12. Novel Bioreactors for Culturing Marine Organisms

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This chapter considers a wide range of novel bioreactor configurations for cultivation of marine organisms for purposes of biomass harvesting/enrichment or synthesis of target metabolites or wastewater treatment. It begins by analyzing biofilm reactors that promote surface-attached growth, including the niche-mimicking types viz. modified roller bottles, air membrane surface bioreactor, and ultralow speed rotating disk bioreactor as well as the small-scale extended surface shaken vessel. Photobioreactors (PBR), used mainly for phototrophic algal growth, are discussed next – these include the tubular, plate/panel and stirred tank types on the one hand and vertical column PBRs on the other, the latter mainly comprising airlift (AL) and bubble column (BC) PBRs. Important AL/BC configurations have been described. Membrane bioreactors (MBR) are then taken up, which include, e.g., the anaerobic MBR, ion-exchange MBR, etc. Immobilized cell bioreactors – primarily packed bed bioreactors (PBBR) and their hybrids (e.g., with PBR, airlift bioreactor (ALBR), MBR etc.), are reviewed next, followed by hollow fiber bioreactors (HFBR) (e.g., HF–submerged MBR, HF-PBR, etc.) which, technically, are also a class of immobilized-cell bioreactors. This is followed by a brief overview of fluidized bed and moving bed bioreactors, used primarily for wastewater treatment. Finally, the different classes of high-pressure and/or high-temperature bioreactors are considered, which are practically wholly devoted to cultivation of extremophiles (barophiles and/or thermophiles) isolated from the deep sea.

12.1 Biofilm Reactors (BFR)

In their natural habitat, most bacteria exist within biofilms that are anchored to surfaces and are inherently different from bacteria existing in a planktonic state. Many bacterial strains upon attaching to a surface reportedly produce exopolysaccharides (EPS) which mediate the attachment of the bacteria to the surface culminating in the formation of a biofilm. Marine microbial communities often occur as biofilms which are high-density surface-attached aggregates embedded in extracellular biopolymer (EPS) matrices. The microbial biofilm is a common adaptation of natural bacteria and other microorganisms. In the fluctuating environment of intertidal systems, biofilms form protective microenvironments and may structure a range

of microbial processes. Surface attachment and biofilm formation possibly also induce metabolic changes resulting in the production of different metabolites under attached growth conditions. Furthermore, many bacteria, including Bacillus species, are known to produce signal molecules that are used by the cells to monitor cell density, a phenomenon termed *quorum sensing*, which also controls cell metabolism. Cells in biofilms usually grow at much higher cell densities (up to 10 000 times higher) than in liquid suspension cultures, and quorum-sensing mechanisms could affect the production of secondary metabolites by these biofilm cells. Biofilm Reactors essentially promote surface-attched growth of biofilms (Table 12.1).

Yan et al. [12.1] examined the possibility whether allowing biofilm forming bacteria to grow under conditions that mimicked their marine ecological niche could elicit the production of antimicrobial compounds that they do not synthesize in the planktonic state, using modified roller bottle cultures (Fig. 12.1). Now, roller bottles provide a (typical) growth environment to cells anchored directly or indirectly to the inner wall of the bottles, allowing the attached cells to form a biofilm periodically in contact with both the gas and the liquid phase, (i. e., culture medium) – conditions similar to the alternating wetting and air exposure experienced by bacteria growing on seaweeds or other surfaces in an intertidal environment. However, the challenge lay in devising a method for anchoring bacterial cells, directly or indirectly, to the inner wall of the roller bottles. During preliminary studies it was noted that the epibiotic marine bacterial strains, EI-34-6 (*Bacillus licheniformis*) and II-111-5 (*Bacillus subtilis*) isolated from the surface of the seaweed *Palmaria palmata*, when cultivated on agar medium remained attached to the agar surface even when mixed or washed with the corresponding liquid broth. Based on this finding, the authors devised a modified roller bottle cultivation method wherein an agar coating on the inner wall of the roller bottles provides the surface necessary for the bacteria to attach and form a biofilm matrix. Subsequently, the effect of surface attachment/biofilm formation on antibiotic production was examined.

To prepare a surface-attached roller bottle culture, an agar coating was deposited on the inside wall of a 500 mL Duran bottle during solidification of the agar (after autoclaving) by rolling on ice, with one part of the coating intentionally thickened. The thickened portion of the agar coating was intended to serve as a baffle and help in enhancing oxygen transfer to the surface-attached culture. The cell inoculum was spread on the surface of the agar coating using a swab. The Duran bottles were incubated under the static condition to initiate an agar surface culture (for biofilm formation), then 50 mL of the corresponding liquid medium was added and the bottles rolled horizontally at 1 rpm; thus allowing the biofilm developing on the agar surface to be periodically exposed to air, and remain submerged in the liquid media.

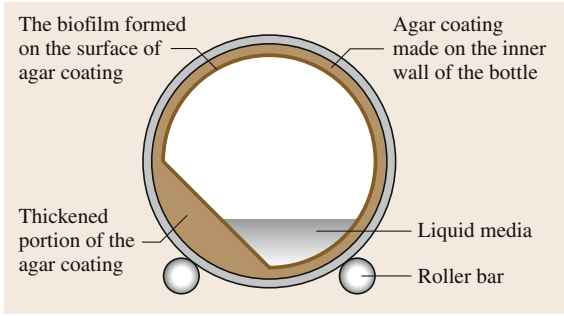

Fig. 12.1 Roller bottle cross section. A layer of agar coating was made on the inner wall of a 500 mL Duran bottle with one fraction thickened. The cell inocula were spread on the surface of the agar coating by a swab and cultivated statically for different times (after *Yan* et al. [12.1])

In suspension cultures, either in a shake flask or in standard roller bottle cultivation, the marine bacteria did not form biofilms on glass surface, which evidently was not conducive for mimicking the ecological niche of the studied strains, i. e., seaweed surface. *B. licheniformis*, strain EI-34-6, characteristically forms colonies that are usually strongly attached to agar surface; thus the agar-coated roller bottle culture makes good use of this phenomenon, facilitating the formation of a strongly bound biofilm. Although the liquid medium provided sufficient nutrients, the cells adhered to the surface of the agar coating, preferring growth in the biofilm even when in the presence of a liquid nutrient source. Furthermore, although the liquid medium provided the same nutrients, cells attached to the agar surface-produced antibacterial compounds that planktonic cells could not. Rolling cultivation for 2 h after the addition of liquid medium showed that antibacterial compounds were produced by surface-attached cells during biofilm growth – in fact detectable antimicrobial activity in the medium was always associated with observed biofilm formation.

Both isolates EI-34-6 and II-111-5 reached very high cell densities (of the order of 10^9 CFU mL^{-1}) even in shaken suspension cultures but without any detectable production of antibiotics, thus pointing to

Table 12.1 Biofilm reactors (BFR)

Sl	Bioreactor	Marine strain and bioprocess	Reference
1	Modified roller bottle cultivation (MRBC)	Antimicrobial compound production by bacterial strains *Bacillus licheniformis* EI-34-6 and *B. subtilis* II-111-5	*Yan* et al. [12.1]
2	Air-membrane surface bioreactor (AMSBR)	Antimicrobial compound production by bacteria *B. licheniformis* EI-34-6	*Yan* et al. [12.2]
3	Ultralow speed rotating disk bioreactor (UL-S-RDBR)	Antimicrobial synthesis by three estuarine/intertidal actinobacteria MS 310, MS 3/20, MS 1/7	*Sarkar* et al. [12.3]
4	ULS-RDBR	Actinomycin-D production by estuarine isolate *Streptomyces* sp. MS 310	*Sarkar* et al. [12.4]
5	ULS-RDBR	Antimicrobial synthesis by estuarine *Streptomyces* sp. MS 1/7	*Sarkar* et al. [12.5]
6	Extended surface shaken vessel (ESSV)	Protease production by intertidal estuarine gamma proteobacterium (DG II)	*Sarkar* et al. [12.6]
7	ESSV	Melanin synthesis by *S. colwelliana* and antibiotic production by *P. rubra* (both bacteria)	*Mitra* et al. [12.7]
8	ESSV	Cellulase and xylanase production by two intertidal filamentous fungi, *C. crispatum* and *G. viride*, respectively	*Mitra* et al. [12.8]

the possibility of either induction mechanisms other than quorum-sensing regulating antibiotic production by the attached cells or mere sensing of the physical attachment by these cells triggering changes in gene expression associated with antibiotic synthesis. The periodic exposure of the growing biofilm to the liquid medium and to air mimics the marine ecological niche of the biofilm-forming microbes on intertidal seaweed, hence the term *niche-mimic bioreactor* for the modified roller bottle cultures.

Yan et al. [12.2], designed a novel bioreactor – the air membrane surface (AMS) bioreactor, that allows the growth of bacteria as biofilms attached/anchored to the surface of a semipermeable membrane disk in contact with air, to investigate the production of antimicrobial compounds by the marine bacterium *B. licheniformis, strain EI-34-6* (used earlier by *Yan* et al. [12.1]) isolated from the surface of a marine seaweed *P. palmata*. The AMS bioreactor (Fig. 12.2) consists of a small, shallow dish filled with sterile liquid medium, with a semipermeable membrane disk placed on top of the dish such that the membrane remains in contact with the medium on one side and with air on the other, is held in place by surface tension. Bacteria were inoculated onto the membrane surface by swabbing, then the inoculated AMS bioreactor was placed in a sterile petri dish during cell growth. *B. licheniformis* produced antimicrobial compounds (the major component identified as bacitracin) when it grew in surface-attached condition as a biofilm at an air–membrane interface in the AMS-BR but not when it was grown planktonically in shake-flask cultures. An unidentified red pigment was also produced by surface-attached cells but not

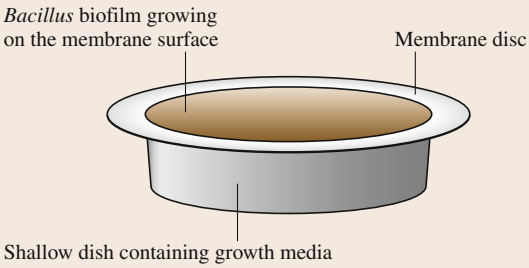

Fig. 12.2 AMS bioreactor. The small chamber beneath the membrane is filled with liquid medium, and the membrane disk is held in place by surface tension. Bacteria were inoculated onto the surface of a semipermeable nylon membrane. The AMS bioreactor was placed in a sterile petri dish during growth to maintain sterility (after *Yan* et al. [12.2])

by suspended cells. Different types of semipermeable membranes with widely varying pore sizes (viz., nylon, cellophane, and flat dialysis membranes) gave similar results, indicating that antibiotic production was not due to the chemical composition of the membrane but rather due to the niche-mimicking environment provided by the AMS bioreactor. Thus, it was established that biofilm formation as well as periodic direct exposure to air are necessary for eliciting production of antimicrobial compounds by the surface-attached cultures of *B. licheniformis*.

Sarkar et al. [12.3], developed an ultralow speed rotating disk biofilm reactor (ULS-RDBR) operated at a rotational speed of one revolution per day at 50% submergence of the rotating discs to mimic the intertidal

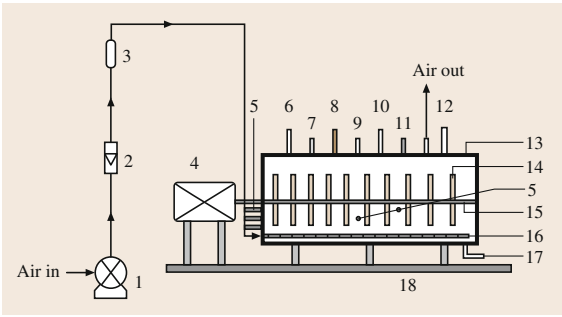

Fig. 12.3 Schematic of the ultralow-speed rotating disk bioreactor (RDBR) (1 – air pump; 2 – rotameter; 3 – air filter; 4 – electrical motor and reducing gear train for speed reduction; 5 – sampling port; 6 – temperature sensor; 7 – antifoam port; 8 – inoculation and medium addition port; 9 – acid port; 10 – pH sensor; 11 – alkali port; 12 – DO sensor; 13 – reactor vessel; 14 – rotating coaxial disks; 15 – shaft; 16 – sparger; 17 – drain; 18 – base plate) (after *Sarkar* et al. [12.4])

estuarine ecological niche of three marine actinobacteria viz. MS 310 (*Streptomyces* sp.), MS 3/20, and MS 1/7. The ULS-RDBR (Fig. 12.3), which facilitates microbial growth in the form of surface-attached biofilms, was designed on the concept of a rotary biological contactor (RBC). The maximum volume of the RDBR is 25 L, and the shaft (on which 10 disks are coaxially mounted) is rotated at an ultralow speed of one revolution per day. When operated with half the volume of the tank filled with liquid medium (i. e., at 50% submergence level), any given point on the disks would remain exposed to air and submerged in the medium alternatively for 12 h; thus mimicking the intertidal conditions of the location from where the microorganism was collected. The ULS-RDBR with its much higher surface-to-volume ratio (compared to a STBR) and niche-mimicking ability, supported good biofilm formation on the extended surface in the reactor (i. e., the rotating disks) and faster attainment of the peak level of antimicrobials metabolized by these isolates. It was also noted that antimicrobial synthesis was growth associated.

The ULS-RDBR (volume 25 L) was employed by *Sarkar* et al. [12.4] for detail investigation of the production of a potentially novel antimicrobial compound by the biofilm-forming marine *Streptomyces* sp. MS 1/7, in particular the effects of pH, aeration rate and disk submergence level (varied at 25, 50 and 75%) on the peak antimicrobial activity (PAMA), peak activity attainment rate (PAAR), and biofilm density (BD). PAMA, PAAR, and BD were all maximized at the intertidal niche-mimic operating condition (1 rev d^{-1}, 50% disk submergence) along with the highest aeration rate provided in the experiment. Furthermore at any aeration rate, PAMA was always highest under the niche-mimic condition – 12 h. alternating cycles of inundation and aerial exposure as in the intertidal conditions characterizing the biofilm-forming microbe's marine ecological niche.

The ULS-RDBR is a horizontal, rectangular parallelepiped shaped vessel (42 cm × 27 cm × 27 cm, volume 25 L), comprising 10 coaxial, equispaced disks (each of 16 cm diameter and 5 mm thickness) mounted on a single rotating shaft. The reactor vessel, the disks as well as the shaft are all made of acrylic (polymethyl methacrylate), which is transparent, corrosion resistant to high salt concentrations, and generally provides a surface conducive to attachment of biofilm. For 50% disk submergence level, i. e., 12.5 L working volume, the ratio of surface area to working volume of the RDBR is 342 $cm^2\,L^{-1}$, considering total surface area of 10 disks only. If, however, submerged wall surface and floor of the tank are also considered together with the disks, then the ratio increases to 552 $cm^2\,L^{-1}$. It was observed (in trial runs) that the roughened acrylic surface allowed substantially more biofilm formation than the smooth surface. Due to this reason, the disk surfaces were roughened using sand paper (Grade 50) to facilitate surface attachment by the biofilm-forming microorganisms. A reducing gear train was designed to reduce the speed of a 7 rpm motor (for driving the rotating shaft) by ca. 10 000 times to the ultralow rotational speed of 1 rev d^{-1}. Air was supplied into the RDBR using an air compressor, passed through a sterilizing air filter, and then uniformly distributed into the fermentation medium using a rectangular sparger (also made of acrylic) having uniformly spaced holes in the downward direction. Ports on the top lid of the RDBR are available for sampling, addition of medium/inoculum/antifoam, pH sensor, DO sensor, temperature sensor, and air exhaust. The fermentation medium was sterilized ex situ and added aseptically to the reactor disinfected both chemically (i. e., by repeated washing with sodium hypochlorite) and by UV radiation.

Sarkar et al. [12.5] found almost similar results in their investigation of actinomycin-D production by the biofilm-forming estuarine isolate MS 310 cultivated in the ULS-RDBR operated at 1 rev d^{-1}. The niche-mimic condition along with maximum permissible aeration was found to be most favorable for antibiotic produc-

tion – peak antibiotic activity (PAA) and peak activity attainment rate (PAAR) simultaneously attaining their highest values at this operating condition – 50% disk submergence. Both PAA and PAAR are observed to increase with increasing aeration at all operating conditions examined. At the niche-mimic condition, a threefold increase in the aeration rate causes PAA to increase by 33%, whereas PAAR increases by 2.5 times, underlining the strong aeration dependence of this actinomycin-D producer. Again, compared to the highest values obtained for antimicrobial production in flask (500 mL) experiments, the corresponding RDBR values were 16% higher for PAA and more than five times higher for PAAR – strong evidence for employing these novel bioreactors for cultivation of antibiotic-producing marine microbes.

It follows from the above discussion that for microorganisms which synthesize metabolites/enzymes at the highest rates only when growing in surface-attached condition, i. e., as biofilms anchored to solid surfaces, special culture conditions are necessary that are conducive to surface attachment and biofilm formation. For this purpose, several typical niche-mimic bioreactors have been developed (e.g., modified roller bottle cultures, AMS-BR, ULS-RDBR, etc.) as described earlier in this section. However, a lack of small-scale shaken vessels with high surface/volume ratio and surface properties favoring attachment of biofilm-forming microbes was noted by *Sarkar* et al. [12.6]. They developed a novel small scale, extended surface shaken vessel (ESSV) in the form of a PMMA acrylic made conico-cylindrical flask (CCF) (volume 500 mL) in which enhanced surface for microbial attachment and biofilm formation was provided by eight equidistantly located vertical rectangular strips radially mounted on the base of the vessel with the base diameter close to that of a 500 mL Erlenmeyer flask (EF) for easy placement in a rotary shaker. The small-scale vessel was designed to allow the use of different internal surface materials – hydrophilic (glass) or hydrophobic (acrylic). Furthermore, protease production by two strains in the ESSV were examined, of which one was marine – an intertidal gamma – Proteobacterium (DG II). Relative to a standard EF with no additional surface, growth and protease synthesis by the marine isolate DG II were 20 and 30% higher, respectively. Again, compared to glass, the use of acrylic surface (hydrophobic) resulted in more than 200% increase in protease production and a dramatic increase (i. e., by 19, 275%) in microbial growth.

Mitra et al. [12.7] cultivated two biofilm-forming marine bacteria in the novel ESSV described above, viz. *Shewanella colwelliana* for melanin production and *Pseudoalteromonas rubra* for antibiotic synthesis. The design allowed comparison of production between (1) CCF with hydrophobic surface (PMMA), (2) ESSV with hydrophilic glass surface, and (3) standard unbaffled EF. Growth and melanin production by *S. colwelliana* were highest in the ESSV with (hydrophilic) acrylic surface, further melanin synthesis increased with increase in surface (for attachment) and increase in biofilm formation and increase in planktonic growth. Growth of *P. rubra* was also highest in the acrylic ESSV but not antibiotic synthesis – it was maximum in the EF without any extended surfaces. Thus antibiotic production was favored by a hydrophilic vessel surface (glass).

Mitra et al. [12.8] examined cellulase and xylanase production in relation to biofilm formation by two intertidal filamentous fungi, viz. *Chaetomium crispatum* and *Gliocladium viride*, respectively, in the novel ESSV (described above) with either hydrophobic (acrylic) or hydrophilic (glass) surface and compared with that in an ordinary EF. Mixed results were obtained with regard to suitability of the EF or the ESSVs for enzyme production by the two filamentous fungi – surface properties as well as surface area of attachment of the cultivation vessel affected biofilm formation and enzyme production.

12.2 Photobioreactors (PBR)–Tubular, Plate/Panel and Stirred Tank Configurations

Photobioreactors, as the name indicates, are specialized bioreactors (Table 12.2) for phototrophic growth of microorganisms – mainly alga but also photosynthetic bacteria; as well as macroorganisms, i. e., macroalgae (*seaweed*). Application of PBRs for microalgal growth has been extensively reviewed [12.19, 20]. PBRs originated as open-air cultivation systems with natural sunlight as the source of illumination that are easy to build and operate – these include natural or artificial ponds/tanks, raceway-shaped culture ponds (which are basically closed-loop recirculation channels) and so-called *inclined surface ponds* driven by paddle wheels. However, open-air PBR systems are prone to evaporative losses and contamination problems and most im-

Table 12.2 Photobioreactors (PBR)-tubular, plate/panel, stirred tank etc.[a]

Sl	Bioreactor	Marine strain and bioprocess	Reference
1	Flat plate PBR	CO_2 removal by green alga *Chlorococcum littorale*	*Hu* et al. [12.9]
2	Double-phase flat panel-type PBR	Microaerobic biohydrogen production by the nonsulfur photosynthetic bacteria *Rhodovulum* sp.	*Matsunaga* et al. [12.10]
3	Flat alveolar panel PBR (FAP-PBR), Green Wall Panel PBR (GWP-PBR)	Oil production by algal strains (e.g., *Nannochloropsis* sp.)	*Rodolfi* et al. [12.11]
4	Tubular loop PBR[a]	Cultivation of red macroalga *Porphyridium* sp.	*Merchuk* et al. [12.12]
5	Tubular recycle PBR	Brown macroalga *L. saccharina* cultivation	*Rorrer* and *Mullikin* [12.13]
6	Stirred tank PBR	Biomass production of microalga *Acrosiphonia coalita*	*Rorrer* and *Zhi* [12.14]
7	Stirred tank PBR	Production of α-tocopherol by microalgae *Euglena gracilis*	*Ogbonna* et al. [12.15]
8	Three-stage serial column-type PBR (CPBR)	*Spirulina* sp. (cyanobacterium) for CO_2 biofixation through photosynthesis	*de Morais* and *Costa* [12.16]
9	Laboratory-scale and pilot-scale PBRs	Harvesting algal biomass of *Euglena gracilis* as potential animal food source	*Chae* et al. [12.17]
10	Serpentine PBR and hybrid flow through-PBR	Biogas production by anaerobic digestion of microalga *P. tricornutum*	*Zamalloa* et al. [12.18]

[a] Also compares the tabulated reactors with airlift and bubble-column reactors, restated in Table 12.4 with discussion in the Sect. 12.3 ALBR and BCBR

portantly, have significantly lower biomass productivity than closed PBRs. The common design configurations of the latter include plate/panel, tubular, stirred-tank and vertical column (including airlift and bubble – column reactors). Tubular PBRs consist of an array of straight, coiled, or looped transparent tubes through which the culture is circulated by pump or by airlift mechanism – the latter having the advantages of permitting exchange of CO_2 and O_2 between the liquid medium and the aeration gas, minimizing shear damage to the cells caused by mechanical pumping and achieving medium circulation without any moving parts. Increase in tube diameter (above 0.1 m) decreases the surface/volume ratio and as culture density increases with growth, the cells begins to shade one another (*self-shading effect*) which results in decreasing volumetric biomass productivity. Again, excessive increase in tube length causes accumulation of O_2 (produced photosynthetically) which, when in excess of the air saturation value, becomes inhibitory for photosynthesis (oxidative photoinhibition). Some common tubular configurations are horizontal/serpentine, near horizontal, helical, conical, and inclined. In general, tubular PBRs have a large illumination surface area but poor mass transfer characteristics.

Flat-plate-type PBRs probably originated from the laminar morphology of plant leaves which are well-evolved natural solar collectors with high surface/volume ratio. They can be horizontal/vertical and posses the advantage of high illumination surface area, high photosynthetic efficiency, and lower accumulation of dissolved oxygen (than tubular PBRs). A tilted flat-plate PBR (e.g., the flat inclined modular PBR – FIMP) may be angled to ensure maximum exposure of the culture to sunlight and facilitates change of light path as and when required. This flexibility is often crucial as individual photosynthetic microorganisms often have an optimum light path that is a compromise between growth inhibition in innermost layers due to insufficient lighting and self-shading, and photoinhibition of growth in outermost layers due to excessive illumination. Stirred tank PBRs have also been used for algal cultures but they are not as common as flat plate/tubular-PBRs. Vertical column PBRs – mainly AL and bubble column (BC) PBRs are discussed in the following section.

PBRs can be externally or internally illuminated. External illumination may be natural (i. e., direct sunlight) or artificial (e.g., fluorescent lamps), internal illumination is necessarily artificial. Optical fiber-based internally illuminated PBRs have the advantage of heat sterilizability and stability to withstand mechanical agitation stresses; however, low light delivery efficiency ($< 50\%$) is a concern.

Hu et al. [12.9] used a modified flat plate PBR (1.4 L) to investigate CO_2 removal by an ultrahigh-cell-

density culture (i. e., with optimal cell density > 10 g DCW/L) of the marine green alga *Chlorococcum littorale*, which shows extended linear growth under light limiting conditions and grows very vigorously in the presence of extremely high CO_2 levels. The remarkably high CO_2 fixation rate reported with this alga is due to the ultrahigh cell density attained in this PBR which has a narrow light path in which intensive turbulent flow is generated by streaming compressed air through perforated tubing into the culture suspension (that produces vigorous air-bubble mixing). The acrylic-made reactor (Fig. 12.4) (height 50 cm, length 38 cm) consists of an outer chamber (width 6 cm) that serves as a temperature regulator and an inner chamber (with a total illuminated area of about 0.16 m^2) of varying width corresponding to the length of the light path (1, 2, and 4 cm) which was optimized in this study for biomass productivity. An air-bubbling tube is placed along the bottom of the inner chamber, through which compressed CO_2-enriched air is streamed to produce turbulence in the culture suspension. The top opening of the inner chamber is covered by silicone rubber together with a thick acrylic lid with a number of openings/ports for various sensors and for air exhaust. A panel with a bank of white fluorescent lamps was installed on each side of the reactor for illumination.

Microaerobic biohydrogen production by the marine, nonsulfur, photosynthetic bacterium *Rhodovulum* species was examined by *Matsunaga* et al. [12.10] in a *double-phase* flat panel PBR consisting of *light* and *dark* compartments. Hydrogen production under microaerobic conditions was found to be four times higher than in anaerobic conditions, mainly due to the much higher ATP accumulation during respiration under microaerobic conditions – ATP accumulated in the dark compartment was utilized for hydrogen production in the light compartment. Double-phase and conventional flat-panel-type PBRs (2 cm × 10 cm × 30 cm) made from 3 mm polyacryl resin sheets, had an illuminated area of 250 cm^2 and a culture volume of 500 mL. Light and dark compartments in the double-phase PBR were obtained by positioning a flat mirror (18 cm × 10 cm). Six fluorescent lamps were used as the source of illumination in this PBR.

Rodolfi et al. [12.11] used two types of PBRs viz., a flat alveolar panel (FAP) PBR (volume 20 L) and green wall panel (GWP) PBR (volume 110 L) for outdoor mass cultivation of the oil-producing marine eustigmatophyte microalga *Nannochloropsis* species. The study was claimed to be the first report of an increase in both lipid content and areal lipid productivity attained in an outdoor algal culture, through nutrient deprivation.

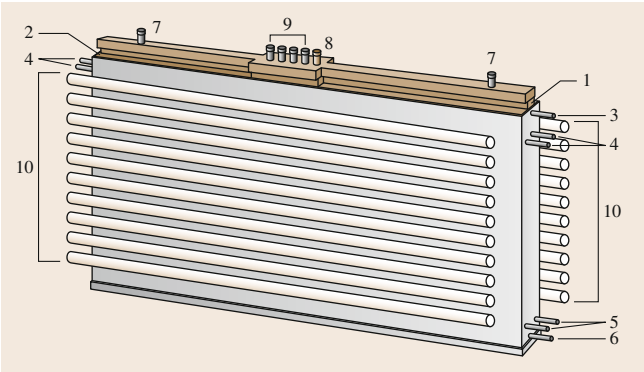

Fig. 12.4 Schematic diagram of the prototype flat-plate photobioreactor. 1 – inner culture chamber; 2 – outer temperature-regulation chamber; 3 – culture overflow; 4 – cooling-water inlets; 5 – cooling-water outlets; 6 – port for compressed-air tubing; 7 – air outlet; 8 – sampling port; 9 – ports for various sensors (pH, temperature, O_2, etc.); 10 – a bank of fluorescent lamps (after *Hu* et al. [12.9])

Merchuk et al. [12.12] studied the effect of light/dark cycles of different frequencies on growth of the red microalga *Porphyridium* sp. in a laboratory-scale tubular loop PBR and compared it with the performances of an AL-PBR and BC-PBR (both of volume 35 L). In the laboratory-scale PBR liquid is circulated by a peristaltic pump at a controlled rate whereas in the column PBRs, which are orders of magnitude larger, liquid movement is pneumatically driven by the injection of gas into the reactors. However, despite basic differences, the light/dark cycles generated, either by the pump of the laboratory-scale tubular bioreactor, or by gas flow in the column PBRs were found to be of the same order. By virtue of the small diameter of the loop and the low concentrations of the biomass used, the performance data from the laboratory-scale tubular loop PBR was free of the effect of self-shading, and therefore, this PBR could be considered as a *thin-film* PBR. The tubular loop PBR (total volume 0.43 L) consisted of a series of glass tubes (I.D. 0.007 m) connected to a small vessel into which 3% CO_2 was bubbled (to provide a carbon source and also to remove O_2) with the loop closed through a peristaltic pump. The cycle time could be varied by manipulating the flow rate in the pump. The ratio of light/dark zones was controlled by darkening different lengths of the tube – the ventilation vessel, being always covered, was part of the dark zone. A bank of fluorescent lamps provided the necessary illumination.

