"Study on long term storage of selected immobilization microalgal species for sustained feed and water quality control for Aquaculture "

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Abstract:

Compared study of long term storage Immobilization impact of entrapped and free microalgae cells SEM observation of cells morphological change and cells viability, metabolic activities, biomass increase measurements and distinguish respective specie biomass contents carbohydrates, proteins, and high lipids content optima for aquaculture for *Scenedesmus quadricauda* in fish culture, *Isochrysis galbana* in clam culture, twelve species of benthic diatoms in post-larval abalone culture.

Keywords:

Immobilized microalgae, diatoms, quantification produced biomass from waste water, aquaculture

INTRODUCTION:

Different applications of microalgae have been studied. In particular, they can be used as waste water filter treatment combined to emission of O_2 that contribute to ponds continuous aeration. Authors (Chen (2001) and Chevalier & de la Noüe (1985) experimented quantitative decrease of Ammonium (NH^+_4–N concentrations), CO_2, phosphate and sludge paired to fast increase of microalgae colonies under favorable conditions. Microalgae have been also applied in aquaculture (Valenzuela-Espinoza et al.1999): microalgae act as sensor to control the water quality, thanks to chlorophyll presence; through photosynthesis and metabolic activities, they fix N, P, K elements by consuming CO_2 to produce lipids, proteins and other valuable byproducts substances to feed shellfish (Chevalier & de la Noüe (1985), Wilkinson et al.,1990, Proulx & de la Noüe, 1988, Garbisu et al., 1991). Many other applications of microalgae exist such as: food additives ; protein complements, that may become a new source of nutrition (Spoeh & Milner) in the future in perspective of climate change enhancement and global depletion of soil, water or resources, to supply either aquaculture, animal or human feed ; production of energy: biogas, coal, or biofuels (biodiesel, bioethanol) ; agricultural inputs: fertilizers and soil conditioners ; pharmaceuticals, cosmetics and other valuable chemicals.(Christi, 2007)

In the present paper, three immobilization applications of microalgae have to be studied for a long term storage (1 to 3 years) for use in aquatic cultivation as feed or/and water quality control: *Scenedesmus quadricauda* (Chlorophyta, Chlorococcale) for fish culture, *Isochrysis galbana* (Haptophyceae) in clam culture, twelve species of benthic diatoms in post-larval abalone culture.
Immobilization is one of the means to circumvent the harvest problem (de la Nouë & de Pams, 1988) and can be considered as a gentle method. Entrapping microalgae in beads (calcium alginate natural algal extract), that can be stored at low temperature for several years, can bring much convenience in aquaculture, under a ready-to-use form, the activities of the cells are maintained during storage without liquid medium, and remain active after long term storage. When put back in liquid medium and normal conditions of temperature, light, atmosphere the stored cells show non differentiate respiratory and metabolic activities or division as normal free cells does when they are in normal conditions. Harvest of the microalgae cells is possible, using the immobilization of the cells in beads. Harvest becomes then very simple and well controlled without loose of materials for aquaculture food control or other microalgae applications such as lipid extraction for bioenergies production. For example, the beads can be gathered in plastic bags and instantaneously removed for further processing, while traditional methods, less efficient and more costly methods , and filtration, or energy intensive centrifugation (Richmond & Becker, 1986, Moha,1988, Oswald, 1998) or autoflocculation method (self-aggregation by stopping aeration and decantation ; i.e. Cyanobacteria, the sedimentation realized through ultrasonic waves and ultrasound (Bosma et al)). Determination of basic requirements and properties of an efficient microalgae system and the ideal matrix for immobilized algae goes through experimentations

on entrapped cells: viability, ability to photosynthesis, high density of cells impact, stability of growth, continuous productivity ability, and proof of immobilization superiority to free cells (Mallick, 2006). Then, numerous reports on immobilized cells concerning algae, bacteria, in-vitro plant cell cultures, etc., have supported the view that cell metabolic activities and efficiencies may remain as they are in normal conditions. During immobilization, algal cells maintain their respiratory and photosynthetic activities. Immobilization prevents algal cells from being washed out or grazed by herbivores. Immobilized algae can, when stored at low temperatures (4℃) in darkness, when put back in normal conditions they show same growth successful after long storage; more than 12 months of immobilization (Faafeng et al., 1994). Practical applications of immobilized algae include nutrient and heavy metal removal from wastewater (Chevalier and de la Nouë, 1985; Wilkinson et al., 1990; Proulx and de la Nouë, 1988; Garbisu et al., 1991). Entrapment, storage and processing into alginate-beads are useful in stock culture management. The preparation of alginate-bead is easier, cheaper and more readily available than other methods, such as cryopreservation (Romo and Pérez-Martınez, 1997).

There are many immobilization techniques that have been experienced. Entrapment is by far a preferred method for microalgae immobilization, using polymers (natural or synthetic offering non toxicity matrix, such as alginates, carrageenan, called biocatalyst beads that are photo-transparent and stable in growth medium. A definition of immobilized cell has been suggested: "A cell that by natural or artificial means is prevent from moving independently of its neighbors to all parts of the aqueous phase of the system under study" (Tampion and Tampion, 1987). Alginates has some affinity and allow interactions with cell surfaces and form a labile structure that allow division inside the beads but resistant enough to do not disrupt: the beads are formed from droplets of a suspension of microalgae in aqueous solution of the gelling material coming out from a nozzle (small orifice) into an interacting salt solution, which stabilize the microalgae into biocatalyst beads with Ca++ for alginate and K+ for carrageenan, entrapping the organism via polymerization or cross linking forming mono-dispersed beads of calcium alginate, sterile (size 0.2-1.0 mm). Six different types of immobilization methods can be distinguished: the covalent coupling, immobilization by affinity, adsorption, confinement in a liquid-liquid emulsion, and capture behind semi-permeable membrane and entrapment in hollow fibers bioreactors and some others not classified.

Immobilization impact on the cells shows some morphological change in many cases.

Immobilized algae are applied primarily in wastewater treatment. There have been few studies that reported on immobilized algal cells for application in aquaculture, such as controlling fish culture water quality. The aim of this first study was to test the feasibility of using an algal immobilization technique to preserve the microalgal species, *Scenedesmus quadricauda*, to determine the algal viability after long-term storage, and to test the use of immobilized algal beads for control of water quality in fish culture.

Isochrysis galbana Parke (Haptophyta) primarily occurs as a unicellular flagellate, but also as palmelloid stages in brackish marine environments with two long isokont flagella emerging from a

gullet-like structure, but no haptonema is present. *I. galbana*, because of its good nutritional qualities (i.e. highly unsaturated fatty acids) and small cell size, is widely used in aquaculture, principally as feed in the early stages of growth of mollusk larvae, fish and crustacean, which cost of production is high due live feed culture cost, up to 30% of the total cost of production (Valenzuela-Espinoza et al., 1999). The aim in this second study is to better understand the microalgae viability and metabolic activities for continuous culture under immobilized microalgae, which could then diminish the cost and bring a convenient ready-for- use form.

Diatoms are important primary producers due to their high lipid content (Chen 2012) and constitute a rich diet for marine organisms, because they are widely distributed in most aquatic habitats. They are unicellular photosynthetic eukaryotes within the class Bacillariophyceae. A peculiar feature of diatoms compared to other microalgae is their siliceous cell wall. The numbers of their genera and species are approximately 250 and 100,000, respectively (Lebeau & Robert, 2003). Lipids are the major metabolites of diatoms, being composed of neutral lipids, polar lipids and traces of sterols. Neutral lipids are their main stored lipids. Benthic diatoms are the main food source for abalone post-larvae (Hahn, 1989a), and monospecific cultures of benthic diatoms have successfully been employed as feed for the culture of abalone post-larvae (Kawamura et al., 1995; Hillebrand and Sommer, 2000). However, in Taiwan, abalone post-larvae grown in hatcheries are usually fed with natural populations of benthic diatoms, following the traditional method of using biofilms of mixed benthic diatoms as the settlement substrata for abalone post-larvae in hatcheries worldwide (Hahn, 1989b; Daume et al., 1997, 1999, 2000; Roberts, 2001; Gallardo and Buen, 2003).

Carbajal-Miranda et al. (2005) recorded the effectiveness of *Navicula incerta* and *Amphiprora paludosa* var. hyaline as feeds for cultivation of post-larval red abalone, *Haliotis rufescens*, and Daume et al. (2000) documented the use of *Navicula* sp., *N. jeffreyi*, *Cylindrotheca closterium*, *Cocconeis* sp. and *Amphora* sp. as feed for post-larvae of *Haliotis rubra*. Gordon et al. (2006) has reported that *Amphora luciae* and *Navicula* cf. *lenzii* supported high post-larval growth and a high survival rate of *Haliotis discus*. Seven species of diatoms (*Achnanthes longipes*, *Nitzschia* sp., *Cocconeis pseudomarginata*, *Navicula britannica*, *N. ramosissima*, *Navicula* sp. and *Nitzschia ovalis*) were used by Kawamura et al. (1998) to culture *Haliotis iris* post-larvae. Mixed diatoms vs. monospecific cultures of *Navicula* were used as feed for *Haliotis asinina* by Gallardo and Buen (2003). Poor and unpredictable performance of abalone postlarvae has been related to the differences in the species of diatoms they have been fed, and the composition of these diatoms (Kawamura et al., 1998), including their lipid, protein and extracellular polymeric substances (EPSs).

The effectiveness of different species of diatoms as feed for the culture of post-larvae of *H. diversicolor is* not well known. Therefore, the aim of this third study is to survey the diversity of diatoms species found in *H. diversicolor* hatcheries, and to isolate and develop a monoculture that could be immobilized and encapsulated in alginate beads for long-term storage as algal stock. Their lipids, proteins, and EPS (soluble EPS, bound EPS, internal carbohydrate and residual carbohydrate)

content are determined and will be compared to distinguish the twelve monospecific phytoplanktons. Their metabolic activities after immobilization as well as their nutritional value for feed use in cultivating the post-larvae of *H. diversicolo is maintained*, to allow the most adapted species through long term storage immobilization, contribute to diminish the cost of the feed with these phytoplanktons used as live feed by the post-larvae, similarly to the above clam study.

MATERIALS AND METHODS:

Different microalgae species have been immobilized (Chen (2001, 2003 & 2006): as per observed,long immobilization storage and morphologic change not affecting the growth or the metabolic activities when placed back in normal liquid medium culture. This method has been used on three microalgae:

- Immobilization microalgae *Scenesdesmus quadricauda* for long term storage and for application for water quality control in fish culture:

- Immobilization microalgae *Isochysis galbana* for long term storage and application for feed and water quality control in clam *(Merextrix lusoria)* culture

- Immobilization of 12 benthic diatoms species for long term storage and as feed for post-larval abalone *(Haliortis diversicolor)*

A) Immobilization *Scenedesmus quadricauda*

1. Microalga culture

Pure *S. quadricauda* cultures were isolated from the fishpond at the Department of Aquaculture, National Taiwan Ocean University, in Keelung, Taiwan. The microalgae were incubated in a 1000-ml flask containing 600 ml of liquid medium (Provasoli's Enriched Freshwater, PEF medium, pH 7.0) (Provasoli and Pintner, 1960) at 100 μmol photons m^{-2} s^{-1} at 24℃ and 12:12 dark:light photoperiods. The media were replaced weekly.

2. Immobilization of the microalga

Microalga entrapment in alginate-beads was performed as follows. A 3% (v/v) sodium alginate (Sigma A-7128) solution was autoclaved for 20 min at 121℃. The *S. quadricauda* strain was concentrated using centrifugation (10 min, 1000×*g* r.c.f.), and then mixed homogeneously into the sodium alginate solution to make the beads. The numbers of algal coenobia 0.1ml of mixture were counted (ca. 2–30,000 coenobia mly1) using a hemacytometer (Bright-Line, improved Neubauer, 0.1-mm deep) under a light microscope. Beads, about 4 mm in diameter, were formed by dropping the alginate–algal mixture into a solution of 0.03 M $CaCl_2$ at room temperature using a burette. In this experiment, 1 ml of the mixture could drop about 10 drops (beads). The beads were hardened in the

$CaCl_2$ solution for 30 min. Subsequently, they were washed 2 several times in autoclaved distilled water to remove excess $CaCl_2$. The wet beads were then directly and immediately stored in a well sealed flask without any liquid medium addition. The flask was wrapped with aluminum foil and then maintained in absolute darkness at 48℃ until used, preventing algal growth. The number of re-cultured cells was also counted, 100 beads were dissolved in a solution of 6 ml of 5% sodium hexametaphosphate and 24 ml of PEF culture medium (Romo and Pérez-Mart´ınez, 1997). The dissolved solution was then used for counting by a hemacytometer.

