ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

MICROALGAE IMMOBILIZATION AND APPLICATIONS

M.Sc. THESIS

Pınar ÖZDEMİR

Department of Environmental Engineering

Environmental Engineering Programme

Thesis Advisors: Prof. Dr. Oya OKAY

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JUNE 2012

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

MİKROALG İMMOBİLİZASYONU VE UYGULAMALARI

YÜKSEK LİSANS TEZİ

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Pınar Özdemir, a **M.Sc.** student of ITU **Graduate School of Science Engineering and Technology** student ID 501081812, successfully defended the **thesis** entitled "**MICROALGAE IMMOBILIZATION AND APPLICATIONS**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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Today I finished my report and I will continue to challenge myself in the future with what I learned. This is not the end but only the start.

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MICROALGAE IMMOBILIZATION AND APPLICATIONS

SUMMARY

Microalgae are unicellular microscopic creatures living in the seas and fresh waters. Algae are very important for the environment and they exist both in their natural environment and can be produced in the culture medium under laboratory conditions. Algae production is the most efficient and economic way for controlling eutrophication, wastewater treatment and transforming the sun's energy into biomass. The micro algae eliminate excess CO_2 and in this way adjust the pH degree of the environment and they also help to control the water quality by removing excess nutrients from any environment. The reason why micro algae are preferred in bio-technologic studies is that they can increase their weight by approximately two times a day; they are easily processed in bio-technologic processes; they are low cost and they can resist to environmental factors. However since the free algae cells are relatively small, problems arise when they are used in the field of bio-technology. For that reason, cell immobilization techniques have been developed. The immobilized algae are used in the generation of those products having a high value (agar, carrageenan, and alginic acid), in the removal of nitrogen, phosphorus, metals and toxic organic substances, in the storage of micro algae and in the development of bio-sensors.

In this study, the cells of *Phaeodactylum tricornutum* (Bohlin, 1897) as microalgae species of the seas were confined in the sodium alginate solution prepared in different concentrations (2.5, 3.5 and 5%) and they were left to harden in the cation solution of 2% and 4%. Then the development of algae that had been immobilized in gels prepared by using different combinations of alginate and cation solutions and the diameters of the beads was monitored and compared with each other in short and long term. At the same time, the development of free algae was monitored and a comparison was made between the immobilized algae and free cultures. Then the beads that were determined in optimum conditions of both algae growth and gel stability.

In short term experiments, the gels prepared by using sodium alginate concentration of 2.5, 3.5 and 5% and cation solutions of 2 and 4% were compared in terms of the development of algae cells and stability of the beads for a period of 15 days. As a result it was found out that the ratio of alginate concentration of 3.5% was better for the development of algae cells confined in gel and that the gel of sodium alginate of 2 and 4% were used. Consequentially, the immobilized algae prepared by using the proper alginate solution concentration (3.5%) and cation solution concentration (2% and 4%) were used in experiments with long term experiments.

In long term experiments, the beads prepared by using a sodium alginate concentration of 3.5% and cation solution of 2% and 4% were used. Inoculation was made during 7 days for a period of 255 days and with intervals of 15 days and after that, the development of algae cells confined in gel and stability of the beads were

examined. In addition to these examinations, cell development and beads stability was measured following an incubation process of 365 days. While the number of the cells from the beads of 3.5% prepared in a cation solution of 4% reached a stabile value and remained nearly same at the end of 60 days, the number of the cells from the beads of 3.5% prepared in a cation solution of 2% continued to increase at a slower rate and cell number reached a higher value at the end of 157 days. After a year, the microalgae in the beads were almost lost their viability. The diameter of algae cells prepared by using two different cation solutions (2% and 4%) was measured as approximately 4mm and no apparent difference was found in the diameter of the cells. The results of this study were used in toxicity tests and nutrient removal studies by using an alginate solution of 3.5% and a cation solution 2%.

The sensitivity of PAH species selected as toxic substance (Phenantrene and Acenaphtene) and of the algae confined in the bead were measured and their reaction to the toxic substance was compared by carrying out synchronous studies on the bacteria (*Vibrio fischeri*, Biotox ^{TM} Kit) and free algae cultures. As a result, no apparent difference was observed on the free algae when the initial concentration of Phen (100 μg/L) and the control group was compared. The effect of other Phen concentrations (400 and 1000 μg/L) was also observed during the latent and logarithmic periods. It was found out that higher concentrations of Ace (500, 1000 and 1500 μg/L) better inhibited the cell development. Then dose-response curve was drawn for Ace and Phen by using these results.

Nutrient (NO_3-N, PO_4-P) uptake studies were carried out in non-continuous systems and the beads were used in four successive applications. In the first application, the net nitrate uptake ratio was determined to be higher when compared to phosphate. The nitrate uptake ratio was determined to be 31% in the first application and it continued to increase in the second, third and fourth applications (51%, 69% and 75% respectively). However, while the net phosphate uptake quantity continued to increase in the first three applications, it showed a reduction in the fourth application (3%, 41%, 74% and 48% respectively). All applications were terminated after four days because sodium alginate gel started to break up after the fourth day and the cells were released to the medium that was isolated from the gel. As a result of these studies, it was found out that alginate beads used in non-continuous systems were better for nitrate uptake when compared to phosphate uptake. Besides that algae cells were not released the beads during treatment. For that reason, they are used again in other treatments since they can easily separate from the treated medium.

In summary, beads prepared using 3.5% Na-alginate concentration and 2% cation solution ($CaCl_2$) were found to be the most stable and suitable to *P. tricornutum* cells. The study shows that algal beads are efficient for toxicity experiments and nutrient (N and P) uptake.

MİKROALG İMMOBİLİZASYONU VE UYGULAMALARI

ÖZET

Mikroalgler deniz ve tatlı su ortamlarında yaşayan tek hücreli, mikroskobik canlılardır. Gerek doğal ortamında bulunan gerekse laboratuvar koşullarında kültürü yapılabilen alglerin önemi büyüktür. Alg üretimi günümüzde ötrofikasyon kontrolü, atıksu arıtımı ve güneş enerjisinin biyokütleye dönüştürülmesinde en etkili ve ekonomik yoldur. Mikroalgler fazla CO_2'i uzaklaştırarak ortamın pH'sını ayarlar ve ortamdaki fazla nutrientin uzaklaştırılmasıyla su kalitesinin kontrolüne yardımcı olmaktadırlar. Biyoteknolojik çalışmalarda mikroalglerin tercih edilme nedenleri; biyoteknolojik işlemlerden geçirilme kolaylıkları, maliyetlerinin düşük olması ve çevresel faktörlere direnç göstermeleri olarak özetlenebilir. Ancak serbest alg hücrelerinin küçük boyutları biyoteknoloji alanındaki kullanımlarda problemler ortaya çıkartmaktadır. Bu nedenle hücre immobilizasyon teknikleri geliştirilmiştir. İmmobilize edilmiş algler yüksek değere sahip ürünlerin üretiminde (agar,karragen, alginik asit vb.), azot ve fosfor ile metal ve toksik organik maddelerin gideriminde, mikroalglerin depolanmasında ve biyosensör geliştirmede kullanılmaktadır .

Bu çalışmada bir deniz mikroalg türü olan *Phaeodactylum tricornutum* (Bohlin, 1897) hücreleri farklı konsantrasyonlarda hazırlanan sodyum alginat (%2.5, 3.5 ve 5) jel içine hapsedilmiş ve %2 ve 4'lük katyon solusyonunda ($CaCl_2$) jelin sertleşmesi sağlanmıştır. Farklı kombinasyonlarda alginat ve katyon çözeltileri kullanılarak hazırlanan ve jel içine hapsedilen alglerin gelişimi ile kürelerin çapları kısa ve uzun vadeli olarak takip edilmiş ve birbirleri ile karşılaştırılmıştır. Eş zamanlı olarak serbest alglerin gelişimi de incelenerek immobilize ve serbest kültürlerin kıyaslaması yapılmıştır. Optimum koşulları tespit edilen küreler daha sonra toksisite deneyleri ve nutrientin hücre içine alımı çalışmalarında kullanılmıştır.

Kısa vadeli deneylerde; %2.5, 3.5 ve 5 sodyum alginat konsantrasyonu ile %2 ve 4 katyon solusyonu kullanılarak hazırlanan jeller, 15 gün süre ile küre stabilitesi ve jel içine hapsedilen alg hücrelerinin gelişimi bakımından kıyaslanmıştır. Buna göre %2.5 alginat konsantrasyonuna sahip küreler, alg hücrelerinin gelişimi bakımından daha uygun bulunurken, %5'lik hazırlanan sodyum alginat jelin daha stabil olması sebebiyle alg gelişimine izin vermediği görülmüştür. Bir sonraki aşamada, %3.5 sodyum alginat konsantrasyonu kullanılarak hazırlanan küreler, %2 ve 4 katyon solusyonları içerisinde sertleştirilerek alglerin gelişimi ve jelin stabilitesi bakımından incelenmiştir. %2 ve 4 katyon solusyonu kullanılarak hazırlanan jellerde hücre gelişimi bakımından belirgin bir farklılık olmazken, buna karşılık %4 katyon solusyonunda hazırlanan kürelerin daha stabil olduğu gözlenmiştir. 15 günlük deney sonucunda %4'lük katyon solusyonunda hazırlanan küreler %24 küçülme gösterirken, %2'lik katyon solusyonunda hazırlanan kürelerde bu oran %35 olarak bulunmuştur. Elde edilen sonuçlar doğrultusunda, en uygun alginat konsantrasyonu (%3.5) ve katyon solusyonu oranı (%2 ve 4) seçilerek hazırlanan immobilize alg küreleri uzun süreli deneylerde kullanılmıştır.

Uzun vadeli deneylerde; %2 ve 4 katyon solusyonunda sertleştirilen %3.5 sodyum alginat konsantrasyonuna sahip küreler kullanılmıştır. 255 gün süre ve 15 gün ara ile 7 günlük aşılama yapılarak, jel içine hapsedilen alg hücrelerinin gelişimi ve kürelerin stabilitesi incelenmiştir. Ayrıca 365 günlük inkübasyon süreci sonunda hücrelerin gelişimi ve küre stabilitesi ölçülmüştür. Deneyin ilk 30 günlük kısmında, %2 katyon solusyonu kullanılarak hazırlanan alg küreleri yaklaşık olarak 100000-125000 arasında hücre sayısına ulaşırken, %4'lük katyon solusyonunda hazırlanan kürelerde bu rakam 18000-35000 arasında kalmıştır. Alglerin büyüme hızı, depolanma süresi arttıkça (her 7 günlük inokulasyon sonunda) gitgide azalan bir eğilim göstermiştir. %4 katyon solusyonunda hazırlanan %3.5 alginat konsantrasyonuna sahip kürelerin hücre sayısı 60 günün sonunda sabit bir değere ulaşıp hemen hemen aynı kalırken, %2 katyon solusyonunda hazırlanan kürelerde hücre sayısı azalarak devam etmiş ve 157 gün sonra daha yüksek değere ulaşmıştır. 365 gün sonunda kürelerin canlılığını kaybettiği görülmüştür. İki farklı katyon solusyonu (%2 ve 4) kullanılarak hazırlanan alg kürelerinin çapı yaklaşık 4mm ölçülmüş ve kürelerin çapı arasında belirgin bir farklılık bulunmamıştır. Bu çalışma sonunda %3.5 alginat konsantrasyonu ve %2 katyon solusyonu seçilerek hazırlanan küreler toksisite deneyinde ve nutrientin hücre içine alımı çalışmalarında kullanılmıştır.

Toksisite deneylerinde önemli çevresel kirleticilerden olan iki farklı PAH (Phenantrene ve Acenaphtene) kullanılarak immobilize algler ve serbest alg kültürleri ile bakteri (Vibrio fischeri, Biotox ^{TM} Kit) üzerindeki etkisi araştırılmıştır. 3 farklı konsantrasyonda Phenantrene (100, 400 ve 1000 µg/L) ve Acenaphtene (500, 1000 ve 1500 µg/L) kullanılmıştır. Buna göre, *Vibrio fischeri* ile yapılan deneyde EC_{20} değeri hesaplanırken, EC_{50} değeri belirlenememiştir. Alg toksisite deneylerinde farklı sistemler kullanılmıştır. Serbest alg kültürleri 10 gün süre ile Phenantrene (100, 400 ve 1000 µg/L) ve Acenaphtene (500, 1000 ve 1500 µg/L) kirleticilerine maruz bırakılarak davranışları gözlenmiştir. Serbest algler üzerinde Phen'in ilk konsantrasyonu (100 µg/L) ile kontrol grubu arasında belirgin bir fark görülmemiştir. Diğer Phen konsantrasyonlarının (400 ve 1000 µg/L) etkisi latent ve logaritmik dönemde gözlenmiştir. Ace konsantrasyonu arttıkça hücrelerin gelişimini engellediği ortaya çıkmıştır. Bir sonraki aşamada, serbest ve immobilize alg hücreleri 4 gün süre ile Phen ve Ace konsantrasyonlarına maruz bırakılarak gelişimleri incelenmiştir. Yapılan deneyler sonucunda, immobilize edilmiş alglerin duyarlı olmadığı görülmüştür. İmmobilizasyon tekniği, jel içine hapsedilen algler üzerinde koruyucu etki sağlamıştır. Fakat bakteri ile kıyaslandığında daha yüksek duyarlılığa sahip olduğu gözlenmiştir. Elde edilen sonuçlar kullanılarak Phen ve Ace için doz-cevap eğrisi çizilmiştir.

Nutrient (NO_3-N, PO_4-P)'in küre içine hapsedilen algler tarafından hücre içine alımı çalışmaları kesikli sistemlerde denenmiş ve küreler birbirini izleyen dört uygulamada kullanılmıştır. Nitrat alım miktarı ilk uygulamada %31, daha sonra sırası ile ikinci, üçüncü ve dördüncü uygulamalarda artarak devam etmiştir (%51, 69 ve 75). Buna karşılık, net fosfat alım miktarı ilk üç uygulamada artarak devam ederken dördüncü uygulamada azalma göstermiştir (%3, 41, 74 ve 48). Sodyum alginat jel içine hapsedilen alg hücrelerinin, jel içinden medyuma taşması sebebiyle her uygulama 4 günde sonlandırılmıştır. Alg küreleri medyumdan kolayca ayrılabilmesi sebebiyle başka arıtımlarda da yeniden kullanılmıştır.

Özetle, *P. tricornutum* hücreleri kullanılarak hazırlanan küreler için hücre gelişimi ve jel stabilitesi bakımından en uygun koşullar bulunmuştur (%3.5 Na-alginat

konsantrasyonu ve %2 $CaCl_2$ solusyonu). Kürelerin toksisite deneyleri ve nutrientin (N ve P) hücre içine alımı çalışmaları için uygun olduğu görülmüştür.

1. INTRODUCTION

1.1 Aim of the Study

One of the objectives of this study was to investigate the stability of alginate beads and growth of immobilized algal cells entrapped in alginate. For that aim, several different concentrations of alginate and hardening agent were used for preparation of the beads to determine the optimum conditions for stability and growth.

The second objective was to determine the stability of algal beads and viability of cells during the long-term storage.

Finally, immobilized algal cells were used to asses the suitability of the algal beads in toxicity testing and nutrient removal studies. During the toxicity tests polycyclic aromatic hydrocarbons (PAHs) of Ace and Phen were used. They are listed as priority pollutants by EPA and found widespread in the environmental matrices.

In this study, pennate diatom *P. tricornutum* is used; The advantages of using pennate diatom P. tricornutum in laboratory and in situ studies have been indicated by several authors (Moreno-Garrido et al., 2005; Morelli and Scarano, 2001; Wiegman et al., 2002; Moreira dos Santos et al., 2002). Those species are easy to maintain under laboratory conditions, tolerant to salinity differences and widely used in ecotoxicological studies (Moreira et al., 2006; Moreira et al., 2002; Moreno-Garrido et al., 2000). Although various results have been presented about the stability of alginate gel beads in the literature (Smidsrod et al., 1990; Dainty et al., 1986; Martinsen et al., 1989), there was a few study reporting the bead stability in seawater (Moreira et al., 2006) and limited reports were published related to the long-term viability of the microalgae immobilized in alginate beads.

1.2 The Concept: Immobilization

Immobilization is now a well-established technique with many industrial applications. At the end of the sixties, novel techniques for immobilizing enzymes, proteins and cells began to expand in the literature (Papageorgiou, 1987). The main

goal of enzyme immobilization is the industrial re-use of enzymes for many reaction cycles. Immobilization of enzymes and other proteins may also be useful at the laboratory scale for the evaluation of critical properties of enzymes and proteins.

1.3 Immobilized Cells and the History

An immobilized cell is defined as a cell that by natural or artificial means is prevented from moving independently of its neighbours to all parts of the aqueous phase of the system under study (Tampion& Tampion, 1987). Immobilization of microalgae, as part of a global trend of immobilizing microorganisms, is used for a wide variety of biotechnological applications that started over 40 years ago (Bashan and Bashan, 2010). The general principle of immobilization of microalgae is based on keeping the living cells within a gel matrix metabolically active as long as possible, during which time they have very limited mobility (Bashan and Bashan, 2010). Immobilized microalgae in matrices may assist the required biotechnological benefits from the mass culture of the microalgae. Furthermore, immobilization allows storage of cells for a longer period without any loss of viability than in free cells and the preparation of immobilized cells is easier, cheaper and more readily available than other methods (Romo & P´erez-Mart´ınez, 1997).

Microalgae form the basis of the aquatic food chain and play a key role in aquatic ecosystems. They are the primary producers of organic matter on the earth. It is estimated that around 40% of global photosynthesis is performed by microalgae (Falkowsky, 1980). They take in carbon dioxide and promote the immobilization of carbon dioxide in the atmosphere. Microalgae contain a good balance of sugar, protein, fat and minerals and are suitable as feed for livestock for meat. Various substances having bioactive and other useful functions are produced by microalgae. There are many microalgae-related biotechnologies that can be commonly used in various industries covering a wide range of fields, including medicine, health, environment, energy, agriculture, food, cosmetic, aquaculture and fisheries (Sumi, 2009 and Borowitzka and Borowitzka, 1988). Since microalgae are widely distributed in marine and fresh water environments and there are about 100,000 different kinds, it is believed there are many compounds with yet-to-be-discovered biological activities (Sumi, 2009).

1.4 Application of Immobilized Algae

1.4.1 Production of high value products

Microalgae are very efficient solar energy converters producing massive blooms in environments. Those organisms can be used to produce various high-value bioactives (Borowitzka, 1986; Bubrick, 1991; Pulz et al., 2001; Li et al., 2001; Banerjee et al., 2002). Several microalgae synthesize a great variety of useful metabolites of great commercial interest. (Lebeau and Robert, 2006; Moreno-Garrido, 2008). Moreover, the production of agar, carrageenan, and alginic acid from macroalgae are well-established industries (Guisan, J.M., 2006). Production of microalgae-derived products/metabolites requires the processes of culturing the alga (Ben-Amotz and Avron, 1987; Molina Grima, 1999; Molina Grima et al., 1999; Sa´nchez Miro´n et al., 1999;Tredici, 1999; Borowitzka, 1999; Pulz, 2001; Pulz et al., 2001), recovery of the biomass, and purify the products/metabolites from the biomass. Previous studies showed that the immobilization of biomass provide advantages such as reuseability and separation of biomass from the bulk liquid. Reusability of the immobilized biomass after regeneration makes the process also cost effective (Bayramoğlu et al., 2003; Arica et al., 2005; Prakasham et al., 1999).

Some specific examples related to the production of high-value products are given in the following;

- Diatom *Haslea ostrearia* most commonly found in the Atlantic shore (France) synthesize a blue-green hydrosoluble pigment “marennine” which is known to be responsible for the greening of oysters (Gaudin et al., 2006; Robert, 1983). The pigment has also commercial applications in food and cosmetic industries (Gaudin et al., 2006; Yaron & Arad, 1993). It is also expected that the pigment will be used in medicines against carcinoma lines (Gaudin et al., 2006; Carbonnelle et al., 1999). Morphological and physiological characteristics of the algal cultures must be preserved for these various applications to be realized. For that aim, this marine diatom were successfully immobilized and stored up to one month. (Lebeau et al., 1998).
- Because of limited oil reserves and rising prices as well as concern about fuel emissions, many countries are interested in developing their internal biofuel markets and established plans for use of these biofuels. Ethanol, as a clean

and renewable combustible, is considered as a good alternative to replace oil (Mussatto et al.,2010; Bai et al., 2008; Almeida and Silva, 2006). Although the energy equivalent of ethanol is 68% lower than that of petroleum fuel, the combustion of ethanol is cleaner (because it contains oxygen). Burning ethanol instead of gasoline reduces carbon emissions by more than 80% while eliminating entirely the release of acid-rain-causing sulfur dioxide (Mussatto et al.,2010; Lashinky and Schwartz, 2006). Consequently, the emission of toxic substances is lower (Mussatto et al.,2010; Krylova et al., 2008). Ethanol production by using the immobilized microalgae seems a promissing strategy.

- Hydrogen production from microalgae (*C. vulgaris*) is possible and can be improved by immobilization of the algae (Rashid et al.,2011; Hahn et al., 2007). Hydrogen (H_2) can be considered as a possible replacement for fossil fuels (Rashid et al., 2009). One way that hydrogen can be produced biologically is by microalgae exposed to anaerobic conditions (Rashid et al.,2011; Vijayaraghavan et al., 2004).

1.4.2 Removal of N and P

Large-scale production of wastewater is a consequence of all societies. Most wastewaters are usually hazardous to humans and the environment and therefore, they must be treated prior to disposal into water bodies. Removing most nutrients is usually obligatory, even though it is not performed in many cases, especially in developing countries. Secondary effluents from domestic and agro-industrial wastewaters contain large amounts of nutrients (NH_4, NO_3 and PO_4) which have been identified as the main causes leading to eutrophication in rivers, lakes, and seas (Lau et al., 1997; Trepanier et al., 2002). Disposal of partially treated wastewaters also an important threat for freshwater resources on a global scale (Montaigne and Essick, 2002). Especially, the presence of inorganic nitrogen compounds diminishes the quality of drinking water (Fraser and Chilvers, 1981). Presently, there are several methods to remove the nutrients from the effluents (Duenas et al., 2003; Stratful et al., 1999; Van Loosdrecht et al., 1997), but these are costly and produce high sludge content. Microalgae have been proposed as an alternative biological treatment to remove nutrients (Mallick, 2002).

Microalgae are commonly used for tertiary wastewater biological treatment (Noue and De Pauw, 1988; Valderrama et al., 2002). Although nitrogen is commonly removed, only a small fraction of phosphorus can be removed (Hernandez et al., 2006; Bashan and Bashan, 2004; Galarneau and Gehr, 1997). It was also found that the efficiency of removing phosphorus by the co-immobilized microorganisms is increased (Hernandez et al., 2006; Bashan et al., 2004).

One of the limitations for the development of wastewater treatment systems based on microalgae is the harvest of the biomass at the end of the treatment process. This involves the high cost and time-consuming filtration and centrifugation, which are not applicable techniques for a wastewater industry dealing with effluents. However, the idea of entrapping microalgae in spherical gels for easy removal by sedimentation after wastewater treatment gained significant momentum in the last decade. The immobilization of cells also provide some major advantages such as (1) high biomass production that can be used as a byproduct; (2) prevents filtration of the treated wastewater, which can be used as is; (3) high resistance to toxic compounds within the treated wastewater; (4) immobilizing more than one microorganism ; (5) an increase in the cell retention time within bioreactors and higher metabolic activity (6) is simple to apply by nonprofessionals (Tam et al., 1994).

During the treatment, firstly the contaminants are absorbed by the immobilized microalgae in a matrix, and then the cleaner waters diffuse out of the polymer matrix and are collected and reused. The process can be repeated for several cycles. The polymer used for this purpose must be efficient for removing the pollutants and cheap. Regardless of the polymers used, the material must be hydrophilic, allowing wastewater to diffuse into the bead. The most commonly used polymers are the natural polymers alginate and carrageenan (Bashan, 1998; Moreno-Garrido, 2008; Murano, 1998), *Chlorella* immobilized in alginate (Lau et al., 1997); *Scenedesmus obliquus* immobilized in k-carrageenan (Chevalier and De la Noüe, 1985a) and *Scenedesmus intermedius* immobilized in calcium alginate (Jimenez-Perez et al., 2004) were some species used for the secondary effluent treatment systems in the literature.

The cultivation of algae in wastewater offers the combined advantages of treating the wastewaters and simultaneously producing algal biomass, which can further be used

for other puroposes such as energy production such as biogas and fuels, or in agriculture (fertilizers and soil conditioners). Compared to the entirely heterotrophic systems like activated sludge plants, the primary attraction of algal ponds lies in the low-grade technology and in saving of energy, since photosynthetic oxygen production can replace mechanical aeration (Romo and Martinez, 1997).

1.4.3 Metal removal

One of the main interests for microalgae in biotechnology is focused on their use for metal removal from effluents and wastewaters. The sources of heavy metals in the environment may be natural and anthropogenic. Those are battery manufacturing, painting, printing, mining activities, alloy industries, fossil fuels etc. Although most of metals are needed in small amounts by the organisms, the higher amounts of especially arsenic, cadmium, copper, lead, mercury and chromium are hazardous to the environment. Their presence in the aquatic ecosystem poses human health risks. The chemical forms of these metals in water are accessible to the biota through significant accumulation in the food chain and causes harmful effects to living organism in water and also to the consumers of them (Vilar et al., 2005). Although the removal of metals from wastewaters is possible by various methods such as precipitation, membrane filtration, chemical oxidation or reduction, ion exchange and adsorption, these techniques are often ineffective or expensive, especially when concentrations are high. The removal of heavy metal ions by immobilized organisms such as fungi (Bayramoğlu et al., 2003; Arica et al., 2004; Al- Qunaibit et al., 2005; Baik et al., 2002), bacteria (Trevors et al., 1986; Saygideger et al., 2005; Tunali et al., 2006b) and yeast (Lamelas et al., 2005), have been studied extensively.

1.4.4 Removal of toxic organic substances

Conventional and advanced wastewater treatment methods are inefficient in removing some toxic, organic contaminants (Nakada et al., 2007; Bertanza et al., 2010). New, cost-effective methods are needed to effectively remove such contaminants from the contaminated environment. Microalgae has been known to remove toxic, organic contaminants by cell uptake and transform (Semple et al., 1999). For example (Luan et al. 2006) reported that tributyltin (TBT) used as antifouling agent can be removed and biodegraded by alginate-immobilized *Chlorella vulgaris*. Similarly, an alginate-immobilized cyanobacteria, *Phormidium*

was found to be effective in removing dye from dye-rich wastewater (Ertuğrul et al., 2008). Ueno et al. (2008) revealed that n-alkanes were almost completely degraded by *Prototheca zopfii* immobilized in polyurethane foam. Furthermore, a recent study by Gao et al (2011) demonstrates that alginate-immobilized *C. vulgaris* beads were more effective than freely suspended cells in removing and biodegrading nonylphenol (NP) from wastewater under photo-autotrophic conditions. Nonylphenol (NP) poses a serious threat to humans and other organisms because it has estrogenic properties, is persistent in the environment and is highly toxic (Soares et al., 2008).

1.4.5 Microalgal storage (Enhancing storage capacity)

Microalgal storage has been studied for various possible applications such as larvae feeding in hatcheries, synthesis of active biomolecules, water quality control (Cheng, 2003) and for ecotoxicological applications (Frense et al., 1998). Microalgae provide a well balanced mixture of nutrients to higher organisms in the food web because they contain the most important macronutrients (Becker, 2004; Volkman&Brown, 2006). But management of a microalgal collection is labor intensive, expensive, and time consuming (Moreno-Garrido, 2008). Immobilization was found to be a practical way to overcome those problems. During the storage of immobilized algal cells, they stay alive and preserve their nutrient, protein, carbohydrate, and lipid contents. The characteristics particularly size and shape of the strains were also preserved especially during the long-term storage. To preserve the size and shape of the marine diatom during long-term storage, the cells were immobilized in alginate beads and stored at 4 º C under reduced light for up to 4 months (Bashan and Bashan, 2010).