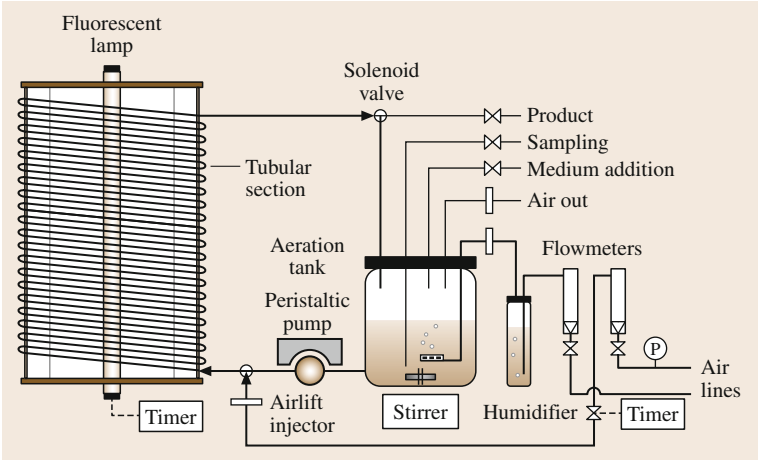

Fig. 12.5 Three-liter tubular photobioreactor, featuring coiled tubular section, aeration tank, and airlift injection system. In batch operation, the product outlet line is closed and the culture continuously recirculates between the aeration tank and the tubular section (after *Rorrer* and *Mullikin* [12.13])

Rorrer and *Mullikin* [12.13] cultivated cell suspension cultures obtained from the marine macrophytic brown alga (*seaweed*) *Laminaria saccharina* in a 3 L tubular recycle PBR (Fig. 12.5) where the culture is recycled between an unaerated coiled tubular section for culture illumination and a nonilluminated aeration tank where absorption of CO_2 (needed for photosynthetic biomass production) and stripping of dissolved O_2 evolved from photosynthesis take place. The tubular recycle PBR could be operated in batch, semicontinuous and continuous-recycle modes. A three-way solenoid valve located at the outlet of the tubular section is used for mode selection. In batch operation, the solenoid valve is closed, and the cell suspension culture leaving the tubular section is recycled back to the aeration tank (total recycle). In semicontinuous or continuous recycle operation, the solenoid valve opens and closes periodically, permitting a portion of the culture to be drawn off as product (while the rest is returned to the reactor), with concurrent feeding of fresh medium.

Rorrer and *Zhi* [12.14] compared the biomass productivities of a semidifferentiated tissue suspension culture of the marine cold-water green macroalga *Acrosiphonia coalita* (a source of oxylipins with potent antimicrobial properties), in two types of PBRs, a stirred tank PBR (ST-PBR) and a BC PBR (BC-PBR). Now, although the parent plant is highly branched and must be anchored to rocky substratum in its marine ecological niche, the tissue culture comprises mainly linear filaments growing in a homogeneous liquid suspension. Toward this end, the inoculum tissue was finely blended to 1–2 mm long filaments but it was observed that only the ST-PBR could provide the agitation necessary for uniform suspension of this tissue culture – in fact, of all the cultivation vessels considered, the highest biomass productivity was obtained in the ST-PBR.

The ST-PBR (Fig. 12.6) (volume 3 L) is actually a jacketed, round-bottomed, glass vessel (I.D. 13 cm, height 24 cm), equipped with a three-blade marine impeller (diameter 4.5 cm, height 6 cm) pitched at an angle of 45°. Ambient air was pumped through a sterile air filter, and then sparged to the culture through an air inlet pipe having seven holes (diameter 1 mm) drilled in a row. Optionally, CO_2 was metered separately and then mixed with the inlet air stream before passing through a sterilizing air filter. Cold water from a low-temperature circulator was pumped through the glass vessel jacket to maintain a constant cultivation temperature. The illumination stage consisted of two light banks positioned on opposite sides of the bioreactor, each comprising three fluorescent tube lamps (each 9 W), mounted horizontally in a parallel array. Each light bank was aligned with the bioreactor vessel so that the length of the lamp coincided with the vessel width, whereas the cumulative height of all the lamps matched the height of the liquid in the reactor vessel. A referencing plate was used to set the distance between the lamp and the vessel surface with high precision so that the desired light intensity (incident on the vessel surface) could be uniformly delivered to the culture. For the ST-PBR, the light sensor was positioned on the inside surface of the vessel to obtain the true incident light intensity to the culture. The light intensity incident on the inner surface of the vessel was fixed at nominally twice the saturation light intensity, to compensate for light attenuation through the culture.

Ogbonna et al. [12.15] studied the production of α-tocopherol by the microalgae *Euglena gracilis* in a con-

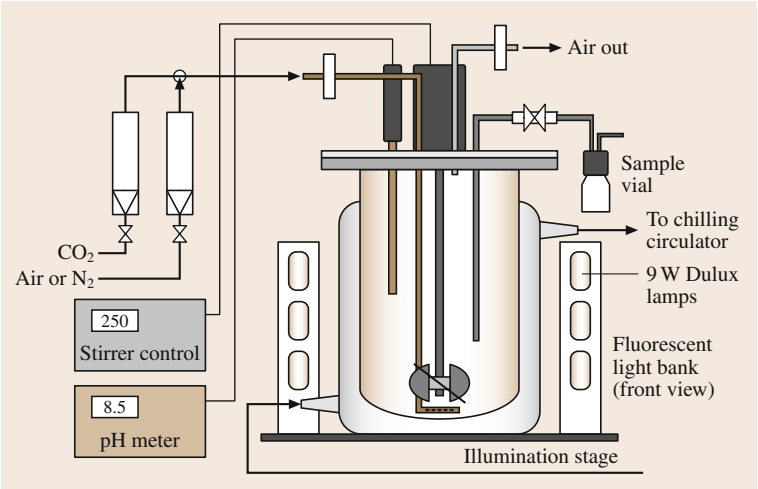

Fig. 12.6 Three-liter stirred-tank photobioreactor, including illumination stage and instrumentation (after *Rorrer* and *Zhi* [12.14])

tinuous sequential heterotrophic–photoautotrophic cultivation system. The cells were continuously cultured heterotrophically in a conventional aerated, stirred mini-jar bioreactor (volume 2.5 L, working vol. 2 L) and the effluent continuously passed through an internally illuminated photobioreactor for the photoautotrophic phase, with α-tocopherol production. The novel sequential continuous process resulted in α-tocopherol productivity (of 100 mg h^{-1}) which is $\approx$ 9.5 and 4.6 times higher than corresponding productivities obtained (individually) in batch photoautotrophic and heterotrophic cultures, respectively.

De Morais and *Costa* [12.16] designed a novel temperature-controlled, three-stage, serial column (i. e., tubular) photobioreactor (volume 2 L, working vol. 1.8 L for each CPBR) to investigate CO_2 biofixation by marine photosynthetic cyanobacteria *Spirulina* species. This study demonstrated the high CO_2 biofixation potential of the cyanobacteria cultivated in the three-reactor cascade CPBR1-CPBR 2-CPBR3. It was also noted that using multiple PBRs in series resulted in much lower CO_2 levels in the final gaseous effluent discharged to the atmosphere. Agitation and aeration were carried out using air from a compressor and a sintered sparger – the effluent air (with or without CO_2) from CPBR1 being fed to the sparger in CPBR2 and the effluent from CPBR2 being fed to CPBR3. The CPBRs were placed in a 30 °C growth chamber under a 12 h dark/light photoperiod with illumination provided by 40 W daylight-type fluorescent lamps during the light period.

Chae et al. [12.17] used two novel PBRs – one laboratory scale (working volume 100 L) (Fig. 12.7) and the other pilot scale (working volume 1000 L) to study single-cell protein production and atmospheric CO_2 biofixation by the microalga *Euglena gracilis*. Besides CO_2 fixation during photosynthesis, algal biomass may be used as a biofertilizer, soil conditioner, and also as feed for terrestrial and aquatic animals. Insofar as the last application is concerned, *E. gracilis* scores highly due to several reasons viz. (1) it has relatively high crude protein content (47% w/w of biomass) and thus higher nutritional quality compared to other microalgae like *Chlorella* and *Spirulina*, (2) its in vitro digestibility is slightly higher than that of casein making it an attractive animal fodder, (3) it grows well under acidic conditions with very little risk of culture contamination. *Euglena gracilis* growth is very sensitive to light intensity. The novel pilot-scale PBR (which uses sunlight as energy source and flue gas from an oil heater as a CO_2 source) minimizes the self-shading effect typical of dense microalgal cultures (that leads to increasing light attenuation with distance from the light source, and thereby to decreasing biomass productivity) and, expectedly, shows much higher biomass yields compared to the laboratory-scale PBR.

The laboratory-scale PBR (90 cm × 20 cm × 70 cm) was provided with a cover and baffles that induced plug flow of the culture medium. Pure CO_2 and air were fed at rates of 0.3 and 2.7 L min^{-1}, respectively, and the mixed gas was passed through a humidifier before entering the reactor. To minimize light attenuation, reactor width was fixed at 20 cm and fluorescent lamps were installed on both sides of the PBR as light sources in parallel. The pilot-scale PBR used sunlight as energy source and flue gas from an industrial oil heater as CO_2

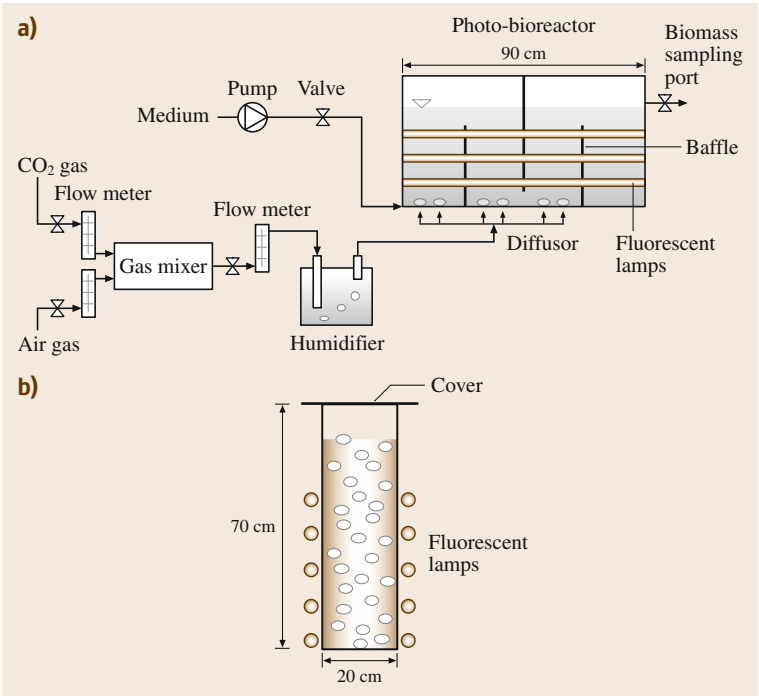

Fig. 12.7a,b Schematic (**a**) and cross-sectional diagrams (**b**) of a laboratory-scale photobioreactor for semicontinuous and continuous culture (after *Chae* et al. [12.17])

source (30 L min^{-1}). The L-shaped PBR (working volume 1000 L) was separated into a dark zone (1.8 m × 0.2 m × 1.4 m) and a light zone (1.8 m × 1.0 m × 0.4 m) of equal working volumes, each 500 L with a cover. To minimize light attenuation, effective height of the light zone was fixed at 20 cm. A scraper was installed for internal circulation of culture medium between dark and light zones.

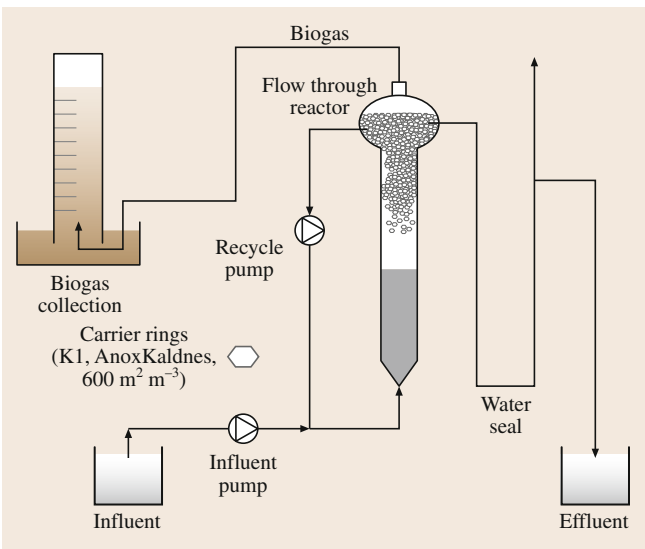

Fig. 12.8 Scheme of the laboratory-scale experimental setup of the hybrid flow-though reactor (after *Zamalloa* et al. [12.18])

Zamalloa et al. [12.18] examined the anaerobic digestibility of the marine microalga *Phaeodactylum tricornutum* for biogas production in a serpentine tubular PBR and a novel, hybrid flow-through anaerobic reactor (HFAR) – the latter essentially a cylindrical tube with a three-phase separator in the upper part (Fig. 12.8). The serpentine PBR comprises of a glass vessel (total volume 80 L, working volume of 65 L) and a transparent tube (diameter 19 mm, length ≈ 80 m). The culture broth was recirculated using a peristaltic pump (10 L min^{-1}). The PBR was installed in a greenhouse at 25 ± 2 °C with illumination provided continuously by fluorescent lamps. Culture pH was controlled (at 8) and regulated by on-demand, automated CO_2 injection. Mixing was achieved by bubbling air through diffusers at an aeration rate of ≈ 0.5 vvm. Each HFAR consisted of a cylindrical tube (diameter 5 cm) having a three-phase separator in the upper part, with a recirculating pump generating an upflow velocity of ≈ 1 m h^{-1}. Biogas production was measured by the liquid displacement method in 10 L airtight calibrated vessels containing water at pH 2 to prevent dissolu-

tion of CO_2. 150 g of plastic carrier rings per reactor were added as carrier material, and an anaerobic filter for increased retention of algal biomass particles, was installed.

12.3 Airlift Bioreactors (ALBR) and Bubble Column Bioreactors (BCBR)

12.3.1 Airlift Bioreactors

Airlift bioreactors (ALBR) (Tables 12.3 and 12.4) are pneumatically agitated gas–liquid or gas–liquid–solid contacting devices characterized by fluid circulation in a defined cyclic pattern through two vertical channels viz. the *riser* for gas–liquid upflow and *downcomer* for downflow) connected at the top (the *gas separator*) and bottom (the *base*). The driving force for recirculation of the fluid is the density difference between the downcomer and the riser which generates the pressure gradient necessary for liquid recirculation [12.21]. Air/gas is usually injected at the bottom of the riser and a portion of the gas disengages in the gas separator. The remaining portion is entrapped by the descending liquid and flows down the downcomer. If the gas residence time in the separator is substantially longer than the time required for disengagement of the gas bubbles, the fraction of gas recirculating through the downcomer would be minimized. In addition to agitation, the gas stream also facilitates exchange of material between the gas phase and the culture medium – oxygen is usually transferred to the liquid and often, metabolic products, from the liquid to the gas phase. ALBRs provide a relatively homogeneous low-shear field for microbial growth. They are further characterized by (a) their total lack of any moving parts and (b) their high aeration efficiency – the latter due to the high rates of oxygen transfer alongside minimal power consumption compared to conventional stirred bioreactor systems. ALBRs may be classified into two basic categories viz. (i) external loop ALBRs, in which fluid circulation occurs through separate and distinct channels, and (ii) internal – loop (baffled) ALBRs – where strategically installed baffles create the channels required for circulation. Configurations of both types may be further modified.

Jiang et al. [12.22] examined simultaneous carbon and nutrient removal from wastewater in a plexiglass bench-scale ALBR (working volume 22 L) by filamen-

Table 12.3 Airlift and bubble column bioreactors (ALBR and BCBR)

Sl	Bioreactor	Marine strain and bioprocess	Reference
1	ALBR	Simultaneous C and N removal from waste water by bacteria *Thiothrix* sp.	*Jiang* et al. [12.22]
2	ALBR	Rhamnolipid production by bacterium *Pseudomonus aeruginosa*	*Jeong* et al. [12.23]
3	Split column ALBR	Decolorization of textile waste water by marine fungi *A. niger*	*Assadi* and *Jahangiri* [12.24]
4	ALBR+ Fiber optics sensors	Filamentous callus induction and microplantlet culture propagation of macroalga *Kappaphycus alvarezzi*	*Munoz* et al. [12.25]
5	AL-PBR	Halogenated monoterpene production by red macroalga *O. secundiramea*	*Polzin* and *Rorrer* [12.26]
6	Concentric tube airlift PBR (AL-PBR)	Cultivation of microalga *P. tricornutum*	*Contreas* et al. [12.27]
7	Triangular configuration inclined tube – AL-PBR	CO_2 fixation from flue gas by green algae *Dunaliella* sp.	*Vunjak-Novakovic* et al. [12.28]
8	Outdoor airlift driven tubular PBR	Production of lutein by green unicellular microalga *Muriellopsis* sp.	*Del Campo* et al. [12.29]
9	BC-PBR	CO_2-fixation and H_2 production by green microalga *Platymonas subcordiformis*.	*Guo* et al. [12.30]
10	BC-PBR	Phototrophic cultivation of microplantlet suspension culture of the red macroalga *A. subulata*	*Huang* and *Rorrer* [12.31]
11	BC-PBR	Photolithotrophic cultivation of cell suspension culture from microscopic, filamentous gametophyte life phase of the complex brown macroalga *L. saccharina*	*Zhi* and *Rorrer* [12.32]

Table 12.4 ALBR and BCBR and/or other PBR – comparative studies

Sl	Bioreactors Compared	Marine strain and bioprocess	Reference
1	ALBR, BCBR	NOx removal by green microalga *D. tertiolecta*	*Nagase* et al. [12.33]
2	AL-PBR, BC-PBR	Harvesting diatom *S. costatum*	*Monkonsit* et al. [12.34]
3	BC-PBR, AL-PBR	Cultivation of red macroalga *Porphyridium* sp.	*Merchuk* et al. [12.12]
4	BC-PBR, AL-PBR	Cultivation of diatom *C. calcitrans*	*Krichnavaruk* et al. [12.35]
5	BC-PBR, split cylinder AL-PBR, draft tube sparged AL-PBR	Harvesting microalga *P. tricornutum*	*Sanchez-Miron* et al. [12.36]
6	BC-PBR, AL-PBR, AL-PBR + helical flow promoter (HFP)	Cultivation of red microalga *Porphyridium* sp.	*Merchuk* et al. [12.37]
7	BC-PBR, AL-PBR, externally illuminated stirred tank PBR, tubular recycle PBR	Culture of red macroalga *A. subulata*	*Rorrer* and *Chenny* [12.38]

tous marine bacteria *Thiothrix* species in a limited filamentous bulking (LFB) state – a repeatable and controllable state that brings about a balance between floc forming and filamentous bacteria. The ALBR with a low height-to-diameter ratio (overall height 1000 mm) consists of a reaction zone (I.D. 160 mm) and an upright settling zone (I.D. 220 mm). Two draft tubes, an upper tube (height 160 mm, dia 130 mm) and a lower tube (height 850 mm, dia 100 mm) were concentrically placed in the ALBR. For sparging gas, 120 holes (dia 0.5 mm) in a perforated pipe were positioned equidistantly around the circle at the middle of the riser. The flow rate of the mixed broth circulating between the annulus and the draft tube could be varied by changing the gas-flow rate. Influent was fed to the reactor through one of the three wastewater inlets located at the bottom and the middle of the riser and the upper part of the annulus, whereas effluent was withdrawn from the liquid in the settling zone. The ALBR and its component tubes were cleaned from time to time to prevent bacterial growth in the lines and on the vessel walls. The authors concluded that under an LFB state in the ALBR, the balance of aerobic and anoxic/anaerobic zones was achieved which is required for enhanced nutrient removal and effluent clarification. Furthermore, the LFB state, which is characterized by low DO levels, causes a reduction in the height-to-diameter ratio of the reactor and thereby in energy requirement for aeration.

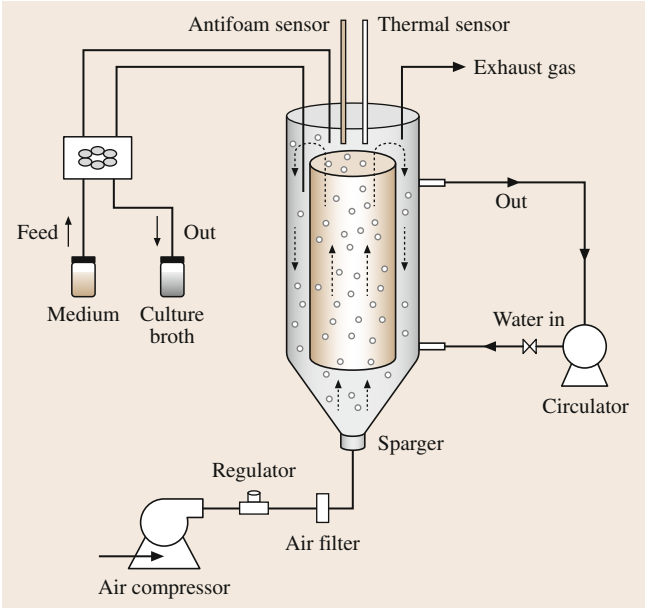

Fig. 12.9 Schematic diagram of an airlift bioreactor (after *Jeong* et al. [12.23])

Jeong et al. [12.23] demonstrated the continuous production of rhamnolipid-type biosurfactants in an ALBR (maximum volume 1.8 L, working volume 1.2 L, diameter 100 mm, height 320 mm) by a marine strain of *Pseudomonas aeruginosa* (isolated from the southern sea of Korea) immobilized, by entrapment in Ca-alginate modified PVA beads (Fig. 12.9). They noted that the medium-to-bead volume ratio is a key parameter for evaluating bioreactor performance and that an optimal value exists considering productivity and economics.

Assadi and *Jahangiri* [12.24] used a split ALBR to obtain a very high level (up to 97%) of decolorization of textile wastewater by a marine strain of *Aspergillus niger* (isolated from Gorgan Bay in the Caspian Sea). The jacketed glass-made ALBR is split by a PTFE strip

(800 mm × 100 mm × 3 mm) and is fitted with a glass condenser to arrest any outgoing moisture. Air is introduced through a chamber (length 100 mm) connecter by a flange to the distributer. 28 holes (1 mm diameter) were drilled (on a square pitch, 6 mm center-to-center) on half of the S.S. sparger that works as a riser. A condenser (area 0.2 m^2) is included to arrest any outgoing moisture.

Munoz et al. [12.25] employed an ALBR fitted with a fiber optic spectrophotometer, for filamentous callus induction and microplantlet culture propagation of the macroalga *Kappaphycus alvarezii* (Doty) which is the largest source of κ-carrageenan in the global phycocolloid industry. Now, ALBRs are usually preferred for macroalgal cell cultivation because they facilitate enhanced gas exchange and light transfer on the one hand and reduced shear damage on the other. Mixing in ALBRs is obtained by pneumatic and/or mechanical agitation in order to maintain a uniform concentration of chemical species in the bulk phase and enhance mass transfer. Since proper mixing is crucial for adequate distribution of cells and nutrients in the liquid phase, a realistic estimation of liquid-phase mixing times is necessary for effective ALBR design. For this purpose, a fiber optic spectrophotometer is used for mixing-time evaluation which has several advantages over other traditional methods viz. faster response without data loss, minimal measurement error, operational flexibility through use of several solutions as tracers, faster in situ measurements and reduced frictional resistance. It is noted that liquid circulation is very sensitive to bioreactor geometry and, on this count, the ALBR described here could provide adequate liquid circulation of the culture media.

The ALBR (effective working volume 1.5 L) externally illuminated with fluorescent lamps, consisted of an acrylic pipe (height 30 cm, I.D. 10 cm) and a draught tube (height 22.5 cm, I.D. 3.8 cm), with inlet air pumped through a flow meter. A fiber optic spectrophotometer was placed 5 cm below the water surface, and a tracer of 1.0 ± 0.3 mL saturated aniline blue aqueous solution was injected over the optical fiber through a Pasteur pipette. Mixing time (defined as the time required to attain a specified mixing intensity at a given scale), was measured from the time at which maximum absorbance was recorded, to the time of minimum absorbance, assuming a completely homogenized medium. It was concluded that filamentous callus production from axenic *K. alvarezii* explants was effectively promoted in the ALBR (with the tested plant growth regulator).

Polzin and *Rorrer* [12.26] used a perfusion airlift photobioreactor, i. e., with continuous liquid medium perfusion, for synthesis of halogenated monoterpenes by regenerated microplantlet suspension cultures of the macrophytic marine red alga (*seaweed*) *Ochtodes secundiramea*, claimed as the *first successful bioreactor production of halogenated monoterpenes from a marine organism*. The bioreactor (Fig. 12.10) is a glass jacketed vessel (working volume 2.1 L, 50 cm high, 7.6 cm I.D.) with the vessel jacket connected to a temperature controlled water circulation bath (maintained at 26 °C). Illumination was provided by four vertically mounted, timer-controlled (14 h on/10 h off) 15 W cool-white fluorescent lamps positioned at 1.0 cm from the vessel to provide a uniform incident light intensity along the reactor surface. CO_2 in the aeration gas served as the sole carbon source for algal growth, supplemental CO_2 (from a CO_2 tank) was mixed with the inlet air which passed successively through a filter (0.2 µm) and a humidifier before entering the reactor through a glass frit (diameter 4 cm, pore size 40–60 µm). Dissolved CO_2 speciates to bicarbonate ion (HCO_3^-) in seawater pH > 7. Fresh medium was continuously pumped into the bottom of the reactor during the 14 h light

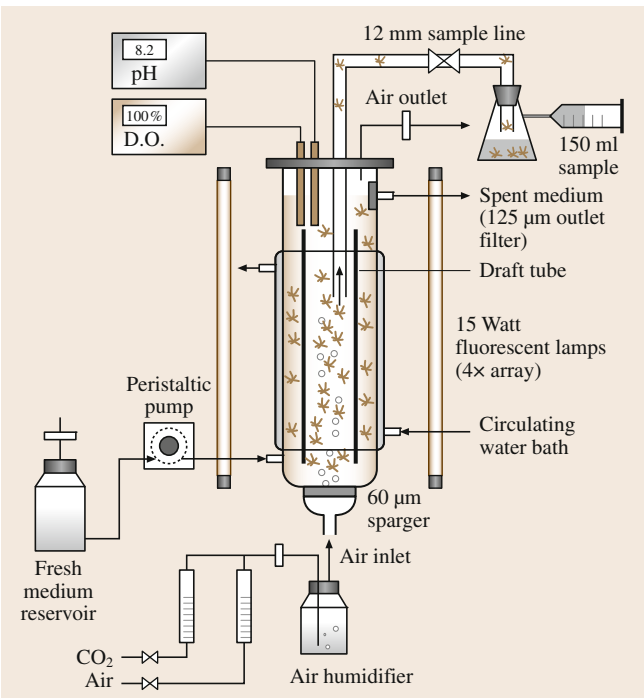

Fig. 12.10 Perfusion airlift photobioreactor (after *Polzin* and *Rorrer* [12.26])

phase. A nylon mesh (125 μm) was affixed on the medium outlet port for biomass retention within the reactor.

Contreras et al. [12.27] employed a plexiglass-made concentric tube airlift PBR (working volume 12 L) for culturing the photosynthetic microalga *P. tricornutum*. The AL-PBR (Fig. 12.11) consisted of a 2 m high outer tube (diameter 0.09 m) within which was installed a 1.5 m high concentric draft tube – the riser, with a cross-sectional area (2.8×10^{-3} m^2) same as that of the downcomer. Continuous illumination was provided by 10 fluorescent lamps, installed around the reactor with an illuminated surface area of 0.471 m^2. Compressed air was passed through an oil separator and a 0.5 mm sterile filter. A cylindrical, sintered glass sparger (diameter 0.02 m, height 0.03 m, pore size 60 mm) located in the riser, was used for aeration. It was noted that the AL-PBR adequately complied with the requirements of microalgal cultivation viz. high mass transfer rates, large surface-to-volume ratio, ease of control, safe sterile operation, and low mechanical shear forces on the cells. The existence of a maximum in μ_{max} of *P. tricornutum* was observed with respect to the gas flow rate as well as the shear rate which is indicative of the presence of a growth limiting or inhibitory effect in the reactor below and above these optimal values.

