Appl Microbiol Biotechnol (2003) 60:612–623
DOI 10.1007/s00253-002-1176-4

MINI-REVIEW

Thierry Lebeau · Jean-Michel Robert

Diatom cultivation and biotechnologically relevant products. Part I: Cultivation at various scales

Received: 24 July 2002 / Accepted: 12 October 2002 / Published online: 13 December 2002

Abstract Biotechnological applications of diatoms are still in development. Further development at the industrial scale will depend on optimisation of the culture process with the aim of reducing costs. Because of the photoautotrophic status of the majority of diatoms, microalgal cultures suffer from the limitation of light diffusion, which requires the development of suitable photobioreactors. Thus, genetically engineered microalgae that may be cultivated in heterotrophic conditions present a new opportunity. Other limiting factors, such as nutrients (phosphate, nitrogen, silicon), pH, temperature, bioturbation and many more must be taken into account. Most of the time, metabolic stress conditions lead to an overproduction of the products of interest, with a decrease in biomass production as a consequence. Outdoor cultures in open ponds are usually devoted to aquaculture for the feeding of shrimps and bivalve molluscs (commercial production), while closed axenic indoor/outdoor photobioreactors are used for biotechnological compounds of homogeneous composition (still at the laboratory scale). In addition to the optimum culture conditions that have to be taken into account for photobioreactor design, the localisation of produced metabolites (intra- or extracellular) may also be taken into account when choosing the design. Microalgal cell immobilisation may be a suitable technique for application to benthic diatoms, which are usually sensitive to bioturbation and/or metabolites which may be overexpressed.

T. Lebeau (✉) · J.-M. Robert
Laboratoire de Biologie Marine,
Institut des Substances et des Organismes de la Mer (ISOmer),
Université de Nantes, 2 rue de la Houssinière, BP 92208,
44322 Nantes cedex 3, France
e-mail: t.lebeau@uha.fr
Fax: +33-3-89202357

Introduction

Diatoms

Diatoms are unicellular photosynthetic eukaryotes (chromophytes), within the class Bacillariophyceae in the Heterokont division, whose peculiarity amongst other microalgae is their siliceous cell wall. Smetacek (1999) argues that this wall acts as a line of defence against various types of grazers. Diatoms are thought to contribute as much as 25% of the global primary productivity (Scala and Bowler 2001) and their population is the largest amongst microalgae in the oceans: amongst the eukaryotic phytoplankton, diatoms are responsible for about 40% of the marine primary productivity (Falkowski et al. 1998). The number of genera and species is in the order of 250 and 100,000, respectively (Norton et al. 1996; Van Den Hoek et al. 1997). Centric diatoms are essentially planktonic microalgae which are found in all open water masses, while pennate are found most of the time in benthic forms, growing on sediments or attached to rocks or macroalgae; and some species can also be found in soil (Lee 1999). In their natural environment, diatoms are abundant at the beginning of spring and autumn, when nutrients are not limiting and when light intensity and day-length are optimal for diatom photosynthesis (Falciatore and Bowler 2002).

Despite their abundance and diversity in nature, few species are cultured in aquaculture or for biotechnology-relevant products.

Biomass production/synthesis of biological products

Diatoms are used for various biotechnological applications, mainly at the laboratory scale, and in aquaculture at a higher scale for commercial outlets. Examples of applications are: (1) silicon production originating from frustules, for technological applications in nanotechnology, pollution remediation or as food in aquaculture thanks to the lipid- and amino-acid-rich algal content, (2)

intracellular metabolites that accumulated in cells, e.g. lipids, particularly eicosapentaenoic acid (EPA) for pharmaceutical applications, or amino acids for cosmetic applications, and (3) extracellular metabolites released into the medium, e.g. various pigments (for chicken and fish feeds) and antibiotics.

Cultivation at various scales

The major advantages and drawbacks of various algal culture techniques, including those devoted to diatoms, are summarised in Table 1. According to Borowitzka (1994), factors to be considered before choosing a culture system are: biology of the alga, cost of land, energy needed, water nutrient, climate if outdoor culture and the type of final product. The choice of culture system then depends on: light utilisation efficiency, ability to control temperature, hydrodynamic stress toward algae, ability to maintain unialgal outdoor axenic conditions and the possibility to scale-up.

Small-scale: closely controlled photobioreactors

Commercialisation of new microalgae and microalgal products requires closed-culture systems, whose major drawback is their cost. Contrary to most algal species currently produced commercially (i.e. *Chlorella*, *Spirulina*, *Dunaliella*), which grow in highly selective conditions with little contamination by other microalgae and protozoa as a consequence, diatoms do not have this selective advantage and must be grown axenically in closed systems (Borowitzka 1994). Closed systems are indoor/outdoor photobiorectors and include bags, tubular photobioreactors and flat-plate reactors. In the latter two cases, light diffusion inside the photobioreactor is improved, the medium is well mixed, gas exchange is higher and the temperature is controlled, which leads to higher biomass productivity and a higher cell density, with reduced harvesting costs as a consequence. Closed indoor photobioreactors are more expensive, nevertheless, because artificial light is used.

A chemostat system that was relatively easy and cheap to construct was utilised by Seasalter Shellfish Co., UK: 400-l polyethylene bags supported by frames were used to grow *Phaeodactylum tricornutum* and *Skeletonema costatum* (Couteau et al. 1996). One drawback of the system was the large diameter of the bags (60 cm) which resulted in self-shading and hence relatively low algal densities. Robinson et al. (1988) designed a new photobioreactor named Biocoil, a helical tubular photobioreactor which consisted of clear plastic tubing with several bands connected together (total volume up to 700 l). It was successfully tested for more than 4 months with *P. tricornutum* and *Chaetoceros* spp. Mixing was uniform and scale-up was easy.

The most important photobioreactor development concerned polyunsaturated fatty acids (PUFA), especially EPA (Lebeau and Robert 2002). EPA was mainly produced by *P. tricornutum* or *Nitzschia laevis* in various photobioreactors: perfusion cell bleeding, glass tank

Table 1 Advantages and disadvantages of various algal culture techniques (from Couteau 1996)

Culture type	Examples	Advantages	Disadvantages
Indoors	Ponds or tanks fertilised with laboratory-grade reagents	Control over illumination, temperature, nutrient level, contamination with predators and competing algae	Expensive
Outdoors	Ponds or tanks fertilised with agricultural-grade fertilisers	Cheap	Little control (difficult to grow specific algal cultures for extended periods, unpredictable algal crashes)
Closed	Tubes, flasks, carboys, bags, etc.	Contamination less likely	Expensive
Open	Ponds, tanks	Cheap	Contamination more likely
Axenic	Sterilisable glassware	Predictable, less prone to crashes	Expansive, difficulty to keep sterility
Non-axenic		Cheap	More prone to crashes
Continuous	Restricted to indoors systems at a small scale	Efficient, provides a consistent supply of high-quality cells, automation, high rate of production (biomass, product) over extended periods	Difficult, usually only possible to culture small quantities, complex, equipment expenses may be high
Semi-continuous	Indoor or outdoor systems	Easy, somewhat efficient, prolong the use of large tank cultures, yields more biomass than the batch method	Sporadic quality, less reliable
Batch	Test-tubes to 25,000-l outdoor tanks	Easiest, most reliable, flexible	At least efficient, quality may be inconsistent (e.g. cells harvested too late), contamination risk during initial inoculation and early growth period, much labour to harvest, clean, sterilise, refill and inoculate the containers

outdoors, tubular photobioreactor, glass tube, helical tubular photobioreactor. Improvement of photobioreactor design led to an enhancement of the cell growth rate, to make EPA production more cost-competitive than fish-oil products (Wen 2001). To alleviate inhibition by metabolic by-products, a one-stage process was applied to continuous cultures, using a cell-recycle technique frequently denoted as perfusion culture in animal-cell cultivation (Wen and Chen 2001a); and this led to a highly enhanced EPA productivity. Transferring the EPA process to other diatoms, like *S. costatum*, the difference in metabolism had to be taken into account: the percentage of EPA increased as the growth temperature decreased, to the detriment of biomass productivity. For this reason, a two-stage process was applied by Blanchemain and Grizeau (1999). Biomass was first produced at optimal conditions in a cylindro-conical system (0.3 m^3) constantly sparged by sterile air; and then EPA accumulated at lower temperatures in coagulated/settled cultures. *Cyclotella cryptica* was cultivated in a 15-l photobioreactor for silica production (Csogor et al. 1999) in careful controlled conditions, to obtain diatoms uniform in shape and size, as required for industrial applications, especially in nanotechnology.