Hertzberg & Jensen (1989) immobilized the marine diatom *Phaeodactylum tricornutum* in alginate beads and those algal alginate-beads kept in the dark at 4 ºC for more than one year and they were capable of growth when transferred to fresh medium. Another study by Chen (2001), showed that immobilized cells of the freshwater alga *Scenedesmus quadricauda* could be stored for more than three years and retained their normal physiological activities. One of the problems during the production of mollusks and crustaceans in the larval stages in laboratory is the high cost of producing live food (microalgae) (Valenzuela-Espinoza et al., 1999). The cost can be reduced by using immobilized algae. The preparation of alginate-beads is easy, cheaper and more convenient than the microalgae produced by traditional methods (Romo and Pérez-Martínez, 1997). For example *Tetraselmis suecica* was

stored and used as food for *Crassostrea gigas* larvae (Montaini et al., 1995; Robert et al., 2001) and the marine microalga *Isochrysis galbana* immobilized in alginate can be used for longterm storage of algal stock and easily applied to clam cultures (Bashan and Bashan, 2010). Tamponnet et al. (1985) and Hertzberg and Jensen (1989) showed that immobilized microalgae maintained ultrastructural integrity and physiological activities for several months, suggesting its application in algal storage.

1.4.6 Biosensor development-Toxicity assessment

The aquatic ecosystem health is a key element in environmental issues. Indeed water is an essential resource needed in most human activities. Protecting water quality requires early warning systems for on line and in situ pollution monitoring (Chouteau et al., 2004). It is well known that microalgae is very sensitive to toxicants in toxicity tests (Radix et al., 2000; Stauber and Florence, 1990), and the key role of these organisms in aquatic ecosystems has been studied extensively. Toxicity tests with microalgae have many advantages with respect to other toxicity tests: they are repeatable, sensitive, cost effective, easy and fast. However , using microalgae for in situ toxicity assessments is relatively rare compared with fish and invertebrates (Moreira-Santos et al., 2010; Burton et al., 2005; Crane et al., 2007) due to the difficulties such as confinement of algal cells at the field sites and their retrieval at the end of the assay. The immobilization of algal cells in a matrix of calcium alginate (beads and films) (Faafeng et al., 1994; Moreira et al., 2006) is an alternative to classic in situ methodologies instead of carrying samples to the laboratory, the immobilized algae is installed in actual locations for a determinate period of time, then, recovered and carried to the laboratory to determine polluted locations (Munawar and Munawar, 1987; Pereira et al., 1999; Moreira dos Santos et al., 2002). For example sediments act as a sink and potential source of xenobiotics, because water solubility of those substances is usually low. But the application of toxicity tests to sediments is a restriction in the integrated toxicity study of coastal management from an environmentalist point of view. On the other hand, toxicity tests applied to extracts of the sediments modify the natural conditions. Direct exposition of immobilized microalgae to sediment would improve in a great way the ecological relevance of the tests (Moreno-Garrido et al., 2003a,b; Adams and Stauber, 2004): Selective enzymatic sensor systems using immobilised single enzymes (Dzadevych et al., 1994; Shul'Ga et al., 1994;Wan et al., 1999; Dennisson

and Turner, 1995; Turner et al., 1987) were developed for detection of toxic compounds such as heavy metals and pesticides in the environment.in environment as well as food and medicine analysis. Detection of different groups of pollutants are possible by developing a multi-enzymatic biosensor (Arkhypova et al., 2001). However, immobilizing different enzymes on a multisensor is not so easy, because all enzymes must work under the same operational conditions simultaneously. Enzyme unstability and their high price are other problems for develepment of multienzyme sensor systems. Since algal cells contain a large number of enzymes, using them for this purpose is a good solution. Moreover using a living organism gives information concerning the ecotoxicological effects of pollutants on these organisms. An algal (*Chlorella*) biosensor has been constructed also to operate in the gas phase for determination of aerosols. Some other toxic compounds such as atrazine, paraquat or methanol which affect the algal photosynthetic activity can also be detected by this biosensor (Naessens and Minh, 1999).

1.5 Immobilization Techniques For Microalgal Cells

Generally, the immobilization techniques could be divided into active and passive. Many microorganisms have a natural tendency to attach to surfaces and grow on them (Robinson et al., 1986). The common supports for adsorption include various membranes, a filter paper, carbon materials and other carriers possess the high absorbance; sometimes the cells have been adsorbed directly onto the electrode surface One of the major advantages of the adsorption is its simplicity; (Reshetilov et al, 2010). Adsorbents for passive immobilization can be natural or synthetic. For example, a natural carrier of loofa sponges are the fibrous support of the fruit of different species obtained from Luffa (*L. cylindrica*). Those sponges are non-toxic, cheap, mechanically strong and stable in long-term cultures (Liu et al., 1998). Akhtar et al. (2004) used loofa sponge to immobilize algal cells of *Chlorella sorokiniana*, to remove nickel(II) from aqueous solutions. On the other hand, polyvinyl and polyurethane are commonly used synthetic materials to immobilize cells.

Active immobilization of cells require the use of some chemical and physical methods for cell fixing and the choose of the most efficient technique is determined by the type of supposed assay and cells' features. Most of the general immobilization techniques for microorganisms can be easily modified and applied to microalgae,

adding a design factor that these are photosynthetic microorganisms that require light.

General features of an immobilized-algal system were shown in Table 1.1.

Table 1.1 : Basic requirements of an useful immobilized algal system and properties of an ideal matrix for immobilization (Mallick, 2002).

Requirements of an useful immobilized algal system	Properties of an ideal matrix for immobilization
Retention of viability	Non-toxicity
Ability to photosynthesize	Phototransparency
High density of cells	Stability in growth medium
Continued productivity	Retention of biomass
Low leakage of cells from matrix	Resistance to disruption by cell growth

Several synthetic (acrylamide, polyurethane, polyvinyl, resins) and natural polymer derivatives of algal polysaccharides (alginate, carrageenan, agar, agarose), and chitosan, an amino polysaccharide derived from chitin, has been experimentally used. Generally synthetic polymers are more stable than natural polymers. On the other hand, natural polymers are easily degraded by microbes, less hazardous to produce (Leenen et al., 1996) and diffusivity is higher. Once the application is clear, guidelines for selecting a polymer exist by several authors (for example Leenen et al., 1996).

Even though different techniques for immobilization of cells in alginate matrixes have been proposed, the entrapment of cells in beads of sodium alginate gel hardened with calcium is one of the most widely used (Smidsrod et al., 1990).

Alginate is a polysaccharide composed of guluronic and mannuronic acid monomers in varying proportions depending on the brown seaweed and the tissue from which they are extracted.

In contrast to other matrices, such as polyurethane foams (Thepenier et al., 1985), calcium alginate is nontoxic and let different type of microorganisms to grow inside. The transparency of small calcium-alginate beads is enough to permit the growth of immobilized microalgae (Codd, 1987; Hertzberg and Jensen, 1989). Additionally it is

an easy, cheap and feasible technique to be used in research laboratories (Papagregoriou, 1987).

The physical properties of alginate gel beads, namely their mechanical strength, susceptibility to shrinkage, porosity and stability towards antigelling cations and chelating agents depend upon both the chemical characteristics of the alginate and the type and concentration of the hardening agent (Dainty et al., 1986; Martinsen et al., 1989).

1.6 General Immobilization Protocol

Most of the immobilization techniques are more or less similar regardless of the polymer matrix that is used. The microalgal suspension is mixed with macromolecule monomers of the polymer. Then, the mixture is solidified to produce a polymeric gel of various shapes. This is done by linking the monomers to each other to form a polymer with as little interaction with the living microorganisms as possible. Polymerization can be achieved by several physical and chemical treatments, depending on the characteristics of polymer. Solidification can be done by linking the monomers with di- and multi-valent cations. The rate of solidification can be increased or decreased by changing the temperature and different chemical and photochemical reactions (Cohen, 2001). This can be a very fast process, as in the case of alginate (Bashan, 1986) or slower for other polymers (Cohen, 2001).

Generally, the mechanical strength of the final polymer increases with the increase in concentration of the monomers and the cross-linking agents used with increase in strength, the pore size of the polymer decreases. Spherical beads, the most common form for application. They are made by slowly dropping the mixture of monomer and microalgae. After production, the beads can be used directly or with secondary multiplication in a growth medium to increase the number of microorganisms within the bead (Bashan, 1986; de-Bashan et al., 2004).

Immobilization of microalgae in polymer matrixes causes a significant stress on the organisms because of chemical forces and interactions between the immobilization matrix The space limitation also affects the metabolism of the microorganisms. On the other hand, immobilization has direct positive effects such as avoiding grazing by aggressive zooplankton (Faafeng et al., 1994) and reduction in competition for

nutrients with other species. Furthermore metabolism, function, and behaviour of the microalgae improved by immobilization.

1.7 Effects of Immobilization on Microalgae

1.7.1 Growth and morphology

In most of the studies, the growth rates of immobilized microalgae are found to be lower than those of the corresponding free cell cultures (Bailliez *et al.* 1985; Robinson *et al.* 1985). However, the studies performed by Chevalier and de la Noüe (1985a) and by Lau *et al.* (1998) with *Scenedesmus obliquus* and *Chlorella* vulgaris respectively showed an popposite trend.

On the other hand, microscopic investigations show that immobilization has a little effect on the morphology of the algal cells (Musgrave *et al.* 1983). For example immobilized *Botryococcus* has more regular shapes and diameters 2.5 times greater than those of free cell cultures (Bailliez *et al.* 1985). Trevan and Mak (1988) also reported that the calcium-alginate-entrapped unicellular *Cholorella* tend to form small colonies (8–30 cells) when released from the immobilized gel.

1.7.2 Metabolism and productivity

Photosynthetic oxygen evolution of free and immobilized cells were observed in several studies (Jeanfils & Collard 1983; Robinson *et al.* 1986; Bailliez *et al.* 1986). A difference in this activity point out a fundamental change of metabolism. In a study with *Chlorella*, no difference in oxygen evolution between free and immobilized cells was observed (Robinson *et al.* 1985). However Bailliez *et al.* (1988) found that oxygen evolution was higher in immobilized state. It was also found that, immobilization is also effective on the chlorophyll metabolism of the cells (Tamponnet *et al.* 1985; Bailliez *et al.* 1986) and respiration rates. The respiration rate of immobilized *Chlorella* was found to be lower than that of free cells Robinson *et al.* (1985) since only a proportion of the immobilized cells in the beads are metabolically active. Studies performed by Kayano *et al.* (1981); Brouers & Hall (1986) showed that hydrogen evolution from the alga *Anabaena* was threefold higher following immobilization. Similarly, ammonia, glycolate and glycerol production by were greatly increased when the cells were immobilized (Brouers & Hall 1986; Santos-Rosa *et al.*,1989; Vilchez *et al.* 1991; Leon &

Galvan,1995). However there are also studies demonstrating a decrease in productivity following immobilization (For example Wilkstrom *et al.* 1982).

2. MATERIALS AND METHODS

2.1 Microalgal Species and Culture

Phaeodactylum tricornutum was used during the experiments. The microalgae was obtained from the culture collection of Ecotoxicology Laboratory at Faculty of Naval Architecture and Ocean Engineering (İstanbul, Turkiye). The cultures were maintained batchwise, in 250 mL erlenmayer flasks containing 100 mL of growth medium in a temperature-controlled room (20 ± 1 ºC) under continuous cool-white flourescent illumination (~3500 lux). The filtered seawater was then autoclaved (10 min 110 ºC), and enriched with Guillard's f/2 nutrient medium (Table 2.1) (Guillard and Ryther, 1962).

Table 2.1 : f/2 Medium

Chemicals	**g/L**
1. $NaNO_3$	75
2. $NaH_2PO_4 \cdot 2H_2O$	5.66
3. $Na_2SiO_3 \cdot 5H_2O$	12.9
4. $CuSO_4 \cdot 5H_2O$	0.005
$ZnSO_4 \cdot 7H_2O$	0.011
$CoCl_2 \cdot 6H_2O$	0.005
$MnCl_2 \cdot 4H_2O$	0.009
5. $FeCl_3 \cdot 6H_2O$	0.909

2.2 The Seawater Used During the Experiments

The seawater (20-22 ppt) was collected from a relatively uncontaminated site of the Istanbul Strait. It was first filtered from activated carbon to remove possible organic contaminanats and then from GFC (Glass-fiber) and membrane filter papers (0.20 μm) and autoclaved (Hirayama HV) for 10 min at 110 ºC.

2.3 Preparation of Immobilized Algal Beads

The principle of the method was described in the Dos Santos Moreira et al.(2005) Four days cells at their log phase were centrifugated at 10000 rpm for 10 min and then mixed with 2.5%, 3.5% and 5% (w/v) sodium alginate solutions to obtain an alginate–cell suspension with a cell density of 500 000 cell mL^{-1} of alginate.

To prepare 100 mL of alginate solution, the alginate was first compeletely dissolved in 70 mL of distilled water and mixed with 30 mL of distilled water containing 3.5 gr sodium chloride (to adjust the salinity to 35 ppt) on a magnetic stirrer.

The algal suspension in alginate solutions was dropped into calcium chloride ($CaCl_2.2H_2O$) solutions prepared at two different concentrations (2% and 4% w/v) placed in an ice bed by using a burette from a height of about 15 cm and at a rate of approximately one drop per second. Cation solutions were prepared in distilled water. Beads were kept stirring in the cation solution for 2 hours to allow complete hardening of the alginate and washed several times with natural sea water to remove the remaining $CaCl_2$ on the beads and stored in fridge at 4 º C until they were used for the experiments. The pH of alginate and $CaCl_2$ solutions were adjusted to 7.5-8.0 by using a 0.1 M NaOH solution.

Blank alginate beads were prepared similarly by using alginate solutions without algal cells. After preparation, the beads were washed several times with filtered seawater.

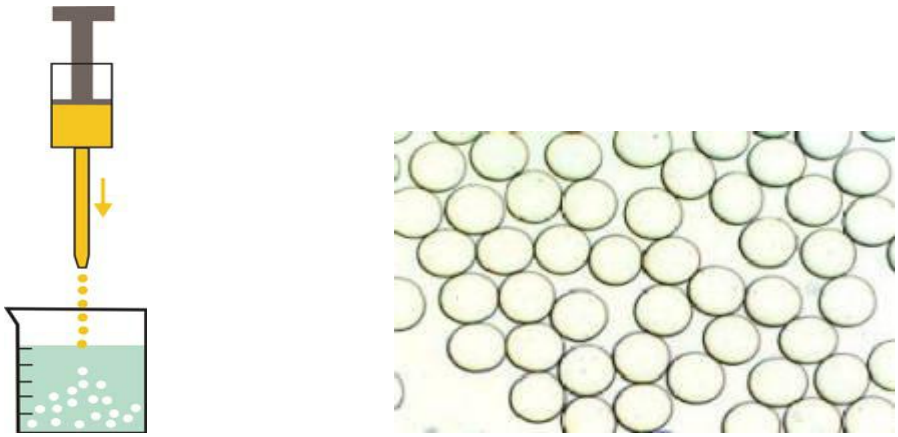


Figure 2.1 : Preperation of alginate beads.

2.4 Determination of Bead Stability and Growth of Algae in the Beads

2.4.1 Short term experiments

Algal beads (400 beads) prepared in several combinations of alginate and $CaCl_2$ (Table 2.2) were grown in 1000 mL erlenmayer flasks containing 400 mL of f/2 medium, at 20±2 ºC, under continuous illumination (3500-4000 lux) for 16 days.

To determine the cell concentrations in the beads, five beads were dissolved in a 50 mL of trisodium citrate solution (pH = 6.5) and counted by using a Coulter Counter. The chlorophyll flourescence (FI) (Ex=430 nm, Em= 663 nm) of the algae in beads was also monitored (after dissolving in trisodium citrate) by measuring the fluorescence intensity with a flourescence spectrophotometer (Perkin Elmer LS 55) at excitation and emission wavelenghts of 430 nm and 663 nm respectively.

The concentration of released cells into the growth medium from the alginate beads were determined by counting as well as by measuring the flourescence intensities.

Table 2.2 : Combinations of alginate and $CaCl_2$ used for immobilization of algal cells.

Sodium alginate concentration (w/v%)	**$CaCl_2$ (w/v%)**
2.5	2
3.5	2
3.5	4
5	2

2.4.2 Long term experiments

The algal beads prepared by using 3.5% alginate and hardened with 2% and 4% $CaCl_2$ were stored in the filtered clean seawater in the fridge (4 ° C) and tested for their growth by inoculating in a fresh f/2 medium every 15 days for a period of 255 days and then once after a year. The experiments were carried out at the same temperature and light conditions in 500 mL erlenmayer flasks containing 200 mL of natural seawater medium and 60 beads. The algal densities in the inoculated beads and in the medium (released) were monitored daily and bead diameters were determined to monitor bead swelling or shrinkage. Five beads were sampled everyday from each flask and the diameters were measured by using an image

analyzing system consisting of a microscope (XSZ single Zoom microscope), a CDD digital camera (TK 1381 EG) and a PC with the data analyzing system Image-Pro Plus.

2.4.3 The growth of free microalgae cells in growth medium

The growth of free algal cultures of *Phaeodactylum tricornutum* was used to compare with the growth of *P. triconutum in* algal beads. The experiment was carried out in 1000 mL erlenmayer flasks containing 400 mL of natural seawater medium. The initial concentration of 15000 cells mL^{-1} algae was inoculated into the medium and incubated at 20±1 º C and under continuous cool-white flourescent light. Algal growth was measured by using coulter counter and fluorescence spectrophotometer every day during 16 days in duplicate.

2.5 Application of Toxicity Tests by Using Algae and Luminescence Bacteria

2.5.1 Toxicity tests

The free and immobilized cells by using 3.5% alginate and hardened with 2% $CaCl_2$ were exposed to 500, 1000 and 1500 µg/L Ace and 100, 400 and 1000 µg/L Phen concentrations for four days and algal densities were determined as described above.

The results were compared with a luminescence bacterial toxicity test performed with *Vibrio fisheri* in an ABOATOX toxicity test system.

Acenapthene (Ace) and Phenthrene (Phen) are polycyclic aromatic hydrocarbons which are listed as priority pollutants by EPA and found widespread in the environmental matrices. The toxicity tests were performed with the concentrations below the solubilities of PAHs. The structures, solubilities and concentrations used for the toxicity tests were indicated in Table 2.3. The preparation of the working PAH concentrations were shown in Table 2.4.

The working PAH concentrations were prepared from the stock solutions in acetone. The same amount of acetone (0.1%) was also added to the control. The final volume of the working solutions were adjusted to 40 mL.

For the algal toxicity tests, a starting concentration of 10000 cells mL^{-1} and 10 beads in 20 mL of PAH solutions (duplicate) in 40 mL of glass vials were used for free and immobilized cell applications respectively. The vials containing free and

immobilized algae were incubated at 20±1 º C and under continuous cool-white flourescent light (3500-4000 lux). Algal growth for free cells were measured by using coulter counter and by determining the flourescence intensity at the beginning and at the end of the experiment. On the other hand, to determine the starting algal density, 10 of the alginate beads before starting the experiment were counted. After four days of exposure, the algal beads were dissolved and counted again. The experiments were carried with two replicates for each set of tests.

Table 2.3 : Structure, water solubility and exposure concentrations of PAHs.

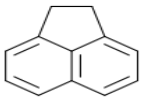
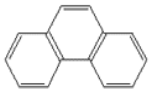
PAHs	Structure	Solubility (µg/L) at 25ºC)	Exposure conc. (1) (µg/L)	Exposure conc. (2) (µg/L)	Exposure conc. (3) (µg/L)
Acenaphthene (Ace)		1930	500	1000	1500
Phenanthrene (Phen)		1200	100	400	1000

Table 2.4 : Preparation of working PAH concentrations from a stock solution of 1000 mg /L.

PAH Concentration (µg/L)	Stock soln(µL)	Acetone (µL)	Seawater (mL)
0 (Control)	0	20	20
Ace 500	5	15	20
Ace 1000	10	10	20
Ace 1500	15	5	20
Phen 100	2	18	20
Phen 400	8	12	20
Phen 1000	20	0	20

2.5.2 Luminescence bacterial toxicity tests

Vibrio fischeri (Biotox ^{TM} Kit) was used as test organisms. The principle of the test depends on the exposure of the luminescent bacteria to several concentrations of test sample and the monitoring of the reductions in the light output of the bacteria reflecting the toxicity of the sample in a temperature-controlled photometer.

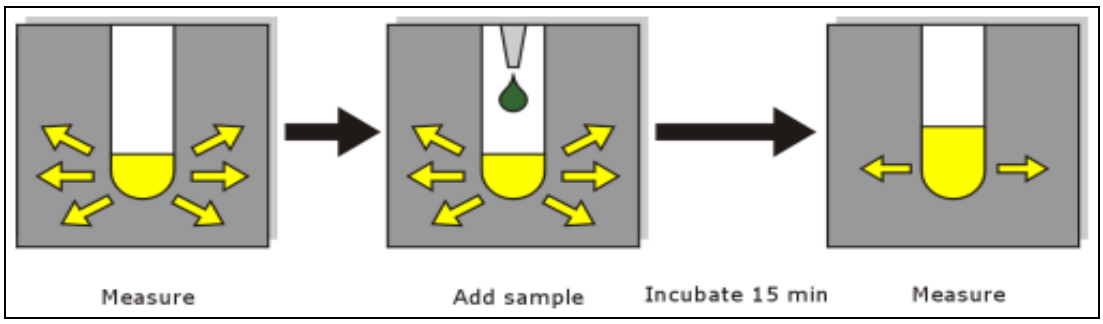


Figure 2.2 : Application of *Vibrio fisheri* toxicity test.

The test endpoint is measured as the effective concentration or inhibitory concentration of a test sample that reduces light emission by a specific amount under defined conditions of time and temperature and expressed as an $EC_{50}(30)$ or $IC_{50}(30)$ which reduces light emission by 50% after 30 minutes at 15 ° C. During the bacterial luminesce test performed in this study, the PAH solutions in dublicate (Table 2.4) were incubated with the bacteria and the results were compared with the results of algal toxicity tests.

2.6 Nutrient Removal Studies with Algal Beads

Removal of NO_3 and o-PO_4 were tested in batch systems. The algal beads were inoculated for four days in a modified f/2 medium containing N and P concentrations of 1500 and 500 µg L^{-1} respectively. After four days the algal beads were removed from the solution and inoculated in a freshly prepared medium and the concentration of the nutrients were measured. This was repeated four times. The results were calculated from dublicate samples.

2.6.1 Nutrient analysis

The samples collected for nutrient analysis were stored at -20 ° C until analysis. The samples were analyzed according to the Standard Methods (Standard Methods for the Examination of Water and Wastewater, 1985). For that aim, a series of calibration

standards were prepared from the stock solutions and the samples were measured against standards in a UV-Vis spectrophotometer (Chebios Optimum-One UV–Vis spectrophotometer) at corresponsding wavelenghts. Blank and seawater corrections were made during the analysis. The samples were measured in dublicate.

2.6.2 Nitrate analysis- Cadmium column reduction method

Nitrate is reduced to nitrite by cadmium and the resulting nitrite determined by formation of an azo dye. In the method, both calibration standards and the samples were passed through a copper-coated cadmium reduction column by means of a peristaltic pump. To prepare the column, Cd granules were treated with copper sulfate ($CuSO_4$) and then packed in a plastic column (2 mm ID). The endings of the tubing were pluged with glass wool. Nitrate in the sample is reduced to nitrite in a buffer solution. The nitrite is then determined by diazotizing with sulfanilamide and coupling with N-1-naphthylethylenediamine dihydrochloride to form a color azo dye. The absorbance measured at 550 nm is linearly proportional to the concentration of nitrate in the sample. Potassium nitrate nitrat was used to prepare the stock and calibration standards.

2.6.3 Phosphate analysis

The principle of the method for the determination of ortho-phosphate depends on the formation of a phosphomolybdenum blue complex. A single reagent solution is used consisting of an acidified solution of ammonium molybdate containing ascorbic acid and a small amount of antimony. The standards and samples were measured at 720 nm wavelength. KH_2PO_4 was used to prepare the stock and calibration standards.

3. RESULTS AND DISCUSSION

3.1 Short-Term Experiments

3.1.1 Bead stability and growth of free algal cultures and algae in the beads

To determine the bead stability and to observe the growth of *Phaeodactylum tricornutum* algal culture, the beads were firstly prepared by using two different alginate concentrations (2.5% and 5%) and hardened with 2% $CaCl_2$. Those algal beads were then inoculated in the growth medium and the effect of alginate concentration both on the shrinkage of the beads and on the growth of the algae in the beads. The results of the diameter measurements of the beads during 15 days inoculation were shown in Figure 3.1 a and b for 2.5% alginate+ 2% cation (a) and 5% alginate+ 2% cation (b) combinations respectively.

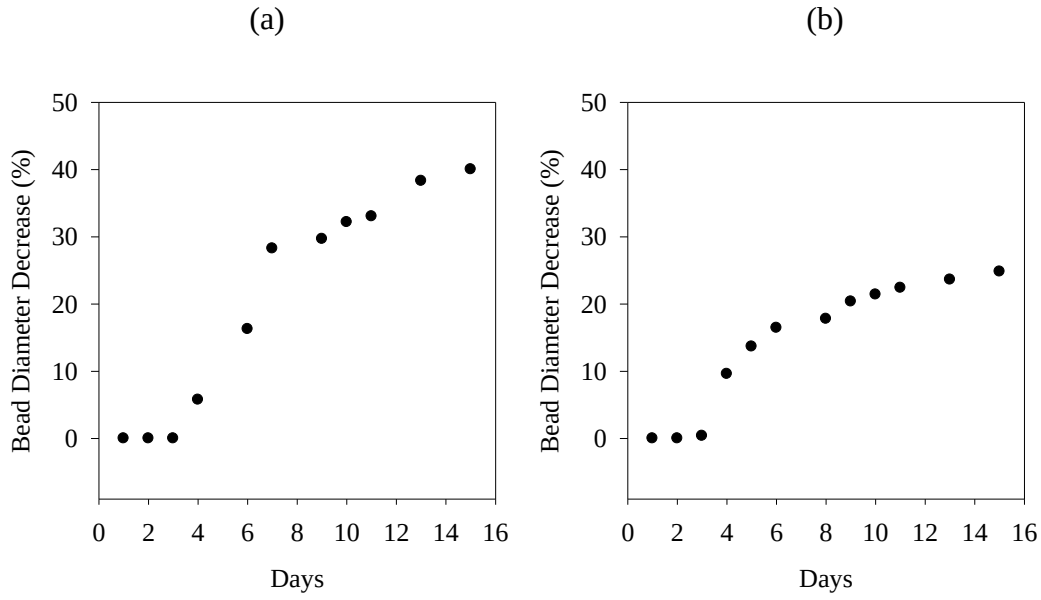


Figure 3.1 : Percentage decrease of bead diameter prepared using (a) 2.5% alginate+ 2% cation and (b) 5% alginate+ 2% cation

The diameters of the beads did not show any change during the first three days following the inoculation, and then started to decrease. The decrease in bead diameter reached 16% for both beads in different alginate solutions after six days. At the end of the experiment, after 16 days of inoculation, those values for the bead diameters were decreased 40 and 24% of their initial values for 2.5 and 5% alginate

solutions, respectively. At the end of the exposure periods, the visual inspection of the beads prepared from 2.5% of alginate solution suggested that alginate matrix was explicitly disrupted and began to lose structure under the experimental conditions. Higher mechanical strength of the beads prepared by using higher (5%) alginate concentration was most probably due to the increase of guluronic monomers in the polymer chain which results in the enhancement of the gellation property (Smidsrod et al., 1990; Martinsen et al., 1989; Draget et al., 1997). The standard deviation (SD) of the beads diameters obtained from the dublicate samples were given in Figure 3.2.

