REMOVAL OF ARSENIC FROM WATER BY FRESHWATER ALGAE

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Alga is reported as a valuable tool for removing metals from polluted water. In this study alga is used to remove arsenic from water. Among the algae obtained from arsenic polluted water in Ron Phibun district, Nakhon Si Thammarat province, the green alga *Chlorella sp.* exhibited the best arsenic (As(V)) tolerant ability. It could survive at 500 mg l^{-1} As(V). The growth of the alga was not affected by As(V) in the medium up to the level of 50 mg l^{-1}. The capacity of As(V) removal by the isolated *Chlorella sp.* was investigated. Approximately 35% As(V) was removed within the 1st day of exposure. The As(V) removal by the isolated *Chlorella sp.* was caused by accumulation of arsenic in the cell. The arsenic accumulated in the cell was directly proportional to the As(V) level in the medium. The higher the As(V) levels in the medium, the higher the arsenic accumulation in the cell. Heat-killed cells did not possess the ability to accumulate arsenic. Factors which were involved in removal and accumulation of As(V) by the isolated *Chlorella sp.* were algal cell concentrations, growth stages of the algae, pH, and phosphate concentrations of the medium. The removal and accumulation was best when using small amounts of cells whose growth was at the middle of the exponential stage. The optimum pH observed was at 5-7. The removal efficiency of the algae was inversely proportional to the concentration of phosphate in the medium. The As(V) removal capacities of the free and immobilized cells of the isolated *Chlorella sp.* were compared. The removal efficiency of the immobilized cells was directly proportional to both cell concentration and exposure time. This was opposite to the free cells which showed inverse relationship after day 1.

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เนื่องจากสาหร่ายมีความสามารถกำจัดโลหะต่างๆออกจากน้ำเสียได้ดี ดังนั้นในการศึกษานี้จึงใช้สาหร่ายในการกำจัดสารหนูออกจากน้ำ ในจำนวนสาหร่ายที่เก็บมาจากแหล่งน้ำซึ่งมีการปนเปื้อนด้วยสารหนูในอำเภอร่อนพิบูลย์ จังหวัดนครศรีธรรมราช สาหร่ายสีเขียวชนิด *Chlorella sp.* ได้ถูกคัดเลือกเพื่อใช้ในการศึกษาเนื่องจากสาหร่ายดังกล่าวมีคุณสมบัติทนต่อสารหนู สามารถเจริญเติบโตได้ดีในอาหารเลี้ยงที่มีสารหนูในปริมาณสูงถึง 500 มิลลิกรัมต่อลิตร ในการศึกษาการกำจัดสารหนูออกจากน้ำโดยใช้สาหร่าย *Chlorella sp.* พบว่าสาหร่ายสามารถกำจัดสารหนูได้35% ภายใน 1 วัน โดยการสะสมสารหนูไว้ในเซลล์และการสะสมจะเพิ่มขึ้นเมื่อมีปริมาณสารหนูในอาหารเลี้ยงสูงขึ้น นอกจากนี้พบว่าการกำจัดสารหนูจะเกิดขึ้นเฉพาะในสาหร่ายที่ยังมีชีวิตอยู่เท่านั้น จากการศึกษาปัจจัยต่างๆที่ส่งผลต่อการกำจัดสารหนูโดยใช้สาหร่าย *Chlorella sp.* พบว่าการใช้สาหร่ายที่มีอายุอยู่ในช่วงกลางของระยะการเจริญเติบโตโดยมีปริมาณเซลล์ค่อนข้างน้อย ภายใต้สภาวะความเป็นกรดด่างระหว่าง 5-7 และปริมาณฟอสเฟตต่ำจะช่วยเพิ่มประสิทธิภาพในการกำจัดสารหนูได้ดียิ่งขึ้น เมื่อทำการเปรียบเทียบความสามารถในการกำจัดสารหนูโดยใช้สาหร่าย *Chlorella sp.* ในลักษณะเซลล์อิสระและเซลล์ที่ถูกยึดไว้บน alginate bead พบว่าเซลล์ที่ถูกยึดไว้สามารถกำจัดสารหนูได้ดีเมื่อมีจำนวนเซลล์เพิ่มขึ้นและระยะเวลาในการกำจัดนานขึ้นตรงกันข้ามกับเซลล์อิสระซึ่งความสามารถจะเพิ่มขึ้นในวันที่ 1 และลดลงอย่างต่อเนื่อง

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CHAPTER I

INTRODUCTION

Arsenic is an interesting element in that it has long affected human life in two contradictory ways : it is an essential as well as a toxic element. The use of several arsenic compounds as insecticides and herbicides has continued from ancient times to the present. These compounds are also found to be used in veterinary medicine (1). In spite of arsenic's excellent properties, its high toxicity has given it a bad reputation. Long-term exposure to inorganic forms of arsenic has toxic effects on a large number of organs. Moreover, these compounds are involved in the development of cancers especially lung and skin cancers in humans (2,3). Environmental pollution with arsenic is considered to be the cause of arsenic poisoning and becomes to be a matter of concern. In some countries arsenic poisoning are usually observed among people who lives in areas where drinking water has an elevated level of inorganic arsenic because of natural mineral deposits or contamination from human activities (4). In Thailand, human health problems resulting from arsenic contamination of domestic water supply have occurred in Ron Phibun district, Nakhon Si Thammarat province (5). Long-term investigation and monitoring of water have confirmed the presence of dissolved arsenic at concentration exceeding the exceptional standard (0.01 mg l^{-1}). Furthermore, the clear spatial correlation between human burdens and arsenic concentrations in drinking water was reported.

There appears to be a great potential in exploiting the intrinsic capacity of biological organisms such as bacteria, fungi, algae, and plants to clean up water. Biological removal of metal from water has received increased attention in recent years because of it has potential to achieve greater performance at lower cost than conventional technologies for metal removal (6). Among biological organisms, algae are known to be capable of sequestering metals and concentrating them up to several thousand times over the surrounding water (7). Thus, they can be effectively useful for metal removal application. In this study algae are used to remove arsenic from the polluted water and the arsenic removal efficiency is investigated. Additionally, the optimum condition is established to improve the removal capacity.

CHAPTER II

OBJECTIVES

2.1 Objectives

2.1.1 Selection of algae which capable to remove arsenic, As(V), from arsenic polluted water.

2.1.2 Establishing an optimum condition which enhance As(V) removal ability of the selected algae.

2.1.3 Study on efficiency of the selected algae in As(V) removal.

2.1.4 Study on mechanism of As(V) removal in the selected algae.

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2.2.5 Study on the efficiency of an immobilized algae on As(V) removal.

CHAPTER III

LITERATURE REVIEW

3.1 Arsenic

Arsenic is classified as a metalloid element. It is a member of the same group in the periodic table as nitrogen, phosphorus, antimony, and bismuth. Its atomic number is 33, its atomic weight is 74.92, its melting point is 817°c and its chemical properties are similar to those of phosphorus (8). Arsenic has four valance states : -3, 0, +3, and +5. Arsines and methylarsines are characteristics of arsenic in the -3 oxidation state (8). Elemental arsenic (0 oxidation state) is a gray, crystalline material (8). Arsenic also exist as arsenite (trivalent or +3 oxidation state), and arsenate (pentavalent or +5 oxidation state). Different arsenic-containing compounds vary substantially in their toxicities. Arsine is the most toxic, followed in order of generally decreasing toxicity by trivalent compounds, pentavalent compounds, and finally elemental arsenic (9).

Arsenic usually occurs in combination with one or more elements such as oxygen, chlorine, and sulfur and is referred to as inorganic arsenic. When arsenic occurs in combination with carbon and hydrogen it is known as organic arsenic (4,10). It is important to maintain a distinction between inorganic and organic arsenic, since the inorganic form is usually more toxic than those in the organic form. Moreover, the inorganic form is more mobile than the organic form (4,10).

3.1.1 Speciation of arsenic

Arsenic is a metalloid which form a large number of inorganic and organic compounds. Each one of these compounds has its own property and affects biological system in its own way (11). Figure 3-1 contains the structural formulae and names of the arsenic compounds that are known to be associated with organisms and which may be encountered in their local environment (11).

Arsine or arsenic trihydride is colorless, extremely poisonous, natural gas. Its melting and boiling points are -117°c and -55°c ,respectively. Arsine is a powerful reducing agent, even for fairly weak oxidizing agents (3). It is produced accidentally as a result of generation of nascent hydrogen in the presence of arsenic. Arsine and its methyl derivatives may be formed from other arsenic compounds by microbial action or inadvertent chemical reaction that generate strong reducing conditions (4).

Arsenite and arsenate are the most common inorganic arsenic compounds in the environment (11). Arsenic, which is a component of sulfidic ores, is weathering to form arsenate although arsenite may be formed under anaerobic conditions. Arsenate is the predominant form of arsenic in water (8).

Methylated arsenic ; methylarsonic acid (MAA), dimethylarsenic acid (DMAA), trimethylarsine oxide (TMAO), and tetramethylarsonium ion are methyl derivatives (12,13). Small amounts of methylarsenic (MMA) and dimethylarsenic (DMA) are found in water, MMA generally present at higher concentrations than DMA. The methyl derivatives are found to be present in aquatic organisms and may concern with arsenic detoxification process (12,13).

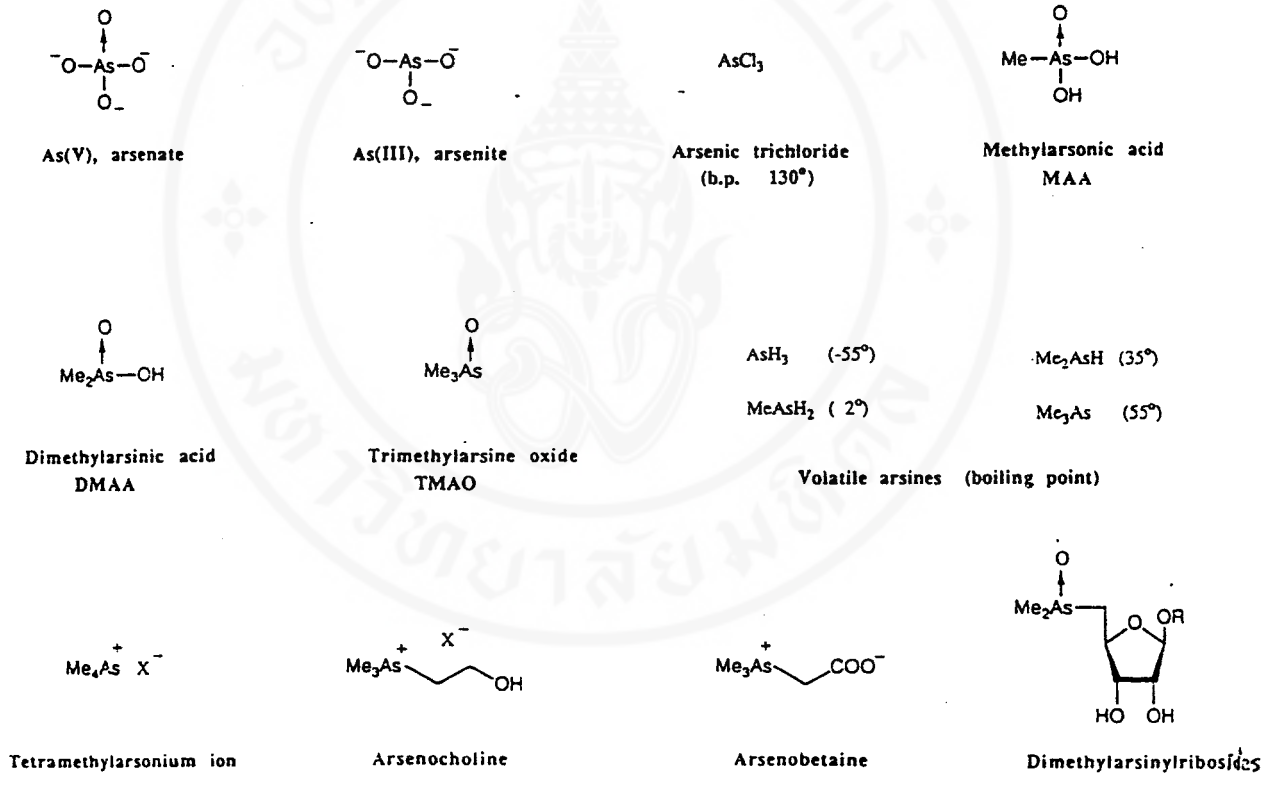


Figure 3-1 : The structure formulae and chemical names of arsenic compound identified in nature.

Arsenosugar or dimethylarsenylribosides are commonly found in marine algae. These arsenic compounds are isolated from the brown alga, *Ecklonia radiata*, *Laminaria japonica*, and also found in marine animal, the giant clam *Tridacna maxima* (11). There are different types of arsenosugar and each type differs only in the precise constituents attached to the sugar at the 1' position (Figure 3-2). Different algal species contain different type of arsenosugars.

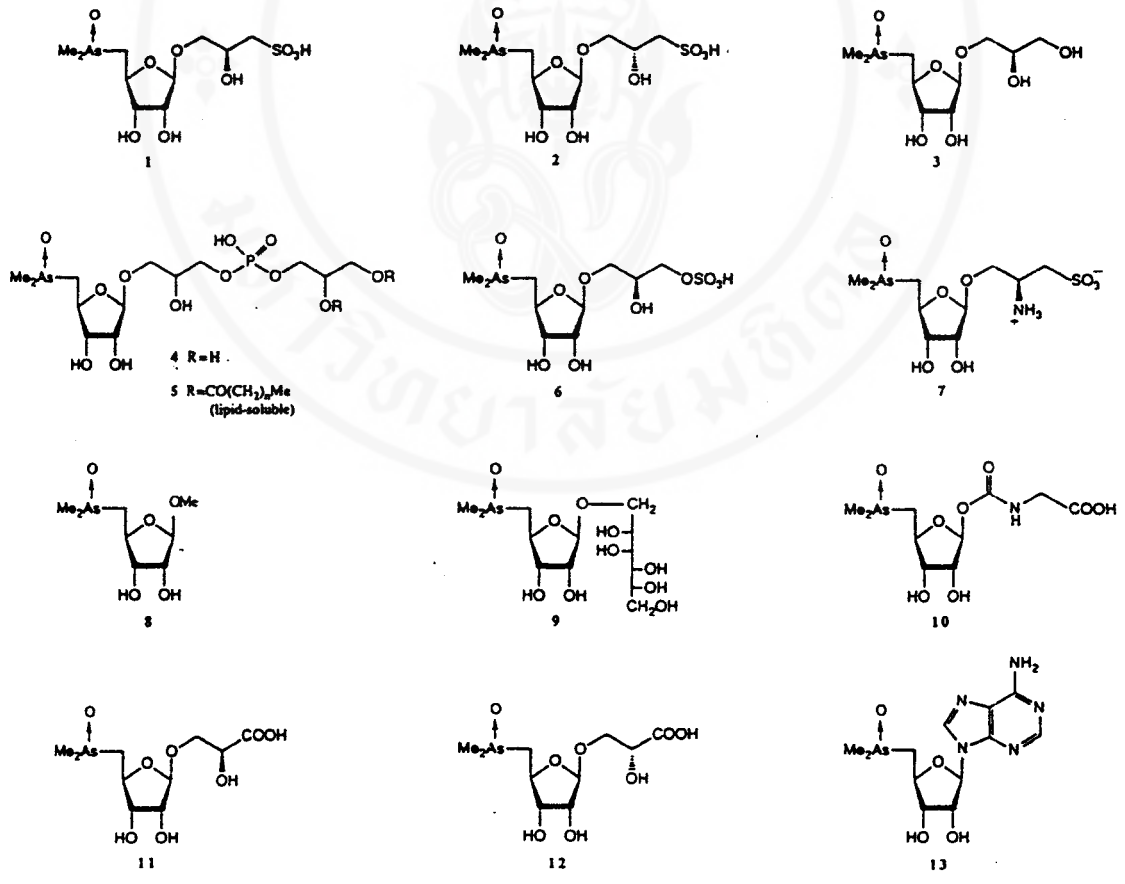


Figure 3-2 : The chemical structures of different types of arsenosugars.

Arsenobetaine is contained at high level in marine invertebrates, especially in crustaceans and mollusks. Arsenobetaine is structurally similar to glycine betaine, but the nitrogen of glycine betaine is replaced by arsenic. Several reports indicated that marine animals accumulated this compound via food chain which is the end product of arsenic cycling in the marine ecosystem (1).

Arsenocholine has the reduced structure of the arsenobetaine. In marine animals, it was shown that glycine betaine could be reduced to choline or the reverse. If this pathway is applicable to the corresponding arsenic compounds, the fact that arsenobetaine is commonly found in marine animals may suggest a wide distribution of arsenobetaine (1).

3.1.2 Occurrence of arsenic

Arsenic is naturally occurring element in the earth's crust. The average crust content of arsenic is estimated to be 1.5 to 3.0 mg kg^{-1} (3). Arsenopyrite (FeAsS) is the most abundant ore of this element. Less widely distributed are arsenolite (As_2O_3), realgar (AsS), orpiment (As_2S_3), mimotite ($Pb_5Cl(AsO_4)_3$), and proustite (Ag_3AsS_3).

3.1.3 Production and uses of arsenic

Annual world production of arsenic is estimated to be about 75,000 to 120,000 tons. Almost all (97%) of the arsenic produced worldwide enter end-product manufacture in the form of arsenic oxide (8,14). The major uses of arsenic are in the production of insecticides and herbicides. Much smaller amounts are used in the manufacture of glass, textiles and in medical and veterinary applications (8,14).

Progressive replacement of arsenic insecticides by organic compounds leads to a decrease in production from up to 70,000 to about 40,000 tons per year. Main producers of arsenic are Sweden, Mexico, France, and the United States (3,15).

The most frequent application of arsenic is in the preparation of insecticides, mainly as lead arsenate and less frequently as calcium arsenate and arsenite, sodium arsenite, cupric arsenite (Scheele's green) and cupric acetoarsenate (Paris green) (3). Arsenicals found other frequent applications as herbicides, desiccants, wood preservatives and growth stimulator for plants and animals (3,16). Other uses of arsenic include the glass industry, electronic applications, colors for digital watches, the textile and tanning industries, and manufacture of pigments and antifouling paints (3,16).

Several arsenic compounds have found applications in veterinary medicine. Arsenic compounds, administered as Fowler's solution (potassium arsenite), Donovan's solution (arsenic iodide) or de Valagin's solution (arsenic trichloride), are employed to treat rheumatism, arthritis, asthma, malaria, trypanosome infections, tuberculosis, and diabetes (3,17).

3.1.4 Effects of arsenic on human

Arsenic is a common toxic substance with exceedingly diverse manifestations of poisoning. Different species of arsenic have different degrees of toxicity. The body's toxic response depends on the route and dose of exposure in addition to individual and local tissue susceptibilities (18).

3.1.4.1 Types of exposure

The two routes of adsorption of arsenic are inhalation and/or ingestion, although there may be some degree of skin absorption (18). If the initial contact is by ingestion, then symptoms caused by gastrointestinal irritation will dominate the early reaction. If inhalation is the route of initial contact, then respiratory irritation will be a major determinant of early symptoms. However, once the arsenic is absorbed, the vascular circulation will ensure contact with other organs, with a wide variety of potential symptoms reflecting the diversity of possible organ damage (18).

3.1.4.2 Arsenic metabolism in human

The metabolism of inorganic arsenic has been studied extensively in both animals and human. Two major metabolic pathways for arsenic have been identified : oxidation-reduction reactions for the interconversion of arsenate and arsenite in the body, and methylation reactions that ultimately convert these compounds to methylated derivatives as metabolic products (4). Essentially, these processes are similar whether the animals or human are exposed orally or by the inhalation of arsenic.

In vitro studies have indicated that the substrate for methylation is arsenic(III), since arsenic(V) is not methylated unless it is first reduced to arsenic(III) (4.19). The main site of methylation appears to be the liver where the methylation process is mediated by enzymes which utilize S-adenosyl-methionine as a cosubstrate. The arsenic dose at which methylation capacity becomes saturated cannot be defined precisely (4).

Human exposure to either arsenate or arsenite usually results in increased levels of inorganic arsenic(III), arsenic(V), MMA and DMA in the urine (4). The relative portions of arsenic compounds in urine varied according to the chemical administered, the amount of time in which samples are analyzed after the initial exposure, the dose, and the animal species. However, DMA is the principal metabolite, followed by the inorganic arsenic and MMA (4).

3.1.4.3 Arsenic poisoning effects

Acute effects

Symptoms of acute intoxication usually occur within 30 minutes of ingestion but may be delayed if arsenic is taken up with food. Initially, a patient may have a metallic taste or notice a slight garlicky odor to the breath associated with a dry mouth and difficulty swallowing. Severe nausea and vomiting, colicky abdominal pain, and profuse diarrhea with rice-water stools abruptly ensue (9). In severe poisoning, the skin becomes cold and clammy, and some degree of circulatory collapse usually occurs along with kidney damage and decrease urine output. Drowsiness and confusion are often seen along with the development of a psychosis associated with paranoid delusions, hallucinations, and delirium. Finally, seizures, coma, and death, usually due to shock, may ensure (9).

Chronic effects

The most prominent chronic manifestations involve the skin, cardiovascular, blood, and neurologic systems (9).

Chronic exposures to arsenic either by ingestion or inhalation will produce a variety of skin insignia of arsenic toxicity (9). An initial persistent erythematous flush from arsenic slowly leads to melanosis, hyperkeratosis, and desquamation. The skin pigmentation is patchy and has been given the peotic description of "raindrops on a dusty road". The hyperkeratosis is frequently punctate and occurs on the distal extremities. A diffuse desquamation of the palms and soles is also seen. Long-term cutaneous complications include the development of multicentric basal cell and squamous cell carcinomas (9).

Arsenic have serious effects on the human cardiovascular system. Arsenic exposure cause altered myocardial depolarization (prolonged Q-T interval and nonspecific ST segment changes) and cardiac arrhythmias that may lead to heart failure. Low-level arsenic exposure may also cause vascular system damage, a classical example of which is blackfoot disease (4).

Anemia and leukopenia are almost universal with chronic arsenic exposure: thrombocytopenia frequently occur. The anemia is usually normochromic and normocytic and caused at least partially by hemolysis. Interference with folate metabolism and DNA synthesis may result in megaloblastic changes (9).

A peripheral neuropathy is the hallmark of chronic arsenic poisoning. The nerves in the limbs are affected, with loss of sensation in the feet and hands. The sense of touch is diminished, and the cornea of the eyes become anaesthetic and easily inflamed and ulcerated. The muscular power is also affected in severe cases (20).

3.1.4.4 Carcinogenic effects

Induction of cancer appears to be the most striking long-term effect of chronic exposure to inorganic arsenic (3). Epidemiological studies have demonstrated an evidence causal relationship between environmental, occupational, and medicinal exposure of man to inorganic arsenic and cancer of the skin and lungs (3,21).

There exists a clear association between pre-cancerous dermal keratosis, epidermoid carcinoma of the skin and lung cancer, and exposure of humans to water-soluble inorganic arsenic through drinking water with a high natural arsenic content (3). The risk of induction of skin cancer has been estimated to be $1x10^{-5}$ per 0.02-0.04 μg As per liter drinking water consumption over 70 years (2,3). WHO also estimated an increased lung cancer incidence of 1% in workers exposed for at least 25 years to 0.250 μg $(m^3)^{-1}$ non-soluble inorganic arsenic, and for the general population of 0.75% to 1 μg $(m^3)^{-1}$ (2,3).