The performance of the photobioreactors used was greatly influenced by their design. For example, with *P. tricornutum*, light diffusion was higher when the tube diameter of the outdoor photobioreactor decreased from 0.06 m to 0.03 m, leading to a 67% increase in EPA productivity (Acién Fernández et al. 2000). At a higher scale, the optimum tube diameter was 0.1 m (Molina Grima et al. 2000). For the same reason, a 2-fold decrease in the agar layer thickness in which the cells of the pennate diatom *Haslea ostrearia* were immobilised led to a 2-fold higher productivity of marennine, a blue-green hydrosoluble pigment (Lebeau et al. 2000). As the design approach developed, Acién Fernández et al. (2001) built a 0.2-m^3 outdoor photobioreactor with a compact degasser in the airlift section that eliminated dead zones and dark zones, while achieving complete separation of gas and liquid.

For immobilised microalgal cells, the only suitable photobioreactor designs were suggested by Lebeau et al. (2000, 2002). The aim of these photobioreactors was to cultivate microalgae, especially benthic diatoms, that suffer from bioturbation. In these photobioreactors, the surface/volume ratio of the matrix (agar or alginate) was maximised to offer the maximal contact between microalgae and nutrient/light.

Large-scale: open air systems

Four major types of microalgal culture exist (Borowitzka 1994): shallow big pond, tank, circular pond with a rotating arm to mix the culture and raceway pond.

Outdoor cultivation of microalgae is mainly devoted to aquaculture. Microalgae are an essential food source in the rearing of all stages of marine bivalve molluscs (clams, oysters, scallops), the larval stages of some marine gastropods (abalone, conch), the larvae of several marine fish species and penaeid shrimp and zooplankton. Table 2 shows the genera of cultured diatoms currently used to feed different groups of commercially important aquatic organisms. Amongst them, favoured genera of diatom for larval feeds include *Chaetoceros* and *Thalassiosira* (Duerr et al. 1998). According to Couteau (1996), *T. suecica*, *T. pseudonana*, *Pavlova lutheri*, *Isochrysis galbana* and *S. costatum* represented over 90% of the volume of algal culture in 1991 for the feeding of bivalve molluscs, compared with the volume of *C. gracilis* used overall by hatcheries.

Table 2 Major genera of diatoms cultured in aquaculture (from Couteau 1996). *BL* Bivalve mollusc larvae, *BP* bivalve mollusc postlarvae, *BS* brine shrimp (*Artemia*), *ML* freshwater prawn larvae, *PL* penaeid shrimp larvae

Genus	Examples of application
Skeletonema	PL, BL, BP
Thalassiosira	PL, BL, BP
Phaeodactylum	PL, BL, BP, ML, BS
Chaetoceros	PL, BL, BP, BS
Cylindrotheca	PL
Bellerochea	BP
Actinocyclus	BP
Nitzschia	BS
Cyclotella	BS

Mass algal cultures in outdoor ponds are commonly applied in Taiwanese shrimp hatcheries, where *S. costatum* is produced successfully in rectangular outdoor concrete ponds holding 10–40 t of water, with a water depth of 1.5–2.0 m (Couteau 1996).

For bivalve hatcheries, all algae at Cost Oyster Co. (Quilcene, Wash., USA), the world's largest oyster-growing company, producing over 30 billion eyed larvae per year (Donaldson 1991), are grown in a batch system designed to maximise yield. To reduce the cost of oyster production, Lefebvre et al. (2000) evaluated the response of the Pacific oyster to a land-based fish-farm effluent comprised mainly of fish faeces and compared the oysters with those obtained with *S. costatum*. It was shown that, in a mixed diet, diatom was preferentially ingested but the food quality did not influence the clearance rates. A two-step system for nursery seed-clam culture was used by Pfeiffer and Rusch (2000): *C. muelleri* was first cultured in covered 550-l tanks, then harvested and provided to clams. However, it seems that higher growth rates and gross growth efficiencies were obtained when *C. neogracile* was fed together with the culture instead of being centrifuged and stored (Couteau et al. 1994). In addition, the limited self-life and/or high prices of the presently available algal pastes (U.S. $ 200 or more per kilogram dry weight) have discouraged many growers from using them, except recently for *T. pseudonana* concentrates, thanks to new preservation techniques that extended shelf-life from 10 days to more than 1 year and made it possible to utilise excess and off-season production (Couteau 1996). Another application is the greening of oysters. Turpin et al. (1999) cultured *H. ostrearia* in

25-m^3 ponds with the aim of allowing constant production of the greening phenomenon by microalgae, with the greening of oyster gills as a result. In this work, oysters and microalgae were in the pond at the same time. An alternative consisted of culturing *H. ostrearia* in tanks before greening the oysters, as currently performed at SOPROMA, a French society (Bouin district, Vendée). An original alternative method for the greening of oysters consisted of high-cell concentrated inocula of *H. ostrearia* immobilised in alginate beads. The beads may be stored for a long time at 4 °C and dissolved with phosphate buffer in oyster ponds. For the greening, natural cell leakage may be promoted after directly introducing beads into the ponds (Lebeau et al. 1998; Lebeau and Robert 1999).

For penaeid shrimp, *C. gracilis* and *S. costatum* are the diatom species most often used. The same-tank method, in which the algae are cultured in the same water as the larvae, using sunlight and fertilisers, was originally developed in Japan for culturing larval *Penaeus japonicus* and was extensively described by Liao et al. (1993). One of the main problems in intensive shrimp-culture systems was the accumulation of dissolved nitrogen, as the result of food addition and the excreta from organisms reared at high density, which affected both food ingestion by shrimps and their growth and survival rates. To keep dissolved nutrient at low levels, large amounts of water had to be exchanged daily, increasing the costs of shrimp production. An alternative consisted in biological treatment with the use of biofilm, mainly composed of pennate diatoms, e.g. *Amphora*, *Campylopyxis*, *Navicula*, *Sinedra*, *Hantschia* and *Cylindrotheca* (Thompson et al. 2002), which maintained a high-water-quality trough uptake of dissolved inorganic nutrients (ammonium, phosphates). Wastewater nutrient was continuously added to *Phaeodactylum tricornutum* for the removal of ammonium and orthophosphate (70–80% removed) under ambient conditions in 20-l mini-ponds (Craggs et al. 1995). This removal reached 100% with *P. tricornutum* and *Oscillatoria* sp. (Craggs et al. 1997).

Diatom hatchery tanks often contain a very large surface area covered by a diatom biofilm. These benthic diatoms greatly influence settlement and post-larval development. Biofilm is relatively easy to establish without specialised equipment, by maintaining microalgal stock cultures regularly subcultured (Roberts et al. 2000). Large-scale batch culture of benthic diatoms may be performed in clear plastic bags or, more commonly, in solid plastic containers. Some benthic diatoms grow in suspension when agitated, while others do not. Couteau et al. (1994) reported special techniques which have been developed by farmers of abalone (*Haliotis* sp.) to provide food for the juvenile stages which feed in nature by scraping coralline algae and slime off the surface of rocks, using their radulae. In culture operations, sessile microalgae are grown on plates of corrugated roofing plastic, which serve as a substrate for the settlement of abalone larvae. After metamorphosis, the spat (tiny baby abalone) graze on the micro-algae until they become large enough to feed on macro-algae. The most common species of micro-algae used on the feeder plates are pennate diatoms (e.g. *Nitzchia*, *Navicula*). The plates are inoculated by placing them in a current of sand-filtered seawater. Depending on local conditions, the micro-algal cultures on the plates take 1–3 weeks to grow to a density suitable for the settling of larvae. As the spat grow, their consumption rate increases and becomes greater than the natural production of the micro-algae. At this stage, the animals are too fragile to be transferred to another plate and algal growth may be enhanced by increasing illumination intensity and/or by the addition of fertiliser.

