goes into the cell might induce the reactive oxygen radical scavenging enzyme to remove the heavy metal to minimize the damage to the chlorophyll. In my case, since the design of Cd concentration is much lower than his, the concentration of Cd ions in the first two groups might not have reached the alert of producing the enzyme for defense. The adsorption is only caused by the digestion of the organisms which contributes to metal-binding protein such as metallothionein.

On the other hand, there is another interesting phenomenon observed during the experiment. Since the concentration, I designed for the first experiment seems to be much lower than the possible limitation of the algae, I did an extra comparison experiment between the survived algae and the same number of uncontaminated algae. Each group was added into 5mg/L Cd ions. The first group survived after two weeks while the uncontaminated one died within one week. This, in other words,

also proved Yin's statement that the defense mechanism of the enzyme is activated and preserved during reproduction, which brings the algae stronger survival ability.

Cu:

The absorption of *S.cap* on Cu^{2+} ions is presented to be faster and more completed under the same environmental conditions comparing to Cd^{2+}. The concentration of copper ions also exhibited to have a clear two-stages reduction. The concentration reduced from 0.10mg/l, 0.18mg/l and 0.45mg/l to 0mg/l, 0mg/l and 0.1mg/l. This could be a piece of evidence that the growth of alga is not suppressed at this range of copper contamination. The recovery of metal from the algae digestion are 0.09mg/l, 0.16mg/l and 0.44mg/l. The corresponding percentages of recovery are:90.00%, 88.89%, 97.78%. The metal ions are proved to be converged in the algae cells.

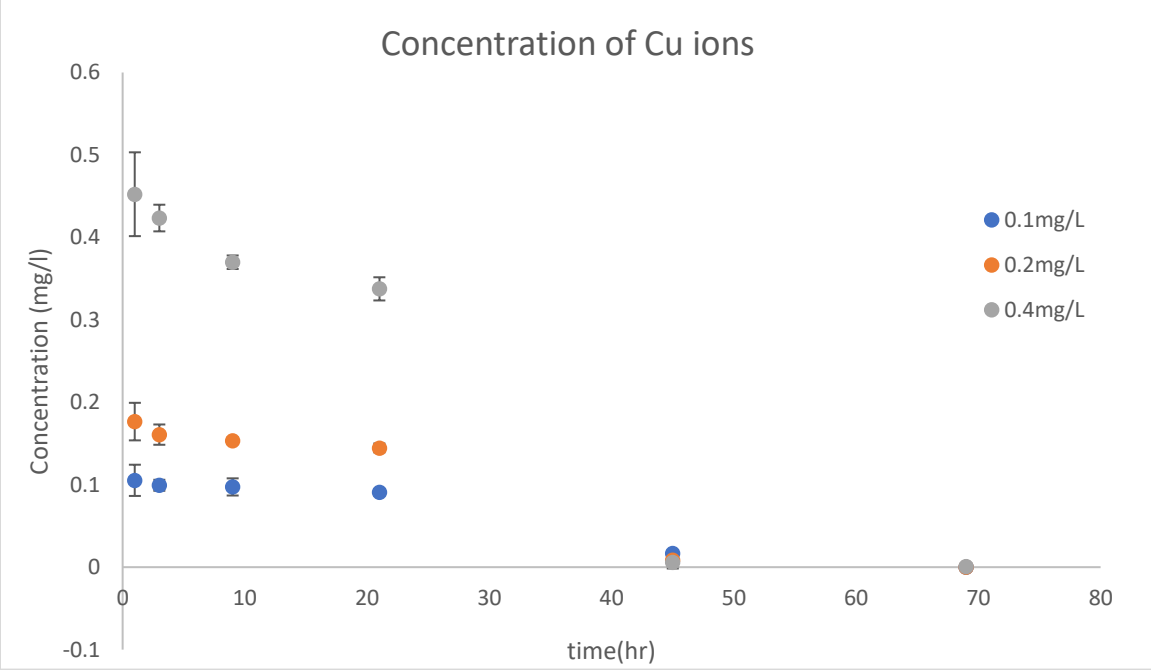


Fig 3. Average Cu^{2+} concentration in the solvent

Cu^{2+} -Cd^{2+} Joint system

The purpose of the study was to determine the influence of different environmental elements on Cadmium and Copper ion removed by *S.Cap*. In general, the heavy metal adsorption process is suppressed with the existence of multiple metal ions. This is proved from the experimental results. The absorption percentage of Cd^{2+} is 40.53% and the one for Cu^{2+} is 94.67% for single metal stress samples. The averaged removal of the same metals declined to 35.56% and 61.86% when added together. The detailed experiment results are presented in Appendix B.