Vunjak-Novakovic et al. [12.28] designed a novel *triangular-configuration* inclined-tube AL-PBR for CO_2 fixation from flue gas by the marine green algae *Dunaliella* sp. When gas enters from the bottom of an inclined tube, the gas bubble travels along the inner upper surface of the tube – this renews the upper surface liquid layer making surface adherence difficult for the growing algae, thereby preventing fouling. As light penetration into the ALBR usually occurs through the upper surface, this self-cleaning feature substantially reduces the need for tube maintenance. Most of the solar radiation incident on the *triangular-configuration* AL-PBR enters through the hypotenuse (3.3 m long), with a circular cross-sectional area. Thus, the cross section has an *intensive light* region, corresponding to that in the annular region of a concentric-tube ALBR. The liquid flow rate is controlled mainly by the feed flow rate of the gas and can be adjusted to give a wide range (seconds to minutes) of retention times within each of the reactor zones. As the algae circulate through the inclined-tube segment, turbulence caused by two gas spargers creates microtrajectories that carry the suspended cells back and forth between zones with different illumination (i. e., closer to the illuminated surface or deeper into the liquid flow with less illumination). The desired throughput for flue gas purification is obtained simply by increasing the number of ALR *triangles* that are connected in parallel. Preliminary studies were carried out in a small-scale laboratory ALBR (volume 7 L) with no internal temperature control, housed in a wedge-shaped greenhouse (temperature-controlled) with a triangular side view resembling the shape of the reactor. A so-called *second*

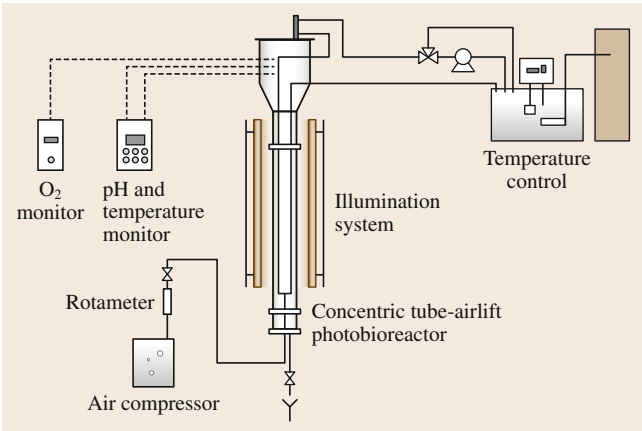

Fig. 12.11 Culture system (after *Contreras* et al. [12.27])

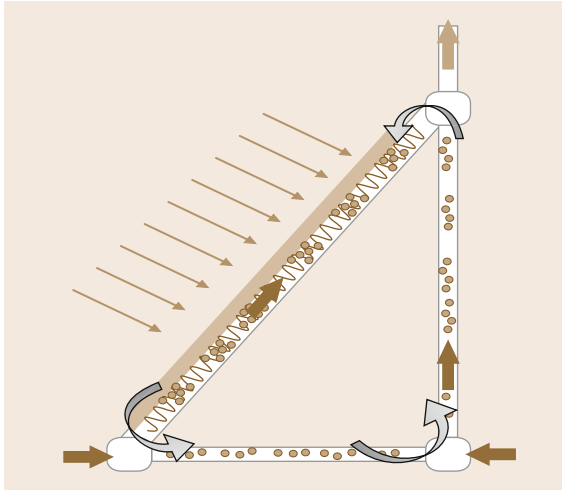

Fig. 12.12 Inclined-tube ALR configuration: Schematic presentation of one ALR triangle. *Solid arrows* indicate the direction of the gas flow, and *open arrows* indicate the direction of the liquid flow (after *Vunjak-Novakovic* et al. [12.28])

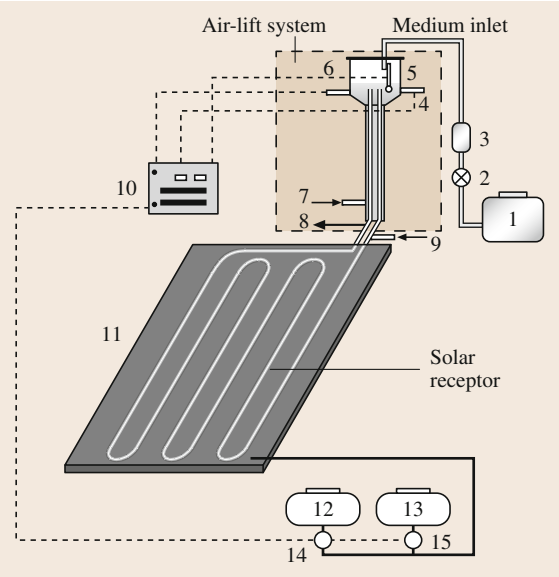

Fig. 12.13 Scheme of outdoor culture system. 1 – Fresh medium; 2, 14, 15 pumps; 3 – sterilization UV lamp; 4 – temperature sensor; 5 – level sensor; 6 – pH probe; 7 – air injection; 8 – sampler; 9 – CO_2; 10 – control unit; 11 – thermostatic water pool; 12 – cool water reservoir; 13 – warm water reservoir (after *Del Campo* et al. [12.29])

pilot-plant unit was also installed that comprised a cascade of 30 ALBRs (volume 30 L each) with a configuration as in Fig. 12.12. These ALBRs were continuously supplied with flue gas from a small power plant to demonstrate CO_2 removal from flue gas by growing algae.

Del Campo et al. [12.29] employed an outdoor airlift-driven tubular photobioreactor (volume 55 L, made of acrylic) for the production of lutein (a valuable carotenoid pigment with a wide range of uses) by *Muriellopsis* species, a chlorophycean microalga. The reactor (Fig. 12.13) has an airlift system to recirculate the cell culture and an external horizontal loop, consisting of tubes (length 90 m, I.D. 2.4 cm, surface area 2.2 m^2) that serve as solar receivers, immersed in a thermostatic pond of water. The airlift consisted of a degasser (in which the pH and temperature probes were inserted) and two 3 m high tubes (i. e., the riser and the downcomer). Compressed air was supplied into the riser to transport the cell suspension through the tubes and create turbulence. The reactor was operated in continuous-flow mode during daylight and in batch mode at night, to prevent culture washout.

12.3.2 Bubble Column Bioreactors (BCBR)

Bubble columns (BC) (Tables 12.3 and 12.4) are also pneumatically agitated vertical column reactors but the main difference with ALRs is in the nature of fluid flow, which depends on the geometry of the reactor. The bubble column is a rather simple vessel into which gas (air) is injected at the bottom and random mixing is produced by the rising bubbles. Now, in contrast to a BC where flow patterns are rather random, the ALR generates a more homogeneous flow pattern that moves suspended cells from the riser to the downcomer. Again cell sedimentation may occur in a BC, but cells remain more uniformly suspended in an ALR.

Guo et al. [12.30] developed an integrated process of CO_2 fixation and biohydrogen photoproduction by the marine green microalga *Platymonas subcordiformis*, grown photoautotrophically in a CO_2 supplemented air BCBR (Fig. 12.14) (volume 600 mL, diameter 50 mm, height 400 mm). CO_2 is required in photosynthesis for algal production of intracellular starch; the latter is then utilized for hydrogen production under anaerobic conditions by mitochondrial respiration to deplete oxygen. In fact, alga with higher starch accumulation shows a substantial increase in rate and duration of hydrogen production. Compressed air and CO_2 were mixed (up to 15 vol. % of CO_2 in air) and

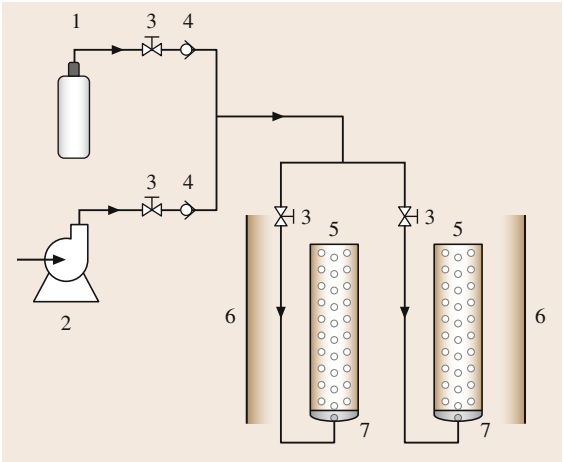

Fig. 12.14 Schematic diagram of the bubble column bioreactor system used for the growth of *P. subcordiformis* cultures under photoautotrophic conditions. 1 – CO_2 bottle, 2 – air compress pump, 3 – manual valve, 4 – gas flowmeter, 5 – bubble column bioreactor, 6 – cool-white fluorescent light, and 7 – porous sieve (after *Guo* et al. [12.30])

metered through calibrated flow meters, and sterilized using membrane filters (pore size 0.22 μm) before entering the reactor (at an aeration rate of 0.2 vvm). The bottom of the bioreactor was filled with a porous quartz sieve (diameter 10 mm), which dispersed the airstream. The cultures were illuminated from two sides with cool white fluorescent lamps under 14 h/10 h light/dark cycle. CO_2 was supplemented in air only during the light phase.

Seven-day-old cell cultures from the BCBR were concentrated by centrifugation and transferred into a 500 mL cylindrical glass bioreactor. They were subjected to the dark anaerobic induction of hydrogenase at 25 °C for 12 h, after continuous flushing for 10 min with N_2 (99.9% purity) through the culture to purge O_2. 15 1M CCCP (carbonylcyanide *m*-chlorophenylhydrazone) was then added, and the culture further incubated in darkness for 20 min, before initiating photobiological H_2 production under continuous illumination. It was demonstrated in this study that the marine green alga *P. subcordiformis* could efficiently convert CO_2 into intracellular starch through photosynthesis and improve H_2 photoproduction through increased accumulation of intracellular starch under CO_2 supplementation in an air bubble column PBR.

Huang and *Rorrer* [12.31] investigated the optimal values of cultivation temperature and diurnal photoperiod for the phototrophic growth of a microplantlet suspension culture derived from the macrophytic marine red alga *Agardhiella subulata* in a BC-PBR. Cultivation temperature and light delivery are two crucial process variables in the design of PBRs for culturing marine microalgae (seaweed). The latter has two components, viz., the light flux intensity and the diurnal photoperiod, i. e., the light/dark (L/D) illumination cycle in 24 h. It was observed in this study that biomass production increased with increasing photoperiod at low fractional photoperiods ($\leq$ 10 : 14 L/D) but at high fractional photoperiods approaching continuous illumination ($\geq$ 20 : 4 L/D) biomass production practically stopped, presumably due to photodamage that inhibits growth). The optimal photoperiod for biomass production was found to be 16 : 8 L/D.

An externally illuminated BC-PBR (same working principle as that used in *Zhi* and *Rorrer* [12.32], cited immediately afterward) (effective cultivation volume 250 mL) consisted of a 12.7 cm straight section (diameter 4.5 cm) and a 15.2 cm conical riser section (inner diameter 1.3 cm at the base). The small vessel diameter minimized light attenuation through the microplantlet suspension. Air was metered, humidified in a bubbler, sterilized through an autoclaved filter (0.2 μm pore size), and then introduced into the base of the riser section through a glass frit (diameter 1.3 cm, pore size 40–60 μm). The liquid suspension culture in the vessel was uniformly agitated and aerated by rising air bubbles (nominal diameter 1.0 mm). Ambient CO_2 in the aeration gas (normally 350 ppm) served as the carbon source for photosynthetic biomass growth. The illumination stage consisted of two 6.0 W cool-white fluorescent lamps vertically mounted on opposing sides of the glass vessel. A referencing plate set the distance between each lamp and vessel wall to deliver the desired incident light flux intensity to the culture and a programmable timer set the photoperiod for each lamp. The PBR was maintained at 24 °C within a temperature-controlled room. Two identical BC-PBRs described above were operated in parallel, each inoculated with microplantets from a common inoculum source.

Zhi and *Rorrer* [12.32] demonstrated the feasibility of the photolithotrophic cultivation of a cell suspension culture derived from the microscopic, filamentous gametophytic life phase of the brown marine macroalga *L. saccharina* in an illuminated BC-PBR, at 13 °C using CO_2 in air as the sole carbon source for growth. Two illuminated glass BCBRs were employed with effective cultivation volumes of 280 and 900 mL, respectively. Each BCBR system, comprising the bioreactor assembly, aeration unit, and illumination arrangement, was housed in a low-temperature incubator fitted with two auxiliary convection fans to maintain uniform temperature, and fresh air was supplied to maintain a constant ambient CO_2 concentration in the incubator gas space (vol. 500 L). Four identical 280 mL bioreactor systems and two 900 mL bioreactor systems were used for batch experiments under different conditions using a common inoculum. The bioreactors of a given volume were all located within the same low temperature incubator.

The 280 mL BCBR (Fig. 12.15) consisted of a 12.70 cm straight section (I.D. 4.45 cm) and a 15.24 cm conical riser section (base I.D. 1.27 cm), and was sealed to the head plate with two G-rings, one above and one below the flange of the glass body. The head plate had two ports, for the sampling assembly and for air outlet, respectively, with the latter connected to a sterilizing filter (0.2 μm). Agitation was provided by rising air bubbles introduced into the reactor base through the sparger assembly. Ambient air from an aquarium pump was metered, filter – sterilized (0.2 μm), and then bubbled through a ster-

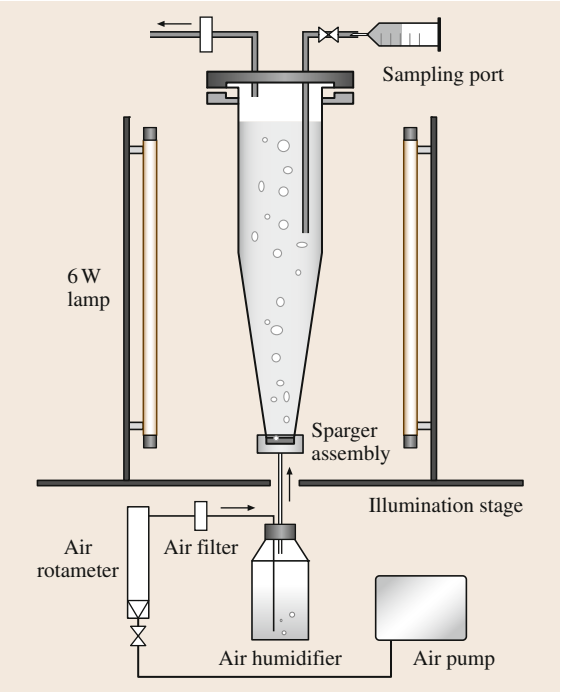

Fig. 12.15 Schematic of 280 mL bubble-column bioreactor (after *Zhi* and *Rorrer* [12.32])

ilized humidifier, before introducing into the culture through the sparger assembly fitted with a removable glass frit (pore size 40–60 µm, diameter 1.27 cm). The sparger generated air bubbles with diameter ranging from 0.2–0.7 mm. The conical riser section improved the fluid circulation provided by the rising air bubbles. CO_2 naturally present in the ambient aeration gas (nominally at 350 ppm) served as the sole carbon source for biomass growth. Illumination was provided by two vertically opposed, timer-controlled 6 W fluorescent lamps mounted on plexiglass plates. A referencing plate set the distance between each lamp and the vessel wall, so that the desired incident light intensity could be applied uniformly to both sides of the reactor. The larger BCBR had the same headplate assembly, sparger assembly, and aeration system as the smaller one but the straight section of the 900 mL glass reactor vessel was 48.26 cm long. Also, the light stage consisted of four 6 W fluorescent lamps mounted vertically, with two per side.

Initial cell density had a profound effect on the final biomass density of the clumped cell suspension, not on the specific growth rate. Increasing the aeration rate somewhat enhanced the specific growth rate and final biomass density, but the culture was not CO_2-transport limited. Initial nitrate concentrations above a certain threshold value had no significant effect on the specific growth rate and final biomass density.

12.3.3 ALBR and BCBR and/or other PBR – Comparative Studies

Nagase et al. [12.33] investigated the removal of NO (present in fossil fuel flue gas) by the marine green algae *Dunaliella tertiolecta* cultured in bubble column and air-lift-type bioreactors. Several alternative means of enhancing NO removal were examined in this study, the first of which was to reduce the bubble size in order to increase the gas–liquid contact surface and thus obtain a higher rate of NO dissolution. For this purpose, S.S. tubes of different gauges, and glass-ball filters (g.b.f.) of varying particle sizes were examined as spargers to give varying bubble diameters – of which the so-called No. 3 g.b.f. generated the smallest bubbles. However on actual use of No. 3 g.b.f in the parallel-flow ALBR, the algal cells became concentrated at the top of the reactor due to froth flotation and could not be cultured further. Secondly, for increasing the NO removal rate, the idea of using a longer column or a modified reactor configuration, viz., the counterflow ALBR, was considered, to increase the gas–liquid contact time. In fact, the counterflow ALBR improved gas liquid contact time by decreasing the rising rate of bubbles (i. e., by increasing gas holdup). With a No. 3 g.b.f. used in the counterflow ALBR, by which a simultaneous increase in gas–liquid contact area and gas holdup was accomplished, a remarkable enhancement in the NO removal rate was observed – there was no growth – inhibition by froth flotation in the reactor and the algal culture remained well mixed. The parallel-flow and counterflow-type ALBRs employed for algal NO removal (shown in Fig. 12.16) were fitted with a draft tube located centrally in the column. The NO containing flue gas was fed outside of the draft tube, where photosynthetic oxygen was produced vigorously. The draft tube was cut near the two parts connected by a fine-mesh stainless steel netting, thereby preventing the mixing of bubbles inside and outside the draft tube and facilitating circulation of the culture medium in the reactor column. The highest NO removal, i. e., 96%, was attained with a counterflow-type ALBR (when 100 ppm NO was aerated with smaller bubbles). The authors concluded that the NO removal ability of a counterflow-type ALBR was threefold higher than a simple BCBR.

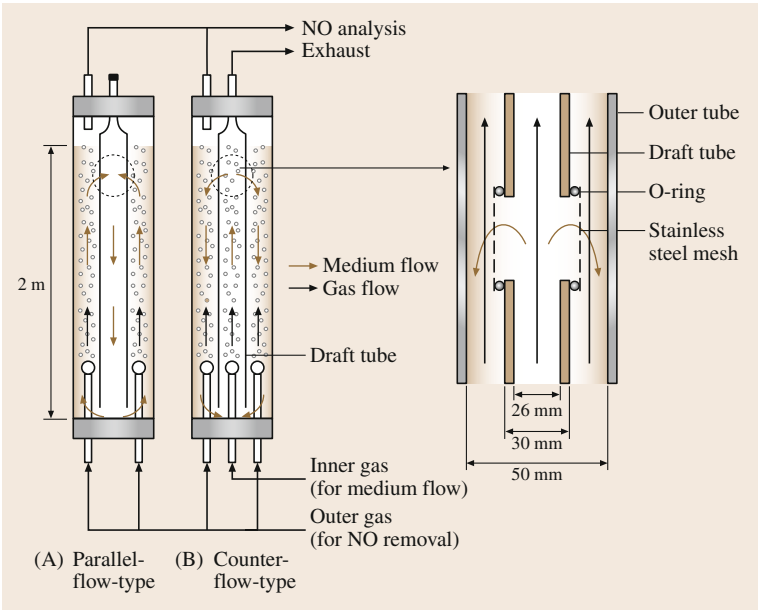

Fig. 12.16 Schematic diagrams of NO removal systems using airlift reactors (after *Nagase* et al. [12.33])

A comparative evaluation of the performance of an AL-PBR and BC-PBR (both 3 L volume, the same column diameter and height) was undertaken by *Monkonsit* et al. [12.34] for the cultivation of the marine diatom *Skeletonema costatum*, which is used as food for shrimp larvae in the first protozoea stage. Maximum cell concentration, specific growth rate, and biomass productivity were higher in the AL-PBR than in the BC-PBR, both having been operated under identical aeration rates and light intensity. The superior performance of the AL-PBR was attributed to its circulatory flow that prevents cell precipitation and improves light utilization efficiency. The optimal reactor operating parameters for cell growth in terms of:

1. Ratio of downcomer-to-riser cross-sectional area (3.27)
2. Superficial gas velocity (1.5 cm s^{-1})
3. Incident light intensity (34 μmol ph m^{-2} s^{-1}) were determined.

The AL-PBR (height 60 cm, column I.D. 9.4 cm) was equipped with a draft tube (height 40 cm) installed centrally in the column. For both reactors, compressed air was passed through a flowmeter and sterilized through a filter (0.45 μm) before entering the reactor bottom. Illumination was provided by 18 W fluorescent lamps installed at the side, along the length of each column (two lamps per column). The reactors were kept in an air-conditioned enclosure with temperature maintained between 25 and 30 °C.

Merchuk et al. [12.12] considered the effect of light/dark cycles of different frequencies on growth and polysaccharide production by the red microalga *Porphyridium* sp. in a laboratory-scale tubular loop PBR (total volume 0.43 L) and compared it with the performance of an AL-PBR and BC-PBR (both of volume 35 L). Whereas the loop device is a small-scale laboratory reactor in which liquid is circulated by a peristaltic pump at a controlled rate, the column reactors (bubble column and air-lift) are orders of magnitude larger, and the liquid movement is driven by the injection of gas into the reactor. However, despite basic differences, the light/dark cycles generated, either by the pump of the laboratory-scale tubular reactor, or by gas flow rate in the much larger BC and ALR, were found to be of the same order.

Under low light intensity and high gas flow rates, the BC and the ALR performed almost identically. However, with high light intensity and low gas flow rates, both growth and polysaccharide production were higher in the ALR. The interactions of photosynthesis and photoinhibition with the fluid dynamics in the bioreactors allowed interpretation of the differences in the performance of the BC-PBR and the AL-PBR. It was posited that the cyclic distribution of dark periods in the AL-PBR facilitates better recovery from the photoinhibition damage suffered by the cells. Due to the

small diameter of the loop and the low biomass concentration involved, the laboratory-scale tubular loop PBR performance data could be considered free of the effect of self-shading, and in this regard, the TL-PBR could be considered a *thin-film* photobioreactor. The larger column reactors were used to examine the effect of mixing under conditions of high optical density, where self-shading and fluid dynamics were responsible for the periodicity of exposure to light.

The tubular loop reactor (volume 0.43 L) consisted of a series of glass tubes (I.D. 0.007 m) connected to a small vessel into which air containing 3% CO_2 was bubbled (to supply a carbon-source and remove O_2); the loop was closed through a peristaltic pump. The ratio of illuminated/dark zones was controlled by darkening different lengths of the tube. The ventilation vessel, which was always covered, formed part of the dark zone. Illumination was provided from a bank of fluorescent lamps.

The column reactors (both 2 m high, column diameter 0.180 m) were also supplied with air containing 3% CO_2 and were housed in a temperature-controlled room at 25 °C. The difference in construction between the AL-PBR and the BC-PBR was that a draft tube (height 1.5 m, diameter 0.09 m) was installed coaxially in the AL-PBR. The draft tube plays a pivotal role in transforming the flow of the liquid, which is approximately random in the bubble column, into more ordered patterns in the ALR. The liquid rises through the central riser due to the difference in hydrostatic pressure, and descends through the annular downcomer, engaging part of the gas. The extent of this gas carryover depends on the operating conditions.

Krichnavaruk et al. [12.35] used a small-scale glass BC-PBR (2.5 L) as well as an AL-PBR and a BC-PBR (both 17 L volume) to investigate the growth of the chlorophyll containing marine diatom *Chaetoceros calcitrans* which is commonly used as feed for shrimp larvae. For the large-scale column reactors, run in batch mode, both maximum specific growth rate and maximum cell concentration were higher in the AL-PBR by about 18 and 16%, respectively. For the AL-PBR operated in semicontinuous mode, maximum specific growth rate increased further by 30%. Experiments to determine the optimal growth conditions of *C. calcitrans* were performed in a small-scale glass bubble column (volume 2.5 L). Compressed air (flowing 3.8 L min^{-1}) entered the bottom of the column. Illumination was provided through 250 W lamps, and incident light intensity was controlled by varying the distance between the lamps and the column. Both large-scale bioreactors were acrylic-made (diameter 15 cm) but the AL-PBR was equipped with a draft tube installed centrally in the column. The ratio between the cross-sectional areas of downcomer and riser was 2.63. Compressed air was supplied at the bottom of the draft tube and a 5 cm gap was left between the bottom of the draft tube and the column to allow liquid circulation. Aeration was controlled by a calibrated rotameter – superficial gas velocity in the riser was controlled in a range of 2–5 cm s^{-1}. Light was supplied through 12 fluorescent lamps placed at the side, along the length of the columns and temperature was controlled ≈ 30 °C (±2 °C).

The superior performance of the ALBR vis-a-vis the BCBR may be attributed to a well-defined flow pattern in the ALBR that allows more effective light utilization by the diatom. In the BCBR, proper recirculation of cells is not possible since aeration only superimposes random motion with no net liquid movement – whereas some cells are exposed to high light intensity in close proximity of the illuminated column walls, those centrally positioned in the column are exposed to much lower light intensity causing ineffective photosynthesis and consequently low biomass growth. However, uneven fluid density in the riser and downcomer sections of the ALBR, induces a well-defined flow pattern – upward liquid movement in the riser and downward in the downcomer. Consequently diatoms in the riser, would, after lapse of a certain time, flow to the downcomer where light is directly incident, implying exposure to more uniform light density than in the BC. Furthermore, liquid movement in the ALR prevents cell accumulation at the bottom of the column (and an uneven cell density along the length of the column arising therefrom) by facilitating cell circulation even at high cell density. Cell accumulation at the bottom of the column may potentially cause starvation, death, and even culture contamination, thereby resulting in an overall reduced growth rate.

Sanchez-Miron et al. [12.36] undertook a comparative evaluation (mainly in terms of hydrodynamics and transport phenomena) of three air-agitated photobioreactors, viz., a bubble column, a split-cylinder or split-column ALR and a concentric draft-tube sparged ALR (Fig. 12.17). Their focus was on fractional gas holdup, liquid circulation velocity, and the overall gas–liquid oxygen mass transfer coefficient and the interdependence of these variables in regimes relevant to microalgal cultures. Comparative evaluation of reactor performance was presented for the culture of the photo-

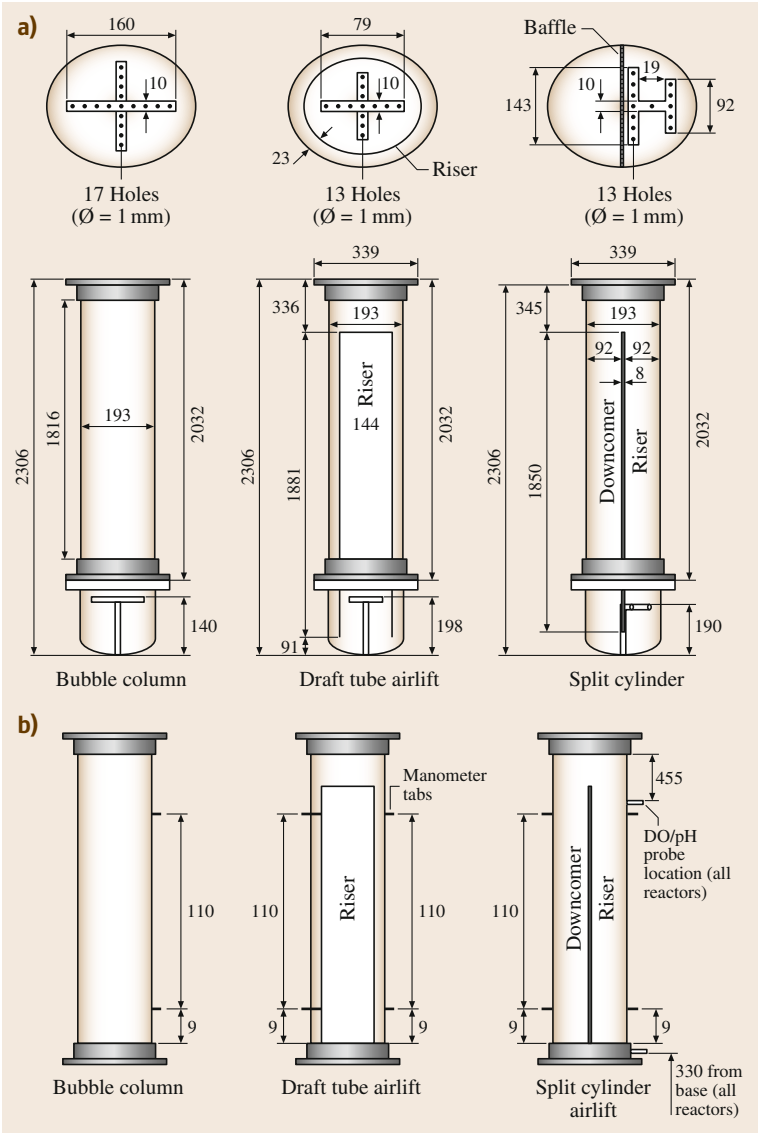

Fig. 12.17a,b Reactors: **(a)** vessel dimensions and air sparger details; **(b)** location of dissolved oxygen (DO) and pH electrodes. All dimensions in mm (after *Sanchez-Miron* et al. [12.36])

synthetic marine microalga *P. tricornutum*, a potential source of certain omega-3 polyunsaturated fatty acids of therapeutic value. Based on the findings of this study, it was noted that the performance of all three reactors were equivalent and that the results did not indicate a clear preference for any reactor – a maximum specific growth rate of 0.022 h^{-1} and a final algal biomass concentration of 4 kg m^{-3} was obtained for all the three reactors.