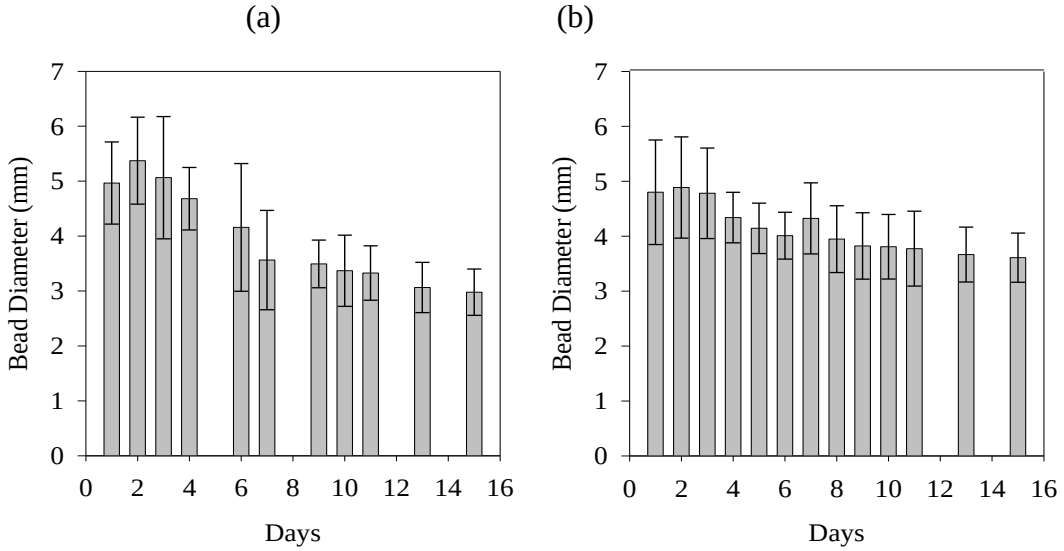


Figure 3.2 : Change in diameter (mm) of beads prepared using (a) 2.5% Alginate+ 2% cation and (b) 5% Alginate+ 2% cation

The growth of algae in the beads were quite different in 2.5 and 5% alginate solutions (Figure 3.3 a and b); after a four days of lag period, the algae showed a rapid growth and maximum production was reached by around 3000000 cells in the 2.5 alginate solution, while the maximum number of cells were around 800000 in 5% alginate solution. During the experiments the florescence intensity of the cells in suspension were also measured. Those results were given in Figure 3.4 a and b. The flourescence intensities of the cultures increase first, reach to the maximum after 8 days of inoculation and then decrease in both cases. The difference between the cell numbers and flourescence intensities of the cells is due to the cell counts correspond to the the total number of alive and dead cells together.

As a result, when two inoculations were compared, the growth of *Phaeodactylum* was found more favorable in 2.5% alginate solution, but the stability of those beads were rather low. On the other hand, the beads from 5% alginate were more stable

compared to the beads from 2.5%, but the growth was inhibited mainly probaby due to the restriction of light penetration and the diffusion of nutrients through the higher concentration of alginate.

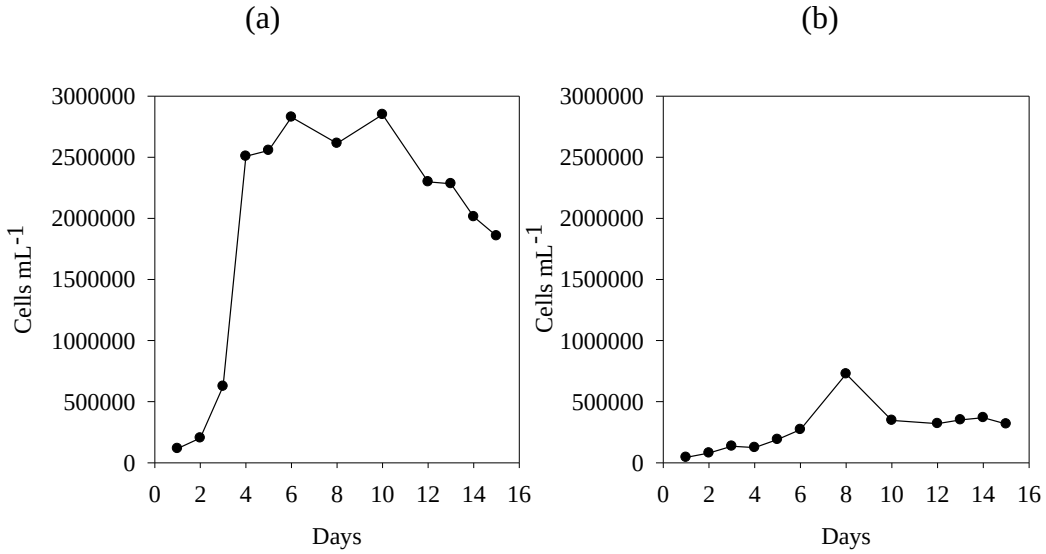


Figure 3.3 : The growth curve of beads prepared using (a) 2.5% alginate+ 2% cation and (b) 5% alginate+ 2% cation

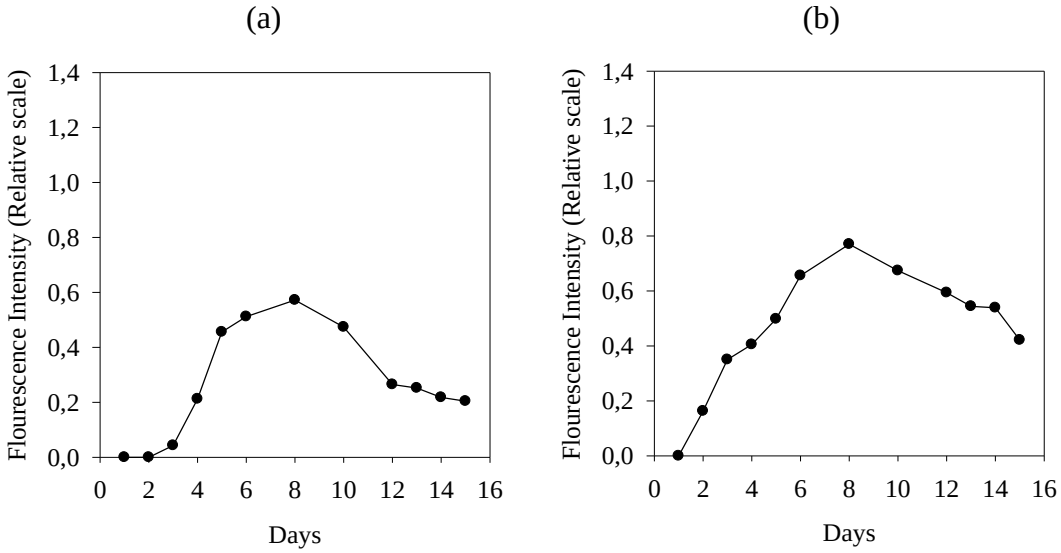


Figure 3.4 : Change in flourescence intensity of beads prepared using (a) 2.5% alginate+ 2% cation and (b) 5% alginate+ 2% cation.

To increase the strength of the alginate beads, the usage of several divalent cations in different concentrations were also proposed in the literature. Of those cations showing high affinity for alginate, calcium and strontium were the most frequently used as hardening agent in the alginate immobilization studies (Smidsrod et al, 1990; Videroe and Danielsen, 2001; Gaserod et al., 1999). Thus, as a next step in the experiment, an alginate concentration in between (3.5%) was selected and hardened

with 2% and 4% $CaCl_2$ to find out the appropriate combination to be used in further studies (long term experiments, toxicity testing and nutrient removal studies). The change in diameters for 15 days inoculation of the beads were shown in Figure 3.5.

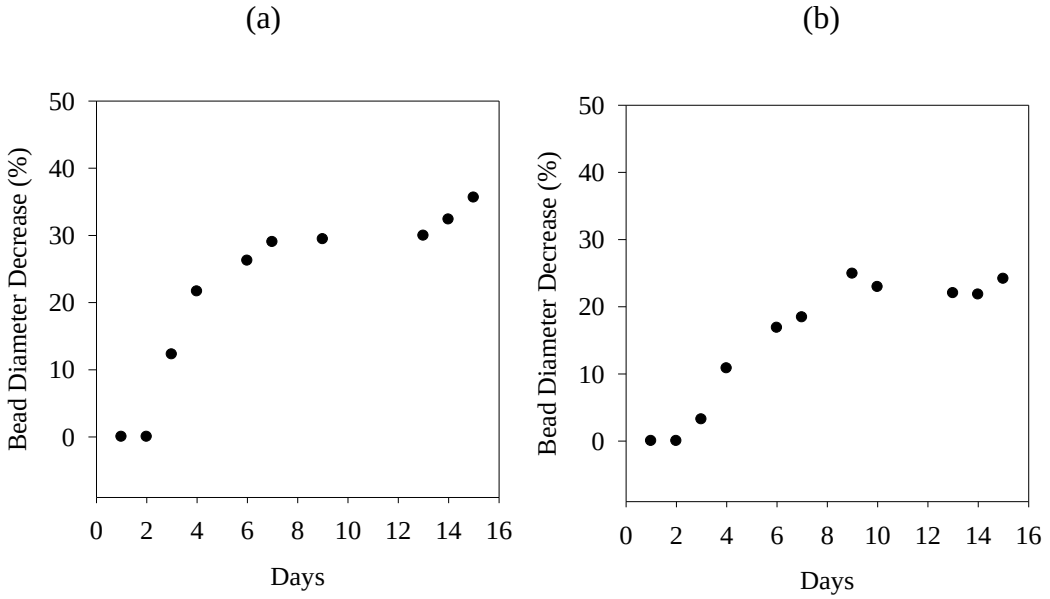


Figure 3.5 : Percentage decrease of bead diameter prepared using (a) 3.5% alginate+ 2% cation and (b) 3.5% alginate+ 4% cation.

The SD values in dublicate samples from the beads diameters were also given in Figure 3.6. Generally, the SD values for the beads from 3.5% alginate (for both cation combinations) were smaller compared to the SD obtained for 2.5% and 5% alginate beads showing the homogenity in the dimensions of the beads.

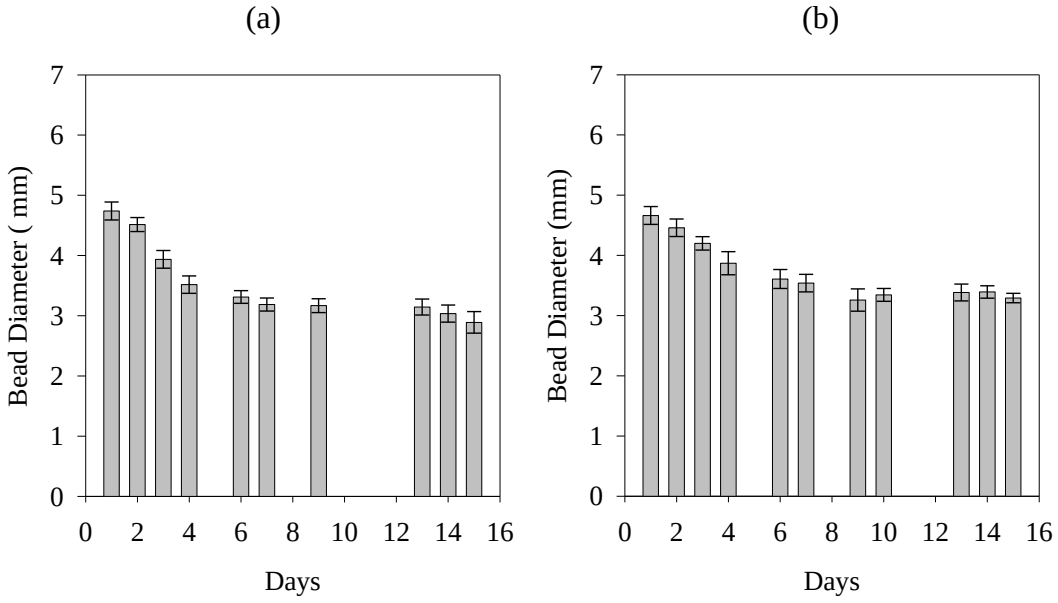


Figure 3.6 : Change in diameter (mm) of beads prepared using (a) 3.5% alginate+ 2% cation and (b) 3.5% alginate+ 4% cation.

The growth of the cells determined by counting the cells and by measuring the fluorescence intensity were given in Figures 3.7 and 3.8, respectively.

No clear differences in the growth of algae were observed for beads in 3.5% alginate hardened both with 2 and 4% $CaCl_2$, although, as expected, beads hardened using 4% rather than 2% of calcium solution were found to be more stable. At the end of the experiment, the decrease in bead diameters in 16 days of inoculation was quantified as 24 and 35% for alginate solutions hardened with 4% and 2% $CaCl_2$ respectively.

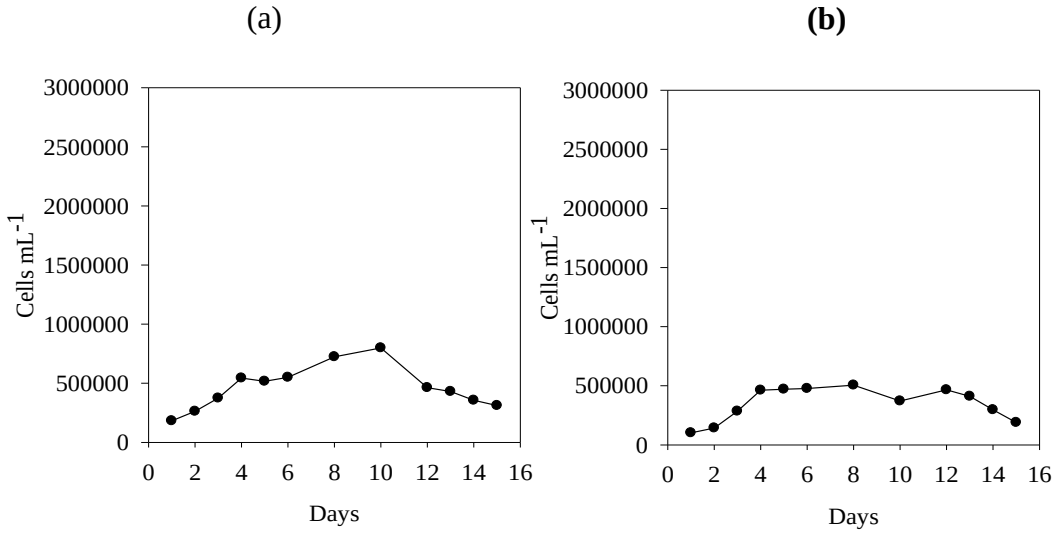


Figure 3.7 : The growth curve of beads prepared using (a) 3.5% Alginate+ 2% cation and (b) 3.5% Alginate+ 4% cation.

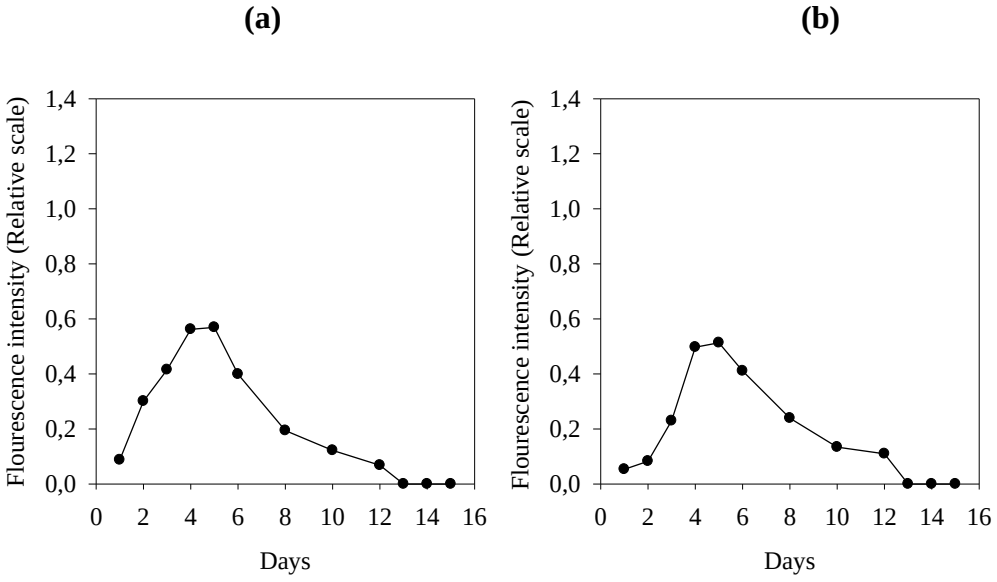


Figure 3.8 : Change in fluorescence intensity of beads prepared using (a) 3.5% Alginate+2% cation and (b) 3.5% Alginate+4% cation.

In a seperate experiment, the free Phaeodactylum cultures were also inoculated in the medium and their growth were monitored to compare the results obtained from the

immobilized cells. The cell numbers and fluorescence intensities of the free cells were given in Figures 3.9 a and b. The maximum cell number in those cultures were found as 3000000 cells/mL. This result is the same with the cell concentration in beads obtained for 2.5% alginate and 2% cation. All other cell concentrations in the rest of alginate+cation beads were smaller than that value. Another important difference between the free cells and the immobilized cells is the synthesis of chlorophyll-a. The fluorescence intensities obtained for the free cells are much higher compared to the intensities measured for the immobilized cells.

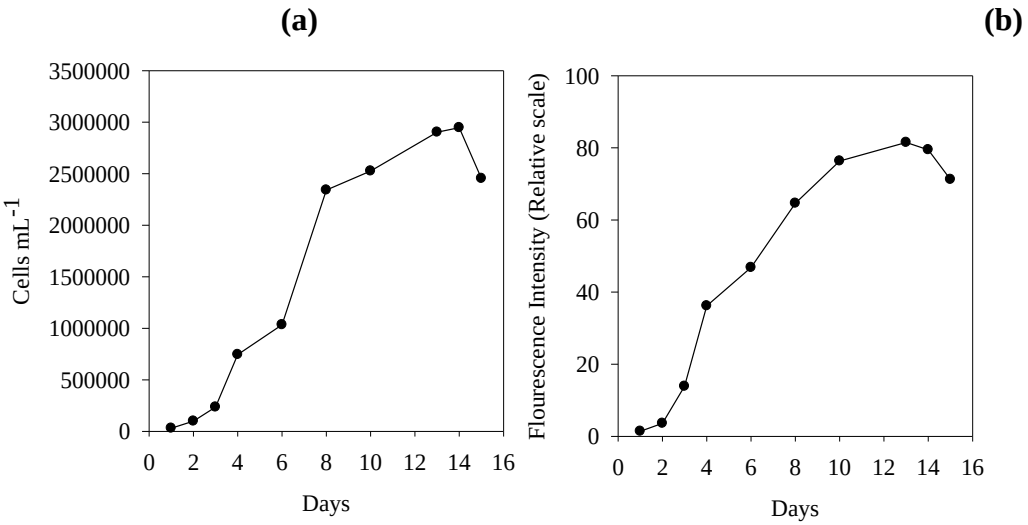


Figure 3.9 : The growth of free algal cells in the medium.

When inoculated to a freshly prepared medium, algal cells starts to grow. The growth curve of an algal culture can be divided into five phases (Figure 3.10).

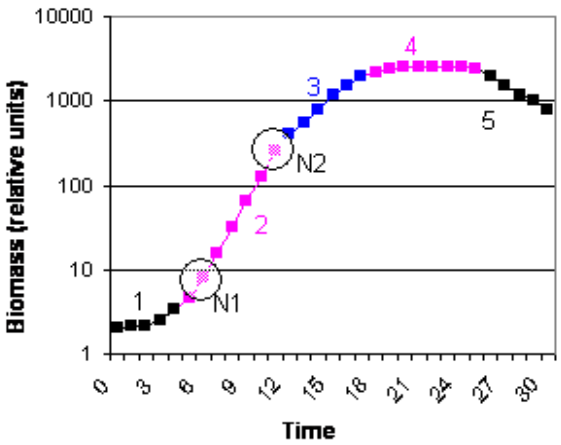


Figure 3.10 : General pattern of microalgal growth in batch cultures.

The first phase is called as lag phase. During the lag phase, algae adapt to growth conditions. It is the period where the individual they are maturing and not yet able to

divide. The growth rate of a microalgal population is the amount of increase that a specific variable has gained within a specific period. This period is determined from the second phase (exponential phase). Declining growth (third phase) becomes in cultures when a specific requirement for cell division is limiting or inhibiting reproduction. In this phase of growth biomass is frequently very high and exhaustion of a nutrient salt, limiting carbon dioxide or light limitation become the primary causes of declining growth. Cultures enter stationary phase (fouth phase) when net growth is zero, and within a matter of hours cells may undergo dramatic biochemical changes. The nature of the changes depends upon the growth limiting factor. Nitrogen limitation may result in the reduction in protein content and relative. While the measured light intensity within the culture will decrease with increasing biomass if the incident illumination is maintained relatively high then a large proportion of cells may become stressed, photoinhibit and the culture can be pushed into the death phase. This is especially the case if the culture is also nutrient stressed. The shut down of many biochemical pathways as stationary phase proceeds means that the longer the cells are held in this condition the longer the lag phase will be when cells are returned to good growth conditions. The final growth phase (fifth phase), during which nutrients have been depleted and cell number decreases. Cultures of some species will lose their pigmentation and appear cloudy. However, cells of other species may disappear but the culture colour will be maintained.

Growth rate is one important way of expressing the relative ecological success of a species or strain in adapting to its natural environment. The duration of exponential phase in cultures depends upon the size of the innoculum, the growth rate and the capacity of the medium and culturing conditions to support algal growth. When the growth phase has been plotted, two points, N_1 and N_2, at the extremes of this linear phase are taken and growth rates can be calculated by using the following formula.

μ (g. g^{-1}. h^{-1}) : Specific growth rate: $\mu = \ln (N_2 / N_1) / (t_2 - t_1)$

Where N_1 and N_2 = biomass at time$_1$ (t_1) and time$_2$ (t_2) respectively; Levasseur *et al* (1993).

Div. per day^{-1}: Divisions per day: $Div.day^{-1} = \mu / \ln 2$

Divisions per day and the generation time can also be calculated once the specific growth rate is known.

Gen't (hours) : Generation time: $Gen' t = 1 / Div.day^{-1}$

The growth rates, divisions/day and generation times of the free and immobilized cultures were calculated and given in Table 3.1.

Table 3.1: The growth rates, divisions/day and generation times of the free and immobilized cultures.

	2.5% Alginate+2% Cation	**5% Alginate+2% Cation**	**3.5% Alginate+4% Cation**	**3.5% Alginate+2% Cation**	**Free Algae**
μ(g. g^{-1}. h^{-1})	1.32	0.32	0.45	0.63	1.58
Div. per day^{-1}	1.92	0.46	0.65	0.91	2.28
Gen't (hours)	12.5	51.4	36.4	26.2	10.5

Among the calculated growth rates, the highest was found for the free cells. The ranking of the growth rates were as follow:

$$\mu_{\text{free cells}} > \mu_{2.5A+2C} > \mu_{3.5A+2C} > \mu_{3.5A+4C} > \mu_{5A+2C}$$

In terms of growth rates, the maximum value was obtained for the algal beads prepared by using 2.5% alginate + 2% cation solution. When two beads prepared by using 3.5% alginate, but different concentrations of cation solutions, it is seen that the increase in the cation concentration resulted in also an increase in growth rate.

The fluorescence intensity per cell is also a useful way to compare the cells metabolism and efficiency. The results are given in Table 3.2. The general trend in all cultures including free cells, the fluorescence intensity per cell values first increase, reach a maximum and then decrease. The maximum values were obtained for the immobilized cells after 2 and 8 days of inoculation. The synthesis of the chlorophyll-a per cell in free cultures were much higher in free cultures compared to the immobilized ones. The alginate as well as the cation surronding the alginate beads restrict the penetration of light. When the average values for the immobilized cells were compared, the maximum values were found for the algae in 5% alginate and 2% cation. As was indicated before the minimum and maximum algal productions were found for 2.5% alginate+ 2% cation and 5% alginate + 2% cation respectively. But there were no clear difference in fluorescence intensities of those cultures. This means that the smaller number of algal cells can produce the same chlorophyll-a, and results in a higher fluorescence intensity per cell. Another words, the increase in the amount of alginate prevents the algal production, but not synthesis of chlorophll-a in the individual cells. On the other hand, when two 3.5% alginate prepared by using different concentrations of cation solutions were compared, there

was no difference was found in the average fluorescence intencity/cell values (Table 3.2).

Table 3.2: The flourescence intensity/cell of beads prepared from 2.5, 3.5 and 5% *P.tricornutum* alginate and hardened using 2 and 4% solutions of $CaCl_2$.

Days	2.5% Alginate 2%Cation F.intensity/Cell X10^{-6}	5% Alginate 2% Cation F.intensity/Cell X10^{-6}	3.5% Alginate 4% Cation F.intensity/Cell X10^{-6}	3.5% Alginate 2% Cation F.intensity/Cell X10^{-6}	Free cells F.intensity/Cell X10^{-6}
1	0	0	0.52	0.48	36.5
2	0	0.20	0.58	1.15	58.8
3	0.07	0.26	0.81	1.11	48.5
4	0.09	0.32	1.08	1.03	45.2
5	0.18	2.62	1.09	1.10	-
6	0.18	2.42	0.86	0.73	27.5
8	0.22	2.22	0.47	0.27	30.2
10	0.17	2.10	0.36	0.15	32.4
12	0.12	1.69	0.24	0.15	-
13	0.11	1.44	0	0	28.1
14	0.11	1.34	0	0	26.9
15	0.11	1.14	0	0	29.0
AV	**0.11**	**1.31**	**0.50**	**0.51**	**36.3**

3.2 Long-Term Studies

3.5% alginate solutions were used to prepare the alginate beads and inoculated with *P. tricornutum* to find out the long-term viability and growth. Although, the stability of the beads hardened with 4% $CaCl_2$ were preferable and the growth of algae were nearly the same in the beads hardened with 2% and 4% $CaCl_2$, both hardening concentrations were used to see if this makes a difference in the bead stability and algal growth during this long term storage of the beads. Figure 3.11 and Figure 3.12 show the cell numbers in the beads for 7 days inoculations for the beads prepared by using 3.5% alginate + 2% $CaCl_2$ and 3.5% alginate + 4% $CaCl_2$ respectively. They scales (cell number) were adjusted as to be the same range to observe the difference between the inoculations clearly.