Statistical analysis:

F-test is made to analyze the null hypothesis of justifying whether pH, hardness interaction with other heavy metal ions will be significant. Based on the calculation of mean and standard error of adsorption percentage of each level of environmental factor, we determined the probability of observing the standard normal value. The results[†]* are shown in the graph below:

[†] More details can be find in Appendix C

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	30.82161825	6.16432365	10.58	<.0001
Error	30	17.47107897	0.58236930		
Corrected Total	35	48.29269722			

R-Square	Coeff Var	Root MSE	data Mean
0.638225	21.77092	0.763131	3.505278

Source	DF	Type I SS	Mean Square	F Value	Pr > F
hardness	1	0.00666944	0.00666944	0.01	0.9155
PH	1	8.41966944	8.41966944	14.46	0.0007
hardness*PH	1	0.10780278	0.10780278	0.19	0.6701
Culevel	2	22.28747659	11.14373829	19.14	<.0001

Table 3. F-test result on Cadmium adsorption

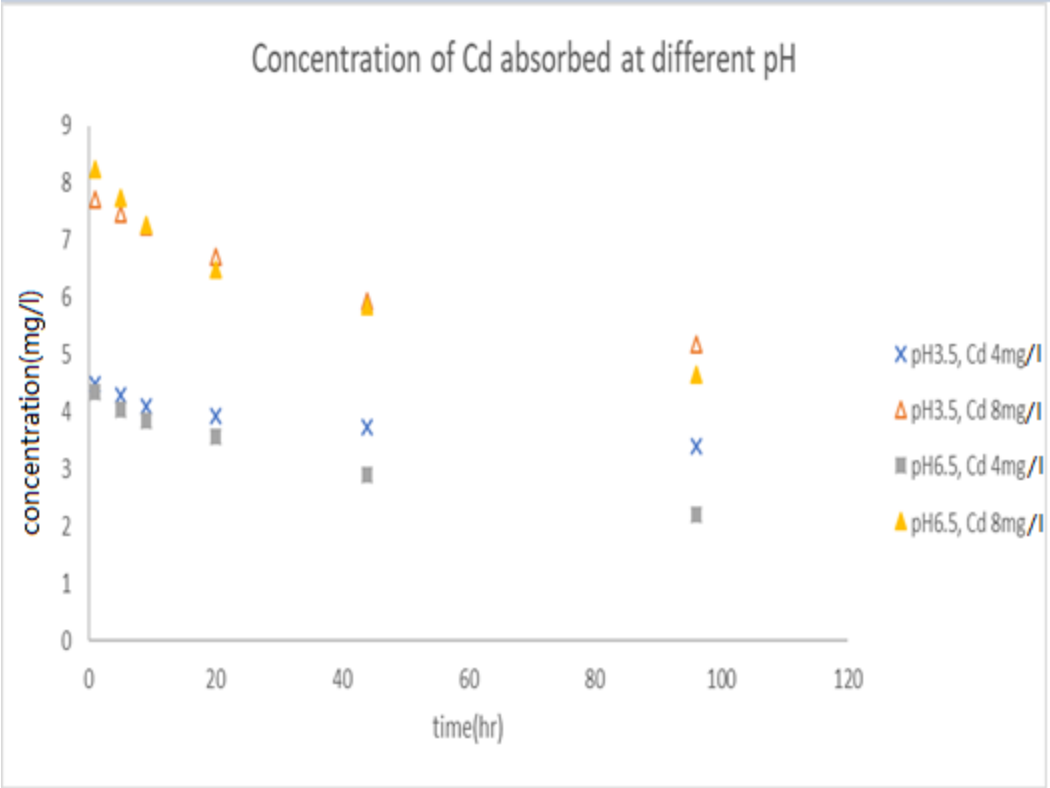
Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.12715625	0.02543125	36.75	<.0001
Error	30	0.02076250	0.00069208		
Corrected Total	35	0.14791875			

R-Square	Coeff Var	Root MSE	data Mean
0.859636	43.54341	0.026307	0.060417

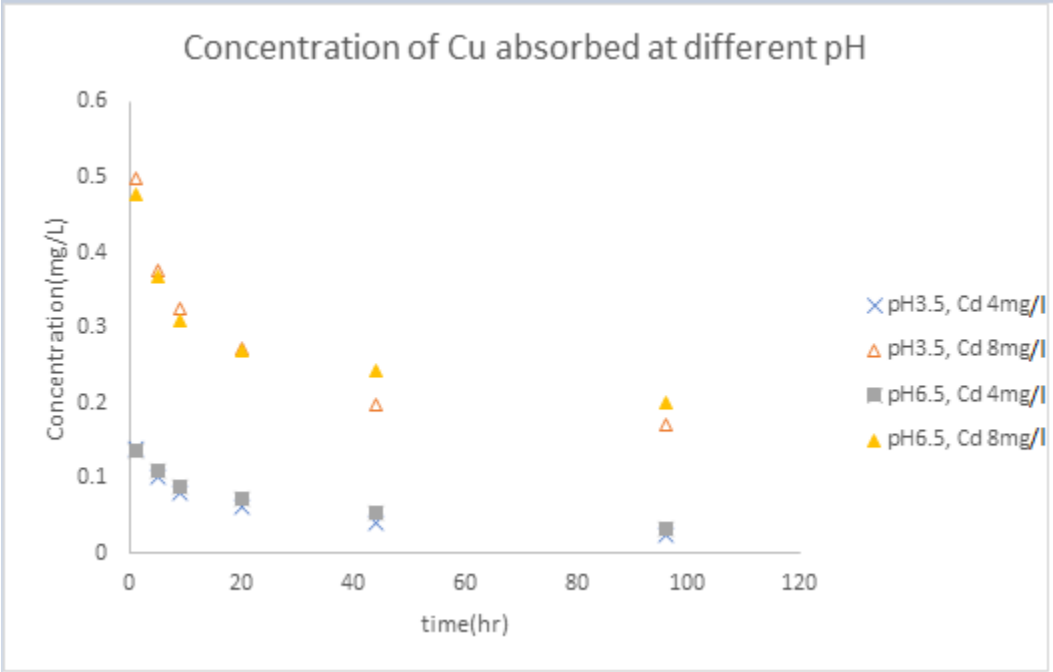
Source	DF	Type I SS	Mean Square	F Value	Pr > F
hardness	1	0.01460069	0.01460069	21.10	<.0001
PH	1	0.00293403	0.00293403	4.24	0.0483
hardness*PH	1	0.00036736	0.00036736	0.53	0.4719
Cdlevel	2	0.10925417	0.05462708	78.93	<.0001