3.1.5 Limiting concentration

According to World Health Organization (WHO) drinking water should not contain more than 50 μg l^{-1}, and daily intake through food should not exceed 50 μg kg^{-1} body weight. This value is probably too high, because it would correspond to a daily

intake of 3.5 mg arsenic for an adult of 70 kg (2,3). As a result of this, the recent potable water standard of 10 $\mu g\ l^{-1}$ has been developed by WHO (5).

Environmental Protection Agency (EPA) has established a Maximum Contaminant Level (MCL) of 50 $\mu g\ l^{-1}$ for arsenic in drinking water (22). EPA has also set the effluent standard of arsenic for industries, in addition to restriction of arsenical pesticides (22).

There is sufficient evidence to confirm that inorganic arsenic compounds are human carcinogens. EPA has thus developed the unit cancer risk estimates, e.g., for inhalation is 50 $\mu g\ kg^{-1}\ day^{-1}$ (assuming that 30% of inhaled arsenic is absorbed) and for oral exposure is 15 $\mu g\ kg^{-1}\ day^{-1}$ (3,22).

3.2 Arsenic in the environment

Arsenic is relatively common in environmental sources such as air water and soil. It is also found in living organisms (8). Natural arsenic cycle entails a constant shifting of arsenic between environmental compartments (8). Arsenic is ubiquitous in living organisms and is constantly being oxidized, reduced, or otherwise metabolized. In soil, insoluble or slightly soluble arsenic compounds are constantly being resolubilized, and the arsenic is being presented for plant uptake or reduction by organisms and chemical processes. Human beings reportedly have been modified the arsenic cycle only by causing localized high concentration. The speciation of arsenic in the environment is affected partly by indiscriminate biological uptake, which consume about 20% of the dissolved arsenic pool and resulted in measurable concentration of reduced and methylated arsenic species (8). The overall arsenic cycle is similar to phosphate cycle,

however, regeneration time for arsenic is much slower-in the order of several months (8,23).

Arsenic in soil exists as both trivalent and pentavalent states. In aerobic soil, the dominant arsenic species is As^{+5}, and small quantities of arsenite and MAA are present in mineralized areas. In anaerobic soils, As^{+3} is the major species (8). The trivalent arsenic species are generally considered to be more soluble and more mobile than As^{+5} and thus poses greater problems by leaching into surface and groundwater (8). Soil microorganisms metabolize arsenic into volatile arsine derivatives. Depending on condition, 17 to 60% of the total arsenic presented in soil may volatilized (8,10).

In water, arsenic occurs in both inorganic and organic forms and in dissolved and gaseous states. The form of arsenic in water depends on Eh, pH, organic content, suspended solids, dissolved oxygen, and other variables (8). Arsenic in water exists primarily as a dissolved ionic species ; particulates account for less than 1% of the total measurable arsenic (8,24). Arsenic is rarely found in water in the elemental state, and it is found in the -3 state only at extremely low Eh values (8). Common forms of arsenic encountered in water are arsenate, arsenite, MAA, and DMA. The formation of inorganic pentavalent arsenic, the most common species in water, is favored under condition of high dissolved oxygen, basic pH, high Eh, and reduced organic material content (8,10). The opposite conditions usually favor the formation of arsenite. Physical processes play a key role in governing arsenic bioavailability in aquatic environments. For example, arsenates are readily sorbed by colloidal humic material under condition of high organic content, low pH, low phosphate, and low mineral

content (8,14). Arsenates also coprecipitates with, or adsorb on , hydrous iron oxides and form insoluble precipitates with calcium, sulfur, and aluminium compounds (8,14).

In reduced environments such as sediments, arsenate is reduced to arsenite and methylated to MAA and DMA. These compounds may be further methylated to trimethylarsine or reduced to dimethylarsine, and they may be volatilize to the atmosphere (8). Arsenates are more strongly adsorbed to sediments than other arsenic forms, the adsorption processes depending on arsenic concentrations, sediment characteristics, pH, and ionic concentration of other compounds (8,14).

3.2.1 Arsenic in aquatic environment

In general, background concentrations of arsenic in water is less than 10 $\mu g\ l^{-1}$ (8,10). Natural weathering of rocks and soils added about 45,000 tons of arsenic to the oceans annually, accounting for less than 0.01 $mg\ l^{-1}$ on a global basis (8,10). However, arsenic inputs to oceans increased as a result of commercial uses and production of arsenic compounds. Estimates of residence times of arsenic are 60,000 years in oceans and 45 years in freshwater lakes (8,10).

In the aquatic environment, arsenic exists in both inorganic and organic forms. Inorganic arsenic occurs predominantly as arsenate. The main organic species in freshwater are MAA and DMA, and there are usually present in lower concentrations than arsenites and arsenates. Inorganic arsenic is more toxic to aquatic biota than organo arsinicals, and trivalent species are more toxic than pentavalent species (8).

Background arsenic concentrations in freshwater biota are usually less than 1 mg kg^{-1} fresh weight (8). These levels are higher in biota live in arsenic contaminated areas. Marine organisms, however, normally contain high arsenic concentrations. Arsenic appears to be elevated in marine biota because of their abilities to accumulate arsenic from seawater and food sources, not because of localized pollution (8). The great majority of the total arsenic in organisms is present as organic derivatives, which are often found at concentrations exceeding those of inorganic arsenic by an order of magnitude or more. In freshwater algae, DMA is the dominant arsenic species (25). In marine biota, arsenic exists as organoarsenicals including arsenosugars in algae, and arsenocholine and arsenobetaine in animals (1). Generally, the arsenic content in freshwater algae is lower than in marine algae (25).

Algae constitute an important source of organoarsenic compounds in aquatic food webs (8). In the food chain composed of the alga *Dunaliella marina*, the grazing shrimp *Artemia salina*, and the carnivorous shrimp *Lysmata seticaudata*, organic forms of arsenic are derived from *in vivo* synthesis by *Dunaliella marina* and efficiently transferred, without magnification, along the food chain (8,26).

Arsenic is bioaccumulated from water by many organisms, however, there is no evidence of biomagnification in aquatic food chains (8,10). In a freshwater food chain composed of algae, daphnids, and fish, water concentrations of 0.1 mg l^{-1} arsenic produced residues after 48 h of 4.5 (mg As kg dry $weight^{-1}$) in algae, and 3.9 in daphnids, but only 0.09 in fish (8).

3.2.2 Biotransformation of arsenic in algae

Arsenic undergoes a series of biological transformations in algae, yielding a large number of compounds especially organoarsenicals (27). Arsenate, the dominant species of arsenic in water, is taken up by algae due to its chemicals similarity to phosphate (28). There seem to be an active regulatory mechanism which maintains the cellular arsenic concentration at non toxic levels (27). At relatively low concentrations of arsenate in water, the algal cell can reduced most of it and send it through the reducing-methylating pathway to neutralizing its harmful effects and to facilitate its excretion into the environment. When arsenate reaches high levels in water, the reducing power of the algal cell becomes insufficient and some of the arsenate replaces phosphate in sugar metabolites, stop glycolysis, and is toxic to the cell (27).

In reducing-methylating pathway, arsenate is reduced to arsenite prior to methylation of the latter to produce MMA and DMA, the methylated derivatives. Mostly DMA is accumulated in the algal cell, however, a small part of this compound is excreted after produced (27). Dimethylarsenic compound is present in both freshwater and marine algae. It seems to be the most stable arsenic form in freshwater algae, whereas in marine algae, it acts as a precursor to be used in the synthesis of arsenosugar (12,13,29). Figure 3-3 shows the biotransformation of arsenic in marine algae.

Arsenosugar is the major form of arsenic which is found in the marine algae. This compound may be an intermediate in the production of arsenocholine and arsenobetaine that are present in marine organisms. Figure 3-4 shows a possible biochemical pathway from arsenosugar to arsenocholine and arsenobetaine.

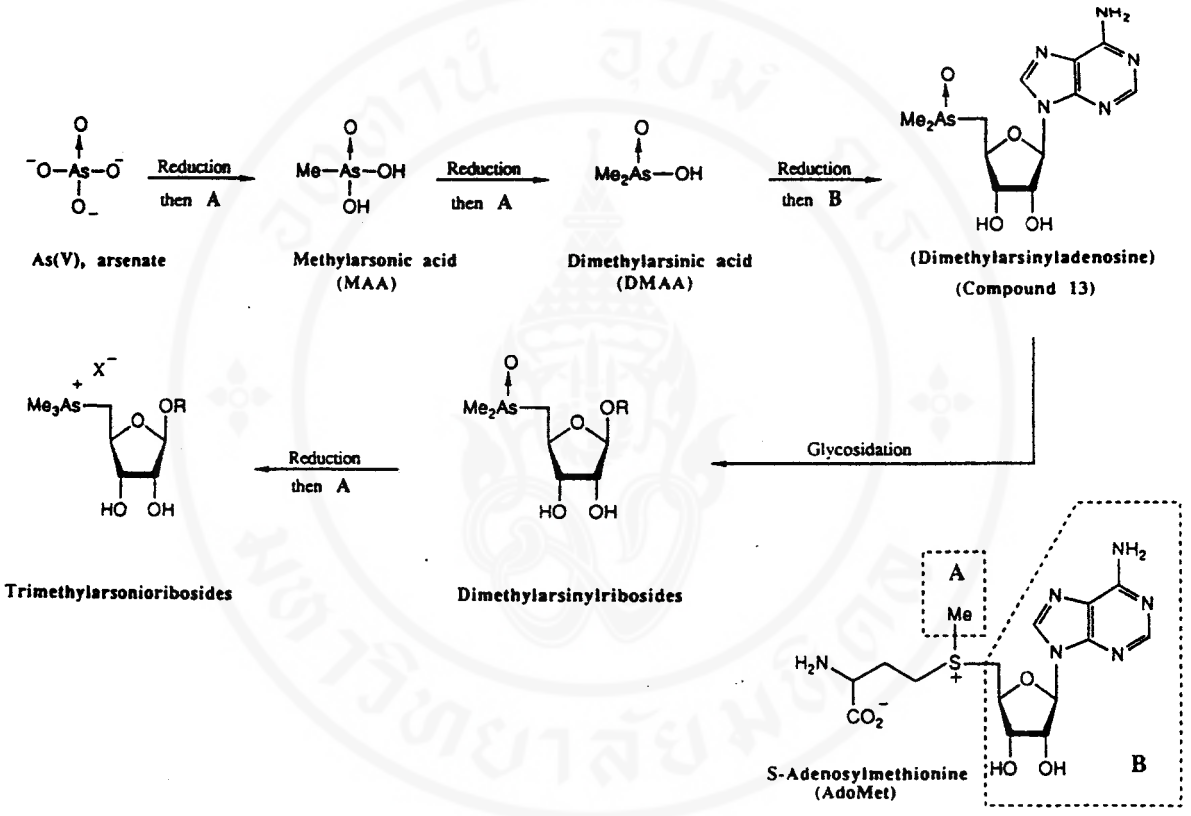


Figure 3-3 : Biotransformation of arsenic in marine algae.

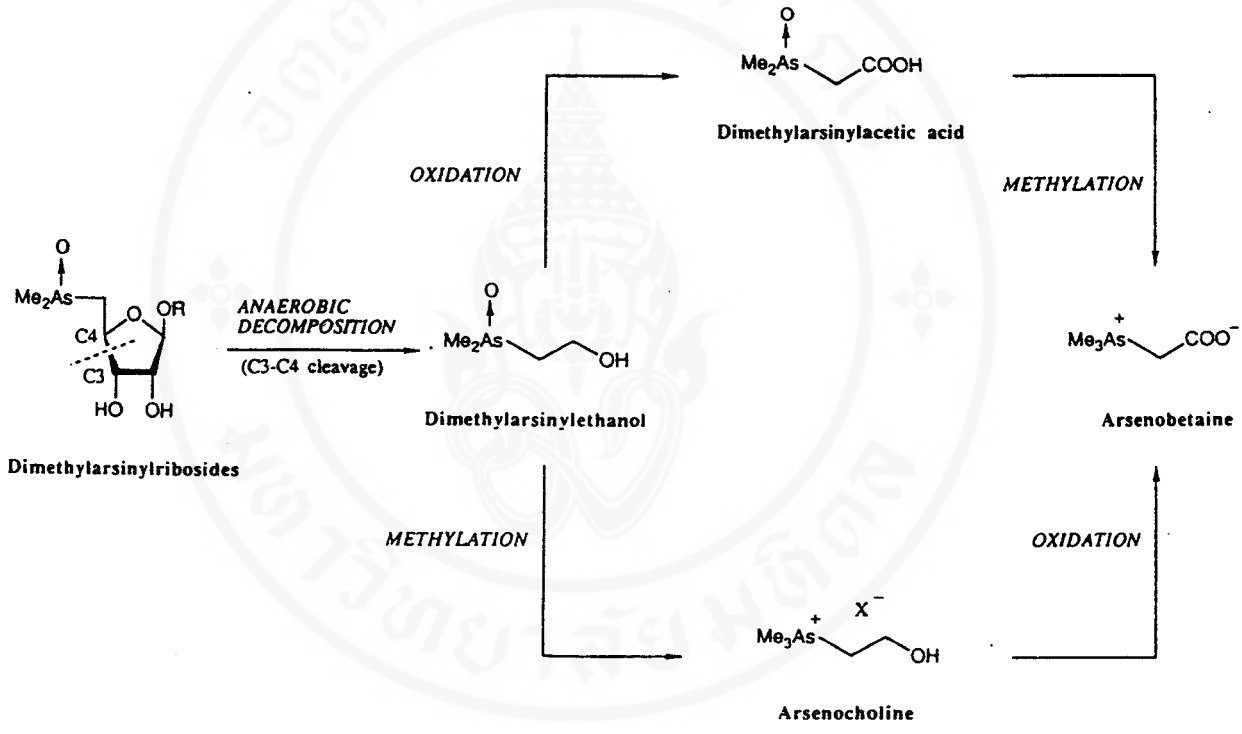


Figure 3-4 : A possible chemical pathway from arsenosugar to arsenocholine and arsenobetaine.

3.3 Case of arsenic poisoning : Chronic arsenic poisoning in Ron Phibun district, Nakhon Si Thammarat province, southern Thailand.

Ron Phibun district is located in Nakhon Si Thammarat province, southern part of Thailand. It consists of 8 subdistricts and 65 villages (5). Main incomes of this district are from mining and farming. In the western part of the district is a wide mountainous area, Ron Na-Suang Chan mountain subrange. This subrange is a component of Khao Luang Range, a series of north-south-trending en-echelon ridges that forms a mountainous backbone to peninsular Thailand. In the eastern part of the district is agricultural plains cultivated with rubber plantations and rice paddies (5). Both surface and groundwater drainage systems are water sources in Ron Phibun. Surface drainage systems are orientated predominantly west-east, with headwaters in the Ron Na-Suang Chan mountains. Groundwater drainage systems including two types of aquifers, a shallow aquifer with a depth of less than 10 m, and a deeper carbonate-hosted aquifer at depth of more than 15 m (5).

Ron Phibun is part of the South-East Asian Tin Belt with respect to its geology (5). The geology of this area is characterized by S-type biotite and biotite-muscovite granitoids of Triassic age, with abundant pegmatitic veining. Cassiterite and wolframite mineralization, with abundant arsenopyrite and pyrite, occurs in pegmatites. Mining and mineral processing activities existed in Ron Phibun during the past 100 years (5). Over 20 bedrock mining and alluvial mining were held in Ron Na-Suang Chan mountain range. Placer deposits at the foot of the Ron Na-Suang Chan range were worked by dredging and open pitting. Furthermore, casual prospecting for cassiterite by residents of the Ron Phibun district was widespread.

The occurrence of human health problems attributable to arsenic contamination of water supplies in Ron Phibun were first recognized in 1987 (5). A preliminary survey was initiated by the Ministry of Public Health in 1988 confirmed approximately 1,000 cases of arsenic-induced skin disorders and skin cancer. Concentrations of arsenic in hair and fingernails were found to be elevated in 80% of the school age population. A follow-up study of 2,400 school pupils in 1992 showed 89% to have excess blood arsenic concentrations, with a 22% incidence of arsenical skin manifestations (5).

According to long-term investigation and monitoring of surface and groundwater, arsenic concentrations in water was exceed the WHO potable water standard (10 $\mu g\ l^{-1}$) (5). Surface drainage was contaminated from adit mines on the mountain to alluvial mines in the lowland. The average concentration during 1992-1997 was observed as 0.077-0.508 mg l^{-1} with the maximum concentration 1.005 mg l^{-1} (5). At the same vicinity, revealing improved condition, arsenic content was reduced to 0.588 mg l^{-1} in 1998 and 0.29 mg l^{-1} in 1999. Shallow groundwater showed the highest concentration in the range of 0.050-5.114 mg l^{-1}. In deep carbonate aquifer, water appeared less contamination. Three wells were contaminated with arsenic concentration in the range of 0.290-1.167 mg l^{-1}. Arsenic speciation trends in the surface and shallow groundwater systems showed a marked dominance of arsenate, 70-95% of total arsenic. In reducing environment of deep aquifer, up to 40-95% of total arsenic were found as arsenite (5).

Mining activities are considered to be a major cause of arsenic contamination of surface drainage and groundwater systems in Ron Phibun (5). The potential contaminant sources of arsenic have been identified by the Department of Mineral

Resources, Ministry of Industry (5). The high-grade arsenopyrite waste piles of the bedrock mines are considered to be particularly critical, as they occupy the main headwater tributaries of the surface drainage system and could thus plausibly contaminate streams and groundwater throughout the entire catchment area. The high-grade waste piles hold arsenic concentrations of 1-30% and are the products of in situ flotation at sites of primary tin extraction. Another sources of arsenic are sub-ore grade waste rock piles, sulphide-rich wastes from ore-dressing plant, dissiminated sulphide waste from small scale prospecting and flotation activities, and alluvial tin workings (5).

Since the recognition of health hazards related to arsenic contaminated water supplied in Ron Phibun, several mitigation measures have been carried out by the Department of Mineral Resources to alleviate arsenic contamination problems. These mitigation measures were comprised of ; providing clean water from deep boreholes, giving official orders to miners and concentrators to implement, issuing Ministeral Announcement to prohibit mining activities and collecting arsenic-rich residues to bury in secure landfill (5). Furthermore, regular environmental monitoring programs and collaborative research projects have also been undertaken to evaluate the efficiency of mitigation measures and ensure the safety of the environmental as well as implement further remedial action.

3.4 Treatment technologies for removal of metal from water

Pollution of water resources by metals is of concern because of the considerable potential that metals cause hazards to human health, harm to living organisms and ecological system, damage to structural or amenity, or interference with legitimate uses of water (30). In order to mitigate adverse effects of metal pollutants in water the treatment of these pollutants has attracted considerable attention. The most appropriate method of treatment depends upon the form of the metal and its concentrations. The major technologies, chemical and physical, currently used to treat metal-containing water include, the addition of chemical for precipitation of metals and the use of ion exchange resins to bind the metals to a substrate. Other, less frequently used, processes include activated carbon adsorption, electrodialysis, and reverse osmosis (30).

3.5 Biological removal of metal from water

Treatment technologies for the removal of metal, as previously mention, are often ineffective and/or very expensive. New technologies that can reduce metal concentrations to environmental acceptable levels at affordable costs are required. Biological removal has potential to greatly contribute to the achievement of this goal (6).

Biological removal of metal is defined as the use of organisms for removal of metal ions from metal polluted environment. When this method is compared with the conventional methods, several potential advantages are apparent (6). These advantages are

- use of naturally abundant renewable biomaterials that can be cheaply produced.
- ability to treat large volumes of water.
- reduction of metal to environmentally acceptable levels.
- high selectivity in terms of removal of specific heavy metal.
- ability to handle multiple heavy metals and mixed wastes.
- operation over a wide range of physiological conditions including temperature, pH and the presence of other ions.
- greatly reduce volume of hazardous wastes produced.

Biological removal processes are conceptually simple. A suitable organisms are added to, cultivated in, or otherwise contacted with aqueous solution containing a metal ion. The contacting process is allowed to proceed for a sufficient time for the organisms to sequester the metal ions after which the organisms are separated from the liquid phase. The liquid phase is then discharged and metal-containing organisms are either regenerated (by eluting the metal as a concentrated solution) or disposed of in an environmentally acceptable manner (6).

Alga, an aquatic organism, stands in the front line of the metal pollution of water. Responses of algae to metal ions in water have received considerable attention. Much of the research on metals pollution emphasized on the use of algae as bioindicators of

metal pollution as well as the use of algae to remove metals from the water system due to their ability to concentrate metals. The superiority of algae over bacterial and fungal biomass for bioremoval has been established (6). Algae use light as an energy source, facilitating the maintenance of metabolism in the absence of organic carbon sources and electron acceptor required by bacteria and fungi. Thus, the use of metabolically active algae may be more readily achieved. Also, algae cultures can be cultivated in open ponds or large-scale laboratory culture, providing a reliable and consistent supply of biomass for such study and eventual scale-up work (6).

3.6 Algae

Algae are thallophytes (plants lacking roots, stems, and leaves) that have a photosynthetic system based on chlorophyll a and their reproductive structures consists of cells which are all potentially fertile, and algae do not form embryos (31).

Algae have become a very diverse group of photosynthetic organisms. They may range in size from microscopic single cells as small as one micrometer to large seaweeds that may grow to over 50 meters in length. They show considerable variation in their cellular structures, in the arrangement of their cells to form multicellular bodies or thalli, and in their pigments for photosynthesis (31).

The algae are divided into eight major groups or divisions. Algal divisions differ in their photosynthetic pigments, carbohydrate reserves, and cell structures (Table 3-1). In addition to chlorophyll a, algae may posses other chlorophylls, carotinoids and phycobiliproteins. Polysaccharide reserves are polymers of glucose. They differ in the way the glucose units are linked together (31).

Table 3-1 : Photosynthetic pigments and carbohydrate reserves in algae (31)

Division	Principal photosynthetic pigments	Carbohydrate reserves
Cyanophyta	Chlorophyll a ; Phycocyanobilin, Phycoerythrobilin	Starch (α-1, 4-glucan)
Prochlorophyta	Chlorophyll a, b	Starch
Chlorophyta	Chlorophyll a, b	Starch
Chrysophyta	Chlorophyll a, c_1, c_2 ; Fucoxanthin	β-1, 3-glycans
Pyrrophyta	Chlorophyll a, c_2 ; Peridinin	Starch
Cryptophyta	Chlorophyll a, c_2 ; Phycocyanobilin, Phycoerythrobilin	Starch
Euglenophyta	Chlorophyll a, b	Paramylon (β-1, 3-glycan)
Rhodophyta	Chlorophyll a ; Phycoerythrobilin	Starch

Each major group of algae may contains one or more morphological types, including flagellated solitary cells, colonies of flagellated cells, palmelloid aggregations, non-flagellated cells and colonies, amoeboid cells, filaments and parenchymatous thalli (31).

Algae occur in every kinds of water habitat (freshwater, brackish and marine) as members of planktonic (floating) and benthic (associated with substrates) communities. However, they can be also found in almost every other environment on earth-in soils, permanent ice, snow field, hot spring, and hot and cold deserts (32).