3. Fixation of algal materials for electron microcopy studies

The freshly made algal beads, the stored algal beads and the re-cultured algal beads were gently crushed individually into fractions using forceps (tongs). These fractions and normal culture (free-living) *S. quadricauda* cells were collected in 15-ml centrifuge tubes followed by separated fixation in 0.1 M sucrose solution containing 4% glutaraldehyde and 0.1 M sodium cacodylate buffer (pH 7.0) at 4℃ for 2 h. They were then rinsed twice with a 0.1 M sodium cacodylate buffer containing 10 mM $CaCl_2$, and sucrose 2 concentrations successively reduced to 0.05 M. This treatment was followed by two rinses by a pure (sucrose-free) 0.1 M sodium cacodylate buffer containing 10 mM $CaCl_2$.

Post-fixation was performed with 2% OsO_4 in 0.1 M sodium cacodylate buffer containing 10 mM $CaCl_2$ for 1 h at 4℃. Thereafter, all materials were rinsed four times with a sodium cacodylate buffer containing 10 mM $CaCl_2$, three times with aqueous ethanol (50%) and gradually dehydrated in ethanol (50, 70, 85, 95, 100%). Dehydrated materials were prepared for transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

For TEM, some dehydrated materials were rinsed in propylene oxide (three times, 30 min each), followed by infiltration in propylene oxide–Spurr's resin in a decreasing ratio from 2:1 (two parts propylene oxide: one part Spurr's resin) to 1:1, each for 4 h. Samples were then suspended in pure Spurr's resin for 2 days at 48C in darkness before embedding in Spurr's resin (Spurr, 1969). The thin-sections were stained with uranyl acetate and lead citrate according to Smith and Croft (1991).

For SEM, some dehydrated materials were dropped onto specimen holders and then dried with a critical-point-drying machine (Hitachi-HCP-1). Finally, they were coated onto an ion coater (Joel, JCF-1100E) for 220 s.

4. Algal bead application for controlling fish culture water quality

Four glass jars (60×55×33 cm), each containing 100 l of freshwater, were assigned as groups 1, 2, 3 and 4. Ten male tilapia, *Oreochromis mossambicus* (average body length 14 cm), were cultured in each jar. Groups 1 and 2 were cultured under continuous light of 100 μmol photons m^{-2} s^{-1} irradiance at 24℃. Groups 3 and 4 were cultured under the same irradiance but with 12:12 dark/light photoperiods (light at 8:00 and dark at 20:00) at 24℃.

Freshly made algal beads (ca. 2–3000 coenobia in each bead) (Figure 1-1) were cultured in PEF medium for 2 weeks. The beads were subsequently packaged into three nylon net bags (pore size was

ca. 2=3 mm in diameter) (Figure 1-2), each containing 4000 algal beads. They were then hung in groups 2, 3 and 4, respectively. For group 4, the nylon bag was removed from the jar when the light turned off at 20:00 (the initial darkness), and replaced until the light was turned on at 8:00 (the initial light). Group 1 was the control group, which only contained fishes. Tilapias in each jar were fed with 2.5 g of commercial feed (Abon, Taiwan) everyday. The feed contained 45% of protein, 5% fat, 3% crude cellulose, 10% ash and 10% water. A pH meter (Suntex, Taiwan), a digital oxygen meter (Lutron DO-5510, Taiwan) and colorimetric test kit (Aquamerck Ammonium, Merck, Germany) were used to test the pH value, dissolved oxygen level (DO) and NH^{+}_{4}–N concentration, respectively. The water quality of groups 1 and 2 was tested 4 once daily. However, groups 3 and 4 were tested twice daily (at 8:00 and 20:00) as the light came on at 8:00 and turned off at 20:00. All cultures were adequately aerated during the period between days 1 to 13. Aeration ceased from day 14 to 18.

B) Immobilization *Isochrysis galbana*:

1 Culture and isolation of alginate beads

The cultures were isolated from the marine fishpond at the Department of Aquaculture, National Taiwan Ocean University, Keelung, Taiwan. *I. galbana* was cultivated in 1000-mL flasks containing 600 mL liquid medium (modified Provasoli's Enriched Seawater, PES medium, salinity = 20) (Provasoli & Pintner, 1960) at 100 μmol photon m−2·s−1 at 24 ◦C and a 12:12, dark: light photo-period. The medium was replaced weekly.

2 Entrapment of I. galbana in alginate-beads was performed similarly as for the *Scenedesmus quadricauda* as exception for *Isochrysis galbana* strain was concentrated by centrifugation (1000 × g,20 min), the cell density count (ca. 6–7 × 106 cells mL−1). Beads, about 4–5 mm diameter. modified PES medium (20‰salinity) to remove excess $CaCl_2$., (Chen, 2001). The resulting solution was then used for counting the cells.

3. Use of algal beads for controlling clam culture water quality and for feed (Figure 5/ Figure 6)

Method similar to S. quadricauda

C) Immobilization of twelve benthic diatom species

1 Collection and preparatory cultures of benthic diatoms

Diatoms from small abalone hatchery ponds were scraped from abalone post-larvae settlement substrates (plastic plates), collected in l L collection bottles, placed in a cool icebox, and then transported to the Algal Laboratory of the National Taiwan Ocean University, Keelung, Taiwan. All the collected materials were cultured in 1 L flasks with 500 mL of autoclaved (at 1.1 atm and 121 ℃ for 20 min) Provasoli's Enriched Seawater (PES) (Provasoli, 1968) addition with 0.03 g/L of NaSiO3 and 0.0013 g/L H2SeO3 and henceforth called 'modified PES medium'. All collected materials were incubated in a plant growth chamber (Firstek, RI-201, Taiwan) with the culture conditions held at 24 ℃/22 ℃ (L/D), 12/12 (L/D cycle) and 122 μmol photons m−2 s−1 light intensity.

.2 Isolating diatom species for monospecific cultures

2.1 Streaking method of agar plate

Agar plates were made by using 100 mL of modified PES medium with 2 g Baco-agar in a 250 mL flask. The opening of the flask was sealed with a cotton plug and the contents autoclaved at 1.1 atm and 121 ℃ for 20 min. When the solution was still hot, it was poured and dispensed into the γ-ray sterile plastic Petridishes (150×14.5 mm, Alpha Plus, Taiwan) on a clean bench (Laminar flow hood, High Ten, 3HT-24, Taiwan). The covers of the Petri dishes were sealed with parafilm. The Petri dishes were then stored at 4 ℃ for further use.

Algal solutions from the preparatory cultures were streaked onto the agar plates using an inoculating platinum loop sterilized with a Bunsen burner. After inoculation, the Petri dishes were cultured in the plant growth chamber with the same conditions as previously described, and were checked each day under an inverted microscope. When clear colonies appeared on the surface of the agar plates, the colonies were individually removed from the plates to 250 mL flasks containing 150 mL of modified PES medium using the flamed, sterile, inoculation method. The algal solutions derived from the isolated colonies were checked using a microscope for identifying the species. If the cultured algal solutions still contained mixed diatom species, then the streaking methods were repeated until monospecific cultures emerged.

2.2 Dilution method

As it was likely that some of the diatoms would not survive when streaked onto solid agar plates, the dilution method was also used as follows: 0.01 mL algal solution of the preparatory cultures were separately diluted into 100 test tubes (16×100 mm), each containing 5 mL of modified PES medium. The test tubes were cultured in a plant growth chamber under the same culture conditions as previously described. One drop of the diluted solution from each tube was checked every week. If the test tubes contained mixed algal species, the dilution method was repeated using the algal solution from the test tubes until monospecies appeared. Once the test tubes contained monospecies of diatom, the algal solution was transferred from the test tube to a 250 mL flask containing 150 mL of modified PES medium, and was then cultured in the plant growth chamber as mentioned earlier.

The size of the diatoms was determined using a microscope equipped with micro-rule (1 μm scales in the eye pieces).

2.3 Using a Scanning Electron Microscope for confirming the diatom species classification

Monospecies from the 250 mL flasks were collected in 15 mL centrifuge tubes, pre-fixed in modified PES medium with 2% glutaraldehyde at 4 ℃ for 2 h, and then fixed in a 0.1 M sucrose solution containing 4% glutaraldehyde and 0.1 M sodium cacodylate buffer (pH 7.0) at 4 ℃ for 4 h. They were then rinsed twice with a 0.1 M sodium cacodylate buffer containing 10 mM CaCl2, and the sucrose concentration was successively reduced to 0.05 M. This was followed by two rinses in sucrose-free 0.1 M sodium cacodylate buffer containing 10 mM CaCl2.

Post-fixation was performed with 2% OsO4 in 0.1 M sodium cacodylate buffer containing 10 mM CaCl2 for 1 h at 4 ℃.

All materials were then rinsed four times with a sodium cacodylate buffer containing 10 mM CaCl2,

three times with aqueous ethanol (50%) and gradually dehydrated in ethanol (50, 70, 85, 95, and 100%). Those dehydrated materials were dropped onto specimen holders and then dried with a critical-point-drying machine (Hitachi-HCP-1). Finally, they were coated onto an ion coater (Hitachi E101, Japan) for 220 s. They were observed using a SEM (Hitachi S2400, Japan) (see Chen, 2001, 2003). Identification was based on Patrick and Reimer (1966), Jin et al. (1985), Round et al. (1990) and Witkowski et al. (2000).

2.4 . Immobilization of the monospecies diatoms it is similar as for S. quadricauda, except for:
each monospecies was concentrated through centrifugation (20 min, 1000 ×g), and then mixed homogeneously into the sodium alginate solution to make the beads. The number of algal cells per mL of mixture was counted (ca. 106–107 cells per mL) Beads, about 3–4 mm in diameter,
Table 1

2.5 Cultivation of post-larvae with the twelve immobilized monospecific diatoms

The immobilized algal beads (100 beads of each of the 12 species) were gently crushed individually into small fractions using forceps. Those fractions were separately collected in twelve 500 mL flasks with 250 mL of modified PES media. These flasks were then cultured in a plant growth chamber under the same culture conditions as previously described. When the algal biomass of those flasks exceeded 105 cells/mL, the algal solutions were separately transferred to twelve 20 L Carboy plastic buckets, each containing 12 L of modified PES medium. These Carboys were cultured in the green house of our laboratory. If the sunlight was insufficient (less than 100 μmol photons m−2 s−1 light intensity), two 40 W natural white fluorescent lights were positioned about 50 cm above the Carboys. When the diatom biomass of those Carboys reached 1×106 cells/mL, the algal solutions were then inoculated into 50 L square PVC tanks containing 45 L of filtered fresh seawater using a 1 μm pore filter bag, to which 0.03 g/L of sodium silicate (Na_2SiO_3) had been added. These square tanks were also cultured in the green house and were equipped with white fluorescent lights. Seven plastic plates, each measuring 25 cm×20 cm were hung vertically in each tank. All the tanks were well aerated using an air pump. After 4–7 days of culture, when the surfaces of both sides of the plastic plates were covered by more than 80% of benthic diatoms (a prefixed level of diatoms presence for the experimentation), the seawater of each tank was refreshed with filtered seawater and inoculated freshly fertilized eggs of small abalone (obtained from the small abalone hatchery) at a density of 200 fertilized eggs/L. About 3 days later the fertilized eggs reached the stage of post-larvae ready to settle on the surfaces of the plastic plates, and the filtered seawater was then refreshed, and every 3 days thereafter. The post-larvae were photographed using a stereomicroscope equipped with a digital camera to record the numbers settling on the plastic plates, and their growth. This procedure was repeated 5 times between April 16, 2005 and May 26, 2006.

2.6. Protein and lipid analysis of the twelve monospecies of diatoms

Following the methods of the Association of Official Analytical Chemists (AOAC, 1990), protein was determined with a Kjeltec semi-auto analyzer model 1007. Lipid was determined by the chloroform-methanol (2:1, v/v) extraction method (Folch et al., 1957).

2.7 Isolation and analysis of extracellular polymeric substances (EPSs)

The procedures for the fractionation of intra- and extra-cellular carbohydrates followed those of De Brouwer and Stal (2002). Soluble EPS was obtained by centrifuging 10 mL of diatom culture at 1000 ×g for 20 min at room temperature. The supernatant was transferred to a centrifuge tube containing 30 mL of cold ethanol (96%) and the soluble EPS was allowed to precipitate overnight at − 20 °C. After centrifugation (20 min, 1000 ×g), the EPS pellet was dried under a flow of nitrogen and subsequently re-suspended in 300 μL of distilled water. A volume of 200 μL was used for carbohydrate analysis. Bound EPS was extracted by resuspending the culture pellet in 2 mL of distilled water. The cell suspension was thoroughly stirred and incubated for 1 h at 30 ℃. After centrifugation at 1000 ×g for 20 min, the bound EPS was isolated.