Table 4. F-test result on Copper adsorption

Therefore, for Cadmium, pH is captured in the alternative hypothesis, that is the change of pH will significantly influence the adsorption rate. The presenting of Cd^{2+} will also significantly influence the adsorption rate of Cu^{2+}. On the other hand, hardness plays significant influence on Cu^{2+} adsorption and the presenting of Cd^{2+} also convincingly influenced it. Moreover, interaction graphs are made to determine the main effect of the factors. As we can see from the graphs, interactions of different factors occurred more frequently for Cd^{2+} ion adsorption. The PH, hardness and the present of another metal ion (Cu^{2+}) are all presence as main effects. Interactions occurred between pH and hardness, pH and the present of Cu^{2+} ions when analyzing the adsorption factors of Cadmium. Therefore, the factors are dependent on each other in this scenario.



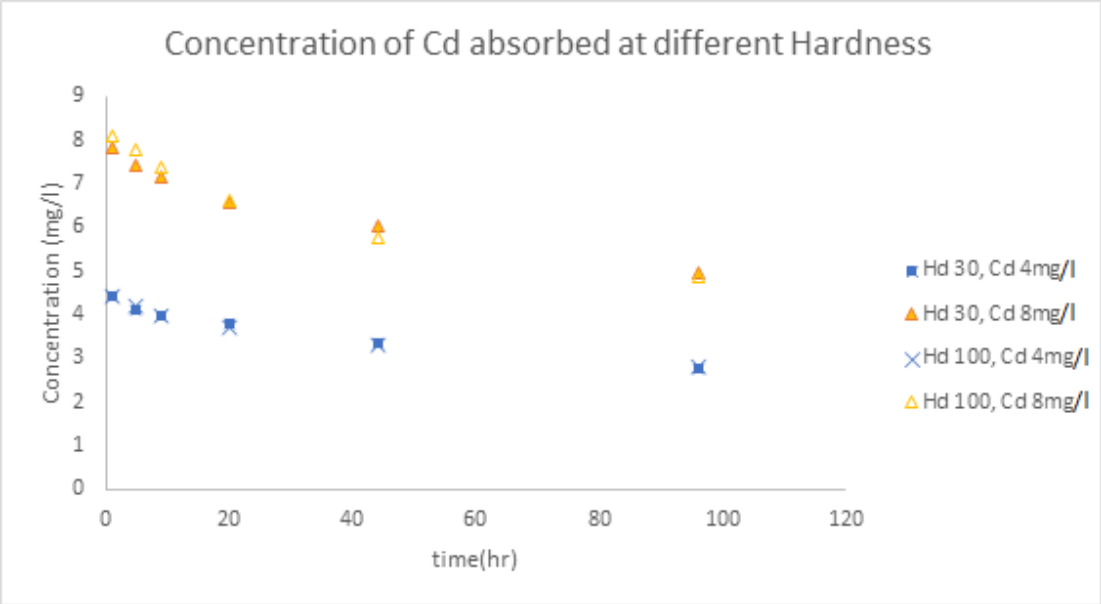
(a)



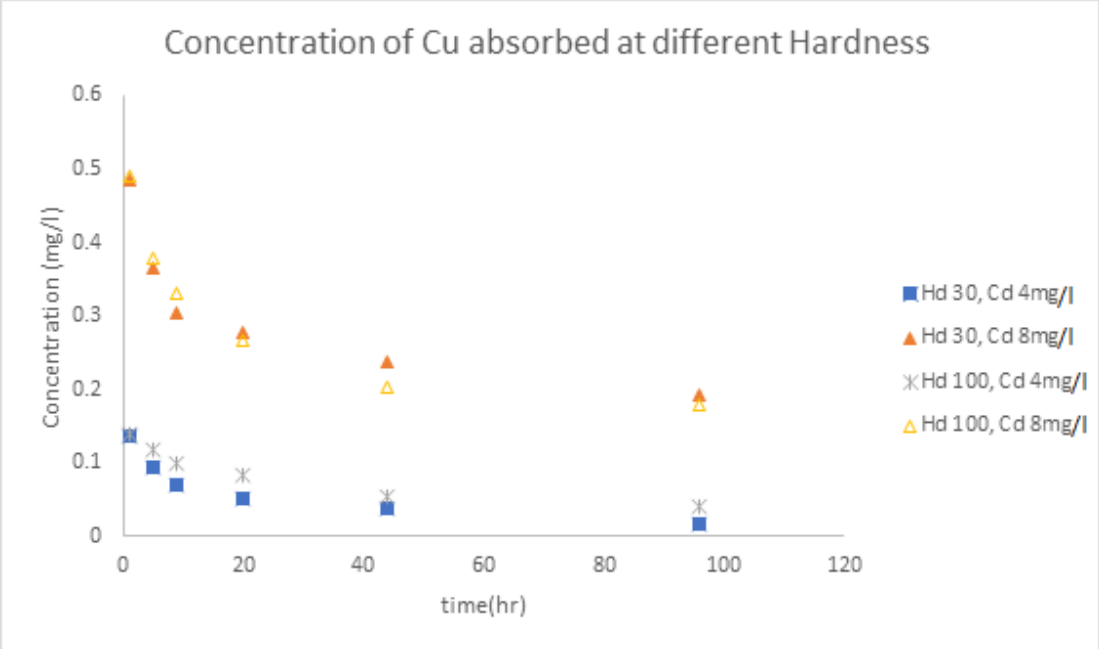
(b)