All the reactor vessels (diameter 0.193 m) were made of acrylic (3.3 mm thick) but for the lower 0.25 m sections, which were made of stainless steel. The ratio of the riser-to-downcomer cross-sectional area for the split cylinder ALBR and draft-tube ALBR were 1.0 and 1.24, respectively. The draft tube (I.D. 0.144 m) and the baffle were located at, respectively 0.091 m, and 0.096 m from the reactor bottom. The gas-free liquid level was about 2 m in all cases.

Merchuk et al. [12.37] considered three types of bench scale, pneumatically agitated photobioreactors for culturing the red microalga *Porphyridium* species – a BCBR, an ALBR, and a modified ALBR fitted with

helical flow promoters (ALBR + HFP). Now, in an earlier study *Merchuk* et al. [12.12] comparing BC and ALR-type photobioreactors for *Porphyridium* growth, it was concluded that the more ordered flow in the ALBR leads to the exposure of the growing cells to light and darkness in more homogenous cycles, resulting in the superior reactor performance.

The HFP is a static device consisting of several fins or baffles that causes fluid flow in a helical path along the downcomer. It effectively modifies the fluid flow path from a rectilinear trajectory along the axis to a helix. Although the HFP may be installed at any point along the riser or the downcomer, a much preferred location is the top of the downcomer – wherefrom a helical flow is generated in the downcomer, which becomes a swirl at the bottom and a corkscrew-like path in the riser. The helical fluid motion causes secondary flow which leads to an enhanced radial mixing, and thus more homogeneous distribution of light among liquid elements and suspended particles. This increases the likelihood of all fluid elements getting the same exposure to light. In this study, the HFPs were positioned at the upper rim of the downcomer. As the ALBRs were illuminated through the external walls, aeration from the bottom of the draft tube was preferred in order to minimize the quantity of bubbles in the downcomer, which would cause loss of efficiency due to light scattering. Installing the HFP at the downcomer inlet maximizes the fluid dynamic effects (i. e., secondary flow) in the photosynthesis zone of the reactor.

The reactors (I.D. 0.13 m, height 1.5 m) were made of flexible polyethylene sleeves with a conical bottom. For operation as an ALBR, an acrylic draft tube (external diameter 0.09 m, height 1.05 m) was inserted. For the ALBR + HFP the same draft tube was used but with the HFP installed. Selection of the draft tube diameter was based on the fluid residence time in the riser as time spent in the dark zone. The HFP comprises three fins at 45° from the axis, placed at the upper 0.05 m of the downcomer, with a width equal to the gap between draft tube and the external wall. Gas entered the reactors through a sparger installed 0.03 m above the bottom. The sparger was a ring of 0.05 m diameter made of a stainless steel tube of 0.005 m diameter, with 15 holes (diameters 0.001 m) equidistantly positioned on its upper face. Now, the gas flow rate to the reactor strongly influences the mixing of medium, the distribution of cells, their nutrient availability, and CO_2 absorption. It was observed for the ALR + HFP that the more ordered flow and the additional secondary circulation due to the HFP provide sufficient mixing and better cell distribution at a lower gas flow rate, without too many cells adhering to the reactor walls. Operability at lower gas flow rates leads to lower consumption of air and CO_2 resulting in lowered costs of air compression and CO_2, thereby improving the efficiency of CO_2 uptake for algal photosynthesis – the basis for superior performance of the ALR + HFP relative to the other reactors considered.

Rorrer and *Cheney* [12.38] have compared four major photobioreactor configurations for microplantlet suspension cultures of photolithotrophic marine macroalgae *A. subulata*, viz., bubble column, airlift (internal draft tube), externally illuminated stirred tank and tubular recycle (helical array) photobioreactors in both batch and medium perfusion modes of macronutrient delivery. Of these four reactors considered, the ST-PBR and tubular recycle PBR have been used earlier by *Rorrer* and *Zhi* [12.14] and *Rorrer* and *Mullikin* [12.13], respectively, and have already been discussed at length in Sect. 12.2. These four PBRs were compared on four major parameters, viz., mixing and biomass suspension, aeration, and gas exchange, light transfer and potential for shear damage. Both BC and ALR-type PBRs have low shear damage potential and very good aeration capabilities, and while mixing and light transfer are rated as adequate for BC, they range from good to very good for ALR. The stirred tank PBR, on the other hand, has high shear damage potential and poor light transfer facilities but with regard to mixing and aeration it is rated as excellent. Finally, for the tubular recycle PBR, while light transfer is excellent and aeration adequate, mixing and shear damage are problem areas. Certain essential common features of these PBR systems are worth noting: the bioreactor control volume being externally illuminated by artificial light source(s), transparency of at least a part of the bioreactor vessel is necessary – this requires the reactor to be made out of glass/polycarbonate for BC/ALR and translucent silicone tubing for tubular PBR; also, the nonfriable nature of the macroalgal cell and tissue precludes continuous culture, because dilution of the culture suspension by continuous addition of fresh medium will dilute out these cell clumps. It may be noted that in microplantlet tissue cultures, since the biomass is compacted into ball-like nonfriable multicellular tissue and not dispersed in the liquid medium as single cells, light attenuation through the culture suspension is low relative to microalgal suspension cultures at the same cell mass density. Again, microplantlets are easily suspended but also easily separated from the culture broth thus facilitating biomass harvesting.

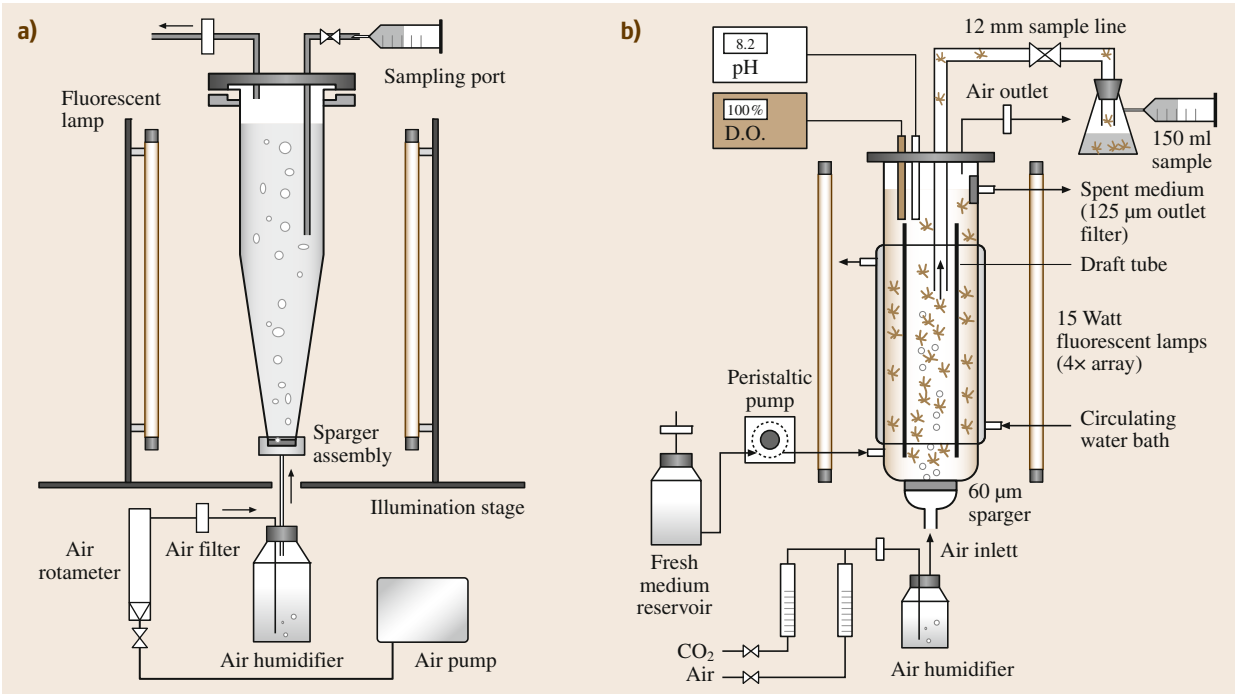

Fig. 12.18a,b Representative bench-scale bubble-column and airlift photobioreactors for macroalgal cell and tissue culture: **(a)** 250 mL bubble-column photobioreactor and **(b)** 3.0 L airlift photobioreactor equipped for continuous medium perfusion (after *Rorrer* and *Chenny* [12.38])

The PBRs discussed (Fig. 12.18), are designed to provide mixing, illumination, gas exchange, and dissolved macronutrients to the macroalgal suspension culture under controlled conditions. In a BCBR, the culture is mixed and suspended by the rising air bubbles, whereas in an ALBR biomass suspension is improved by a *draft tube* (of diameter d_i) which fits within a cylindrical vessel of width d ($d > d_i$). The draft tube provides a defined liquid circulation pattern where the suspension ascends within the draft tube (riser) and descends outside of the draft tube (downcomer). Draft tube ALBRs also provide enhanced light transfer to the culture broth as it moves down the downcomer because the suspension remains in close proximity to the illuminated reactor wall and is confined within a short light path ($d - d_i$). Aerated stirred tank bioreactors improve mixing and biomass suspension. Cell-lift and marine-blade impellers provide axial circulation patterns which help to suspend macroalgal cell clumps and tissues. Moreover, the rotating impeller breaks up and disperses the air bubbles to improve interphase mass transfer for CO_2 delivery. In general, cell cultures derived from terrestrial plants are prone to damage by hydrodynamic shear forces generated by the stirred tank impeller rotation. However, the nonfriable cell and tissue cultures of marine macroalgal plants are not susceptible to shear damage in stirred tank photobioreactors. Light delivery is an important design parameter for PBRs made for macroalgal cell and tissue suspension culture, where growth becomes light saturated at relatively low light intensities compared to microalgae because in the rocky, intertidal, benthic ecological niche microalgae are exposed to much low light intensity. Therefore, as long as the light path through the culture is short and the reactor vessel is transparent, external illumination of the bioreactor with cool-white fluorescent lamps is generally adequate for bench-scale or pilot-scale reactors for macroalgal culture.

For macroalgal culture suspensions that can be pumped, the tubular recycle photobioreactor (TR-PBR) offers excellent light transfer and low light attenuation, because the path length for light transfer is reduced down to the diameter of the coiled tubing, and the light source is placed within the center of the coil. TR-PBR was found to successfully cultivate *L. saccharina* gametophyte cell clump suspensions. It is not suitable for

microplantlet cultures, however since their large tissue size makes it difficult for them to pass through the tubing without settling. Continuous bubbling aeration of the culture with CO_2 in air simultaneously serves four purposes, viz. (1) transfer of CO_2 to the culture; (2) maintenance of the dissolved inorganic carbon level in the culture medium; (3) pH control; and (4) removal of dissolved O_2 produced by photosynthesis.

Although macroalgal suspension cultures grow rather slowly (with specific growth rates less than 0.20 day^{-1}) and k_La values in aerated PBRs are high, it is challenging to supply CO_2 at flow rates that can avoid the CO_2 limited growth rate condition at high cell density unless CO_2 is added to the aeration gas. This requires CO_2 to be present in the aeration gas at 10 times its normal ambient concentration. If the culture suspension is continuously supplied with light and CO_2, then the cumulative biomass production will be limited ultimately by available macronutrients dissolved in the liquid medium, mainly N in the form of nitrate, and P in the form of phosphate. Macronutrient delivery depends on the mode of cultivation. In batch mode, macronutrients are initially provided in the growth medium at culture inoculation. Nitrate and phosphate concentrations in the medium decrease with time until one (or both) becomes zero. At this point, the *limiting nutrient* is exhausted, and cell growth stops even if light and CO_2 are still being continuously supplied. In perfusion cultures, liquid medium containing dissolved macronutrients is continuously supplied to the bioreactor culture suspension. The spent medium leaves the bioreactor but the biomass is retained inside. Under these conditions, cumulative biomass production continues, but its rate is ultimately limited by the rate of CO_2 delivery, or the attenuation of light through the dense culture suspension. The final limit to biomass production is space. If the tissues completely fill up the bioreactor control volume, then biomass production will stop even in the presence of an infinite supply of nutrients (light, CO_2, and macronutrients).

12.4 Membrane Bioreactors (MBR)

An MBR is basically a membrane filtration unit (membranes ranging between microfiltration, and ultrafiltration) coupled or integrated with a suspended phase bioreactor (Table 12.5). Depending on whether the membrane module is located inside or outside the bioreactor, MBRs are categorized as internal (i. e., *submerged*-type) or external (i. e., *cross-flow* type). In a cross-flow MBR medium is recirculated between the membrane module and the BR by pumping it through the membranes. In a submerged MBR, the membrane filtration unit is immersed inside the culture medium in the BR (or sometimes in a separate tank connected to the BR). Some common membrane configurations employed in MBRs are:

i) Hollow fiber
ii) Spirally wound
iii) Plate-and-frame (i. e., flat sheet)
iv) Tubular.

Insofar as wastewater treatment applications are concerned, MBRs provide a host of advantages compared to traditional activated sludge processes that include the total separation of hydraulic retention time (HRT) and solids retention time (SRT), high efficiency of pollutant removal and no requirement of a secondary clarification unit.

Zamalloa et al. [12.39] employed an anaerobic membrane bioreactor (AnMBR) for biomethanation of the marine microalgae *P. tricornutum*. In fact, this report was claimed as the pioneering study to demonstrate the feasibility of an AnMBR for digestion of algal biomass. In conventional, continuously stirred anaerobic digesters, where the SRT is identical to the HRT, a high SRT required for effective destruction of volatile solids necessitates a large, often prohibitively high reactor volume. Now, in an AnMBR, the solids are separated from the sludge suspensions by a membrane so that biomass wasting rates are low – therefore, an AnMBR routinely allows operation at SRTs as high as 50 d or even higher. This facilitates the growth of slow-growing microorganisms, e.g., methanogens, and also increases the fraction of fermented organic matter. The AnMBR employed in this study consists of (a) a reactor (working volume 8 L) made of acrylic panels and (b) a microfiltration membrane module arranged in parallel. Two peristaltic pumps were used to feed influent into the anaerobic reactor and separately withdraw permeate from there. For mixing and membrane scouring to control cake formation, biogas recirculation was carried out using a diaphragm gas pump through a diffuser located just below the membrane module. Biogas production was measured

Table 12.5 Membrane bioreactors (MBR)

Sl	Bioreactor	Marine strain and bioprocess	Reference
1	Anaerobic membrane biorector (AnMBR)	Biomethanation of microalgae *P. tricornutum*	*Zamalloa* et al. [12.39]
2	AnMBR	Biohydrogen production by bacterial consortia	*Oh* et al. [12.40]
3	Ion-exchange MBR (IEMBR)	Nitrate removal from saline water by bacterial consortium	*Matos* et al. [12.41]
4	Submerged-MBR (sMBR)	Harvesting anaerobic methanotrophic archaea (ANME)	*Meulepas* et al. [12.42]

by means of a gas meter. Transmembrane pressure (TMP) was measured with an analogical gauge installed between the membrane module and the permeate pump.

Oh et al. [12.40] studied biohydrogen production by bacterial consortia dominated by marine (viz., *Clostridiaceae*) and nonmarine (viz., *Flexibacteraceae*) bacterial families in an anaerobic MBR (AnMBR) – basically a cross-flow membrane coupled to a chemostat. According to the authors, this was the pioneering study on biohydrogen production in an MBR. The facilities/accessories of the reactor (Fig. 12.19) (volume 2 L, working volume 1 L) include constant stirring, flow level controller, pH controller, three peristaltic pumps (feed, waste, and recirculation – bioreactor side), a centrifugal pump (recirculation – membrane side), a membrane housing, liquid flow meter, three pressure gauges, and a backpulse unit with a control cabinet. The feed tank was pressurized using nitrogen gas. The recycle loop around the membrane module was operated at a flow rate of 378 L h^{-1} producing a membrane cross-flow velocity of 2.8 m s^{-1}. The recycle flow rate from the reactor to the high recycle loop was set at 51 mL min^{-1}. The working volume of the reactor was maintained by a level probe connected to the feed pump. To maintain the SRT, solids were purged from the MBR intermittently using a timer and the peristaltic pump, and medium (without glucose) was simultaneously fed into the reactor at the same rate to maintain a constant liquid level. Three alumina membranes of various pore sizes were used. Pressures were measured at the inlet, outlet, and permeate side of the membrane to determine the TMP. A constant permeate flow was obtained by adjusting the peristaltic pump on the permeate side of the membrane reactor. To limit membrane fouling, intermittent backpulsing (every 10–30 s) was done using a piston that injected nitrogen gas into the permeate side of the membrane module for a very short period of time (0.5–1 s). This backpressure caused a reversal in flow through the membrane thereby removing solids off the membrane surface.

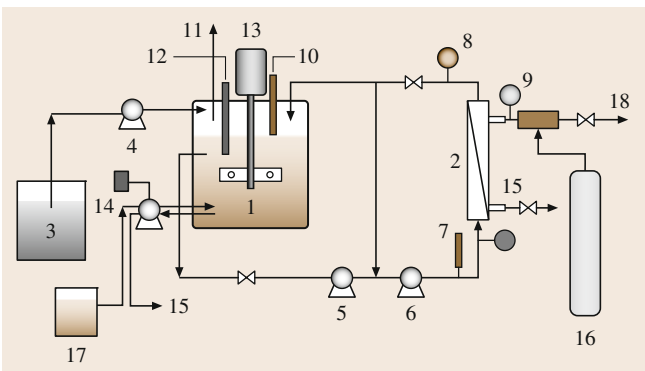

Fig. 12.19 Schematic diagram of the membrane bioreactor for hydrogen production. 1 – anaerobic reactor; 2 – cross-flow membrane; 3 – influent purged with nitrogen; 4 – feed pump; 5 – recirculation pump; 6 – high recirculation pump; 7 – flow meter; 8 – manometer; 9 – backpulsing; 10 – level controller; 11 – gas monitor; 12 – pH-controller; 13 – motor; 14 – timer; 15 – waste; 16 – nitrogen gas; 17 – medium w/o organics; 18 – effluent (after *Oh* et al. [12.40])

A common problem in biohydrogen production is that some of the bacteria in the consortium may consume hydrogen thereby lowering the overall hydrogen productivity, e.g., methanogens that convert H_2 to methane (which has less than half of the specific energy content of H_2). Of the several strategies to control or arrest the growth of methanogens, which are typically slow growing, the one that was deemed most effective was to maintain short residence times in continuous-flow reactors. However, this, in turn, reduces the efficiency of substrate utilization by hydrogen-producing bacteria and thereby that of the overall process.

Matos et al. [12.41] employed the ion exchange membrane bioreactor (IEMBR) for nitrate removal from highly saline water in a closed marine system, viz., a marine aquarium (oceanarium). Ammonia released by catabolism of reduced nitrogen compounds by aquatic animals is converted by nitrifying organisms into nitrate, the latter accumulating in a closed system like an aquarium up to levels potentially toxic for many marine species (which are generally much more sensitive to nitrate than freshwater ones) as well

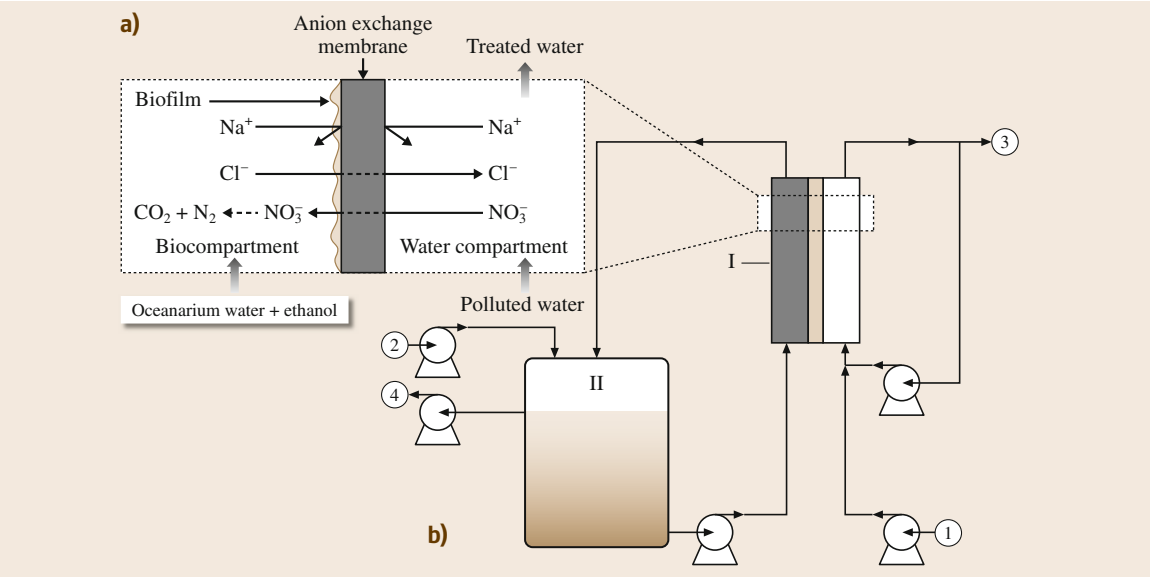

Fig. 12.20 **(a)** Schematic diagram of nitrate transport and bioreduction in the IEMB. **(b)** IEMB setup: 1 – feed water; 2 – biofeed; 3 – treated water; 4 – biocompartment effluent; aI, membrane module with two compartments separated by an anion-exchange membrane; bII – bioreactor vessel (after *Matos* et al. [12.41])

as posing problems related to its discharge into the external environment. The IEMBR concept is based on the integration of membrane separation technology with biological denitrification – nitrate is transported through a dense, nonporous anion-exchange membrane and subsequently converted into molecular nitrogen by a microbial consortium in a separate *biocompartment* isolated from the water stream (by the nonporous membrane *barrier*). This isolation allows for independent adjustment of the HRT in the biocompartment without affecting the production rate of the treated water and prevents the potential risk of secondary contamination of the treated water by excess carbon source (ethanol in this study). The transport across the membrane follows the principles of Donnan dialysis and may be increased by adding a suitable counter-ion (viz., chloride) to the biocompartment. An IEMBR is thus based on biological wastewater treatment avoiding secondary contamination of the treated water by complete isolation of the microbial culture through use of a membrane while preserving the water composition with respect to other ions.

The IEMBR (Fig. 12.20) consists of a membrane module with two identical rectangular channels, separated by a mono-anion permselective membrane (working area 39 cm^2). One channel was connected to an external loop where the aqueous phase was recirculated (at a flowrate of 97.2 $L\,h^{-1}$, $N_{Re} = 3000$). This *water compartment* was continuously fed with oceanarium water containing high levels of nitrate. The other module channel was connected to a stirred vessel through another recirculation loop ($N_{Re} = 3000$). This vessel, inoculated with 100 mL of the enriched microbial culture, was continuously fed (at a flow rate of 0.0048 $L\,h^{-1}$) with nitrate-free saline water from the oceanarium to which ethanol was added as a carbon source. This *biocompartment* was operated at a HRT of 5 d. To lower the volume of effluent produced in the biocompartment, a relatively high HRT was chosen. Lower HRTs could be set without affecting the nitrate reduction rates, which would, however, increase the volume of liquid waste discharged, which is undesirable.

Vis-a-vis other membrane treatment technologies for wastewater, e.g., reverse osmosis (RO), it was noted that the IEMBR provides for conversion of nitrate into harmless N_2 whereas in RO-based processes, the nitrate removed accumulates in a brine stream which usually requires further treatment. Again, in the IEMBR, the ion balance of the treated water is unaffected (other than nitrate) but in RO, demineralized water is obtained and all required salts have to be added to the treated water for it to be reused.

Meulepas et al. [12.42] used a novel, well-mixed, ambient-pressure, submerged-membrane bioreactor

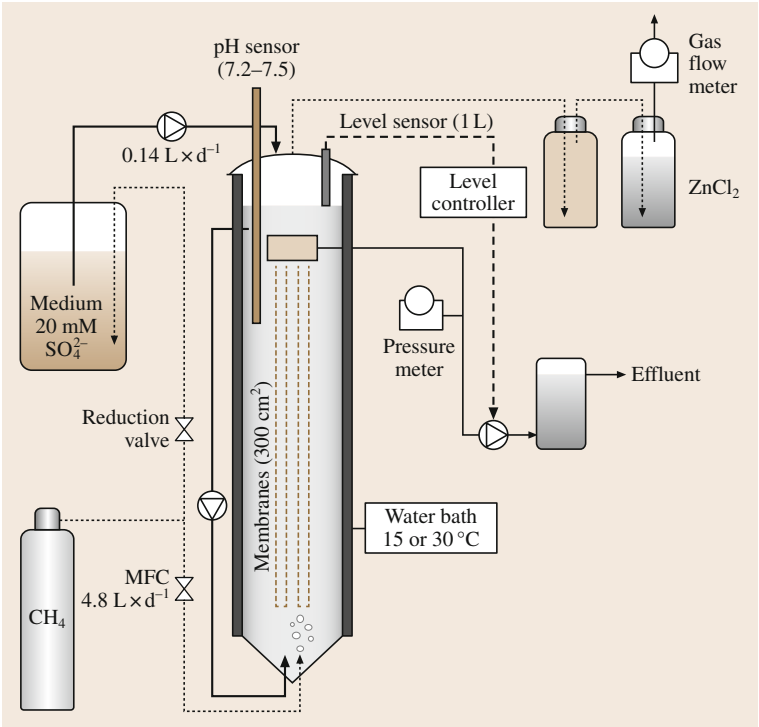

Fig. 12.21 Schematic overview of submerged-membrane bioreactor used for the enrichment experiments (after *Meulepas* et al. [12.42])

(sMBR) for enrichment of anaerobic methanotrophic (ANME) archaea that mediate anaerobic oxidation of methane (AOM) in marine sediments, which is coupled to sulfate reduction (SR) mediated by sulfate-reducing bacteria (SRB) that exist in consortia with the ANME. The sMBR (shown in Fig. 12.21) consists of a cylindrical glass vessel (total volume: 2.0 L; covered with opaque plastic to prevent phototrophic conversions), equipped with sampling ports for the headspace and the culture suspension, and connected to diaphragm metering pumps that continuously feed medium to the reactor. Each reactor was equipped with 4 polysulfone membranes, through which the effluent was extracted by means of a peristaltic pump. The membrane pore size guaranteed complete cell retention. Transmembrane pressure was monitored using a pressure sensor. The effluent pump was controlled by a level switch, which maintained the working liquid volume at 50% of the total volume (1.0 L). Each reactor was equipped with a water-jacket, through which water, cooled or heated in a thermostatic water bath, was recirculated to maintain a constant reactor temperature. CH_4 gas (purity 99.9995%), was supplied through a gas sparger at the reactor bottom to (a) aid the growth of the microorganisms, (b) to promote reactor mixing, (c) to strip off the sulfide, and (d) to prevent membrane fouling. The influent CH_4 flow was measured and controlled by a thermal mass flow controller. The exit gas from the reactor contained hydrogen sulfide (H_2S) and carbon dioxide (CO_2) stripped from the liquid, it was passed through two gas cleaning bottles, the first meant for collecting liquid entrained in the reactor exit gas, whereas the second (filled with a 0.5 M zinc chloride solution), intended to selectively retain H_2S was placed on a magnetic stirrer. The reactor suspension was recirculated from top to bottom to provide additional mixing and to suspend the sediment/biomass. Notably, the reactors in this study were not operated at *niche-mimicking* conditions but rather at conditions that supported high conversion rates. It was found that the AOM rate increased exponentially and a very high enrichment 1.0 mmol (g VSS d)$^{-1}$ was attained – the sMBR could thus be deemed an excellent system for growth of microorganisms mediating AOM-coupled-SR. In contrast to their marine ecological niche, the microorganisms grown in the sMBRs were continuously exposed to high shear forces due to liquid recirculation and gas sparging, and further, were suspended in the liquid phase. However, these factors did not prevent the observed exponential increase in AOM rate.