The internal sugars were extracted by adding 1 mL 0.05MH2SO4 to the cell pellet. The suspension was mixed every 30 min for a period of 2 h. After centrifugation (20 min, 1000 ×g) 200 μL of the supernatant was used for carbohydrate analysis. For determination of the residual carbohydrates, what was left of the cell pellet after the extraction of the internal sugars was re-suspended in 400 μL of distilled water. A volume of 200 μL of this suspension was used for analysis.

Carbohydrates were measured in four different fractions (soluble EPS, bound EPS, internal carbohydrates, and residual carbohydrates) using the phenol/ H2SO4 assay (Dubois et al., 1956).

Results

A) Scenedesmus quadricauda

1. Storage, culture and electron microscopy studies of the immobilized S.quadricaudas

Freshly made algal beads (Figure 1/ Figure 1-1) contained an average of 2470±460 *S. quadricauda* coenobia in each bead. The wet beads without liquid medium were stored in darkness at 4℃ for 3 years. The algal coenobia did not lose their reproduction ability within the beads after long-term storage, and the number of coenobia was the same as those of fresh algal beads. The amount of coenobia increased tremendously to average $9\times10^4\pm$ 2300 coenobia in each stored bead (Figure 1/ Figure 5), following culture in PEF medium for 4 weeks.

Thin-sections of freshly made algal beads (Figure 1/ Figure 7) showed that a large chloroplast, filling most of the cell, was the predominant organelle. The chloroplasts were typically composed of stacks of thylakoids. The simple internal suspended pyrenoids were normally closely surrounded by caps reserve materials. The ultrastructures of freshly immobilized algal cells were the same as those of free-living algal cells. In contrast, in thin-sections of the long-term stored immobilized algal cells, the chloroplast pyrenoids had disappeared, although a chloroplast filled most of the cell (Figure 1/ Figure 9). However, the pyrenoids were reconstructed within a week after those cells were re-cultured in PEF medium at 100 μmol photons m^{-2} s^{-1} irradiance and 12:12 dark:light photoperiods at 24℃.

The SEM observations of the fractions of stored algal beads showed that the morphology of the immobilized *S. quadricauda* coenobia (Figure 1/ Figure 10) was nearly the same as normal coenobia (Figure 1-6), except that coenobia spines were modified into bent shapes.

2. Application of algal alginate-beads for fish culture water quality control

2.1. The NH^+_4 –N concentrations of fish cultures

As shown in Figure 2, NH^+_4–N concentration of group 1 (control group: only fish without microalgae beads added) increased quickly to 17 mgl^{-1} within a week. The freshwater in group 1 was replaced on day 7, 20:00. However, NH^+_4–N concentration still showed an increasing trend from 1.5 to 15 mgl^{-1} during the period from day 8 to 18 after water was replaced. (A colorimetric test kit/ Aquamerck Ammonium, Merck-Germany/ was used to control NH^+_4–N concentration)

In contrast, groups 2, 3 and 4 showed low concentration levels of NH^+_4–N (total 4 average was 5.01 mgl^{-1}) during the period from day 1 to 13. The average NH^+_4–N concentrations of groups 2, 3 and 4 were 4.85, 4.96, and 5.24 mgl^{-1}, respectively. After aeration ceased during the period between day 14 to 18, the average NH^+_4–N concentrations of groups 2, 3 and 4 were 0.66, 2.25, and 2.38 mgl^{-1}, respectively. In group 2. 3 and 4, presence of S. quadricauda microalgae proved that the physiological capabilities of the immobilized algal cells were not excessively disrupted by the immobilization. Thus, the immobilized algae can be used successfully to control the water quality in the cultures. Chen (2001) and Chevalier & de la Noüe (1985) also reported that *Scenedesmus* cells immobilized in beads were as efficient as free cells in taking up ammonium.

2.2. Fish culture DO levels

DO level of 4 cultures was compared and helped to follow the evolution of fish culture groups without or with Scenedesmus quadricauda. As shown in Figure 3, DO levels of all culture groups were around 7 mgl^{-1} with aeration. However, there were obvious differences in DO levels between the four culture groups when aeration ceased. After ceasing aeration (from days 14 to 18), group 2 showed the highest DO level of 4.8 mgl^{-1}, (4.16 mgl^{-1} on average). The DO levels of group 3 varied (1.7"0.8 mgl^{-1}). The DO levels of group 4 were stable: 2.2 mgl^{-1} on average. The DO levels of group 1, while the control group without microalagae, was the lowest, with only 0.96 mgl^{-1} on average. It is obvious that the algae affected the DO level when no aeration was provided.(DO was controlled by a digital oxygen meter/ LutroDO-%%10-Taiwan)

2.3. Fish culture pH values

As shown in Figure 4 pH values of group 3 were the most changed when aerated. However, the average pH values of the four groups were very close. The average pH values of groups 1, 2, 3 and 4 were 7.72, 7.78, 7,70 and 7.71, respectively. In contrast, after aeration was suspended, the pH values of all groups decreased. The average pH value of group 1 was 7.46. In contrast, the pH values of culture groups with algal beads were near to pH 7.0, in which, groups 2, 3 and 4 were 7.16, 6.96 and 7.07, respectively (a pH meter / Suntex-Taiwan/ was used to test the pH value]. This result showed that the algae controlled water quality to neutral when aeration ceased.

2.4. Body length of tilapia (the growth of tilapia)

After 18 days of culture, the average body length of group 1 was 14.5±0.3 cm, the poorest in growth. Group 2 was 16.3±0.2 cm, the best growth. Group 3 was 15.1±0.3 cm and group 4 was 15.5±0.2 cm.

3.Discussions for S.quadricauda

The alginate-beads method has the following advantages: (1) bead preparation is easier and cheaper than cryopreservation, (2) the beads can be stored in refrigerators, and (3) immobilized cells can be rapidly introduced into liquid cultures. Romo and PérezMart´ınez (1997) reported that they found similar satisfactory results when this technique was used with the chlorophyte *S. obliquus* (Turp) Kütz. The present study also confirmed that the technique is suitable for *S. quadricauda*. Moreover, algal alginate beads without liquid medium directly kept in darkness at 4℃ for more than 3 years were capable of growing and initiated new cultures when transferred into a fresh medium. The amount of *S. quadricauda* coenobia was 9×10^4 per bead. This was the same amount as found in a normal culture. Tamponnet et al. (1985) had similar results. They reported that immobilized *Euglena gracilis* alga was kept for more than 2 years in alginate-beads. It is important to point out that growth is possible within the alginate-beads under suitable culture, which reveals that the algal viability is maintained within the beads.

The reason that *S. quadricauda* remained alive within the beads in the dark at low temperatures for an extended period may be due to the caps reserve of the pyrenoids that present materials for maintaining their basic requirements. TEM observations showed that the ultrastructure of *S. quadricauda* cells lost the pyrenoids after extended storage. The lost pyrenoids were reconstructed when the algae were re-cultured in an aqueous medium with light. However, SEM observations showed that the morphology of *S. quadricauda* cells were not altered extensively when they were entrapped in the alginate-bead, with the exception of bent spines in contrast to the straight spines of normal free-living *S. quadricauda* cells.

Ammonium (NH^+_4–N. in fish cultures with algal beads was utilized by the immobilized microalga as nutrient and resulted in lower concentrations (ca. 5 mgl^{-1}). The biomass of the immobilized algae increased ca. 15 times in 18 days of culture in this experiment. This proved that the physiological conditions of the immobilized algal cells were not interrupted by the immobilization. Thus, the immobilized algae can be used successfully to control the water quality in fish cultures. Chevalier and de la Nouë (1985) also reported that *Scenedesmus* cells immobilized on k-carrageenan beads are as efficient as free cells in taking up ammonium.

Aeration maintained DO and pH at acceptable levels for tilapia. When aeration was suspended, the algal beads were able to increase the DO and they kept pH levels near 7.0 in water. In contrast, ammonium concentrations of tilapia cultures with algal beads (group 2, 3 and 4) were at low levels (i.e. 1.76–5.01 mgl^{-1}) regardless of whether the cultures were aerated. However, when aeration ceased, the ammonium concentration of the fish cultures with algal beads showed lower concentrations than those cultures with aeration (cf. the control group 1: no microalgae introduced)Aeration and variation of

dissolved CO2 and O2 impacts are not further studied in this experience

The average pH values of culture groups with algal beads were near 7.0, in which pH values of group 3 were the most affected. This is in contrast to pH values of group 4, which was more stable than those of group 3 when aeration ceased. These two groups were cultured under the same photoperiods, 12:12 dark:light. However, the algal beads of group 4 were taken away from the jar following darkness. In this study, all immobilized algal cells could be removed from the water easily and immediately, just by taking away the algal containers (the nylon net bags), to avoid the algal respiratory effects. In contrast, it is impossible to remove all the free-living and suspended microalgae from the water of normal cultures.

By judging the data on ammonium, DO and pH (Figures 2,3,4)?) and the results of tilapia growth clearly indicated that the water quality in cultures with algal beads (groups 2, 3 and 4) were better than the culture without algal beads (group 1). The algal alginate-beads could control the water quality to be suitable for tilapia growing.

In conclusion, the microalga *S. quadricauda* previously entrapped into alginate-beads maintained their normal physiological activities and grew within the beads, just like free-living algal cells, no interruption during the immobilization, and the biomass of the immobilized microalgae has increased 15 times in 18 days of culture in this experiment. Therefore, this technique is very suitable for preserving microalgal species and the application of microalgae in aquaculture for controlling water quality in aquaculture application (groups 2,3 and 4 can manage to contribute to Tilapia optimum growth, while in the group 1 no microalgae introduced, only the fish Tilapia, pH went highest to reach 7.72, and fish body length the shortest.

B) Immobilization *Isochrysis galbana*:

Storage, culture and electron microscopy studies of immobilized cells.

Freshly made algal beads contained an average 6.8 × 105± 2.2 × 104 cells per bead. The wet beads without liquid medium were stored in darkness at 4 ◦C for a year. The immobilized cells did not lose their ability to grow after long-term storage, and cell number in the beads was the same than that of fresh algal beads. Cell number rose to 2.1× 107± 5.4× 106 cells in each stored bead following culture in modified PES medium for 5 weeks.

Thin sections of freshly made beads (Figure 6) showed that a large chloroplast filled most of the cell. The conspicuous chloroplasts were typically composed of stacks of thylakoids in groups of three. The multilayered cell coverings (organic scales) (Figure 6,7), which were separated by amorphous electron dense material were laid outside the distinct cytoplasmic membrane. The ultrastructure of freshly immobilized cells was the same as that of free-living *Isochrysis* (Figure 7). However, the cell covering in the latter was more developed. By contrast, after long-term storage of immobilized cells, the organic scales were minimal or had disappeared (Figure 8), the cytoplasmic membrane was less distinct and the pyrenoids were smaller than those of normal cells. However, the organic scales and

pyrenoids appeared normal again within a week after cells had been cultured in modified PES medium at 100 μmol photon m−2s−1 irradiance and 12:12, dark: light photoperiods at 24 ◦C.

SEM sections of immobilized cells, which had been stored for an extended period (1 year) and then re-cultured for five weeks, showed inconspicuous gullet-like structures (Figure 10). Flagella protruded from these structures, and were lost when the organism was no longer alive. The morphology was nearly the same as that of normal cells (Figure 10). In addition, cells located at the surface of the bead showed two long flagella (Figure 11). These cells escaped from the beads and feed the clams (as per explained in the following discussion) into the culture water after 5~6 weeks of reculture. Because of the small size of these cells (ca. 3∼5 μm diameter), the flagella of most *I. galbana* were 'burned' away by the high voltage electron beam during SEM observation. However, some flagella on the immobilized beads were protected by the alginate, and could therefore be observed (Figure 11).

Use of algal alginate-beads for clam culture

The ammonium concentration of group 1 increased to 9.5 mg L−1 at day 30 of culture and remained at this high value. During the 30 days of culture the ammonium-N concentration was 5.92 ± 3.1 mg L−1, the highest among Isochrysis galbana groups.

In contrast, groups 2, 3 and 4 showed low concentrations of ammonium during the 30 days of culture. The ammonium concentration of group 2 increased to 3.5 mg L−1 at day 15, and then remained around at 3.3 to 3.5 mg L−1 from days 15 to 30 of culture. The ammonium concentration of group 3 fluctuated with a slow trend toward increase, but remained between 4.3 to 4.5 mg L−1 during light periods and between 5 to 5.3 mg L−1 during dark periods after day 19 of culture. The ammonium concentrations of group 4 fluctuated with a trend towards increase, from 0.3 to 5.1 mg L−1 during light periods and from 0.1 to 6.3 mg L−1 during dark periods during the 30 days of culture. In the present study ammonium of clam cultures was utilized as a nutrient by the immobilized alga, which resulted in lower concentrations (average 2.75 mg L−1). The cell numbers of the immobilized alga increased ca. 32 times in 35 days of culture in this experiment. This proved that the physiological capabilities of the immobilized algal cells were not excessively disrupted by the immobilization. Thus, the immobilized algae can be used successfully to control the water quality in clam cultures (Chen, 2001 and Chevalier & de la Noüe, 1985).