Fig 4(a~b). the average concentration of Cd^{2+} and Cu^{2+} in different groups at different pH

Solution acidity has a significant influence on the activity of metal absorption. Although the algae are successfully domesticated under pH3.5. The Chlorophyll pigment losses obviously. The average efficiency of Cd^{2+} removal from the solvent is about 46.45% at the pH of 6.5, which is comparatively much higher than the efficiency, 28.73%, in pH3.5 samples. On the other hand, the Cu^{2+} ion removal has shown the opposite result with the influence of sample acidity. Along with the pH increased from 3.5 to 6.5, the average percentage of Cu^{2+} removal decreased from 74.52% to 66.90%.

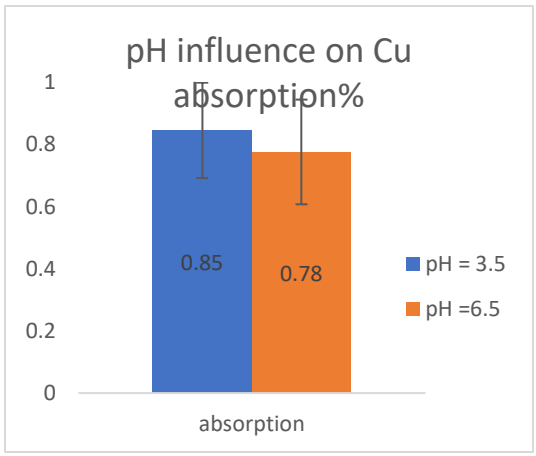


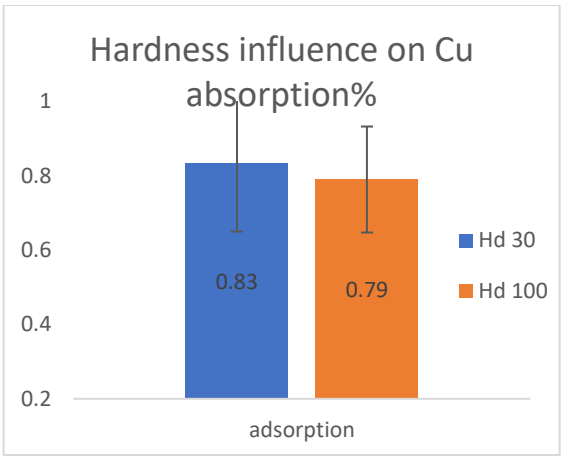
(a)



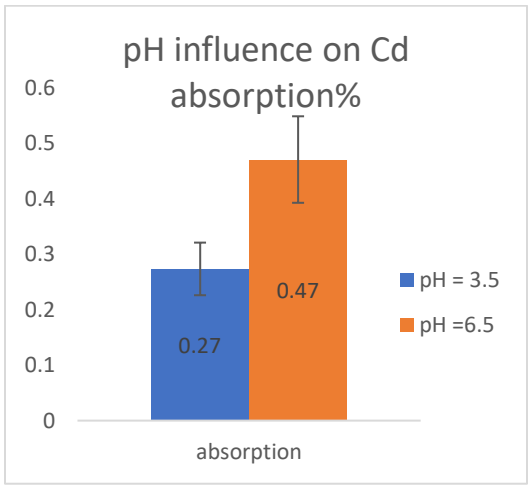
(b)

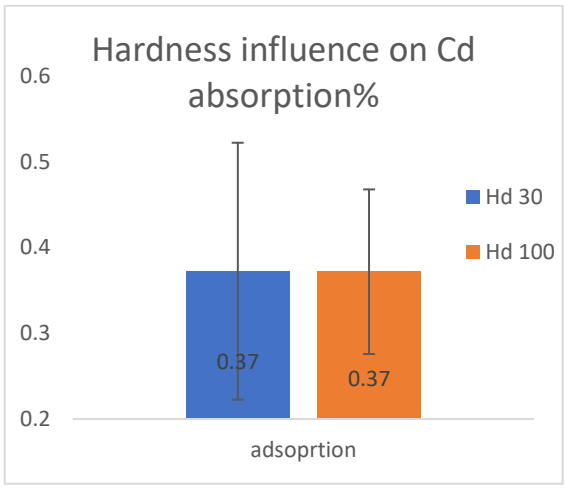
Fig 5(a~b). the average concentration of Cd and Cu^{2+} in different groups at different hardness