Walsh et al. (1987) estimated the production costs of various diatoms in continuous flow cultures (240 m^3) at U.S. $ 167 kg^{-1} dry weight. To reduce the production cost of microalgae used in aquaculture, underground water was used for the cultivation of *Chaetoceros calcitrans* (Yusoff et al. 2001) or *Skeletonema costatum* (Baud and Bacher. 1990).

In addition to diatom culture for aquaculture, Veloso et al. (1991) cultivated *P. tricornutum* for EPA production in outdoor PVC ponds. A total of 4.0 g m^{-2} day^{-1} in ash-free dry weight (AFDW) were obtained in semi-continuous mode; and EPA production was 3.9% of ADFW.

Culture conditions

The most important parameters regulating algal growth are nutrient quantity and quality, light, pH, turbulence, salinity and temperature; and broad tolerance ranges are summarised in Table 3 (Couteau 1996).

Heterotrophytic/photosynthetic conditions

Diatoms are basically photosynthetic microalgae, i.e. they assimilate inorganic carbon for conversion into organic

Table 3 A generalised set of conditions for culturing microalgae (from Couteau 1996)

Parameters	Range	Optima
Temperature (°C)	16–27	18–24
Salinity (g l^{-1})	12–40	20–24
Light intensity ($\times 10^{12}$ quanta cm^{-2} s^{-1})	8.5–85.0 (depends on volume and density)	21.3–42.5
Photoperiod (light:dark, in hours)	–	16:8 (minimum)
	–	24:0 (maximum)
pH	7.0–9.0	8.2–8.7

matter. But several species may use organic nutrients with or without any light. In microalgal mass culture, a heterotrophic culture mode is preferred, as it eliminates the requirement for light and hence offers the possibility of greatly enhancing cell density, by using high-cell-density culture techniques, as currently used in fermentation. Cultures of *Nitzschia inconspicua* supplemented with glucose or acetate can attain higher cell densities than photoautotrophic cultures (Chu et al. 1996). As a consequence, the production of the metabolite of interest may be enhanced. For example, *N. laevis* is a good EPA-producer in heterotrophic conditions (Chor Koon Tan and Johns 1996) when cultivated on glucose as source of carbon and energy. Glucose concentration (with various silicate levels) at 20 g l^{-1} led to highest cell weight, EPA content, yield and productivity (Wen and Chen 2000a). Wen and Chen (2001c) showed also that tryptone and yeast extract – with a positive interaction when combined – enhanced EPA production, in comparison with nitrate or urea, which were found to be the preferred nitrogen sources for both cell growth and EPA content. Berman and Chava (1999) reported other sources of organic nitrogen as the only source of nitrogen (i.e. hypoxanthine, urea, guanine, ornithine, glucosamine, lysine) on which *Cyclotella* grew well. Nevertheless, heterotrophic conditions may result in a drastic change in the gross composition and a reduced (*n*–3) highly unsaturated fatty acids (HUFA) content, as compared with light-grown algae (Couteau 1996).

Mixotrophic conditions seem to be more suited than heterotrophic for higher cell growth and EPA production with *N. laevis* (Wen and Chen 2000b), *Navicula saprophila* in the presence of acetic acid (Kitano et al. 1997) improved with a CO_2-enriched atmosphere (Kitano et al. 1998) and *P. tricornutum* (Céron García et al 2000) improved with glycerol, leading to 9- and 8-fold higher biomass concentration and biomass productivity than in photoautotrophically cultured cells.

In aquaculture, heterotrophic algal culture is one option being studied to increase production and reliability. Cost estimates, based on laboratory experiments, are projected to be U.S. $ 2–25 kg^{-1} dry weight while, at U.S, $ 50 kg^{-1} dry algae equivalent, there is significant pressure to replace algae with prepared diets (Duerr et al. 1998). Projected costs for producing algae within industrial fermentors range over U.S. $ 5–25 kg^{-1} dry weight (Gladue 1991). Unfortunately, heterotrophic mass-production techniques have only been realised for a few algal species; and most of the species that are known to be of high nutritional value (e.g. *Chaetoceros*, *Skeletonema*, *Thalassiosira*) are not capable of growing in the dark.

Genetically engineered microalgae represent progress toward the use of fermentation technology for the large-scale commercial exploitation of algae by reducing the limitations associated with light-dependency, growth and costs as a consequence (Zaslavskaia et al. 2001).

Light

Light is the source of energy which drives photosynthesis and, in this regard, the availability and intensity of light (and the spectral quality and photoperiod) are the major factors controlling the productivity of photosynthetic cultures (Lee and Low 1992; Pulz and Scheibenbogen 1998). Couteau (1996) summarised in his technical paper some interested considerations about microalgae, including diatoms: (1) light intensity varies greatly with culture depth and density of algal culture (8.5×10^{12} quanta cm^{-2} s^{-1} is suitable for Erlenmeyer flasks, but from 4.3×10^{13} quanta cm^{-2} s^{-1} to 8.5×10^{13} quanta cm^{-2} s^{-1} is required for larger volumes), (2) overheating due to both natural and artificial illumination should be avoided, (3) fluorescent tubes emitting either in the blue or red light spectrum should be preferred, as these are the most active portions of the light spectrum for photosynthesis, (4) the duration of artificial illumination should be a minimum 18 h light day^{-1}.

The optimisation of light illumination in a photobioreactor is not easy; and an homogeneous light intensity is required to ensure that all cells of the population are equally exposed to the light, for the standardisation of metabolite production in terms of quantity and quality. The use of immobilised cells complicates this, due to the appearance of a notable gradient of light intensity inside the matrix, a consequence of the diffusional limitation of light, which leads to heterogeneous cell distribution in the matrix. This phenomenon was shown by Lebeau et al. (2000) with *Haslea ostrearia* immobilised in a tubular gel layer: on the side away from the light source, the cell concentration was high and the cells were alive and weakly pigmented [which revealed, according to Schubert et al. (1995), no cellular stress from light] while, in the area close to the light source, blue-pigmented cells were mixed with dead cells. In free-cell cultures, it was also shown that the cultures were clearly light-limited, due to self-shading, when the biomass concentration of *P. tricornutum* exceeded about 1 g l^{-1} (Sánchez Mirón et al. 1999) or 18×10^9 cells l^{-1} (Chrismadha and Borowitzka 1994). For a high cell density, a heterotrophic culture mode is preferred, as it eliminates the requirement for light and hence offers the possibility of greatly enhanced cell density. Heterotrophic algal cultures can attain up to 1,000 times higher densities than photoautotrophic cultures (Couteau 1996).

The change in light intensity may turn the metabolism of microalgae towards higher synthesis of the metabolite of interest. For example, with *P. tricornutum* cultivated in a outdoor tubular photobioreactor, the biomass productivity is limited by low light availability, although the EPA content is maximum in winter. In summer, EPA content diminishes by photoinhibition and the biomass productivity is higher than with a higher EPA productivity (Acién Fernández et al. 2000). In tubular photobioreactors, the maximum EPA productivity was at the maximum irradiance, i.e. 1×10^{17} quanta cm^{-2} s^{-1} (Chrismadha and Borowitzka 1994). Molina Grima et al. (1995) showed

that the maximum photosynthetic activity of *P. tricornutum* was around 2×10^{17} quanta cm^{-1} s^{-1}.