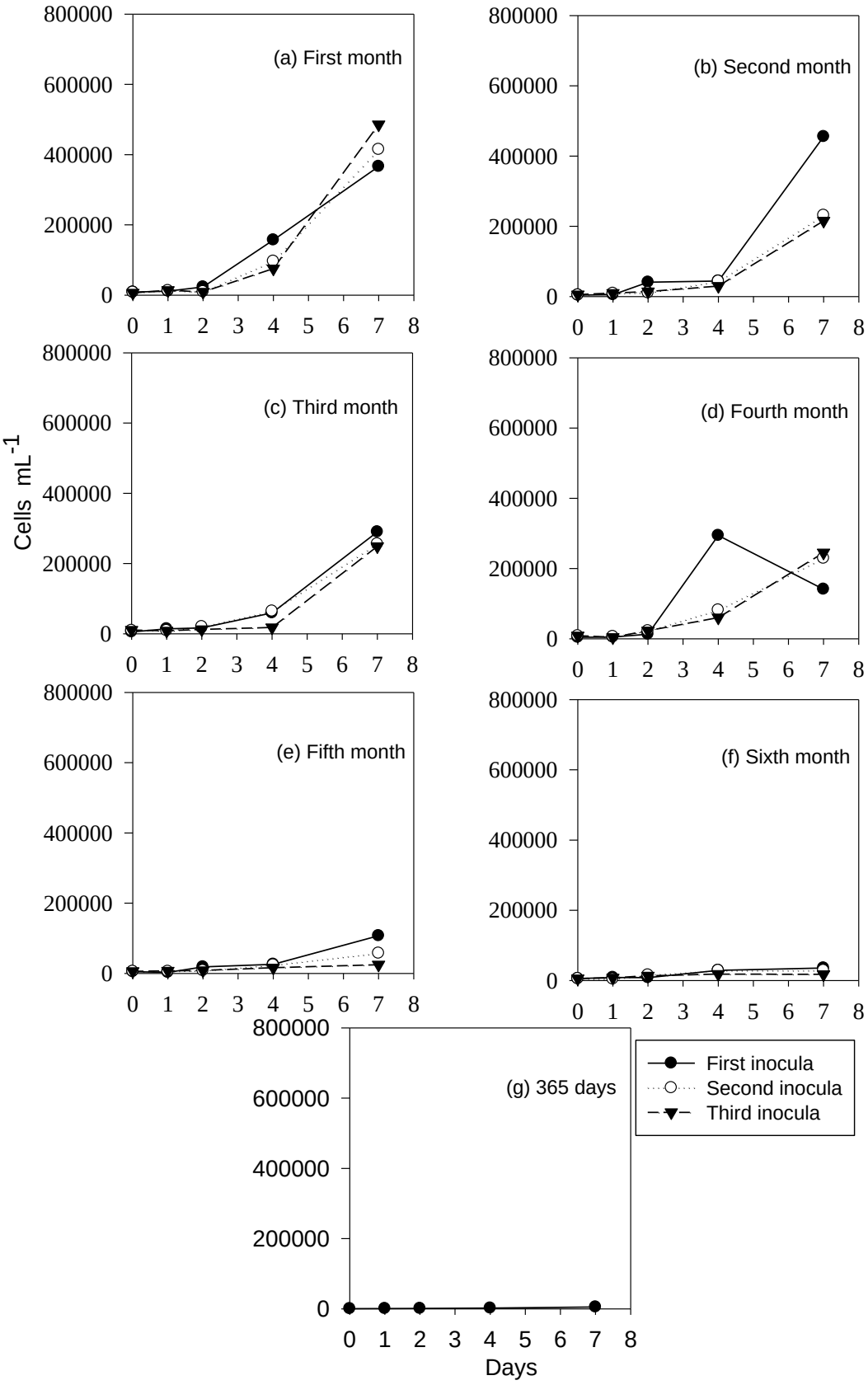


Figure 3.11 : The growth curve of beads prepared using 3.5% alginate + 2% CaCl2.

Each graph from (a) to (f) contains curves for three inoculations initiated at 15 days of intervals. For example the first graph indicates the growth curves of algae in the beads inoculated to the freshly prepared medium firstly following the preparation and 15 and 30 days after the preparation of the algal beads. The last graph (g) contains only the incubation of the algal beads after 365 days. The algal beads after preparation were stored in the fridge at 4 º C. Similarly, the second graph represents the growth curves of the algae in the beads after 45, 60 and 75 days of storage at 4 º C after preparation. At the first stage of the experiment (First 30 days), the maximum number of algae in the alginate beads hardened with 2% cation solution was found approximately between 400000 and 500000 cells, while the cell numbers reached only 100000-200000 in the beads hardened with 4% cation solution.

Both the growth rate and maximum number of the algae at the end of inoculation days (after 7 days) exhibit a diminishing trend when the storage times of the algal beads increase.

The cell numbers in 3.5% alginate beads hardened with 4% $CaCl_2$ reached nearly a constant value after 55-60 days and stayed roughly the same throughout the experiment. On the other hand, the diminishing trend of the cell numbers continued for the beads hardened with 2% $CaCl_2$, although even 157 days, the cell numbers in those beads were still higher compared to the cell number in the beads hardened with 4% $CaCl_2$. Both alginate beads hardened with different cation concentrations attained the same cell concentration after 200 days of storage and following seven days of inoculation. After a year, the microalgae in the beads were almost lost their viability.

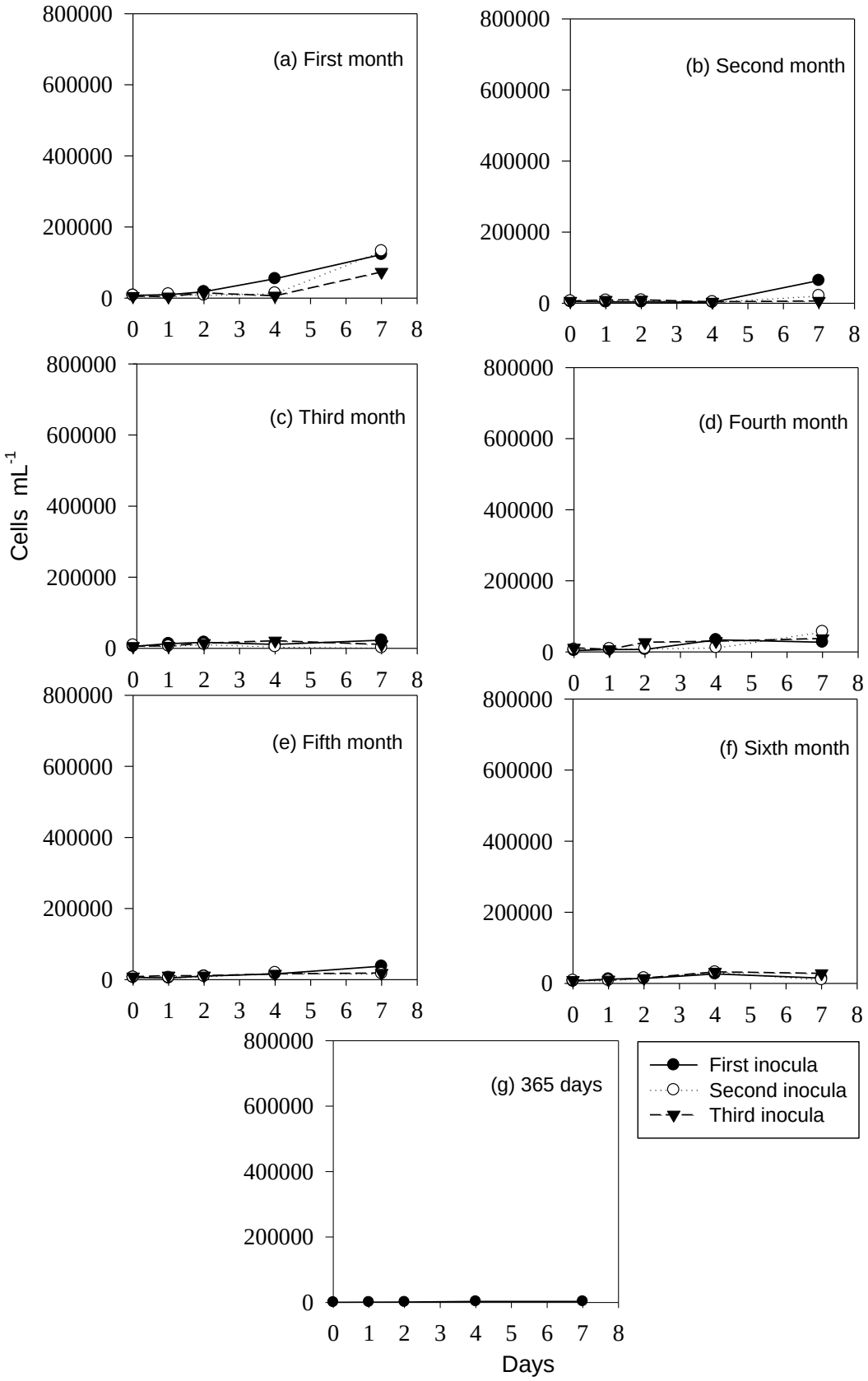


Figure 3.12 : The growth curve of beads prepared using 3.5% alginate + 4% $CaCl_2$.

Figure 3.13 shows some photo examples taken from the beads. The first row shows the photos of the beads after one month storage following the preparation. As clearly seen from the photos, the colors are light due to a little number of algae in the beads (before inoculation) and the shapes of the beads are spherical. After 7 days of inoculation (second row), due to the algal production, the colors change into brownish, and the shapes lost their spherical forms for both beads hardened with 2% and 4% $CaCl_2$. On the other hand, six months storage of the beads resulted in the beads hardened by using 4% cation solution to be in elliptical shape. After 7 days of inoculation, although, it is more significant in the beads hardened with 4% cation solution, the diameters of those beads -for both cases- stored for 6 months were smaller and the shapes were deteriorated.

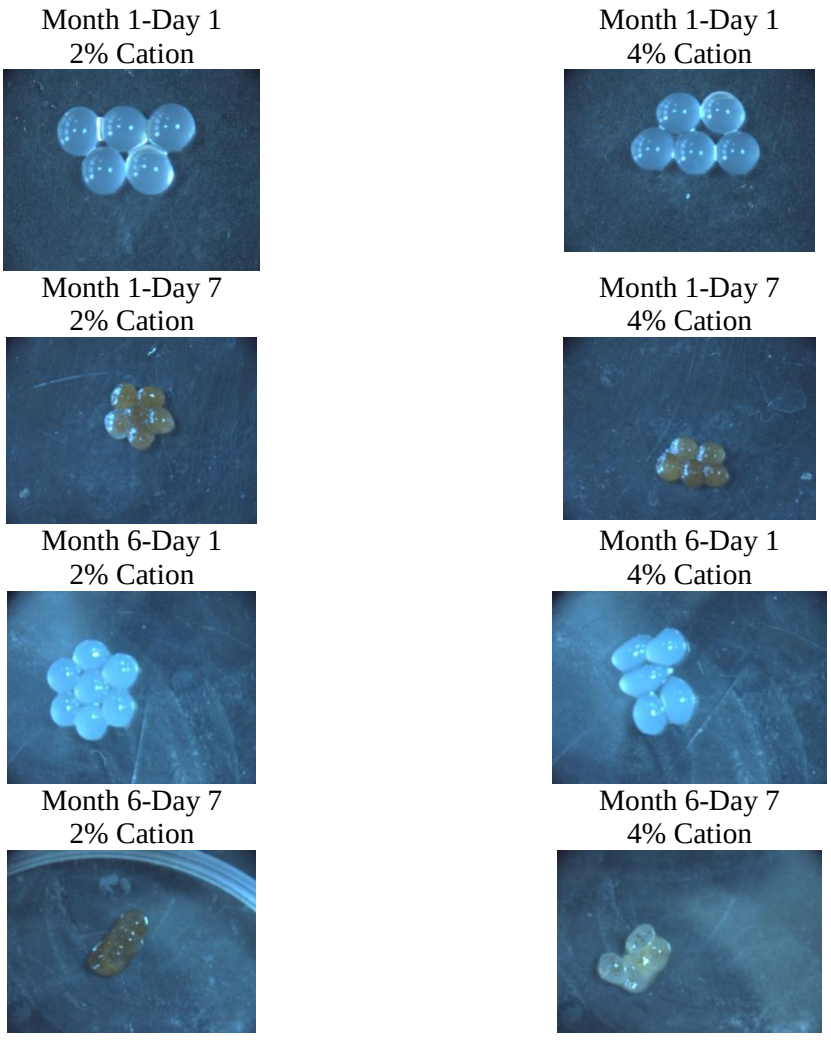


Figure 3.13 : The images of beads prepared using 3.5% alginate+ 2% cation and 3.5% alginate+ 4% cation.

Average growth rates, divisions/day and generation times for the immobilized cultures were shown in Table 3.3 and 3.4 for the algae in beads prepared by using

3.5% alginate+2% cation and 3.5% alginate+4% cation respectively. The average values rates were calculated for each set using the values obtained for three seperate inoculations. Although, the values were higher for the algae in 3.5% alginate+2% cation beads, all those average values calculated for the sets decrease steadily when the storage time increases for both cases. Finally, during a year storage, the average growth rate for the algae in 3.5% alginate+2% cation and in 3.5% alginate+4% cation were found as 0.71 and 0.57 respectively.

Table 3.3: The growth rates, divisions/day and generation times of beads prepared from 3.5% alginate *P. tricornutum* and hardened using 2% solutions of $CaCl_2$.

Months		**μ**	**Div. per day**	**Gen't (hours)**	**$\mu_{Ave.}$**	**Div. per $day_{Ave.}$**	**Gen't ave. (hours)**
0-30 days	First inocula	1.01	1.45	0.69	1.09	1.57	0.69
	Second inocula	1.53	2.20	0.45			
	Third inocula	0.73	1.05	0.95			
45-75 days	First inocula	1.13	1.64	0.61	0.83	1.20	0.92
	Second inocula	0.52	0.76	1.33			
	Third inocula	0.84	1.21	0.83			
90-120 days	First inocula	0.47	0.68	1.48	0.74	1.07	1.07
	Second inocula	0.26	0.37	2.69			
	Third inocula	1.49	2.15	0.47			
135-165 days	First inocula	2.18	3.14	0.32	1.04	1.5	1.25
	Second inocula	0.64	0.93	1.08			
	Third inocula	0.29	0.43	2.34			
180-210 days	First inocula	0.28	0.39	2.51	0.19	0.27	1.63
	Second inocula	0.29	0.42	2.37			
	Third inocula	0	0	0			
225-255 days	First inocula	0.54	0.78	1.28	0.62	0.89	1.14
	Second inocula	0.74	1.07	0.94			
	Third inocula	0.58	0.83	1.20			
365 days	1 inocula	0.49	0.70	1.43	0.49	0.70	1.43
	AVERAGE				0.71	1.03	1.16

Table 3.4: The growth rates, divisions/day and generation times of beads prepared from 3.5% alginate *P. tricornutum* and hardened using 4% solutions of $CaCl_2$.

Months		μ	Div. per day	Gen't (hours)	$\mu_{Ave.}$	Div. per dayAve.	Gen't ave.(hours)
0-30 days	First inocula	0.29	0.43	2.34	0.89	1.29	1.17
	Second inocula	1.14	1.65	0,61			
	Third inocula	1.24	1.78	0.56			
45-75 days	First inocula	1.93	2.78	0.36	0.78	1.12	0.68
	Second inocula	0.41	0.59	1.68			
	Third inocula	0	0	0			
90-120 days	First inocula	0.91	1.31	0.76	0.75	1.09	0.95
	Second inocula	0.59	0.86	1.17			
	Third inocula	0.76	1.09	0.91			
135-165 days	First inocula	0.85	1.23	0.82	0.88	1.27	0.97
	Second inocula	0.44	0.64	1.57			
	Third inocula	1.35	1.95	0.51			
180-210 days	First inocula	0.83	1.19	0.84	0.42	0.60	4.74
	Second inocula	0.06	0.09	11.5			
	Third inocula	0.37	0.53	1.89			
225-255 days	First inocula	0.12	0.18	5.58	0.16	0.24	4.76
	Second inocula	0.25	0.37	2.73			
	Third inocula	0.12	0.17	5.97			
365 days	1 inocula	0.14	0.21	4.82	0.14	0.21	4.82
AVERAGE					0.57	0.83	2.58

The results were also presented in terms of fluorescence intensity in Figures 3.14 and 3.15 for 3.5% alginate + 2% $CaCl_2$ and 3.5% alginate + 4% $CaCl_2$ respectively. The y scales were again adjusted as to be the same range to observe the difference between the inoculations clearly. The trend of the fluorescence intensity curves for each set of inoculations are -as expected- very similar to the cell number graphs. The fluorescence intensities of the cells decreases during the storage depending on the cell production. One of the important point in those graphs is that an increase in the fluorescence intensity after 365 days of inoculation. According to the cell graphs (Figure 3.11 and 3.12), the cells were not viable, but in those graphs, it is clearly seen that the cells in alginate hardened with 2% $CaCl_2$ still contain colorful chlorophyll-a pigments and they continue the synthesis during the last stage of their 7 days of inoculation. This situation is not very clear for the beads hardened with 4% cation solution.

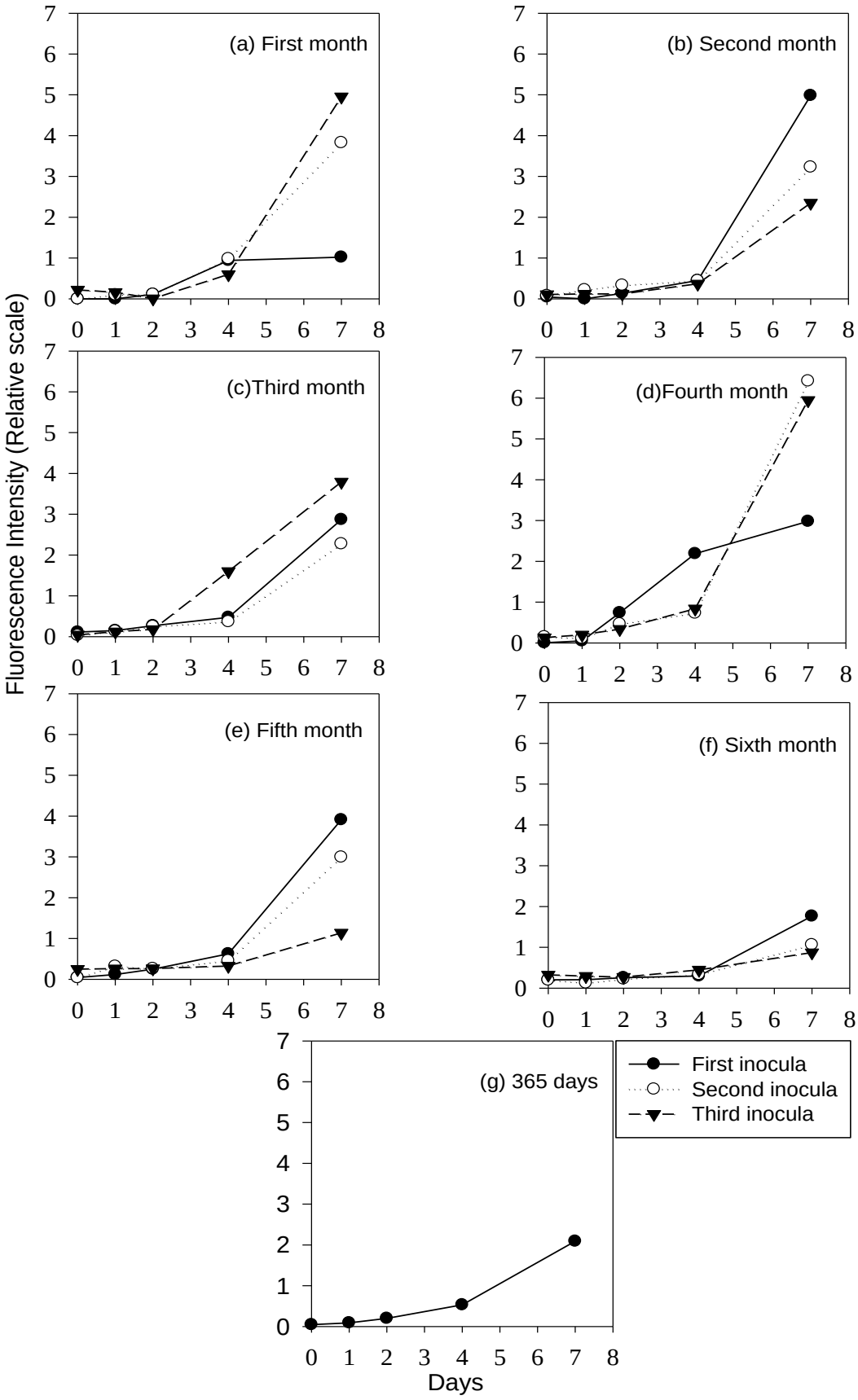


Figure 3.14 : Change in fluorescence intensity of beads prepared using 3.5% alginate + 2% $CaCl_2$.

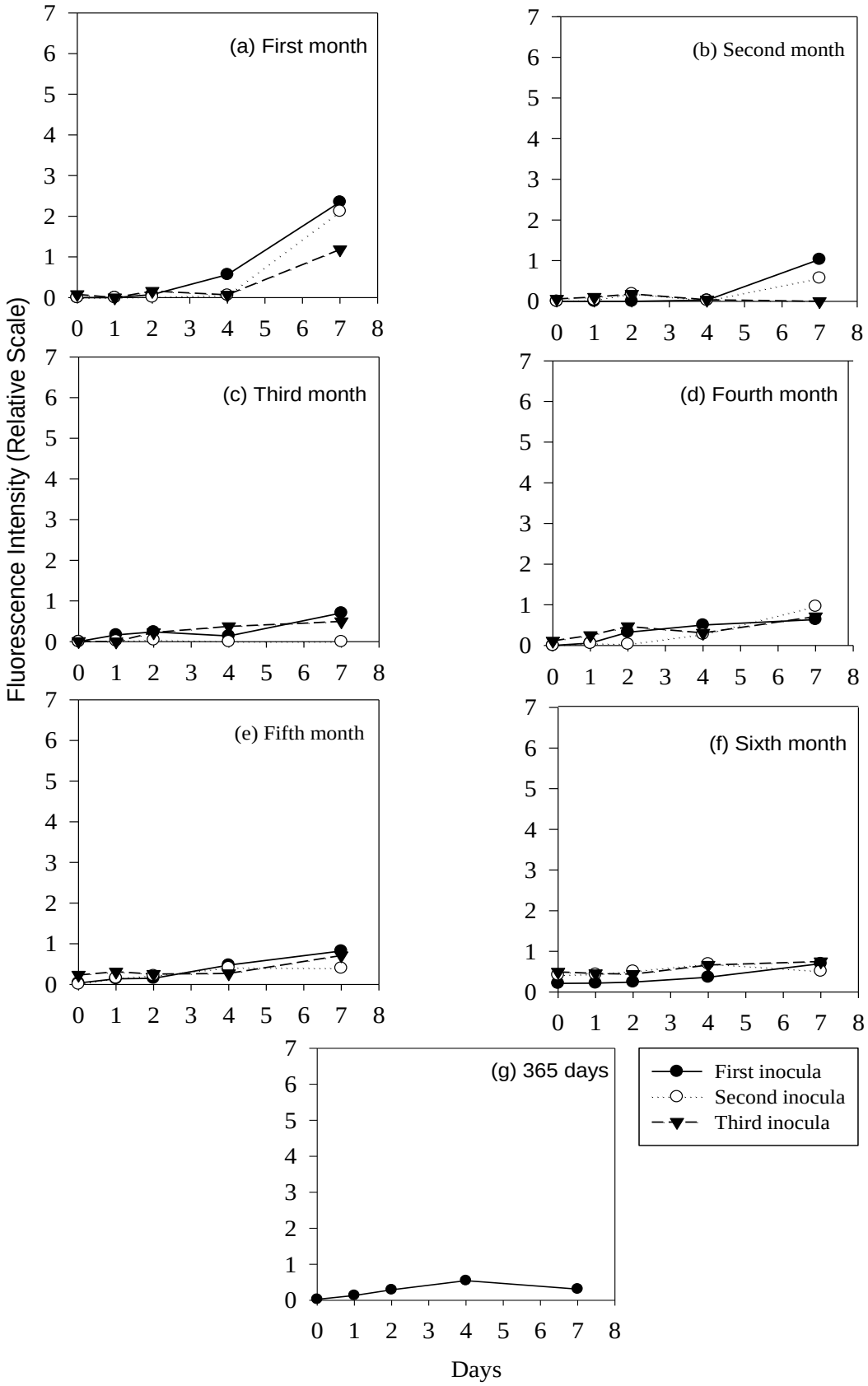


Figure 3.15 : Change in fluorescence intensity of beads prepared using 3.5% alginate + 4% $CaCl_2$.

The shrinkage in the diameter of the beads hardened with 2% and 4% cation solutions was shown in Figure 3.16 and Figure 3.17 respectively. The percent reductions in bead diameters for the first three set of inoculations (a,b,c) were rapid compared to the rest of the sets. This is may be related to the release of hardening agents into the storage solution (seawater) covering the beads during the storage and also during the first inoculations. Then, the alginate started to be released into the solutions.

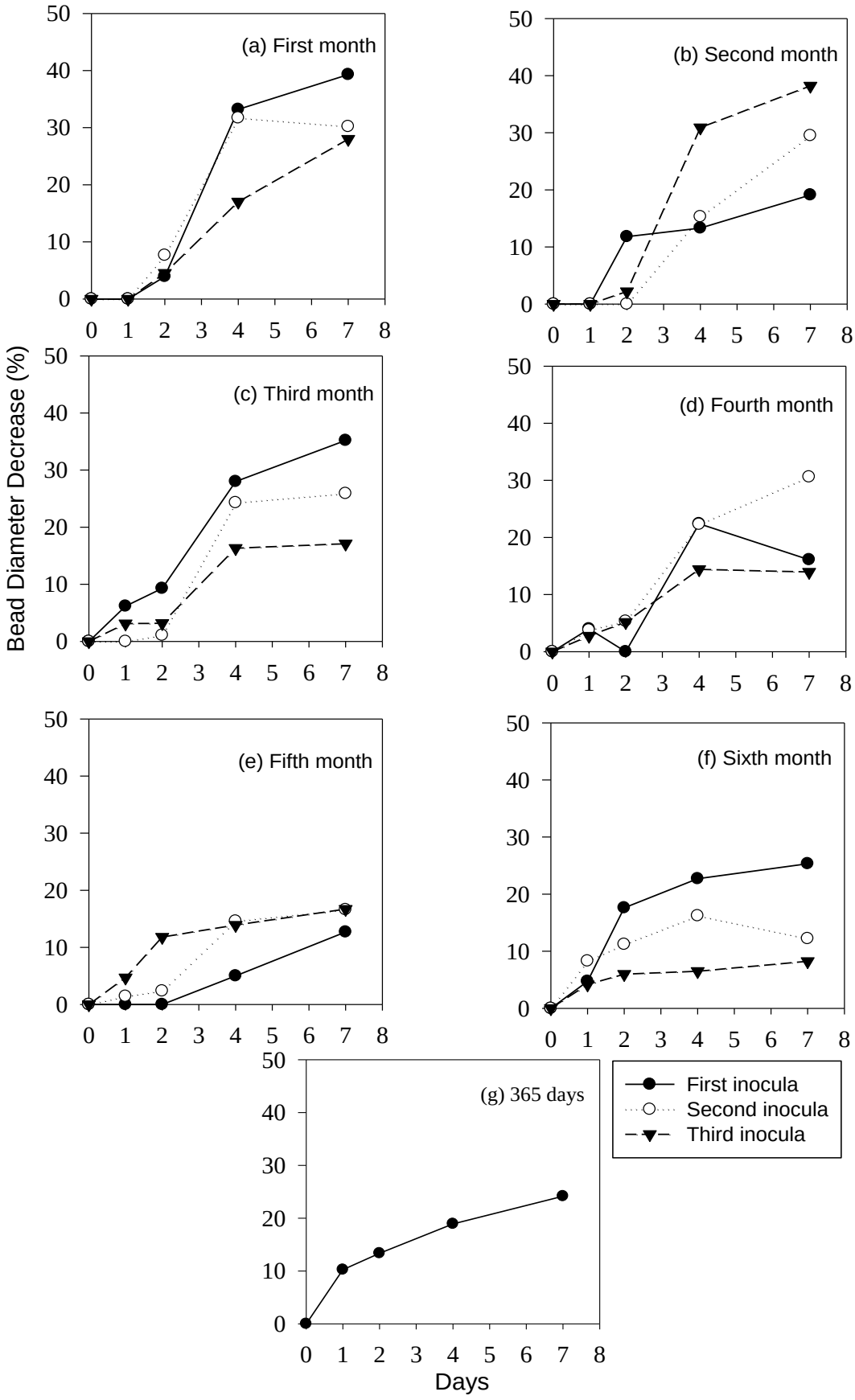


Figure 3.16 : Percentage decrease of bead diameter using 3.5% alginate+2% $CaCl_2$.