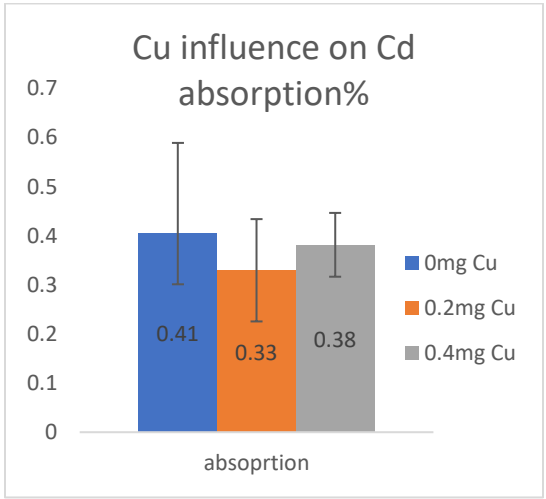


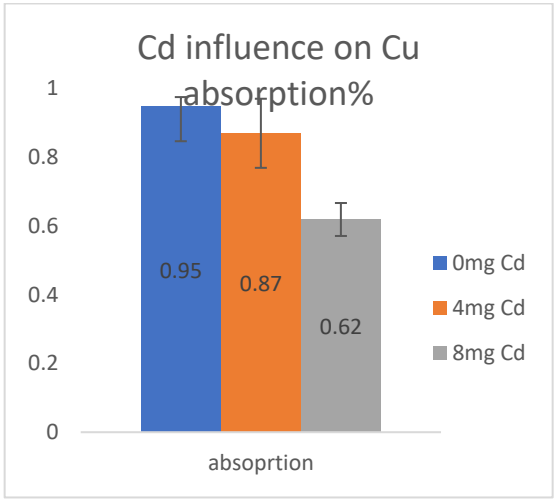
(a) (b)





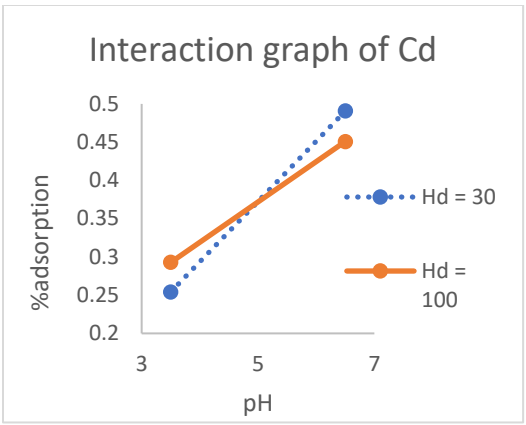
(c) (d)

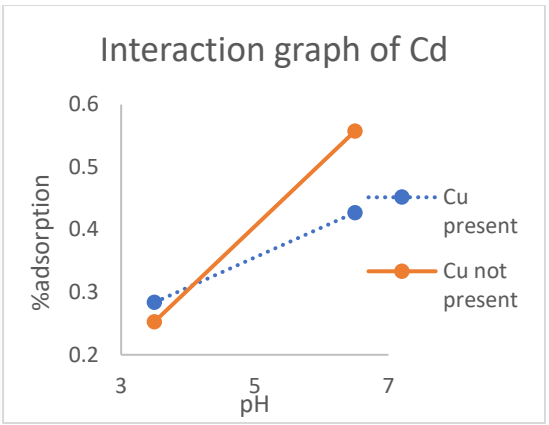




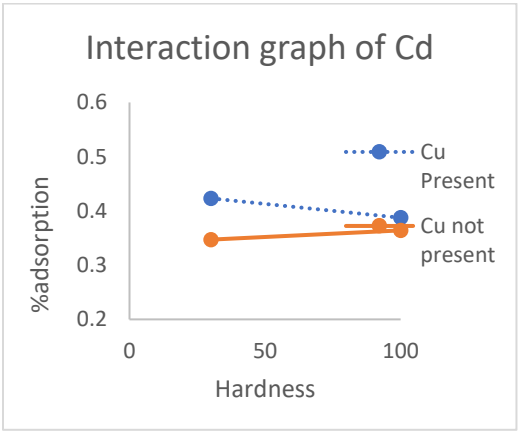
(e) (f)