12.5 Immobilized-Cell Bioreactors

An immobilized cell may be defined as one that, by natural or artificial means is constrained in space such that it cannot independently be in motion relative to cells in its immediate vicinity. The common immobilization techniques are covalent coupling, affinity immobilization, adsorption, confinement in liquid–liquid emulsion, retention by semipermeable membranes and entrapment, of which the last named is the most frequently adopted technique for all immobilization. In immobilization by entrapment, cells are confined in a three-dimensional gel lattice made of either synthetic (e.g., acrylic, polyurethane, polyvinyl etc.) or natural polymer (e.g., collagen, agar, agarose, cellulose, alginate, carrageenan etc.). Substrate/nutrients and products can diffuse through the lattice to/from the cells. The microorganisms are entrapped onto the surfaces of the gel *beads* or *carrier particles* which are normally cross-linked (to obtain a stable immobilization matrix) by polymerization or by other means, e.g., carrageenan droplets are typically cross-linked with K^+.

The most common configuration of an immobilized cell bioreactor (Table 12.6) is the so-called PBBR, where cells immobilized on *carrier particles* or *beads* are *packed* into a matrix or bed that fills the reactor, usually a cylindrical column, and flow of nutrients/gases/products etc. usually occurs longitudinally through the beds. An alternative to cell entrapment is immobilization of cells in semipermeable membranes, which confine and thereby shield the enclosed microorganisms while facilitating transport of soluble matter (nutrients/products) to and from the cells. This technology is employed in HFBR (considered in the following section) where the growing microbial cells are constrained to one side of porous fiber strands and media containing nutrients and products on the other. However, pressure buildup due to excessive accumulation of biomass could lead to membrane rupture/fiber leakage, therefore cell growth in such membranes/fiber immobilized systems must be carefully controlled.

Table 12.6 Immobilized-cell bioreactors

Sl	Bioreactor	Marine strain and bioprocess	Reference
1	Packed bed bioreactor (PBBR)	Photo-evolution of hydrogen by polyvinyl alcohol immobilized cyanobacterium *Phormidium valderianum* (in combination with other strains)	*Bagai* and *Madamwar* [12.43]
2	PBBR	Hydrogen production by Ca-alginate/agar-agar immobilized cyanobacterium *Phormidium valderianum* (in combination with other strains)	*Patel* and *Madamwar* [12.44]
3	PBBR	Continuous production of L-glutaminase by bacteria *Pseudomonas* sp. *BTMS-51* immobilized on Ca-alginate gel	*Kumar* and *Chandrasekaran* [12.45]
4	PBBR	Continuous production of L-glutaminase by Ca-alginate-immobilized fungus *B. bassiana* BTMF S-10	*Sabu* et al. [12.46]
5	PBBR	Nitrification by NBC immobilized on polystyrene beads	*Kumar* et al. [12.47]
6	Serially connected activated PBBR	Nitrification by NBC comprising mainly *Nitrosococcus* sp. immobilized on polystyrene and low-density polyethylene	*Kumar* et al. [12.48]
7	Packed bed external loop ALBR (PBEL-ALBR)	Nitrogen compound removal from marine aquaculture wastewater by mixed (bacterial) consortia immobilized on plastic bioballs	*Silapakul* et al. [12.49]
8	Immobilized Cell Continuous ALBR	Nitification by nitrifying bacterial consortia NBC immobilized on polyvinyl alcohol PVA	*Seo* et al. [12.50]
9	Continuous-flow packed bed-photobioreactor (PB-PBR)	Nitrate removal from water by cyanobacterium *P. laminosum* immobilized on polyurethane foams	*Garbisu* et al. [12.51]
10	PB-PBR	Nitrate removal from water by microalga *S. obliquus* immobilized on polymeric foams	*Urrutia* et al. [12.52]
11	Immobilized-cell tubular PBR	Continuous synthesis of the blue-green pigment marennin by the diatom *H. ostrearia* immobilized in an agar gel layer	*Lebeau* et al. [12.53]
12	Free-cell and immobilized cell membrane photobioreactor (M-PBR)	Production of pigments marennin by the diatom *H. ostrearia*	*Rossignol* et al. [12.54]

The nonheterocystous marine cyanobacterium *Phormidium valderianum* (which is known to produce hydrogen under argon atmosphere) was immobilized in combination with two other nonmarine cells (viz., the extreme halophile *Halobacterium halobium* and salt tolerant *Escherichia coli*) for stable, long-term biohydrogen photo-evolution under an alternating light/dark illumination cycle (6 h on/18 h off) in a PBBR by *Patel* and *Madamwar* [12.44], as well as by *Bagai* and *Madamwar* [12.43].

The former workers used Ca-alginate or agar-agar as the immobilization matrix for cell entrapment in a 50 mL working volume PBBR (basically a packed column) continuously fed with N-free growth medium – comparatively better results were obtained with alginate. The problem of cell and enzyme leakage from alginate beads (leading to loss of H_2 production) was overcome by using glutaraldehyde as a cross-linking agent. H_2 photoevolution rate increased with medium feed rate up to a maximum, and then declined markedly. Stable, long-term H_2 production for 11 d was demonstrated.

Using the same combination of microorganisms, in a similar PBBR, but with a different immobilization matrix, viz., PVA cross-linked with glutaraldehyde, *Bagai* and *Madamwar* [12.43] demonstrated continued H_2 production for over 60 d. In both these studies for photohydrogen production, compared with a free-cell system, the immobilized system was much more productive, stable, and long lasting.

Sabu et al. [12.46] and *Kumar* and *Chandrasekaran* [12.45] all reported the production of the therapeutically and industrially important enzyme L-glutaminase by Ca-alginate immobilized marine microorganisms in glass-column PBBRs, but whereas the former employed the fungus *Beauveria bassiana* the latter used the bacterium *Pseudomonas* for that purpose. Although the PBBRs used in these studies differed in dimensions (i. e., column height and diameter) with the one used by *Kumar* and *Chandrasekaran* [12.45] being larger (height 45 cm and internal diameter 3.6 cm), the basic principle of reactor construction was the same – immobilized viable cell beads were aseptically packed into the column with a perforated Teflon disk placed at the top of the packing to prevent bed expansion during operation.

Sabu et al. [12.46] pumped the medium from the bottom of the packed column and collected the effluent from the top. This was, however, reversed by *Kumar* and *Chandrasekaran* [12.45]. The inlet and outlet flow rates were kept equal to maintain a constant liquid level at just above the bed level. Now *B. bassiana* being aerobic with strong aeration dependence, air was introduced through a sparger at the bottom of the packed column after passing through a bacterial filter. Remarkably, *Sabu* et al. [12.46], noted that the maximum enzyme production rate was observed in the absence of forced aeration, and any increase in aeration rate caused a decrease in enzyme productivity. In order to explain this finding, the authors posited that with external aeration, air bubbles fill the void spaces of the packing beads, thus reducing the contact between the cell-laden beads and the liquid medium. They also found that enzyme productivity increased with increasing bed height/decreasing flow rate, leading them to conclude that increase in residence time either with increase in bed height or with decrease in flow rate, in turn increases the contact time between the immobilized beads and the medium, thereby causing an increase in productivity.

Kumar and *Chandrasekaran* [12.45] reported that enzyme production declined with an increase in dilution rate, irrespective of the substrate concentration used. Again, at all dilution rates examined, enzyme yield increased with increase in substrate concentration. It was noted that their continuous-flow PBBR system could be operated for 120 h without any decline in enzyme productivity.

Kumar et al. [12.47] developed a nitrifying PBBR immobilized with an indigenous NBC (nitrifying bacterial consortium) (comprising predominantly marine species, e.g., *Nitrosospira*) for rapid nitrification in brackish water and marine hatchery systems. They examined nitrification performances of the PBBR integrated into a recirculating aquaculture system (RAS) viz., a *Penaeus monodon* (a marine crustacean – shrimp) recirculating maturation system. The RAS (Fig. 12.22) comprises a shrimp maturation tank (MT), an overhead tank (OHT), Six PBBRs (designated as R1 through R6) and a collection tank (CT). Fluid from the MT was pumped into the OHT from where it flowed through the reactors (R1–R6) connected serially by gravitation and finally collected in the CT, from where the treated water was recirculated back to the MT. Pumping was controlled by an automated water level controller installed in the OHT. A regulator valve was connected to the OHT to maintain the flow through the reactors.

All six PBBRs (R1–R6) (working volume 20 L each) were constructed identically, consisting of a fiberglass shell with a perforated Perspex plate carrying nine equidistantly fixed polyvinylchloride (PVC) pipes (air-

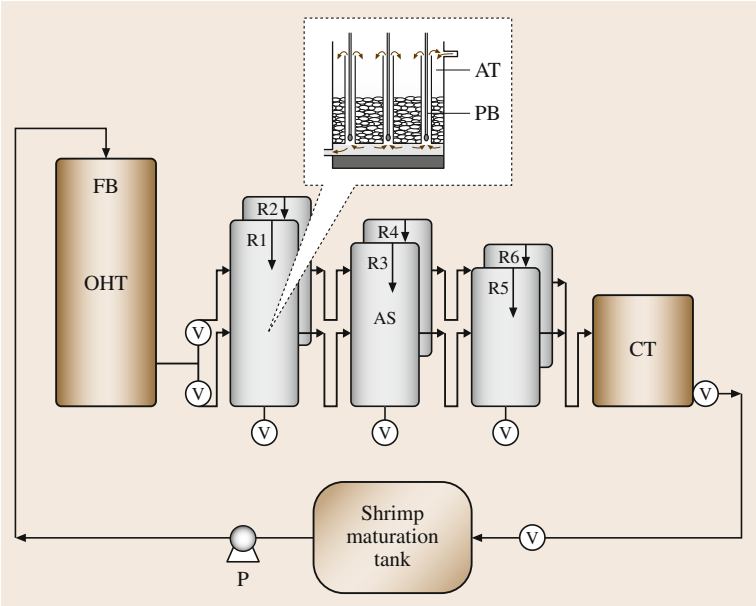

Fig. 12.22 Packed bed bioreactor connected to a shrimp maturation system. AS – aeration supply; AT – aeration tube; CT – collecting tank; FB – filter bags; OHT – overhead tank; PB – polystyrene beads; P – pump; R1–R6 – reactor R1–R6; V – valves (after *Kumar* et al. [12.47])

lift pumps) installed at the base of the reactors. On passing air, the area filled with the support medium surrounding each airlift pump works as an aeration cell. The baseplate is at an elevated location (5 cm) from the bottom, supported by PVC pipes (length 5 cm, diameter 3 cm). An inlet pipe is fixed at a water discharge height of 35 cm from the reactor base. The outflow pipe, on emerging from the base of the reactor, bends upward at a water discharge height of 35 cm from the base to the next reactor. Polystyrene beads (diameter 5 mm, surface area 0.785 cm^2) with spikes on the surface were used as the immobilization matrix – each reactor was packed with 60 000 beads. The reactors were equipped with a valve for periodical backwashing, located at the bottom. A bag filter placed in the OHT was used to filter debris from the incoming water from the MT. Significant nitrification was observed with the immobilized PBBRs integrated into the marine RAS for shrimp (*P. monodon*) maturation, over a wide range of substrate concentrations and flow rates examined. Nitrification was effective even after 70 d of operation as evident from the consistently reducing levels of total ammoniacal nitrogen (TAN).

Kumar et al. [12.48] employed two serially connected activated immobilized-cell PBBRs – one an ammonia oxidizing bioreactor (AOB) activated by ammonia-oxidizing bacterial consortia immobilized on polystyrene (PS) beads and the other a nitrite-oxidizing bioreactor (NOB) activated by nitrite-oxidizing bacteria immobilized on low-density polyethylene (LDPE), for rapid nitrification in a RAS – a brackish water hatchery system for larva production of the marine crustacean *Macrobrachium rosenbergii* (Fig. 12.23). Upon integration of the activated PBBRs with the hatchery system (thereby enabling it to operate in closed recirculating mode), ammonia removal was obtained up to BDL (beyond detectable limit) levels and consequently a substantially higher percentage survival of larvae was

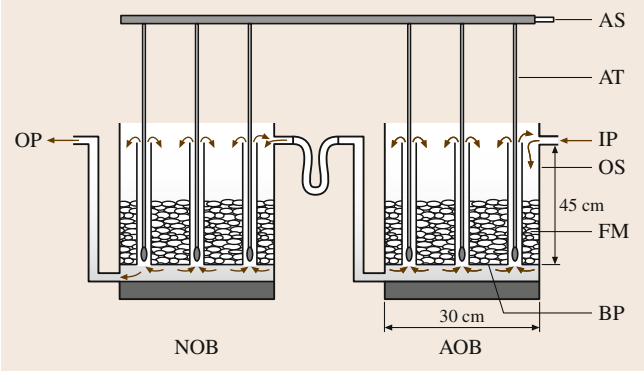

Fig. 12.23 Cross-sectional view of the bioreactors connected serially (AOB – ammonia-oxidizing bioreactor, NOB – nitrite-oxidizing bioreactor, BP – base plate, FM – filter media, OS – outer shell, IP – inlet pipe, OP – outlet pipe, AT – aeration tubes, AS – air supply) (after *Kumar* et al. [12.48])

observed. The PBBR system was energy optimized for continuous operation by limiting energy input to a single stage pumping of water and aeration to the aeration cells.

Both the PBBRs have the same configuration and are identical to the PBBRs used in *Kumar* et al. [12.47], as are the PS beads used for immobilization. The AOB and NOB reactors were connected in series. Water flowed from a large OHT into the AOB and therefrom into the NOB by gravity. The outflow from the NOB flows into a CT and from there into the larval rearing tank (LRT). From the LRT, water is pumped out into the OHT. The major components of the nitrifying bacterial consortia (NBC) were the marine genus, viz., *Nitrosococcus* and *Nitrobacter*. A noteworthy operational flexibility of the PBBRs was that they were interchangeable between prawn (salinity $15\,g\,L^{-1}$) and shrimp (salinity $30\,g\,L^{-1}$) larval rearing systems simply by changing the NBC based on salinity.

Silapakul et al. [12.49] examined a novel packed bed external loop airlift bioreactor (PBEL-ALBR) (volume 60 L) as an integrated system with simultaneous nitrification/denitrification for treatment of marine aquaculture wastewater containing ammonia and nitrate compounds. The PBEL-ALBR consisted of both aeration and nonaeration zones in the same unit, that served as nitrification and denitrification compartments (where ammonia and nitrate were biodegraded, respectively), which were packed with plastic bioballs to increase the surface area for microbial attachment, on the surface of which nitrifying and denitrifying microorganisms were immobilized.

The reactor (Fig. 12.24) consists of one aerated column (riser) interconnected by conduits with two unaerated columns (downcomer) – the cross-sectional area of the downcomers being almost 10 times larger than the riser to ensure adequate retention time for denitrification which is known to require $\approx 5-10$ times longer reaction time than nitrification. The aerated and unaerated columns were packed with 200 and 2000 bioballs (per column), respectively. A porous gas sparger for air dispersion was located at the bottom of the aerated column. The airflow rate was determined as a minimum that could induce liquid circulation between the aerated and unaerated sections. The recirculation of water was driven from the aerated riser where water moved up as aeration was provided and down through the unaerated downcomer. In the riser, nitrifying bacteria fed on wastewater containing high dissolved oxygen, oxidizing ammonia to nitrate. The low dissolved oxygen effluent from the riser flowed through the downcomer where denitrifying bacteria removed the nitrate. The authors noted that no nitrite/nitrate accumulation occurred in any experiment, indicating rapid and effective denitrification. Overall, the reactor performance was satisfactory and comparable with other treatment systems, however, where the PBEL-ALBR stands out is in providing nitrification and denitrification in a single setup without requiring a two-reactors-in-series cascade.

Seo et al. [12.50] examined the nitrification performance of an NBC (comprising mainly *Nitrosomonas* species) immobilized in boric acid treated PVA beads, for a marine RAS, by employing a continuous immobilized-cell ALBR (volume 45 L) for acclimation of the NBC from activated sludge and estimation of ammonia removal rate. In addition, four three-phase, completely mixed, fluidized bed-type bioreactors (FBBR) (volume 2.5 L each) were also employed for acclimation of the immobilized nitrifiers from freshwater to seawater system, with increasing salt concentration. Immobilization in support gel is an effective technique for maintaining high cell density and preventing washout of slow-growing autotrophic nitrifiers (ATN) under a low water temperature or high water flow rates. Although ATNs are sensitive to low temperatures, immobilization (a) improves tolerance to low temperatures and also, (b) provides protection against various toxic agents. In the operational range of values for the HRT, an optimum HRT was found for the marine nitrification process at which the highest ammonia removal rate was reached.

The four reactors were used to determine nitrification activity at four different salt concentrations. Each FBBR consisted of two separate compartments – one for beads settling and the other for airlift of the beads. Air was supplied through acrylic pipes (0.1 vvm). Influent NH_3 concentration was maintained at $10\,mg\,L^{-1}$. Nitrification activity decreased during the immobilization process due to centrifugation, transportation by pump, shear stress arising out of stirring, toxicity from the immobilization support material, and low pH of the boric acid solution. However, nitrification activity gradually recovered with operating time. It was observed that, nitrification activity of recovered nitrifier beads was higher than that of free nitrifiers due to high ammonia loading.

Polysaccharide-based gels and reticulate foams are usually considered as the preferred immobilization matrices for photosynthetic cell systems. Although five major immobilization techniques (viz., entrapment, microencapsulation, covalent coupling, aggregated cells, and adsorption) are in vogue, for growth of photosyn-

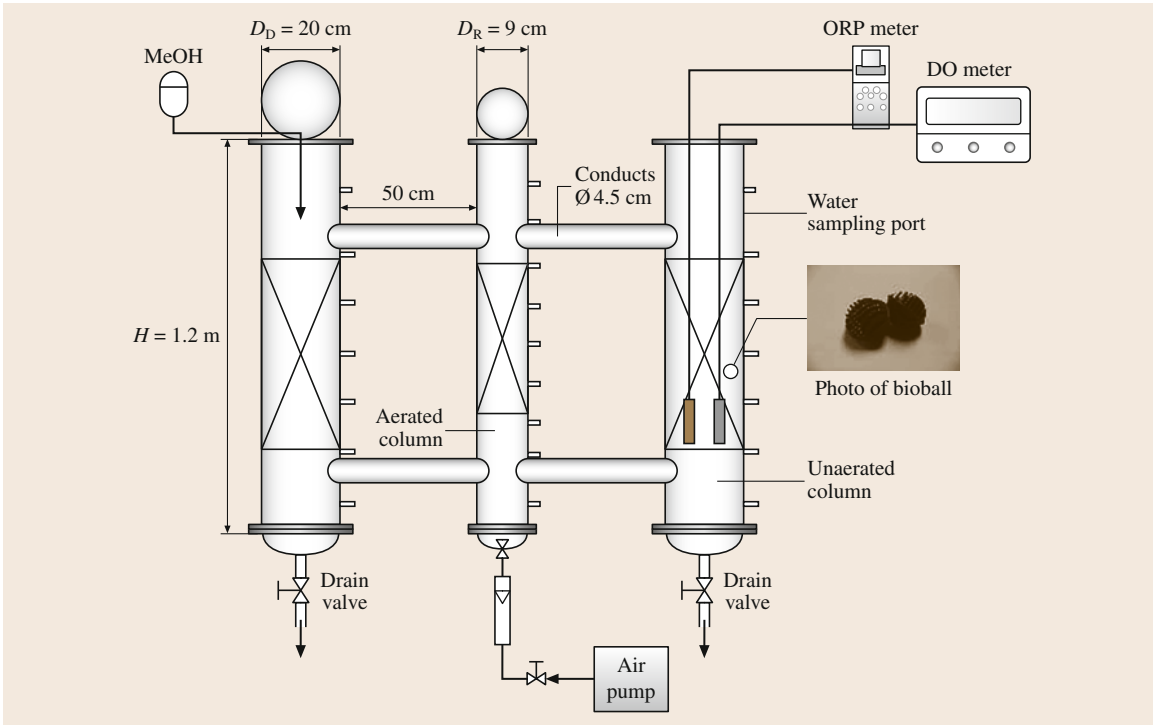

Fig. 12.24 Experimental setup (after *Silapakul* et al. [12.49])

thetic cyanobacteria in foam pieces, immobilization is often carried out by adsorption of cells on the foam surface in addition to entrapment in foam cavities. A continuous-flow packed bed photobioreactor (PB-PBR) was employed by *Garbisu* et al. [12.51] to study nitrate removal from water by cells of the non-N_2-fixing, filamentous, thermophilic cyanobacterium *Phormidium laminosum* immobilized in (a) polyurethane (PU) foams either by (i) absorption, or by (ii) entrapment in the PU prepolymer followed by polymerization, as well as in (b) polyvinyl (PV) foams by adsorption. Although entrapment repeatedly caused toxicity problems leading to rapid cell death, cells could be effectively immobilized by adsorption onto PU/PV foams and maintained their photosynthetic electron transport activities for at least 7 weeks. *P. laminosum* being thermophilic grows at 45 °C, a temperature too high for most other contaminating species, and further, has the ability to utilize either nitrate, nitrite or ammonium as the sole N-source – thus affording great flexibility and efficiency in the water treatment process. It was demonstrated in this study that *P. laminosum* immobilized on polymer foams is of potential value for biological nitrate removal (BNR) from water in a continuous-flow packed bed column PBR.

Urrutia et al. [12.52] obtained almost similar results (similar to *Garbisu* et al. [12.51]) in investigating BNR from water in a continuous packed bed column PBR with immobilized cells, but employing the green, mesophilic, unicellular, chlorophycean microalga *Scenedesmus obliquus*. Cells were immobilized by (a) entrapment, using urethane prepolymer as well as by (b) adsorption in preformed PU/PV foams, the latter deemed more convenient and effective, particularly when cells were subjected to nitrogen starvation, which also caused substantial increase in N uptake rate of immobilized cells. The study established the effectiveness of *S. obliquus* cells immobilized on hydrophilic polymeric foams grown in packed column photobioreactors, in N removal from water. Notably, polymeric foam immobilization has the particular advantage that at any point, after deimmobilizing the cells by simply squeezing the foam, it can be recycled and reused for further cell immobilization almost indefinitely.

Lebeau et al. [12.53] reported the continuous synthesis of the blue-green pigment marennine (with potential anticancer applications) by the marine diatom

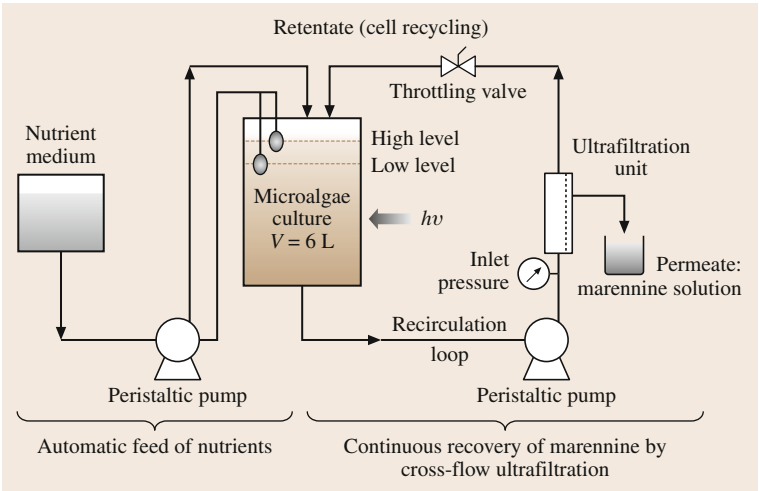

Fig. 12.25 Schematic of the free-cell M-PBR (after *Rossignol* et al. [12.54])

Haslea ostrearia in an immobilized-cell tubular membrane PBR (with internal illumination by optical fiber), described by *Rossignol* et al. [12.54]. Now, in its marine ecological niche, as the diatom migrates from the planktonic to the benthic phase, the number of pigmented cells increase, becoming maximum in the benthic phase where the cells are immobilized in a natural matrix formed from their own exopolysaccharides. Artificial immobilization, as reported in this study, was essentially an attempt to mimic this ecological niche.

The PBR was operated with 10 L of media. Agar immobilized cells were fed continuously (at a dilution rate $= 0.025\,d^{-1}$ during the first experiment and at $D = 0.25\,d^{-1}$ during the other two experiments). The PBR was placed in an air-conditioned room with the temperature maintained at 15 °C. The immobilized-cell layer was illuminated by a barrel of optical fibers connected to a 150 W light generator, with a 14 h/10 h light illuminated cycle. The reactor has a large surface/volume ratio and efficient lighting is provided by the evenly distributed optical fibers. The *cold* light source is particularly suitable for *H. ostrearia* whose cultivation temperature is quite low.

Rossignol et al. [12.54] have compared two membrane photobioreactors (MPBRs), with free and immobilized cells, respectively, for the exocellular production of the blue-green, hydrosoluble pigment, marennine by the marine diatom *H. ostrearia*. In the former, cells are free and grown in a recycle PBR with total biomass recycling coupled to a membrane ultrafiltration system (external loop). In the latter, i. e., the immobilized cell tubular membrane PBR, essentially a glass and S.S. cylinder (volume 10 L) cells are immobilized in a tubular agar gel layer (surface area 955 cm^2, volume 550 cm^3) placed in liquid culture medium with an optical-fiber barrel (as a source of artificial illumination) running through the inner annular space of the tubular agar gel and an external microporous membrane forming an outer sheath around the gel layer. Major drawbacks of the free cell continuous recycle system are membrane fouling and hydrodynamic shear stress on the shear-sensitive microalgal cells, which may be somewhat overcome by immobilization that essentially mimics the marine ecological niche of these cells – the number of pigmented cells increase as the algae migrate from the planktonic to the benthic phase becoming maximum as the cells are immobilized in a biofilm matrix formed by their excreted exopolysaccharides. PBRs were housed in a 15 °C air-conditioned room and illumination followed a 14 h/10 h light/dark cycle. The respective reactor setups are shown in Figs. 12.25 and 12.26. The free-cell photobioreactor (FCB) consisted of a glass cylinder integrated with an external flat – membrane module (to perform tangential filtration) comprising a plane polyacrylonitrile (PAN) membrane (MWCO 40 kDa, area 100 cm^2). Although the retentate loop was closed, sampling could be done as scheduled. To maintain a constant volume, sterile nutrient solution was fed into the bioreactor, as the permeate was extracted, using a peristaltic pump. Product (marennine) concentrations and volumetric productivity were found to be much higher for the free-cell system, however specific (i. e., cell number based) productivity was larger for the immobilized cell system due to the decreased cell numbers in the latter at the end of the culture.

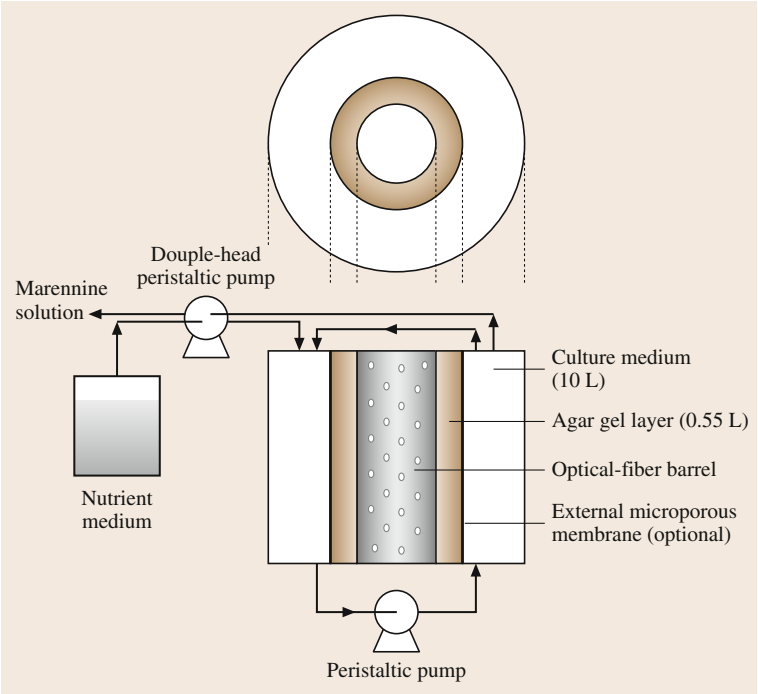

Fig. 12.26 Schematic of the immobilized-cell M-PBR (after *Rossignol* et al. [12.54])

12.6 Hollow Fiber Bioreactors (HFBR)

In an HFBR (Table 12.7), hundreds of thousands of hair-like hollow fibers (which are essentially semipermeable membranes) are bundled together within a tubular/cylindrical casing to form a cartridge. The cartridge is then linked to a perfusion system which circulates nutrient media for cell growth continuously through the fibers. Cells are usually inoculated into the so-called *extra capillary space* or shell-side space external to the fibers within the cartridge, whereas culture media is circulated through the *lumen* of the fibers (i. e., the middle of the fibers), allowing nutrients, dissolved gases and metabolic wastes to diffuse across the fiber walls. HFBRs are characterized by extremely high surface-to-volume ratio (> 150 cm^2) and support surface-attached growth of cells bound to a porous matrix whose MWCO (molecular weight cut-off) may be varied. Hollow-fiber reactors may be operated in direct mode, where growth media is passed through the fiber lumen, or in the transverse mode as done by *Lloyd* et al. [12.55], where growth medium is passed over immobilized cells, and spent medium with products collected after passage of the liquid through the semipermeable membrane of the hollow fibers. Cells are retained in the HFBR unless the hollow fibers rupture or the end-seals fail.