Algae are the major primary producer of organic compounds, and they play a central role as the base of the food chain in aquatic systems. Besides forming the basic food source for these food chains, they also from the oxygen necessary for the metabolism of the consumer organisms (32).

Reflecting well the environmental conditions and their changes, algae have been observed for a long time as indicators of shifting ecological balances and alterations in natural nutritional conditions, as well as of even toxic effects of substances originating from man's activities. Natural populations of algae can readily respond to and have actually been used to monitor the degree of pollution in the aqueous environment (33).

Algae can alter the fate of metals entering aquatic environment. They can alter the form of occurrence of metals through oxidation, reduction, complexation, absorption, and accumulation. Thus, algae affect the bioavailability of metals in aquatic system, and these processes affect the movement of the metal up the food chain (34). Factors which affect the ability to alter chemical form of the metal are bioavailable concentration of the metal, the types and numbers of the algae present, the time period of the algae's exposure to the metal, and the physiocochemical parameters of the environment (34).

3.7 Removal of metal by algae

Alga is a preferred subject to be used for heavy metals removal from polluted water because of two reasons. One is that many algae are known to be capable of sequestering heavy metals and concentrating them up to several thousand times over their environment. The second reason concerns the ecological and nutritional importance of these organisms. Algae are located at the bottom of the aquatic food chain. Thus the accumulation of metals can be magnified by passage up the food chain, thereby posing a grave threat to higher organisms (7,27,35).

The ability of algae to accumulate metal ions from water is characterized by two mechanisms, metabolism-independent (passive) and metabolism-dependent (active) mechanisms (35). The first mechanism is rapid, reversible, and is not influenced by light, temperature, or the presence of metabolic inhibitors. Another mechanism, active process is slow and is inhibited by low temperature, absence of energy sources (light), metabolic inhibitors, and uncouples, and may influenced by the health of the cells (35).

Metabolism-independent mechanism, also called biosorption, which metal ions are bound or adsorbed to cell surface, thereby making the metals unavailable for internalization (35). This process is rapid, occurring immediately after initial contact with the metal and usually lasting for less than 5 to 10 minutes (32). The two principal mechanisms involved in biosorption appear to be : (1) ion exchange phenomenone wherein ions such as Na, Mg, and Ca become displaced by heavy metal ions, and (2) complexation between metal ions and various functional groups such as carboxyl, hydroxyl, amino, and thiol groups that can interact in coordinated way with heavy metal ions (35).

Metabolism-dependent mechanism is process which metal ions are transported, across cell membrane, into the cell. Once the metal ion is inside the cell, it can be handled by internal detoxification (36) as follow,

- Binding of metal ions with proteins or polysaccharides in the interior of the cell.
- Accumulation of metal ions in polyphosphate bodies which are highly charged anions and thus adhere strongly to cations.
- Isolating of metal ions within vacuoles.
- Intracellular precipitation.
- Oxidation and/or reduction processes which a more toxic form of metal can enzymatically be converted to less toxic form.
- Methylation of metal ions, producing metal-organic compounds which have less toxic than inorganic form, for example, many algae convert arsenic to DMA.
- Volatilization by means of converting metal to the volatile chemical species, for example, volatilization of mercury.
- Detoxification via metabolic shunt through which cell avoid the process that the metal would otherwise inhabit.

Figure 3-5 summarizes the route of metals uptake by the algal cell and with this, identifies the sites where tolerance and detoxification mechanisms could operate and the ways which metals affect algal growth (35). The metal normally has to be in labile (chemically active) form for uptake. Biosorption of metals occurs rapidly to organic metal ligands on the cell wall. Algae that produce a mucilage may have a certain

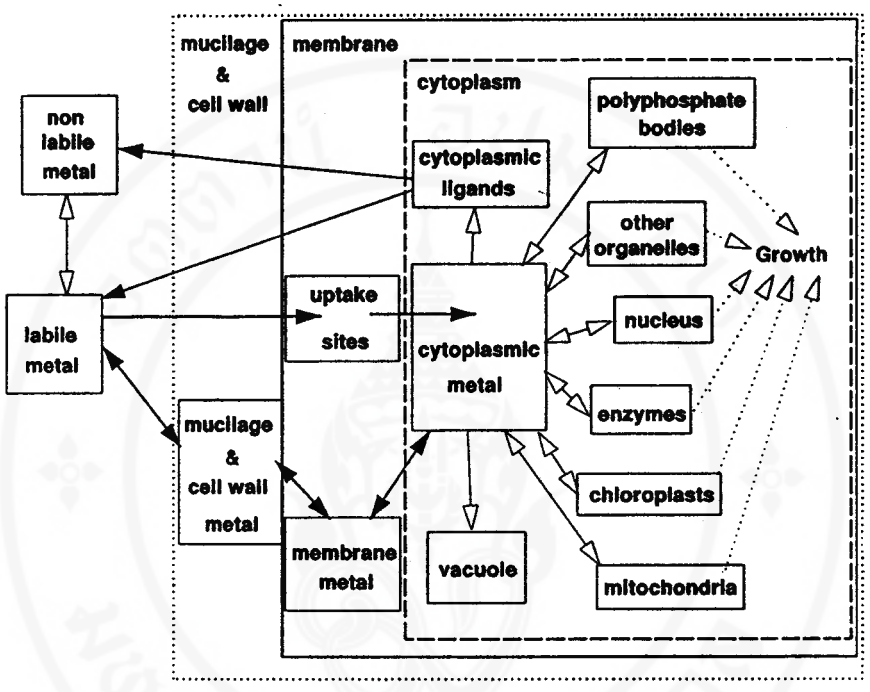


Figure 3-5 : Schematic model of pathways of metal taken up by an algal cell. Solid arrows represent direct pathways between dissolved metal and algal surfaces and cytoplasm. Open arrows with solid lines are pathways between forms of dissolved metal and between cytoplasm and intracellular structures. Dotted arrows are factors that affect algal growth. The sizes of the boxes and arrows are not scaled to represent the imporance of different components (35).

amount of metal adsorb to it. The metabolism-dependent phase of metal uptake involves specific uptake sites, and it may involve diffusion through cell membrane into the cytoplasm. Cytoplasmic metal has many fates. Some metal is sequestered to the vacuole or is bound to cytoplasmic ligands, where it no longer alters metabolism. Algal cell also synthesize the extracellular ligands which either transport the metal outside or probably bind external metal and thus prevent the cellular uptake. If metal is stored in polyphosphate bodies then metal may be released to the cytoplasm when polyphosphate reserves are depleted. Biologically active metal may alter enzyme systems and organelles and ultimately affect metabolism (photosynthesis and respiration) and factors like growth, reproduction and development (35).

Metal accumulation by algae is influenced by a number of abiotic and biotic factors (32).

Abiotic factors : specific traits of metal (affinity to binding site, electronegativity), metal concentration, duration of exposure, concentration of other ions (e.g. Ca, Mg, P other heavy metals), pH, complexing and chelating agents, redox conditions, temperature, light, turbulance.

Biotic factors : species-specific characteristics (cell wall, mucilage, cellular composition), algal biomass concentration, extracellular products, stage of development, cellular activity.

3.8 Bioaccumulation and biotransformation of arsenic by algae

Many algae, both freshwater and marine algae, are reported to be able to accumulate arsenic. Generally, the arsenic content in freshwater algae is lower than in marine algae (12). However, some freshwater algae accumulate arsenic to a large degree. Three freshwater algae (green algae : *Chlorella pyrenoidosa* ; blue green algae : *Oscillatoria rubescens* ; diatom : *Phaeodactylum tricornutum*) and three marine algae (green algae : *Chlorella ovalis* ; diatom : *Phaeodactylum sp.* and *Skeletonema costatum*) accumulated arsenic from an aqueous phase containing 1-30 mg l^{-1} of arsenic and concentrated it by factors of 240-2,800 and 710-2,900, respectively (25,37).

Seaweeds were analyzed for arsenic accumulation. Seaweeds have taken up arsenic from water and accumulated it at high average levels ranging from 1.43 to 10.30 mg kg^{-1} (36). More than 50% of the arsenic accumulated by these seaweeds was in organic form. Most algae exhibited relatively low concentrations of inorganic arsenic, with much greater levels of organic forms of the element contributing to high total concentrations of arsenic (25).

Arsenic-tolerant freshwater algae were screened from algae which had been sampled at sites polluted with arsenic from a geothermal electric power plant and old mines and smelters of arsenic-containing ores (36,37,38). Four algae, i.e., green algae ; *Chlorella vulgaris* and blue green algae ; *Nostoc sp., Phormidium sp.* and *Hydrocoleum sp.* were isolated (37). The isolated *Chlorella vulgaris* grew better in a medium containing levels of pentavalent arsenic up to 2,000 μg g^{-1} and accumulated this form of arsenic at levels of up to 50,000 μg As g dry $cell^{-1}$ (25,36,37,39).

Arsenic bioaccumulation by the *Nostoc sp.* and *Phormidium sp.* were investigated. Arsenic concentration of the cells increased with an increase of the surrounding arsenate concentrations up to 1,000 μg g^{-1} for *Nostoc sp.* and 7,000 μg g^{-1} for *Phormidium sp.*, respectively (40,41).

Another study which using arsenite instead of arsenate reported that arsenite was more toxic to *Chlorella vulgaris*, whose growth dropped off at concentrations above 10 μg g^{-1} and whose cells were cytolyzed at levels higher than 40 μg g^{-1} (25,42).

No arsenic was bioaccumulated by cell of *Chlorella vulgaris* and *Phormidium sp.* that had been pretreated with ethanol or with heat (39,41). Phosphate competitively inhibited arsenic accumulation by the two isolated algae (39,41). In *Chlorella vulgaris* cells acclimated to the high level of arsenic accumulated arsenic at a higher concentration than non-acclimated cells. The tolerant behavior of *Chlorella vulgaris* to arsenic was in striking contrast to that of the same *Chlorella vulgaris* to copper ions which the non-tolerant strain was four times as sensitive to copper ions but accumulated 5 to 10 times more metal compared to the tolerant strain. This tolerance to copper ion was attributed to the exclusion of copper from the cell. Therefore, different mechanisms are likely to be involved in the tolerance of *Chlorella vulgaris* to arsenic and copper (25).

Algae have been found to biotransform inorganic arsenic into organoarsenic compounds. Methylation of inorganic arsenic compounds by algae is a detoxification process both in freshwater and marine ecosystems. In methylation process arsenate is reduced to arsenite which can be methylated to form monomethyl- and dimethyl-arsenic compounds (25). Baker et.al. (28) isolated four freshwater algae

(*Ankistrodesmus sp.*, *Scenedesmus sp.*, *Chlorella sp.* and *Selenastrum sp.*) which capable to methylating sodium arsenite in lake water and Bold's basal medium into MMA, DMA, and TMAO. The levels of methylated arsenic compounds were always significantly higher when the algae were grown in lake water. This may be due to the lower phosphate concentration in the lake water than in Bold's basal medium.

Four arsenic-tolerant freshwater algae (*Chlorella sp.*, *Nostoc sp.*, *Phormidium sp.*, and *Hydrocolium sp.*) which had been isolated from an arsenic polluted environment were studied for the biotransformation of arsenic compounds accumulated by them from the aqueous phase (43). Methylated arsenic compounds were found in all algal cells. The predominant arsenic species in the cells, however, were non-methylated arsenic compounds. These compounds were found mainly in the lipid-soluble fractions and the major form was a dimethylarsenic compound. Trimethyl-and monomethyl-arsenic compounds were detected but at very low levels (43).

In the growth phase of *Chlorella vulgaris*, a small part of arsenic accumulated in the cells was first transformed to monomethyl- and dimethyl- arsenic compounds during the early exponential phase, and after a short time a part of these compounds was transferred to trimethylarsenic species (44). Another study by Maeda reported that *Chlorella vulgaris* can be taken up not only inorganic arsenic but also methylated arsenic compounds, and that the methylated arsenic compounds taken up were further biomethylated but not demethylated. The dimethylarsenic compound seems to be the most stable form in the cells (25) and this compound is not have toxic effects to algae (45).

In marine algae dimethylarsenic compounds were transformed further to arsenosugar (12,13). Arsenosugars were identified as the major arsenic compounds present in marine algae (29). Four arsenosugars have been isolated from seaweed *Ecklonia radiata* and these compounds correspond to 81% of the total arsenic content in the seaweed (12,13,36). The arsenosugars were present in other marine algae, for example *Undaria pinnatifida, Sargassum thunbergii, Porphyra tenera* and *Codium fragile* (12,13). In addition to arsenosugar, marine algae may incorporate arsenic into lipids. Three arsenolipids were isolated from the unicellular marine phytoplankton *Dunaliella tertiolecta*. These compounds were identified as arsenite-lipid complexes (46).

CHAPTER IV

MATERIALS AND METHODS

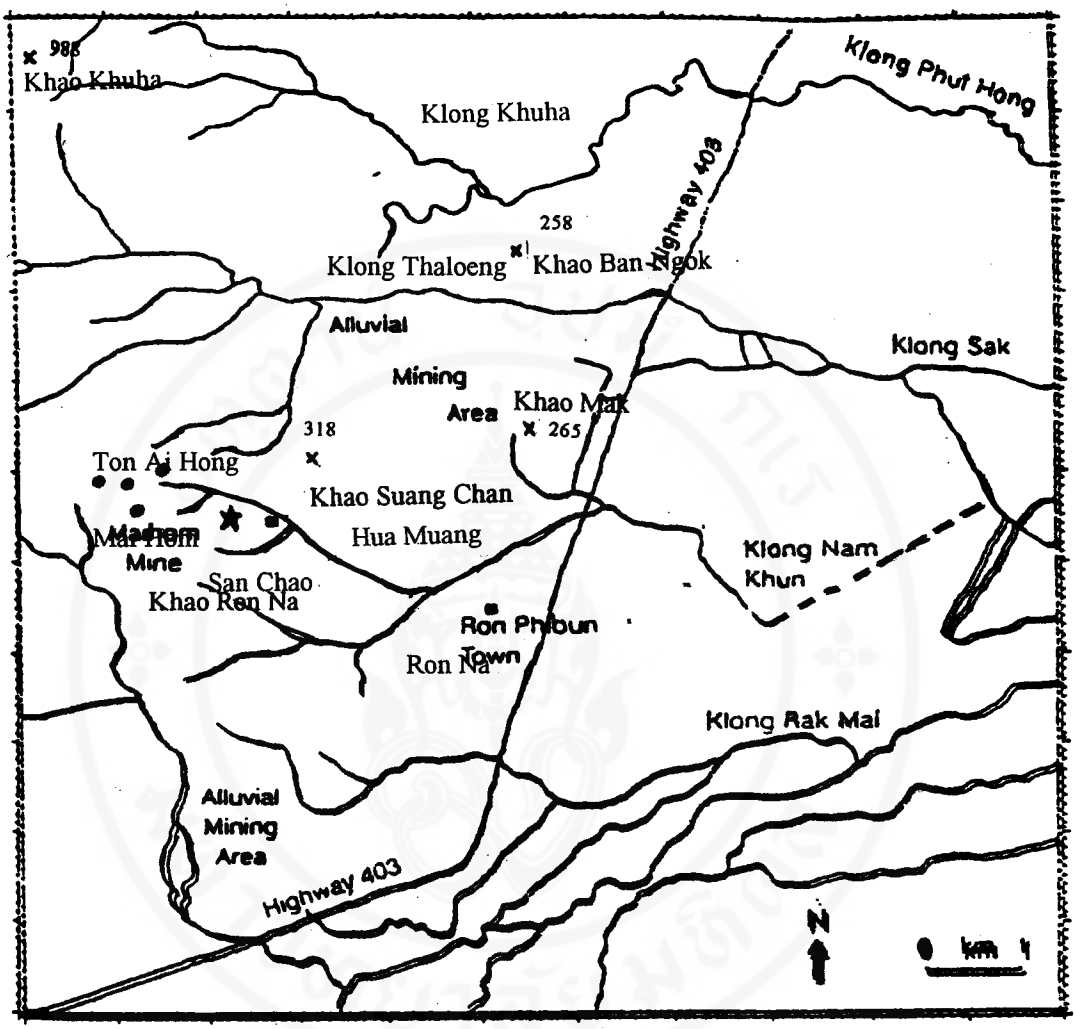
4.1 Determination of total arsenic concentration in water samples.

4.1.1 Water sampling

Water samples were collected from 5 locations in the old mining areas in Ron Phibun district (Figure 4-1). Collection of samples were carried out in October 1998, February 1999, and October 1999. The containers used for water sampling were polyethylene bottles. Water samples were filtered with Whatman No.4. Duplicate 500 ml water samples were collected and the concentrated nitric acid (0.5 ml) was added into one of them to preserve arsenic. The samples were stored at 4°c to preserve the physical and chemical properties of water. Temperature, pH (using pH meter scan 3 ±0.05 pH at 25°c) and conductivity (using YSI model 30/10 FT) were recorded at time of sampling.

4.1.2 Arsenic determination

Analytical techniques used for determination of arsenic concentration in water samples were graphite furnace atomic absorption spectrometry (GFAAS), hydride generation atomic absorption spectrophotometry (HGAAS) and inductive couple plasma mass spectrometry (ICP-MS).



● Mine site & waste plies

■ Ore dressing plant

★ Secure landfill

x Mountain peak

(numbers represent elevation in meters)

Figure 4-1 : Surface drainage system in Ron Phibun district, Nakhon Si Thammarat province. This figure shows the water sampling locations.

4.2 Identification and isolation of algae

4.2.1 Collection of algae

Algae were collected by hand. Water containing phytoplanktons was collected in the plastic bottles. For algae attached to the surface of the substrates, collection can be done by scraping off the surface film of algae and kept in the plastic bottles with some sample water. Algae which used for identification were preserved with fixative (copper acetate dissolved in 40% formaldehyde).

4.2.2 Identification of algae

Microscopic analysis was used for algal identification. The morphological differences of algae were observed under light microscope. The references used as key for identification are

- Prescott GW. How to know freshwater algae. Dubuque(IA): Wm C Brown ; 1978.

- Lund HC, Lund JWG. Freshwater algae : their microscopic world explored. Bristol: Biopress ; 1995.

4.2.3 Isolation of algae

The objective of the isolation of algae was to establish unialgal culture. There were 2 methods which can be used to isolate algae (47). One was plating and streaking of algae onto agar plate, allowing colonies to develop, and the individual cells were transferred to the liquid medium (48). This method could be used to isolate algae that can survive on agar plate. Another method was picking up the single cell with a

micropipette while observing under stereomicroscope, then the alga was transferred to the culture medium (49). The unialgal culture was established by repeated subculturing of the algae. Culture media preparation and culture condition for algal maintenance are summarized in APPENDIX 1.

All experiments describe in the following part were performed by using unialgal culture of alga as test organism. This alga was subcultured in the arsenic free medium for 2 weeks before used. The experiments were conducted using deionized water. The glass apparatuses were washed for 24 hr. in 10% (v/v) nitric acid, rinsed with deionized water and dried in the oven before used. The reagent and standard metal preparation were shown in APPENDIX 2. All experiments were carried out in triplicate and the control without algae was performed. The total arsenic concentration in all samples were examined by graphite furnace atomic absorption spectrophotometer (GFAAS Perkin-Elmer Analyst model 100). The sample preparation and furnace operating condition for arsenic analysis were shown in APPENDIX 3.

4.3 Screening for arsenic (As(V)) tolerance algae.

Unialgal cultures of the isolated algae were used. They were grown in the vessels containing 30 ml culture medium and various concentration of arsenic i.e., 0, 5, 50, and 500 mg l^{-1} . Arsenic was used in the form of sodium arsenate (As(V) or $Na_2HAsO_4 \cdot 7H_2O$). The amounts of chemical added into the medium was adjusted to the required concentration of elemental arsenic. The algal growth was recorded. The algae which exhibited good growth after 2 weeks of cultivation were selected as As(V) tolerant strain.

4.4 Effects of concentration and chemical forms of arsenic (As(V)/As(III)) on growth of As(V) tolerant algae.

The As(V) tolerant algae were inoculated into the vessels containing 30 ml culture medium which arsenic levels varied from 0 to 5, 50, and 500 mg l^{-1} and culture for 2 weeks. Arsenic can be used as sodium arsenate (As(V) or $Na_2HAsO_4 \cdot 7H_2O$) or sodium arsenite (As(III) or $NaAsO_2$). Culture solution was drawn everyday at the constant time intervals to determine the cell concentration by measuring the absorbance at 660 nm with spectrophotometer (UV-1201). The absorbance at various levels of arsenic was plotted against time to obtain growth curves. The growth curves of algae at different levels and chemical forms of arsenic were compared.

All experiments describe in the following part were performed using only sodium arsenate (As(V) or $Na_2HAsO_4 \cdot 7H_2O$) because of As(V) is the dominant form of arsenic exist in freshwater environment (8,10) and this form has less toxic effects to algae than As(III) form (25,42).

4.5 Study on As(V) removal ability by the As(V) tolerant algae.

The As(V) tolerant algae were inoculated into a 100 ml Erlenmayer flask containing 50 ml culture medium and As(V) at concentration of 1 mg l^{-1} and cultured on a rotary shaker set up at 150 rpm for 2 weeks. Control was similar performed but without an inoculum. At the beginning and at the end of the exposure time, the algae were harvested and analyzed for the total arsenic concentration. The culture media were also analyzed for the remaining concentration of arsenic. The removal ability of the As(V) tolerant algae was observed

4.6 Establishing the optimum condition for As(V) removal by the As(V) tolerant algae.

The experiment was carried out to study the effects of various factors, i.e., concentrations and types of algal cell, growth stages, pH, and phosphate levels on As(V) removal by the As(V) tolerant algae. The condition which showed the highest arsenic removal and accumulating efficiency was selected as the optimum condition.

4.6.1 Algal cell concentrations

The effects of algal cell concentrations on As(V) removal by As(V) tolerant algae were investigated. In this study different volumes of algal suspension were inoculated into 50 ml culture medium containing As(V) at concentration of 1 mg l^{-1} in a 100 ml Erlenmayer flask. The concentrations of cells were determined by using spectrophotometer at 660 nm. The cell concentrations at the absorbance of 0.2, 0.4, and 0.8 were used in the experiment. Each cell concentration had its own control which deionized water was added instead of algal suspension into the medium. The algae were cultured on a shaker set up at 150 rpm for 2 weeks before harvesting. The algal cells and the remaining media of both experiment and control were analyzed for the total arsenic concentration. The removal and accumulation of As(V) at different concentrations of cell were compared and the optimum cell concentration was obtained.

4.6.2 Types of cell

This experiment was conducted by inoculating either living or heat-killed cells of the As(V) tolerant algae into 50 ml culture medium containing 1 mg l^{-1} As(V) in a 100 ml Erlenmayer flask. The algal cells were heated at 95°c for a hour prior to use as the heat-killed cells. After inoculation, the cultures were shaken at 150 rpm on the rotary shaker for 2 weeks. The algal cells were harvested, afterthat the algal cells and the media were analyzed for total arsenic concentration. The As(V) removal and accumulation were compared and the one that showed the better capacity was selected as the optimum type of cell.