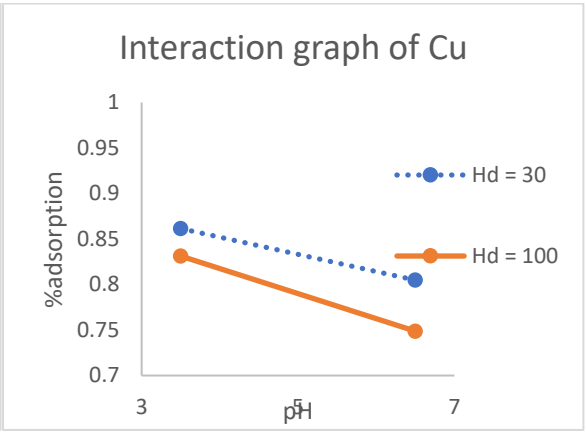
Fig 6. （a~f） Adsorption box charts



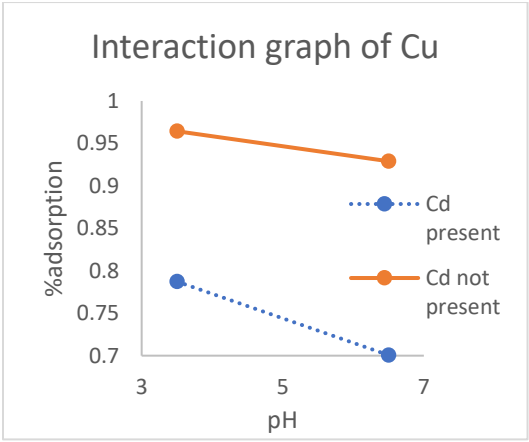


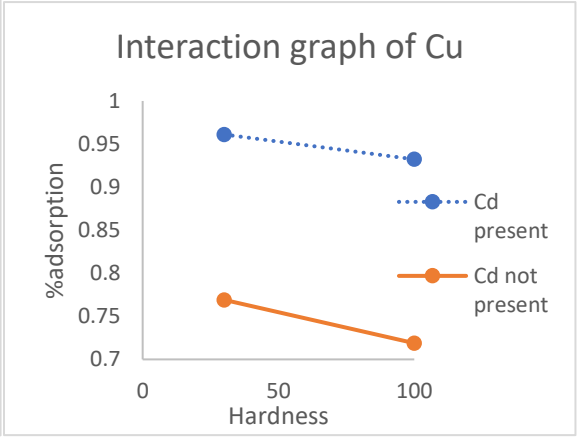
(a) (b)





(c) (d)





(e) (f)

Fig 7(a~f). interaction effect on analyzing the crossing effect of different environmental factors on Cd and Cu adsorption.

Conclusion:

This paper discussed the single and binary toxicity of Cadmium (II) and Copper (II) ions against *Selenastrum Capricornutum* with involving of environmental factors of pH and water hardness. The conclusion is made as below:

(1) *S. Cap* is verified with its ability to adsorb Cu^{2+} and Cd^{2+} ions.

Under the single Cd^{2+} stress, the maximum adsorption of heavy metal observed at 0.4mg/l. At this concentration, the adsorption rate is 93.70%. This value is normal since is obviously much higher than other samples. An assumption of the active enzyme site is made and needed to be further justified. Consulting with Yin's optimal concentration of Cd^{2+}, the maximum adsorption of the metal is 61.78% at Cd^{2+} = 4mg, Cu^{2+} = 0mg, pH =6.5 and hardness = 30 mg/l as Ca^{2+}. In joint toxicity conditions, the adsorption of Cd declined but not significantly. There are no data shown a significant difference of algae absorption suppressed when the concentration of Cd concentration increased from 4mg/l to 8mg/l and Cu^{2+} concentration increased from 0.2 mg/l to 0.4mg/l at all other condition remain the same. Under the single Cu^{2+} stress, the adsorption of heavy metal is completed at lower concentrations. Consulting with Yin's optimal concentration of Cd^{2+}, the maximum adsorption of the metal is 98.66% at Cd^{2+} = 0mg, Cu^{2+} = 0.2mg, pH =3.5and hardness = 30 mg/l as Ca^{2+}. In joint toxicity conditions, the adsorption of Cd^{2+} declined but not significantly. Significant declining of

algae absorption when the concentration of Cd^{2+} concentration increased from 4mg/l to 8mg/l and Cu^{2+} concentration increased from 0.2 mg/l to 0.4mg/l at all other conditions remain the same.

Hardness, presented by the concentration of Ca2+, doesn't seem to have a significant influence on the total consumption of either Cadmium or Copper. A similar result is also found in Venkat's (38 2016) Silver stress experiment. As he noticed, exposure of the more bioavailable silver in very soft water could have resulted in the algae inhibiting silver uptake by downregulating transport proteins or by increasing silver excretion. In soft, moderately hard and hard water, however, there is no additional protection against silver toxicity was observed. In other word, the reaction of heavy metal toxicity is not much influenced in a more bioavailable environment. One assumption might be helpful to discuss this phenomenon: The enzyme, as the defense mechanism, is induced to change its structure so that it binds to certain heavy metals under conditions considered more bioavailable. The inhibiting of heavy metal uptaking under very soft water will be the consequence of the degeneration of enzyme protein during reproduction.

On another hand, when considering the influence between the two different metal ions, as the concentration of heavy metals increases, the amount of enzyme should be affected. We, however, noticed that the ability to absorb Cadmium is reinforced at a higher concentration of binary-metal-stress. The average absorption of Cd seems to be novel. All the samples at

8mg/l Cd^{2+} -0.4mg/l Cu^{2+} shown an increase of reaction completion from the 4mg/l Cd-0.2mg/l Cu^{2+} groups. An average increasing of adsorption rate is about 15.70%.

This is also verified with Yin's experiment result. As his conclusion, when the concentration is Cd^{2+} is at 8mg/l to 32mg/l, the Chlorophyll fluorescent intensity of S. Cap decreased and rose again. The possible reason can be the PSII photosystem of S. Cap gradually adapted to the toxicity at a lower concentration and showed certain stress resistance (Yin et al., 2014). Considering Chlorophyll fluorescent intensity can be a good index of cell activity, the enzyme proteins can be affected in this similar mechanism.