Table 12.7 Hollow fiber bioreactors (HFBR)

Sl	Bioreactor	Marine strain and bioprocess	Reference
1	HF-MBR	Production of recombinant toxoid by bacteria *Vibrio* sp. immobilized on hollow-fiber membrane.	*Lloyd* et al. [12.55]
2	HF-sMBR	Growth of marine rotifer *Brachinus plicatilis* immobilized on Ca-alginate	*Rombaut* et al. [12.56]
3	HF-sMBR	Oily water treatment by bacterial consortium	*Soltani* et al. [12.57]
4	Microfiltration HFBR	Enhancing cell yield of thermoacidophillic archaeon *S. solfataricus*	*Schiraldi* et al. [12.58]
5	HF-PBR	Nitrate and phosphate removal by non-N_2-fixing cyanobacterium *P. laminosum* immobilized on hollow fibers	*Sawayama* et al. [12.59]

An HFBR permits high cell densities to be attained in a practically nonshear environment with mild nutrient perfusion. Bacterial cell densities between 100 and 200 g L^{-1} have been commonly attained in HFBRs. However, the exceptionally high density of 550 g L^{-1} was reported [12.60] for a rifamycin-producing strain of *Nocardia mediterranei* (later renamed *Amycolatopsis rifamycinica*) grown in a dual hollow-fiber bioreactor. It is this ability to establish high cell densities while operating with continuous nutrient supply and product removal, that gives rise to higher product yields. HFBRs have been shown to work well with bioprocesses that result in the synthesis of products from major metabolic pathways, e.g., glycolysis, but, they do not work very well with processes that involve metabolic pathways of apparently less significance to the producer microbe.

Lloyd et al. [12.55] used an HFBR for production of a recombinant toxoid by a marine *Vibrio* species and compared it with recombinant protein synthesis in traditional batch and chemostat cultures. The type of fiber used significantly affected productivity, both with regards to maintenance of reactor integrity and by allowing passage of the toxoid through the selectively permeable membrane. A hollow-fiber-based system was selected for this comparative study because it offers easy immobilization and growth of recombinant cells, in addition to the potential to select fibers with permeability properties that could help improve the purity of the product obtained in the outflow from the reactor, thus maximizing the exploitation of the secretory capabilities of the *Vibrio* species cultured. Fibers were embedded in polyacetal end plugs, but spe-

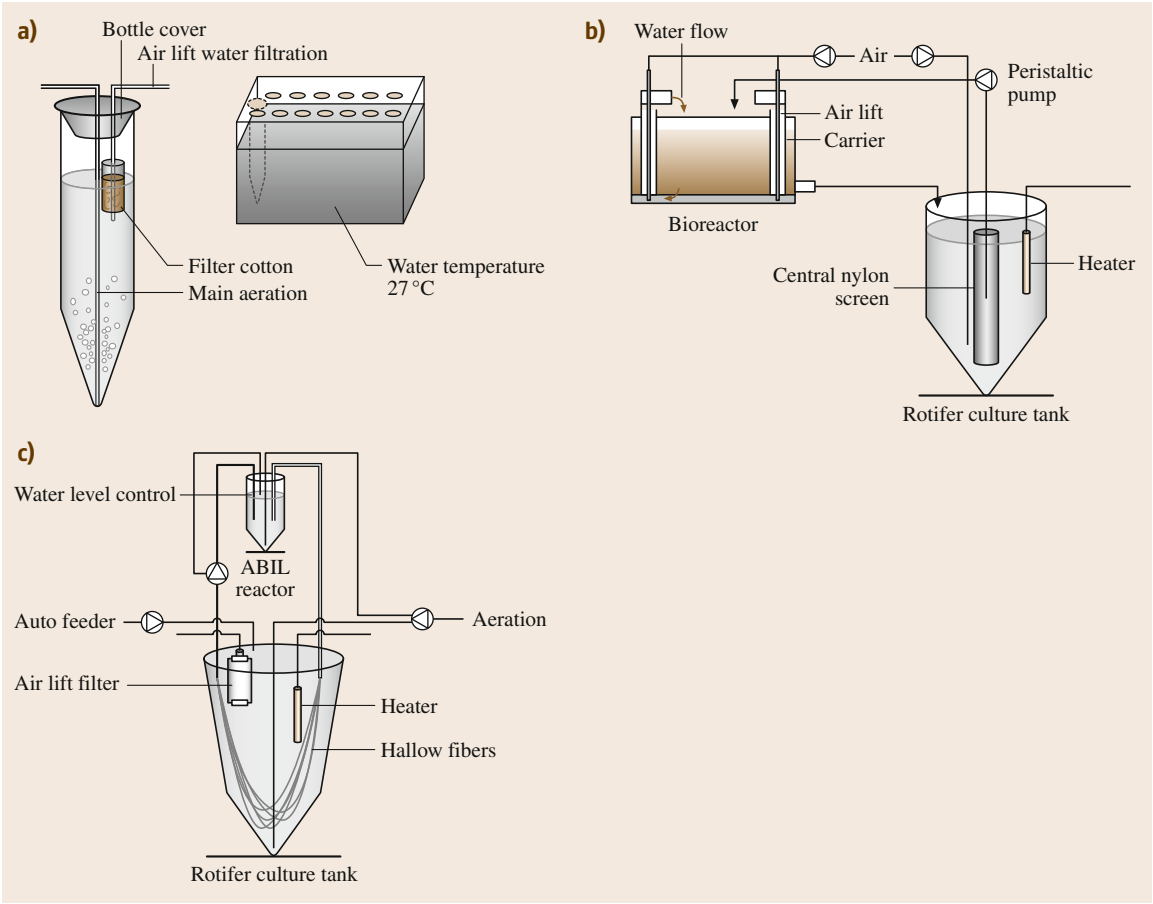

Fig. 12.27a–c Schematic of the experimental setup: **(a)** Batch reactor to test ABIL for rotifers. **(b)** Bioreactor setup with four different carrier materials. **(c)** Integration of an ABIL reactor with hollow fibers to the rotifer culture system (after *Rombaut* et al. [12.56])

cific microfibers were embedded in S.S. tubes (length 2 cm, I.D. 10 mm) using adhesive resin for holding the fibers in place during reactor operation. Medium supply was through the shell-side port. Air was supplied in a transverse mode through the fiber lumen. (It may be noted that air supply may also be done in direct mode through the shell side space.) Growth was evaluated by using a pressure transducer, which monitors the pressure differential between the inside and outside of the fibers and by measuring the protein content of fibers at the end of an incubation.

Rombaut et al. [12.56] used a dense, nitrifying inoculum culture (ABIL) for stimulating growth of the marine rotifer *Brachionus plicatilis* in several bioreactor systems, viz., batch culture, continuous bioreactors packed with carrier materials (a PVC matrix, gravel and $CaCO_3$ were evaluated separately) and a submerged hollow-fiber membrane bioreactor (HF-MBR).

A series of rectangular shaped bioreactors (Fig. 12.27) (total volume of one tank, 10 L) were each filled with 4 dm^3 of carrier material and 4 L of artificial seawater (25 $g\,L^{-1}$ salinity). The bottom of each tank was fitted with a cover (with holes drilled on it) for retaining the carrier material, through which two airlifts were installed for oxygen supply and for recirculation of the aqueous phase. $CaCO_3$ stones (2.0 ± 0.7 mm), gravel (16 ± 2 mm), a mixture of both types of stones, and a PVC-based carrier material (viz., Bionet) were examined as biomass carrier materials, however for the rotifer production periods, only the crushed $CaCO_3$ stones were used. After the start-up period of 11 d, water from the different bioreactors was drained out of the system and replaced by new diluted seawater. Except for the reactor packed with Bionet, the bioreactors were connected to a cylindro-conical culture tank of 10 L, well aerated to ensure good oxygenation and uniform distribution. The culture-rearing tank was filled with artificial seawater and maintained at a constant temperature ($25 \pm 1\,°C$). A central nylon screen (mesh size 33 µm) sieve was installed in the center of each tank to retain the rotifers and also to enable pumping of the culture water (using a peristaltic pump) to the bioreactors. The water returned by gravity to the rotifer culture tanks.

For the experiments with HF-MBR, conical PVC tanks were used for rotifer culture which were filled with 10 L of artificial seawater. Hydrophilic hollow fibers (nominal pore size 0.2 µm; wall thickness 450 µm; inner diameter 1800 µm), adjusted to pieces of 1 m and 10 fibers (total surface area = 0.078 m^2), were installed in each culture tank. The ends of the hollow fibers were connected to the ABIL reactor, inside which filter cotton was placed at the bottom to collect large debris. Water and bacteria from the ABIL reactor were pumped through the hollow fibers (using a peristaltic pump). The rotifer feed was stored at temperatures $< 10\,°C$ and was fed (15 $min\,h^{-1}$) by a peristaltic pump. The rotifer culture tanks were continuously aerated and the temperature was controlled by a heater at 28 °C. Results showed that concentrations of the major nitrogenous waste, viz., TAN in the control batch culture was much higher than that in the HF-MBR recirculating system. Since the principal objective of the bioreactor systems evaluated is the removal of TAN, thereby facilitating vigorous rotifer growth, the improved water quality obtained with the HF-MBR system resulted in much higher rotifier densities compared to the normal batch culture.

Soltani et al. [12.57] demonstrated that a hollow-fiber submerged MBR (HF-sMBR) could be used very effectively for oil removal from oilfield wastewaters using a marine bacterial consortium isolated from sea sediments and adapted to growth environments containing high amounts of salt and oil. The reactor setup (Fig. 12.28) consisted of a hollow fiber membrane module (made from polypropylene with pore size ≈ 0.2 µm) submerged in a feed tank. Permeate was withdrawn by a vacuum pump, its flow rate monitored by a rotameter and adjustable by operating a valve placed before the pump. Oxygen necessary for microbial growth and aeration of the membrane were supplied by two air diffusers, placed below the membrane module. Remarkably, 100% oil removal in the permeate was accomplished. Furthermore, although isolated from a typically saline marine ecological niche, the bacteria didn't lose their oil-degrading ability even in the total absence of salt in the culture medium – leading the authors to classify them as halotolerant.

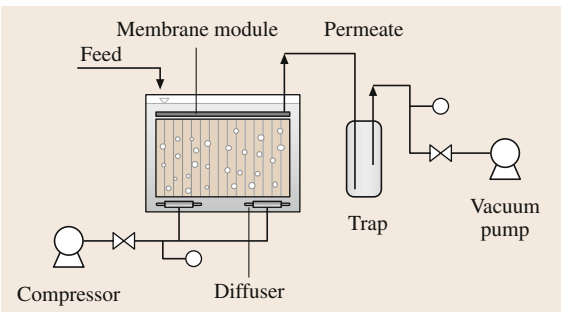

Fig. 12.28 A schematic of the HFBR experimental setup (after *Soltani* et al. [12.57])

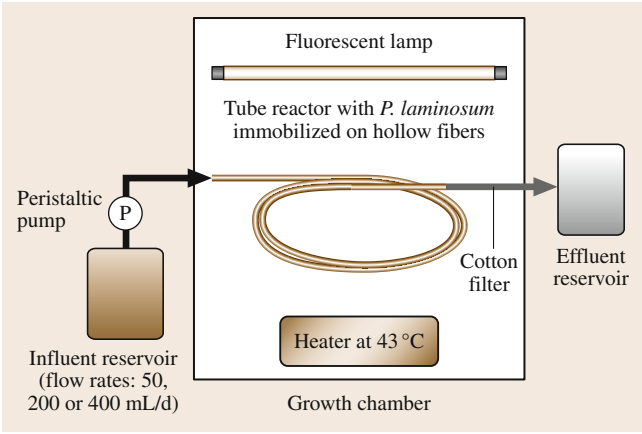

Fig. 12.29 Schematic diagram of the hollow-fiber photobioreactor (after *Sawayama* et al. [12.59])

Schiraldi et al. [12.58] used a hollow fiber microfiltration (MF) bioreactor (HF-MFBR) for high cell density growth of the thermoacidophilic archaeon *Sulfolobus solfataricus* – with the MF-hollow fiber module located inside a traditional fermentation vessel. They obtained biomass yields about 15–20 times higher than in batch culture, and about 6 times that of fed-batch culture. Furthermore, the MF module was found to be highly resistant to the extreme environments typical of thermoacidophile fermentation. Despite the high cell densities attained, transmembrane flux was consistently maintained (with repeated backflushing) at 75% of the maximum which is quite remarkable compared to usual crossflow filtration where flux declines to 20% of the maximum within 30 h of operation. To obtain the same amount of biomass, whereas at least 2000 h of batch time is necessary (ignoring the turnaround time between batches) only about 15% of this time is required with the HF-MFBR.

The polypropylene membrane used consisted of a long capillary (5–10 m) with an inner diameter of 0.2 mm and a cutoff of 0.22 µm. A number of 15 cm-long capillaries were obtained from the original membrane and were assembled together with a silicon adhesive; one side was closed and the other was sealed to a connector. The module was connected to a peristaltic pump equipped with a silicon tube (I.D. 2 mm), which provided the driving force for transmembrane flux. Considering the geometry of the module, the total filtering area measured 1.47×10^{-2} m^2. Before operating the module, the membranes were treated with a 70% ethanol solution for 1 h to make them hydrophilic. The microfiltration module was then placed inside the fermentation vessel and fixed vertically to a baffle in order to expose it (i. e., the module) to high turbulence near the filtering surface for minimizing fouling.

Sawayama et al. [12.59] studied nitrate and phosphate removal from water by the non-N_2-fixing cyanobacterium *P. laminosum* immobilized on cellulose hollow fibers in a tubular photobioreactor at 43 °C, continuously supplying dilute growth medium for 7 d and then secondarily treated sewage (STS) for 12 d. Cyanobacteria can grow much faster than higher plants under appropriate conditions, therefore, cyanobacteria-based inorganic nutrient removal systems are worth exploring. Furthermore, with thermophilic cyanobacteria, contamination can be avoided since it is tolerant of high-temperatures permitting sewage treatment at temperatures (≈ 45 °C), which cannot be tolerated by most other contaminating microbial species. Hollow fiber-immobilized cyanobacterial systems are easy to construct and immobilization is relatively simple. The bioreactor (Fig. 12.29) comprised a transparent PVC tube containing cellulose hollow fibers (used as the immobilization matrix), a peristaltic pump, a heater and two cool, white fluorescent lamps. Cells were immobilized on 600 cellulose hollow fibers (length 0.5 m, internal diameter 0.18 mm, total surface area 0.19 m^2) and placed in a PVC tube (length 0.5 m, I.D. 16 mm) with a total reactor volume of 100 mL. Immobilization was carried out by addition of cell culture (2 mg total chlorophyll) to the reactor using a peristaltic pump. Two days after cell inoculation, diluted medium (at a flow rate of 200 or 400 mL d^{-1}) or STS (at a flow rate of 50 mL d^{-1}) was passed through the HFBR at 43 °C (±1 °C) continuously illuminated with fluorescent light. The quantity of immobilized cyanobacterial chlorophyll in the bioreactor was 1.7–1.9 mg after 24 h of influent flow. *P. laminosum* cultured in the continuous HF-PBR removed nitrate and phosphate ion from water quite efficiently, however nitrate ions present in STS could not be removed satisfactorily. Again, compared to chitosan immobilized cells used earlier, P removal was higher (more than double) in this HF-PBR but N removal was significantly lower (less than half).

12.7 Fluidized Bed and Moving Bed Bioreactors (FBBR and MBBR)

12.7.1 Fluidized Bed Bioreactors (FBBR)

An FBBR (Table 12.8) is a reactor consisting of a *bed* of particles/carriers such that the particles are not in constant contact with each other (i. e., they are in motion resembling that of fluid particles) due to the flow of a fluid through them. If the component particles of the *bed* are indeed in continuous contact with each other, the bed is referred to as a *packed bed*. Often, the term *expanded bed* is used to describe a bed that has expanded just marginally above the corresponding *packed bed* height, i. e., above the *settled height* – the condition when the bed particles have *settled down* and are NOT in motion. The term fluidized bed is usually used for beds that have undergone significant expansion, so that *fluidization* of the bed has occurred – i. e., the bed particles are in continuous motion as in case of a fluid. Fluidization involving liquids and solid particles is commonly particulate characterized by smooth expansion of the bed, as opposed to *aggregative* characterized by violent bubbling and particle motion typical of gas–solid fluidization. FBBRs are essentially immobilized-cell reactors. Individual cells being very small and light and with a settling velocity less than the superficial liquid velocity are washed out, whereas carrier particles with cells immobilized on them have much larger settling velocity and are retained in the bed, which allows operation of FBBRs at dilution rates much higher than the cell specific growth rate. Compared with stirred-tank immobilized reactors, the shear at the particle surface is much lower in an FBBR, which actually poses a problem with bacteria and yeast because they can continue to grow as a biofilm around the particle. Increasing biofilm volumes forces the bed to expand and the steady state, where cell growth equals the rate that cells are washed off by surface shear is attained, only with a very large bed height.

Cytryn et al. [12.61] investigated the diversity of microbial communities correlated to physiochemical parameters in a digestion basin (DB) of a zero-discharge experimental mariculture system that integrated conventional nitrification with a combination of sludge digestion and denitrification. The integrated system comprises an intensively stocked fish basin with two parallel biofiltration loops (Figs. 12.30 and 12.31): (1) a trickling filter, where ammonia is oxidized to nitrate; and (2) a digestion/sedimentation basin connected to an FBBR for organic degradation and denitrification. Surface water from the basin was pumped over the trickling filter in one loop while bottom water was recirculated through a sedimentation basin followed by the FBBR in the other. Ammonia oxidation to nitrate in the trickling filter and organic matter digestion coupled with nitrate reduction in the combined sedimentation basin–FBBR loop, allowed zero-discharge operation of the integrated system. Chemical activity and microbial diversity were higher in the sludge layer than in the overlying aqueous layer of the basin. Chemical parameters in sludge samples close to the basin inlet indicated enhanced microbial activity relative to other sampling areas with evidence of both nitrate and sulfate reduction.

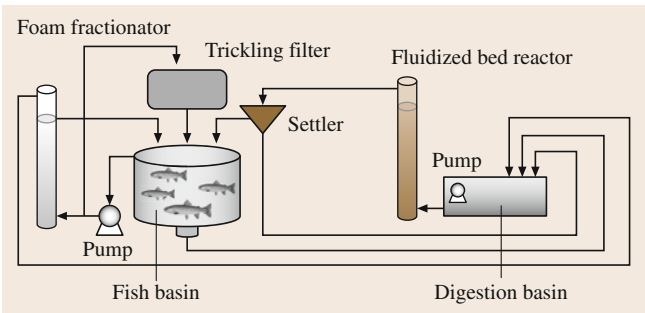

Fig. 12.30 Schematic diagram of the recirculating zero-discharge mariculture system (after *Cytryn* et al. [12.61])

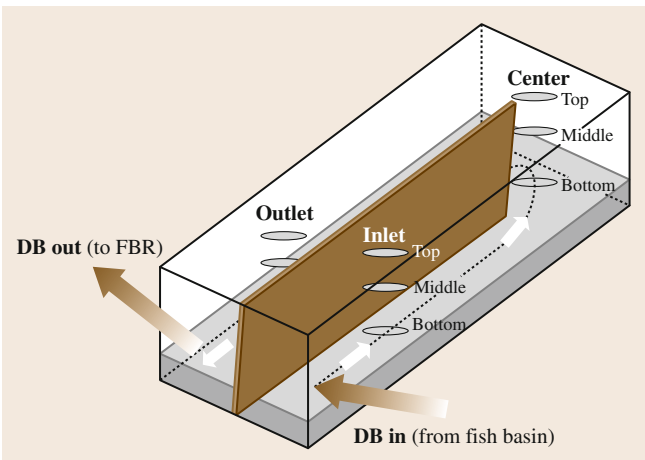

Fig. 12.31 Schematic diagram of the digestion basin. Sampling stations (inlet, center, and outlet) and sampling depths (top, middle, and bottom) are depicted by *ellipses*. *Gray area* indicates the underlying sludge layer of the basin (bottom 5 cm) and the *clear area* indicates the overlaying aqueous phase (top 10 cm). *Arrows* and *dotted lines* indicate the flow direction (after *Cytryn* et al. [12.61])

Cytryn et al. [12.62] integrated microscopic and molecular microbial analyses to characterize rapidly developing white filamentous tufts in an FBBR (used earlier by *Cytryn* et al. [12.61]) for nitrate removal from a marine recirculating aquaculture (fish culture) system – RAS. The dominant constituents of these tufts were identified as filamentous, gram-negative bacteria with densely packed intracellular sulfur granules belonging to the marine *Thiothrix* genus. Water from the upper part of a double-drain fish basin (2.3 m^3), stocked with Gilthead Seabream (fish) to a final density of 20–50 $kg\,m^{-3}$, was recirculated at a rate of 1–2 fish basin volumes per hour over a trickling filter. Simultaneously, water from the bottom center of the fish basin flowed into a digestion basin (DB) (working volume 0.4–0.5 m^3) at a rate of 0.1–0.25 fish basin volumes per hour. Water from the upper layers of the DB outlet was pumped (5–7 $L\,min^{-1}$) into the cylindrical FBBR through a vertical pipe that extended from the top center to ≈ 3 cm above the base of the FBBR. The FBBR (working volume 6.26 L) was filled with sand (average diameter 0.7 mm), as carrier material for growth of microbial biofilm. Upflow from the inlet pipe caused the flocs (biofilm-attached sand grains) to float within the column. Water from the FBBR outlet at the top of the cylinder was drained back to the fish basin after passing through a settler for removal of particulate matter, which was funneled back into the DB.

Schramm et al. [12.63] analyzed the change of activity and abundance of *Nitrosospira* and *Nitrospira* spp. along a bulk water gradient in a nitrifying FBBR, by a combination of microsensor measurements and fluorescence in situ hybridization (FISH), which facilitated a detailed analysis of the in situ structure and function of the nitrifying bioreactor on a microscale. The conical, continuous-upflow FBBR (originally developed by *de Beer* et al. [12.64]) (Fig. 12.32) (volume 360 mL, height 0.8 m, I.D. 1 cm at the bottom, 3 cm at the top) was covered with black paper to prevent phototrophic algal growth. It was fed with mineral medium containing 72 mM NH_4^+ and the liquid phase was recirculated at a rate of 1.8 $mL\,s^{-1}$. The conical shape of the vertical reactor column generates a flow velocity gradient that stabilizes nitirifying bacterial aggregates of different diameters and densities at fixed positions in the column according to their settling velocity. Nitrification occurred in a narrow zone of 100–150 mm on the surface of the aggregates, that contained a very dense community of nitrifying bacteria. The central part of the aggregates was inactive, containing significantly lesser number of nitrifiers. Since conditions in the reactor were ammonium limiting, it was completely oxidized to nitrate within the active layer of the aggregates, the rates decreasing with increasing reactor height. Thus, this study, as well as the one carried out by *de Beer* et al. [12.64] demonstrated the successful operation of a two-phase (liquid–solid) fluidized-bed nitrification reactor with an external aerator, for development of nitrifying bacterial aggregates. Most importantly, the much lower shear forces generated, relative to those in three-phase systems, facilitated development of bacterial aggregates without addition of carrier materials.

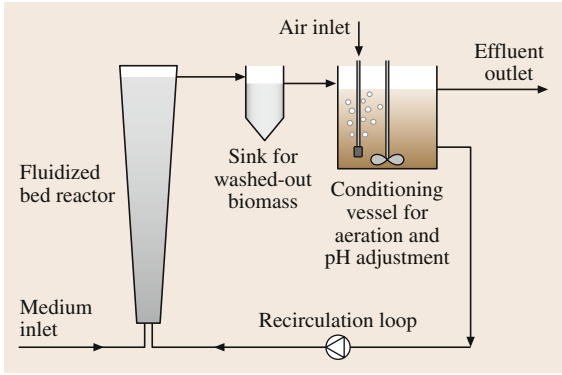

Fig. 12.32 Scheme of the nitrifying reactor setup (after *de Beer* et al. [12.64])

Garbisu et al. [12.65] used free and polyvinyl foam-immobilized cells of the cyanobacteria *P. laminosum* for phosphate removal from water in batch and flow-type FBBRs as well as in continuous flow PBBR. Phosphate uptake by nitrogen starved free-living cyanobacteria showed that N-starvation led to lower uptake rates, but addition of nitrate significantly increased the uptake. Phosphate uptake by free as well as immobilized cyanobacteria was inhibited in the dark and simulated by the presence of calcium ions or bicarbonate. Addition of a chelating agent, such as ethylenediaminetetraacetic acid to the cultures, led to zero phosphate uptake. Although high phosphate uptake was not observed with cyanobacteria immobilized on polymer foams, concurrent removal of nitrate and phosphate from water using nitrogen-starved immobilized cyanobacteria showed sufficient potential for future development.

12.7.2 Moving Bed Bioreactors

Although FBBRs ensure homogeneous environmental conditions for all biofilm surfaces and enhance mass transfer rates, nevertheless, both for upward and down-

ward fluidization, the active volume is low and fluidization performance may deteriorate over time and may not remain at the optimum level, primarily due to the changing density of the carriers with biofilm growth on their surfaces. An effective alternative to an FBBR is an MBBR (Table 12.8), which has carrier particles that are continuously in motion. As the liquid circulates, the carriers move through the liquid between two retention grids at two ends of the reactor and are agitated either mechanically or hydrodynamically by liquid jets. High shear stress from water circulation coupled with interparticle collision prevents uncontrolled proliferation of biofilm on the carrier surface and thereby stabilizes the reactor system.

An MBBR is thus *self-cleaning* and has low head loss. In fact, MBBRs with up to 60% of the working volume occupied by carriers have been used and found to perform satisfactorily.

A pilot scale, submerged MBBR was installed at the Montreal Biodome (a 3.25 million closed-circuit mesocosm) to deal with the constraints of seawater denitrification by an attached-growth process, with an aim to optimize a large-scale MBBR. *Labelle* et al. [12.66], evaluated the seawater treatment performance of the reactors, using methanol as carbon source at different C/N ratios and examined its efficiency in preventing sulfate reduction by limiting biofilm thickness, dead mixing zones and residual substrate in the outflow.