The average ammonium concentrations of groups 2, 3 and 4 were 2.75 ± 1.1, 3.65 ± 1.5 and 3.94 ± 1.7 mg L−1, respectively. However, there was no significant difference in ammonium concentration between groups 2, 3 and 4 during light periods. There were obvious differences in DO concentrations among the four culture groups during the 30 days of culture. The DO concentrations of group 1 showed a decreasing trend, from 7.2 to 6.2 mg L−1. DO concentrations of group 2 showed the highest values, from 7.3 increasing to 8.7 mg L−1. Groups 3 and 4 (especially the latter) showed fluctuating values. Average DO concentration for groups 1, 2, 3, and 4 was 6.8 ± 0.36, 8.2 ± 0.5, 7.5 ± 0.58 and 6.6 ± 0.77 mg L−1, respectively.

The pH of group 1 was 6.5 at the end of the experiment, the lowest among all groups. The values for group 2 were steady at around pH 8.4. The values for group 4 showed the most change. The

average pH values for groups 3 and 4 were around 8.0 and 7.7, respectively.

Initially, cells were rare in the liquid medium of groups 2, 3 and 4. After 1 week of culture some cells escaped from the alginate-beads, becoming active and swimming freely in the water. The cell number in groups 2, 3 and 4 ranged between 8 × 102 and 3 × 103 cells mL−1 during the 30 days of culture. In group 2 some escaped cells assumed the palmelloid morphology and adhered to the wall of the jar in brown film-like layers (the biomass of those palmelloid cells was not assessed). In the algal bead re-culture experiments, the concentration of escaped cells was ca. 5 × 104 cells mL−1 at week 6 in a 5-L flask containing 3 L modified PES medium. This value continued to rise until reaching 5.6 × 107 cells mL−1.

After 30 days of culture, the average clam length for groups 1, 2, 3 and 4 was 2.65 cm, 3.0 cm, 2.9 cm and 2.8 cm, respectively. The clam survival rate for groups 1, 2, 3 and 4 was 25%, 85%, 80% and 70%, respectively.

The biomass of the escaped algae varied, possibly due to the physiology of the clams that ate the algae, as well as the environmental conditions affecting algal growth, such as the concentrations of ammonium and DO and pH value and light or CO2 dissolved. Consumption by the clams reduced algal biomass, presumably by cells escaping from the surface of the beads due to continuous division inside the beads. The space inside the beads is limited; therefore the cell volume expanded and extruded excess cells out from the surface of the beads into the culture water. The escaped cells did not seem to affect the water quality. This finding provides a sustainable clam culture system without the need to add live feed repeatedly.

3.Discussion for Isochysis galbana

This study confirmed that the technique reported by Romo & Pérez-Martínez (1997) and Chen (2001) with the non-motile freshwater green alga *Scenedesmus* is also suitable for the motile marine alga, *Isochrysis galbana.* Moreover, those algal alginate-beads without liquid medium kept in the dark at 4 ◦C for more than one year were capable of growth and initiated new cultures when transferred to fresh medium and suitable growth conditions.

TEM showed that the pyrenoid size was reduced after extended storage. The cell covering component was also reduced, perhaps to save and store materials for cell survival. However, ultrastructural observations showed that the structures of *I. galbana* cells were not altered extensively when they were entrapped in the alginate-beads.

The pH values of culture groups 2, 3 and 4 with algal beads were in the range 7.7 to 8.4. The values for group 2 were more stable than those of groups 3 and 4, which were cultured under the same irradiance and photoperiods. The algal beads of group 3 were removed from the jar during darkness to avoid the algal respiratory effects. The slight fluctuation in pH may therefore be due to the clam respiration during the periods without the alga.

DO concentrations of group 2 were always higher than those of the other groups. The film-like palmelloid *Isochrysis galbana* layer in this group may have also aided in maintaining good quality

culture water. In group 3 the algal beads were removed during darkness, whereas the beads of group 4 were not so, leading to lowest values for both pH and DO concentrations.

Judging from the results of ammonium, DO and pH values and the clam growth and survival rates, the water quality in cultures with algal beads (groups 2, 3 and 4) was better than the cultures without algal beads (group 1). The algal alginate-beads could maintain the water quality suitable for clam culture.

The number of cells was ca. (2.18×107) per bead after re-culture was similar to that found in a normal culture. Hertzberg & Jensen (1989) immobilized the marine diatom *Phaeodactylum tricornutum* in alginate beads and had similar results. However, the present study required only 5 weeks to reach that amount of cells in contrast to the study by Hertzberg & Jensen, which required 5.5 months. It is important to point out that growth is possible within the alginate-beads under suitable culture conditions, therefore the method should reduce considerably the cost of clam culture compared to traditional culture methods, because the algal beads can act as a convenient 'auto-feed' system to reduce the cost of clam culture,

C) Immobilization of twelve benthic diatom species

1. Classification, isolation and immobilization of the benthic diatom species

In the survey of the diatom species from the H. diversicolor post-larvae hatcheries (from East-north Taiwan and Penghu county), 29 species of pinnate diatom (belonging to 19 genera) were identified, in addition to 4 species of centric diatom (belonging to 4 genera). The diatom species are listed in Table 1. Among these diatoms, 12 species of pennate diatoms were successfully isolated to monospecies, either by streaking or by dilution methods, and survived re-culture under laboratory conditions. These isolated monospecies were further observed via SEM to confirm their species (Fig. 12,13,14). They were also successfully immobilized in alginate beads (Figure 12, A to F, Figure 13 G to L and Figure 14).

When the algal beads were stored in a 4 ℃ refrigerator for more than 1 year, the cell numbers of the diatoms remained at more than 105 cells/bead, capable of initiating new cultures. When the algal beads were then re-cultured in modified PES media to check their viabilities, all the diatom species in the beads were still alive and increased in biomass once again (Table 2).

2. Post-larval survival and growth rates

Twelve species of benthic diatoms were used as feed for post-larvae of *H. diversicolor*. This work was repeated five times between April 16, 2005 and May 26, 2006. During that time the range of the average temperatures was 19.7 to 29.2 ℃ and the range of the average light intensities was 228.7 to 2743.7 μmol m^{-2} s^{-1}. These environmental conditions are suitable for the culture of small abalone fertilized eggs, larvae and post-larvae, according to Chen (1989). The detailed data of dates,

temperatures and light intensities of the five experiments in the green house are listed in Table 3. Because abalone larvae hatch without a shell gland and die within 1 or 2 days when the fertilized eggs are reared at 30.5 ℃ or above (Chen, 1989), no larvae were cultivated during the hot summer season (July and August) when the temperatures rise to above 31 ℃.

The abalone survival (harvest) rates, growth rates, diatom sizes and diatom composition are summarized in Table 4. All 12 species of diatom attracted settlement of the small abalone post-larvae and could successfully be used as feed for the post-larvae, which will grow to 3–4 mm shell lengths after 40 to 54 days of culture, and can then be transferred from the hatchery to farmers rearing juvenile abalone. The reddish colors of the shells of small abalone were not identical. Some shells were brown-red in appearance, and some were fresh red, depending on the particular species of diatom they had been fed. The post-larvae fed with *Cocconeis scutellum* and *Cylindrotheca* sp. had pale areas on their shells and were noticeably different from other postlarvae. There were big differences in the average numbers of post-larvae harvested from cultures fed different species of diatoms, ranging from 7.7 to 112.6 post-larvae/1000 cm^2. *Nitzschia grossestriata* resulted in the best average harvest of 112.6 post-larvae/ 1000 cm^2 and the best average daily growth rate of 91.13 μm/day. In contrast, *C. scutellum* resulted in the lowest average harvest of 7.7 post-larvae/1000 cm^2 and the slowest average daily growth rate of 65.33 μm/day. However, the growth rates (78.13–91.13 μm/day) were not significantly different between the diatom species except (65.33–66.43 μm/day) for the last three listed in Table 4 (*Cylindrotheca* sp., *Fragilaria schulzii* and *C. scutellum*).

3. EPS and biochemical composition of the diatoms

The EPS (soluble EPS, bound EPS, internal carbohydrates, and residual carbohydrates) of the diatom species varied. The soluble EPS had a positive effect on the survival of abalone larvae (Figure 15 A). The protein content of the diatoms was fairly uniform, mostly in the range of 10–14% of dry weight, with *Cylindrotheca* sp. lowest at 8.8%, and *Caloneis platycephala* highest at 17.4%. In contrast, lipid contents (4.7 to 20.3% of dry weight) showed a larger variation, indicating a relationship with the survival and growth of post-larvae, with higher lipid contents resulting better survival and growth (Figure 12, Figure 1-2 and 1-3). The lipid content of *Cylindrotheca* sp. was the lowest at only 4.4% of dry weight, while *Seminavis gracilenta* was the highest at 20.3% and its bound EPS was also the highest at 1190 pg/ 100 $μm^2$. *S. gracilenta* resulted in the second-highest harvest number (102.9 post-larvae/1000 cm^2), with the best harvest rate (112.6 post-larvae/1000 cm^2) being obtained by using *N. grossestriata* as the feed. Lipid, bound and soluble EPS contents of N. grossestriata were 13.7%, 173.9 pg/100 μm2, and 212.31 pg/ 100 $μm^2$, respectively. The amount of soluble EPS of N. grossestriata was the highest among the diatom species. The average diatom cell sizes, the average contents of internal and residual carbohydrates were also varied between the diatom species, but showed no relation to the larvae harvest (survival) rate as well as the bound EPS (Figure 12, Figure C and D).

*4. Discussion on twelve benthic diatoms species immobilization with compared quantitative efficiency of each species***:**

In the survey of the diatom species from the small abalone hatcheries, 33 species of diatom were identified and 12 species of benthic diatoms were successfully isolated to generate monospecific cultures. Therefore, this paper focused on isolating and culturing the benthic pennate diatoms. The present study confirms technical reports by Romo and Pérez-Martínez (1997) and Chen (2001, 2003) showing that immobilized methods are also suitable for the benthic diatoms. In addition, benthic diatom alginate beads that were held without liquid medium and in the dark at 4 ℃ for more than 1 year were capable of growth and initiating new cultures when transferred to fresh medium and suitable growth conditions. The numbers of cells of those diatoms were ca. 2.7×106 to 2.6×107 cells per bead after re-culture, similar to the values for a normal culture. Hertzberg and Jensen (1989) immobilized the marine diatom *Phaeodactylum tricornutum* in alginate beads and obtained similar results. However, the present study required only 4 weeks to reach that amount of cells, in contrast to the study by Hertzberg and Jensen, which required 5.5 months. It is important to point out that growth is possible within the alginate beads under suitable culture conditions (Chen, 2003).

The immobilized monospecies were subsequently applied as feed for the culture of post-larvae of the small abalone, *H. diversicolor*, and yielded a good post-larval harvest (up to 112.6 post-larvae/1000 cm2). The traditional *H. diversicolor* culture using natural growth mixed diatoms had a similar harvest (Chen, 1989). However, in the present study post-larvae required only 40–54 days to reach a harvestable size of 3–4 mm, whereas those reared with traditional methods required 2–3 months to reach the same size.

In the present study the average daily growth rates were 65.33–91.13 μm/day which were substantially faster than those recorded by Kawamura et al. (1998) for Haliotis iris post-larvae fed with 8 monospecific cultures of diatoms (11.8 to 35.3 μm/day). Underwood et al. (2004) described the amount of EPS in diatom species using the unit of weight/cell (pg/cell). However, the cell sizes vary among diatom species, making it impossible to compare the EPS contents among species with accuracy. Therefore, in the present study the cell surface area was used for the denominator of the unit (pg/100 μm^2) to describe the amount of EPS in each diatom species.

Various factors may influence the nutritional value of diatom cultures, including the nature and quantity of their extracellular polysaccharide (EPS), the health of the diatom culture, the associated microbial flora (Kawamura, 1996) and the biochemical composition of the diatom cell contents (Dunstan et al., 1994, 1996). Many of these characteristics will vary among diatom species. The present study has shown that even when cultured under the same laboratory conditions, the 12 species all had different cell components (influencing their nutritional values).

Daume et al. (2000) suggested that extracellular polysaccharides (EPS) produced by diatoms are responsible for the attachment of the cells and that well attached *Navicula* sp. produced the largest number of post-larvae with the highest survival rate. However, the present study found that sufficient lipid (ca. 7–20.3% of dry weight) and soluble EPS (ca. 30–212 pg/100 μm^2) collectively improved the

survival rate (harvest rate) of small abalone post-larvae.