At the same time, the absorption of Cu^{2+} ions seems to be presenting a simpler hypothesis: As the complexity and concentration of metal ions increases, the removal decreases. One possible explanation might need to be further studied: Copper ions, as Cu^{2+}, disrupt metabolic pathways, such as photosynthesis, respiration, ATP production, and pigment synthesis, and inhibits cell division (39. Johnson et al., 2007, as cited in Expósito, 2017). The toxic effect of copper on the growth of S. Cap was about 22(Yin et al., 2014) times that of cadmium, the defense mechanism can be more towards the Cadmium side.

Therefore, as a conclusion, the total adsorption of cation was increased in a binary stress condition. Especially the adsorption of Cd^{2+} is obvious. It is

observed that an antagonistic affect existed between Cd^{2+} and Cu^{2+}. Further research can be studied.

(2) At a binary system of Cd^{2+} and Cu^{2+}, the amount of metal removed by a unit of biomass is:

Condition / Metal	pH = 3.5 Hardness= 30mg/l Ca^{2+}	pH = 6.5 Hardness= 30mg/l Ca^{2+}	pH = 3.5 Hardness= 100mg/l Ca^{2+}	pH = 6.5 Hardness= 100mg/l Ca^{2+}
Cd^{2+} (mg/g biomass)	11.5	17.48	13.82	18.42
Cu^{2+} (mg/g biomass)	1.57	1.35	1.70	1.44

Table 5. mass of metal ions removed by biomass of *S.cap* (mg/g *S.cap*)

Assume 5 kg of each metal required to be moved, the amount of biomass required is:

Condition / Biomass For:	pH = 3.5 Hardness= 30mg/l Ca^{2+}	pH = 6.5 Hardness= 30mg/l Ca^{2+}	pH = 3.5 Hardness= 100mg/l Ca^{2+}	pH = 6.5 Hardness= 100mg/l Ca^{2+}
5 kg of Cd^{2+} (kg)	434.78	286.04	361.79	271.44
5 kg of Cu^{2+} (kg)	3184.71	3703.00	2941.18	3472.22

Table 6. biomass of *S.cap* (kg) required to remove 5 kg of metal ions

Therefore, if we picked the best scenario we achieved, we need 271.44 kg of *S.cap* to remove 5 kg of Cd^{2+}, which is about 135.72 m^3. This is a feasible volume to achieve in wastewater plant. Considering about the reaction processed under a 96-hour interval. If this approach of biosorption is proved to be realistic at a larger scale, *S.cap* could be an efficient biomass for the specified heavy metal treatment.

(3) PH is the most significant factor at both single and binary toxicity conditions for Cd adsorption. At pH = 6.5, the adsorption is much higher than the ones at pH = 3.5. This is justified with the null hypothesis analysis. However, it is not as significant observed during Cu^{2+} adsorption. Further research can be studied.

(4) Hardness is not presented to be significantly influencing the metal adsorption at a bio-available condition. The adsorption of Cu^{2+} is slightly reduced at a higher hardness (100mg/l Ca^{2+}).

(5) Changing from a lower concentration of Cu^{2+} and Cd binary system to a higher group, the absorbing of Cadmium is slightly increased while the one for Copper decreased obviously. The toxicity effect of these two metals on *S. Cap* might not be simply understood as the antagonistic effect.

(6) The algae showed the potential of surviving and the ability to remove heavy metal ions during this experiment. It is worth to persuade further study on the mechanism and possible applications.

Future Works:

The research provided considerations of heavy metal removal using microalgae. It exhibited the combined effect of toxicities on the algae which might influence the overall removal-ability.

In the future, some considerations of the aqueous speciation can be further studied. For example, the relative importance of OH^-. Cl^-, NO_3^- and other anions can be studied for their impact on the algal biosorption. As many anions are proved destructing on algal cell wall and membrane. A combination with domestication of algae can be very important and helpful to demonstrate the biosorption ability.

Moreover, this research only discussed the combination stress of extraneous heavy metal ions. In the future, experiment with longer time scale can be used to justify the results. Also, as the conclusion of observing an antagonistic affect, domesticate of *S. cap* under lower concentration of Cu^{2+} might be an approach to increase the adsorption ability on Cd^{2+} since the concentration of Cu^{2+} is much smaller than Cd^{2+}.

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Appendix A: Bold's Basal Medium

Stock	*Stock solution*	*mL/L*
1. KH_2PO_4	8.75 g/500 mL	10 mL
2. $CaCl_2 \cdot 2H_2O$	12.5 g/500 mL	1 mL
3. $MgSO_4 \cdot 7H_2O$	37.5 g/500 mL	1 mL
4. $NaNO_3$	125 g/500 mL	1 mL
5. K_2HPO_4	37.5 g/500 mL	1 mL
6. NaCl	12.5 g/500 mL	1 mL
7. $Na_2EDTA \cdot 2H_2O$	10 g/L	1 mL
KOH	6.2 g/L	
8. $FeSO_4 \cdot 7H_2O$	4.98 g/L	1 mL
H_2SO_4 (concentrated)	1 mL/L	
9. Trace Metal Solution	See below	1 mL
10. H_3BO_3	5.75 g/500 mL	0.7 mL
11. F/2 Vitamin Solution (optional)	See below	1 mL