Two well-mixed bioreactors in series (Fig. 12.33) (working volume 110 L each, diameter 43 cm and with conical bottom) were used for deoxygenation and denitrification, respectively. Methanol was fed to both reactors (C-source) and a trace element solution to the second one, the latter intended to maintain iron, manganese, and copper concentrations over 150, 80 and 10 $\mu g\,L^{-1}$, respectively. Indigenous biomass in seawater was used to colonize carriers in the two-stage bioreactor system. The first stage, a pretreatment deoxygenation unit, was actually a packed bed biofilter packed to 80% of its total volume with 63 mm random plastic carriers (specific surface area 180 $m^2\,m^{-3}$, void fraction 91%, specific gravity ≈ 0.90). The second stage, a denitrification unit, consisted of a submerged MBBR configured to minimize media fouling and dead

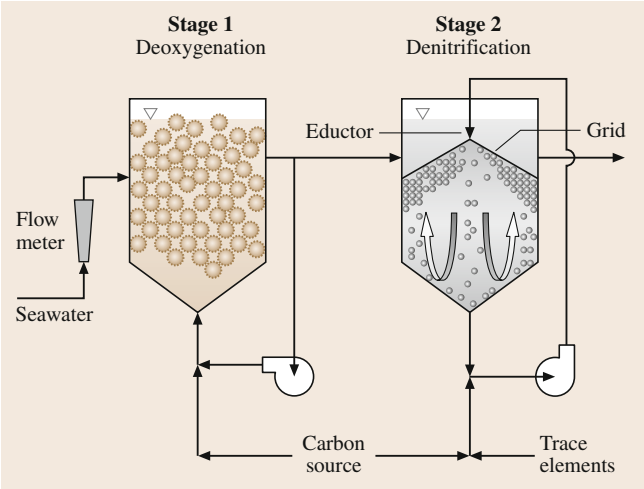

Fig. 12.33 Schematic diagram of experimental setup: Two CFSTR in series for deoxygenation and denitrification, respectively (after *Labelle* et al. [12.66])

Table 12.8 Fluidized bed and moving bed bioreactors (FBBR and MBBR)

Sl	Bioreactor	Marine strain and bioprocess	Reference
1	FBBR	Digestion of marine waste by mixed bacterial consortium	*Cytryn* et al. [12.61]
2	FBBR	Cultivation of a novel filamentous bacteria *Thiothrix* sp.	*Cytryn* et al. [12.62]
3	FBBR	Nitrification by *Nitrosospira and Nitrospira* sp., immobilized in bacterial aggregates.	*Schramm* et al. [12.63]
4	Batch and continuous-flow FBBR	Nitrate removal from water by cyanobacterium *P. laminosum*	*Garbisu* et al. [12.65]
5	Submerged MBBR	Denitrification of seawater in a closed circuit mesocosm	*Labelle* et al. [12.66]
6	Downflow MBBR	Denitrification of seawater by mixed bacterial consortia from a marine mesocosm	*Dupla* et al. [12.67]
7	MBBR	Harvesting anaerobic ammonium-oxidizing (anammox) bacteria in biological filter and waste sludge compartments of a marine RAS	*Tal* et al. [12.68]
8	Sequencing MBBR	Denitrifying biological phosphorus removal (BPR) in wastewaters by mixed bacterial consortia	*Vallet* et al. [12.69]

mixing zones. The 2.5 cm spherical polyethylene carriers used in the second stage had a fill ratio of 25%, a surface area of 278 $m^2\,m^{-3}$, a void fraction of 90%, and a specific gravity of 0.89. Effective surface area for biofilm attachment was estimated at ≈ 35% of the total surface area (i. e., 97 m^2/m^3) since no growth was observed on the outer parts of the moving carriers. Carriers were kept submerged by a conical welded stainless steel wire mesh (5 mm openings, wire diameter of 0.56 mm) and mixing was ensured by the downflow jet of a 9.5 mm glass-filled polyethylene eductor positioned vertically over the conical grid. The single-jet configuration was designed to generate adequate hydrodynamic shear to prevent carrier fouling and to create a hydraulic movement minimizing the surface diffusion of oxygen in the MBBR. High sulfate concentration in seawater complicates denitrification in attached-growth processes. Natural, anoxic marine environments where sulfate reduction occurs are associated with N_2O production. However, the study demonstrated that a pilot-scale sMBBR could efficiently denitrify seawater/saline water with a high sulfate concentration.

In seawater aquariums, nitrates are produced in the biological aerobic treatment units by nitrification of excreted ammonia, which is toxic in its unionized form. The recommended level of nitrates in an artificial seawater ecosystem is 50 mg NO_3-N L^{-1}, which is low compared to its generation rate in a closed system. *Dupla* et al. [12.67] investigated the hydrodynamic behavior and denitrification capacity of a downflow self-cleaning moving bed bioreactor (MBBR) using floating packed-bed carriers. A deoxygenation unit was installed upstream from the denitrification unit, consisting of an MBBR (Fig. 12.34) (working volume 110 L) packed with plastic carriers placed randomly. The denitrification unit comprised two identical cylindrical reactors made of PVC (height 40 cm, diameter 30.5 cm, height of conical section 15 cm). Each reactor (including pipes) contained 33 L of water and was equipped with a recirculation loop and piping that allowed tangential recirculating water inflow, causing rotational movement of the liquid. Welded stainless-steel grid cones (10°, 45° or 60° inclination) were used to retain the carriers (5 mm grid openings, wire diameter 0.56 mm). Two jet nozzles with outlet diameter of 4.8 mm (small) and 8 mm (large) were installed to generate different water recirculation velocities at the nozzle i. e., 18.5 and 6.6 m s^{-1}, respectively. Eleven types of plastic packed-bed carriers commonly used in biological processes were considered and carrier selection was based on specific surface area, density, and size. The MBBR appeared to control biofilm development and could easily be scaled up to denitrify seawater or freshwater systems. Due to hydrodynamic control by means of the jet nozzle, the carriers did not clog and a thin biofilm was maintained throughout.

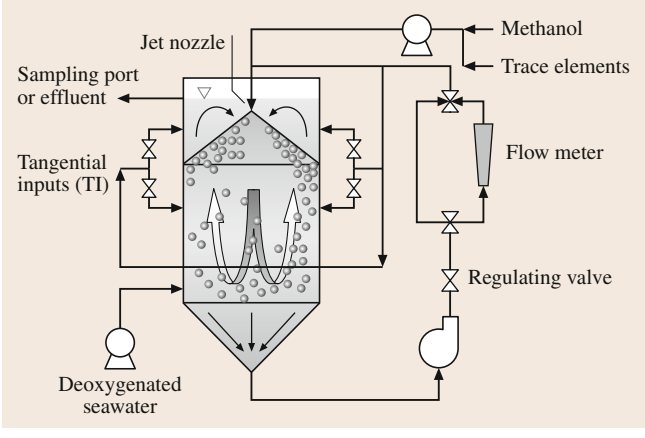

Fig. 12.34 Schematic diagram of the MBBR used in denitrification experiments (*arrows* show water circulation) (after *Dupla* et al. [12.67])

Tal et al. [12.68] examined the microbial communities in the biological filter and waste sludge compartments of a marine RAS to determine the presence and activity of anaerobic ammonium-oxidizing (anammox) bacteria. Fluorescence in situ hybridization (FISH) studies using an anammox-specific probe confirmed the presence of anammox *Planctomycetales* in the microbial biofilm from the denitrifying biofilters, and anammox activity was observed in these biofilters, as detected by the ability to simultaneously consume ammonia and nitrite.

Two tanks (vol. 4.2 m^3 each) containing the fish gilthead seabream (*Sparus aurata*) were operated at a density of 10–50 kg m^{-3} and a feeding rate of 1–1.6% body weight d^{-1}. The tanks were connected to a 2 m^3 nitrifying MBBR filled with 1 m^3 of polyethylene beads (diameter 1 cm, surface to volume ratio 500 $m^2\,m^{-3}$). A flow rate of 16 $m^3\,h^{-1}$ was used to obtain two exchanges of tank water per hour through the nitrifying biofilter. Attached to each tank as a denitrification side loop was a cylindrical up-flow fixed-bed biofilter (volume 0.3 mL) filled with 0.2 m^3 of polyethylene beads. The flow rate for this anaerobic biofiltration compartment was 0.1 $m^3\,h^{-1}$, and the water was supplied by two sources, direct water from the fish tank and water recovered from the sludge tank. Sludge was collected with a drum screen filter using a backwash system in

which tank water was used. Sludge and backwash water were collected in a rectangular tank (volume 0.3 m^3) packed with 0.1 m^3 beads that provided a means for solids removal, as well as a substrate for bacterial colonization. Water from the sludge tank was pumped back into the system through the anaerobic biofilter, and high-density sludge was collected and removed. In view of stricter environmental regulations for effluent discharge from aquaculture facilities there is increasing emphasis on closing the nitrogen cycle in the water treatment system by utilizing the anammox process as a preferred alternative to heterotrophic denitrification, especially in marine recirculating systems in order to reduce effluent volumes as well as nitrogen loads. The results of this study show that anammox bacteria and their activity are associated with the consortia of a denitrifying biofilter, which makes this approach feasible for implementation.

Enhanced biological phosphorus removal (EBPR) in wastewater treatment is based on the fact that phosphorus accumulating organisms (PAOs) can accumulate intracellular phosphorus as polyphosphate (poly-P). In EBPR, the biomass is exposed to cyclically occurring anaerobic and aerobic/anoxic conditions. Under anaerobic conditions, PAOs store rapidly biodegradable substrate in their cells as poly-β-hydoxyalkanoates (PHA) by using intracellular poly-P and glycogen as energy sources. Under aerobic/anoxic conditions, PAOs use PHA as a carbon source to refill poly-P and glycogen stocks. Poly-P synthesis allows bulk phosphate to accumulate in the biomass. The phosphate is then removed with the biomass during aerobic/anoxic conditions. Another group of bacteria, the glycogen accumulating organisms (GAOs), also grow under cyclic alternation of anaerobic and aerobic/anoxic conditions, using only glycogen as energy source and therefore consuming substrate but not removing phosphorus. Thus to optimize EBPR, anaerobic conditions must be strictly controlled, poly-P storage maximized, and GAOs growth minimized. Poly-P storage is maximized by using acetate as a carbon source because it increases the production of poly-P compared to other volatile fatty acids during aerobic periods. Environmental conditions known to favor PAOs over GAOs are temperature $< 25\,^{\circ}C$ and pH > 7.5. Aquariums and fish farms run in closed circuit face major accumulation of phosphate and nitrate which cause, respectively, excessive algal growth and toxicity to aquatic fauna. As an alternative to regularly changing the water that causes highly polluted discharge to the surroundings, denitrifying biological phosphorus removal (DN-BPR) may be applied to this system without producing excessive sludge.

The Montreal Biodome operates a 3250 m^3, closed circuit, cold (10 °C) marine mesocosm named St. Lawrence Mesocosm (SLM) containing very high nitrate and phosphate levels. Earlier workers (e.g., *Labelle* et al. [12.66], *Dupla* et al. [12.67]) have demonstrated the feasibility of denitrifying the SLM water using an MBBR with methanol as the C-source. *Vallet* et al. [12.69] modified the process to implement DN-BPR to promote the anaerobic growth of PAOs in the reactor – and additional C-source was required in view of low concentration of organic matter in the SLM water (2–4 mg C/L). The process developed is based on the alternation of anaerobic and aerobic/anoxic conditions in the MBBR with biofilm growth occurring on the surfaces of plastic carriers. A sequential complete exchange of two waters thus takes place in the MBBR – (1) the influent containing nitrate and phosphate and (2) the anaerobic water stored in a stock tank. Therefore, the environmental conditions in the MBBR selectively favor the growth of PAOs only. An additional advantage is the accumulation of phosphate in a stock tank thereby allowing an easy recovery of P for use as fertilizer.

Two identical reactor setups (used earlier by *Labelle* et al. [12.66]) were operated in parallel under identical conditions. Each setup comprised a well-mixed bioreactor (volume 110 L) filled with 35 L of plastic carriers, and a stock tank (volume 220 L). The packed bed was moved using a circulation pump thereby allowing operation as an MBBR. Water was pumped from the stock tank to the MBBR through a three-way transfer valve, while the MBBR was filled with the pressurized influent through a solenoid valve. A pH probe, a redox-potential probe, an oxygen probe, and a UV nitrate analyzer were installed on the recirculation line. This study demonstrated the inhibition of BPR by seawater presumably due to the high calcium levels in seawater. However, in freshwater, a biofilm was developed with a phosphorus removal efficiency rate of 20% of which 80% was attributed to biological removal and 20% to chemical precipitation.

12.8 High-Temperature and/or High-Pressure Bioreactors (HP-/HTBR)

High-pressure and/or high-temperature bioreactors (Table 12.9) are practically wholly devoted to cultivation of extremophiles (barophiles and/or thermophiles) isolated from the deep sea. Most, though not all, HP/HT bioreactor systems may be expected to fall into one of the following categories:

i) One-phase batch systems without gas enrichment (e.g., *Canganella* et al. [12.70], *Kallmeyer* et al. [12.71]).
ii) Processing of microbial samples without depressurization (e.g., *Parkes* et al. [12.72]).
iii) Two-phase batch systems with gas enrichment from free gas (includes studies by the group of Prof. Douglas Clark at the University of California, Berkeley e.g., *Miller* et al. [12.73], *Miller* et al. [12.74], *Miller* et al. [12.75], *Nelson* et al. [12.76], *Nelson* et al. [12.77], *Hei* and *Clark* [12.78], *Park* and *Clark* [12.79]).
iv) High-pressure fed-batch/continuous incubation (e.g., *Girguis* et al. [12.80], *Houghton* et al. [12.81]).
v) High-pressure fed batch/continuous incubation with gas-enrichment medium without free gas (e.g., *Deusner* et al. [12.82], *Zhang* et al. [12.83]).

Besides the above HP/HT bioreactor systems, *Marteinsson* et al. [12.84, 85] used pressurized gas-

Table 12.9 High-temperature and/or high-pressure bioreactors (HP-/HTBR)

Sl	Bioreactor	Marine strain and bioprocess	Reference
1	HPHTBR	Cultivation of thermophilic archaebacterium *T. peptonophilus*	*Canganella* et al. [12.70]
2	High-pressure thermal gradient system (HPTGS)	Cultivation of sulfate-reducing bacteria (SRB)	*Kallmeyer* et al. [12.71]
3	Deep-IsoBUG System	Enrichment and isolation of deep subsea floor prokaryotes	*Parkes* et al. [12.72]
4	High-pressure-high-temperature bioreactor (HPHTBR)	Effect of pressure on methanogenic archaea *M. jannaschii*	*Miller* et al. [12.73]
5	HPHTBR	Study of pressure–temperature relationship on growth and productivity of methanogenic archaea *M. jannaschii*	*Miller* et al. [12.74]
6	HPHTBR + fiber optics sensors	Assay of thermostable hydrogenase from methanogenic archaea *M. jannaschii*	*Miller* et al. [12.75]
7	HPBR	Effect of hyperbaric He pressure on the growth of archaeon ES 4	*Nelson* et al. [12.76]
8	HPBR	Comparing effects on hyperbaric and hydrostatic pressure on thermophile archaeon ES4	*Nelson* et al. [12.77]
9	Anaerobic HTBR	Pressure stabilization of protein from methanogenic archaea *M. jannaschii*	*Hei* and *Clark* [12.78]
10	HPHTBR	Cultivation of methanogenic archaea *M. jannaschii*	*Park* and *Clark* [12.79]
11	Continuous-flow anaerobic methane incubation system (AMIS)	Growth and methane oxidation of anaerobic methanotrophic archaea in marine sediment samples	*Girguis* et al. [12.80]
12	HPLC column flow-through bioreactor	Cultivation of nitrate and sulfate reducing thermophiles	*Houghton* et al. [12.81]
13	HPBR	Anaerobic oxidation of methane (AOM) by bacterial consortium	*Deusner* et al. [12.82]
14	Continuous HPBR	Anaerobic oxidation of methane in deep-sea sediments	*Zhang* et al. [12.83]
15	Sterilized gas-tight glass syringe in HPHT incubation system	Cultivation of a hyperthermophillic species (archaea) *P. abyssi* GES	*Marteinson* et al. [12.84]
16	Glass Syringes in HPHT incubation system	Cultivation of a novel strain archaeon *Theemococcus barophilus*	*Marteinson* et al. [12.85]
17	High-pressure bioreactor (HPBR)	Study on pressure tolerance of marine bacterial strains isolated from surface water	*Wright* et al. [12.86]

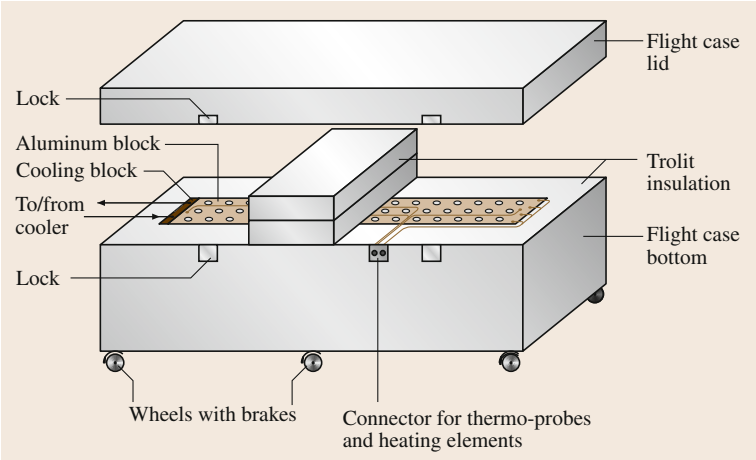

Fig. 12.35 Schematic view of the thermal gradient system. For better viewing, only parts of the Trolit blocks that cover the top are shown. The case has eight heavy-duty handles (not shown) (after *Kallmeyer* [12.71])

tight syringes for cell cultivation, placed in an HPHT incubation system. Again, *Wright* et al. [12.86] investigated the pressure tolerance of barotolerant (rather than barophilic) strains isolated from surface waters (and not from the deep sea).

Canganella et al. [12.70] investigated the effects of high temperatures and elevated hydrostatic pressures on the physiological behavior and viability of the extremely thermophilic deep-sea archaeon *Thermococcus peptonophilus* (isolated from deep-sea hydrothermal vents in the western Pacific ocean at a depth of 1380 m) in a high-pressure/high-temperature bioreactor (HPHTBR). Growth was fastest at 30 and 45 MPa without any noteworthy increase in cell yields, growth at 60 MPa was slower. The optimal growth temperature shifted from 85 °C at 30 MPa to 90–95 °C at 45 MPa. Cell viability during the stationary phase was also enhanced under high pressure. A trend toward barophily at pressures greater than those encountered in situ at the sea floor was observed at increasing growth temperatures. Relative to that at atmospheric pressure, the viability of cells during starvation, at high temperature (90, 95 °C), as well as at low temperature (10 °C) increased at 30 and 45 MPa. These results established that the extremely thermophilic archaeon *T. peptonophilus* was a barophile. The HPHTBR used in this study was designed to work within a temperature range of 0–300 °C and up to 68 MPa pressure and was suitable for continued sampling without any perturbation of the culture.

Kallmeyer et al. 2003 [12.71] developed a high-pressure thermal gradient system (HPTGS) consisting of a thermal gradient block (TGB) and a high-pressure unit (HPU) for simultaneous incubation of multiple microbial samples over a wide range of temperatures and pressures. To maintain the pressure independent of the thermal expansion of sample vessels, a back-pressure system (BPS) with a constant small leakage was also incorporated into the HPTGS. The HPTGS was used to study the pressure–temperature characteristics of sulfate-reducing bacteria (SRB) isolated from hydrothermal vent sediments (from Guyamas Basin, Gulf of California) and measure bacterial sulfate reduction rates which were found to increase with increasing pressure and showed maximum values at pressures higher than in situ.

The HPTGS consists of two main units: the TGB and the high-pressure unit (HPU). The TGB (Fig. 12.35) consists of an aluminum block (Al Cu Mg Pb F34; 1185 × 150 × 150 mm) with three rows of 15 equidistant holes (140 mm deep × 36 mm diameter) insulated by a 20 cm thick layer of Trolit (proprietary solid material, made from mineral fibers) with very good thermal insulation characteristics, temperature resistant to > 1000 °C, also robust and mechanically stable. The temperature gradient is almost linear, independent of the minimum and maximum temperatures. At incubation temperatures > 200 °C, the outside of the unit is at ≤ 25 °C. For ease of transportation, the whole block is housed in a custom-made case on wheels. The Trolit and the aluminum block are held in place only by the case to allow for thermal expansion of the material and easier dismantling. At one end of the block, a cooling element is attached, milled from a solid piece of aluminum (150 × 150 × 60 mm) with a tortuous internal channel where coolant is circulated. The inlet and outlet are on top of the unit and connected to a cooling unit mechanically fixed to the block with four screws, and

sealed with heat-resistant elastic sealant. The coolant was chosen according to the desired temperature and for very low temperatures, the hoses connecting the cooler and the TGB were thermally insulated to avoid precipitation of moisture and buildup of ice. At the opposite end of the TGB, four electrical heating elements (6.4 mm diameter × 88.9 mm, 200 W) were placed into 6.5 mm diameter holes in the block. The maximum temperature attainable is 220 °C and the steepest thermal gradient is 160 °C, providing ≈ 10 °C temperature difference between each row of containers. Over the length of the block, four thermo-probes (PT 100, −60 to +400 °C) were installed to continuously monitor the temperature, which were connected to an electronic multicontroller for on–off switching of the heating elements.

The HPU (Fig. 12.36) is essentially a back-pressure system with a constant small leakage. Pressure is created by two HPLC pumps that can operate in a higher pressure range (from 400 to 600 bar). The pumps run in constant pressure mode with variable flow rate. In case of any thermal expansion during the warm-up, pressure remains below the specified threshold. The back-pressure system comprises five used HPLC columns (250 × 4.6 mm) in line. At 450 bar pressure, ≈ 0.7–0.8 mL leaks through. During the warm-up period, thermal expansion may cause the pressure to rise faster than the back-pressure system can release it, therefore opening of one of the connections between the HPLC columns smoothly increases the outflow of the system in a controlled manner. After thermal equilibrium is achieved, the connection is fastened again. The outflow of the back-pressure system flows back into the eluant vessel for subsequent reuse.

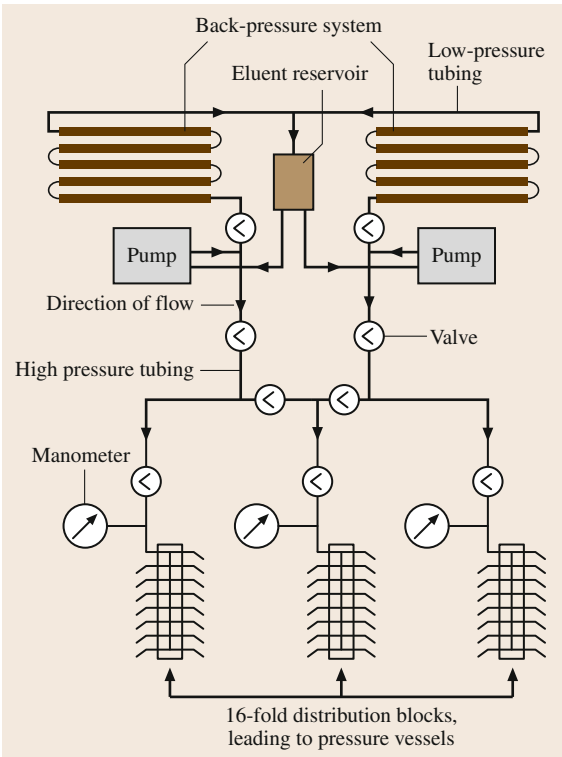

Fig. 12.36 Schematic view of the high-pressure system. The *thin lines* are high-pressure connections; the *bold lines* are low-pressure connections (after *Kallmeyer* et al. [12.71])

The pressure distribution system consists of pumps connected to a manifold of valves which allows connection of each pump to any of the three lines of pressure vessels, or for use of the pumps in parallel. Each line has a Bourdon-type manometer (16 cm diameter) to dampen the pulsation of the pump. A custom-made 16-fold distribution block connects the pressure containers of each line to the valve manifold. Small pockets in the Trolit insulation house the distribution blocks. Pressure vessels consist of large standard S.S. HPLC columns (I.D. 20 mm, 120 mm long), one end having a normal HPLC $\frac{1}{16}$ in fitting, the other end closed with a steel cap. The pressure containers are sealed with copper rings that last for several incubations but must be replaced periodically. Custom-made screw-cap tubes (O.D. 18 mm, 50 mm long) are used as sample vials, two of which fit in each pressure vessel, thereby providing exact duplicates in pressure and temperature.

The pressure vessels are placed in a bucket filled with demineralized water and the sample vials dropped into them. The caps are screwed on the pressure vessel under water to avoid trapping of air bubbles. After all pressure vessels are connected to the distribution block, ≈ 50 bar is applied to check for leaks. When no leak is noted, the vessels are inserted into the TGB and allowed to attain thermal equilibrium. The buildup of pressure is carefully monitored and released if necessary, then, after 1–2 h, the pressure is set to the desired value.

Parkes et al. [12.72] developed the novel DeepIsoBUG system for handling sediments (that contain depressurization sensitive anaerobic, piezophilic prokaryotes) under elevated pressure (up to 25 MPa) for enrichment, growth, and isolation of prokaryotes at pressures up to 100 MPa. When coupled with acquisition of pressurized subsurface cores using the HYACINTH pressure retaining drilling and core storage system and the PRESS core-cutting system, Deep-

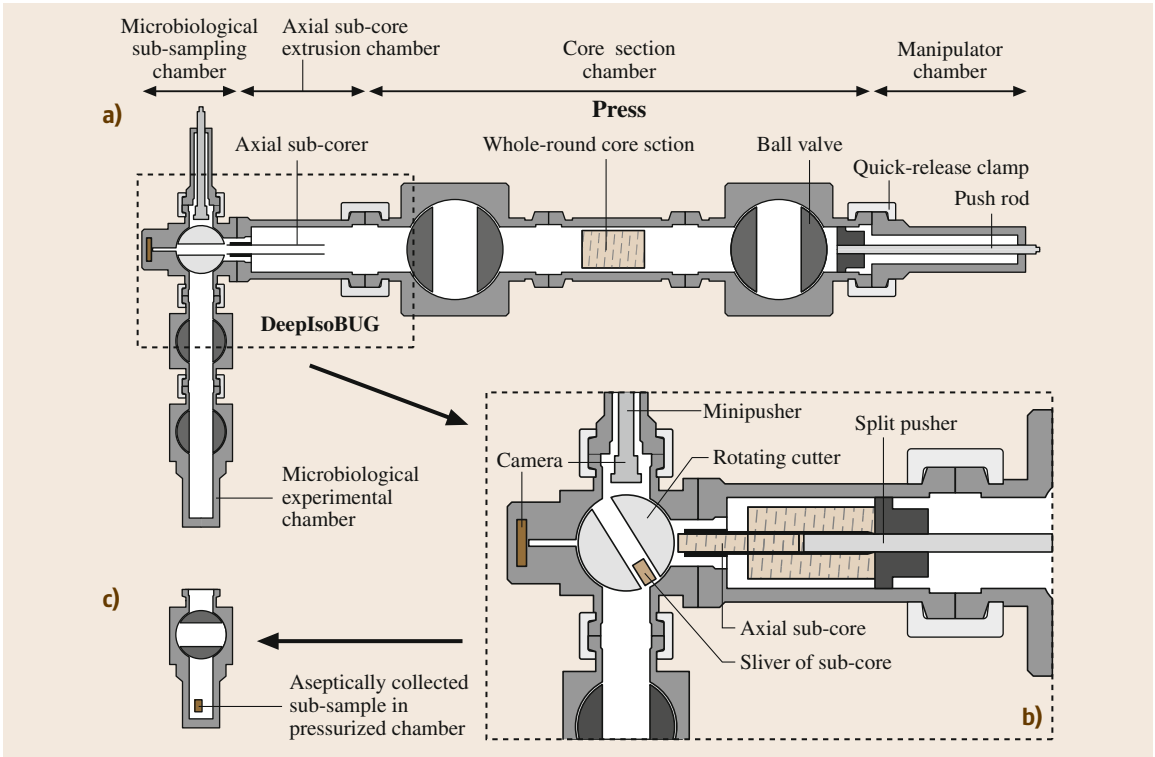

Fig. 12.37a–c Schematic of subcoring and slicing procedure using the coupled PRESS and DeepIsoBUG system to obtain an uncontaminated central core slice for prokaryotic enrichments and other experiments. **(a)** Complete system connected after cutting and transfer of core section. **(b)** Blow-up showing the subcoring and slicing operation. **(c)** Sediment slice isolated in pressure vessel (after *Parkes* et al. [12.72])

IsoBUG enables the recovery and handling of cores at in situ pressures (up to 25 MPa) and subsequent enrichment and isolation of prokaryotes at a range of pressures without depressurization. Using subsurface gas hydrate containing sediments (obtained from the Indian Continental Shelf, the Gulf of Mexico, and the Cascadia Margin), it was examined whether enrichment and isolation of subseafloor anaerobic prokaryotes under elevated pressure, without depressurization, would enable acquisition of different culturable prokaryotes compared with the normal procedure of using depressurized sediments and atmospheric pressure handling and isolation. They found that, in general, highest cell concentrations in enrichments occurred close to in situ pressure (14 MPa) in various growth media, although growth was observed at pressures up to 80 MPa.

The DeepIsoBUG system consists of a subcoring and slicing system (Fig. 12.37), a transfer chamber, an isolation chamber and pressure vessels, described below. The subcoring and slicing system (made of S.S. 316) enables a central subcore (20 mm) to be obtained from a core section and to be sequentially sliced (using a manually rotated blade), with each slice being transferred (using a rotating central section) to a low pressure (up to 25 MPa) vessel (with a large ball valve) containing anaerobic, mineral salts medium, vigorous shaking of which produces a sediment slurry for use as inocula. Two cameras behind sapphire windows allowed viewing of the extrusion process.

Through the transfer chamber, the sediment slurry is transferred from the low-pressure vessel (5 mL slurry aliquots) to a number of high-pressure (up to 100 MPa) culture vessels containing enrichment medium. The chamber also contains a filter ($\approx$ 100 mm) to prevent transfer of large particles that could damage ball valves.