Another factor to be considered is the light quality. Tremblin et al. (2000) cultivated two prominent diatoms encountered in oyster-ponds, *H. ostrearia* and *S. costatum*, under light of different spectral quality: white, blue or blue-green. The last corresponded to white light modified by a water-soluble pigment, marennine, produced by *H. ostrearia*. The growth rate of both species showed little variation with respect to light quality. The parameters for photosynthesis vs irradiance curves were very similar in *H. ostrearia* grown under the three light conditions whereas, for *S. costatum*, the maximum photosynthetic capacity was significantly reduced under blue-green light. We may then assume that marennine production is of constant quality during the synthesis of this pigment in photobioreactor, even when the light quality evolves during incubation.

In outdoor production, changes in weather (e.g. light intensity, temperature) may lead to a poor reproducibility of the successive cultures and unpredictable culture crashes, i.e. cultures die (Couteau 1996); and culture productivity is all the more dependent on light as the scale of the operation increases. In the case of tubular closed photobioreactors, difficulties also arise with scale-up, because the relative volumes of light and dark zones change as the tube diameter increases (Molina Grima et al. 1999). These same authors recommend the use of a pipe diameter of no more than 0.1 m, a continuous run length of about 80 m and a flow velocity of 0.3–0.5 m s^{-1}.

In aquaculture, light intensity must also be controlled. For example, diatoms influence the settlement and post-larval development of abalone, which requires a natural succession of diatom communities, controlled to some extent by light (moderate light levels avoid overstacked diatoms, keeping diatom films young and healthy) to favour useful diatoms (Roberts et al. 2000). Battaglene et al. (1999) also showed that light reduced by shading favoured the diatoms and epiphytic algae that were important sources of food for cultured juvenile sea cucumbers, *Holothuria seabra*.

The design of the photobioreactor influences the light regimen, e.g. vertical bubble columns or airlift reactors vs horizontal tubular loops (Sánchez Mirón et al. 1999). One responsible factor is the culture depth: typically 0.02–0.06 m in horizontal tubes but much greater (e.g. 0.2 m or more) in vertical columns. Self-shading may occur, which is the case when *P. tricornutum* or *S. costatum* are grown in bags of large diameter (60 cm; Shellfish Co., UK; Couteau 1996). At constant external illumination, small changes in culture depth are known to generally affect the productivity of microalgal cultures (Kobayashi and Fujita 1997), including those of *P. tricornutum*. Because of the lesser depth and because light intensity declines exponentially with depth according to Beer–Lambert law, the light level is always greater in the relatively thin horizontal tubes than in the larger diameter vertical column, when the systems are compared at an identical orientation of light source relative to reactor surface. Nevertheless, the maximal biomass productivity of *P. tricornutum* was similar, whatever the photobioreactor used (Camacho et al. 1999). The higher photosynthetic efficiency in the vertical one was attributed to less photoinhibition (a vertical arrangement avoids the high irradiances in summer and spring and at noon all through the year).

Light availability in bubble columns and airlift devices depends not only on light quantity and quality, but is also influenced by aeration rate, gas holdup and liquid velocity (mixing, turbulence) within the photobioreactor (Sánchez Mirón et al. 2000).

Nutrients

Several macronutrients are necessary for diatoms, including nitrogen, silicate and phosphate (Facialtore and Bowler 2002), and micronutrients consisting of various trace metals (particularly dissolved iron; Kudo et al. 2000) and the vitamins thiamine (B1), cyanocobalamin (B12) and sometimes biotin. A few enrichment media have been used extensively: Walne (Couteau 1996), Provasoli (Provasoli 1968) and F/2 (Guillard 1982) media. The cell yields of *Chaetoceros* sp. may be also improved by more than 30% by the addition of 10 mg l^{-1} of experimental zeolithic products (natural Al silicates with one or more alkaline or terrous-alkaline metals) to the medium (Voltolina et al. 1997). Zeolites remove ammonia and this was recently shown to be an important role in nitrification and other biological processes (López Ruiz 1995). Nevertheless, their cost often hampers their use for large-scale cultures. An alternative to the mass production of microalgae in large-scale extensive systems consists of using enriched seawater with only the most essential nutrients. Turpin et al. (1999) prepared a N+P+Si simplified medium for the greening of oysters by *Haslea ostrearia*. A simpler medium containing the soluble fractions of wheat flour, rye flour and boiled potato was used by Fabregas et al. (1996, 1997) to semi-continuously cultivate *P. tricornutum*. The simplest medium consisted of interstitial water – rich in total nitrogen and phosphorus – extracted from the bottom sediment of a shrimp-culture pond for the cultivation of *C. calcitrans* (Yusoff et al. 2001). Moreau (1996) also showed that iron and manganese were involved in the fertility of underground water. Voltolina et al. (1998) underlined the importance which should be given to algal cell acclimation to culture media.

Nitrogen is an important parameter. Chelf (1990) showed that nitrogen concentration was the variable with the greatest effect on neutral lipid and AFDW accumulation by *C. muelleri* and *Navicula saprophila*. In a chemostat, a fixed rate of nitrogen may be applied to keep the growth rate constant while the cell density is not kept constant. For marennine or lipid production, a voluntary nitrogen deficiency acted as a cell stress and led to a higher synthesis of these products (Lebeau et al. 2002; McGinnis et al. 1997; Sriharan et al. 1990). Nevertheless,

this nutrient must be regularly supplied to the algal cells for their basal metabolism and turnover. Wen and Chen (2001b) showed that *Nitzschia laevis* used various sources of nitrogen, with a preference for a mixture of tryptone and yeast, while *Cyclotella* (Berman and Chava 1999) grew well on most dissolved organic nitrogen sources (e.g. hypoxanthine, urea, guanine, ornithine, glucosamine, lysine). The EPA content of the total fatty acids produced by *P. tricornutum* increased with increasing concentrations of nitrate and urea (Yongmanitchai and Ward 1991).

Because silicate is the essential compound in the diatom cell wall, this nutrient has an positive effect on growth (Turpin et al. 1999). It had a more or less negative effect on lipid production from *Hantzschia* and *N. laevis* (Sriharan et al. 1990; Wen and Chen 2000a), while Chu and al. (1996) showed the opposite effect (range of silicate level 8.8–176.0 μM) on cell growth, biochemical content and fatty acid composition with *N. inconspicua*. The highest cell concentration, growth rate, EPA content and productivity were obtained with various silicate concentrations.

pH, temperature, gas, salinity

The pH range for most cultured microalgae is between 7 and 9. In the case of a high cell density, the addition of CO_2 or $NaHCO_3$ in the medium (Couteau 1996; Lebeau et al. 1999) allows a correction for increased pH. It buffers the water against pH changes as a result of the CO_2/HCO_3 balance. Acidic pH is important in: (1) the silicification of diatoms (Martin-Jezequel et al. 2000; Vrieling et al. 1999), because it facilitates a fast nucleation and aggregation of silica particles, thus increasing the rate of formation of mature frustules, (2) EPA production (Wen and Chen 2001b; Yongmanitchai and Ward 1991), which was optimised at pH 7.5 and pH 8.5 for *N. laevis* and *P. tricornutum*, respectively, and (3) amino acid production by *S. costatum* (Taraldsvik and Myklestad 2000), in which cellular concentration varied from about 62 mM to 8 mM over the pH range 8.0–9.4. The bubble photobioreactor allows a relatively homogeneous culture environment, that leads to a more uniform and better controlled pH (Sánchez Mirón et al. 1999).