4.6.3 Growth stages of algae

The effects of stages of growth on As(V) removal by the As(V) tolerant algae were determined. The algae were cultured in As(V)-free medium and their growth was measured by using spectrophotometer at 660 nm. The growth curve of the algae is shown in Figure 4-2. The algae were exposed to As(V) at concentration of 1 mg l^{-1} at time when the growth reached early, middle, and late exponential stages (Figure 4-2). After exposure to As(V) for 2 weeks, the algal cells were separated and the media were analyzed for the remaining arsenic concentration. The percentages of As(V) removal at different stages of growth were compared and the algal growth stage which gave the highest efficiency in As(V) removal was obtained

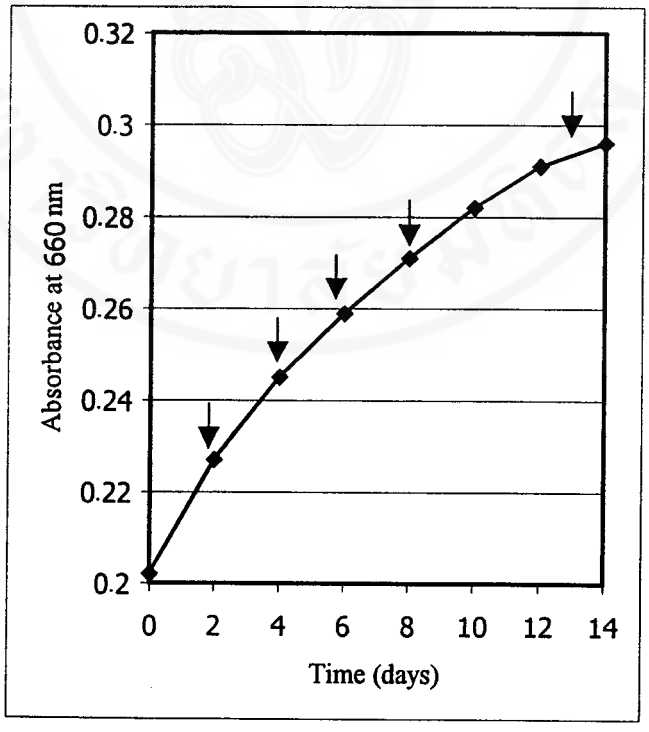


Figure 4-2 : Growth curve of the isolated *Chlorella sp.* cultures in arsenic-free medium. Arrows indicate algal growth stages which are subjected to testing the efficiency in As(V) removal.

4.6.4 pH

In this experiment the algae were inoculated into 50 ml culture medium containing 1 mg l^{-1} As(V) in a 100 ml Erlenmayer flask. Various pH values, i.e., pH 2, pH 5, pH 7, and pH 9 were used. The medium pH was adjusted with 1N HCL and 2N NaOH after being autoclaved. The control flask for each pH value was set up similarly to the experimental flask but without inoculum. After 2 weeks, the algal cells were harvested. All samples were analyzed for total arsenic concentration. The pH value which gave the highest efficiency in removing and accumulating As(V) by the algae was selected as the optimum pH value.

4.6.5 Phosphate concentrations

Phosphate was another factor of concern because of chemical similarity between phosphate and arsenate, thus it may influence the arsenic removal ability of the algae. To confirmed this, the study on As(V) removal at various phosphate concentrations was performed. The algae was inoculated into 50 ml culture medium containing As(V) at concentration of 1 mg l^{-1}, and the phosphate at various concentration of 0, 1, 10, and 100 mg l^{-1} in a 100 ml Erlenmayer flask. The control for each phosphate concentration was also set up similarly but without inoculum. All flasks were shaken at 150 rpm on the rotary shaker for 2 weeks. Then the algal cells were harvested. All samples were analyzed for total arsenic concentration. The effects of phosphate on As(V) removal were determined by comparing the ability of the algae in removing and accumulating As(V) at different phosphate concentrations.

4.7 Effects of As(V) concentrations on the removal and accumulation by the As(V) tolerant algae.

The algae were cultured in 50 ml culture medium containing As(V) at concentration of 1, 5, 10, and 50 mg l^{-1} in a 100 ml Erlenmayer flask. The culture was shaken at 150 rpm on the rotary shaker for 2 weeks. There was the control for each As(V) concentration. Afterwards the algae were harvested and all samples were analyzed for total arsenic concentration. The amount of arsenic accumulated in the algal cells and remaining in the medium were used to calculate the bioaccumulation factor (BCF) (50).

$$\text{Bioaccumulation factor (BCF)} = \frac{\mu g \text{ compound removed/g dried weight}}{\mu g \text{ compound in solution/ml solution}}$$

This value is used to characterize the metal uptake. High BCF value, high ability on removal metal from water. The BCF value for each As(V) concentration was calculated. The effects of As(V) concentration on removal and accumulation were determined by comparing on either the percentages of removal or BCF values.

4.8 Kinetic of As(V) removal by the As(V) tolerant algae.

The algae were grown in 50 ml culture medium containing arsenate at concentration of 1 mg l^{-1} in a 100 ml Erlenmayer flask and shaken at 150 rpm on the rotary shaker. This experiment was set up at the optimum condition obtained from the previous studied except the phosphate concentration which was still used at the concentration described in the culture medium. Samples were withdrawn every 2 days after exposure to As(V). The algal cells were separated and the medium was analyzed

for the remaining arsenic concentration. The samples from the control flasks were withdrawn only at the beginning and at the end of exposure time. These samples were also analyzed for the remaining arsenic concentration. During the experiment, growth of the algae was also monitored by measuring the absorbance at 660 nm using spectrophotometer. The As(V) content in the samples and the growth of the algae were plotted against time in order to obtain the As(V) removal ability during the growth of the algae.

4.9 Kinetic of the As(V) removal by the arsenic tolerant algae with and without phosphate.

This experiment was designed to study the effects of phosphate on the kinetic of As(V) removal. The experiment was performed by inoculating the algae into a 100 ml Erlenmayer flask containing 50 ml deionized water and As(V) at concentration of 1 mg l^{-1} and phosphate at concentration of 0 or 1 mg l^{-1}. The cultures were shaken at 150 rpm for 2 weeks. Samples were withdrawn at 0, 4, 7, 9, and 11 days after exposure to As(V). The algal cells were separated and the medium was analyzed for the remaining arsenic concentration. The percentages of As(V) removal for with and without phosphate were compared.

4.10 Determination of arsenic in various cell fractions of the As(V) tolerant algae.

The algae were grown in 100 ml Erlenmayer flask containing culture medium and As(V) at concentration of 10 mg l^{-1} on a shaker set up at 150 rpm for 2 weeks. There were 5 replications in this experiment. At the end of the exposure time, the algal cells were harvested and the supernatant (medium) was analyzed for the remaining arsenic concentration. For the first 2 replications, the algal cells were washed three times with the diluted medium to remove the remaining arsenic which might be adsorbed on the algal cell surfaces. After each washing, the supernatant was separated and analyzed for total arsenic concentration. To determine whether arsenic was attached to the algal cell components, the cell pellets were suspended in 1 ml deionized water and the suspensions were disrupted by sonicating for 5 min (45 times per min) by using sonicator model VC100 (Sonics & Material Inc., Danbury, Connecticut, USA). After sonication, the suspensions were centrifuged (10,000 g, 10 min), and the supernatant and the cell debris were used for the determination of total arsenic concentration. For the other 3 replications, the algal cells were washed with the diluted medium and the supernatants and the pellets containing algal cells were analyzed for total arsenic concentration.

4.11 Removal of As(V) by the immobilized algae.

Immobilization by entrapment of the As(V) tolerant algae in calcium alginate was performed. The algae were centrifuged at 3,000 g for 10 min and the pellet was resuspended in water. The cell suspension was then mixed with an equal volume of 2%(w/v) sodium alginate solution (ratio1:1 v/v). The mixture was added, dropwise, into 2% (w/v) calcium chloride solution. The alginate beads prepared by this method had a diameter of 5 mm. The immobilized cells were maintained in 2% calcium chloride for 2 hr., then washed twice with deionized water and kept in the culture medium for 1 week before used in the experiment.

In the experiment various numbers of beads ranged from 300 to 600 beads were packed into the column of 3 cm internal diameter and 50 cm length. The As(V) solution (1 mg l^{-1}), 50 ml, was added to the column. The controls were the As(V) solution without the bead and the As(V) solution containing the beads but no algae. After the experiment had been set up, the sample solutions were collected every 2 days and then analyzed for the remaining arsenic concentration.

4.12 Efficiency on removal of As(V) by the free and the immobilized algae.

The objective of this experiment was to compare the ability of the free and immobilized algae on As(V) removal. In this experiment algal cell concentration in both free and immobilized conditions was the same. The free and immobilized algal cells were inoculated into 50 ml As(V) solution (1 mg l^{-1}) in a 100 ml Erlenmayer flask. The solutions were collected at 0, 1, 3, 5, 7, and 9 days after exposure to As(V) and then analyzed for the remaining arsenic concentration.

4.13 Statistical analysis

One way analysis of variance (one-way ANOVA) was used to test the difference between the treatment and the control in all experiment. A difference in arsenic removal among the treatment in the experiment about the optimum condition was also tested by one-way ANOVA and that between each treatment was tested by using Duncan multiple range test. The analytical data are shown in APPENDIX V. Two-way ANOVA was used to estimated the difference in As^{5+} removal of an immobilized algae which the number of beads was varied.

CHAPTER V

RESULTS

5.1 Arsenic concentration in water samples collected from the arsenic polluted surface water in Ron Phibun district.

Concentrations of arsenic and other environmental parameters (pH, temperature, and conductivity) in water samples taken from Ron Phibun in October 1998, February 1999, and October 1999 are shown in Table 5-1.

Water samples collected from 5 locations in the old mining areas, contain high arsenic level. The range of arsenic concentration in water, range from 70 to1,000 $\mu g\ l^{-1}$, with the highest value in the Ron Na stream. When comparing between two techniques of water samples collection, it is found that the acid treated water samples give higher total arsenic concentration than the untreated water samples (Table 5-1). This may be from the activity of concentrated acid in preserving arsenic ions in water. Concentrations of arsenic in surface water are compared with those obtained during 1992 to 1997 (51). As illustrate in Figure 5-1, concentrations of arsenic in surface water is gradually decreased according to time.

Table 5-1 : Total arsenic concentration of water samples collected from 5 locations in Ron Phibun district.

Sampling times / Sampling locations	pH	Temp. (°c)	Conductivity (µs)	Total arsenic concentration (µg l^{-1})	
				without acid	with acid
October 1998				ICPMS	HGAAS
Hua Muang	6.48	28.30	70.60	210.00	260.00
Ron Na	7.02	30.00	29.40	810.00	985.00
San Chao	6.71	27.50	103.60	450.00	580.00
Mai Hom	4.58	27.80	63.10	140.00	165.00
Ton Ai Hong	4.29	27.20	73.90	210.00	230.00
February 1999				GFAAS	GFAAS
Hua Muang	6.23	26.40	57.70	152.88	198.42
Ron Na	7.41	28.40	235.60	735.64	805.67
San Chao	6.45	26.80	86.00	219.64	256.98
Mai Hom	4.55	27.30	69.90	105.96	133.61
Ton Ai Hong	4.37	27.40	60.70	149.94	185.83
October 1999				GFAAS	
Hua Muang	7.36	27.00	88.40	93.77	-
Ron Na	7.81	26.80	232.80	322.21	-
San Chao	7.32	26.00	55.30	117.77	-
Mai Hom	4.77	26.00	45.60	76.01	-
Ton Ai Hong	4.65	25.80	56.60	81.36	-

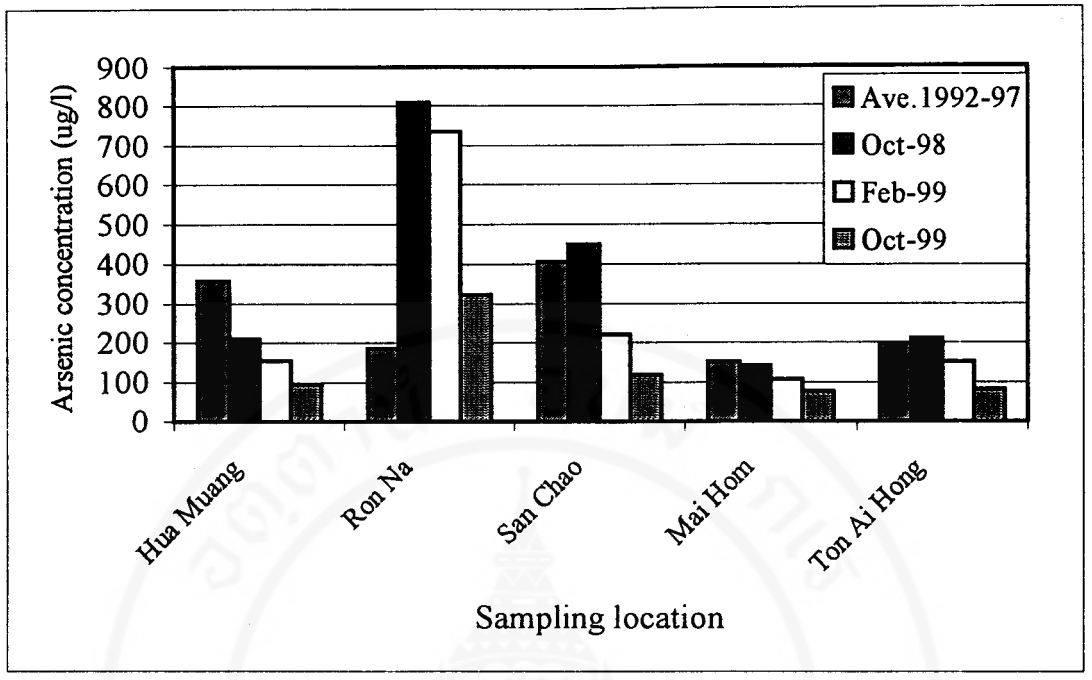


Figure 5-1 : Total arsenic concentration of water samples collected from 5 locations in Ron Phibun district at different collection times.

The conductivity, pH, and temperature of the water samples vary throughout the sampling sites (Table 5-1). The conductivity seem to have the greatest variation either according to sites or times of sampling. In October 1998 San Chao stream shows the highest value whereas in February 1999 the highest value is observed at Ron Na stream. The conductivity is not correlated with arsenic concentration in water (R^2 = 0.2037). The pH of water samples collected at different times are recorded in the same range, i.e., from 4.3 to 7.8 (Table 5-1). Even though there is relatively low correlation value (R^2 = 0.5698) between water pH and arsenic concentration, but at the high pH value, it is found that the arsenic content is also high. Water temperature at all collection sites range from 25 to 30 °c.

5.2 Identification of algae.

The algal samples were collected from the arsenic polluted water and were preserved for identification. Fifteen algal genera were identified based on the cellular structure and pigmentation. These algae are classified, according to their morphological characteristics into 3 major groups ; blue-green algae, green algae, and diatom as shown in Table 5-2. Green alga, division Chlorophyta, is the most diversed group. Eleven genera out of fifteen are belonging to this group, the Chlorophyta. Detailed characteristics of each genera are shown in the APPENDIX 4.

5.3 Isolation of algae

Out of seven algal genera obtained from the non-preserved water samples, three unicellular algae were isolated. The green algae *Chlorella sp.* and *Euastrum sp.* and the diatom *Navicula sp.* could be isolated as unialgal cultures. The unialgal cultures of the isolated algae were subculture in every 2 weeks. These cultures were used for further investigations.

Table 5-2 : The algal genera found in the collecting locations in Ron Phibun district.

Cyanophyta (blue-green algae)	Chlorophyta (green algae)	Chrysophyta (diatom)
*Oscillatoria sp.**	*Chlorella sp.**	*Navicula sp.**
Phormidium sp.	*Oocystis sp.*	*Fragilaria sp.*
	Scenedesmus sp.	
	Chlorococcum sp.	
	*Euastrum sp.**	
	Netrium sp.	
	Spirogyra sp.	
	*Ulothrix sp.**	
	*Microspora sp.**	
	Cladophora sp.	
	*Pseudendoclonium sp.**	

* refers to the algal genera obtained in non-preserved collection

5.4 Screening for As(V) tolerant algae

The three isolated algae were tested for their As(V) tolerance abilities. As shown in Table 5-3, a 5 mg l^{-1} of As(V) could inhibit growth of *Euastrum sp.* At the highest concentration of As(V) tested (500 mg l^{-1}), both *Chlorella sp.* and *Navicula sp.* could still grow. However, *Chlorella sp.* has good growth, while *Navicula sp.* has poor growth. The *Chlorella sp.* which could tolerate 500 mg l^{-1} of As(V) is selected for further studies.

Table 5-3 : Growth of the As(V) tolerant algae which exposure to various concentration of As(V).

Algae	As(V)concentration (mg l^{-1})			
	0	5	50	500
Chlorella sp.	+++	+++	+++	+++
Euastrum sp.	+++	-	-	-
Navicula sp.	+++	+++	++	+

Growth is defined as follows : +++ good growth, ++ medium growth, + poor growth, - no growth

5.5 Effects of concentrations and chemical forms of arsenic (As(V) /As(III)) on growth of the isolated *Chlorella sp.*

To investigate toxicity and tolerance to arsenic by the isolated *Chlorella sp.*, the effects of concentrations and chemical forms of arsenic on algal growth were studied.

5.5.1 As(V)

The effects of various As(V) concentrations on growth of the isolated *Chlorella sp.* are shown in Figure 5-2. The growth of the isolated *Chlorella sp.* seems to be unaffected by high level of As(V) in the culture medium because there is no significant difference on growth of the isolated *Chlorella sp.* In comparison of the algal growth at different levels of As(V), the alga grows rapidly at 50 more than 5 or 500 mg l^{-1} , respectively. The growth of the isolated *Chlorella sp.* at 50 mg l^{-1} is nearly equal to the growth of the control.

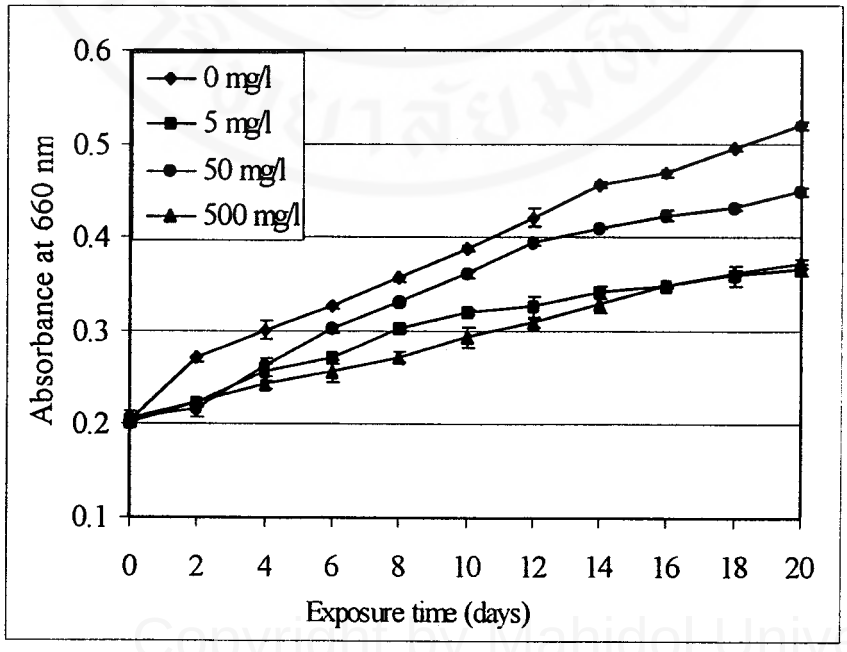


Figure 5-2 : Effects of As(V) on growth of the isolated *Chlorella sp.* Each point is a mean of three replicates and the error bars indicate the standard deviation.

5.5.2 As(III)

The growth curves of the isolated *Chlorella sp.* in the presence of various levels of As(III) are shown in Figure 5-3. The growth is decreased when the alga was exposed to As(III). At concentration of 5 and 50 mg l^{-1}, alga could still grow. At 500 mg l^{-1} As(III), the highest level of As(III) tested, the alga could not survive. These results indicate that As(III) is more toxic to the isolated *Chlorella sp.* than As(V).

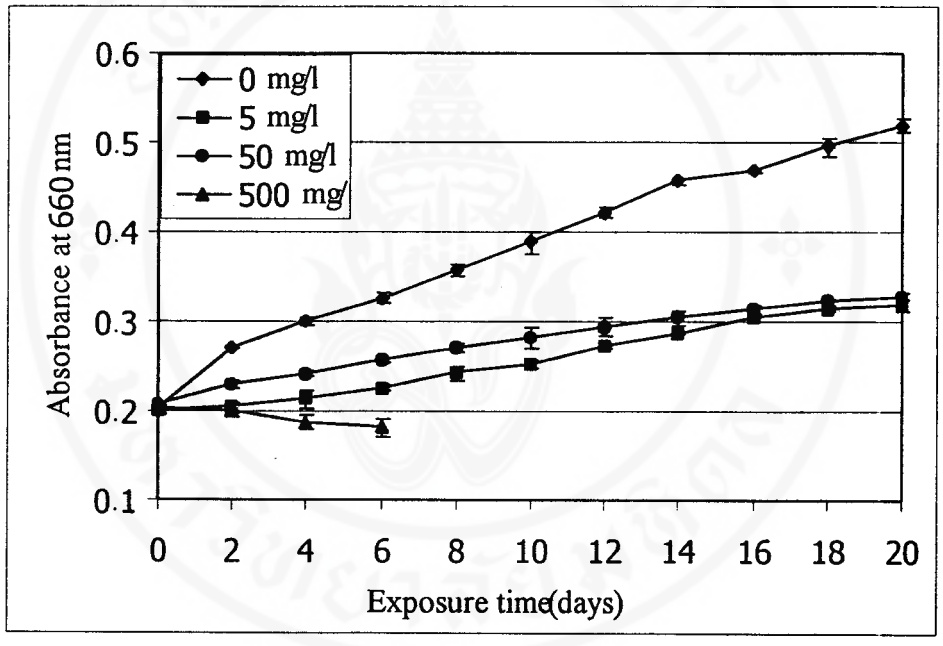


Figure 5-3 : Effects of As(III) on growth of the isolated *Chlorella sp.* Each point is mean of three replicates and the error bars indicate the standard deviation.

5.6 Study on As(V) removal ability of the isolated *Chlorella sp.*

The ability of the isolated *Chlorella sp.* to remove As(V) was assessed by growing the alga in the medium containing 1 mg l^{-1} As(V). The results (Table 5-4) show that the alga could remove approximately 32% As(V) from water within 14 days. This alga could accumulated arsenic in their cells also. The removed As(V) is mostly accumulated in the algal cell (90%).

5.7 The optimum condition for As(V) removal by the isolated *Chlorella sp.*

This experiment was designed to obtain the optimum condition for As(V) removal by the isolated *Chlorella sp.*. The effects of various factors ,i.e., concentrations and types of algal cell, growth stages, pH and phosphate levels on As(V) removal by the isolated *Chlorella sp.* were investigated.