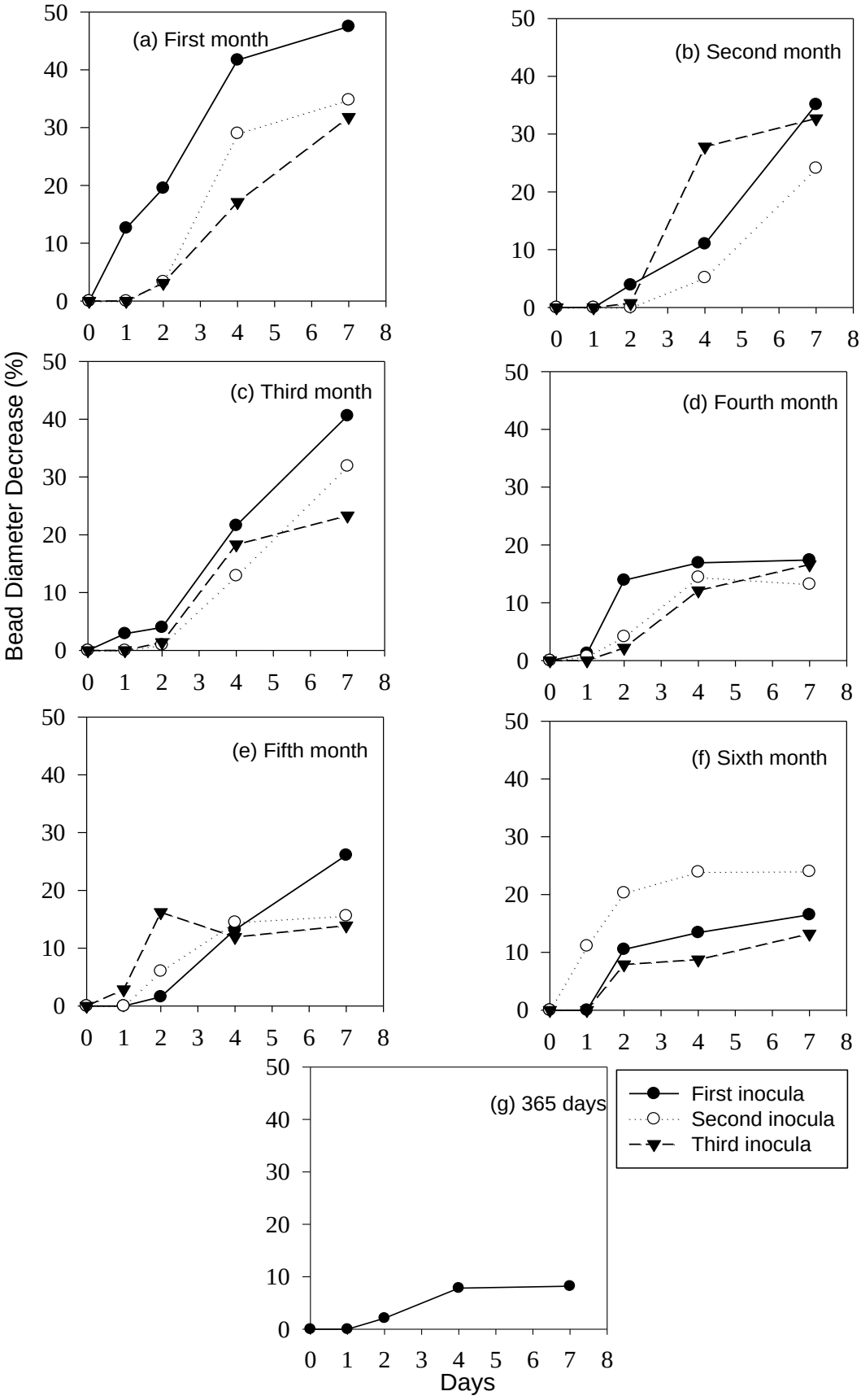


Figure 3.17 : Percentage decrease of bead diameter prepared using 3.5% alginate + 4% $CaCl_2$.

To evaluate the shrinkages in the diameter of the beads more clearly, two different Figures related to the diameters were drawn for both 3.5% alginate+2% $CaCl_2$ and 3.5% alginate+4% $CaCl_2$. Figure 3.18a and 3.19a of those Figures demonstrate the diameter of the beads versus storage time in the fridge, while Figure 3.18b and 3.19b represent the diameters of the beads after when those were inoculated for 7 days. According to those Figures, the shrinkage of the beads were rapid until 25-50 days of storage and then stayed nearly the same. The lines on the graphs demonstrates the mean value of the measurements, thus the mean diameter of the stored alginate beads hardened both with 4 and 2% $CaCl_2$ was about 4 mm. The inoculation of the beads for 7 days resulted in 25% reduction in their diameters. The standart deviation in the beads diameter were higher for the inoculated beads.

(a) (b)

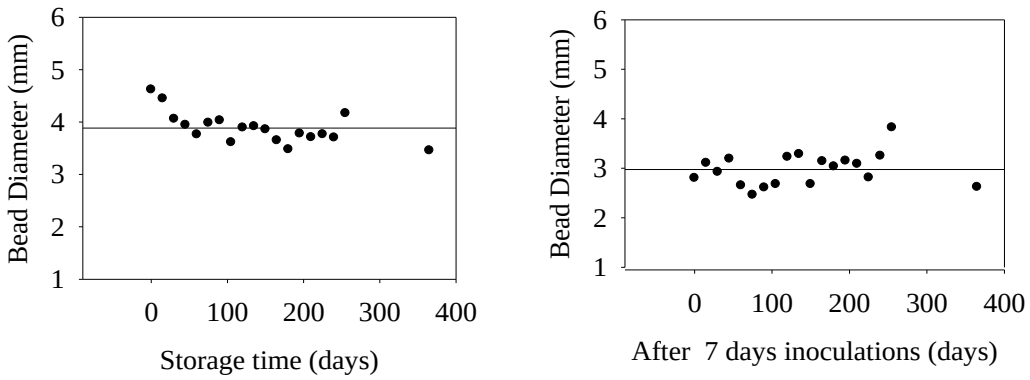


Figure 3.18 : The change in bead diameter of algal beads during storage (a) and after 7 days of inoculations (b) prepared from 3.5% alginate + 2% $CaCl_2$.

(a) (b)

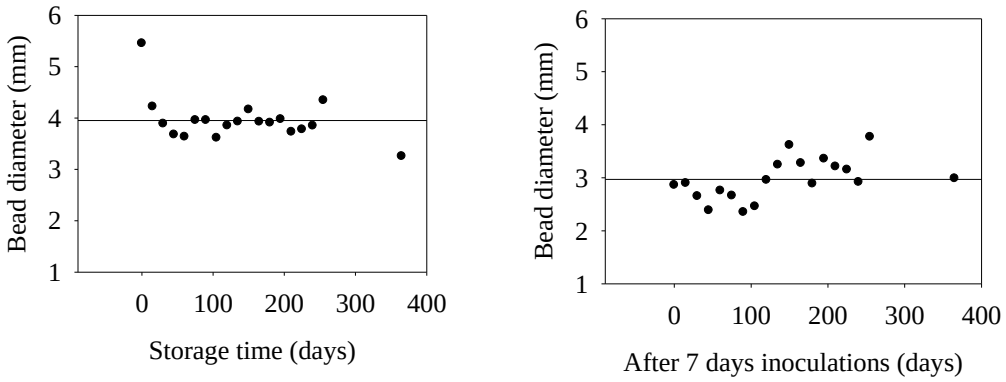


Figure 3.19 : The change in bead diameter of algal beads during storage (a) and after 7 days of inoculations (b) prepared from 3.5% alginate + 4% $CaCl_2$.

When those results regarding to cell production in different alginate and cation combinations and shrinkage of the beads prepared from those combinations were evaluated all together, no clear difference was observed in the diameters of the alginate beads prepared using different concentration of $CaCl_2$ solutions during the long-term storage. On the other hand, the productivity and growth rate of *Phaeodactylum tricornutum* cells immobilized in the beads prepared from 3.5% alginate solution and hardened using a 2% cation solution were found higher Therefore, those beads were selected to be used in toxicity testing and in nutrient removal studies due to the their suitability for the growth of *P. tricornutum* cells.

3.3 Toxicity Testing

Toxicity tests applied to PAHs (Phen and Ace) were accomplished by using two different species; Luminescence bacteria, *Vibrio fisheri* and microalgae, *Phaeodactylum tricornutum*. The dose-response curves for the toxicity tests performed with Ace and Phen were shown in Figure 3.20 and the EC_{20} values found from those curves were given in Table 3.5. The EC_{50} values could not be determined due to the low the effect levels of PAHs used during the experiments.

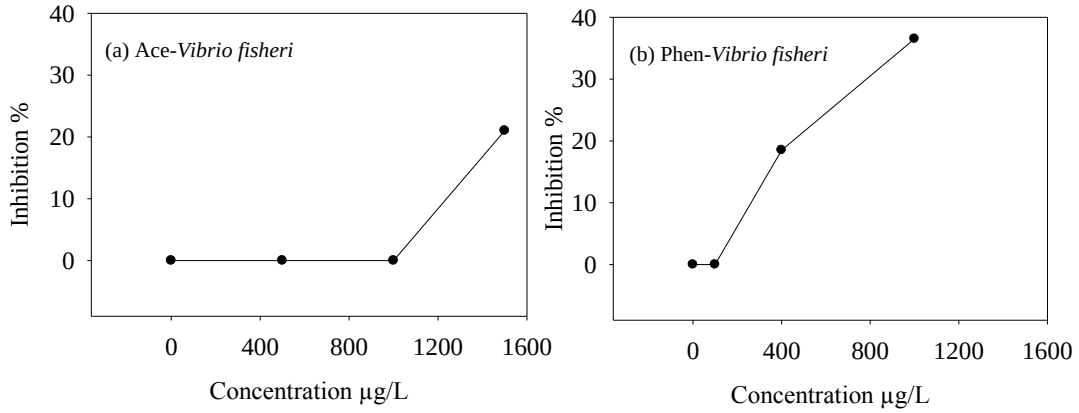


Figure 3.20 : Percentage inhibition graph of *Vibrio fisheri* using Ace (500, 1000 and 1500 µg L^{-1}) and Phen (100, 400 and 1000 µg L^{-1}).

For application of algal toxicity tests, several systems were used. First of all, the free cells of *Phaeodactylum tricornutum* were exposed to three different concentrations of PAH solutions for ten days in 250 mL test containers to see the general behaviour of the cultures in batch cultures. The growth of the cultures determined both by counting the cells and by measuring the fluorescence intensity of the cell suspensions. The growth curves of the cells grown in batch cultures were given in

Figure 3.21a and 3.22a for cell numbers and Figure 3.21b and 3.22b for fluoresecence intensity.

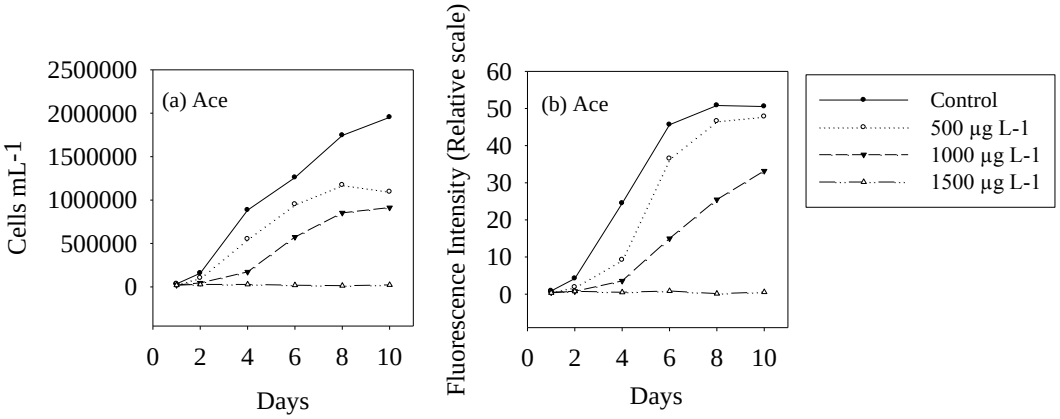


Figure 3.21 : The growth curve of the free cells of *P. tricornutum* were exposed to three different concentrations of Ace.

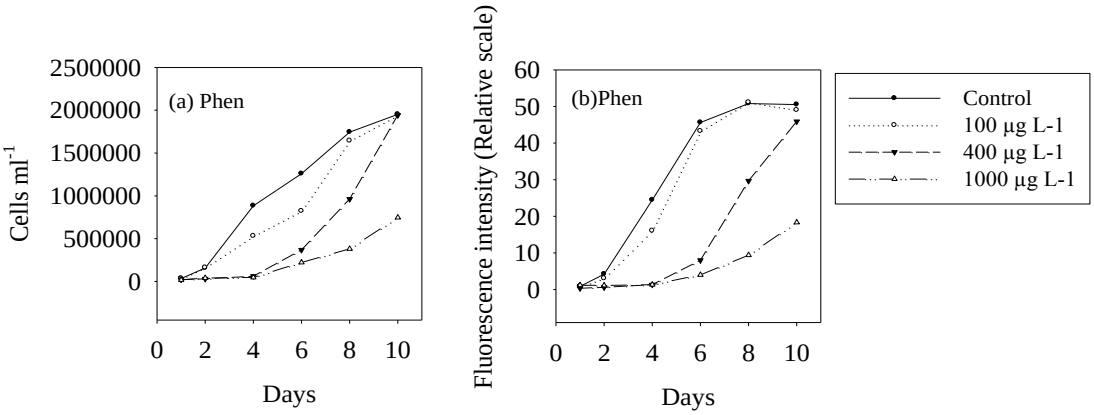


Figure 3.22 : The growth curve of the free cells of *P. tricornutum* were exposed to three different concentrations of Phen.

According to the literature (Rand, G.M., 1995) the effect of the toxic substances may affect the growth four different ways; the lag phase may be extended, the growth rates may be diminished, the total production (maximum number of algae at the stationary phase) may decrease or the cells may not have any chance to increase their numbers (Total toxicity).

Although, the first concentration of Phen (100 µg L^{-1}) did not show a clear affect compared to the control cultures, the other two concentrations showed their effect both in the lag phase and in the exponential phase; the lag phases extended and the growth rates were decreased. The control culture reached the steady state, but because the lag phases extended the two higher concentrations could not reach the

stationary phase until the end of the exposure experiment. Similarly, increasing concentrations of Ace affected the growth, and the cultures exposed to the highest concentrations of Ace were not able to grown during the test duration.

The EC_{50} values for the free cells exposed to PAHs were determined from the dose-response curves (Figure 3.23 and 3.24) by using the 4 days (96 hours) values of cell numbers and fluorescence intensity and given in Table 3.5.

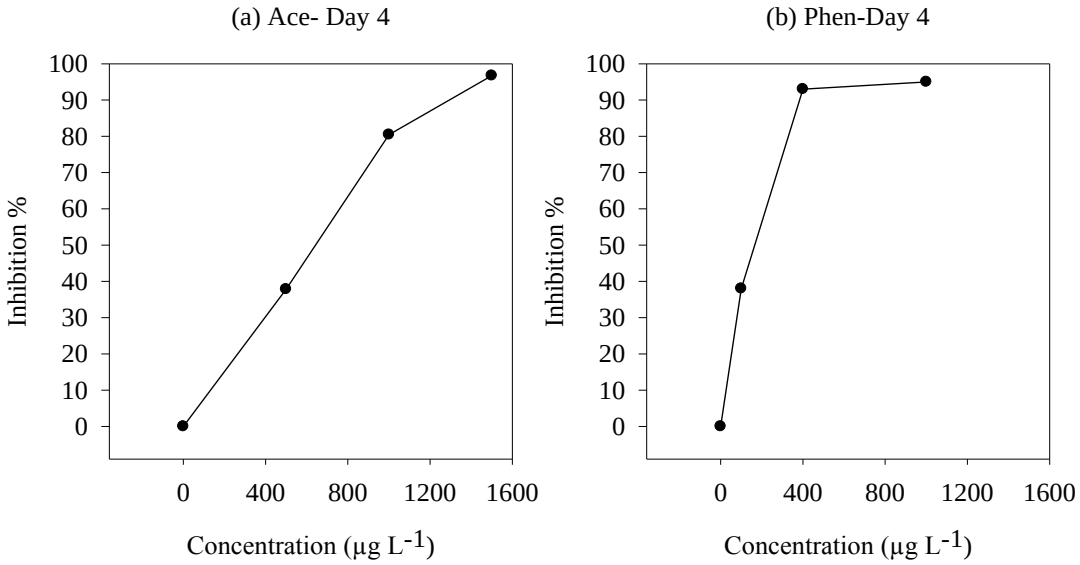


Figure 3.23 : Percentage inhibition values by using the cell numbers of the free cells exposed to Ace (500, 1000 and 1500 µg L^{-1}) and Phen (100,400 and 1000 µg L^{-1}).

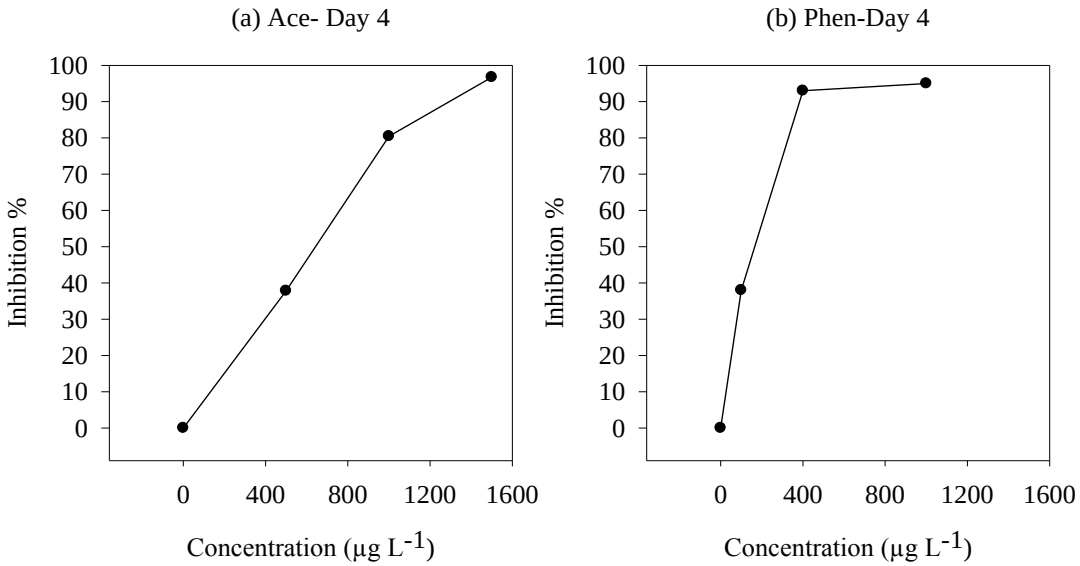


Figure 3.24 : Percentage inhibition values by using the fluorescence intensities of the free cells exposed to Ace (500, 1000 and 1500 µg L^{-1}) and Phen (100,400 and 1000 µg L^{-1}).

Free and immobilized cells were also incubated only for four days in smaller (40 mL) test containers batchwise, then the test was ended. The cell numbers and fluorescence intensity of PAH exposed cells were determined both at the beginning and at the end of the test duration. The results were used to draw the dose-response curves for Phen (Figure 3.26) and Ace (Figure 3.25). The EC 50 values determined from those curves were given in Table 3.5.

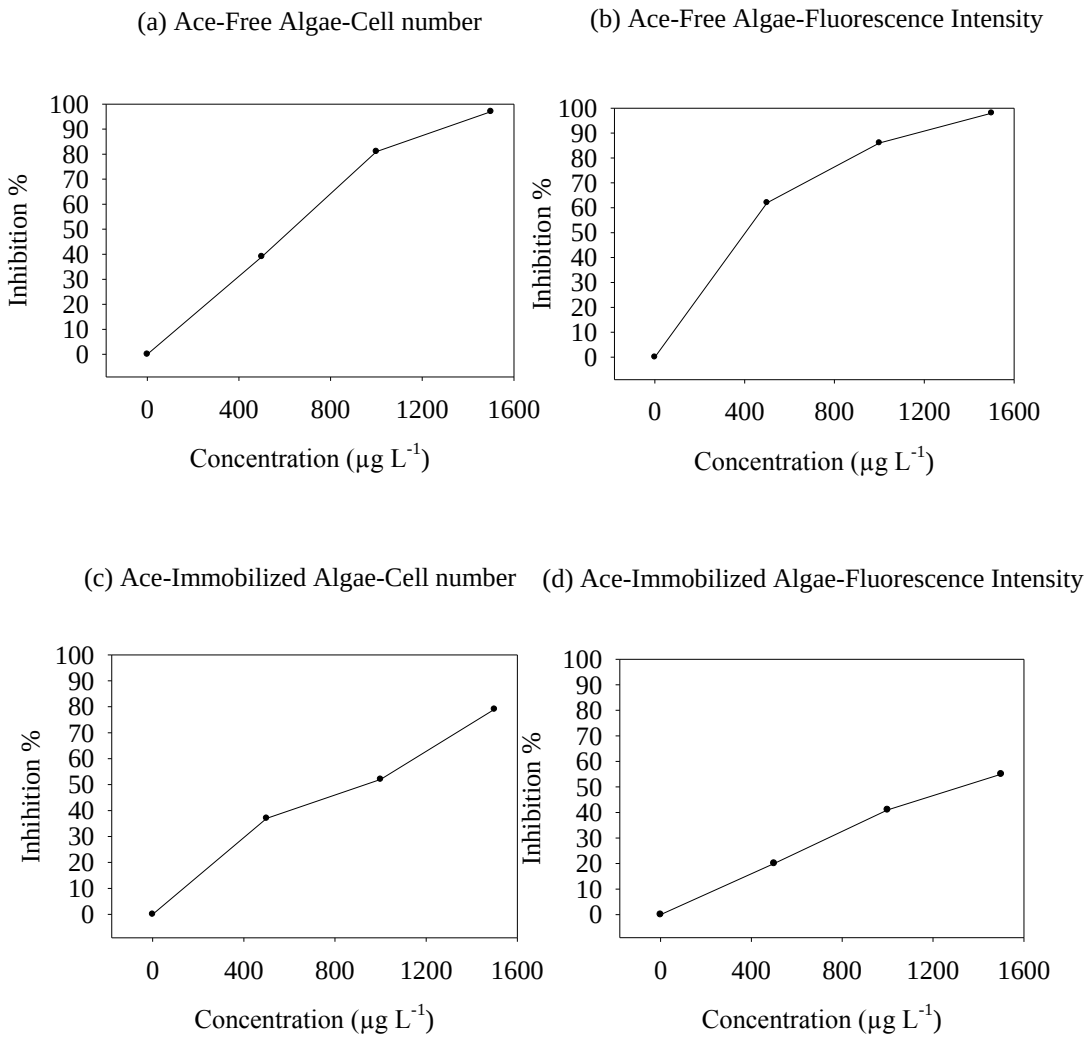


Figure 3.25 : Percentage inhibition values by using the cell numbers and fluorescence intensities of the immobilized and free cells exposed to Ace (500, 1000 and 1500 µg L^{-1}).

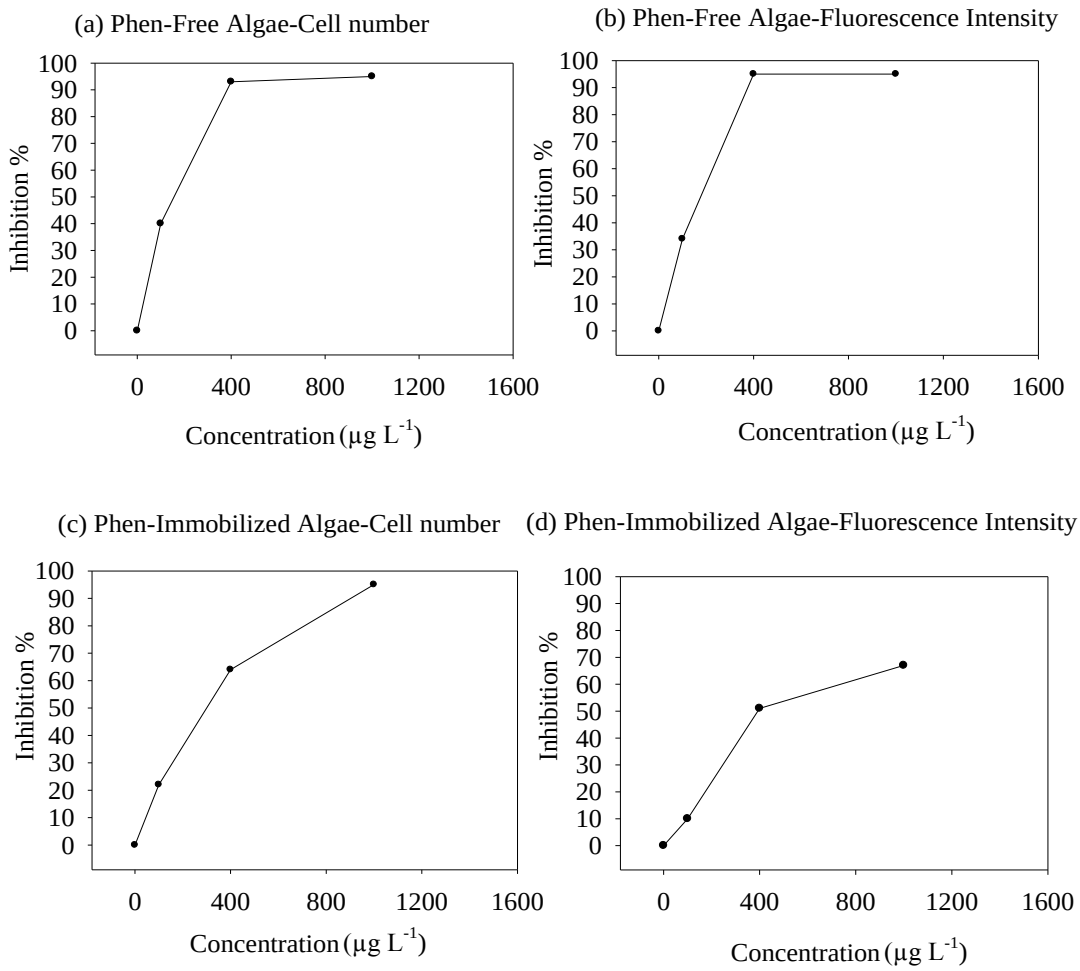


Figure 3.26 : Percentage inhibition values by using the cell numbers and fluorescence intensities of the immobilized and free cells exposed to Phen (100, 400 and 1000 µg L^{-1}).

Table 3.5: The EC_{20} ve EC_{50} values for the bacteria, free and immobilized algaes exposed to PAHs (Ace and Phen).

Organism	**PHEN**				**ACE**			
	EC20		EC50		EC20		EC50	
Vibrio fisheri	457		-		1474		-	
	PHEN (using CN [1])		**PHEN (usingFI[2])**		**ACE (using CN [1])**		**ACE (usingFI[2])**	
	EC20	EC50	EC20	EC50	EC20	EC50	EC20	EC50
Free-Algae (Day 4-Batch)	56	167	63	181	264	644	167	402
Free-Algae (4 days test)	54	152	65	181	253	643	167	401
Immobilized algae (4 days test)	87	303	172	405	274	971	496	1309

1. Cell number 2. Fluorescence intensity

In bacterial testing conducted using *Vibrio fischeri*, no EC_{50} value has been calculated for Ace and Phen. In toxicity testing, it has been observed that immobilized algal beads are not sensitive. Encapculation of algae inside the gel has provided a protective effect, with, however, a higher sensitivity compared with bacteria. It has been observed that both Ace and Phen have a higher toxic effect on free cells compared with the beads.