Temperature plays an important role for cell growth and metabolite synthesis. As summarised by Couteau (1996), the most commonly cultured species of microalgae tolerate temperatures of 16–27 °C. Temperatures lower than 16 °C slow growth down, whereas those higher than 35 °C are lethal for a number of species. If necessary, algal cultures can be cooled by a flow of cold water over the surface of the culture vessel or by controlling the air temperature with air-conditioning units. The maximum growth rate occurred at 20–25 °C in the case of *P. tricornutum* (Kudo et al. 2000; Sánchez et al. 1995; Yongmanitchai and Ward 1991) and at 20–30 °C for *N. closterium* (Renaud et al. 1995), but a broad range of temperatures (20–35 °C) is withstood by *C. muelleri*, which thus has the potential for exploitation as a renewable precursor to liquid fuels or as a lipid source (McGinnis et al. 1997). For growth rate and EPA production, optimal temperatures were respectively 19.8 °C and 18.0 °C with *N. laevis* (Wen and Chen 2001b). With *S. costatum*, the optimal growth temperature was 20 °C, while the EPA content was higher at a lower temperature, i.e. 15 °C (Blanchemain and Grizeau 1999) and lipid production by *Navicula saprophila* was optimal at 10 °C (Kitano et al. 1997). For *Nitzschia paleacea*, (Renaud et al. 1995) and *Nitzschia* sp. (Kitano et al. 1997), EPA production was higher at around 20 °C. Unsaturation in the haslene (polyunsaturated sesterpene oil) produced by *H. ostrearia* increased with increasing algal growth temperature (Rowland et al. 2001). The maximal temperatures tolerated by microalgae may reduce the choice of matrices used for the immobilisation of microalgae. This is the case for agar matrix, which gels at these temperatures, preventing the mixing of the cell suspension with the polysaccharide (Lebeau et al. 1999). Cell viability is more secure with alginate beads (cold gelling). This technique was used with success for *P. tricornutum* and *S. costatum* by Hertzberg and Jensen (1989), *N. obtusa* by Kannapiran et al. (1997) and *H. ostrearia* by Lebeau et al. (1998).

In several photobioreactors, CO_2 gradients in the culture were shown to be responsible for growth-rate limitation during the exponential growth phase. To remedy this drawback, Contreras et al. (1998) optimised the cell growth of *P. tricornutum* using a superficial gas velocity around 0.055 m s^{-1} in a concentric airlift photobioreactor. From 0.5% to 5.0% CO_2 added to the culture of *P. tricornutum*, *N. saprophila* or *N. inconspicua* (Chrismadha and Borowitzka. 1994; Chu et al. 1996; Kitano et al. 1998; Yongmanitchai and Ward. 1991) had a stimulating effect both on biomass and on EPA production. However, with *Nitzschia* sp. EPA production decreased when the temperature decreased (Kitano et al. 1997). In aquaculture, the percentage of CO_2 may reach 10% in bivalve hatcheries with *T. pseudonana*, *Skeletonema* sp., *Chaetoceros* spp, *P. tricornutum* and *Nitzschia* sp. (Duerr et al. 1998). For very dense cultures, the CO_2 originating from the air bubbling through the culture limited the algal growth; and pure CO_2 may be supplemented into the air supply (e.g. at a rate of 1% of the volume of air), as shown by Couteau (1996).

Microalgae generate oxygen during photosynthesis; and, hence, dissolved oxygen levels equivalent to several times air saturation are easily reached in closed cultures. Oxygen concentrations above air saturation generally inhibit photosynthesis in microalgae (Aiba 1982) and lead to a decrease in biomass yield (Acien Fernández et al. 2001; Molina et al. 2001). Bubble columns, split-cylinder airlift devices and concentric draft-tube sparged airlift vessels should easily maintain a dissolved oxygen level, at optimal liquid velocity (Acien Fernández et al. 2001), only a little higher than the air saturation level, with less oxygen inhibition as a consequence (Molina Grima et al. 1995; Sánchez Mirón et al. 2000). Indeed, in one case, the dissolved oxygen level did not exceed the air saturation

value during outdoor culture of *P. tricornutum* in a draft-tube airlift reactor (Contreras 1996).

Most species grow best at a salinity that is slightly lower than that of their native habitat, which is obtained by diluting seawater with tap water. Salinities of 20–24 g l^{-1} have been found to be optimal for several species (Couteau 1996). For *H. ostrearia*, 28 g l^{-1} was found to be optimal (Robert 1983).

Microalgal stresses

One of the major algal stresses results from the mixing of medium, which is necessary to prevent sedimentation of the algae, to ensure that all cells of the population are equally exposed to the light and nutrients, to avoid thermal stratification (e.g. in outdoor cultures) and to improve gas exchange between the culture medium and the air. Mixing is achieved by stirring daily by hand (test tubes, Erlenmeyers), aerating (bags, tanks), or using paddle wheels and jet-pumps. Nevertheless, not all algal species can tolerate vigorous mixing. This is the case for benthic diatoms (e.g. *H. ostrearia*).

Because of their rigid frustule (siliceous cell wall), diatoms may suffer from shear stress. This phenomenon was shown by Contreras et al. (1998) in a vertical bubble column in the absence of gas (to ensure liquid homogenisation), when mechanical agitation was used. Small bubbles bursting at the surface of the culture were apparently the main cause of cell damage in batch cultures in laboratory-scale bubble columns with *P. tricornutum* (Camacho et al. 2001). The maximum specific growth rate (0.023 h^{-1}) increased with increasing aeration velocity in the riser zone, up to 0.055 m s^{-1}. At higher aeration velocities, a hydrodynamic stress appeared, although damage to algal cells was not shown. To overcome this drawback, supplementation of the microalgal culture medium with carboxymethyl cellulose (CMC, a modifier of interfacial properties) at a concentration of at least 0.2% (by weight) was shown to protect the algal cells against aeration-induced hydrodynamic stress. The CMC had a purely physical protective effect and no physiological effect on the algal cells (Camacho et al. 2001). Other shear stress may occur during the circulation of diatoms in tangential-flow filtration systems for a continuous-separation microalgal culture medium. Damage to brittle cells of *H. ostrearia* and *S. costatum* was showed by Vandanjon et al. (1999). Shearing the algal cells is a consequence of the circulation of these cells in valves and pumps. The shear stress depends on the type of pump but not on the number or frequency of loops. Shear may thus be evaluated by an overall parameter (K_v, pressure-drop coefficient) which integrates both the type of valve and its degree of opening. To allow the separation of *H. ostrearia* from medium for the recovery of marennine without any shear stress, a new photobioreactor coupled with an ultrafiltration system (immersed membranes) was investigated (Rossignol et al. 2000b). The cells were not submitted to any shear stress due to pumping or circulation. To achieve this, since the photobioreactor was of the cylindrical type, a membrane module was placed at the bottom of the reactor and the hydrostatic pressure (the height of the water column) was used as the driving force both for permeation and for periodic back-flushing.

Other microalgal stresses of metabolic origin may be induced voluntarily in biotechnological processes. For example, *C. gracilis* displayed an increase in triacylglycerol synthesis in situations of stress (Lombardi et al. 1995), but few differences occurred in lipid-class distribution between log-phase and stationary-phase cultures. Nitrogen is one stress factor, the lack of which led to an enhanced lipid content (Couteau 1996) or marennine production (Lebeau et al. 2000; Neuville and Daste 1972), with nevertheless a reduction in the growth rate as a consequence. Fe is another factor: Fe-stressed cells grew at half the growth rate of Fe-replete cells at 20 °C; and this difference became larger at lower temperatures (Kudo et al. 2000). Light may also act as a stress. The cells of the diatom *H. ostrearia* protect themselves by synthesising and excreting marennine of technological interest (Schubert et al. 1995) into their environment, that acts as a filter.

Cell immobilisation

This culture technique may be used for metabolites synthesised and excreted from microalgae but not for biomass production nor for intracellular metabolites, except when microalgal cells may be recovered. Immobilisation offers a practical alternative to free-cell cultures of microalgae (Lewin 1990). Published investigations on the immobilisation of diatom species are very scarce, e.g. *N. obtusa* (Kannapiran et al. 1997), *P. tricornutum*, *S. costatum* and *T. pseudonana* (Hertzberg and Jensen 1989) for stock culture management and *H. ostrearia* for the greening of oysters (Lebeau et al. 1998) and for the synthesis of marennine, a blue-green pigment (Lebeau et al. 1999, 2000, 2002) of biotechnological interest, with in vitro and in vivo activities against human lung cancer and anti-HIV effects (Carbonelle et al. 1999).