Table 5-4 : Removal of As(V) by the isolated *Chlorella sp.* The values are given as mean ± standard deviation (n=3)

Sample	Amount of As(V) in media (μg)		As(V) removal		As in algae		% As accumulation in algae[b]
	Before treatment	After treatment	Amount (μg)	% [a]	μgAs g^{-1}d.w.	Total amount (μg)	
Control	58.18±0.35	57.68±0.35	0.50±0.00	0.86±0.00	-	-	-
Treatment	58.18±0.35	39.27±0.47	18.42±0.83	31.93±1.24	166.40±7.74	16.48±1.00	89.70±9.47

a : % As(V) removal = [Amount of removed As(V) / Amount of initial As(V)] x 100

b : % As(V) accumulation in algae = [Total amount of As in algae/ Amount of As(V) removal] x 100

5.7.1 Cell concentrations

The effects of the algal cell concentrations on the removal of As(V) by the isolated *Chlorella sp.* is shown in Table 5-5. The concentration of cells were obtained by measuring the absorbance at 660 nm with spectrophotometer. The cell concentrations at the absorbance of 0.2, 0.4, and 0.8 were tested in this experiment. The amount of As(V) removed is inversely proportional to the cell concentration. The highest percentages of As(V) removal (37%) is observed with the cell concentration at the absorbance of 0.2. Arsenic accumulation also shows the maximum value at the same algal cell concentration. Thus, this cell concentration is selected as optimum algal cell concentration for As(V) removal by the isolated *Chlorella sp.*

5.7.2 Types of algal cell

In the study on the effects of types of algal cell on As(V) removal, both living and heat-killed cells were exposed to As(V) for 14 days, then the removal capacities were compared. As shown in Table 5-6, percentage of As(V) removal by the heat-killed cells is very low. The amount of As(V) removal is not significantly different from the control. No arsenic is found to accumulate in the heat-killed cells. On the contrary, As(V) is removed from culture medium and accumulated in the living cells of the isolated *Chlorella sp.*

Table 5-5 : Removal of As(V) by various cell concentration of the isolated *Chlorella sp.* The experiment was conducted at 25°c on a shaker set at 150 rpm for 14 days. The values are given as mean ± standard deviation (n=3).

Abs[a]	Amount of As(V) in media (μg)		As(V) removal		As in algae		% As accumulation in algae[c]
	Before treatment	After treatment	Amount (μg)	% [b]	μgAs g^{-1}d.w.	Total amount (μg)	
Control	141.37±0.77	140.33±1.12	1.04±0.34	0.74±0.25	-	-	-
0.2	141.37±0.77	87.94±2.37[d]	52.39±2.42	37.33±1.67	505.73±30.35	41.47±2.54	79.28±6.30
Control	115.48±1.40	113.69±1.47	1.79±0.90	1.55±0.78	-	-	-
0.4	115.48±1.40	85.78±4.40[d]	27.91±3.09	24.57±2.97	201.55±2.42	18.14±0.24	65.57±7.80
Control	127.49±0.47	125.48±0.93	2.01±0.81	1.58±0.63	-	-	-
0.8	127.49±0.47	107.42±0.59[d]	18.06±0.34	14.39±0.17	131.28±2.70	12.69±0.37	70.28±2.14

a : Algal cell concentration obtained by measuring absorbance at 660 nm.

b : % As(V) removal = [Amount of removed As(V) / Amount of initial As(V)] x 100

c : % As(V) accumulation in algae = [Total amount of As in algae/ Amount of As(V) removal] x 100

d : $P < 0.05$, significant different when comparing with the control.

Table 5-6 : Removal of As(V) by the living vs heat-killed cells of the isolated *Chlorella sp.*

The values are given as mean ± standard deviation (n=3).

Type of cell	Amount of As(V) in media (μg)		As(V) removal		As in algae		% As accumulation in algae[b]
	Before treatment	After treatment	Amount (μg)	%[a]	μgAs g^{-1}d.w.	Total amount (μg)	
Control	114.32±0.87	112.65±1.07	1.67±0.29	1.46±0.26	-	-	-
Living cell	114.32±0.87	67.21±3.46[c]	45.45±2.40	40.36±2.51	361.81±10.37	35.70±1.22	78.76±6.19
Dead cell	114.32±0.87	110.60±0.68	1.44±0.41	1.80±0.93	5.47±1.10	0.53±0.11	20.15±5.08

a : % As(V) removal = [Amount of removed As(V) / Amount of initial As(V)] x 100

b : % As(V) accumulation in algae = [Total amount of As in algae / Amount of As(V) removal] x 100

c : $P < 0.05$, significant different when comparing with the control.

5.7.3 Growth stages of alga

The removal of As(V) by the cells of the isolated *Chlorella sp.* which were exposed to As(V) at times when the growth reached early, middle, and late exponential stage were compared. The results (Table 5-7) show that cells at the middle exponential stage of growth are the best in removing of As(V) from the medium. Thus, culturing of the isolated *Chlorella sp.* in As(V)-free medium for 6 to 8 days (middle stage of growth) before exposure to As(V) is performed in order to obtain high percentage of As(V) removal.

Table 5-7 : Removal of As(V) by the isolated *Chlorella sp.* at different stages of growth. The values are given as mean ± standard deviation (n=3).

Culture in As(V)-free medium	As(V) before treatment		As(V) after treatment		% As(V) removal[a]
	Concentration ($\mu g\ l^{-1}$)	Amount (μg)	Concentration ($\mu g\ l^{-1}$)	Amount (μg)	
Control	1905.97±13.45	95.30±0.67	1888.56±19.15	94.43±0.96	0.91±0.31
2 days	1905.97±13.45	95.30±0.67	1754.23±16.83[b]	87.71±0.84	7.11±0.68
4 days	1905.97±13.45	95.30±0.67	1583.83±45.60[b]	79.19±2.28	16.12±2.94
6 days	1905.97±13.45	95.30±0.67	1236.82±16.83[b]	61.84±0.84	34.50±1.44
8 days	1905.97±13.45	95.30±0.67	1206.97±26.21[b]	60.34±1.31	36.08±1.85
13 days	1905.97±13.45	95.30±0.67	1343.78±65.73[b]	67.19±3.29	28.83±3.93

a : % As(V) removal = (Amount of removed As(V)) / (Amount of initial As(V)) x 100

b : $P < 0.05$, significant different when comparing with the control.

5.7.4 pH

Various pH values were explored for their effects on As(V) removal by the isolated *Chlorella sp.* The results are presented in Table 5-8. The As(V) removal is very low at pH 2 and 9, but the metal is better removed at the pH range from 5 to 7. The arsenic accumulated in the algal cells decrease in the following order : pH 5 > pH 7 > pH 9 > pH 2. The lowest accumulation of arsenic by the alga at pH 2 is due to death of the alga. Thus, pH range from 5 to 7 is selected as optimum pH for As(V) removal by the isolated *Chlorella sp.*

5.7.5 Phosphate concentrations

Another factor to be considered on As(V) removal was the effects of phosphate, which is structurally similar to arsenate. Four different concentrations (0, 1, 10, and 100 mg l^{-1}) of phosphate were used in this experiment. The results, as shown in Table 5-9, indicate that percentage of As(V) removal is inversely proportional to phosphate concentration. The higher the phosphate concentration, the lower the percentage of As(V) removal. In the absence of phosphate in the culture medium, the As(V) removal is the highest. The accumulation of arsenic by the algal cells at various phosphate concentration is in the same order as the As(V) removal. The ability of the isolated *Chlorella sp.* on As(V) removal is inhibited by the presence of high concentration of phosphate in the water, i.e., 10 and 100 mg l^{-1} (Table 5-9).

Table 5-8 : Effects of pH on As(V) removal by the isolated *Chlorella sp.* The values are given as mean ± standard deviation (n=3).

pH	Amount of As(V) in media (μg)		As(V) removal		As in algae		% As accumulation in algae[b]
	Before treatment	After treatment	Amount (μg)	%[a]	μgAs g^{-1}d.w.	Total amount (μg)	
Control	103.91±2.46	103.04±2.19	0.87±0.45	0.83±0.42	-	-	-
2	103.91±2.46	93.46±2.03[c]	9.58±0.16	9.30±0.05	34.14±1.72	2.97±0.15	31.00±1.24
Control	101.97±1.35	101.08±0.91	0.90±0.45	0.87±0.43	-	-	-
5	101.97±1.35	71.27±1.67[c]	29.80±1.74	29.50±1.65	218.73±1.38	19.91±0.34	66.98±4.89
Control	102.64±0.90	102.12±1.15	0.52±0.26	0.51±0.25	-	-	-
7	102.64±0.90	72.43±2.25[c]	29.36±2.60	28.74±2.40	204.87±2.10	18.57±0.15	63.58±5.33
Control	101.52±0.34	100.48±0.81	1.05±0.56	1.03±0.56	-	-	-
9	101.52±0.34	92.57±2.24[c]	7.91±1.49	7.88±1.54	65.18±2.85	5.54±0.25	71.68±13.62

a : % As(V) removal = [Amount of removed As(V) / Amount of initial As(V)] x 100

b : % As(V) accumulation in algae = [Total amount of As in algae / Amount of As(V) removal] x 100

c : $P < 0.05$, significant different when comparing with the control.

Table 5-9 : Effect of phosphate on As(V) removal by the isolated *Chlorella sp.* The values are given as mean ± standard deviation (n=3).

$[PO_4^{3-}]$ (mg l^{-1})	Amount of As(V) in media (µg)		As(V) removal		As in algae		% As accumulation in algae[b]
	Before treatment	After treatment	Amount (µg)	% [a]	µgAs g^{-1}d.w.	Total amount (µg)	
Control	47.47±0.28	47.21±0.26	0.26±0.02	0.56±0.04	-	-	-
0	47.47±0.28	34.91±0.61[c]	12.30±0.37	26.05±0.91	109.61±3.49	10.74±0.34	87.37±2.67
Control	36.60±0.41	36.29±0.41	0.30±0.06	0.83±0.15	-	-	-
1	36.60±0.41	29.83±0.84[c]	6.77±0.43	17.83±1.45	50.94±1.24	4.82±0.09	71.37±3.78
Control	33.62±0.21	33.27±0.37	0.37±0.24	1.09±0.73	-	-	-
10	33.62±0.21	32.37±0.23[c]	0.89±0.14	2.67±0.14	6.81±1.00	0.63±0.09	71.13±1.29
Control	29.89±0.28	29.67±0.38	0.01±0.00	0.72±0.71	-	-	-
100	29.89±0.28	29.09±0.28[c]	0.58±0.14	1.96±0.45	3.92±1.37	0.36±0.13	62.22±16.91

a : % As(V) removal = [Amount of removed As(V) / Amount of initial As(V)] x 100

b : % As(V) accumulation in algae = [Total amount of As in algae / Amount of As(V) removal] x 100

c : $P < 0.05$, significant different when comparing with the control.

5.8 Effects of As(V) concentrations on the removal and accumulation by the isolated *Chlorella sp.*

The isolated *Chlorella sp.* was cultured in the media containing As(V) at concentrations of 1, 5, 10, and 50 mg l^{-1} for 14 days. The effects of various As(V) concentrations on As(V) removal and accumulation are summarized in Table 5-10. The removal of As(V) is unaffected by an increase of metal concentrations in the medium. The percentage of As(V) removal at each As(V) concentration is in the range of 34-36%. However, the arsenic accumulation by the alga is directly depended on the As(V) concentrations in the medium. The higher the As(V) concentration in the medium, the greater the concentration of arsenic accumulation in the algal cells. Furthermore, the percentages of arsenic accumulation in the isolated *Chlorella sp.* decreases with an increase in As(V) concentration in the medium, but the value is quite stable (58-59%) at high As(V) concentration, either 10 or 50 mg l^{-1}. The results obtained in this experiment were used to calculate the bioconcentration factor (BCF). The highest BCF value is observed when the alga was exposed to As(V) at concentration of 1 mg l^{-1}. The alga which was cultured in media containing higher As(V) concentration shows the lower BCF values. However, these values are not significant different when compare with that of As(V) concentrations.

Table 5-10 : Effects of various [As(V)] on the removal capacity of the isolated *Chlorella sp.* The values are given as mean ± SD (n=3).

[As(V)]	Amount of As(V) in media (μg)		As(V) removal		As in algae		% As	BCF[c]
($mg\ l^{-1}$)	Before treatment	After treatment	Amount (μg)	%[a]	$\mu gAs\ g^{-1}d.w.$	Total amount (μg)	accumulation in algae[b]	
Control	77.38±0.79	76.88±1.00	0.50±0.29	0.65±0.38	-	-	-	-
1	77.38±0.79	48.88±1.77[d]	28.00±2.46	36.40±2.85	281.31±9.17	26.37±1.60	94.36±3.01	182.95±5.71
Control	299.29±6.05	296.40±7.51	2.89±2.78	0.97±0.93	-	-	-	-
5	299.29±6.05	196.62±10.34[d]	99.78±4.91	33.69±2.12	791.11±10.72	75.16±1.02	75.44±.373	133.48±1.80
Control	661.00±21.79	658.22±22.75	2.78±0.96	0.42±0.16	-	-	-	-
10	661.00±21.79	431.56±20.57[d]	226.67±8.82	34.45±1.37	1407.95±40.67	133.76±3.86	59.12±4.05	107.05±5.34
Control	3616.11±42.76	3596.67±38.19	19.44±4.81	0.54±0.12	-	-	-	-
50	3616.11±42.76	2280.00±38.19[d]	1361.67±57.74	36.60±1.34	8018.45±283.83	772.67±48.05	58.68±2.24	111.05±4.38

a : [Amount of removed As(V) / Amount of initial As(V)] x 100

b : [Total amount of As in algae / Amount of As(V) removal] x 100

c : Bioconcentration factor = $\frac{\mu g \text{ compound removed / g dried weight}}{\mu g \text{ compound in solution / ml solution}}$

d : $P < 0.05$, significant different when comparing with the control.

5.9 Kinetic study of As(V) removal by the *Chlorella sp.*

The growth pattern of the isolated *Chlorella sp.* and its ability to remove As(V) are shown in Figure 5-4 and Table 5-11. As illustrate in Figure 5-4, the amount of As(V) in the medium is found to be gradually decreased, with the concurrent increase in the growth of the isolated *Chlorella sp.* The As(V) is drastically decreased during the first 2 days of exposure, then gradually decreases afterwards until the 12^{th} day. The amount of As(V) of the control, without alga, does not decrease. The results also indicate that growth of the isolated *Chlorella sp.* is unaffected by the presence of As(V) in the medium at concentration of 10 mg l^{-1}. No lag phase is observed in the growth curve and the growth of algae is steadily increased from the beginning until the end of the experiment. The isolated *Chlorella sp.* is capable to remove approximately 15% As(V) within 2 days of exposure and the percentage of removal is increased to 31% in 14 days (Table 5-11). The percentages of As(V) removal are directly proportional to the exposure times.

Table 5-11 : Kinetic removal of As(V) by the isolated *Chlorella sp.*

The values are given as mean ± standard deviation (n=3).

Time (days)	As(V) before treatment		As(V) after treatment		% As(V) removal[a]
	Concentration ($\mu g\ l^{-1}$)	Amount (μg)	Concentration ($\mu g\ l^{-1}$)	Amount (μg)	
Control	9812.76±43.65	294.38±1.31	9721.81±125.17	291.65±3.76	0.93±0.19
2	9812.76±43.65	294.38±1.31	8310.70±77.67[b]	249.32±2.33	14.51±0.31
4	9812.76±43.65	294.38±1.31	8180.04±251.81[b]	245.40±7.56	15.87±1.82
6	9812.76±43.65	294.38±1.31	7683.13±108.39[b]	230.49±3.25	20.97±1.15
8	9812.76±43.65	294.38±1.31	7559.67±158.38[b]	226.79±4.75	22.24±1.36
10	9812.76±43.65	294.38±1.31	7446.50±108.39[b]	223.40±3.25	23.40±1.28
12	9812.76±43.65	294.38±1.31	7384.78±47.15[b]	221.54±1.41	24.04±0.50
14	9812.76±43.65	294.38±1.31	6705.76±278.35[b]	201.17±8.35	31.03±2.46
16	9812.76±43.65	294.38±1.31	6736.63±175.50[b]	202.10±5.27	30.71±1.64

a : % As(V) removal = (Amount of removed As(V)) / (Amount of initial As(V)) x 100

b : $P < 0.05$, significant different when comparing with the control.

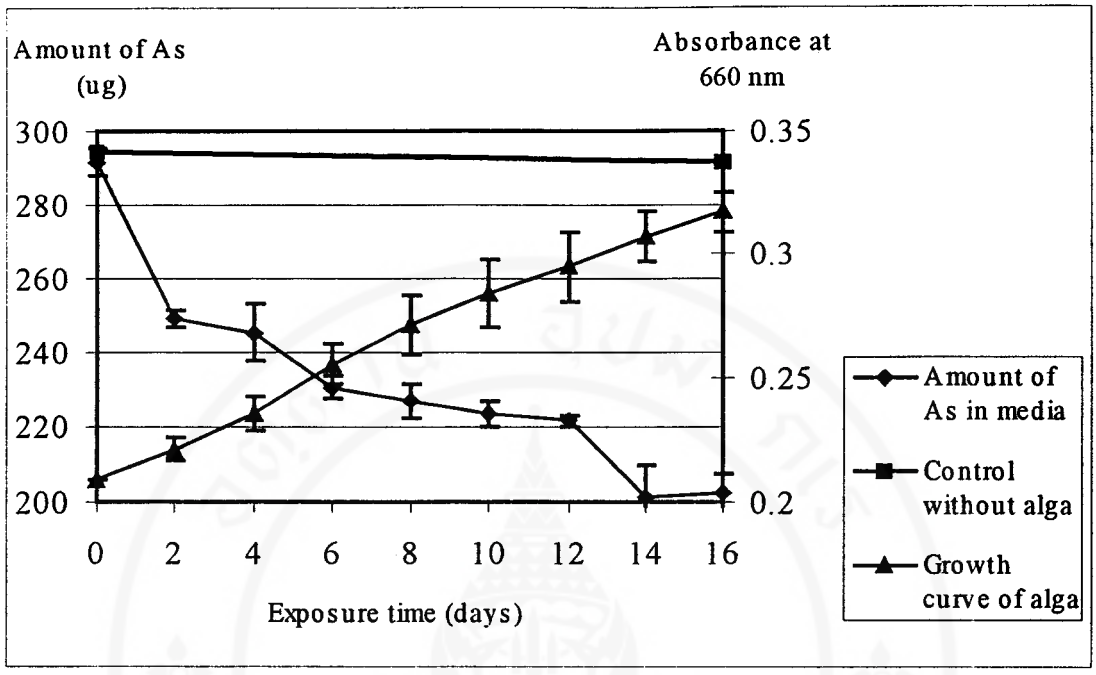


Figure 5-4 : Kinetic of As(V) removal by the isolated *Chlorella sp.* The control (—■—) is the amount of As(V) in media without alga at the beginning and the end of the exposure time. The diamon (—◆—)is the amount of As(V) in media containing alga. The triangle (—▲—) is the growth of the alga exposure to As(V) at level of 10 mg l^{-1}.

5.10 Kinetic of the As(V) removal by the isolated *Chlorella sp.* from the with and without phosphate in the media.

The objective of this experiment is to improve As(V) removal ability of the isolated *Chlorella sp.* by investigating the effects of phosphate, which has been reported as a competitive inhibitor of arsenate uptake and accumulation, on the kinetic of the As(V) removal. Percentages of As(V) removal from the with and without phosphate media during the exposure time were compared. In the presence of phosphate, the gradually increasing of percentage of As(V) removal is observed

(Figure 5-5). The As(V) removal is gradually increased from 16% at the 4th day to 24% at the 11th day of exposure. In the absence of phosphate, percentage of As(V) removal is rapidly increased during the early exposure time and reaches the maximum value (28% removal) at the 4th day, then it is slowly decreased. The removal efficiency is directly proportional to the exposure time in the presence of phosphate in the medium, but it is inversely proportional in the absence of phosphate. The experimental results indicate that the higher As(V) removal efficiency is obtained at the 4th day of exposure in the condition of without phosphate in the medium. This condition could be applied for improving the ability of the isolated *Chlorella sp.* to remove As(V) from water.

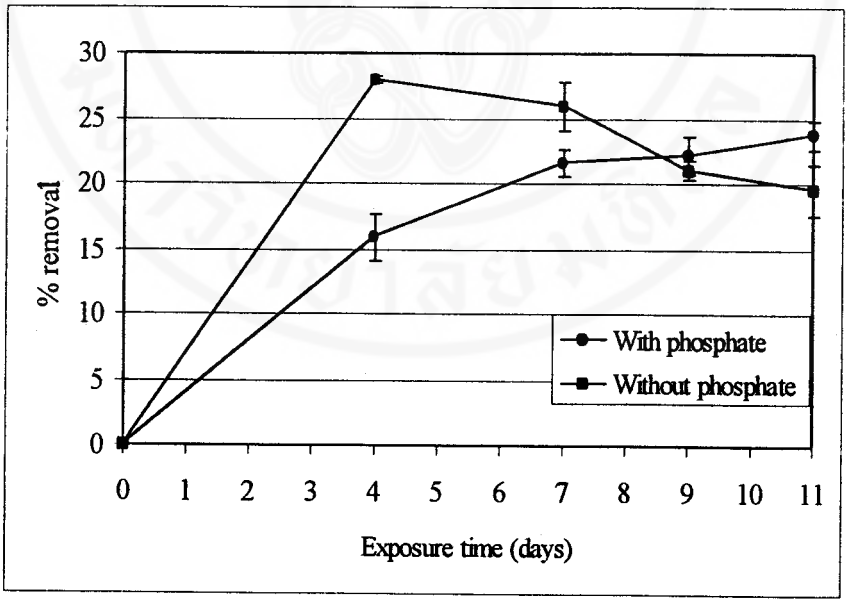


Figure 5-5 : Kinetic of As^{+5} removal by the isolated *Chlorella sp.* from with and without phosphate conditions. The circle (—●—) is the percentage of As(V) removal in the condition of As(V) 1 mg l^{-1} and PO_4^{3-} 1 mg l^{-1}. The square (—■—) is the percentage of As(V) removal in the condition of As(V) 1 mg l^{-1} and PO_4^{3-} 0 mg l^{-1}.

5.11 Determination of arsenic in various cell fractions of the isolated *Chlorella sp.*

The decrease of As(V) in the culture medium containing the isolated *Chlorella sp.* is due to the removal capacity of the alga. The algal cells could uptake and accumulate the metal into their cell, thus removing the metal from water. The experiments were carried out to determine the presence of arsenic in various cell fractions. The cell culture was separated into 4 fractions namely, supernatant, the washing liquid (first, second, and third), cell fragment, and cell debris. The arsenic content in the washing liquid is that adheres to the cell wall. The remained content either in the cell fragment or cell debris is the amount of arsenic accumulates by cell. The results are shown in Table 5-12. The washing liquid contains 14.41 μg arsenic (or 5.73% of total arsenic removal by the alga). Thus, there is very little arsenic bound to the cell wall. Large amount of arsenic is found in the cell fractions, cell fragment contains 46.40 μg (18.45%) and the cell debris contains 60.69 μg (24.13%). This indicates that most of the arsenic removed by the isolated *Chlorella sp.* is accumulated within the algal cells. The results also show that, out of 251.49 μg arsenic removed by the alga, only 121.50 μg (48.31%) could be detected in the cell fractions. The amount of 129.99 μg (51.69%), which could not detected, may either loss during the experimental procedures or being transformed into some other undetectable products. As a result of this, another experiment could be performed to find out the certain amount of arsenic accumulation in the algal cell. In this experiment the cell culture was separated into 3 fractions, supernatant, the washing liquid, and the algal cells. As shown in Table 5-13, the amount of arsenic found in the washing liquid is low

Table 5-12 : The presence of arsenic in the algal cell fractions. The algal cell culture was separated into 4 fractions : supernatant, washing liquid (first, second, third), cell fragment, and cell debris.