3.4 Nutrient Uptake Studies

The results of the nutrient uptake studies performed in batch systems were demonstrated in Figure 3.27. The uptake efficiencies were calculated for both blank beads (containing no algae) and for the algal beads.

The efficiency of the blank beads decreased steadily at each cycle, probably due to the surface area were almost occupied by adsorbed nutrients. Although the net uptake of nitrate was higher than the phosphate during the first run, when uptake by the blank beads were considered, the next three runs removal efficiencies of nutrients were comparable.

The net uptake of nitrate for the first run was 31%, whereas the efficiencies were increased for the next runs and found as 51%, 69% and 75% for the second, third and fourth treatment cycles respectively. On the other hand, net phosphate uptake efficiencies were calculated as 3% , 41% and 74% for the first three runs, but then decreased to 48%.

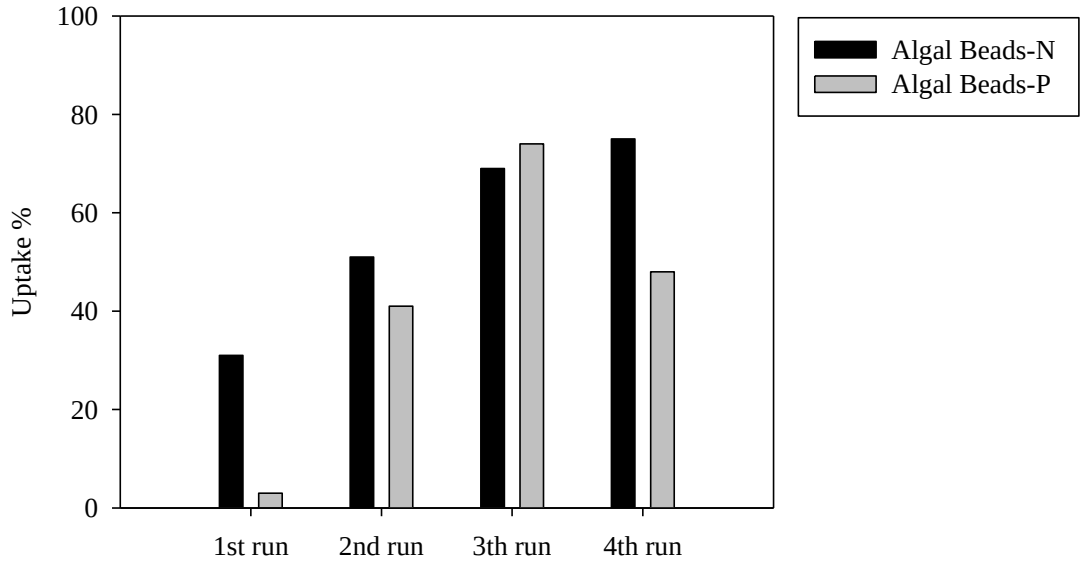


Figure 3.27 : Percentage nutrient (N and P) uptake of algal beads.

During the treatments, the release of the cells from the alginate beads to the medium is unwanted due to the seperation problem of the cells from the treated medium. For that reason, the release of the cells from the alginate beads during the 4-days incubation period was also determined by counting the free cells in the medium. The results showed that no algae was released for these total 16 days.

Although, the results indicate that the uptake of nitrate is more favorable compared to phosphate uptake by calcium alginate beads in a batch system, an efficient three sequenced treatment cycles can be applied to uptake the simultaneous uptake of both nutrients. Furthermore, no algae cells were released from the beads into the solution during the treatments. Thus, the algal beads from each treatment cycle can easily be seperated from the treated liquid (medium/effluent) and can be used for further treatments.

4. CONCLUSION

In short term experiment, firstly, the beads prepared by using two different alginate concentrations (2.5% and 5%) and hardened with 2% $CaCl_2$ were inoculated to determine the bead stability and to observe the growth of *Phaeodactylum tricornutum* algal culture. At the end of the experiment, those values for the bead diameters were decreased 40 and 24% of their initial values for 2.5 and 5% alginate solutions, respectively. The growth of *Phaeodactylum* was favorable in 2.5% alginate solution, but the stability of those beads were rather low. On the other hand, the beads from 5% alginate were more stable compared to the beads from 2.5%, but the growth was inhibited mainly probaby due to the restriction of light penetration and the diffusion of nutrients through the higher concentration of alginate. Thus, as a next step in the experiment, an alginate concentration in between (3.5%) was selected and hardened with 2% and 4% $CaCl_2$ to find out the appropriate combination to be used in further studies (long term experiments, toxicity testing and nutrient removal studies). No clear differences in the growth of algae were observed for beads in 3.5% alginate hardened both with 2 and 4% $CaCl_2$, although, as expected, beads hardened using 4% rather than 2% of calcium solution were found to be more stable. At the end of the experiment, the decrease in bead diameters in 16 days of inoculation was quantified as 24 and 35% for alginate solutions hardened with 4% and 2% $CaCl_2$ respectively. In a seperate experiment, the growth of the free cells were also determined to compare the results obtained from the immobilized cells. The cell numbers and fluorescence intencities of the free cells were found as 3000000 cells/mL. This result is the same with the cell concentration in beads obtained for 2.5% alginate and 2% cation. The fluorescence intensities obtained for the free cells are much higher compared to the intensities measured for the immobilized cells.

In long term experiment, 3.5% alginate solutions were used to prepare the alginate beads and inoculated with *P. tricornutum* to find out the long-term viability and growth. The cell numbers in 3.5 alginate beads hardened with 4% $CaCl_2$ reached nearly a constant value after 55-60 days and stayed roughly the same throughout the

experiment. On the other hand, the diminishing trend of the cell numbers continued for the beads hardened with 2% $CaCl_2$, although even 157 days, the cell numbers in those beads were still higher compared to the cell number in the beads hardened with 4% $CaCl_2$. Both alginate beads hardened with different cation concentrations attained the same cell concentration after 200 days of storage and following seven days of inoculation. After a year, the microalgae in the beads were almost lost their viability. In summary, because no clear difference was observed in the diameters of the alginate beads prepared using different concentration of $CaCl_2$ solutions during the long-term storage, the beads from 3.5% alginate solution and hardened using a 2% cation solution were selected to be used in toxicity testing and in nutrient removal studies due to the their suitability for the growth of *P. tricornutum* cells.

Toxicity tests applied to PAHs (Phen and Ace) were accomplished by using two different species; Luminescence bacteria, *Vibrio fisheri* and microalgae, *Phaeodactylum tricornutum*. The EC_{50} values could not determined for the bacteria, *Vibrio fisheri* by using both Ace and Phen. The free cells of *Phaeodactylum tricornutum* were exposed to three different concentrations of PAH solutions for ten days in 250 mL test containers to see the general behaviour of the cultures in batch cultures. The first concentration of Phen (100 $\mu g\ L^{-1}$) did not show a clear affect compared to the control cultures, the other two concentrations showed their effect both in the lag phase and in the exponential phase; the lag phases extended and the growth rates were decreased. Increasing concentrations of Ace affected the growth, and the cultures exposed to the highest concentrations of Ace were not able to grown during the test duration. Free and immobilized cells were also incubated only for four days in smaller (40 mL) test containers batchwise, then the test was ended. In toxicity testing, it has been observed that immobilized algal beads were not sensitive. Immobilization of algae inside the gel has provided a protective effect, with, however, a higher sensitivity compared with bacteria. It has been observed that both Ace and Phen have a higher toxic effect on free cells compared with the beads.

Nutrient(NO_3-N, PO_4-P) uptake studies were carried out in non-continuous systems and the beads were used in four successive applications. The net uptake of nitrate for the first run was 31%, whereas the efficiencies were increased for the next runs and found as 51%, 69% and 75% for the second, third and fourth treatment cycles respectively. On the other hand, net phosphate uptake efficiencies were calculated as

3% , 41% and 74% for the first three runs, but then decreased to 48%. the results indicate that the uptake of nitrate is more favorable compared to phosphate uptake by calcium alginate beads in a batch system, an efficient three sequenced treatment cycles can be applied to uptake the simultaneous uptake of both nutrients. Furthermore, no algae cells were released from the beads into the solution during the treatments. Thus, the algal beads from each treatment cycle can easily be seperated from the treated liquid (medium/effluent) and can be used for further treatments.

In summary, beads prepared using 3.5% Na-alginate concentration and 2% cation solution ($CaCl_2$) were found to be the most stable and suitable to *P. tricornutum* cells. The study shows that algal beads are efficient for toxicity experiments and nutrient (N and P) uptake. The biotechnology of algal immobilization is still an open area for various researches in different fields.

5. REFERENCES

Adams, MS, Stauber, JL., 2004. Development of a whole-sediment toxicity test using a benthic marine microalga. Environ Toxicol Chem; 23(8):1957–68.

Akhtar, N., J., Iqbal and M. Iqbal, 2004. Enhancment of lead (II) biosorption by microalgal biomass immobilized into loofa (*Luffa cylindrical*) sponge. Wiley Interscience J., 4: 171–8.

Alejandro, Ruiz-Marin, A., Mendoza-Espinosa, L.G., Stephenson, T., 2010. Growth and nutrient removal in free and immobilized green algae in batch and semi-continuous cultures treating real wastewater. Bioresource Technology 101 58–64.

Almeida, ELF, Silva, CMS., 2006. Formação de um mercado internacional de etanol e suas interrelações com os mercados de petróleo e açúcar. XI Congresso Brasileiro de Energia, Rio de Janeiro, Brazil.

Al-Qunaibit, M., Khalil, M., Al-Wassil, A., 2005. The effect of solvents on metal ion adsorption by the algae *Chorella vulgaris*. Chemospheres 60, 412–418.

Arica, M.Y., Bayramoglu, G., Yılmaz, M., Genç, O., Bektaş, S., 2004. Biosorption of Hg^{2+} , Cd^{2+} and Zn^{2+} by Ca-alginate and immobilized wood-rotting fungus *Funalia trogii*. J. Hazard. Mater. 109, 191–199.

Arica, M.Y., Bayramoglu, G., 2005. Cr(IV) biosorption from aqueous solution using free and immobilized biomass of *Lentinus sajorcaju*: preparation and kinetic characterization. Colloids Surf., A Physicochem. Eng. Asp. 253, 203–211.

Arkhypova, V.N., Dzyadevych, S.V., Soldatkin, A.P., El'skaya, A.V., Jaffrezic-Renault, N., Jaffrezic, H., Martelet, C., 2001. Multibiosensor based on enzyme inhibition analysis for determination of different toxic substances. Talanta 55, 919–927.

Bai FW, Anderson WA, Moo-Young M., 2008. Ethanol fermentation technologies from sugar and starch feedstocks. Biotechnol Adv; 26:89-105.

Baik,W.Y., Bae, J.H., Cho, K.M., Hartmeier,W., 2002. Biosorption of heavy metals using whole mold mycelia and parts thereof. Bioresour. Technol. 81, 167–170.

Bailliez C, Largeau C, Casadevall E., 1985. Growth and hydrocarbon production of *Botryococcus braunii* immobilized in calcium alginate gel. Appl Microbiol Biotechnol 23, 99–105.

Bailliez C, Largeau C, Berkaloff C, Casadevall E., 1986. Immobilization of *Botryococcus braunii* in alginate: influence on chlorophyll content, photosynthetic activity and degeneration during batch cultures. Appl Microbiol Biotechnol 23, 361–366.

Bailliez, C., Largeau, C., Casadevall, E., Yang, L. W. & Berkaloff, C., 1988. Photosynthesis, growth and hydrocarbon production of *Hotlyorocrus braunii* immobilized by entrapment and adsorption in polyurethane foams. Appl. Mimobiol. Biotechnol. 29: 141-7.

Banerjee A, Sharma R, Chisti Y, Banerjee UC., 2002. *Botryococcus braunii*: a renewable source of hydrocarbons and other chemicals. Crit Rev Biotechnol; 22:245– 79.

Bashan, Y., 1986. Alginate beads as synthetic inoculant carriers for the slow release of bacteria that affect plant growth. Appl. Environ. Microbiol. 51, 1089–1098.

Bashan, Y., 1998. Inoculants of plant growth-promoting bacteria for use in agriculture. Biotechnol. Adv. 16, 729–770.

Bashan, L. E., Bashan, Y., 2009. Immobilized microalgae for removing pollutants: Review of practical aspects. Environmental Microbiology Group, Northwestern Center for Biological Research (CIBNOR), La Paz, BCS, Mexico. The Bashan Foundation, Corvallis, OR, USA.. Bioresource Technology 101 (2010) 1611–1627.

Bayramoğlu, G., Bektas, S., Arica, M.Y., 2003. Biosorption of heavy metal ions on immobilized white-rot fungus Trametes versicolor. J. Hazard. Mater. 101, 285–300.

Bayramoğlu, G., Tuzun, I., Celik, G., Yilmaz , M., Arica, M.Y., 2006. Biosorption of mercury(II), cadmium(II) and lead(II) ions from aqueous system by microalgae *Chlamydomonas reinhardtii* immobilized in alginate beads. Int. J. Miner. Process. 81 (2006) 35–43.

Becker W., 2004. Microalgae for aquaculture, the nutritional Value of microalgae for aquaculture. In: Richmond A (ed.). Microalgae Culture, biotechnology and applied phycology. Blackwell Science Oxford, pp 380–391.

Ben-Amotz A, Avron M., 1987. The biotechnology of mass culturing of Dunaliella for products of commercial interest. In: Cresswell RC, Rees TAV, Shah N, editors. Algal and cyanobacterial technology. London: Longman; p. 90– 114.

Bertanza, G., Pedrazzani, R., Zambarda, V., Dal Grande, M., Icarelli, F., Baldassarre, L., 2010. Removal of endocrine disrupting compounds from wastewater treatment plant effluents by means of advanced oxidation. Water Sci. Technol. 61, 1663– 1671.

Borowitzka MA., 1986. Microalgae as sources of fine chemicals. Microbiol Sci; 3:372 – 5.

Borowitzka, M.A., Borowitzka, J.L., 1988. Micro-algal biotechnology. Cambridge University Press, Cambridge.

Borowitzka MA., 1999. Commercial production of microalgae: ponds, tanks, tubes and fermenters. J Biotechnol; 70:313– 21.

Brouers M, Hall DO., 1986. Ammonia and hydrogen production by immobilized cyanobacteria. J Biotechnol 3, 307–321.

Bubrick P., 1991. Production of astaxanthin from *Haematococcus*. Biores Technol; 38:237– 9.

Burton GA Jr, Greenberg MS, Rowland C, Irvine CA, Lavoie DR, Brooker JA, Moore L, Raymer DFN, McWilliam RA., 2005. In situ exposures using caged organisms: A multi-compartment approach to detect aquatic toxicity and bioaccumulation. Environ Pollut 134:133–144.

Carbonnelle D, Pondaven P, Moran¸cais M, Mass´e G, Bosch S, Jacquot C, Briand G, Robert JM, Roussakis C., 1999. Antitumor and antiproliferative effects of an aqueous extract from the marine diatom *Haslea ostrearia* (Simonsen) against solid tumors: lung carcinoma (NSCLC-N6), kidney carcinoma (E39) and melanoma (M96) cell lines. Anticancer Res 19:621–624.

Chen, Y.C., 2001. Immobilized microalga *Scenedesmus quadricauda* (Chlorophyta, Chlorococcales) for long-term storage and application for water quality control in fish culture. Aquaculture 195, 71–80.

Chen YC., 2003. Immobilized *Isochrysis galbana* (Haptophyta) for long-term storage and applications for feed and water quality control in clam (*Meretrix lusoria*). J Appl Phycol 15:439–444.

Chevalier, P., de la Noue, J., 1985. Wastewater nutrient removal with microalgae immobilized in carrageenan. Enzyme Microb. Technol. 7, 621–624.

Chouteau, C., Dzyadevych, S., Chovelon, J.M., Durrieu, C. 2004. Development of novel conductometric biosensors based on immobilised whole cell *Chlorella vulgaris* microalgae. Biosensors and Bioelectronics 19 1089–1096.

Codd, G.A., 1987. Immobilized micro-algae and cyanobacteria. Br. Phycol. Soc. Newslett. 24.

Cohen, Y., 2001. Biofiltration – the treatment of fluids by microorganisms immobilized into the filter bedding material: a review. Bioresour. Technol. 77, 257–274.

Crane M, Burton GA, Culp JM, Greenberg MS, Munkittrick KR, Ribeiro R, Salazar MH, St.-Jean SD., 2007. Review of aquatic in situ approaches for stressor and effect diagnosis. Integr Environ Assess Manag 3:234– 245.

Dainty AL, Goulding KH, Robinson PK, Simpkins I, Trevan MD. 1986. Stability of alginate–immobilized algal cells. Biotechnol Bioeng; 28:210–6.

de-Bashan LE, Bashan Y., 2004. Recent advances in removing phosphorus from wastewater and its future use as fertilizer (1997–2003). Water Res; 38:4222–46.

de-Bashan LE, Hernandez JP, Morey T, Bashan Y., 2004. Microalgae growth-promoting bacteria as "helpers" for microalgae: a novel approach for removing ammonium and phosphorus from municipal wastewater. Water Res; 38:466–74.

de la No¨ue J, De Pauw N., 1988. The potential of microalgal biotechnology: a review of production and uses of microalgae. Biotechnol Adv; 6:725–70.

Dennisson, M.J., Turner, A.P.F., 1995. Biosensors for environmental monitoring. Biotechnol. Adv. 13, 1–12.

Draget KI, Skjak-Bræk G, Smidsrod O., 1997. Alginate based new materials. Int J Biol Macromol; 21:47–55.

Duenas, J.F., Alonso, J.R., Rey, A.F., Ferrer, A.S., 2003. Characterisation of phosphorous forms in wastewater treatment plants. J. Hazard. Mater. 97, 1–3.

Dzadevych, S.V., Shul'Ga, A.A., Patskovsky, S.V., Arkhipova, V.N., Soldatkin, A.P., Strikha, V.I., 1994. Thin-film conductometric sensors for enzyme biotransducers. Russ. J. Electrochem. 30 (8), 987–991.

Ertugrul, S., Baklr, M., Dumez, G., 2008. Treatment of dye-rich wastewater by an immobilized thermophilic cyanobacterial strain: *Phormidium sp.* Ecol. Eng. 32, 244–248.

Faafeng BA, Van Donk E, Ka¨llqvist ST., 1994. In situ measurement of algal growth potential in aquatic ecosystems by immobilized algae. J Appl Phycol 6:301–308.

Falkowsky, P.G., 1980. Primary productivity in the sea. In: Falkowski, P.G. (Ed.), Environmental Science Research, vol. 19. Plenum Press, New York/London.

Fraser P, Chilvers C., 1981. Health aspects of nitrate and drinking water. In: Van Lelyveld H, Zoeteman BCJ, editors. Water Supply and Health. Oxford: Elsevier; 103–16.

Frense D, M¨uller A, Beckmann D., 1998. Detection of environmental pollutants using optical biosensor with immobilized algae cells. Sens Actuators B-Chem 51:256–260.

Galarneau E, Gehr R., 1997. Phosphorus removal from wastewaters: experimental and theoretical support for alternative mechanisms. Water Res; 31:328–38.

Gao, Q.T., Wong, Y.S., Tam, N.F.Y., 2011. Removal and biodegradation of nonylphenol by different *Chlorella species*. Mar. Pollut. Bull. 63, 445–451.

Gao, Q.T., Wong, Y.S., Tam, N.F.Y., 2011. Removal and biodegradation of nonylphenol by immobilized *Chlorella vulgaris*. Bioresource Technology 102 10230–10238.

Garbayo, I., Vigara, A.J., Conchon, V., Martins Dos Santos, V.A.P., Vılchez, C., 2000. Nitrate consumption alterations induced by alginate-entrapment of *Chlamydomonas reinhardtii* cells. Process Biochemistry 36 459–466.

Gaserod O, Sannes A, Skjak-Bræk G., 1999. Microcapsules of alginate–chitosan. II. A study of capsule stability and permeability. Biomaterials; 20:773–83.

Gaudin, P., Lebeau, T., Robert, J.M., 2006. Microalgal cell immobilization for the long-term storage of the marine diatom *Haslea ostrearia*. Springer Science+Business Media B.V. J Appl Phycol 18:175–184.

Guillard, R.R.L., Ryther, J. H., 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* (Hustedt) and *Detonula confervacea* (Cleve). Can. J. Microbiol. 8, 229-239.

Guisan, J. M., 2006. Immobilization of Enzymes and Cells. Institute of Catalysis, CSIC Campus UAM–Cantoblanco Madrid, Spain. Humana Press Inc. 999 Riverview Drive, Suite 208 Totowa, New Jersey 07512.

Hahn, J.J., Ghirardi, M.L., Jacoby, W.A., 2007. Immobilized algal cells used for hydrogen production. Biochemical Engineering Journal 37, 75–79.

Hertzberg, S., Jensen, A., 1989. Studies of alginate-immobilized marine microalgae. Bot. Mar. 32, 267–273.

Hiller UW, Park RB., 1969. Photosynthetic light reactions in chemically fixed *Anacystis nidulans*, *Chlorella pyrenoidosa* and *Phormidium cruentum*. Physiol Plant 44, 535–539.

Jeanfils J, Collard F., 1983. Effect of immobilized *S. obliquus* cells in a matrix in oxygen evolution and fluorescence properties. Eur J Appl Microbiol Biotechnol 17, 254–257.

Jimenez-Perez, M.V., Sanchez-Castillo, P., Romera, O., Fernandez-Moreno, D., Perez- Martinez, C., 2004. Growth and nutrient removal in free and immobilized planktonic green algae isolated from pig manure. Enzyme Microb. Technol. 34, 392–398.

Kayno H, Karube I, Matsunaga T, Suzuki S, Nakayama O., 1981. A photochemical fuel cell system using *Anabaena* N-7363. Eur J Microbiol Biotechnol 12, 1–5.

Krylova AY, Kozyukov EA, Lapidus AL. 2008. Ethanol and diesel fuel from plant raw materials: a review. Solid Fuel Chem; 42:358–64.

Lamelas, C., Avaltroni, F., Benedetti, M., Wilkinson, K.J., Slaveykova, V.L., 2005. Quantifying Pb and Cd complexation by alginates and the role of metal binding on macromolecular aggregation. Biomacromolecules 6, 2756–2764.

Lashinky A, Schwartz ND., 2006. How to beat the high cost of gasoline.

Lau PSA, Tam NFY, Wong YS., 1997. Wastewater nutrients (N and P) removal by carrageenan and alginate immobilized *Chlorella vulgaris*. Environ Technol; 18:945–51.

Lau PS, Tam NFY, Wong YS., 1998. Effect of carrageenan immobilization on the physiological activities of *Chlorella vulgaris*. Bioresource Technol 63, 115–121.

Lebeau T, Moan R, Turpin V, Robert JM, 1998. Alginateentrapped *Haslea ostrearia* as inoculum for the greening of oysters. Biotechnol Tech 12:847–850.

Lebeau, T., Robert, J.M., 2006. Biotechnology of immobilized micro-algae: a culture technique for the future? In: Rao, S. (Ed.), Algal Cultures, Analogues of Blooms and Applications. Science Publishers, Enfield, NH, pp. 801–837.

Leenen, E.J.T.M., dos Santos, V.A.P., Grolle, K.F.C., Tramper, J., Wijffels, R.H., 1996. Characteristics of and selection criteria for support materials for cell immobilization in wastewater treatment. Water Res. 30, 2985–2996.

Leon R, Galvan F., 1995. Glycerol photoproduction by free and calcium-entrapped cells of *Chlamydomonas reinhardtii*. J Biotechnol 42, 61–67.

Li HB, Chen F, Zhang TY, Yang FQ, Xu GQ., 2001. Preparative isolation and purification of lutein from the microalga *Chlorella vulgaris* by high-speed counter-current chromatography. J Chromatogr A; 905:151–5.

Liu, Y.K., Seki, M., Tanaka, H., Furusaki, S., 1998. Characteristics of loofa (*Luffa cylindrica*) sponge as a carrier for plant cell immobilization. J. Ferment. Bioeng. 85, 416–421.

Luan, T.G., Jin, J., Chan, S.M.N., Wong, Y.S., Tam, N.F.Y., 2006. Biosorption and biodegradation of tributyltin (TBT) by alginate immobilized *Chlorella vulgaris* beads in several treatment cycles. Process Biochem. 41, 1560–1565.

Mallick, N., 2002. Biotechnological potential of immobilized algae for wastewater N, P and metal removal: a review. Biometals 15, 377–390.

Martinsen A., Skj°ak-Bræk G, Smidsrød O., 1989. Alginate as immobilization material. I. Correlation between chemical and physical properties of alginate gel beads. Biotechnol Bioeng; 33:79–89.

Molina Grima E., 1999. Microalgae, mass culture methods. In: Flickinger MC, Drew SW, editors. Encyclopedia of bioprocess technology: fermentation, biocatalysis and bioseparation, vol. 3. New York: Wiley; p. 1753– 69.

Molina Grima E., Acie´n Ferna´ndez FG, Garcia Camacho F, Chisti Y., 1999. Photobioreactors: light regime, mass transfer, and scale-up. J Biotechnol; 70:231– 48.

Montaigne, F., Essick, P., 2002. Water pressure. Natl. Geogr. 202, 2–33.

Montaini E., Chini Zittelli G., Tredici MR., Molina Grima E., Fern´andez Sevilla JM., S´anchez P´erez JA., 1995. Long-term preservation of *Tetraselmis suecica*: influence of storage on viability and fatty acide profile. Aquaculture 134:81–90.

Moreira dos Santos, M., Moreno-Garrido I., Gonc alves F., Soares A.M.V.M., Ribeiro R., 2002. An in situ bioassay with microalgae for estuarine environments. Environ. Toxicol. Chem. 21 (3), 567– 574.

Moreira dos Santos M, Moreno-Garrido I, Gonc¸alves F, Soares AMVM, Ribeiro R., 2002. An in situ bioassay for estuarine environments using the microalga *Phaeodactylum tricornutum*. Environ Toxicol Chem; 21:567–74.

Moreira SM., Guilhermino L., Ribeiro R., 2006. An in situ assay with the microalga *Phaeodactylum tricornutum* for sediment-overlying water toxicity evaluations in estuaries. Environ Toxicol Chem 25:2272–2279.

Moreira S.M., Moreira-Santos, M., Guilhermino, L., Ribeiro, R., 2006. Immobilization of the marine microalga *Phaeodactylum tricornutum* in alginate for in situ experiments: bead stability and suitability. Enzyme Microb. Technol. 38, 135–141.

Morelli E., Scarano G., 2001. Synthesis and stability of phytochelatins induced by cadmium and lead in the marine diatom *Phaeodactylum tricornutum*. Mar. Environ. Res. 52, 383–395.

Moreno-Garrido I, Lubi´an LM, Soares AMVM., 2000. Influence of cellular density in determination of EC_{50} in microalgal growth inhibition tests. Ecotoxicol Environ Saf; 47:112–6.

Moreno-Garrido I, Hampel M, Lubián LM, Blasco J., 2003a. Sediment toxicity tests using benthic marine microalgae *Cylindrotheca closterium* (Ehremberg) Lewin and Reimann (Bacillariophyceae). Ecotoxicol Environ Saf; 54:290–5.

Moreno-Garrido I, Hampel M, Lubián LM, Blasco J., 2003b. Marine benthic microalgae *Cylindrotheca closterium* (Ehremberg) Lewin and Reimann (Bacillariophyceae) as a tool for measuring toxicity of linear alkylbenzene dulfonate (LAS) in sediments. Bull Environ Contam Toxicol; 70(2):242–7.

Moreno-Garrido I., Campana O., Lubia´n, L.M., Blasco, J., 2005. Calcium alginate immobilized marine microalgae: Experiments on growth and short-term heavy metal accumulation. Mar. Pollut. Bull. 51, 823–929.