Appendix B: Adsorption raw results

Set NO.	Initial Cu^{2+} Concentration (mg/l)	Initial Cd^{2+} Concentration (mg/l)	Final Cu^{2+} Concentration (mg/l)	Final Cd^{2+} Concentration (mg/l)	Cu^{2+} Recovery (%)	Cd^{2+} Recovery (%)
1 pH=3.5 hd=30	0.21	None	0	0	98.66	None
	0.27	None	0.01	0		
	0.27	None	0	0		
2 pH=6.5 hd=30	0.21	None	0.02	0	93.55	None
	0.25	None	0.02	0		
	0.17	None	0.01	0		
3 pH=3.5 hd=100	0.22	None	0	0	94.20	None
	0.22	None	0.03	0		
	00.26	None	0.01	0		
4 pH=6.5 hd=100	0.22	None	0.01	0	92.25	None
	0.29	None	0.03	0		
	0.21	None	0.02	0		
5 pH=3.5 hd=30	None	4.22	0	3.39	None	22.85
	None	4.16	0	3.52		
	None	4.67	0	3.16		
6 pH=6.5 hd=30	None	4.29	0	2.12	None	61.77
	None	4.74	0	1.01		
	None	5.33	0	2.36		
7 pH=3.5 hd=100	None	4.35	0	3.17	None	27.74
	None	4.29	0	3.21		
	None	4.77	0	3.31		
8 pH=6.5 hd=100	None	4.19	0	2.22	None	49.74
	None	4.63	0	2.01		
	None	4.43	0	2.43		
9 pH=3.5 hd=30	0.25	4.48	0	3.49	96.67	22.70
	0.23	5.14	0.01	3.46		

(con't)	Initial Cu^{2+} Concentration (mg/l)	Initial Cd^{2+} Concentration (mg/l)	Final Cu^{2+} Concentration (mg/l)	Final Cd^{2+} Concentration (mg/l)	Cu^{2+} Recovery (%)	Cd^{2+} Recovery (%)
	0.28	4.14	0.02	3.69	96.67	22.70
10 pH=6.5 hd=30	0.27	3.82	0.02	2.66	90.85	42.72
	0.25	4.70	0.04	2.19		
	0.26	3.77	0.02	2.16		
11 pH=3.5 hd=100	0.26	4.36	0.04	3.21	87.16	25.33
	0.22	4.53	0.03	3.43		
	0.27	4.89	0.03	3.65		
12 pH=6.5 hd=100	0.23	4.07	0.05	2.73	73.04	41.15
	0.22	4.34	0.07	2.43		
	0.26	4.01	0.08	2.16		
13 pH=3.5 hd=30	0.5	7.20	0.2	5.29	63.09	30.72
	0.5	7.73	0.13	5.35		
	0.49	7.57	0.22	4.96		
14 pH=6.5 hd=30	0.49	7.67	0.25	5.01	57.04	42.70
	0.47	8.49	0.22	4.66		
	0.46	8.41	0.14	4.42		
15 pH=3.5 hd=100	0.5	8.17	0.16	5.33	68.00	34.77
	0.52	8.21	0.13	4.21		
	0.48	7.46	0.19	5.01		
16 pH=6.5 hd=100	0.46	7.79	0.25	4.95	59.31	44.41
	0.51	8.29	0.19	4.60		
	0.48	8.80	0.15	4.28		

Appendix C: SAS® DATA

Cu:

The GLM Procedure

Dependent Variable: data

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.12715625	0.02543125	36.75	<.0001
Error	30	0.02076250	0.00069208		
Corrected Total	35	0.14791875			

R-Square	Coeff Var	Root MSE	data Mean
0.859636	43.54341	0.026307	0.060417

Source	DF	Type I SS	Mean Square	F Value	Pr > F
hardness	1	0.01460069	0.01460069	21.10	<.0001
PH	1	0.00293403	0.00293403	4.24	0.0483
hardness*PH	1	0.00036736	0.00036736	0.53	0.4719
Cdlevel	2	0.10925417	0.05462708	78.93	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
hardness	1	0.01460069	0.01460069	21.10	<.0001
PH	1	0.00293403	0.00293403	4.24	0.0483
hardness*PH	1	0.00036736	0.00036736	0.53	0.4719
Cdlevel	2	0.10925417	0.05462708	78.93	<.0001

Cd:

The GLM Procedure

Dependent Variable: data

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	30.82161825	6.16432365	10.58	<.0001
Error	30	17.47107897	0.58236930		
Corrected Total	35	48.29269722			

R-Square	Coeff Var	Root MSE	data Mean
0.638225	21.77092	0.763131	3.505278

Source	DF	Type I SS	Mean Square	F Value	Pr > F
hardness	1	0.00666944	0.00666944	0.01	0.9155
PH	1	8.41966944	8.41966944	14.46	0.0007
hardness*PH	1	0.10780278	0.10780278	0.19	0.6701
Culevel	2	22.28747659	11.14373829	19.14	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
hardness	1	1.87809534	1.87809534	3.22	0.0826
PH	1	8.41966944	8.41966944	14.46	0.0007
hardness*PH	1	0.10780278	0.10780278	0.19	0.6701
Culevel	2	22.28747659	11.14373829	19.14	<.0001