Gordon et al. (2006) indicated that the nutritional value of microalgae as feed is influenced to a great extent by their lipid. The protein composition of microalgae is generally conservative and is unlikely to account for major difference in the nutritional value of particular species. The present study also found that the protein contents of the 12 diatom species examined fell within a limited range, and were not significantly different. In contrast, the lipid and soluble EPS contents of these same diatom species were significantly different, and appeared to be the major factors affecting the abalone post-larvae survival (Figure 12 A,B and C)

Hahn (1989c) reported that 'good' diatoms are defined as ones that produce large amounts of secretion and are smaller than 10 μm. The present study had a similar result in that *N. grossestriata* secreted the highest amount of soluble EPS (212.31 pg/100 μm^2), and this may well attract abalone larvae to settle on areas containing this diatom. The sizes of the diatom species bore no relationship to the abalone post-larval harvest rate. The fact that *C. scutellum* resulted in the worst post-larval harvest rate contradicts the conclusions of Hahn (1989c). This diatom was the smallest of the diatom species examined, measuring 7 μm in length and 5 μm in width. However, Hahn (1989c) further indicated that *Navicula* spp. and *Nitzschia* spp. produce good growth rates. The present study yield similar results, with *N. grossestriata* and *N. panduriformis*, as well s *S. gracilenta* (similar to *Navicula* spp.), producing good post-larval harvest rates.

In conclusion, using the microalgal immobilization technique for long-term storage of monospecific cultures of diatoms and then subsequently re-culturing them for use as feed for the cultivation of post-larvae of the small abalone, *H. diversicolor*, proved to be a viable means of increasing the survival rate of the post-larvae, provided that the species of diatom employed had sufficient lipid content and a large amount of soluble EPS. This procedure can considerably reduce the cost of abalone post-larval culture compared to the traditional methods, using microalgae which constitutes up to 30% of the total cost of production (Valenzuela-Espinoza, Millan-Nunez & Nunez-Cebrero, 1999), as peculiar species of diatoms lipids composition shows higher effectiveness impact the growth and survival rates of marine organisms.

III) Conclusions

In general the morphologic change observed in the cell (Spine bent,…), seems not to have affected their metabolism and the physiological conditions of the algal cells not interrupted with capacity to grow, and multiply in aquaculture medium after few weeks acclimatization of the chloroplast the Pyrenoid has reappeared. The period of storage was particularly long and successful for most of the immobilized microalgae. The gel of Ca-Alginate constitutes an ideal matrix for the microcells due the affinity with microalgae. It is offering a labile and mild interactive structure, allowing retention of cells inside the beads, non toxicity, photo-transparency, stability and mobility in liquid growth medium, offering a good resistance to a possible disruption of the beads by cells division,

allowing this way extrusion of excess cells out from the surface of the beads as seen on electronic microscope (Brouers et al, 1989). It can preserve the microalgae species during storage and serves as a continuous feed for the culture.

- *S. quadricauda* previously entrapped into alginate-beads maintained their normal physiological activities and grew within the beads, free-living algal cells. The algal alginate-beads could control the water quality to be suitable for tilapia growing. Therefore, this technique is very suitable for preserving microalgal species.

- *I.galbana* alginate-beads could maintain the water quality suitable for clam culture too, those algal alginate-beads deprived of medium or of light, can keep aliveness after one year time and initiate new cultures when transferred to suitable growth conditions. The number of cells found was normal and growing very fast (30 to 40 times).
- *H. diversicolor*. -Immobilization technique for long-term storage showed similar favorable results for the most nutritious Diatoms monospecific species for the post-larvae of the small abalone,

Certainly the environmental conditions needs to be further determined to find optima growth (ammonium concentration, DO, pH, light intensity, density of the cells (some experiments suggesting that higher density would accelerate reaction rates), respiratory activity effects in parallel to photosynthetic activities culture cycle, temperature variation, day and night differences in ponds culture, seasons difference, and high probability of heterotrophic presence during the culture. Also the incidence of immobilization on cells wall permeability, cells division and the leaking from the beads, could be studied.

Then the three studies information can crosscheck, and match up with authors references, it even goes for a longer storage time, and pushed to quantification of microcells contents, while the use of TEM and SEM helped to bring in images of the immobilized cells in bead, during and after immobilization. It allowed following the life development of the microalgae and diatoms and clarifying each phase of the study and confirming the advantages of biocatalyst entrapment in these cases studied.

1) *Scenedesmus quadricauda* immobilization : Tab & Figures

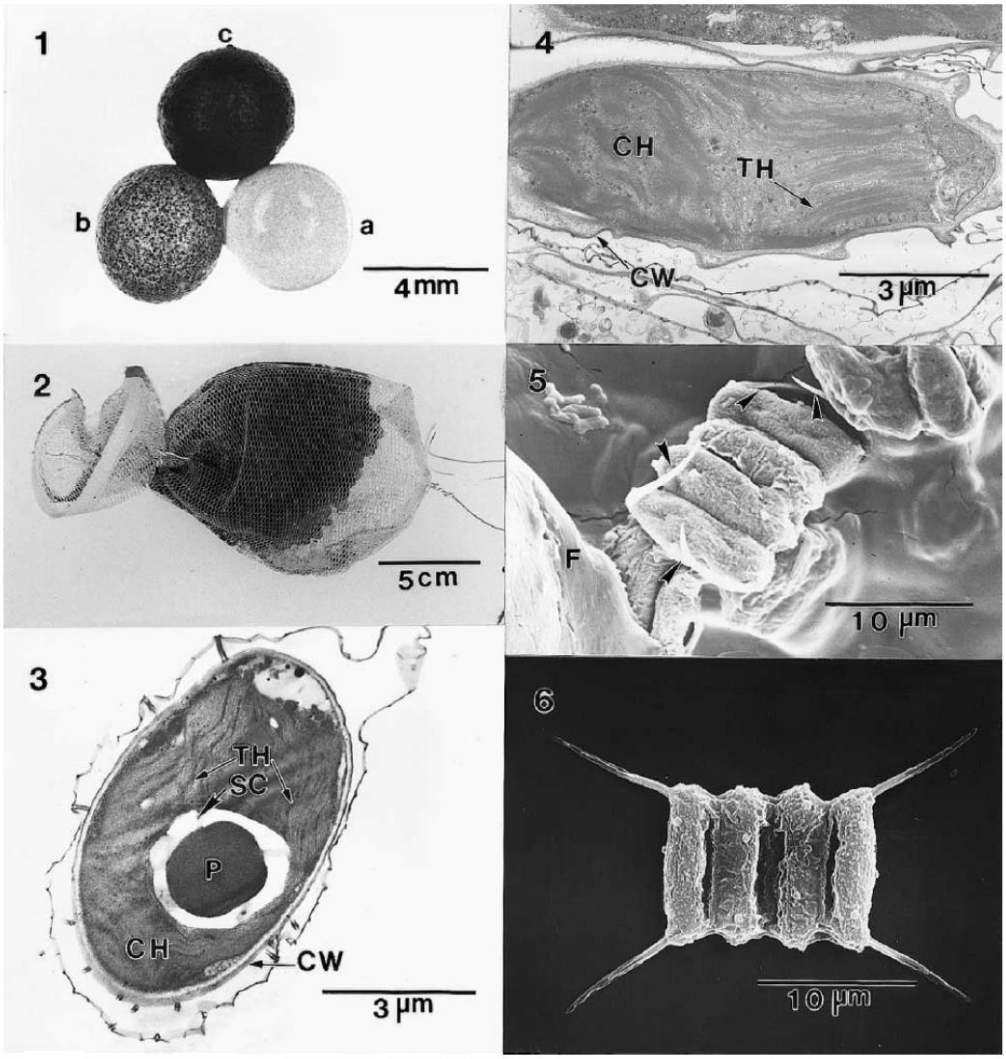

Figure 1. (1-6). (1–2) The algal alginate-beads: (1a) The long-term stored algal bead that contained ca. 2500 algal coenobia. (1b) The stored bead that contained ca. 20,000 algal coenobia had been re-cultured in PEF medium with light for 2 weeks. (1c) The stored bead that contained ca. 90,000 algal coenobia had been re-cultured in PEF medium with light for 4 weeks. (2) The algal beads were packaged in a nylon-net bag for subsequently hanging in the water of fish culture. (3–6) The ultrastructure of algal cells: (3–4) Observations under TEM: (3) A thin-section of an algal cell showing thylakoids (TH) composed of chloroplast (CH) fills most of the cell, in which a pyrenoid (P) is normally suspended. CW: cell wall. SC: starch cap. (4) Thin-section of long-term stored algal cell. The chloroplast (CH) was simply composed of thylakoids (TH) without the pyrenoid. CW: cell wall. (5–6) Observation under SEM: (5) *S. quadricauda* coenobium located within the fractions (F) of alginate-bead. The spines (arrow heads) were modified into bent shapes. (6) Free-living *S. quadricauda* coenobium with straight spines.

Figure 1

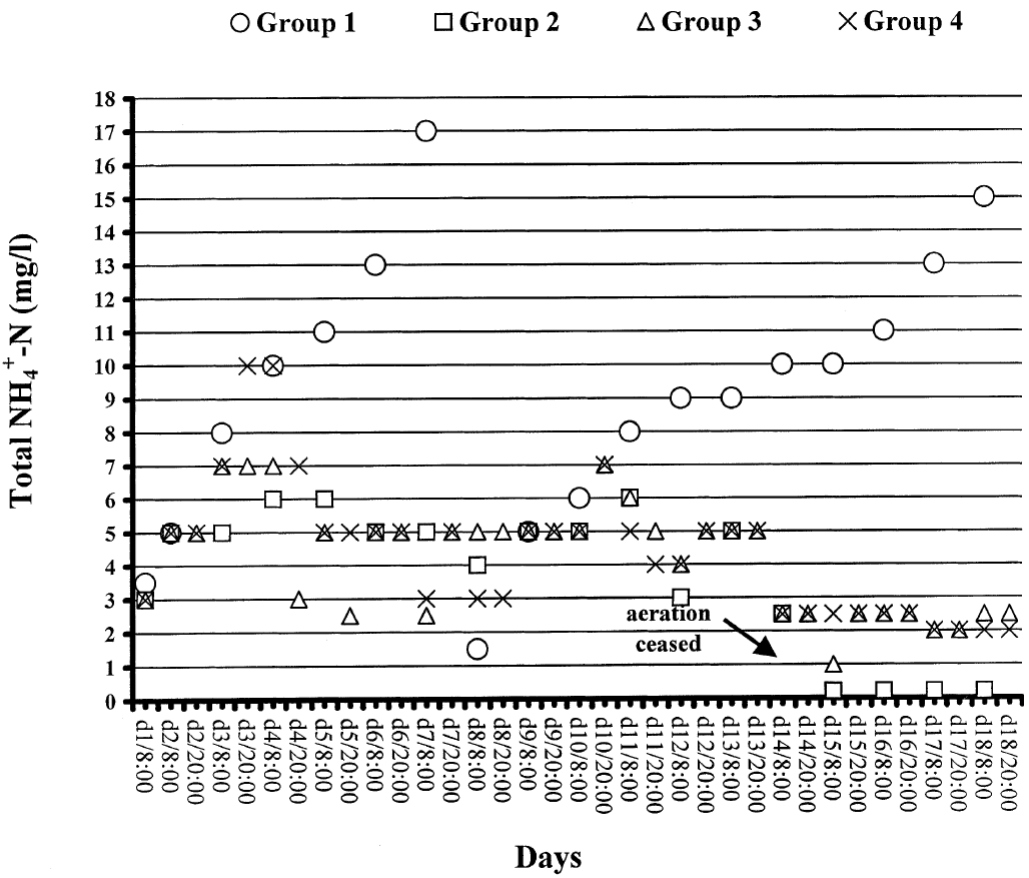

Figure 2. Total NH_4^+ –N concentrations of the tilapia culture in all the groups.