Moreno-Garrido I., Lubián, L.M., Blasco, J., 2007. Sediment toxicity tests involving immobilized microalgae (*Phaeodactylum tricornutum* Bohlin, 1897). Environment International 33 481–495.

Moreno-Garrido I., 2008. Microalgae immobilization : Current techniques and uses: a review. Bioresource Technology 99 3949–3964.

Moreno-Garrido I., 2008. Microalgae immobilization: current techniques and uses. Bioresour. Technol. 99, 3949–3964.

Moreira-Santos M., Antunes S.C., Goncalves F., Soares A., and Riberio R., 2011. An In Situ Toxicity Assay With The Local Phytoplankton Community. Environmental Toxicology and Chemistry, Vol. 30, No. 1, pp. 196–205.

Munawar M., Munawar IF., 1987. Phytoplankton bioassays for evaluating toxicity of in situ sediment contaminants. Hydrobiologia; 149:87-105.

Murano, E., 1998. Use of natural polysaccharides in the microencapsulation techniques. J. Appl. Ichthyol. 14, 245–249.

Musgrave SC, Kerby NY., Codd GA., Stewart WDP., 1983. Structural features of calcium alginate entrapped cyanobacteria modified for ammonia production. Eur J Appl Microbiol Biotechnol 17, 133–136.

Mussatto S. I., Dragone G., Guimarães P.M.R., Silva, J.P.A., Carneiro L.M., Roberto I.C., Vicente A., Domingues L., Teixeira J.A., 2010. Technological trends, global market, and challenges of bio-ethanol production. Research review paper. Biotechnology Advances 28 817–830.

Naessens M., Tran-Minh C., 1999. Biosensor using immobilized *Chlorella* microalgae for determination of volatile organic compounds. Sensors and Actuators B 59; 100–102.

Nakada N., Shinohara H., Murata A., Kiri K., Managaki S., Sato N., Takada H., 2007. Removal of selected pharmaceuticals and personal care products (PPCPs) and endocrine-disrupting chemicals (EDCs) during sand filtration and ozonation at a municipal sewage treatment plant. Water Res. 41, 4373–4382.

Papageorgiou G.C., 1987. Immobilized photosynthetic microorganisms. Photosynthetica 21, 367–383.

Park RB, Kelly J, Drury S, Sauer K., 1966. The Hill reaction of chloroplasts isolated from glutaryldehyde-fixed spinach leaves. Proc Natl Acad Sci USA 55, 1056–1062.

Pereira AMM, Soares AMVM, Gonçalves F, Ribeiro R., 1999. Test chambers and test procedures for in situ toxicity testing with zooplankton. Ecotoxicol Environ Chem; 18(9):1956–64.

Prakasham, R.S., Sheno Merrie, J., Sheela, R., Saswathi, N., Ramakrishna, S.V., 1999. Biosorption of chromium VI by free and immobilized *Rhizopus arrhizus*. Environ. Pollut. 104, 421–427.

Pulz O, Scheibenbogen K, Gross W., 2001. Biotechnology with cyanobacteria and microalgae. In: Rehm H.-J., Reed G, editors. Biotechnology, vol. 10. Weinheim: Wiley-VCH; p. 105–36.

Pulz O., 2001. Photobioreactors: production systems for phototrophic microorganisms. Appl Microbiol Biotechnol; 57:287– 93.

Radix P., Léonard M., Papantoniou C., Roman G., Saouter E., Gallotti-Schmitt S., 2000. Comparison of four chronic toxicity tests using algae, bacteria and invertebrates with sixteen chemicals. Ecotoxicol Environ Saf; 47:186–94.

Rai LC., Mallick N., 1992. Removal and assessment of toxicity of Cu and Fe to *Anabaena doliolum* and *Chlorella vulgaris* using free and immobilized cells. World J Microbiol Biotechnol 8, 110–114.

Rashid N., Song W., Park J., Jin H.-F., Lee, K., 2009. Characteristics of hydrogen production by immobilized cyanobacterium *Microcystis aeruginosa* through cycles of photosynthesis and anaerobic incubation. Journal of Industrial and Engineering Chemistry 15, 498–503.

Rashid N., Lee K., Mahmood Q., 2011. Bio-hydrogen production by *Chlorella vulgaris* under diverse photoperiods. Department of Environmental Engineering and Biotechnology, Myongji University, Yongin, Republic of Korea Department of Environmental Sciences, COMSATS Institute of Information Technology, Abbottabad 22060, Pakistan. Bioresource Technology 102 2101–2104.

Reshetilov A.N., Iliasov P.V. and Reshetilova T.A., 2010. The Microbial Cell Based Biosensors. Institute of Biochemistry & Physiology of Microorganisms, Russian Academy of Sciences Russian Federation.

Robert JM., 1983. Fertilit´e des eaux de claires ostr´e¨ıcoles et verdissement: utilisation de l'azote par les diatom´ees dominantes. D Sc thesis University of Nantes France.

Robert R., Parisi G., Rodolfi L., Poli BM., Tredici MR., 2001. Use of fresh and preserved *Tetraselmis suecica* for feeding *Crassostrea gigas* larvae. Aquaculture 192:333–346.

Robinson PK., Dainty AL., Goulding KH., Simpkins I., Trevan MD., 1985. Physiology of alginate-immobilized *Chlorella*. Enzyme Microb Technol 7, 212–216.

Robinson, P.K., Mak, A.L., Trevan, M.D., 1986. Immobilized algae: a review. Process Biochem. (August), 122–126.

Romo S, P´erez-Mart´ınez C., 1997. The use of immobilization in alginate beads for long-term storage of *Pseudoanabaena galeata* (Cyanobacteria) in the laboratory. J Phycol 33:1073–1076.

Ruiz-Marin, A., Mendoza-Espinosa, L.G., Stephenson, T., 2010. Growth and nutrient removal in free and immobilized green algae in batch and semi-continuous cultures treating real wastewater. Bioresour. Technol. 101, 58–64.

Sa´nchez Miro´n A., Contreras Go´mez A., Garcı´a Camacho F., Molina Grima E., Chisti Y., 1999. Comparative evaluation of compact photobioreactors for large-scale monoculture of microalgae. J Biotechnol; 70:249–70.

Santos-Rosa, F., Galvan, F., Vega, J.M., 1989. Photoproduction of ammonium by *Chlamydomonas reinhardtii* cells immobilized in barium alginate: a reactor feasibility study. Appl. Microbiol. Biotechnol. 32, 285–290.

Saygideger S., Gulnaz O., Istifli E.S., Yucel N., 2005. Adsorption of Cd(II), Cu(II) and Ni(II) ions by Lemna minor L.: effect of physicochemical environment. J. Hazard. Mater. 126, 96–104.

Semple K.T., Cain R.B., Schmidt S., 1999. Biodegradation of aromatic compounds by microalgae. FEMS Microbiol. Lett. 170, 291–300.

Shul'Ga A., Dzyadevych S.V., Soldatkin A.P., Patskovsky S.V., Strikha V.I., El'Skaya A.V., 1994. Thin-film conductometric biosensors for glucose and urea determination, Biosens. Bioelectron. 9.

Smidsrød O., Skj°ak-Bræk G., 1990. Alginate as immobilization matrix for cells. Trends Biotechnol; 8:71–8.

Soares A., Guieysse B., Jefferson B., Cartmell E., Lester J.N., 2008. Nonylphenol in the environment: a critical review on occurrence, fate, toxicity and treatment in wastewaters. Environ. Int. 34, 1033–1049.

Stauber, JL, Florence, TM., 1990. Mechanism of toxicity of zinc to the marine diatom *Nitzschia closterium*. Mar Biol; 105:519–24.

Standard Methods for the Examination of Water and Wastewater, 16th Edition, APHA, AWWA, WPCF, 1985.

Stratful, I., Brett, S., Scrimshaw, M.B., Lester, J.N., 1999. Biological phosphorus removal, its role in phosphorus recycling. Environ. Technol. 20, 681–695.

Sumi, Y., 2009. Microalgae Pioneering the Future Application and Utilization. Life Science Research Unit. Quarterly R eview N o. 3 4.

Tam, N.F.Y., Lau, P.S., Wong, Y.S., 1994. Wastewater inorganic N and P removal by immobilized *Chlorella vulgaris*. Water Sci. Technol. 30, 369–374.

Tampion, J, Tampion, MD., 1987. Immobilized Cells: Principles and Applications. Cambridge UK: Cambridge University Press; 257 pp.

Tamponnet, C., Costantino, F., Barbotin, J. N. & Calvayrac, R., 1985. Cytological and physiological hehaviour of *Euglena gratillis* cells entrapped in a calcium alginate gel. Physiol. Plant. 63:277-83.

Thepenier, C., Gudin, C., Thomas, D., 1985. Immobilization of *Porphyridium cruentum* in polyurethane foams for the production of polysaccharide. Biomass 7, 225–240.

Tredici MR., 1999. Bioreactors, photo. In: Flickinger MC, Drew SW, editors. Encyclopedia of bioprocess technology: fermentation, biocatalysis and bioseparation, vol. 1. New York: Wiley; p. 395– 419.

Trepanier, C., Parent, S., Comeau, Y., Bouvrette, J., 2002. Phosphorus budget as a water quality management tool for closed aquatic mesocosms. Water Res. 36, 1007–1017.

Trevan, MD, Mak AL., 1988. Immobilized algae and their potential for use as biocatalysts. Trends Biotechnol 6, 68–73.

Trevors, J.T., Stratton, G.W., Gadd, G.M., 1986. Cadmium transport, resistance, and toxicity in bacteria, algae, and fungi. Can. J. Microbiol. 32, 447–464.

Tunali, S., Akar, T., Özcan, A.S., Kiran, I., Özcan, A., 2006a. Equilibrium and kinetics of biosorption of lead(II) from aqueous solutions by Cephalosporium aphidicola. Sep. Purif. Technol. 47, 105–112.

Turner A.P.F., Karube I., Wilson G.S., 1987. Biosensors, Fundamentals and Applications. Oxford University Press, Oxford.

Ueno, R., Wada, S., Urano, N., 2008. Repeated batch cultivation of the hydrocarbondegrading, micro-algal strain *Prototheca zopfii* RND16 immobilized in polyurethane foam. Can. J. Microbiol. 54, 66–70.

Valderrama LT, Del Campo CM, Rodriguez CM, de-Bashan LE, Bashan Y., 2002. Treatment of recalcitrant wastewater from ethanol and citric acid production using the microalga *Chlorella vulgaris* and the macrophyte *Lemna minuscula*. Water Res; 36:4185–92.

Valenzuela-Espinoza E, Millán-Núñez R, Núñez-Cebrero F., 1999. Biomass production and nutrient uptake by *Isochrysis aff.* Galbana (clone T-ISO) cultured with low cost alternative to the f/2 medium. Aquacul. Engng. 20: 135–147.

Van Loosdrecht, M.C.M., Hooijmans, C.M., Brdjanovic, D., Heijnen, J.J., 1997. Biological phosphate removal processes. Appl. Microbiol. Biotechnol. 48, 289–296.

Vijayaraghavan, K. et al., 2004. Trends in hydrogen production – a review. Journal of Integrative Environmental Sciences 3, 255–271.

Vilar, V.J.P., Botelho, C.M.S., Boaventura, R.A.R., 2005. Influence of pH, ionic strength and temperature on lead biosorption by *Gelidium* and agar extraction algal waste. Process Biochem. 40, 3267–3275.

Vilchez C, Vega JM., 1994. Nitrate uptake by *Chlamydomonas reinhardtii* cells immobilized in calcium alginate. Appl Microbiol Biotechnol 41, 137–141.

Volkman JK, BrownMR., 2006. Nutritional Value of Microalgae and Applications. In: Rao S (ed.). Algal cultures, analogues of blooms and applications. Science Publishers Inc New Hampshire, pp 407–457.

Wan, K., Chovelon, J.M., Jaffrezic-Renault, N., Soldatkin, A.P., 1999. Sensitive detection of pesticides using ENFET with enzymes immobilized by cross linking and entrapment method. Sens. Actuators B 58, 399–408.

Wideroe H, Danielsen S., 2001. Evaluation of the use of Sr^{2+} in alginate immobilization of cells. Naturwissenschaften; 88:224–8.

Wiegman, S., Termeer, J.A.G., Verheul, T., Kraak, M.H.S., De Voogt, P., Laane, R.W.P.M., Admiraal, W., 2002. UV absorbance dependent toxicity of acridine to the marine diatom *Phaeodactylum tricornutum*. Environ. Sci. Technol. 36 (5), 908–913.

Wilkstrom P, Swajcer E, Brodelius P, Nilsson P, Mosbach K., 1982. Formation of α-keto acids from amino acids using immobilized bacteria and algae. Biotechnol Lett 4, 153–158.

Yaron A, Arad SM., 1993. Natural pigments from red microalgae for food and cosmetics. In: CharalambousG(ed), Foods flavors, ingredients and composition. Elsevier Science Publishers B.V., Amsterdam, pp 835–839.

APPENDICES

Short Term Experiments

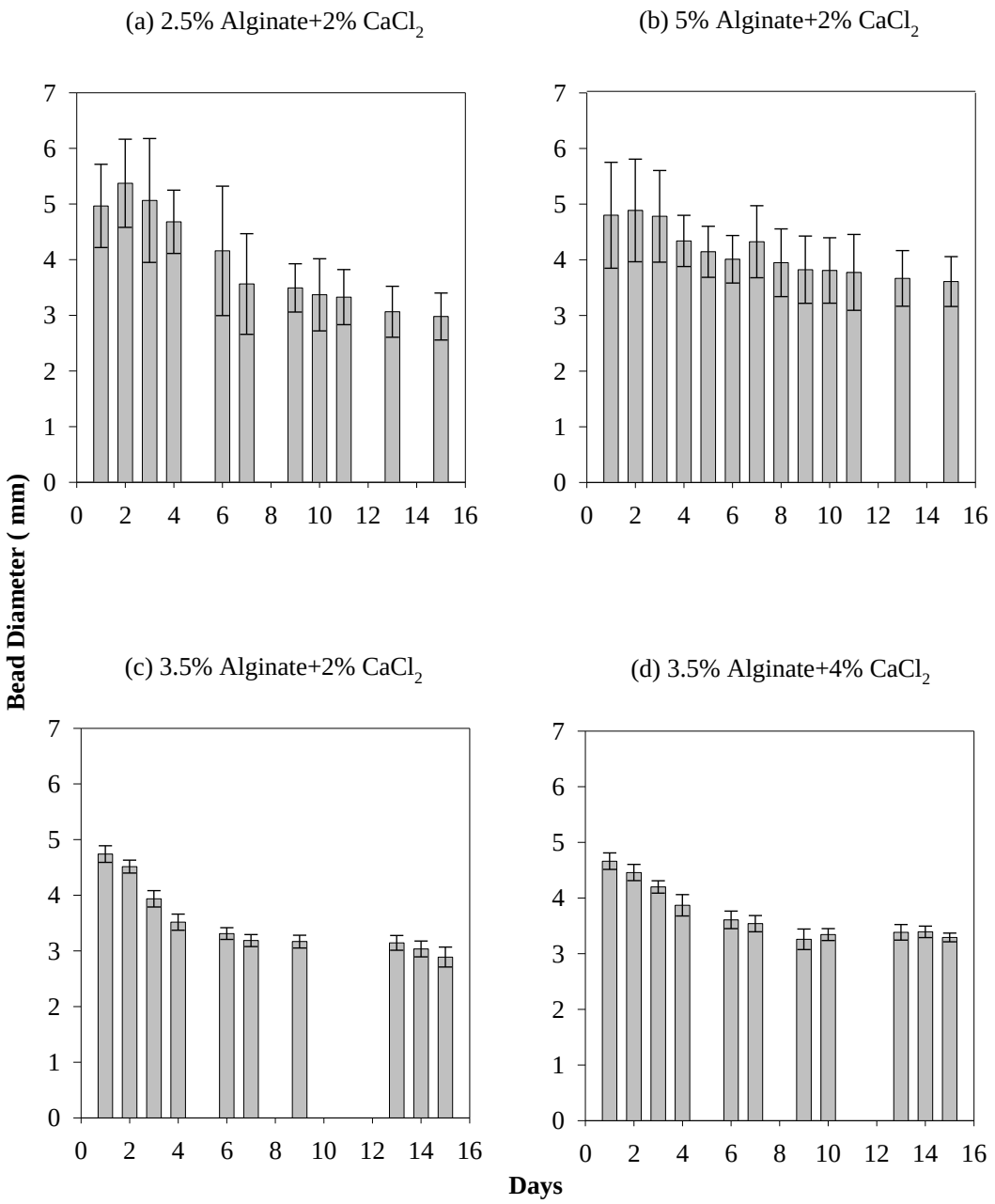


Table A.1 : Change in diameter (mm) of beads prepared using (a), (b), (c) and (d)

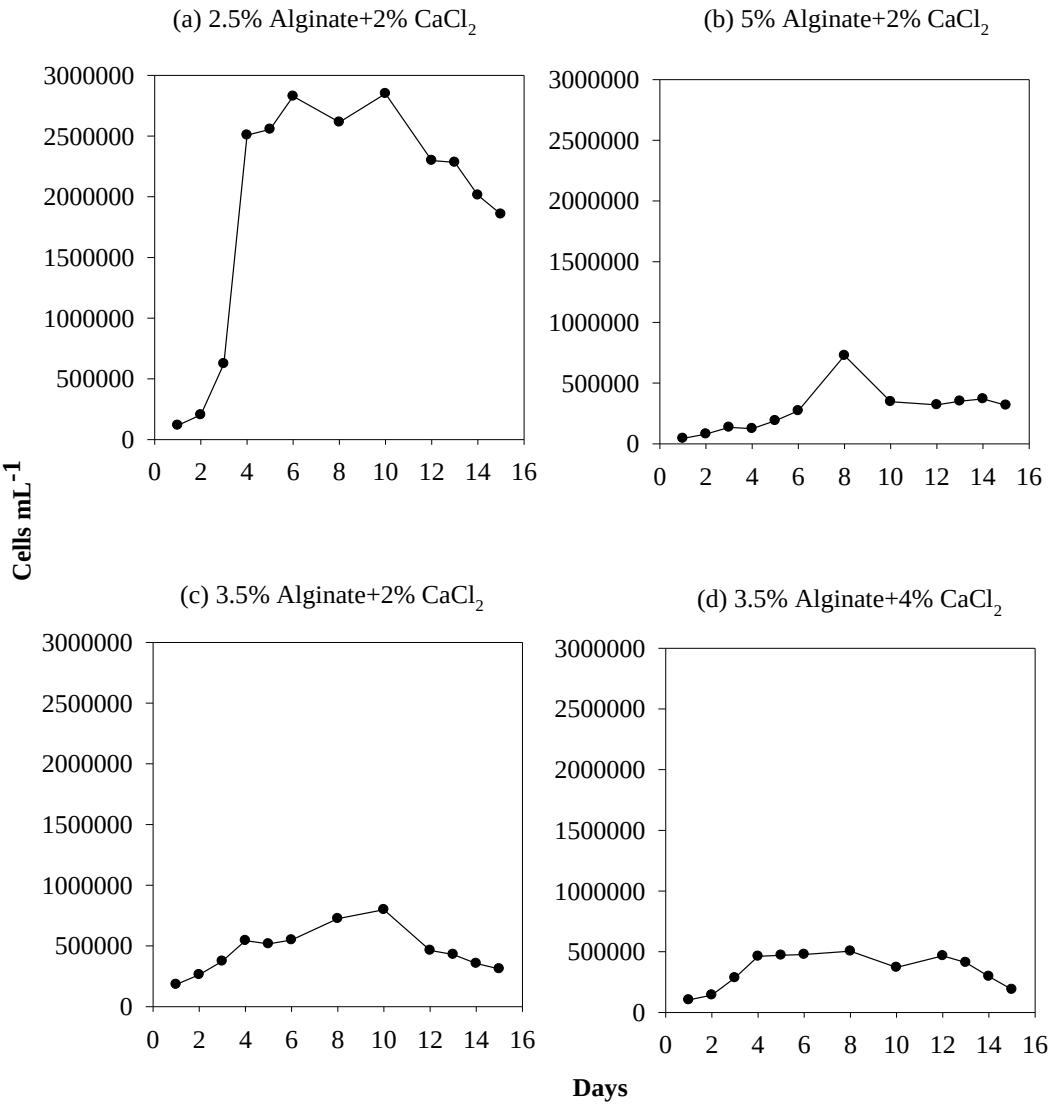


Table A.2 : The growth curve of beads prepared using (a), (b), (c) and (d).

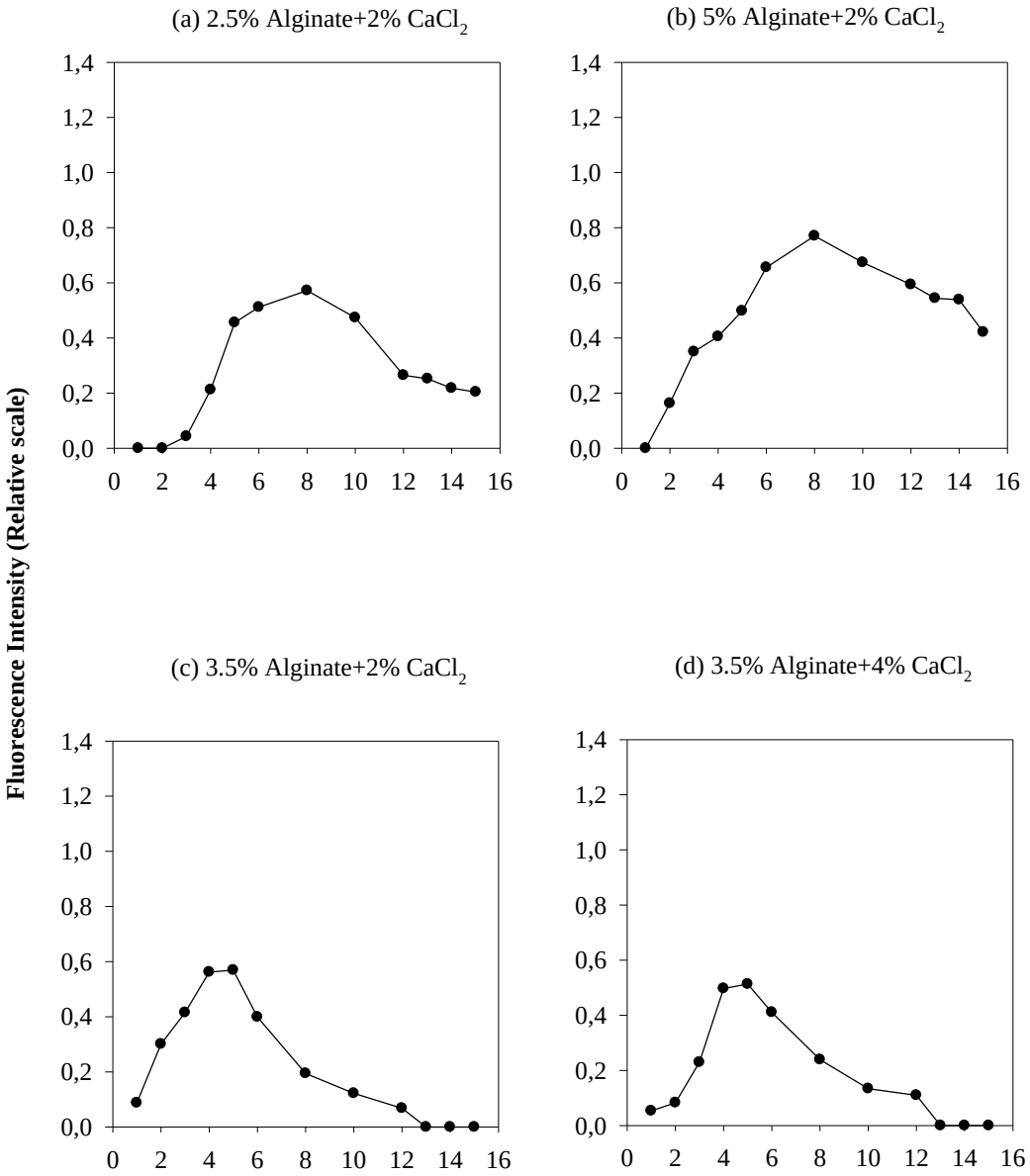


Table A.3 : The change in fluorescence intensity of beads prepared using (a), (b), (c) and (d).

Long Term Experiments

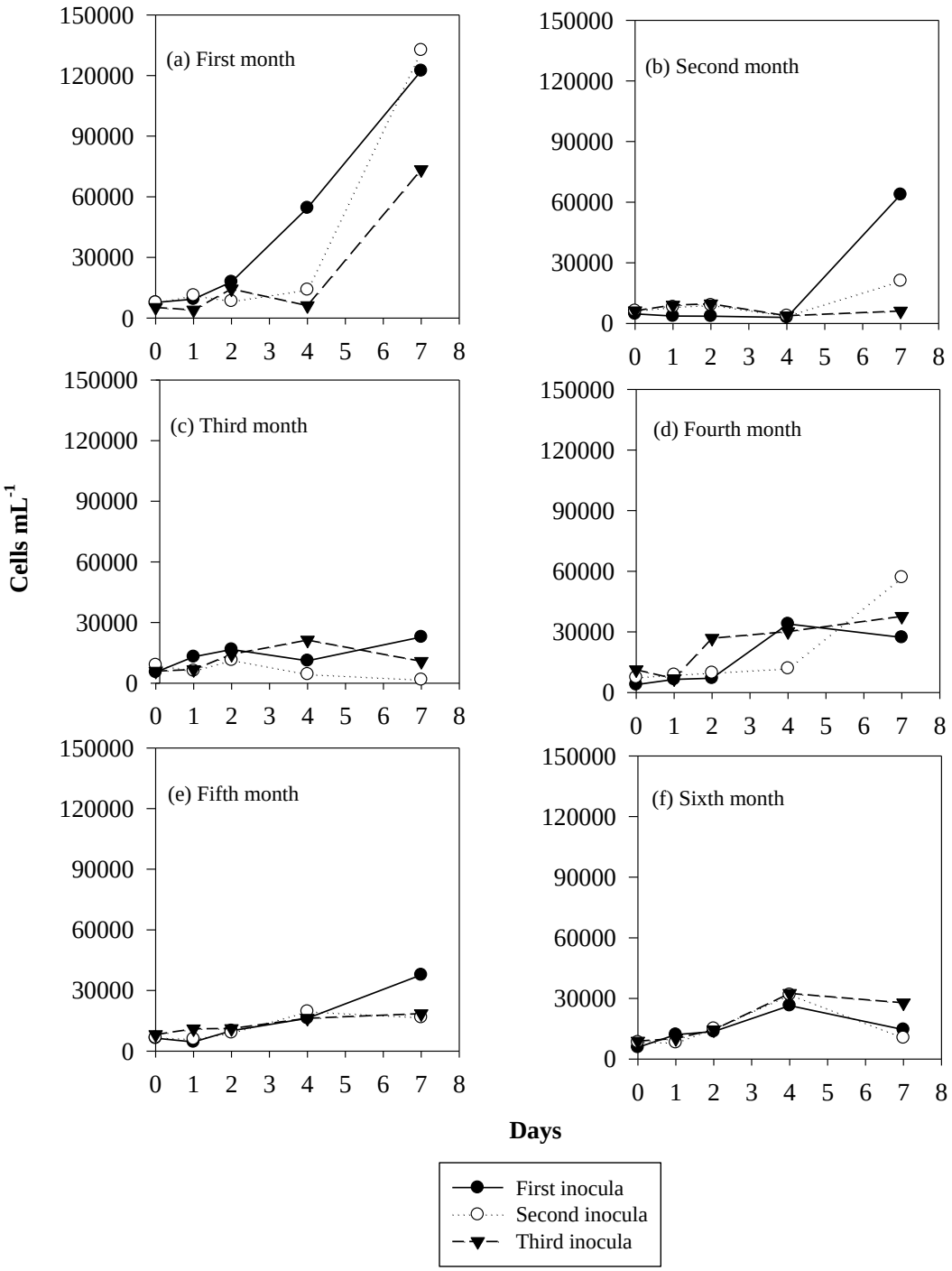


Table A.4 : The growth curve of beads prepared using 3.5 % alginate+4 % $CaCl_2$ on the same scale.