Artificial immobilisation is of particular interest for benthic diatoms that are sensitive to disturbance and whose products of interest may be naturally over-expressed during the benthic stage, where algal cells are immobilised in their own exopolysaccharides. For example, marennine production increased during the migration of algae from the planktonic to the benthic compartment and became maximum during the benthic stage (Robert 1983). The artificial network of agar polysaccharide reproduced well the extracellular polysaccharides synthesised by *H. ostrearia* (Rincé et al. 1999).

H. ostrearia was entrapped in an agar gel layer or in alginate beads for marennine production. On an identical basis, the specific productivity of marennine was higher using photobioreactors with immobilised cells rather than free cells (Rossignol et al. 2000a). Photobioreactors

dedicated to immobilised photosynthetic cells (Junter et al. 1989; Lebeau et al. 2002) were used for marennine production by *H. ostrearia*. The advantages of these photobioreactors were the following: (1) operational stability, (2) low cell leakage from gel matrices, due to low cell growth inside the matrix, avoiding marennine purification afterwards, (3) high cell concentration in comparison with that obtained with free cells, which is the case for diatoms with low cell growth (Rossignol et al. 2000a) and (4) no cell wash-out of the photobioreactor during continuous processes, whatever the dilution rate, thanks to the cell immobilisation. The cell metabolism may be turned onto marennine synthesis, which is a product from secondary metabolism. Nevertheless, drawbacks for such photobioreactors exist: (1) metabolite by-product accumulation, due to high density, that may lead to cell toxicity (Schürgel 2000) and (2) diffusional limitation of light and nutrients that leads to a heterogeneous cell distribution in the matrix. Reducing the agar layer thickness by a factor of two (Lebeau et al. 2000) leads to a more homogeneous cell distribution, an increase in the total biomass of *H. ostrearia* and a higher volumetric productivity of marennine.

Genetically engineered microalgae

Compared with other micro-organisms (bacteria, yeasts), few works refer to genetically engineered microalgae, particularly diatoms. However, genetic transformation finally occurred in 1995 with transgenic lines of *C. cryptica* and *N. saprophila* (Dunahay et al. 1995).

Most microalgae are obligate photoautotrophs and their growth is strictly dependent on the generation of photosynthetically derived energy. *P. tricornutum* can be genetically engineered (Zaslavskaia et al. 2001) to thrive on exogenous glucose in the absence of light, through the introduction of a gene encoding a glucose transporter (*glut1* or *hup1*). This represents progress toward the use of fermentation technology for large-scale commercial exploitation of algae, by reducing the limitations associated with light-dependent growth and reducing costs as a consequence. A mutagenesis was performed, using *P. tricornutum* Bohlin UTEX 640 as parent strain (Alonso et al. 1996). The putative mutant II242 exhibited a 44% higher EPA content, compared with the wild type (38.6 mg EPA g^{-1} dry biomass; 4.98 mg l^{-1} day^{-1}). Another example is the lipid producer *C. cryptica*, which was genetically engineered for further biodiesel production (Dunahay et al. 1996). In the future, engineered diatoms could potentially be used as vectors to introduce vaccines and improve the nutritional value of feedstuff for aquacultured fish and crustaceans (Gladue and Maxey 1994).

Concluding remarks

Cultures of diatoms of biotechnological interest are still at the early stage of development, except for aquaculture (commercial exploitation). Further development will depend on the use of new diatom species less restricting in cultivation with relation to light (for example because of diffusional limitations that may compromise scale-up) and optimisation of photobioreactor design. However, careful control conditions can be applied to microalgal culture (with the aim of obtaining homogeneous production, in terms of quality and quantity) in contrast to higher plant cultivation, which requires a considerable surface area and is largely dependent on climate. Since several diatom species are benthic, specific culture techniques should be developed, such as cell immobilisation. Finally, the future for microalgal cultures will be determined by the production cost for an equivalent efficiency (e.g. diatom feeding in aquaculture vs artificial diets).

Acknowledgement The authors would like to thank Pierre Gaudin for his technical assistance.

References

Acién Fernández FG, Sánchez Pérez JA, Fernández Sevilla JM, García Camacho F, Molina Grima E (2000) Modeling of eicosapentaenoic acid (EPA) production from *Phaeodactylum tricornutum* cultures in tubular photobioreactors. Effects of dilution rate, tube diameter, and solar irradiance. Biotechnol Bioeng 68:173–183

Acién Fernández FG, Fernández Sevilla JM, Sánchez Pérez JA, Molina Grima E, Chisti Y (2001) Airlift-driven external-loop tubular photobioreactors for outdoor production of microalgae: assessment of design and performance. Chem Eng Sci 56:2721–2732

Aiba S (1982) Growth kinetics of photosynthetic microorganisms. Adv Biochem Eng 23:85–156

Alonso DL, Del Castillo CIS, Grima EM, Cohen ZJ (1996) First insights into improvement of eicosapentaenoic acid content in *Phaeodactylum tricornutum* (Bacillariophyceae) by induced mutagenesis. J Phycol 32:339–345

Battaglene SC, Seymour JE, Ramofafia C (1999) Survival and growth of cultured juvenile sea cucumbers, *Holothuria scabra*. Aquaculture 178:293–322

Baud JP, Bacher C (1990) Use of saline ground water for intensive rearing of *Ruditapes philippinarum* juveniles in a nursery system. Aquaculture 88:157–178

Berman T, Chava S (1999) Algal growth on organic compounds as nitrogen sources. J Plankton Res 21:1423–1437

Blanchemain A, Grizeau D (1999) Increased production of eicosapentaenoic acid by *Skeletonema costatum* cells after decantation at low temperature. Biotechnol Tech 13:497–501

Borowitzka MA (1994) Commercial production of microalgae: ponds, tanks, tubes and fermenters. J Biotechnol 70:313–321

Camacho FG, Contreras Gómez A, Acien Fernández FG, Fernández Sevilla J, Molina Grima E (1999) Use of concentric-tube airlift photobioreactors for microalgal outdoor mass cultures. Enzyme Microbiol Technol 24:164–172

Camacho FG, Grima EM, Mirón AS, Pascual VG, Chisti Y (2001) Carboxymethyl cellulose protects algal cells against hydrodynamic stress. Enzyme Microb Technol 29:602–610

Carbonnelle D, Pondaven P, Morançais M, Massé 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

Céron García MC, Fernández Sevilla JM, Acien Fernández FG, Molina Grima E, García Camacho F (2000) Mixotrophic growth of *Phaeodactylum tricornutum* on glycerol: growth rate and fatty acid profile. J Appl Phycol 12:239–248

Chelf P (1990) Environmental control of lipid and biomass production in two diatom species. J Appl Phycol 2:121–130

Chor Koon Tan MR, Johns MR (1996) Screening of diatoms for heterotrophic eicosapentaenoic acid production. J Appl Phycol 8:59–64

Chrismadha T, Borowitzka MA (1994) Effect of cell density and irradiance on growth, proximate composition and eicosapentaenoic acid production of *Phaeodactylum tricornutum* grown in a tubular photobioreactor. J Appl Phycol 6:67–74

Chu WL, Phang SM, Goh SH (1996) Environmental effects on growth and biochemical composition of *Nitzschia inconspicua* Grunow. J Appl Phycol 8:389–396

Contreras A (1996) Caracterización de una columna de burbujeo con recirculación interna. Aplicación al cultivo de *Phaeodactylum tricornutum*. PhD thesis, University of Almería, Almería

Contreras A, García F, Molina E, Merchuk JC (1998) Interaction between CO_2-mass transfer, light availability, and hydrodynamic stress in the growth of *Phaeodactylum tricornutum* in a concentric tube airlift photobioreactor. Biotechnol Bioeng 60:317–325

Couteau P (1996) Manual on the production and use of live food for aquaculture. (FAO fisheries technical paper 361) FAO, Rome

Coutteau P, Curé K, Sorgeloos P (1994) Effect of algal ration on feeding and growth of juvenile Manila Clam *Tapes philippinarum* (Adams and Reeve). J Shellfish Res 13:47–55