Cell fractions	Amount of As (μg)[a,b]	% As removal
Control	790.249±3.76	-
Supernatant	534.78±7.39	-
Total As remove	255.47±7.39[c]	100.00
Wash liquid 1	8.48±2.36	3.37
Wash liquid 2	3.66±0.53	1.46
Wash liquid 3	2.27±0.14	0.90
Cell fragment	46.40±7.92	18.45
Cell debris	60.69±4.77	24.13
Total As in cell fractions	119.99±8.59[d]	48.31

a : Total amount of As in each fraction.

b : All values were the averages of the two replicates.

c : Total As remove =

Amount of initial As (control) - Amount of removed As (supernatant)

d : Total As in cell fractions =

Summation of the amount of As found in washing liquids, cell fragment, and cell debris

Table 5-13 : The presence of arsenic in the algal cell fractions. The algal cell culture was separated into 3 fractions : supernatant, washing liquid, and algal cells.

Cell fractions	Amount of As (μg)[a,b]	% As removal
Control	790.249±3.76	-
Supernatant	523.58±4.48	-
Total As remove	266.67±3.76[c]	100.00
Wash liquid	33.45±0.81	12.73
Algal cell	176.42±3.68	67.16
Total As in cell fractions	209.87±3.01[d]	79.89

a : Total amount of As in each fraction.

b : All values were the averages of three replicates.

c : Total As remove =

Amount of initial As (control) - Amount of removed As (supernatant)

d : Total As in cell fractions =

Summation of the amount of As found in washing liquids and algal cells.

(only 33.45 μg or 12.73%) as compares with that found in the algal cells (176.42 μg or 67.16%). Furthermore, there are altogether 209.87 μg (79.89%) arsenic which could be detected in both washing liquid and the algal cells. When these values are compared with the total arsenic removal (262.69 μg), a few different is observed. So, it could be confirmed that the isolated *Chlorella sp.* have ability to remove As(V) from water by accumulation the metal into their cell.

5.12 Removal of As(V) by the immobilized *Chlorella sp.*

This experiment was designed to investigate the possibility of application of immobilized *Chlorella sp.* to remove As(V) from water. Immobilization of algae in a polymeric matrix, calcium alginate, can yield an insoluble alginate bead containing the entrapped algal cells. The As(V) removal capacity of these beads was tested. Various numbers of beads, ranged from 300 to 600 beads, were used in the experiment and the removal capacity were determined. These results are shown in Figure 5-6. The results show that the removal capacity increases with an increase in numbers of alginate beads. The highest percentage of As(V) removal, approximately 37%, is observed with 600 beads. Additionally, the removal capacity is directly proportional to the exposure time. The longer the time exposed to As(V), the higher the level of metal removed. The removal ability of the alginate beads contain no algae is very low. There was very small leakage of algal cells from the immobilized bead during the experiment. However, a small air bubble was found in some beads and resulted in the floatation of these beads to the surface of the medium. The experimental results indicate the possibility in utilizing the immobilized *Chlorella sp.* to remove As(V) from water.

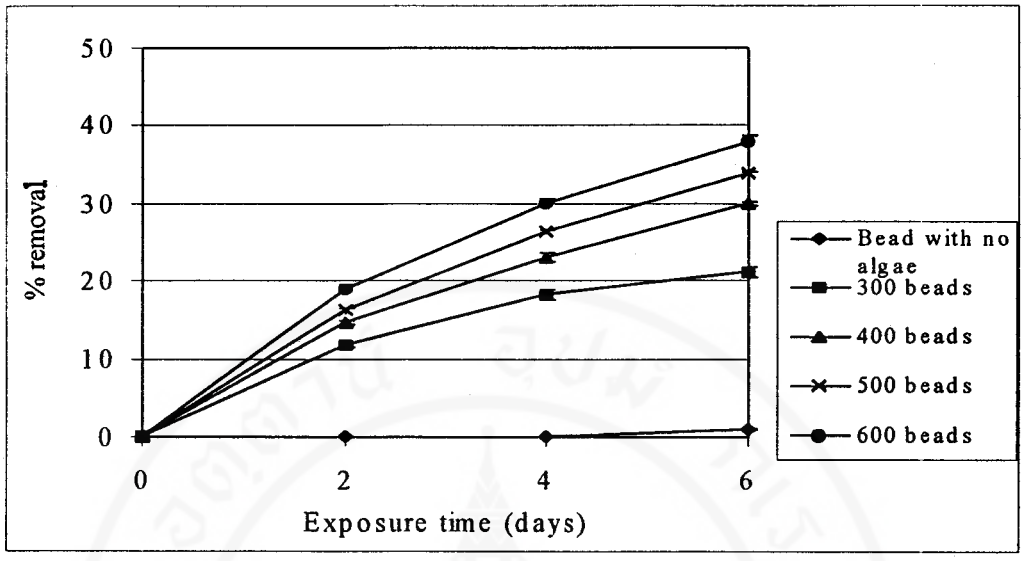


Figure 5-6 : The As(V) removal capacity of various nembers of the alginate beads entrapped cells of the isolated *Chlorella sp.*

5.13 Efficiency on removal of As(V) by immobilized and free *Chlorella sp.*

In this experiment the As(V) removal capacity of the free cells and the immobilized cells of the isolated *Chlorella sp.* were compared. Cell concentration in both free and immobilized conditions was constant. The results, as shown in Figure 5-7 and Table 5-14, reveal that at the early exposure time, the ability of algal free cells is better than the immobilized cells in removing As(V), then it is steadily decreased to 8% at the 9th day. This is opposite to the immobilized cells which the removal capacity is drastically high at the 1st day of exposure (28%) and then steadily increases to 49.84% at the 9th day. The removal efficiency at the constant cell concentration is directly proportional to the exposure time in the immobilized algal cell, but is inversely proportional in the free cells.

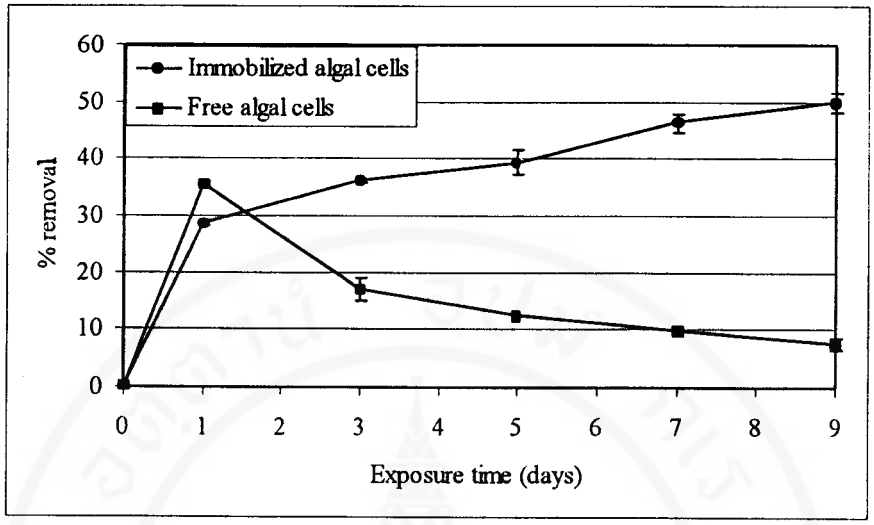


Figure 5-7 : Comparison on the As(V) removal capacities between the free and the immobilized cells of the isolated *Chlorella sp.*

Table 5-14 : Kinetic removal of As(V) by the immobilized and the free cell of the isolated *Chlorella sp.*

The values are given as mean ± standard deviation (n=3).

Time (days)	Immobilized cells			Free cells		
	Amount of As(V) before treatment (μg)	Amount of As(V) after treatment (μg)	% As(V) removal[a]	Amount of As(V) before treatment (μg)	Amount of As(V) after treatment (μg)	% As(V) removal[a]
Control	71.10±0.21	70.61±0.21	0.69±0.16	99.29±0.21	98.65±0.61	0.64±0.83
1	71.10±0.21	50.37±0.21[b]	28.67±0.46	99.29±0.21	65.66±0.96[b]	35.44±0.57
3	71.10±0.21	45.17±0.31[b]	36.03±0.24	99.29±0.21	81.94±1.63[b]	16.94±2.07
5	71.10±0.21	42.82±1.43[b]	39.36±2.11	99.29±0.21	86.40±0.44[b]	12.42±0.08
7	71.10±0.21	37.92±1.37[b]	46.31±1.63	99.29±0.21	89.09±1.10[b]	9.69±0.79
9	71.10±0.21	35.42±1.12[b]	49.84±1.67	99.29±0.21	91.20±1.61[b]	7.58±1.02

a : % As(V) removal = [Amount of removed As(V) / Amount of initial As(V)] x 100

b : $P < 0.05$, significant different when comparing with the control.

CHAPTER VI

DISCUSSION

The occurrence of human health problems resulting from arsenic contamination of domestic water supplies were recognized in Ron Phibun district, Nakhon Si Thammarat province, southern Thailand, where had an extensive history of bedrock and alluvial mining (5). The long-term investigation of water showed the presence of arsenic at concentration exceeding the exceptional standard (0.01 mg l^{-1}) as recommend by the WHO (5). The average concentration of surface water during 1992 to 1997 was in the range of 0.077-0.508 mg l^{-1} (51). Interestingly, the result of water analysis in this study showed that the degree of contamination was declined. Arsenic contents of water samples collected between October 1998 and October 1999 were reduced when compare to the average concentration during 1992 to 1997 (Figure 5-1). The reduction may be considerably resulted from the effective mitigation measures to alleviate arsenic contamination which have been carried out by the Department of Mineral Resources, Ministry of Industry. Although the progressive decreasing in arsenic level in surface water is recorded, this level still exceed the WHO potable water standard of 0.01 mg l^{-1}. So, arsenic contamination of the surface water in Ron Phibun continues to be a matter of concern and requires environmental monitoring to ensure that the arsenic contamination is within the standard limit of WHO.

Apart from water collection for arsenic concentration analysis, algae were also collected from arsenic polluted water in Ron Phibun. The most important aspect of collection is the maintenance of the algal species composition and the survival of the algae (47). Algae usually living in highly dynamic communities. The collection of algae actually changes the environmental condition which suitable for their survival. As a result of this, some algae die and the high diversity of species initially present in a collection can declined rapidly, leaving only few species from which to collect and isolate. In order to avoid this problem, the collection regime was divided into 2 groups, the first one was preserved with the fixative for identification, another one was collected without preservative for culturing purpose. The preservative was used to preserve all algal species present at each collection site and the species composition was known after identification. Fifteen algal genera were obtained, eleven genera out of these were green-algae and the others were blue-green algae and diatom. However, seven genera were also observed in the non-preserved samples. These algal genera may have ability to grow under the prolonged collection or enrichment procedure used, while the other eight might loose these ability and died during the collection. Only 3 unicellular algae, *Chlorella sp.*, *Euastrum sp.*, and *Navicula sp.*, were isolated and the axenic cultures of the algae were established. It was very difficult to isolate the filamentous algae because they form mass growth, whether adhere together as a compact mat floating on the surface of the medium or coating as a greenish film on the surface of the container.

The green alga, *Chlorella sp.* exhibited a good growth when exposed to As(V) even at the highest concentration (500 mg l^{-1}). As a result of this, the isolated *Chlorella sp.* was selected as the As(V) tolerant alga. It is not surprised because the algae in this genera usually have great tolerant ability for many metals, including Cd, Zn, Cu, Pb, and Mn (7,52). The As(V) tolerant ability of this algal genera was earlier observed by other investigators. *Chlorella vulgaris* was found to survive in As(V) concentration up to 10,000 mg l^{-1} (39). The As(V) tolerance was also reported by other freshwater algae such as *Phormidium sp.* (41), *Nostoc sp.* (40), *Anabaena variabilis* (53), and *Synechococcus leopoliensis* (53) and the considerable variation between algae in As(V) tolerance was suggested. The effects of concentrations and chemical forms of arsenic (As(V) / As(III)) on growth of the isolated *Chlorella sp.* were investigated. The results indicated that As(III) was more toxic to the alga than As(V). The algal growth was unaffected by As(V) up to the level of 50 mg l^{-1}, but the growth was reduced and inhibited when exposed to As(III) at the same concentration. This finding agreed with earlier observation by Maeda et al. (39,42). They reported that the growth of *Chlorella vulgaris* increased with an increase in As(V) concentration up to 2000 mg l^{-1}, but decreased at As(III) concentration higher than 10 mg l^{-1}. The study which used other algal genera by Budd et al. (53,54) reported similar result that As(III) inhibited growth of *Synechococcus leopoliensis* while As(V) did not.

The results obtained in this experiment showed that the isolated *Chlorella sp.* could remove As(V) from water and accumulate in their cells. In order to achieve an effective removal of As(V) from water, all factors of concern including, concentrations and types of cells, growth stages, pH, and phosphate levels should be optimized.

The experimental results indicated the optimum condition which generate high percentages of As(V) removal by the isolated *Chlorella sp.* This condition is the use of living algal cells at the middle stage of growth, at cell concentration equals to the absorbance 0.2 to remove As(V) from water which has pH ranged from 5 to 7 and in the absence of phosphate.

Arsenic (As(V)) removal and accumulation by the isolated *Chlorella sp.* was occurred only by the living cells. These results correspond with the previous reports for *Chlorella vulgaris* (39) and *Phormidium sp.* (41). Heat-killed and ethanol-killed cells of the *Chlorella vulgaris* and *Phormidium sp.* did not accumulate arsenic *in vitro*. It might be suggested that accumulation of arsenic by these algae was not due to adsorption on the cell surfaces, but that the metal was uptake into the algal cells.

The maximum As(V) removal and accumulation was observed in the culture contained small amount of algal cells. This condition might be favored growth of the algae as they could grow without competition for the nutrient and remove large amount of As(V) from water. Additionally, the optimum growth stage of the isolated *Chlorella sp.* which yield the highest As(V) removal was the middle exponential stage. The algal cells were active and grew well during this stage. However, the prior study by Maeda et al. (42) did not produce the same result as they were reported that the early exponential stage was the optimum growth stage for *Chlorella vulgaris*. This discrepancy may occur form the different in either the algal species or the exposure concentration, *Chlorella vulgaris* was exposed to higher As(V) concentration (10 mg l^{-1}) than the current study by the isolated *Chlorella sp.* (1 mg l^{-1}).

In addition to the factors concerning about the algae, another environmental factor to be considered was the pH of water. The optimum pH should be the pH that suitable for the algal growth and thus the more active biomass which has great potential for metal removal purpose was produced. The highest ability of the isolated *Chlorella sp.* to remove and accumulate As(V) was observed in the pH range from 5 to 7. So, this pH range was the optimum pH for the isolated *Chlorella sp.*

The concentration of phosphate in water directly influences the As(V) removal because of the chemical similarities between phosphate and arsenate. The results in this study indicated that As(V) removal by the isolated *Chlorella sp.* was competitively inhibited by phosphate as the As(V) uptake and accumulating efficiency was inversely proportional to the phosphate concentration. The previous study with *Chlorella vulgaris* by Maeda et al. (39,42) also reported similar finding. The arsenic accumulation capacity of the *Chlorella vulgaris*, which exposed to As(V) at concentration of 10 mg l^{-1} and various concentration of phosphate (KH_2PO_4), decreased with an increase in phosphate concentration in the medium (42). The percentages of As(V) removal between conditions with and without phosphate were compared (Figure 5-5). The greatest removal efficiency for a short exposure time was observed in the absence of phosphate. However, the percentages of As(V) removal in the absence of phosphate decreased according to the exposure time. This may be caused by lacking of phosphate for the algal growth. Phosphate is an important nutrient which algae require for their growth. As a result, the condition with low phosphate concentration may favor growth of the alga and may not inhibit As(V) removal by the alga. So, the low concentration

of phosphate may be an optimum condition for As(V) removal by the isolated *Chlorella sp.*

In general, arsenate and phosphate usually compete for being uptaken by algae (55). Most alga is unable to differentiate between these two compounds. During phosphate limiting condition, high level of arsenate is taken up and interferes with phosphate metabolism (35). As a result of this, the growth of the sensitive algae is inhibited. Arsenate toxicity may be reduced by increasing phosphate concentration in water (45). In arsenic tolerant algae the detoxification mechanisms are evolved to cope with the problem of arsenate uptake (55). However, some algae are capable of discriminating between these two compounds and they can be survived in water with raised arsenate levels because their ability to either reject or rapidly excrete arsenate (53,55).

The experimental results revealed that the amount of As(V) in the medium decreased with time while the isolated *Chlorella sp.* grew in number (Figure 5-4). The decreasing was very rapid and large amount of As(V) could be removed during the early exposure time. No lag phase was observed, so the algal growth was unaffected by As(V). Approximately 31% of As(V) was removed by the isolated *Chlorella sp.* within 14 days. So, the isolated *Chlorella sp.* was judged to have high ability to remove As(V) from water. The experiment also showed that the percentage of As(V) removal was not much different (33-36%) at concentration of 1, 5, 10, and 50 mg l^{-1} of As(V) in the medium. In contrast, the amount of arsenic accumulated in the algal cells increased according to increasing level of As(V) in the medium. The result of this study agreed with that of Maeda et al. who studied with *Chlorella vulgaris* (39) and *Phormidium sp.*

(41). The arsenic accumulation in the *Chlorella vulgaris* increased with an increase in As(V) in the medium (0.1-1,000 mg l^{-1}). The other study using the *Phormidium sp.*, exposed to As(V) at concentration of 1 to 7,000 mg l^{-1}, also produced the similar result. The percentages of arsenic accumulation in the isolated *Chlorella sp.* decreased when the alga was exposed to As(V) at high concentration. However, at As(V) concentration of 10 or 50 mg l^{-1}, the percentage of As(V) removal exhibited similarity (58-59%).

The bioconcentration factor (BCF) value which is used to characterize the metal uptake rate was another concern. The highest BCF was observed at the lowest concentration (1 mg l^{-1}) of As(V), but this value was not increased with an increase in As(V) level. However, there was a few different between the BCF values when compared with that of the As(V) concentration. Therefore, the As(V) uptake by the isolated *Chlorella sp.* was high when the alga was exposed to the low As(V) concentration. At the higher concentrations the uptake of As(V) by the algae occurred nearly to a constant rate.

Studies on the presence of arsenic in various cell components (Table 5-13) indicated that the quantity of arsenic found in the washing liquid was smaller than that in the algal cells. The arsenic content in the washing liquid was the amount of arsenic adsorbed on the cell surfaces. The remainder that was found in the algal cells was the accumulated arsenic. Thus, it can be concluded that the isolated *Chlorella sp.* can remove As(V) from water by accumulating the metal into their cells. The prior studied by Maeda et al.(42), which using *Chlorella vulgaris*, also reported the same result that the small quantity of arsenic taken up by the algal cells (23%) was adsorbed on the cell

surfaces, but the large amount of arsenic (77%) was accumulated by the cell. They also reported that both adsorbed and accumulated arsenic concentration increased with an increase in As(V) of the aqueous phase. In the present study there was some arsenic that could not be detected in the cell components. This may be occurred by the arsenic lost during experimental procedures or tightly adsorption of arsenic onto the algal cells that the extracting solvent could not extract them from the cells as well as the transformation of arsenic into other metabolites. There is some evidence to show that arsenic can be methylated by algae. Baker et al. (28) reported the 4 freshwater algae which capable of methylating arsenate into methylated arsenic compounds. Similar compounds have been found in the lipid soluble fractions of freshwater algae isolated from arsenic-polluted environment (43). In addition to methylated arsenic compounds, the other compound arsenosugar is also found in some freshwater algae which are exposed to high level of arsenic (56).

The immobilization technique which entrapped the *Chlorella sp.* in calcium-alginate bead showed high efficiency in arsenic removal. Approximately 37% of As(V) was removed by the immobilized *Chlorella sp.* within 6 days. The removal capacity was directly proportional to both numbers of beads and exposure time. With the finding in this study, the immobilized algae is useful for removal of As(V) from water.

The immobilization technique may be suitable for household application as it is cost-effective for As(V) removal and it does not produce hazadous wastes. Alginate costs only 9 bath per gram and this amount could make around 1,000 beads at 0.5 cm size. There are several ways to improve the removal capacity of the immobilized algal cells. These include the use of a countercurrent (from the bottom) feed flow, decreasing

the size of the immobilized algal beads, and increasing the porosity of the immobilized algal beads (52).

When the activities of the free and immobilized cells were compared, it was found that the removal efficiency was directly proportional to the exposure time in the immobilized algal cells, but was inversely proportional in the free cells after day 1 (Figure 5-7). The percentage of As(V) removal by the immobilized algae was slowly increased until reached the highest value (50%) in the 9^{th} day. This may be due to the reason that the As(V) has to penetrate into the beads before being removed by the algal cells which are inside the beads. In addition, the beads have been left in the culture medium for a week before exposure to As(V). During this time, the alginate beads may absorb phosphate from the medium and thus supporting growth of the entrapped algal cells. In the opposite, the percentage of As(V) removal by the free algal cells was gradually decreased after day 1. This may be the result of lacking phosphate for growth of the algal cells as the experiment was conducted for 9 days.

CHAPTER VII

CONCLUSION

From the experimental result of this study, the conclusion can be drawn as follows :

1. The *Chlorella sp.* isolated from the arsenic polluted water can tolerate high level of As(V) (500 mg l^{-1}) and remove 35% of As(V) from water within the 1st day of exposure.
2. The optimum condition which enhance the As(V) removal ability of the isolated *Chlorella sp.* is the used of living algal cells in the middle stage of growth, at cell concentration equals to the absorbance of 0.2 under the pH range of 5 to 7 and in low level of phosphate.
3. The removal ability is high only at the low As(V) level, but that for the higher As(V) level it is quite stable.
4. The isolated *Chlorella sp.* can remove As(V) from water by accumulation of arsenic in their cells, but not by adsorption on the cell surfaces.
5. The immobilization that entrapped the isolated *Chlorella sp.* in calcium-alginate bead can remove 50% of As(V) from water within 9 days of exposure. The removal efficiency is directly proportional to both cell concentration and exposure time. However it is opposite to the free cells which showed inverse relationship after day 1.

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APPENDIX I

Preparation of culture media

Recipes of media for cultivation of algae.