Figure 2

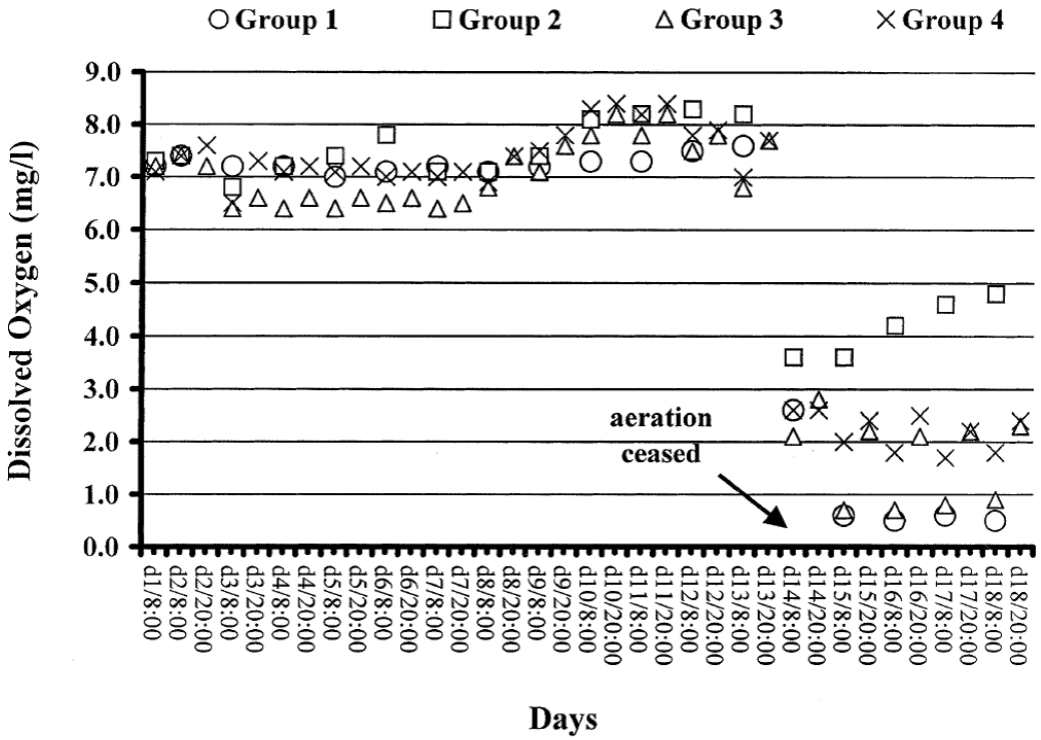

Figure 3. Dissolved oxygen of the tilapia culture in all the groups.

Figure 3

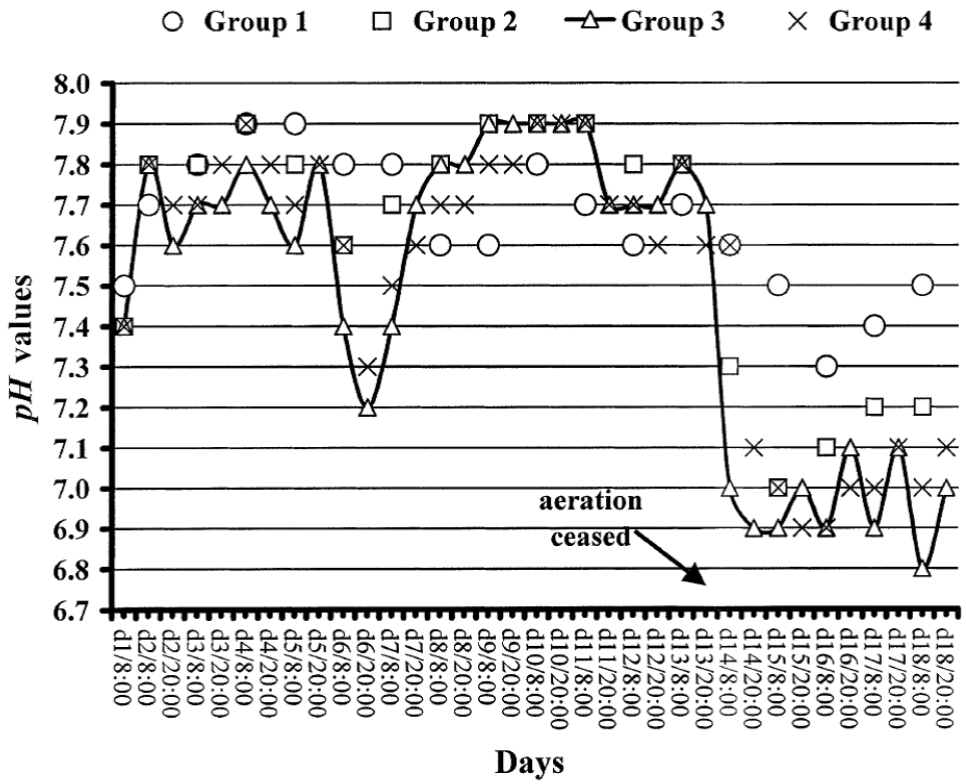

Figure 4. pH values of the tilapia culture in all the groups.

Figure 4

2) *Isochrysis galbana* immobilization Figures and Tab

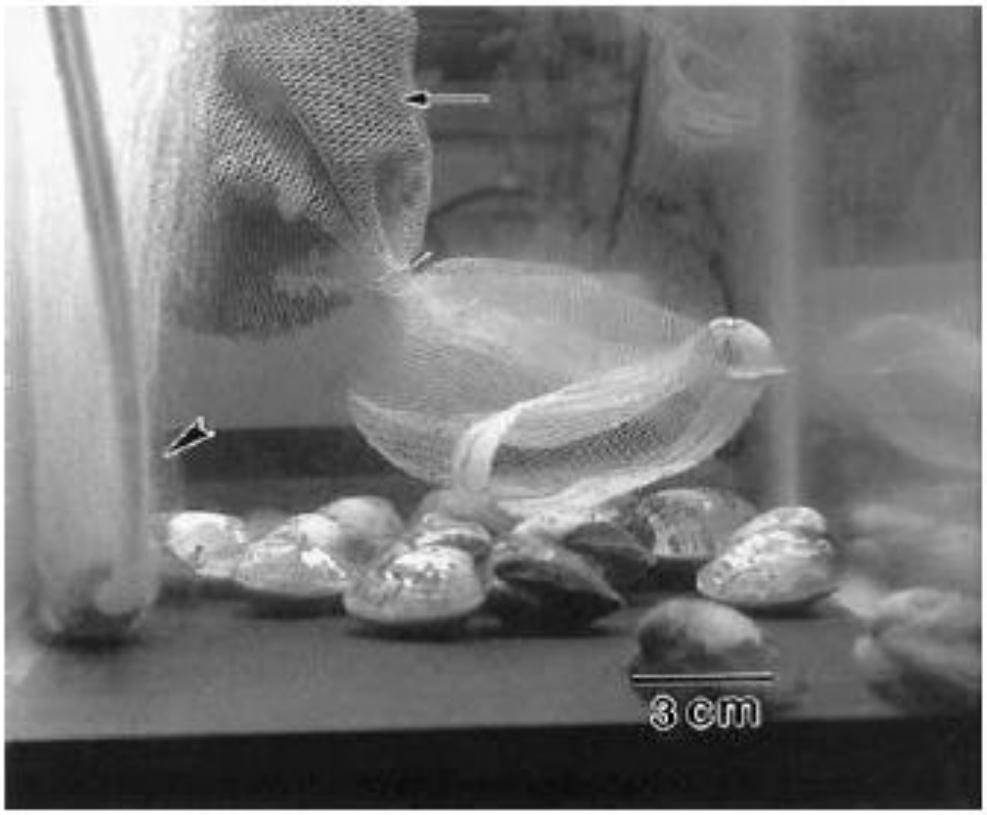

Figure 5. Clam culture with algal beads. Arrow indicates the nylon net bag with algal beads inside. Arrowhead indicates the aeration system.

Figure 5

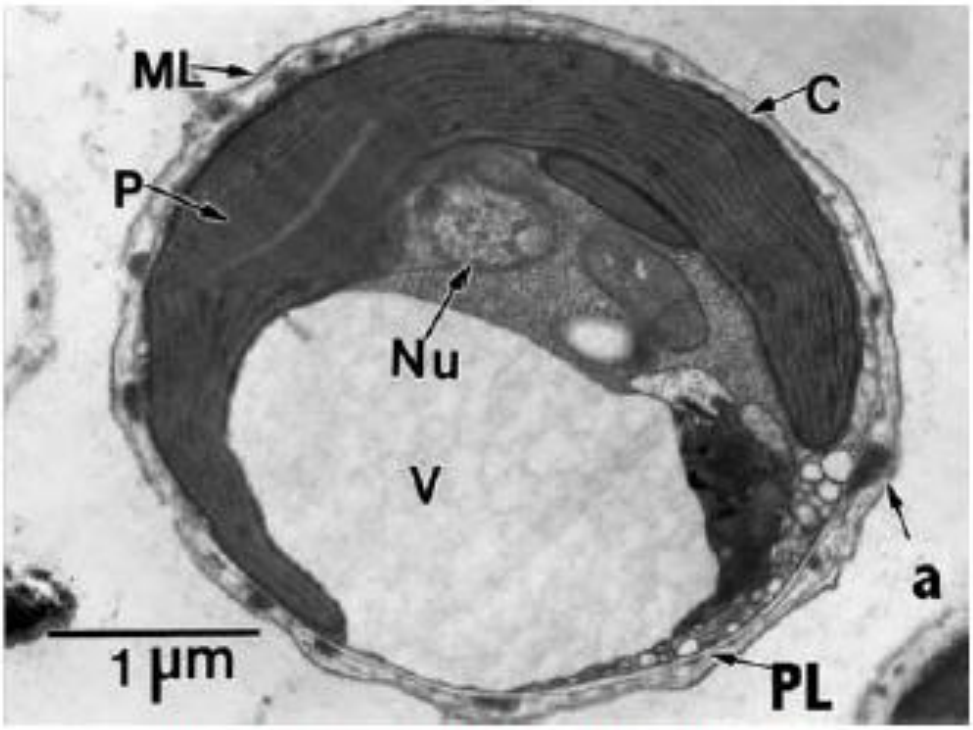

Figure 6. Thin-section of freshly immobilized *Isochrysis galbana* cell. A chloroplast (C) with prominent thylakoids in which a pyrenoid (P) is normally suspended, fills most of the cell. Thylakoids are typically grouped as threes. a: amorphous electron-dense material. ML: multi-layers cell covering. V: vacuole. PL: Plasmalemma. Nu: nucleus.

Figure 6

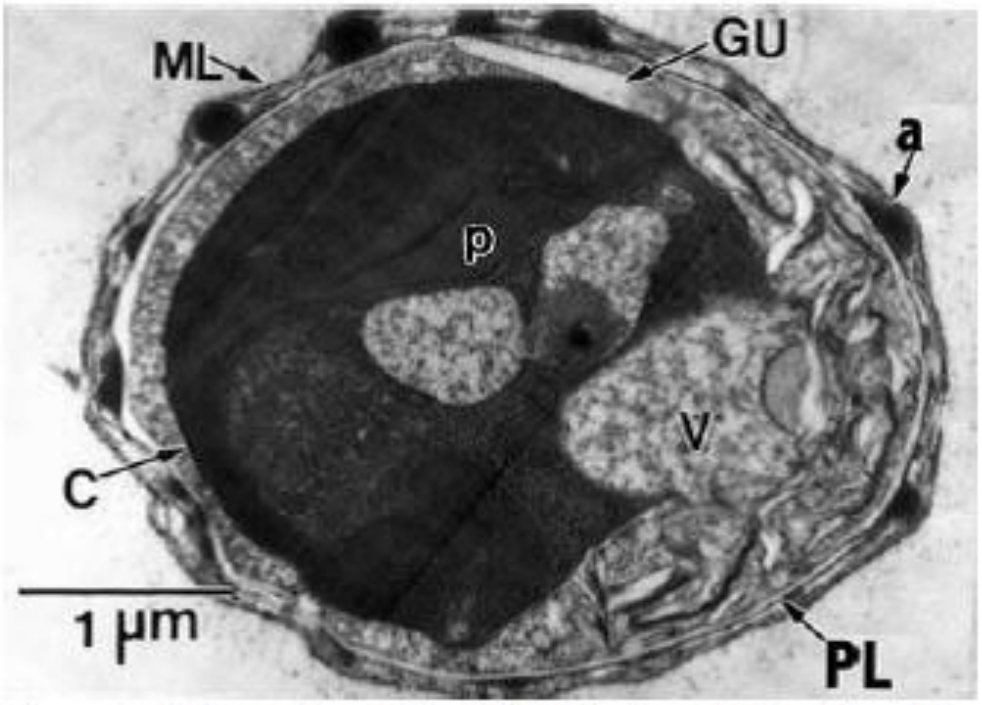

Figure 7. Thin-section of free living algal cell. The chloroplast again composed of thylakoids and a prominent pyrenoid. The cell has obvious PL and cell covering (ML). GU: gullet-like structure.