Craggs RJ, Smith VJ, McAuley PJ (1995) Wastewater nutrient removal by marine microalgae cultured under ambient conditions in mini-ponds. Water Sci Technol 31:151–160

Craggs RJ, McAuley PJ, Smith VJ (1997) Wastewater nutrient removal by marine microalgae grown on a corrugated raceway. Water Res 31:1701–1707

Csogor Z, Melgar D, Schmidt K, Posten CJ (1999) Production and particle characterisation of the frustules of *Cyclotella cryptica* in comparison with siliceous earth. J Biotechnol 70:71–75

Donaldson J (1991) Commercial production of microalgae at Coast Oyster Company. In: Fulks W, Main KL (eds) Rotifer and microalgae culture systems. (Proc US/Asia Workshop) The Oceanic Institute, Honolulu, Hawaii, pp 229–236

Duerr EO, Molnar A, Sato V (1998) Cultured microalgae as aquaculture feeds. J Mar Biotechnol 7:65–70

Dunahay TG, Jarvis EE, Roessler PG (1995) Genetic transformation of the diatoms *Cyclotella cryptica* and *Navicula saprophyla*. J Phycol 31:1004–1012

Dunahay TG, Jarvis EE, Dais SS, Roessler PG (1996) Manipulation of microalgal lipid production using genetic engineering. Appl Biochem Biotechnol 57/58:223–231

Fabregas J, Morales E, Polanco N, Patino M, Otero A (1996) Soluble fractions of *Solanum tuberosum* enhance cell and pigment production of semi-continuous cultures of the microalga *Phaeodactylum tricornutum*. Lett Appl Microbiol 23:223–226

Fabregas J, Morales E, Lamela T, Cabezas B, Otero A (1997) Mixotrophic productivity of the marine diatom *Phaeodactylum tricornutum* cultured with soluble fractions of rye, wheat and potato. World J Microbiol Biotechnol 13:349–351

Falciatore A, Bowler C (2002) Revealing the molecular secrets of marine diatoms. Annu Rev Plant Biol 53:109–130

Falkowski PG, Barber RT, Smetacek V (1998) Biogeochemical controls and feedbacks on ocean primary production. Science 281:200–206

Gladue R (1991) Heterotrophic microalgae production: potential for application to aquaculture feeds. In: Fulks W, Main KL (eds) Rotifer and microalgae culture systems. (Proc US/Asia Workshop) The Oceanic Institute, Honolulu, Hawaii, pp 276–286.

Gladue RM, Maxey JE (1994) Microalgal feeds for aquaculture. J Appl Phycol 6:131–141

Guillard RRL (1982) Culture of phytoplancton for feeding marine invertebrates. In: Smith WL, Charley MH (eds) Culture of marine invertebrate animals. Plenum Press, New York, pp 108–132

Hertzberg S, Jensen A (1989) Studies of alginate-immobilised marine microalgae. Bot Mar 32:267–273

Junter GA, Labbé M, Mignot L, Guérout P, Paporé F (1989) ANVAR–CNRS. French patents 89-02129, 89-02130

Kannapiran E, Kannan R, Kannan L (1997) Immobilisation of *Nitzchia obtusa* W. Smith for stock culture management. Seaweed Res Util 19:67–73

Kitano M, Matsukawa R, Karube IJ (1997) Changes in eicosapentaenoic acid content of *Navicula saprophila*, *Rhodomonas salina* and *Nitzschia* sp. under mixotrophic conditions. Appl Phycol 9:559–563

Kitano M, Matsukawa R, Karube IJ (1998) Enhanced eicosapentaenoic acid production by *Navicula saprophila*. Appl Phycol 10:101–105

Kobayashi K, Fujita K (1997) Tube diameter on tubular photobioreactor for microalgal culture and its biomass productivity. J Chem Eng Jpn 30:339–341

Kudo I, Miyamoto M, Noiri Y, Maita Y (2000) Combined effects of temperature and iron on the growth and physiology of the marine diatom *Phaeodactylum tricornutum* (Bacillariophyceae). J Phycol 36:1096–1102

Lebeau T, Robert JM (1999) Conditionnement et procédé de conditionnement de cellules vivantes de l'algue *Haslea ostrearia* Simonsen. French patent 98-09270

Lebeau T, Robert JM (2002) Diatom cultivation and biotechnologically relevant products. Part II: Current and putative products. Appl Microbiol Biotechnol DOI 10.1007/s00253-002-1177-3

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

Lebeau T, Junter GA, Jouenne T, Robert JM (1999) Marennine production by agar-entrapped *Haslea ostrearia* Simonsen. Bioresour Technol 67:13–17

Lebeau T, Gaudin P, Mignot L, Junter GA (2000) Continuous marennine production by agar-entrapped *Haslea ostrearia* using a tubular photobioreactor with internal illumination. Appl Microbiol Biotechnol 54:634–640

Lebeau T, Gaudin P, Moan R, Robert JM (2002) A new photobioreactor for the continuous marennine production with a marine diatom: influence of the light intensity and the immobilised-cell matrix (alginate beads or agar layer). Appl Microbiol Biotechnol 59:153–159

Lee RE (1999) Heterokontophyta, Bacillariophyceae. In: Lee RE (ed) Phycology. Cambridge University Press, Cambridge, pp 415–458

Lee YK, Low CS (1992) Productivity of outdoor algal cultures in enclosed tubular photobioreactors. Biotechnol Bioeng 40:1119–1122

Lefebvre S, Barillé L, Clerc M (2000) Pacific oyster (*Crassostrea gigas*) feeding responses to a fish-farm effluent. Aquaculture 187:185–198

Lewin RA (1990) Phycotechnology: yesterday, today and tomorrow. Bull Mar Sci 47:256–257

Liao I, Su HS, Lin JH (1993) Larval foods for penaeid prawns. In: McVey JP (ed) Crustacean aquaculture, 2nd edn. (CRC handbook of mariculture, vol 1) CRC Press, Boca Raton, Fla. pp 29–59

Lombardi A, Wangersky PJ (1995) Particulate lipid class composition of three marine phytoplankton *Chaetoceros gracilis*, *Isochrysis galbana* (Tahiti) and *Dunaliella tertiolecta* grown in batch culture. Hydrobiologia 306:1–6

López Ruiz J, García García R, Ferreiro Almeda MS (1995) Diferentes acciones de productos de naturaleza zolítica en procesos acuícolas. Proc Nat Congr Aquacultura 5:959–963

Martin-Jezequel V, Hildebrand M, Brzezinski MA (2000) Silicon metabolism in diatoms: implications for growth. J Phycol 36:821–840

McGinnis KM, Dempster TA, Sommerfeld MR (1997) Characterisation of the growth and lipid content of the diatom *Chaetoceros muelleri*. J Appl Phycol 9:19–24

Molina E, Fernández J, Acién FG, Chisti Y (2001) Tubular photobioreactor design for algal cultures. J Biotechnol 92:113–131

Molina Grima E, Sánchez Perez JA, García Camacho F, Fernandez Sevilla JM, Acien Fernandez FG, Urda Cardona J (1995) Biomass and eicosapentaenoic acid productivities from an outdoor batch culture of *Phaeodactylum tricornutum* UTEX 640 in an airlift tubular photobioreactor. Appl Microbiol Biotechnol 42:658–663

Molina Grima E, Acién Fernández FG, García Camacho F, Chisti Y (1999) Photobioreactors: light regime, mass transfer, and scaleup. J Biotechnol 70:231–247

Molina Grima E, Acién Fernández FG, García Camacho F, Camacho Rubio F, Chisti Y (2000) Scale up of tubular photobioreactors. J Appl Phycol 12:355–368

Moreau C (1996) Des eaux souterraines salées en baie de Bourgneuf pour la production de microalgues en aquaculture: l'azote ammoniacal, le fer et le manganèse, causes de la variabilité de la fertilité potentielle par trois diatomées-tests. DSc thesis, University of Nantes, Nantes