CHU NO.10 (Stein, 1973)

- $Ca(NO_3)_2$	40	mg
- $K_2HPO_4 \cdot 7H_2O$	10	mg
- $MgSO_4 \cdot 7H_2O$	25	mg
- Na_2CO_3	20	mg
- Na_2SiO_3	25	mg
- $FeCl_3$	1	mg
- deionized water	1000	ml

pH 6.5

Hoagland solution (Rechcigl, 1977)

- $Ca(NO_3)_2 \cdot 4H_2O$ 940 mg
- KNO_3 660 mg
- $MgSO_4 \cdot 7H_2O$ 520 mg
- $NH_4H_2PO_4 \cdot H_2O$ 120 mg
- $FeSO_4 \cdot 7H_2O$ 70 mg
- Trace element 0.1 ml
    - H_3BO_3 28 g
    - $MnSO_4 \cdot H_2O$ 34 g
    - $ZnSO_4 \cdot 7H_2O$ 2.2 g
    - $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 1.0 g
    - $CuSO_4 \cdot 5H_2O$ 1.0 g
    - H_2SO_4(concentrated) 5.0 ml
    - deionized water 1000 ml
- deionized water 1000 ml

pH 6.0

Modifies Detmer (Maeda, 1983)

- KNO_3 1000 mg
- $CaCl_2$ 100 mg
- $MgSO_4 \cdot 7H_2O$ 250 mg
- NaCl 100 mg
- $K_2HPO_4 \cdot 7H_2O$ 250 mg
- $FeSO_4 \cdot 7H_2O$ 20 mg
- Trace element 1 ml
    - H_3BO_3 2.86 g
    - $MnCl_2 \cdot 4H_2O$ 1.81 g
    - $ZnSO_4 \cdot 7H_2O$ 0.22 g
    - $CuSO_4 \cdot 5H_2O$ 0.08 g
    - Na_2MoO_4 0.021 g
    - H_2SO_4(concentrated) 1 drop
    - deionized water 1000 ml
- deionized water 1000 ml

pH 7.0

The media were prepared with dissolve all ingredient in deionized water, adjusted the pH by using 1N HCl and 2N NaOH, and then sterilized by autoclaving at 121°c with 15 lbs pressure for 15 minutes. If a solid medium was used, 15 g of agar was added into the liquid medium before sterilization.

The medium used for culturing *Navicula sp.* was CHU NO.10 because this medium had silica (as Na_2SiO_3) which necessary for the diatom. Hoagland solution was used to culture green and blue-green algae. The medium used for culturing the *Chlorella sp.* and used in all experiments was Modified Detmer. Stock culture of the *Chlorella sp.* was grown in the medium containing arsenate at concentration of 0.5 mg l^{-1} in order to maintain the arsenic tolerant ability of the algae.

Condition of cultivation

All culture were grown at temperature 25°c and under 12 hr light period with a light intensity of 2,600 lux. A day light fluorescence (TL40W/54) was used. During the course of cultivation, culture containers were manually swirled every two to three days. The algae was subcultured every three weeks.

APPENDIX II

Standard preparation

Standard arsenate (As(V)) solution

Standard of As(V) solution at concentration of 1,000 mg l^{-1} was prepared by dissolved 0.416 g sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$) with deionized water in a 100 ml volumetric flask, and used as stock solution. Working solutions used in all experiments were prepared from the stock solution.

Standard arsenite (As(III)) solution

Standard of As(III) solution at concentration of 1,000 mg l^{-1} was prepared by dissolved 0.174 g sodium arsenite ($NaAsO_2$) with deionized water in a 100 ml volumetric flask, and used as stock solution. Working solutions used in all experiments were prepared from the stock solution.

Standard phosphate solution

Standard of phosphate solution at concentration of 1,000 mg l^{-1} was prepared by dissolved 0.220 g potassium dihydrogen phosphate (KH_2PO_4) with deionized water in a 100 ml volumetric flask, and used as stock solution. Working solutions used in all experiments were prepared from the stock solution.

APPENDIX III

Procedures for sample preparation for arsenic determination.

After exposure time, the algal cells were harvested by centrifugation (3,000 g, 10 min). The supernatant was collected and analyzed for arsenic concentration. The pellets containing algal cells were washed three times with the diluted medium and analyzed for arsenic concentration.

Preparation of water samples

All water samples, i.e., control, medium, and washing liquid (in the cell fraction experiment), were diluted with 1% nitric acid to desired concentration and store in the refrigerator until analysis.

Preparation of algal sample

The algal cell dry weights were obtained by adding the cells to the glass vials which preweighed and drying at 80°c for 16 hr and then weighed. Concentrated nitric acid was added to each sample. The samples in glass vials were heated at 70°c on water bath for 2 hr and then cooled. After all the samples had cooled, the solution remaining was filtered and diluted to 10 ml with deionized water and store in the refrigerator until analysis.

Standard arsenic solution

For calibration purpose, 100 μg l^{-1} arsenic solution was prepared by diluted standard 1,000 mg l^{-1} sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$) with 1% nitric acid.

Matrix modifier (Palladium 1,000 mg l^{-1})

An accurate 0.1 g of Pd metal was dissolved in 10 ml of nitric acid and the minimum volume of hydrochloric acid. This was then diluted to 100 ml.

Determination of total arsenic concentration in sample.

Graphite furnace atomic absorption spectrometry (GFAAS) measurements were performed with a Perkin-Elmer Analyst 100 (Norwalk, CT, USA) equipped with a deuterium-arc background corrector and HGA-800 heated graphite atomizer. The sample was introduced by AS-72 autosampler. The cooling system HGA also used to allow the temperature of the atomizer to cool down more rapidly. The atomic signals were monitored by a Compac computer. The radiation source was an arsenic electro discharge lamp operated at not over 300 mA, and the 193.7 nm wavelength was monitored. The spectral bandwidth used was 0.7 nm. Graphite furnace operating conditions for analysis are shown in Table III-1.

Table III-1 : Furnace operating conditions for analysis of sample by GFAAS using aqueous sample introduction.

Step	Temperature (°c)	Ramp/Hold Time (sec)	Argon gas flow (ml/min)
1. Drying	120	10/30	250
2. Pyrolysis	1,200	10/30	250
3. Cooling	20	5/5	250
4. Atomization	2,300	0/5	Stop flow
5. Clean up	2,400	1/5	250

APPENDIX IV

The characteristics of each algal genera are summarized as follows :

Cyanophyta : blue-green algae

The principal characteristics of Cyanophyta

Blue-green algae are procaryotic organisms. They occur as unicellular, colonial, and filamentous forms. The cell wall of blue-green algae are composed of murein or peptidoglycan. The cells are often embedded in gelatinous sheaths or mucilage. Blue-green algae resemble other eubacteria in lacking both nucleus and complex organelles, such as chloroplasts, mitochondria, golgi apparatus, and endoplasmic reticulum. The photosynthetic pigments of blue-green algae are associated with thylakoids. The reaction centers of chlorophyll a are in the membrane of a thylakoid. On the outer surface of the thylakoid membrane are granular structures called phytobilisomes, which are composed of phycobiliproteins. The four phycobiliproteins in blue-green algae are phycoerythrin, phycocyanin, allophycocyanin, and phycoerythrocyanin. Phycocyanin and allophycocyanin occur in all blue-green algae, and phycocyanin is usually responsible for giving cells their distinctive blue-green color. Blue-green algae can only reproduce asexually. They occur in marine, freshwater and terrestrial habitats.

Oscillatoria sp. (Figure IV-1)

Division Cyanophyta

Class Cyanophyceae

Order Oscillatoriales

Family Oscillatoriaceae

Genus *Oscillatoria*

Characteristics : *Oscillatoria sp.* is an unbranched filamentous alga. Its disc-shaped cells are arranged in a single series. All cells are alike except for a slight modification in the shape of terminal cells. One of the chief characteristic of species is their oscillating movement of filament and another is their lack of a definite firm sheath. *Oscillatoria sp.* grows by production of new cells that lengthen an existing filament. A new filament is initiated when part of the filament breaks off. Often this is a short segment from the end of a filament which is called a hormogonium-a short pieces of trichome that become detaches from the parental filament and moves by gliding. More than 100 species are known and these are widely distributed in the sea, freshwater, hot springs and areas affected by sewage effluents.

Phormidium sp.

Division Cyanophyta

Class Cyanophyceae

Order Oscillatoriales

Family Oscillatoriaceae

Genus *Phormidium*

Characteristics : *Phormidium sp.* is filamentous alga. The filaments commonly are within a sheath. The sheath is thin and sticky so that the plants often adhere together, forming compact mat, or a skein over submersed surfaces or on dripping rocks. The plants are blue- or black-green and feel slimy or slippery in the touch. *Phormidium sp.* is particularly common in wet or damp terrestrial habitats. There are numerous species. They are different in size, sheath characteristics, and the morphology of the apical cell.

Chlorophyta : green algae

The principal characteristics of Chlorophyta

The members of the Division Chlorophyta have similar pigments, reserved foods, and chloroplast structures. In the green algae the principal photosynthetic pigments are chlorophyll a and b, and in some algae the pigments are siphonaxanthin or lutein. The reserve is usually starch which is formed in the chloroplast. The green algal chloroplast is surrounded by double-membrane envelope and contains thylakoids joined in groups of two to six. No chloroplast reticulum occurs around the chloroplasts. Cell walls of green algae generally have cellulose as the main structural polysaccharide. The Chlorophyta are primarily freshwater, and only about 10% of the algae are marine.

Chlorella sp. (Figure IV-2)

Division Chlorophyta

Class Chlorophyceae

Order Chlorococcales

Family Oocystaceae

Genus *Chlorella*

Characteristics : *Chlorella sp.* is a common unicellular algae. Its cell is small spherical or oval shape. *Chlorella sp.* generally occurs on soil and in freshwater. It can also occur as endosymbionts in invertebrate animals. *Chlorella sp.*has cup-shaped chlopoplast. It reproduces by internal cell division, in this instance forming non-motile autospores which morphologically like the parent.

Oocystis sp. (Figure IV-3)

Division Chlorophyta

Class Chlorophyceae

Order Chlorococales

Family Oocystaceae

Genus *Oocystis*

Characteristics : *Oocystis sp.* has free-floating solitary ellipsoidal cells sometimes held in loose colonies by the remains of the parent cell wall. Most species have a number of discoid chloroplast, and there is a characteristic nodular thickening on the wall at each pole, but absence in some species. Reproduction is usually by the formation of 2 to 16 daughter cells enclose within the mother-cell wall which enlarges so that it often appears as a gelatinous sheath. The daughter cells can live separately after released from the parent cell wall or living together as a group within the parent cell wall. *Oocystis sp.* are usually found as the planktonic flora of small freshwater lakes and ponds.

Scenedesmus sp. (Figure IV-4)

Division Chlorophyta

Class Chlorophyceae

Order Chlorococcales

Family Oocystaceae

Genus *Scenedesmus*

Characteristics : The individual cells of this algae are elliptical to spindle-shaped and in many species they bear spines. The cells are connected together side by side, usually 2, 4, 8, or 16 of them, to form a flat colony. *Scenedesmus sp.* are common in fresh and brackish waters. More than 100 species have been described, the species differs mostly in the number and type of spines on the cells and the texture of the wall.

Chlorococcum sp. (Figure IV-5)

Division Chlorophyta

Class Chlorophyceae

Order Chlorococcales

Family Chlorococcaceae

Genus *Chlorococcum*

Characteristics : The cells of *Chlorococcum sp.* vary considerably in size, young cells having a thin cell wall and older cells a thick one. The chloroplast in young cells is a massive parietal cup shape with a single pyrenoid. In older cells the chloroplast become diffuse. *Chlorococcum sp.* is common in freshwater environment, and also occurs in abundance on dampsoil or brickwork.

Spirogyra sp. (Figure IV-6)

Division Chlorophyta

Class Chlorophyceae

Order Zygnematales

Family Zygnemataceae

Genus *Spirogyra*

Characteristics : *Spirogyra sp.* is filamentous alga which has one or more chloroplasts which spirally arranged periphery of its cell wall. A series of pyrenoids extend along each chloroplast. *Spirogyra sp.* is often common in ponds and other freshwater habitats. It grows attached to the bottom by rhizoids from cells at the base of filaments but may become detached and form masses of elongate filaments.

Euastrum sp. (Figure IV-7)

Division Chlorophyta

Class Chlorophyceae

Order Zygnematales

Family Desmidiaceae

Genus *Euastrum*

Characteristics : *Euastrum sp.* cells are always compressed and have a narrow but deep central isthmus. The cells have a linear to retuse median incision of the apex of each semicell. The semicells are trapezoidal and their margins bear 2 or 4 broad indentation which are symmetrically placed. About 150 species are known, almost all of them are in acidic water. Most species can be identified by the polar notch and by the protrusions or swellings on the face of the semicell.

Netrium sp. (Figure IV-8)

Division Chlorophyta

Class Chlorophyceae

Order Zygnematales

Family Mesotaeniaceae

Genus *Netrium*

Characteristics : *Netrium sp.* cells are elongate and elliptical in outline. The cell wall is smooth and there are two axial chloroplasts, one in each half of the cell. The chloroplasts are usually distinctly ridged with fimbriate margins. There are several pyrenoids in each chloroplast.

Ulothrix sp. (Figure IV-9)

Division Chlorophyta

Class Chlorophyceae

Order Ulotrichales

Family Ulotrichaceae

Genus *Ulothrix*

Characteristics : *Ulothrix sp.* is an unbranched filamentous alga which its cells arranged end to end. The only specialization is a holdfast cell for attachment. Otherwise all the cells are similar in function and structure. Cells vary from quadrate to long cylinder and also has a band-shaped or girdle chloroplast wrap around the periphery. In some species chloroplast is parietal band completely encircle the cell. Other species have chloroplasts which encircle 2/3 or 3/4 of a circle. Several pyreniods may be present.

Ulothrix sp. is found in quiet of running freshwater and occasionally on damp rocks or soil.

Microspora sp. (Figure IV-10)

Division Chlorophyta

Class Chlorophyceae

Order Ulotrichales

Family Microsporaceae

Genus *Microspora*

Characteristics : *Microspora sp.* is unbranched filamentous alga which has quadrangular to short-cylindric cells. The chloroplast varies greatly in appearance, even within the same filament, being either a parietal, folded, discontinuous plate, or reticulate. The wall is in 2 sections which overlap in the midregion. The overlapping is conspicuous in thick-walled species, but at least can be determined in the thin-walled species by examining the broken ends of filaments where the sections of the terminal cell protrude. When the filaments fragment or cells dissociated, H-shaped sections are found. Because of the wall features and the nature of the chloroplast *Microspora spp.* are placed in their own family.

Cladophora sp.

Division Chlorophyta

Class Chlorophyceae

Order Cladophorales

Family Cladophoraceae

Genus *Cladophora*

Characteristics : *Cladophora sp.* is a branching filamentous alga. Each cell in is surrounded by a thick wall, which lacks a sheath of mucilage (usually rough to touch). Most of the volume of a cell is filled by a large vacuole. The cytoplasmic layer surrounding the vacuole contains a net-like chloroplast and numerous nuclei which divide synchronously. *Cladophora sp.* is common and often abundant in rivers, lakes, and small bodies of water. It is also common in estuaries and oceans.

Pseudendoclonium sp. (Figure IV-11)

Division Chlorophyta

Class Chlorophyceae

Order Chaetophorales

Family Chaetophoraceae

Genus *Pseudendoclonium*

Characteristics : The filament of *Pseudendoclonium sp.* is irregularly branching, partly prostrate and partly erect, with all cells approximately the same size.

Division Chrysophyta : Class Bacillariophyceae

The principal characteristics of the Bacillariophyceae (Diatoms)

Diatoms are common in both marine and freshwater, as parts of benthic and planktonic communities. They occur as either solitary cells or in small colonies. A wall, the distinguishing feature of diatoms, consists of a frustule of silica surrounded by mucilaginous material. The frustule is composed of two valves and girdle bands. The larger valve, called the epitheca, fits over the small valve, or hypotheca, much like the parts of a petri dish. Additionally, there are often loops of silica inserted between the valves which are called the girdle bands. The principal photosynthetic pigments of diatom are chlorophyll a, b, c_1, c_2 , and the carotinoid fucoxanthin which makes cell become golden brown. The carbohydrate reserve is chrysolaminarin (leucosin). The reserve is present in cytoplasmic vacuoles rather than in chloroplast. The taxonomy of diatoms is largely based on the structure and markings of the valves. There are distinct markings on the valve as a result of the presence of pores, thickenings and extensions. Diatoms are divided into two groups based on the symmetry of the markings. Centric diatoms have radially arranged markings, while the marking on pennate diatoms are bilaterally arranged about the long axis of the valve.

Navicula sp. (Figure IV-12)

Division Chrysophyta

Class Bacillariophyceae

Order Pennales

Family Naviculaceae

Genus *Navicula*

Characteristics : This is a large genus in respect to the number of species, occurring in both fresh and marine water, benthic or in the planktonic from, and also terrestrial. The cells vary considerably in shape, especially in the valve view but mostly they are naviculoid or cigar-shaped, with narrowly rounded or capitate poles. In girdle view the cells are rectangular. There is a raphe in each valve. There may be internal plates but no complete septa. Striae are composed of puncta, sometimes very coarse and form what appear to be costae. The striae or rows of puncta are convergent toward the central, clear area of the axial field is distinctly enlarged in the midregion.

Fragilaria sp. (Figure IV-13)

Division Chrysophyta

Class Bacillariophyceae

Order Pennales

Family Fragilariaceae

Genus *Fragilaria*

Characteristics : The frustules are narrowly elongate, fusiform in valve view ; rectangular in girdle view and usually showing intercalary bands. The pseudoraphe is broad and distinct and occurs in each valve. The prominent striae actually are composed of rows of puncta. The most common species forms ribbons with the valves attached side by side.

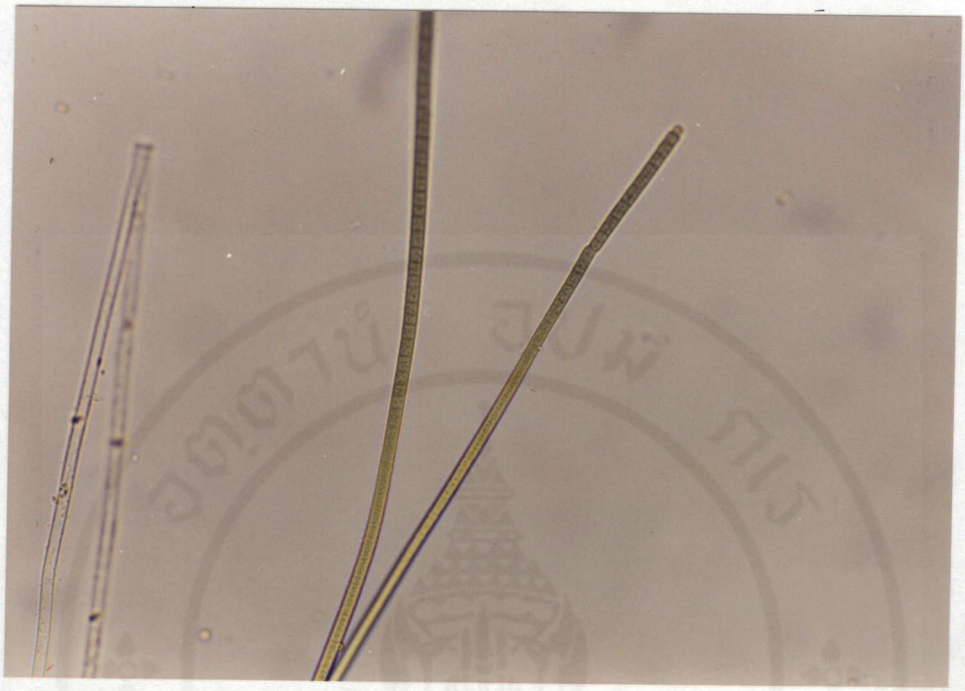

Figure IV-1 : *Oscillatoria sp.* (x20)

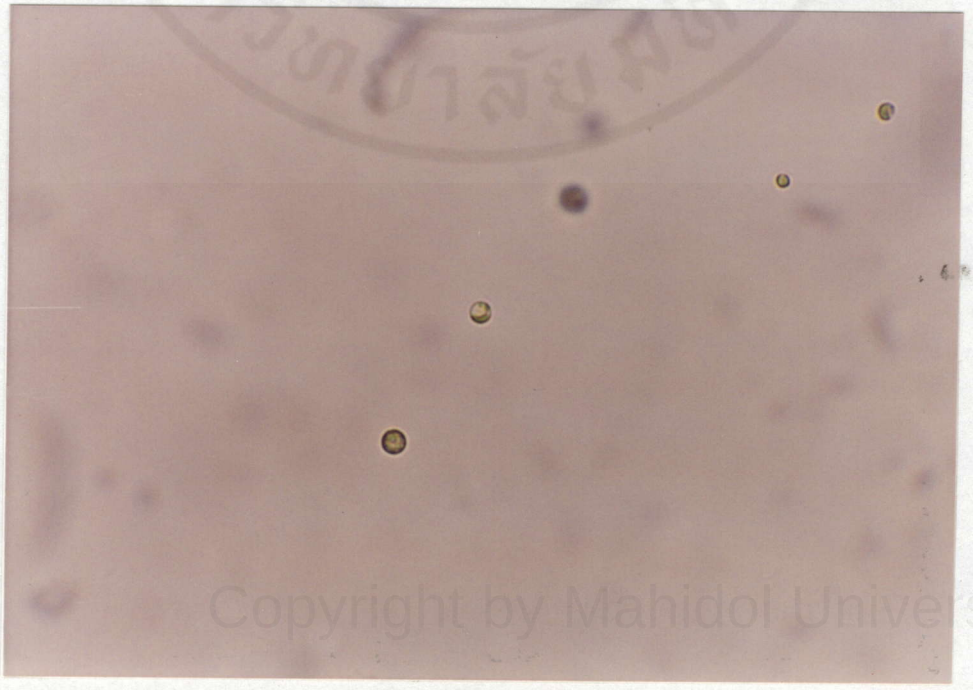

Figure IV-2 : *Chlorella sp.* (x40)

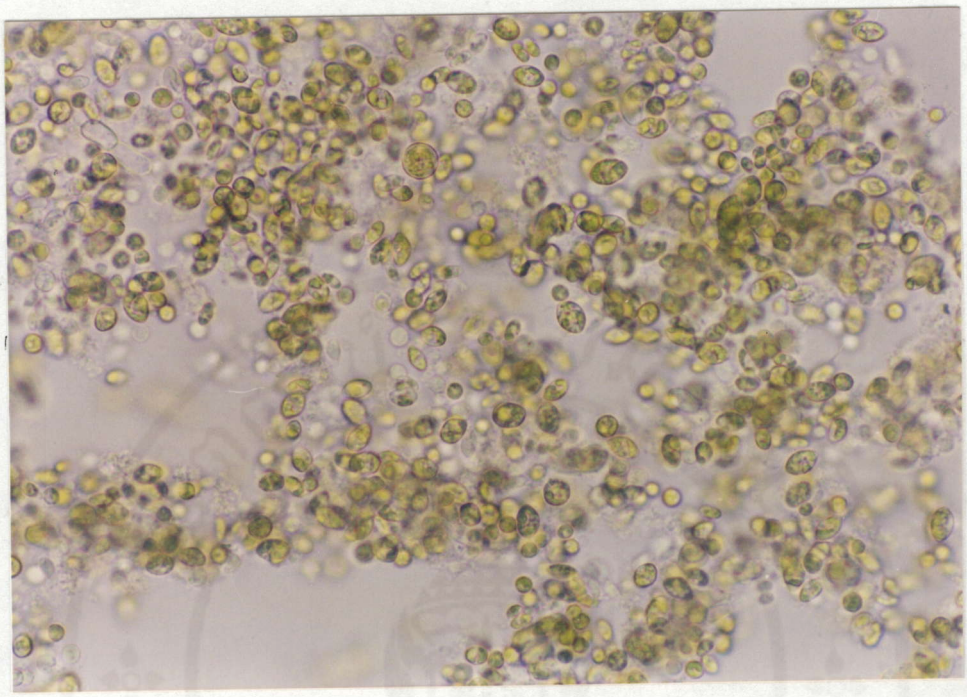

Figure IV-3 : *Oocystis sp.* (x40)