Figure 7

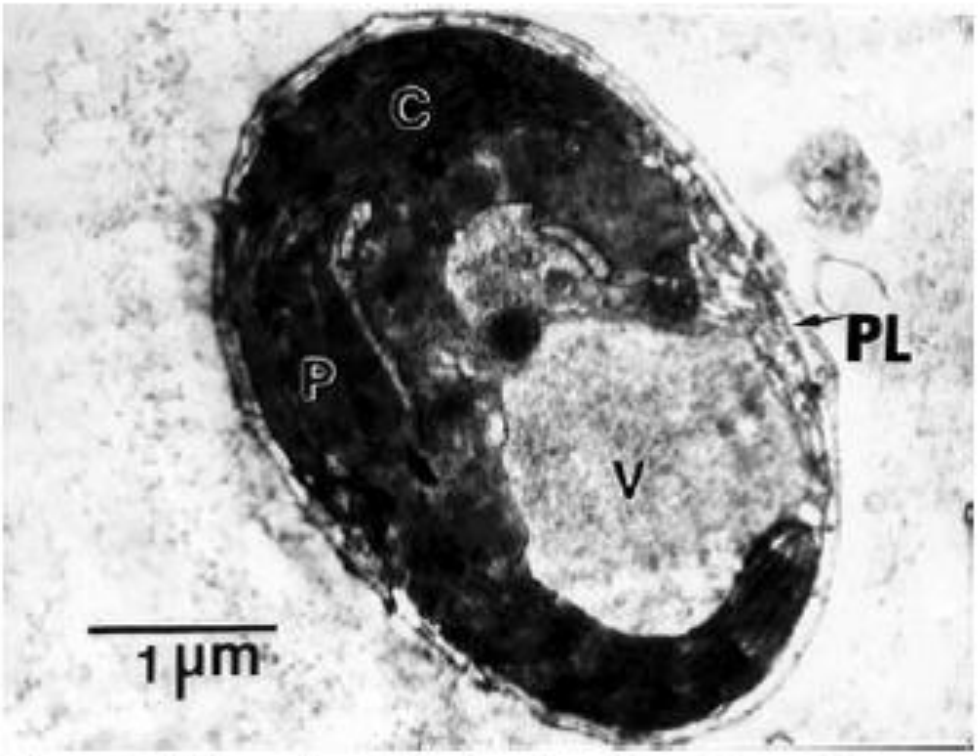

Figure 8. Thin-section of *Isochrysis galbana* from a long-term storage bead. The pyrenoid is reduced, the cell covering (ML) disappeared and the plasmalemma was not conspicuous.

Figure 8

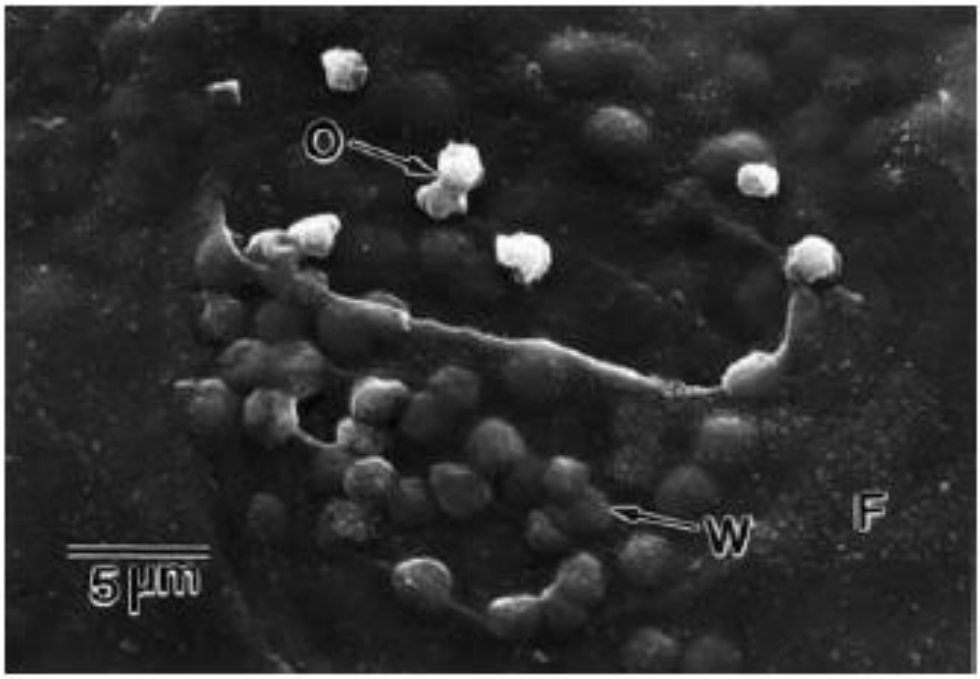

Figure 9. *Isochrysis galbana* on and within surface of a fragment (F) of a recultured long-term storage algal bead. O: Newly extruded *I. galbana* on the surface of the bead. W: entrapped *I. galbana* protruding from the surface.

Figure 9

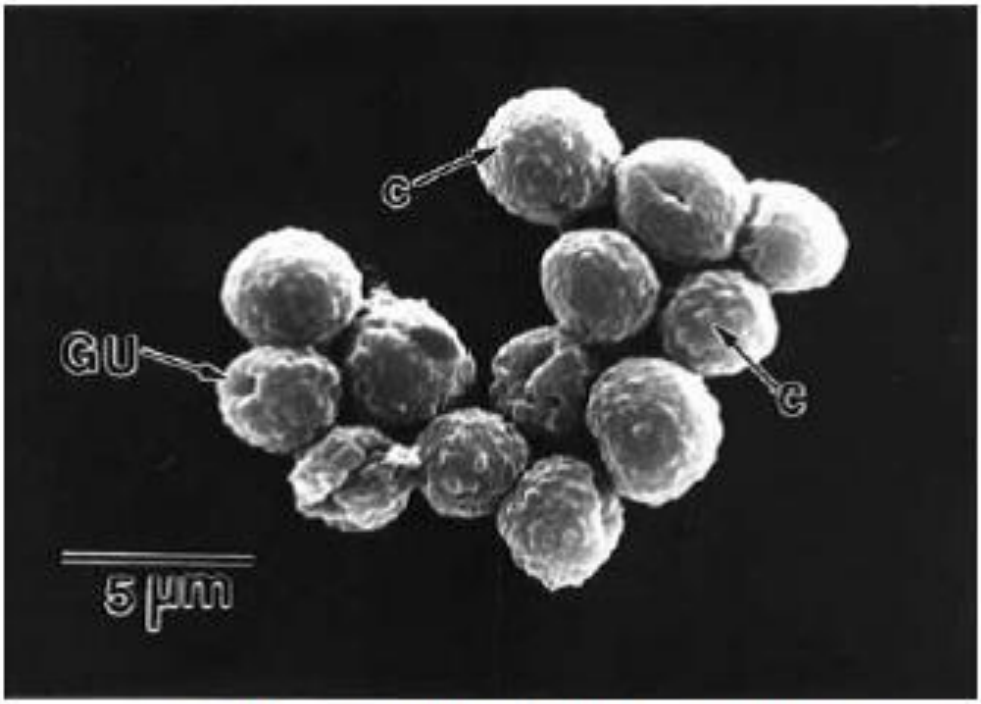

Figure 10. Free-living *I. galbana*. GU: gullet-like structure. c: cell covering (organic scale).

Figure 10

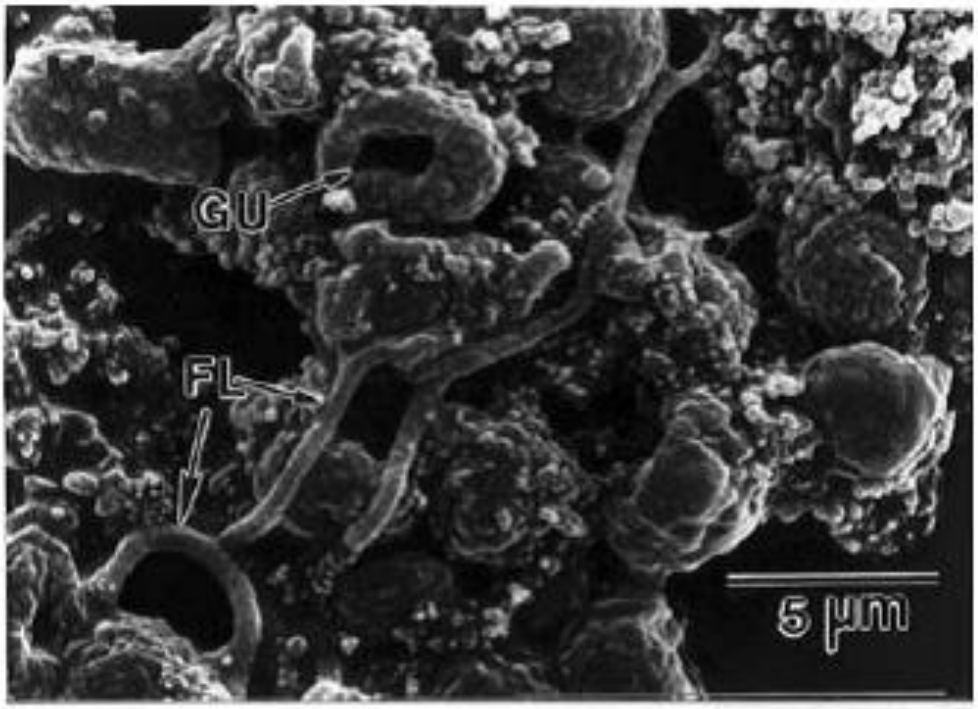

Figure 11. Flagella (FL) of *I. galbana* on the surface of the bead.

Figure 11

3) Twelve immobilized monospecific benthic diatoms Figures and Tab

Table 1

Table 1
The diatom species found from the abalone post-larvae hatcheries

Pennate diatoms	Centric diatoms
Achnanthes citronella	*Biddulphia* sp.
Achnanthes javanica	*Chaetoceras* sp.
Amphora exigua	*Melosira nummuloides*
Amphora bigibba	*Skeletonema costatum*
Asterionella sp.	
Caloneis platycephala	
Climacosphenia sp.	
Cocconeis scutellum	
Cylindrotheca closterium	
Cylindrotheca sp.	
Cymbella sp.	
Diploneis sp.	
Entomoneis sp.	
Fragilaria schulzii	
Fragilaria sp.	
Licmophora sp.	
Navicula lyra	
Navicula tuscula	
Nitzschia lanceolata	
Nitzschia panduriformis	
Nitzschia grossestriata	
Pleurosigma normanii	
Seminavis gracilenta	
Synedra fulgens	
Synedra sp.	
Stauroneis sp.	
Trachyneis minor	

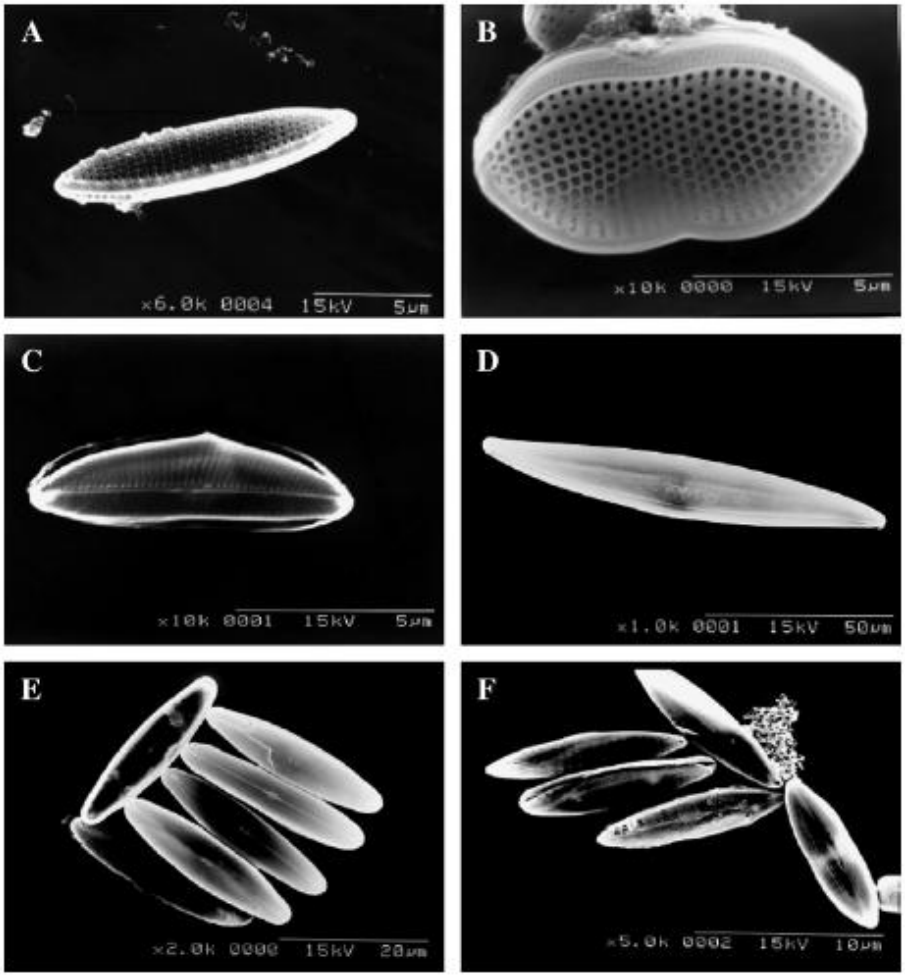

Figure 12. The scanning electron micrographs of the twelve diatom species. Names of the species: A, *Nitzschia grossestriata*. B, *Nitzschia panduriformis*. C, *Seminavis gracilenta*. D, *Pleurosigma normanii*. E, *Caloneis platycephala*. F, *Trachyneis minor*. G, *Synedra fulgens*. H, *Cylindrotheca* sp. I, *Amphora biggiba*. J, *Fragilaria schulzii*. K, *Amphora exigua*. L, *Cocconeis scutellum*.