Neuville D, Daste P (1972) Production du pigment bleu par la diatomée *Navicula ostrearia* (Gaillon) Bory, maintenue en culture uni-algue sur milieu synthétique carencé en azote. CR Acad Sci Ser D 374:2030–2033

Norton TA, Melkonian M, Andersen RA (1996) Algal biodiversity. Phycologia 35:308–326

Pfeiffer TJ, Rusch KA (2000) An integrated system for microalgal and nursery seed clam culture. Aquacult Eng 24: 15–31

Provasoli L (1968) Media and prospect for the cultivation of marine algae. In: Watanabe A, Hattori A (eds) Cultures and collections of algae. Jpn Soc Plant Physiol, Hakoue, pp 63–75

Pulz O, Scheibenbogen K (1998) Photobioreactors: design and performance with respect to light energy input. Adv Biochem Eng Biotechnol 59:123–152

Renaud SM, Zhou HC, Parry DL, Thinh LV, Woo KC (1995) Effect of temperature on the growth, total lipid content and fatty acid composition of recently isolated tropical microalgae *Isochrysis* sp. *Nitzschia closterium*, *Nitzschia paleacea*, and commercial species *Isochrysis* sp. (clone T.ISO). J Appl Phycol 7:595–602

Rincé Y, Lebeau T, Robert JM (1999) Artificial cell-immobilisation: a model simulating immobilisation in natural environment? J Appl Phycol 11:263–272

Robert JM (1983) Fertilité des eaux de claires ostréïcoles et verdissement: utilisation de l'azote par les diatomées dominantes. DSc thesis, University of Nantes, Nantes

Roberts R, Kawamura T, Takami H (2000) Diatoms for abalone cultures: a workshop for abalone farmers. Int Abalone Symp 4:1–28

Robinson LF, Morrison AW, Bamforth MR(1988) Improvements relating to biosynthesis. European patent 261,872

Rossignol N, Lebeau T, Jaouen P, Robert JM (2000a) Comparison of two membrane-photobioreactors, with free or immobilised cells, for the production of pigments by a marine diatom. Bioprocess Eng 23:495–501

Rossignol N, Jaouen P, Robert JM, Quemeneur F (2000b) Production of exocellular pigment by the marine diatom *Haslea ostrearia* Simonsen in a photobioreactor equipped with immersed ultrafiltration membranes. Bioresour Technol 73:197–200

Rowland SJ, Belt ST, Wraige EJ, Massé G, Roussakis C, Robert JM (2001) Effects of temperature on polyunsaturation in cytostatic lipids of *Haslea ostrearia*. Phytochemistry 56:597–602

Sánchez S, Martinez Ma E, Molina E, De la Casa JA (1995) The influence of temperature on the growth and fatty-acid composition of *Skeletonema costatum* in a batch photobioreactor. J Chem Technol Biotechnol 62:148–152

Sánchez Mirón A, Contreras Gómez A, García Camacho F, Molina Grima E, Chisti Y (1999) Comparative evaluation of compact photobioreactors for large-scale monoculture of microalgae. J Biotechnol 70:249–270

Sánchez Mirón A, García Camacho F, Contreras Gómez A, Molina Grima E, Chisti Y (2000) Bubble-column and airlift photobioreactors for algal culture AIChE J 46:91872–1886

Scala S, Bowler C (2001) Molecular insights into the novel aspects of diatom biology. Cell Mol Life Sci 58:1666–1673

Schubert H, Tremblin G, Robert JM, Sagert S, Rince Y (1995) In vivo fluorescence measurement of photosynthesis of *Haslea ostrearia* Simonsen in relation to marennine content. Diatom Res 10:341–349

Schügerl K (2000) Integrated processing of biotechnology products. Biotechnol Adv 18:581–599

Smetacek V (1999) Diatoms and the ocean carbon cycle. Protist 150:25–32

Sriharan S, Bagga D, Sriharan TP (1990) Effects of nutrients and temperature on lipid and fatty acid production in the diatom *Hantzschia* DI-60. Appl Biochem Biotechnol 24/25:309–316

Taraldsvik M, Myklestad SM (2000) The effect of pH on growth rate, biochemical composition and extracellular carbohydrate production of the marine diatom *Skeletonema costatum*. Eur J Phycol 35:189–194

Thompson FL, Abreu PC, Wasielesky W (2002) Importance of biofilm for water quality and nourishment in intensive shrimp culture. Aquaculture 203:263–278

Tremblin G, Cannuel R, Mouget JL, Rech M, Robert JM (2000) Change in light quality due to a blue-green pigment, marennine, released in oyster-ponds: effect on growth and photosynthesis in two diatoms, *Haslea ostrearia* and *Skeletonema costatum*. J Appl Phycol 12:557–566

Turpin V, Robert JM, Goulletquer P (1999) Limiting nutrients of oyster pond seawaters in the Marennes-Oleron region for *Haslea ostrearia*: applications to the mass production of the diatom in mesocosm experiments. Aquat Living Resour 12:335–342

Vandanjon L, Rossignol N, Jaouen P, Robert JM Quemeneur F (1999) Effects of shear on two microalgae species. Contribution of pumps and valves in tangential flow filtration systems. Biotechnol Bioeng 63:1–9

Van Den Hoek C, Mann DG, Johns HM (1997) Algae. An introduction to phycology. Cambridge University Press, Cambridge

Veloso V, Reis A, Gouveia L, Fernandes HL, Empis JA, Novais JM (1991) Lipid production by *Phaeodactylum tricornutum*. Bioresour Technol 38:115–119

Voltolina D, Nieves M, Ruiz JL (1997) Zeolitic products as enrichment for cultures of a marine microalga. Aquacult Eng 16:1–5

Voltolina D, Nieves M, Navarro G, Oliva T, Peraza D (1998) The importance of acclimation for the evaluation of alternative media for microalgae growth. Aquacult Eng 19:7–15

Vrieling EG, Gieskes WC, Beelen TPM (1999) Silicon deposition in diatoms: control by the pH inside the silicon deposition vesicle. J Phycol 35:548–559

Walsh DT, Withstandley CA, Kraus RA, Petrovits BJ (1987) Mass culture of selected marine microalgae for the nursery production of bivalve seed. J Shellfish Res 6:71–77

Wen ZY (2001) A high yield and productivity strategy for eicosapentaenoic acid production by the diatom *Nitzschia laevis* in heterotrophic culture. PhD thesis, University of Hong Kong, Hong Kong

Wen ZY, Chen F (2000a) Heterotrophic production of eicosapentaenoic acid by the diatom *Nitzschia laevis*: effects of silicate and glucose. J Ind Microbiol Biotechnol 25:218–224

Wen ZY, Chen F (2000b) Production potential of eicosapentaenoic acid by the diatom *Nitzschia laevis*. Biotechnol Lett 22:727–733

Wen ZY, Chen F (2001a) A perfusion-cell bleeding culture strategy for enhancing the productivity of eicosapentaenoic acid by *Nitzschia laevis*. Appl Microbiol Biotechnol 57:316–322

Wen ZY, Chen F (2001b) Optimisation of nitrogen sources for heterotrophic production of eicosapentaenoic acid by the diatom *Nitzschia laevis*. Enzyme Microb Technol 29:341–347

Wen ZY, Chen F (2001c) Application of statistically-based experimental designs for the optimisation of eicosapentaenoic acid production by the diatom *Nitzschia laevis*. Biotechnol Bioeng 75:159–169

Yongmanitchai W, Ward OP (1991) Growth of and omega-3 fatty acid production by *Phaeodactylum tricornutum* under different culture conditions. Appl Environ Microbiol 57:419–425

Yusoff FM, Matias HB Khalid, ZA, Phang SM (2001) Culture of microalgae using interstitial water extracted from shrimp pond bottom sediments. Aquaculture 201:263–270

Zaslavskaia LA, Lippmeier JC, Shih C, Ehrhardt D, Grossman AR, Apt KE (2001) Trophic conversion of an obligate photoautotrophic organism through metabolic engineering. Science 292:2073–2075