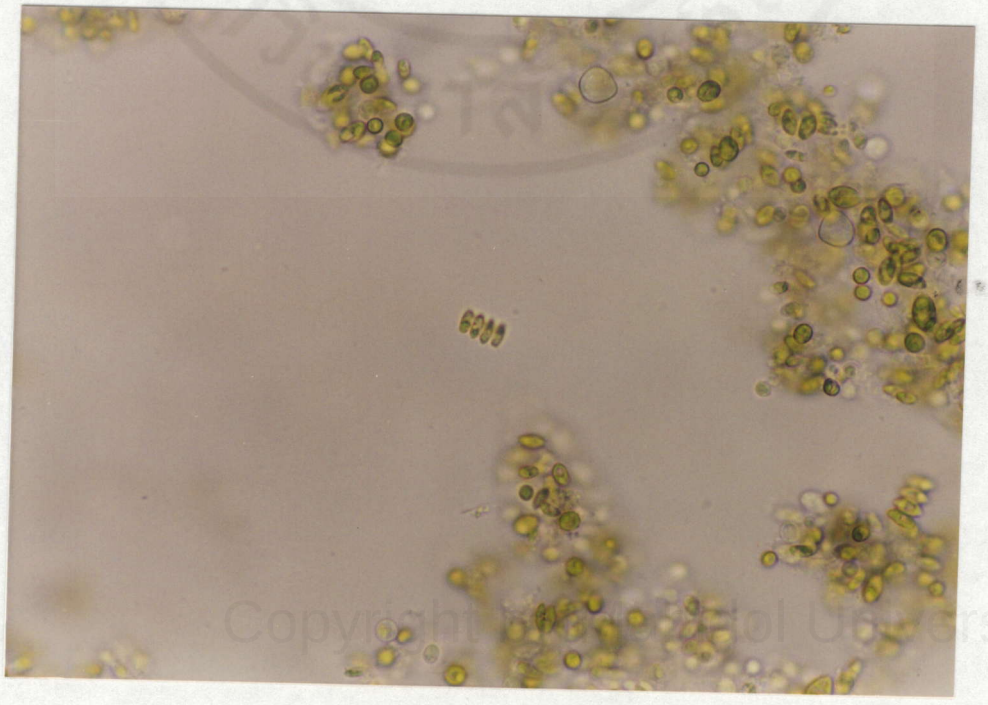

Figure IV-4 : *Scenedesmus sp.* (x40)

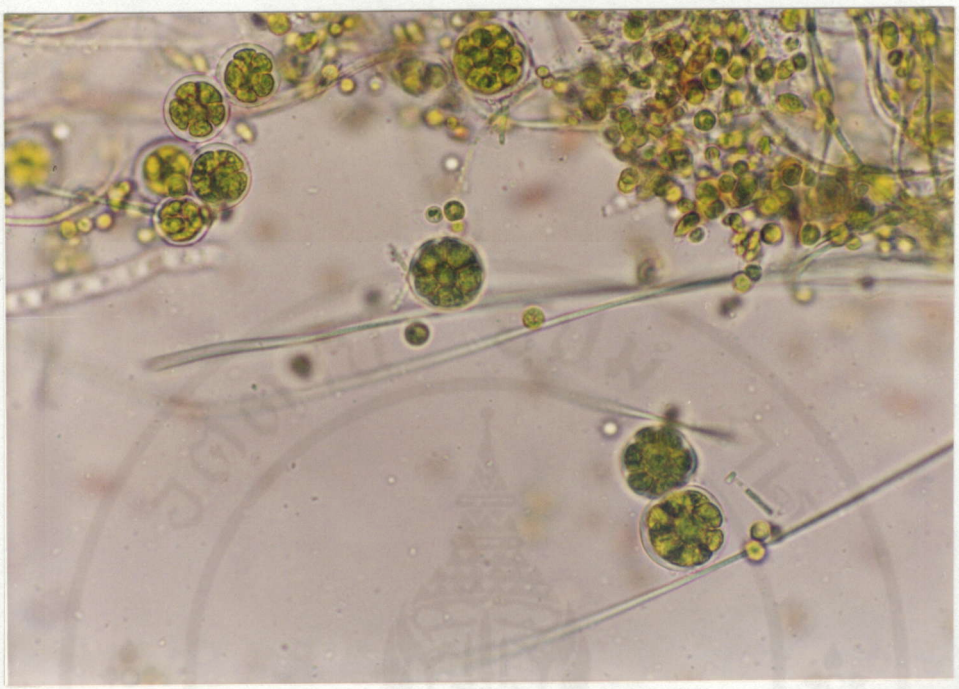

Figure IV-5 : *Chlorococcum sp.* (x40)

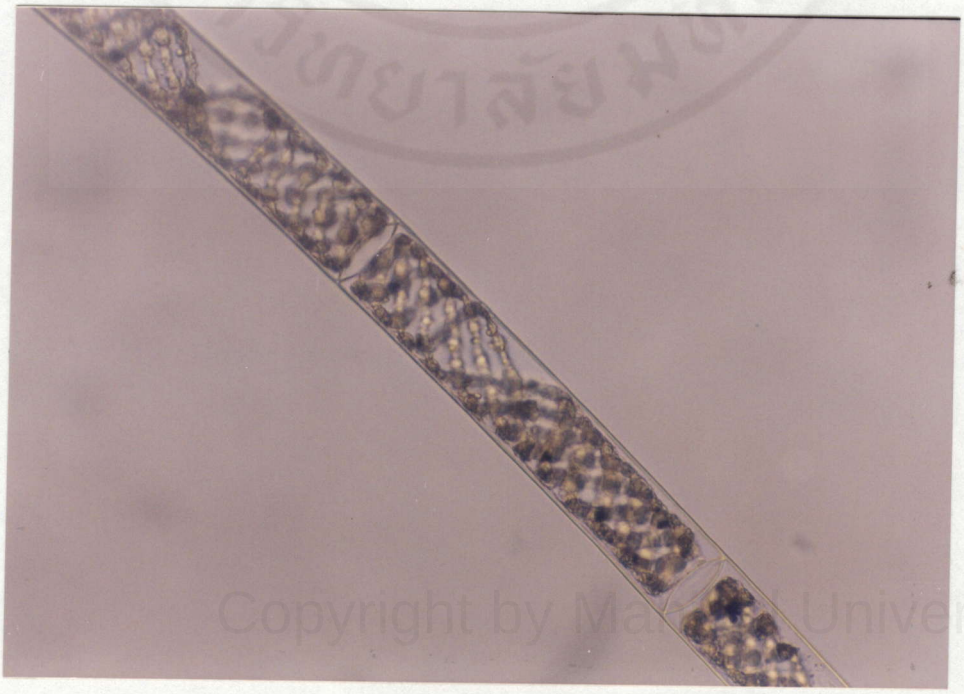

Figure IV-6 : *Spirogyra sp.* (x40)

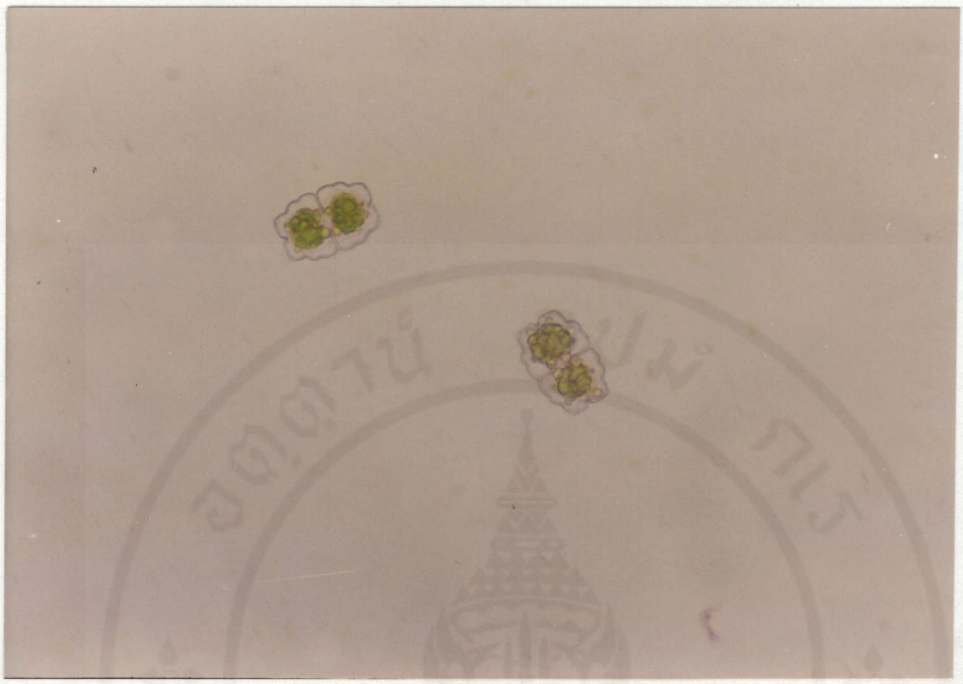

Figure IV-7 : *Euastrum sp.* (x40)

Figure IV-8 : *Netrium sp.* (x40)

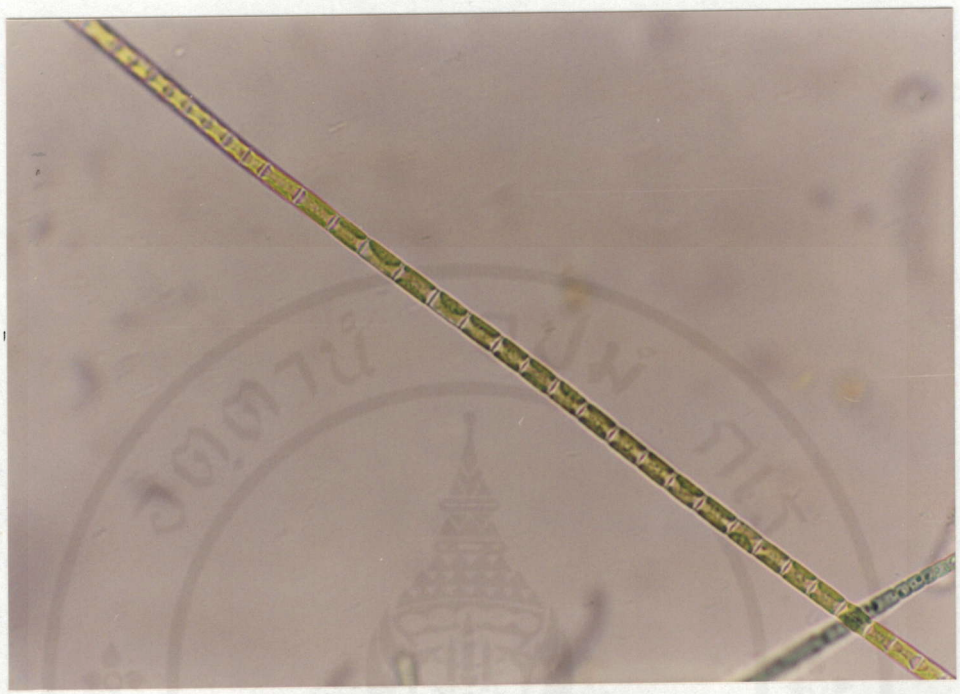

Figure IV-9 : *Ulothrix sp.* (x40)

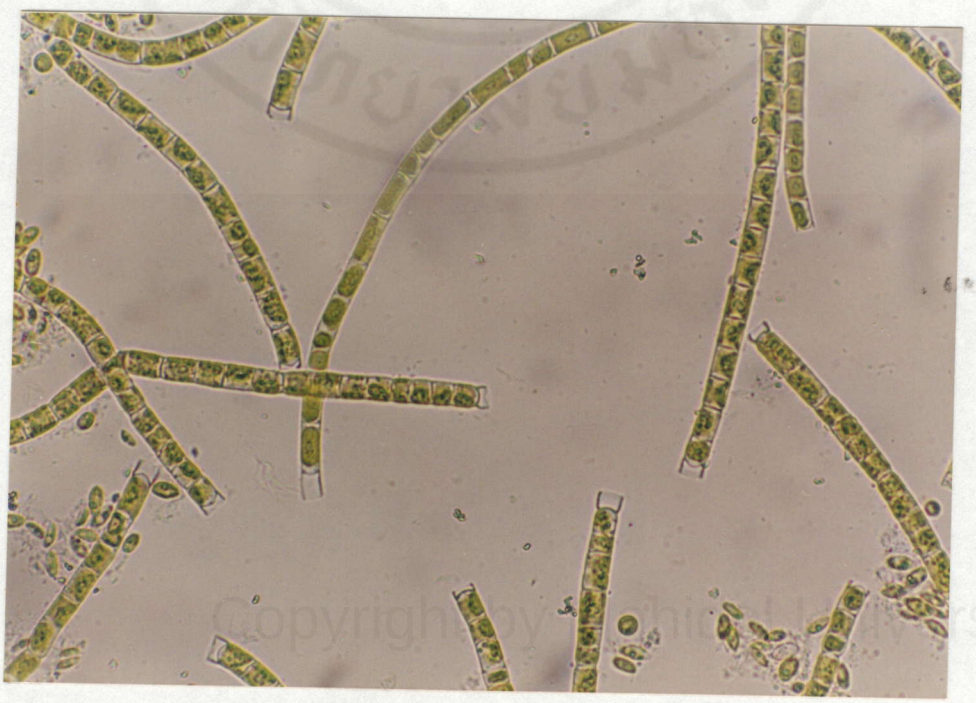

Figure IV-10 : *Microspora sp.* (x40)

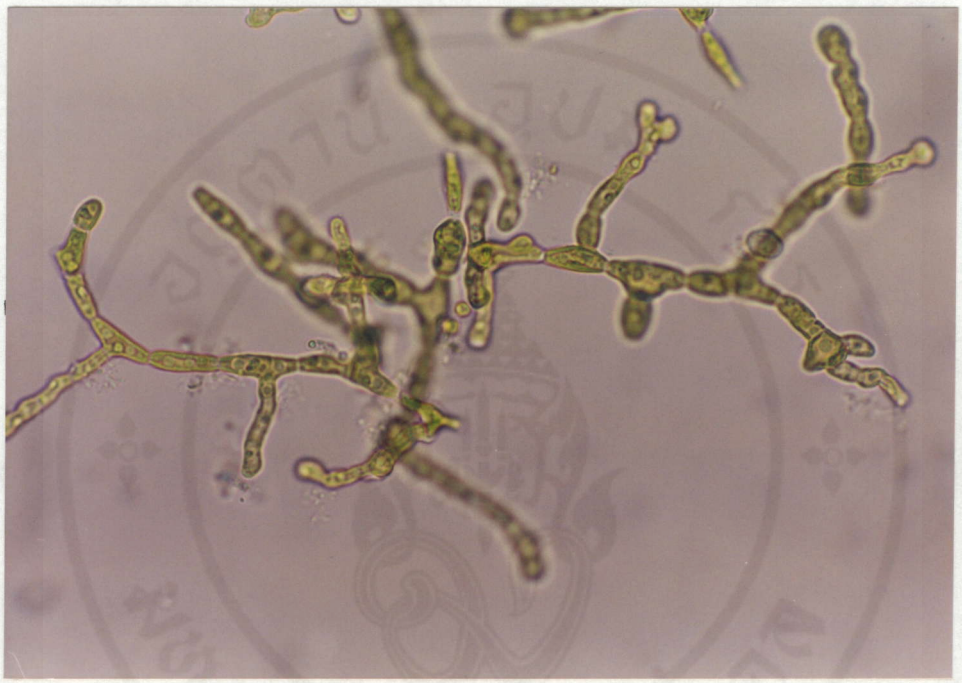

Figure IV-11 : *Pseudendoclonium sp.* (x40)

Figure IV-12 : *Navicula sp.*(x40)

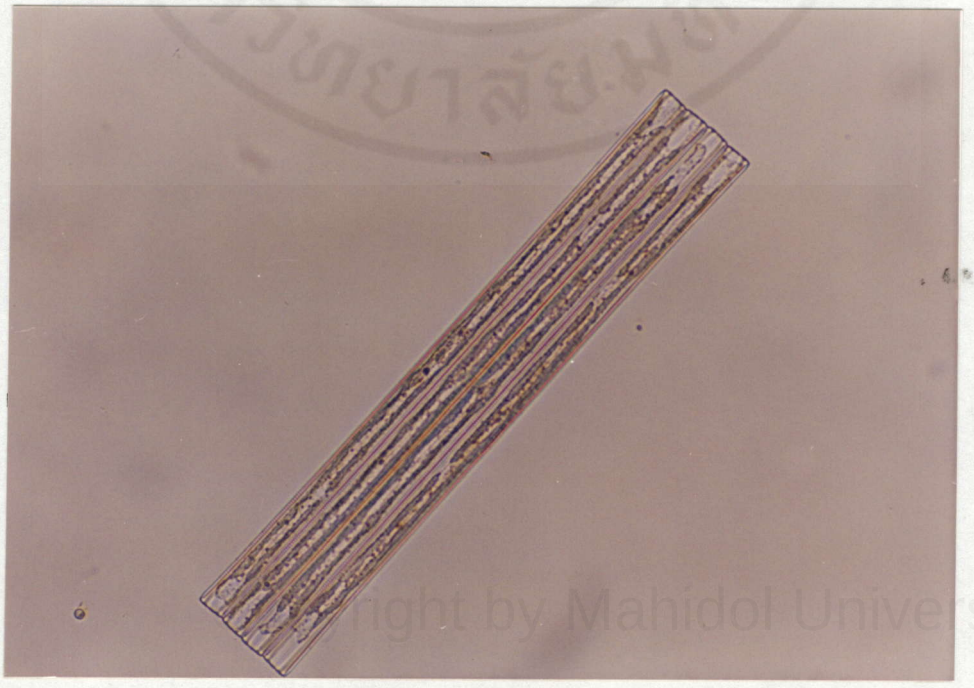

Figure IV-13 : *Fragilaria sp.* (x20)

APPENDIX V

STATISTICAL ANALYSIS

Cell concentrations

One Way Analysis of Variance for arsenate removal by various cell concentrations of the *Chlorella sp.* (with significant level 0.05).

Source of variance	Sum of square	Degree of freedom	Mean square	F value	Significant of F value
Between group	520,730	2	260,365	181.166	0.000
Within group	8,623	6	1,437.167		
Total	529,353	8			

Duncan Multiple Range Tests

The different between two means is significant if

Mean (J) - Mean (I) >= Range MSE/n with the following values for Range

Step 2 3

Range 3.461 3.587

I	J	Significant
Abs.0.2	Abs.0.4	Yes
	Abs.0.8	Yes
Abs.0.4	Abs.0.2	Yes
	Abs.0.8	Yes
Abs.0.8	Abs.0.2	Yes
	Abs.0.4	Yes

Growth phase

One Way Analysis of Variance for arsenate removal by the *Chlorella sp.* at different growth phases (with significant level 0.05).

Source of variation	Sum of square	Degree of freedom	Mean square	F value	Significant of F value
Between group	669,502.6	4	167,375.6	66.787	0.000
Within group	25,061	10	2,506		
Total	694,563.6	14			

Duncan Multiple Range Tests

The different between two means is significant if

Mean (J) - Mean (I) >= Range MSE/n with the following values for Range

Step	2	3	4	5
Range	3.151	3.293	3.362	3.430

I	J	Significant
2 days culture	4 days culture	Yes
	6 days culture	Yes
	8 days culture	Yes
	13 days culture	Yes
4 days culture	2 days culture	Yes
	6 days culture	Yes
	8 days culture	Yes
	13 days culture	Yes

I	J	Significant
6 days culture	2 days culture	Yes
	4 days culture	Yes
	8 days culture	No
	13 days culture	Yes
8 days culture	2 days culture	Yes
	4 days culture	Yes
	6 days culture	No
	13 days culture	Yes
13 days culture	2 days culture	Yes
	4 days culture	Yes
	6 days culture	Yes
	13 days culture	Yes

pH

One Way Analysis of Variance for arsenate removal by the *Chlorella sp.* at different pH (with significant level 0.05).

Source of variation	Sum of square	Degree of freedom	Mean square	F value	Significant of F value
Between group	363,016.3	3	121,005.4	144.719	0.000
Within group	6,689.1	8	836		
Total	369,705.4	11			

Duncan Multiple Range Tests

The different between two means is significant if

Mean (J) - Mean (I) >= Range MSE/n with the following values for Range

Step	2	3	4
Range	3.261	3.399	3 .460

I	J	Significant
pH 2	pH 5	Yes
	pH 7	Yes
	pH 9	No
pH 5	pH 2	Yes
	pH 7	No
	pH9	Yes
pH 7	pH 2	Yes
	pH 5	No
	pH 9	Yes
pH 9	pH 2	No
	pH 5	Yes
	pH 7	Yes

Phosphate concentrations

One Way Analysis of Variance for arsenate removal by the *Chlorella sp.* at the different phosphate concentrations (with significant level 0.05).

Source of variation	Sum of square	Degree of freedom	Mean square	F value	Significant of F value
Between group	110,190.3	3	367,30	947.605	0.000
Within group	310.1	8	38.8		
Total	110,200.4	11			

Duncan Multiple Range Tests

The different between two means is significant if

Mean (J) - Mean (I) >= Range MSE/n with the following values for Range

Step	2	3	4
Range	3.261	3.399	3.460

I	J	Significant
Phosphate 0 mg l^{-1}	Phosphate 1 mg l^{-1}	Yes
	Phosphate 10 mg l^{-1}	Yes
	Phosphate 100 mg l^{-1}	Yes
Phosphate 1 mg l^{-1}	Phosphate 0 mg l^{-1}	Yes
	Phosphate 10 mg l^{-1}	Yes
	Phosphate 100 mg l^{-1}	Yes
Phosphate 10 mg l^{-1}	Phosphate 0 mg l^{-1}	Yes
	Phosphate 1 mg l^{-1}	Yes
	Phosphate 100 mg l^{-1}	No
Phosphate 100 mg l^{-1}	Phosphate 0 mg l^{-1}	Yes
	Phosphate 1 mg l^{-1}	Yes
	Phosphate 10 mg l^{-1}	No

Immobilized algal cell (1)

Two Way Analysis of Variance for arsenate removal capacity of various numbers of alginate beads entrapped the *Chlorella sp.* (with significant level 0.05).

Source of variation	Sum of square	Degree of freedom	Mean square	F value	Significant of F value
Main effect					
Day (treatment)	64.431	2	32.216	14.278	0.007
Bead (block)	49.224	3	16.408	7.272	0.020
Residual	13.538	6	2.256		
Total	127.193	11			

Immobilized algal cell (2)

Two Way Analysis of Variance for arsenate removal capacity of various numbers of alginate beads entrapped the *Chlorella sp.* (with significant level 0.05).

Source of variation	Sum of square	Degree of freedom	Mean square	F value	Significant of F value
Main effect					
Day (treatment)	214.397	2	107.199	154.662	0.000
Bead (block)	51.161	3	17.054	24.605	0.001
Residual	4.159	6	0.693		
Total	269.717	11			

BIOGRAPHY

NAME	Miss Piralada Bunnag
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