Figure 12

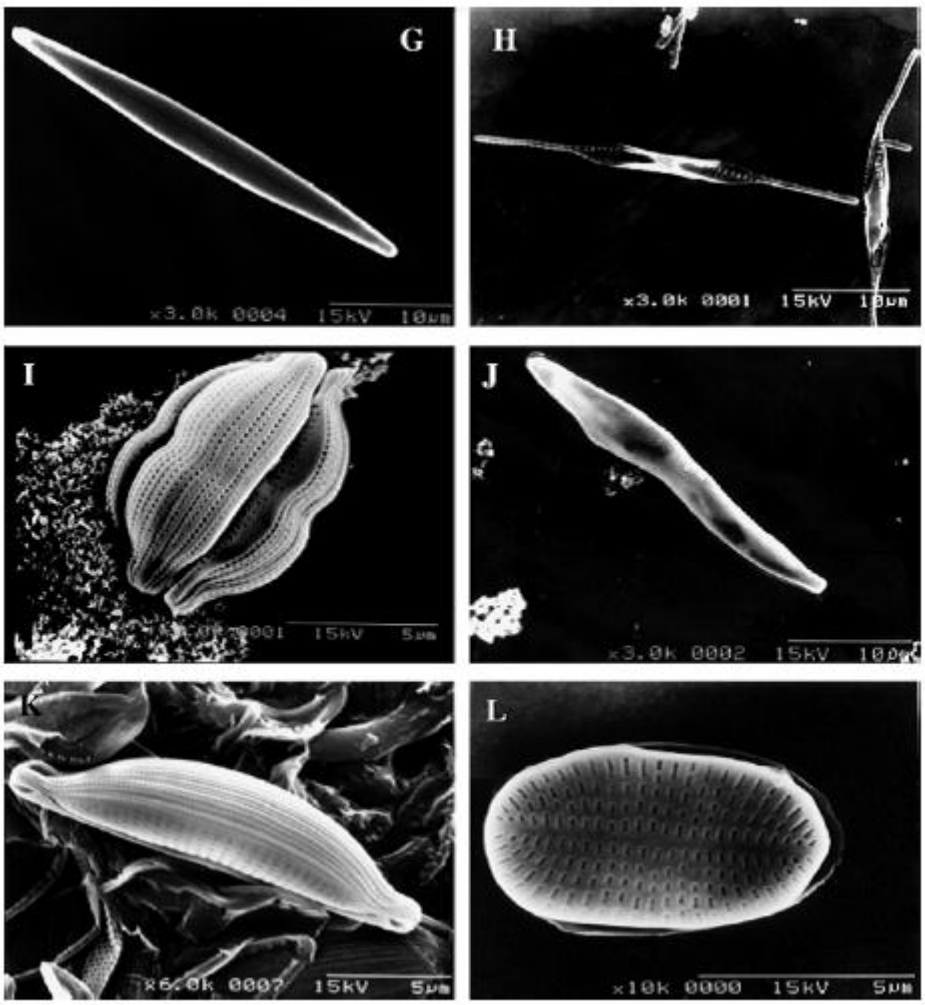

Figure 13. (*continued*).

Figure 13

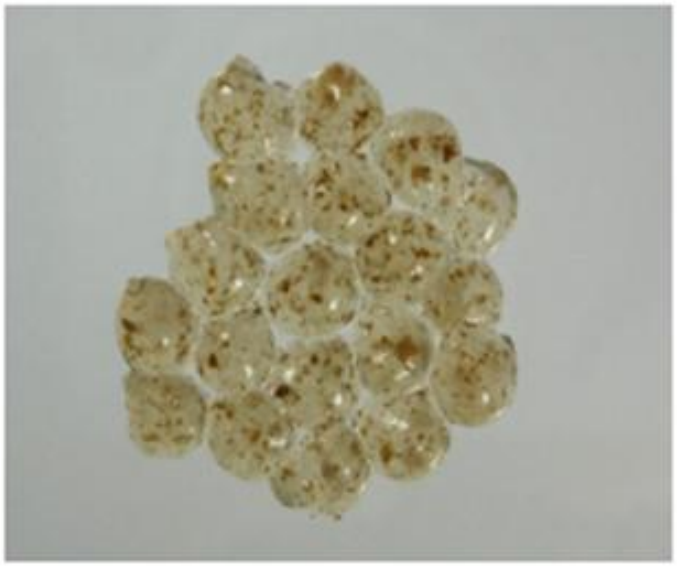

Figure 14. The algal beads of the diatom after being re-cultured for 4 weeks. The diameters of the beads measured about 3–4 mm.

Figure 14 The algal beads of the diatom after being re-cultured for 4 weeks. The diameters of the beads measured about 3-4 mm.

Table 2
Cell numbers of diatoms of immobilized algal beads of twelve diatom species

Diatom species	Cell number in bead	Cell number in bead after storage *	Cell number per bead after re-culture
Amphora bigibba	2.7×10^{5}	2.0×10^{5}	8.5×10^{6}
Amphora exigua	2.1×10^{5}	1.7×10^{5}	2.7×10^{6}
Caloneis platycephala	9.5×10^{5}	1.0×10^{5}	1.3×10^{6}
Cocconeis scutellum	8.7×10^{6}	1.8×10^{5}	6.6×10^{6}
Cylindrotheca sp.	3.9×10^{5}	2.1×10^{5}	9.0×10^{6}
Fragilaria schulzii	2.7×10^{5}	1.4×10^{5}	2.0×10^{6}
Nitzschia grossestriata	1.3×10^{7}	4.5×10^{5}	4.0×10^{6}
Nitzschia panduriformis	1.5×10^{6}	5.3×10^{5}	2.6×10^{7}
Pleurosigma normanii	3.8×10^{5}	3.0×10^{5}	6.0×10^{6}
Seminavis gracilenta	3.9×10^{5}	1.6×10^{5}	1.5×10^{6}
Synedra fulgens	8.2×10^{5}	1.3×10^{5}	2.6×10^{6}
Trachyneis minor	6.3×10^{5}	5.4×10^{5}	7.4×10^{6}

* Storage in 4 °C for more than 12 months.

Table 3
The dates of the five small abalone culture experiments were performed, and the temperatures and light intensities of the green house

Date of experiment were performed	Average temperature (°C)		Range of light intensities (μmol m^{-2} s^{-1})		Average light intensity (μmol m^{-2} s^{-1})	
	0900 h	1500 h	0900 h	1500 h	0900 h	1500 h
Experiment 1 (4.16.2005–6.8.2005)	25.7	26.4	11.4–5887.5	11.4–3789.4	1909.5	927.6
Experiment 2 (9.8.2005–10.25.2005)	26.9	29.2	83.4–6448.0	23.8–6021.2	2743.7	2167.4
Experiment 3 (11.23.2005–1.4.2006)	19.7	20.7	24.8–3010.9	5.0–1517.9	609.9	228.7
Experiment 4 (3.20.2006–5.9.2006)	23.5	24.8	22.3–1393.9	30.8–2322.6	460.2	469.3
Experiment 5 (4.17.2006–5.26.2006)	25.3	26.7	22.3–4553.6	42.2–3392.9	876.9	774.2

Table 4
The average number of abalone larvae harvest rates, survival rate, days of harvesting and diatom cell sizes, and EPS (soluble EPS, bound EPS, internal carbohydrate and residual carbohydrate), lipid, and protein contents of the 12 diatom species

Diatom species	Harvest rate *	Survival rate (%)	Daily growth rate (μm)	Cell lengths (μm)	Cell widths (μm)	Soluble EPS (pg/100 μm^2)	Bound EPS (pg/100 μm^2)	Internal carbohydrate (pg/100 μm^2)	Residual carbohydrate (pg/100 μm^2)	Protein (% dw)	Lipid (% dw)	Days
Nitzschia grossestriata	112.6	8.76	91.13	15.56	4.71	212.31	73.9	44.21	25.92	11.3	13.7	40
Seminavis gracilenta	102.9	8	83.17	12.19	4.31	51.58	1188.24	46.36	39.57	14.4	20.3	40
Caloneis platycephala	90.4	7.03	79.26	29.69	8.44	49.87	81.49	28.45	99.53	17.4	11.3	40
Nitzschia panduriformis	83.3	6.48	89.06	12.0	7.29	40.63	52.81	25.26	81.28	10.9	10.1	40
Pleurosigma normanii	69.3	5.39	78.13	73.75	15.31	37.51	54.02	23.13	85.37	11.8	9.2	40
Trachyneis minor	67.6	5.26	79.05	18.19	2.81	36.81	74.15	10.96	73.57	10.4	7.0	40
Synedra fulgens	65.6	5.1	88.21	39.31	5.56	32.07	97.5	27.27	24.52	11.0	6.7	40
Amphora biggiba	59.4	4.62	84.38	10.94	5.81	29.89	174.32	27.08	161.99	12.3	9.9	40
Amphora exigua	42.7	3.32	86.31	18.31	7.31	32.37	252.82	27.49	117.81	11.0	9.3	40
Cylindrotheca sp.	18.4	2.39	65.71	32.63	2.95	39.85	43.64	66.12	340.19	8.8	4.4	54
Fragilaria schulzii	16.4	2.32	66.46	38.06	5.23	14.22	42.4	122.83	104.45	10.5	5.4	53
Cocconeis scutellum	7.7	0.28	65.33	7.0	5.05	27.24	46.76	52.05	48.66	9.7	4.7	54

* Unit of the average harvest rate: larvae/1000 cm^2.

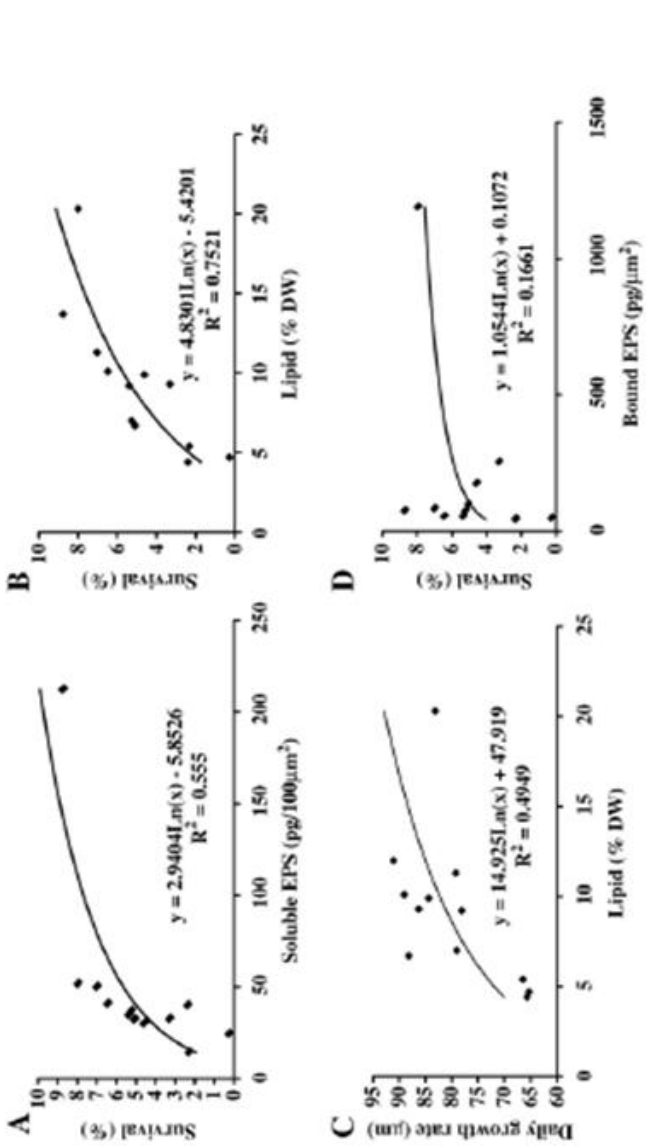

Figure 15. The relationship between soluble EPS contents of diatoms and the survival of post-larvae, showed a positive relation. B, The relationship between lipid contents of diatoms and the survival rate of post-larvae, showed a positive relation. C, The relationship between lipid contents of diatoms and the daily growth rate of post-larvae, showed a positive relation. D, The relationship between bound EPS contents of diatoms and the survival rate of post-larvae, showed no relation between them.

Figure 15

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