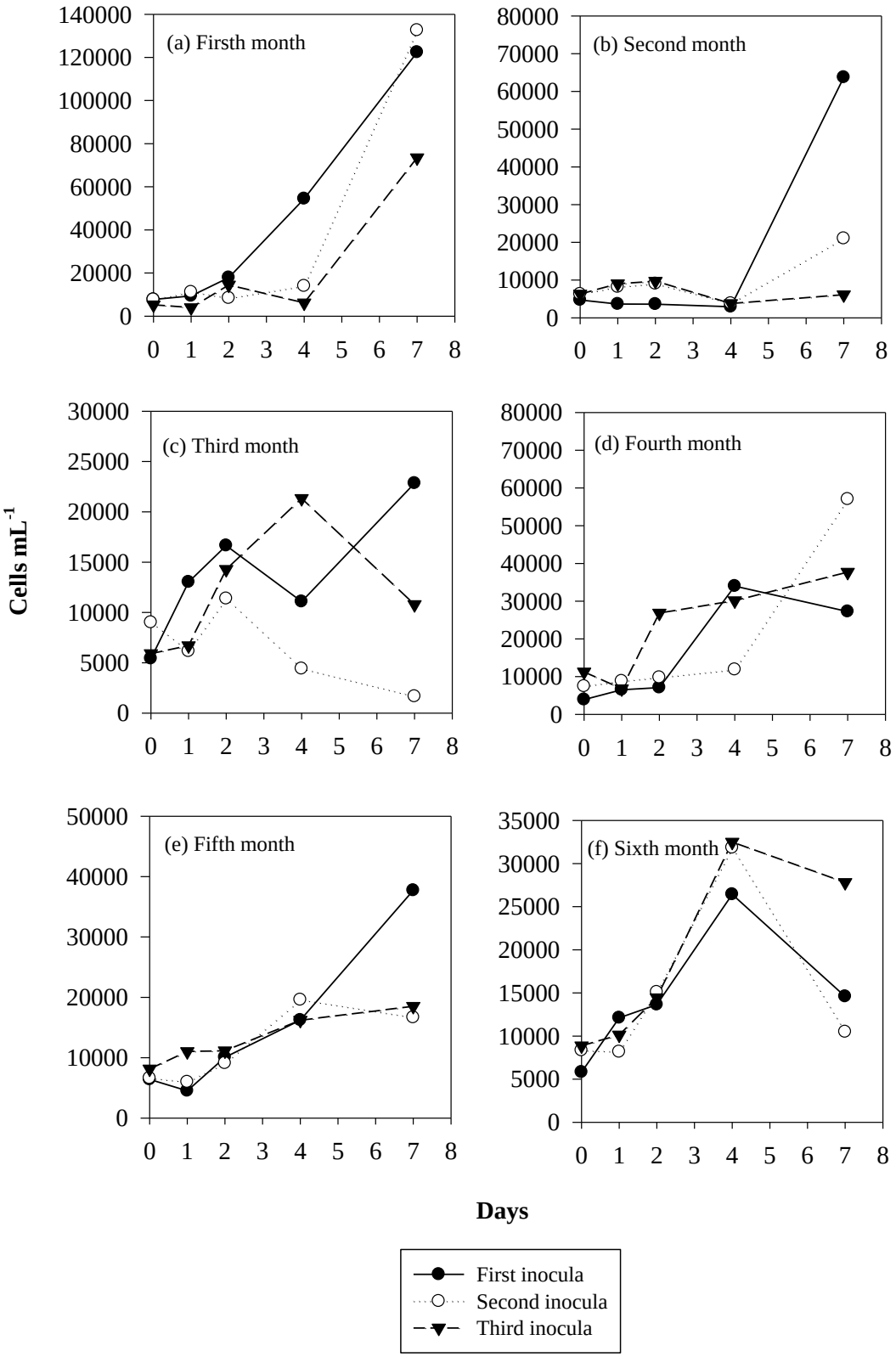


Table A.5 : The growth curve of beads prepared using 3.5 % alginate+4 % $CaCl_2$ on different scales.

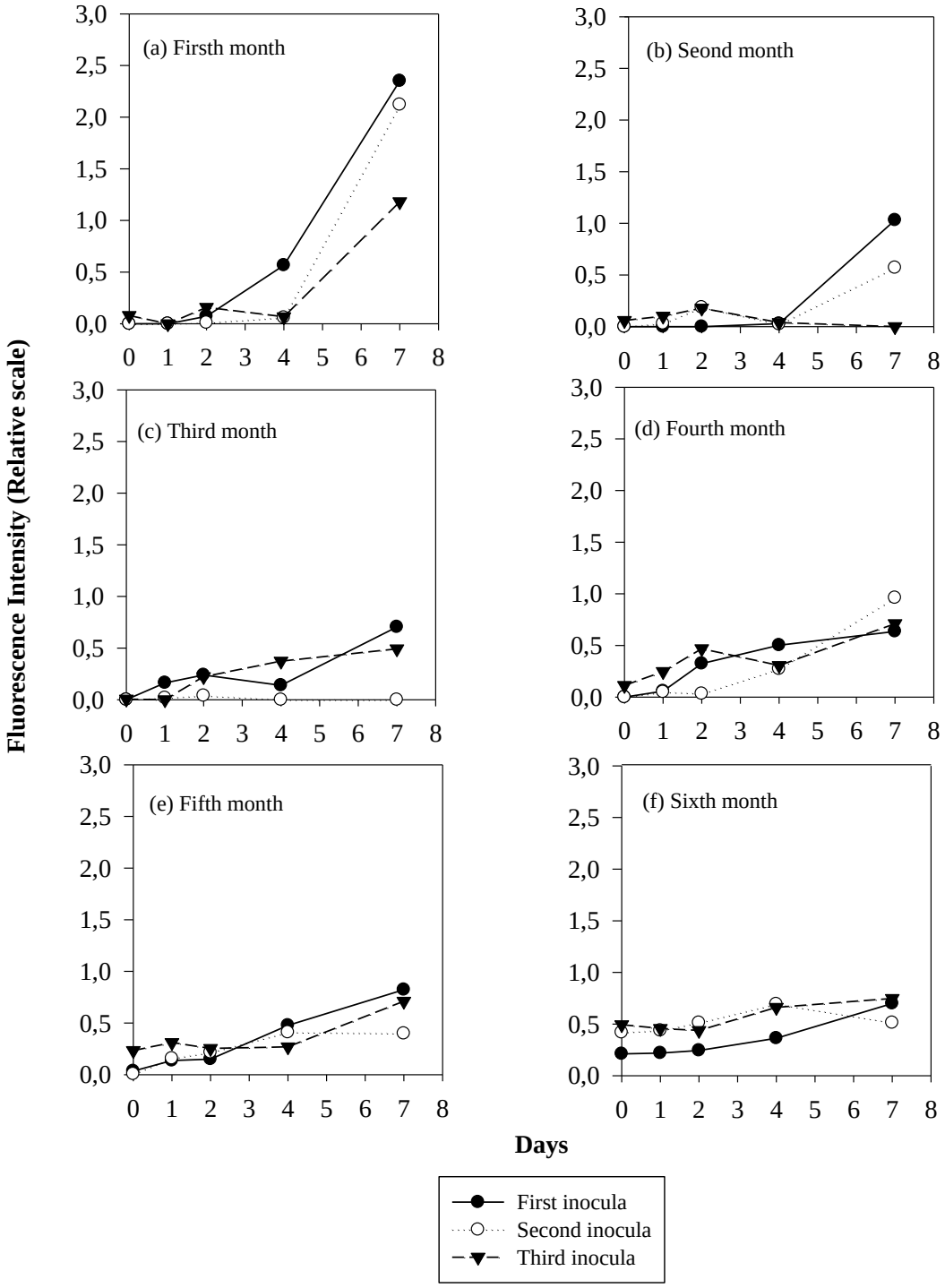


Table A.6 : Change in fluorescence intensity of beads prepared using 3.5 % alginate+4 % $CaCl_2$ on the same scale.

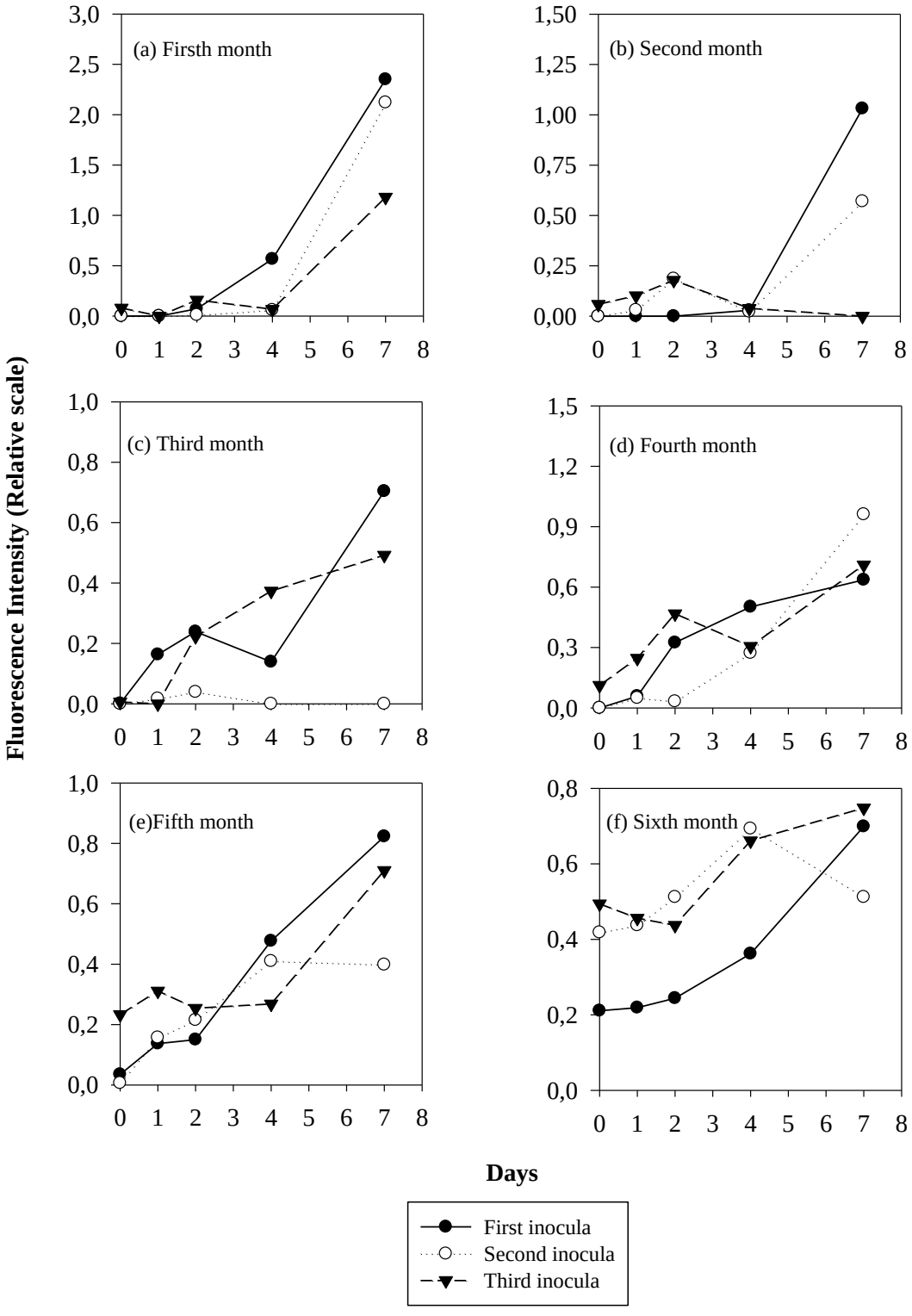


Table A.7 : Change in fluorescence intensity of beads prepared using 3.5 % alginate+4 % $CaCl_2$ on different scales.

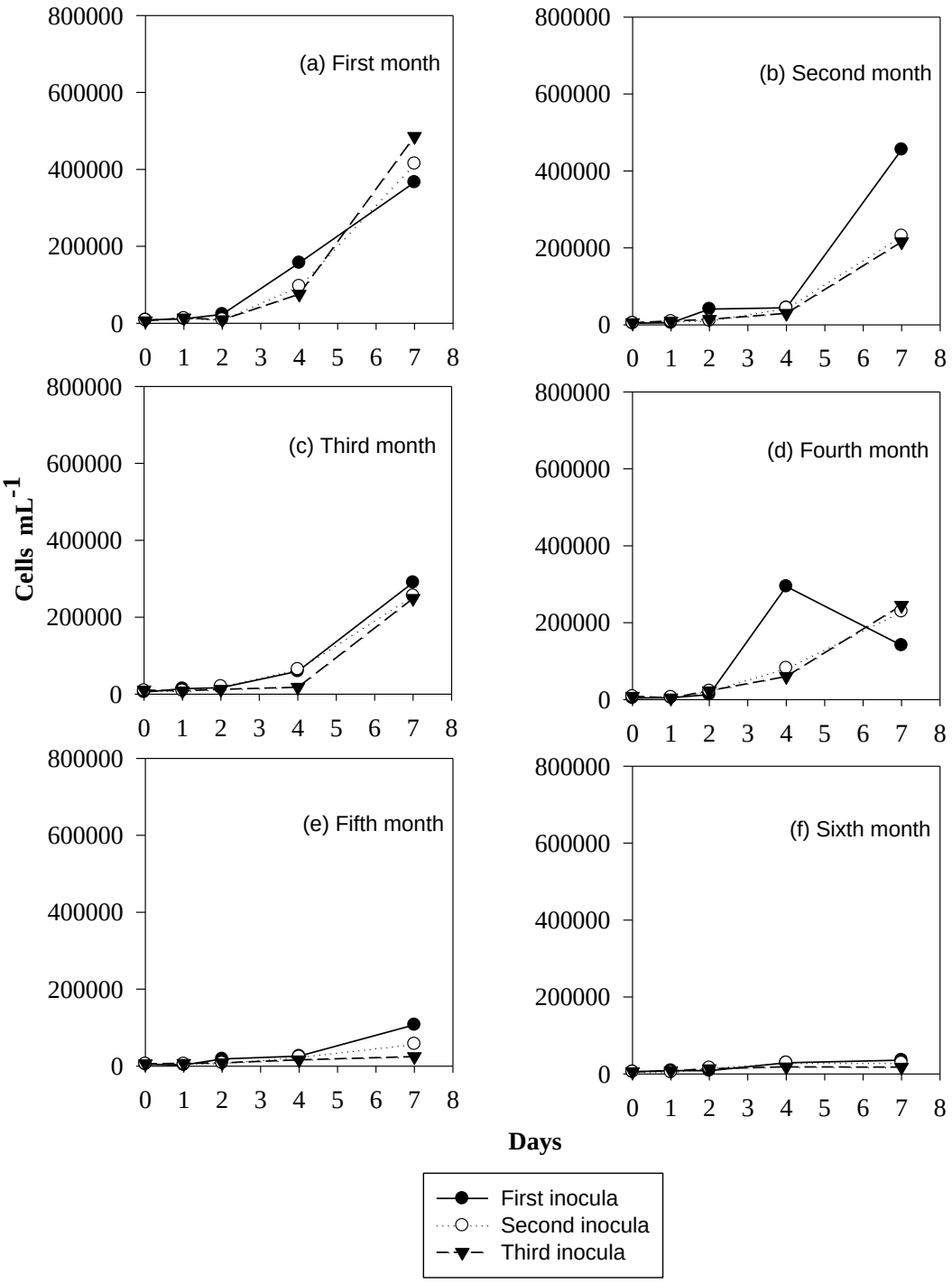


Table A.8 : The growth curve of beads prepared using 3.5 % alginate+2 % $CaCl_2$ on the same scale.

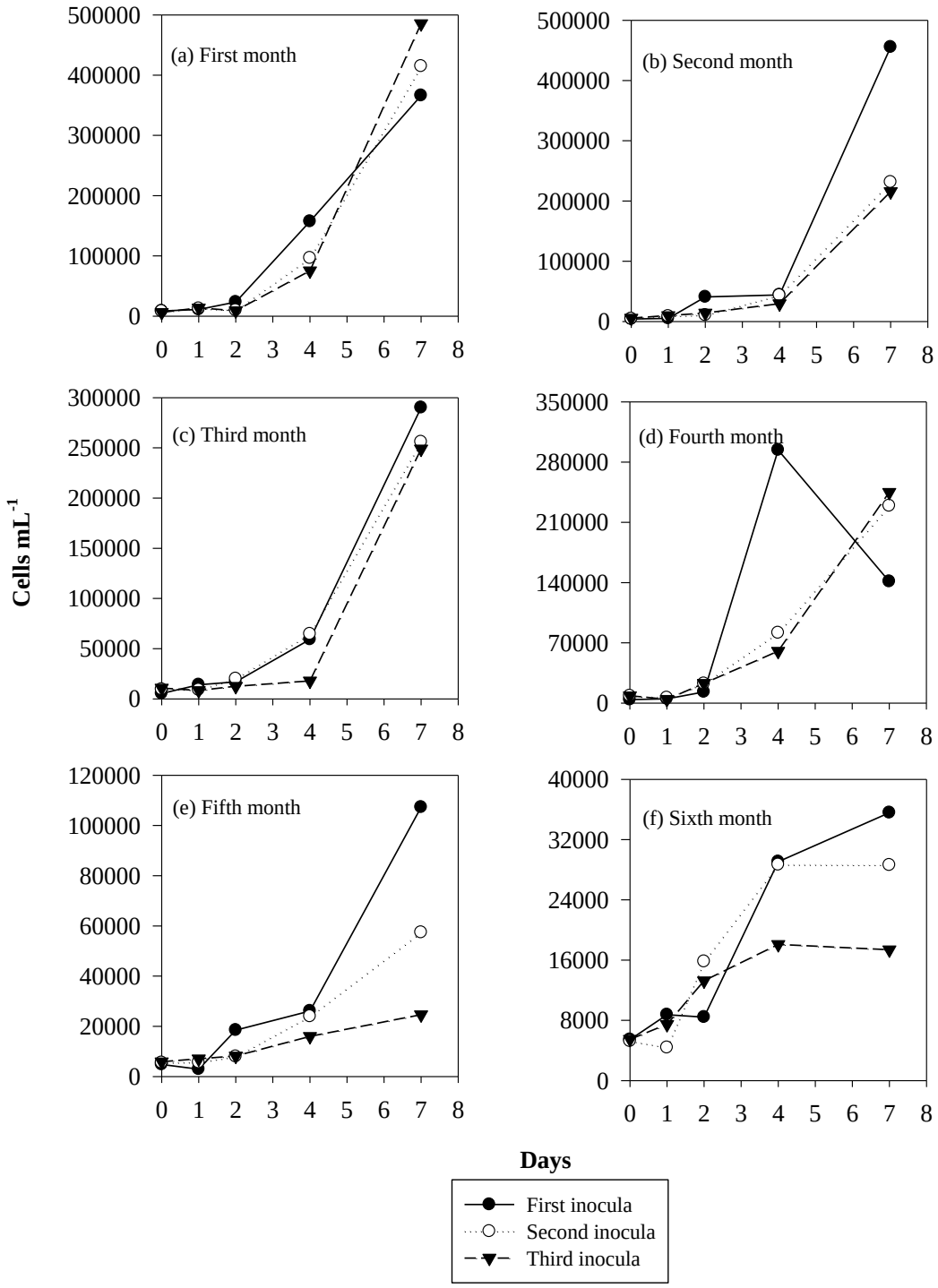


Table A.9 : The growth curve of beads prepared using 3.5 % alginate+2 % $CaCl_2$ on different scales.

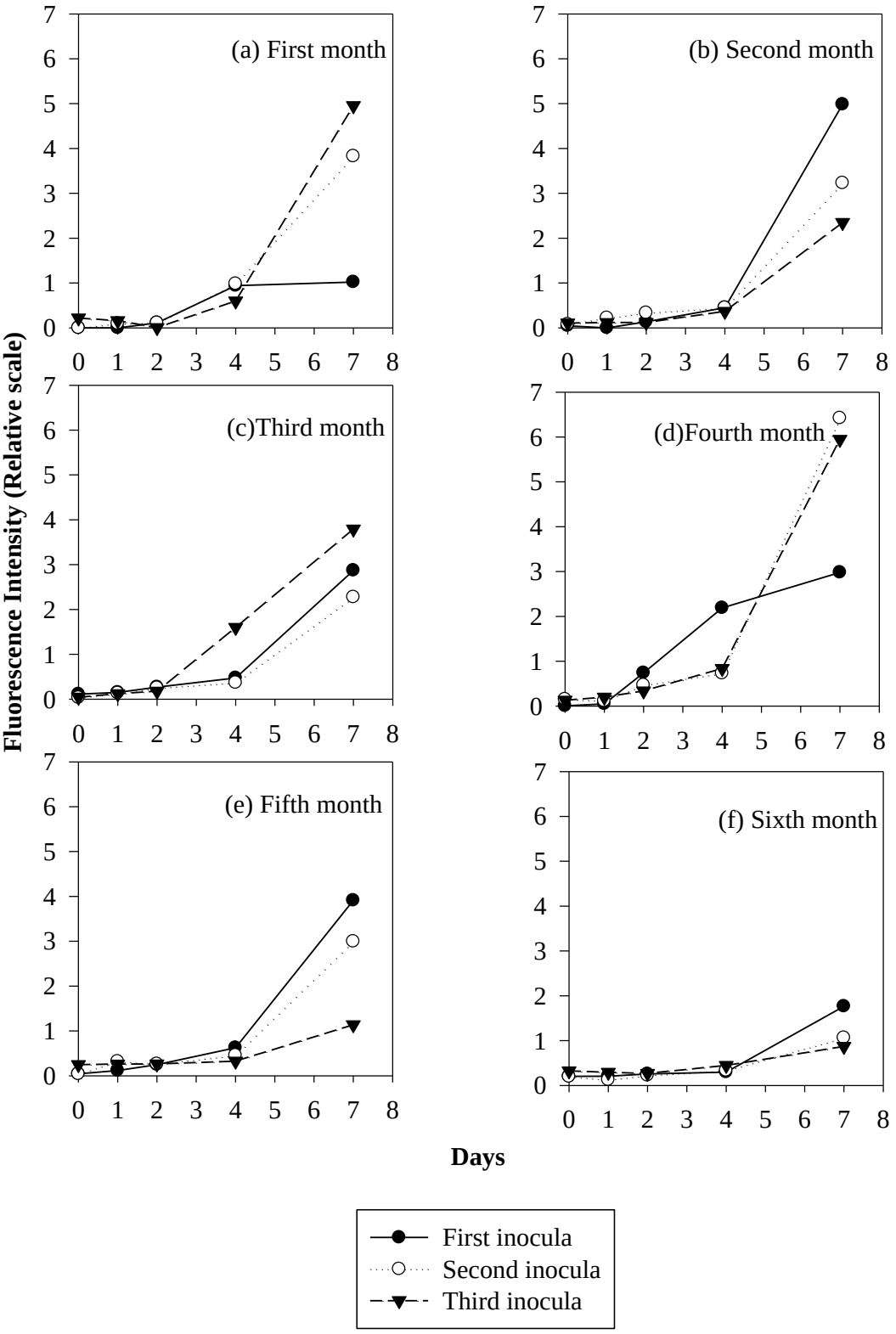


Table A.10 : Change in fluorescence intensity of beads beads prepared using 3.5 % alginate+2 % $CaCl_2$ on the same scale.

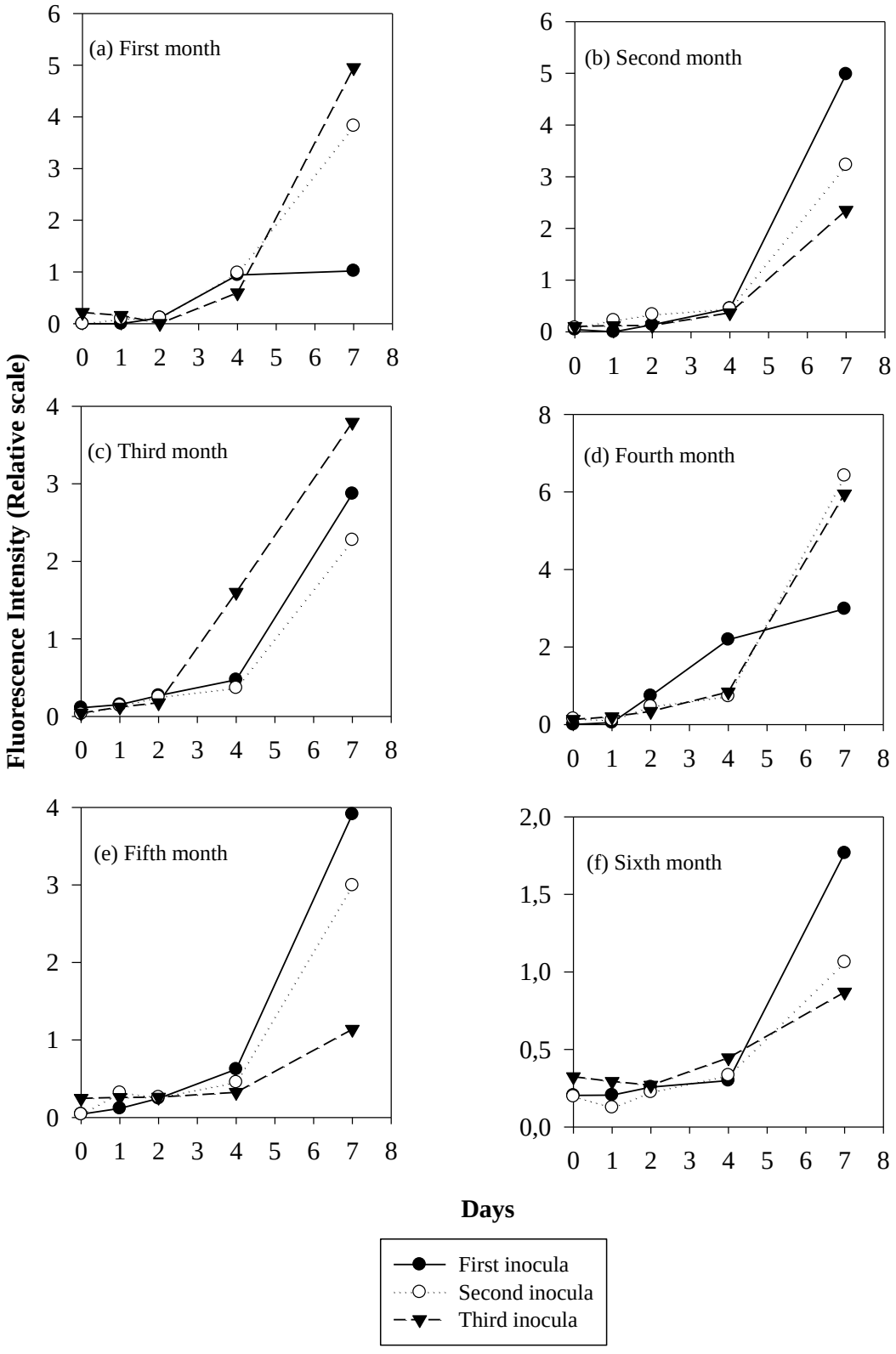


Table A.11 : Change in fluorescence intensity of beads beads prepared using 3.5 % alginate+2 % $CaCl_2$ on different scales.

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