The isolation chamber has 12 agar plates attached to an electric motor-driven chain allowing individual plates to be selected. The chain drive lifts out of the isolation chamber and plates detach so that anaerobic

media can be prepared and the system assembled in an anaerobic chamber and transferred (via an anaerobic bag) to the presterilized and gas-flushed chamber body. The chamber also contains eight detachable cells (up to 15 mL in volume) that allows microbial growth in liquid medium within the chamber and also transfer in and out of the isolation chamber. Enrichments for isolation were transferred into an individual growth cell within the isolation chamber. A motorized robotic arm in the isolation chamber enabled a sterile inoculation loop (electrically heated in situ) to be dipped into the growth cell and its contents to be streaked onto an agar plate. After incubation, individual colonies could then be picked off and transferred to other cells containing sterile media for further growth. Transfer of these purified cultures out of the isolation chamber into high-pressure incubation chambers permitted further subculture and physiological tests to be conducted on isolates. All manipulations in the isolation chamber were viewed through a sapphire window via an endoscope attached to a digital camera and monitor.

Pressure vessels (100 cm^3 total volume, 80 cm^3 medium) made of titanium, used for enrichment and cultivation have a body and a lid, attached together with a screw thread. They are sealed by a male cone in the lid and a complementary female cone in the body, plus O-ring seals. The low-pressure enrichment vessels have a small-bore ball valve (4 mm) on the lid for gas input and liquid transfer, as well as a large-bore ball valve (32 mm) for sediment slice transfer. The high-pressure, incubation vessels have just two small-bore ball valves. Preprepared sterile anaerobic media were transferred into sterile pressure vessels within an anaerobic chamber. For high-pressure liquid transfer between systems, a small pressure differential ($\approx$ 2 MPa) between vessels was maintained. Ball valves were used to prevent shear stresses and possible cell rupture during culture transfer by pressure differential. For routine high-pressure sampling of pressure vessels and subculture into vessels with fresh medium, a bored-out *T*-piece and a series of valves were used, that enabled transfers without depressurization.

The study accomplished the following, viz.:

a) The direct enrichment of subseafloor prokaryotes under elevated pressure.
b) Enrichment of sedimentary, anaerobic prokaryotes without depressurization.
c) High-pressure cultivation from subsurface gas hydrate sediments.

Results showed that many subseafloor prokaryotes from gas hydrate containing sites could grow (anaerobically) up to at least 80 MPa in various media which is a much higher pressure than that previously recorded for subseafloor prokaryotes. As 70% of the ocean is at a pressure of 38 MPa or higher and the average depth of sediments is 500 m, growth at 80 MPa would enable the majority of the subseafloor environment to be populated by prokaryotes. This study was claimed as the first report on the isolation of *Carnobacterium*, *Marinilactobacillus*, *Acetobacterium*, *Clostridium*, and *Bacteroidetes* species from deep, subseafloor, gas hydrate deposits.

Miller et al. [12.73] developed a novel HPHTBR to investigate pressure–temperature relationships (up to 260 °C and 350 bar) in the growth and productivity of the extremely thermophilic methanogenic archaea *Methanococcus jannaschii* isolated from a deep sea hydrothermal vent. The corrosion-resistant reactor system permits direct sampling (of both liquids and gases) from a transparent culture vessel without affecting the internal growth conditions. It was found that increasing the pressure from 7.8 to 100 bar accelerated the production of methane and unicellular protein at 90 °C; raised the maximum temperature for growth from 90 to 92 °C but further increase in pressure did not affect growth. The reactor vessel or pressure cell (internal volume $\approx$ 43 mL) is a transparent synthetic single crystal sapphire tube (I.D. 0.7″, O.D. 1.25″, length 6″) which allows direct visual observation of the growth medium. It may be sealed by two different configurations – one for working at 100 bar consisting of a spring-loaded Teflon gasket held onto the tube ends by flanges which, again, are held in place by six spacer rods. In the other sealing technique, the distance between the flanges is reduced, thus compressing the Teflon rings which, on application of pressure, are further compressed thereby maintaining the seal.

The pressure cell is connected to a magnetic pump to recirculate vapor through the liquid, which serves two purposes, viz. (1) agitation of the liquid and (2) to promote vapor–liquid equilibrium. The pump (I.D. 0.375 in, internal length 2 in) is actuated by a stack of ceramic ring magnets located around the pump body that are oscillated up and down through a motor driven crank and cable. A piston with a plug travels the length of the pump. On the upstroke, gas is pushed out of the pump into the pressure cell, on the down stroke, gas fills the pump for the next stroke. A crank valve at each end of the pump prevents flow in the opposite direction.

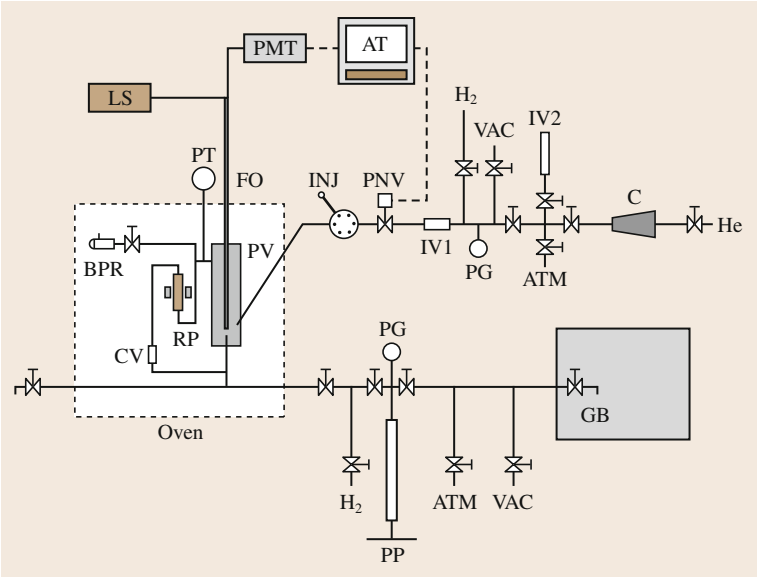

Fig. 12.38 Schematic diagram of the apparatus: PV – stainless steel pressure vessel, RP – magnetically driven vapor recirculation pump, CV – check valve, BPR – back pressure regulator, PT – pressure transducer, FO – fiber optic probe, LS – light source, PMT – photomultiplier tube, water-cooled housing, and photometer, AT – computer, PNV – pneumatic valve, INJ – six-port valve, IV1 – 1 mL injection vessel, HJ – hydrogen supply, PG – pressure gauge, IV2 – 100 mL injection vessel, C – two-stage compressor, He – helium supply, GB – anaerobic glove box, and PP – piston pump. The abbreviations ATM and VAC refer to atmosphere and vacuum, respectively (after *Miller* et al. [12.75])

The pressure cell and magnetic pump are housed in an oven (maximum temp. 260 °C) equipped with a viewing window. The oven temperature is measurable by a thermocouple and a digital thermometer. Pressure is maintained by a triple range strain gauge transducer coupled to a digital voltmeter for display.

All gas entering the high-pressure system is passed through a trap to strictly exclude molecular O_2. For experiments performed at pressures above the cylinder pressure of the gases, a two-stage diaphragm compressor is used to raise the gas pressure. For liquid supply to the reactor vessel, the microbial suspension is drawn (from the anaerobic glove box) into the screw-actuated piston pump, which is operated to raise the pressure on the microbes to the value in the reaction vessel. When the pressure in the pump equals that in the vessel (20 psi initially), the valve is opened for solution inflow to the vessel. In case of methanogenesis, a pressure drop accompanies methane formation – this necessitates use of a pressure regulator for injecting inert gas into the reaction vessel to maintain constant pressure. The regulator also maintains constant pressure when samples are withdrawn. Both the liquid and gas lines are connected to a vacuum system so that lines can be evacuated or purged before material is passed through them into the pressure cell. In this way, molecular oxygen is excluded from the pressure vessel. To prevent corrosion and H_2 embrittlement, all metal parts in contact with high-pressure fluid are made of S.S. 316.

Miller et al. [12.74] cultivated the deep-sea thermophilic archaea *M. jannaschii* in a high-temperature, high-pressure bioreactor (HTHPBR) at high temperatures and hyperbaric pressures of helium. The bioreactor used was similar to a prototypical device described earlier in *Miller* et al. [12.73] modified as follows – the synthetic sapphire pressure vessel (volume 90.63 cm^3) was replaced with a larger S.S. 316 vessel (internal volume, 167 cm^3) capable of withstanding pressures up to 1000 atm; the screw-actuated piston pump was replaced with a higher throughput pneumatic pump to allow rapid addition of fresh medium to the reactor vessel. High hyperbaric pressure of He up to 750 atm was observed to increase the growth rate of *M. jannaschii* at both 86 and 90 °C but did not extend the upper temperature limit for growth. However, increased pressure did extend the maximum temperature for methanogenesis by *M. jannaschii* from < 94 °C at 7.8 atm to 98 °C at 250 atm, indicating a possible pressure-stabilizing effect on enzymes facilitating methane synthesis.

Miller et al. [12.75] designed a HPHTBR for measuring enzyme activity (by UV-visible spectrophotometry/gas chromatography) at temperatures up to 260 °C and pressures up to 667 atm. Initial studies focused on the pressure-enhancement of methyl viologen-reducing hydrogenase activity in crude extracts of *Methanococcus jannaschii*. The oven of this reactor system (Fig. 12.38) contains a S.S. 316 cylindrical

reaction vessel and a magnetically driven pump to recirculate gas through the liquid phase. A fiber optic probe is inserted into the reaction vessel for spectrophotometric measurements (Fig. 12.39). A six-way valve is used to inject substrate or enzyme into the vessel. The rest of the setup is used for transferring fluids and for maintaining constant pressure. The reaction vessel is a custom-built S.S. 316 cylinder (O.D. $3'' \times$ I.D. $\frac{5}{8}'' \times$ length $5\ \frac{1}{2}''$) topped by a high pressure female opening (I.D. $1\ \frac{1}{2}''$, length $\frac{1}{2}''$, threaded 1 in deep, 12 threads in^{-1}) above a $\frac{3}{8}$ in long $60''$ taper. In addition, there is a high-pressure female opening (O.D. $\frac{1}{8}''$) at the bottom of the vessel. Two side arms extend from the vessel: one leads from the injection valve to the liquid phase, and the other from the gas phase to the magnetically driven pump. The pump draws vapor from the headspace and bubbles it up through the liquid. Pressure in the vessel is maintained by a back pressure regulator and measured by a Bourdon tube transducer coupled to a digital voltmeter for display. Temperature is controlled by the oven, which may be operated up to 260 °C.

Nelson et al. [12.76] investigated the effects of hyperbaric helium pressures on the growth and metabolism of the deep-sea hyperthermophilic archaeon, strain designate ES4 (that grows up to 110 °C) in a HPHT bioreactor. Growth studies performed at pressures up to 500 atm in a stainless steel vessel with and without a glass lining indicate that the behavior of ES4, specifically, its ability to grow and its response to elevated hyperbaric pressure, depends overwhelmingly on its environment. ES4 was grown at hyperbaric pressures in an HPHTBR originally developed by *Miller* et al. [12.73] but modified in this study as detailed below. Three pneumatically actuated valves were installed in the gas sampling line for automated sampling. The compressed-air supply to these valves was controlled by electronically actuated solenoid valves, which were in turn controlled by an integrator through a sample/event control module. The integrator was programmed to withdraw samples at any desired interval with less than a 0.75% drop in total system pressure at 500 atm. (The pressure drop was $\approx 2.5\%$ with each sample at 8 atm.) The sample loop had an internal volume of 0.20 cm^3 for pressures up to 10 atm; for higher pressures, its volume was 0.03 cm^3. The larger sample volume at lower pressures was necessary to purge all lines. At each sampling time, two samples were taken: the first to purge the sample lines, and the second for analysis. The lines were evacuated with a vacuum pump between

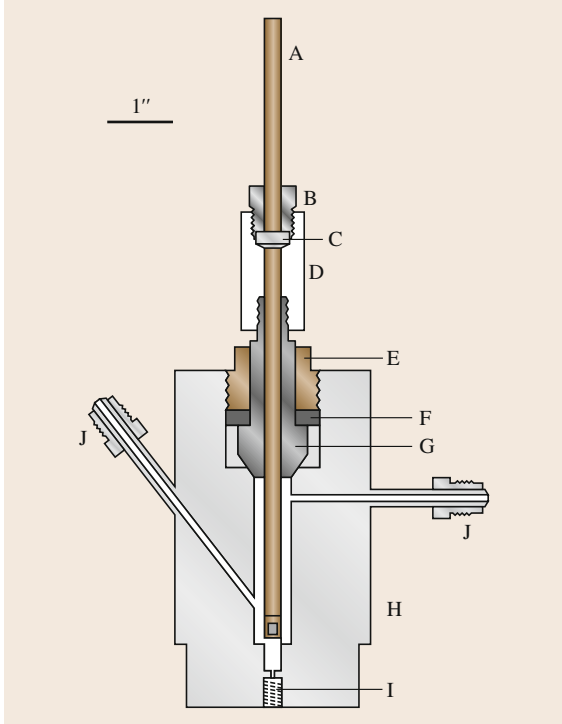

Fig. 12.39 Reaction vessel and fiber optic probe assembly: A – SS316 tube containing optical fibers, B – $\frac{1}{4}$ in. O.D. tube taper-seal gland, C – $\frac{1}{4}$ in. O.D. tube taper-seal sleeve, D – modified $\frac{1}{4}$ in. O.D. tube taper sealto- $\frac{1}{4}$ in. NPT coupling, E – custom $\frac{7}{8}$ in. high-pressure gland, F – washers, G – $\frac{7}{8}$ in. O.D. $\times \frac{1}{4}$ in. I.D. high-pressure plug with $\frac{1}{4}$ in NPT connector (H) SS316 reaction vessel, (I) $\frac{1}{8}$ in. high-pressure female opening, and J – $\frac{1}{4}$ in. high-pressure collar and gland (after *Miller* et al. [12.75])

all samples. For pressures > 10 atm, a 20 cm^3 reservoir was included between the gas chromatograph and the reactor to allow decompression of the gas sample before it entered the G.C. A back-pressure regulator maintained the gas sample pressure at 120 kPa while the temperature was constant at 100 °C. Helium was used to pressurize the system, and the concentrations of CO_2 and H_2S were measured with a G.C. The final modification was the incorporation of a liquid sampling device that allowed the slow decompression of liquid samples before their removal from the reactor system.

An S.S. 316 high-pressure syringe (internal volume 2.5 mL) was used with a maximum working pressure > 1000 atm. A polycarbonate piston equipped with two

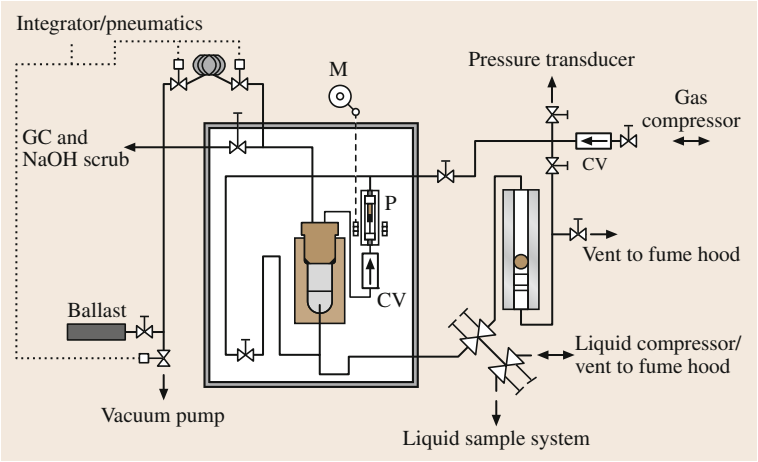

Fig. 12.40 Schematic diagram of modified high-pressure system. CV – check valves; P – gas recirculation pump; M – motor-driven crank; GC – gas chromatograph (after *Nelson* et al. [12.77])

O-rings, separated the culture broth from the hydraulic fluid. A minimum pressure drop of 100 psi across the piston is required for its movement. A second high-pressure syringe (maximum working pressure 250 atm, internal volume 1 mL) was equipped with polycarbonate windows for direct viewing of the sample during decompression.

Nelson et al. [12.77] designed a bioreactor for precise determination of the growth rate as a function of hyperbaric and hydrostatic pressures, simultaneously. The effects of hyperbaric and hydrostatic pressures of up to 500 atm on the growth rate of the deep sea isolate ES4 (*Nelson* et al. [12.76]) were compared at 95, 100, and 105 °C. Results indicate that the microbial growth rate at elevated pressures could have a remarkable dependence on the mode of pressurization.

A previously used high-pressure reactor system (*Nelson* et al. [12.76]) was modified in this study to facilitate studies on hydrostatic pressure concurrently with hyperbaric experiments (Fig. 12.40). A second high-pressure vessel was connected to the gas and liquid lines feeding the hyperbaric system. The temperature and pressure of each vessel were controllable independently, or the pressure could be equilibrated between the two. Temperature was microprocessor-controlled with two types of J thermocouples (one internal and one external) as the input source for the controller. Although He is used as a pressurizing gas, a Teflon piston equipped with O-rings provided a barrier against the direct liquid–gas contact. A ferromagnetic steel ball enabled magnetic mixing of the liquid as required and also marked the location of the piston. The ball could be magnetically lifted to promote mixing prior to sampling, or a stack of ceramic ring magnets could be oscillated up and down by a motor-driven crank and cable if continuous mixing was desired. The hydrostatic vessel (total internal volume 58 mL) has a working volume of 50 mL. The reactor consists of an S.S. 316 tube (length 18″, O.D. 1″, I.D. $\frac{1}{2}$″), with a maximum working pressure of 15 000 psi and a 24 karat gold plated interior surface to reduce corrosion and consequent leaching. The interior of the hyperbaric system was also treated similarly to minimize differences between the two systems. All wetted thermocouples and the steel ball were also gold plated. To minimize erosion of the gold due to piston motion, subsized O-rings (nominal O.D. $\frac{3}{8}$″) were used on the piston, which were stretched to provide a seal. Since the pressure drop across the piston was less than 1 atm when static (up to 10 atm during sampling), little leakage across the piston was detected. It was also possible to use standard $\frac{1}{2}$″. O-rings with deeper-than-standard grooves in the piston to minimize compression of the O-ring. This approach has an advantage of longer O-ring life but could reduce the lifetime of the piston at high pressure. Vertical alignment of the vessel facilitated the removal of trapped gases and containment of sulfur when required.

Hei and *Clark* [12.78] studied the pressure stabilization of enzymes, including a hydrogenase from *M. jannaschii*, an extremely thermophilic deep-sea methanogen, in an anaerobic HTHPBR. *M. igneus, M. jannaschii, M. thermolithotrophicus, and M. maripaludis* (all thermophilic archaea) were grown in artificial seawater medium with a continuous anaerobic gas phase of 20% CO_2 and 80% H_2. The bioreactor (internal volume 10 mL) and all tubings and fittings were constructed of S.S. 316, pressurized with oxygen-

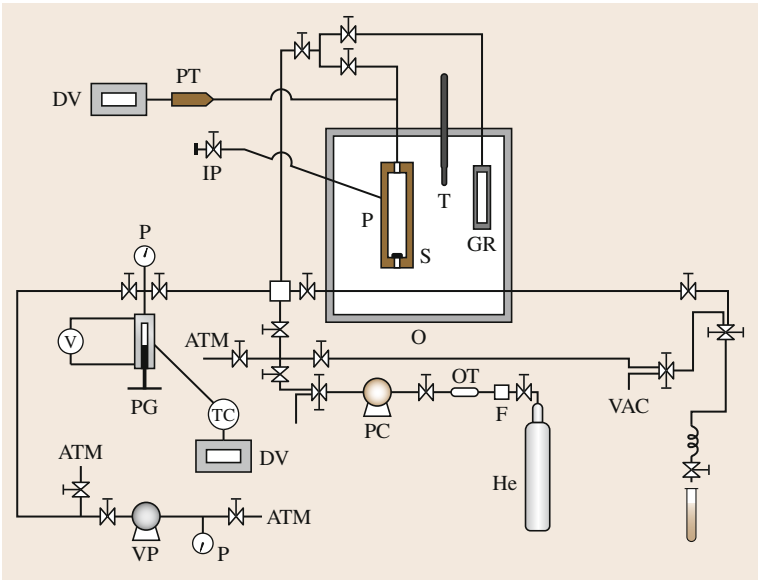

Fig. 12.41 Schematic diagram of the high-pressure reactor used in pressure stability studies. Components shown: P – stainless-steel pressure vessel; GR – gas reservoir; S – water-driven magnetic stirrer; T – ASTM thermometer; O – oven; IP – injection port; PT – pressure transducer; DV – digital voltmeter; P – pressure gauge; PC – pneumatic compressor; F – filter; OT – oxygen trap; PG – pressure generator; TC – thermocouple; V – power supply to heating tape; VP – vacuum pump; ATM – line to atmosphere; VAC – line to vacuum (after *Hei* and *Clark* [12.78])

free helium compressed with a pneumatic compressor and pressure controlled with a manually operated pressure generator (Fig. 12.41). The reactor and the reservoir holding the pressurizing gas were enclosed in a forced-air oven that allowed temperature control to $\pm 0.1\,^{\circ}C$. Agitation of reactor liquid was done by a magnetic stir bar driven by a water-powered magnetic stirrer.

The effect of decompression on the structure of *M. jannaschii*, an extremely thermophilic deep-sea methanogen, was examined in a novel HPHTBR by *Park* and *Clark* [12.79]. The cell envelope of *M. jannaschii* appeared to rupture upon rapid decompression (ca. 1 s) from 260 atm of hyperbaric pressure. However, when decompression from 260 atm was performed slowly over 5 min, the proportion of ruptured cells decreased significantly. In contrast to hyperbaric decompression, decompression from a hydrostatic pressure of 260 atm did not cause cell lysis.

A confined gasket-closure reactor (Fig. 12.42) used as the hyperbaric bioreactor (volume 1.15 L) could be operated up to 200 °C and 590 atm. A thermocouple inserted into an S.S. 316 well extending three-fourths of the way into the bioreactor provided the signal to a PID controller for precise temperature control through a heating belt. A diaphragm compressor was used to supply H_2, CO_2, and He at high pressures. An oxygen trap was installed between the diaphragm compressor and the gas cylinder. The gas atmosphere in the hyperbaric bioreactor was H_2/CO_2 (4 : 1) up to 7.8 atm, and He was used for pressurization > 7.8 atm. For precise pressure control, an air-actuated backpressure regulator was placed in the exit line of the gas loop.

A tubular reactor (volume 65 mL) was used as the hydrostatic bioreactor which was first pressurized with H_2/CO_2 (4 : 1) to 7.8 atm, then pressure was increased to 260 atm hydrostatically by pumping in cells and growth medium. The medium (45 mL) was anaerobically inoculated in a serum bottle with 20 mL of exponentially growing *M. jannaschii* cells, followed by transfer into the hydrostatic reactor through the liquid pump until internal pressure reached 260 atm. Temperature control was achieved by incubating the pressurized hydrostatic bioreactor in an oven. The reactor was reconnected to the system and repressurized to 260 atm every 2 h throughout the incubation period, during which the pressure did not fall by > 10%. After 10 h of cultivation, the hydrostatic bioreactor was depressurized in 1 s and the culture was withdrawn for further analysis.

Girguis et al. [12.80] incubated both marine hydrocarbon seep sediments and marine nonseep (i. e., aerobic) sediments in a continuous-flow anaerobic methane incubation system (AMIS) that simulates the majority of in situ conditions and supports the metabolism and growth of anaerobic methanotrophic archaea. Methane oxidation rates and population growth of MOA (methane oxidizing archaea) were measured over the course of the incubation (24 weeks), thus allowing the evaluation of growth and metabolism of MOA in

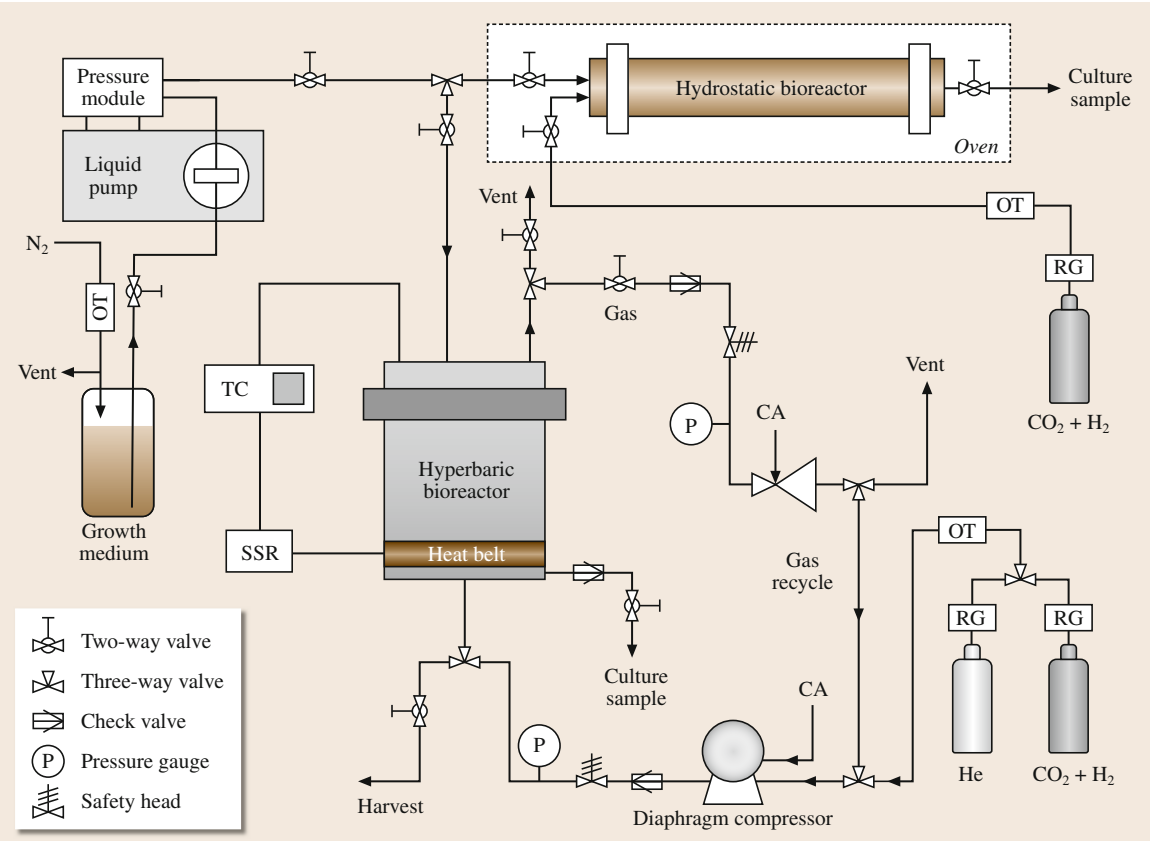

Fig. 12.42 Schematic diagram of a high-pressure, high-temperature bioreactor system. The *dotted lines* signify an oven into which the pressurized hydrostatic bioreactor was placed after being disconnected to control the temperature. SSR – solid-state relay; TC – temperature controller; OT – oxygen trap; CA – compressed air; RG – regulator (after *Park* and *Clark* [12.79])

both seep and nonseep sediments both prior and subsequent to incubation on AMIS.

Conditioned seawater was pumped out of the conditioning column by a diaphragm-metering pump through a capsule filter and rigid polypropylene tubing (I.D. 1.5 cm, O.D. 2.5 cm) into a schedule 80 (PVC) manifold (the *lower* manifold) on which four sediment cores were incubated with a gas- and water-tight seal (using double O-ring seals) maintained on the bottom of each sediment core sleeve. Gas-tight PVC ball valves were installed on both the inlet and the outlet, allowing the manifold to be sealed to maintain anaerobic conditions even during servicing. During operation, the backpressure in the lower PVC manifold was maintained at (200 kPa) by a PVC backpressure valve or by running a length of PVC pipe vertically to create a seawater pressure head at the outlet. After circulating through the manifold, seawater returned to the 25 L reservoir and was recirculated through the conditioning column. The *upper* PVC manifold consisted of PVC tees connected by rigid PVC tubing – here each sediment core sleeve was also sealed to a PVC tee fitting with O-ring seals. The upper PVC manifold was connected to a chilled seawater system (5 °C) and could be sealed at both the inlet and outlet by gas-tight PVC ball valves. During operation, the entire manifold assembly and seawater reservoir were maintained in a cold room at 5 °C. The AMIS system thus successfully stimulated the maintenance and growth of anaerobic methanotrophic archaea, and possibly their syntrophic, sulfate-reducing partners as well.

Houghton et al. [12.81] developed a *PEEK-HPLC column flow-through bioreactor* for continuous enrichment of nitrate and sulfate reducing thermophiles under

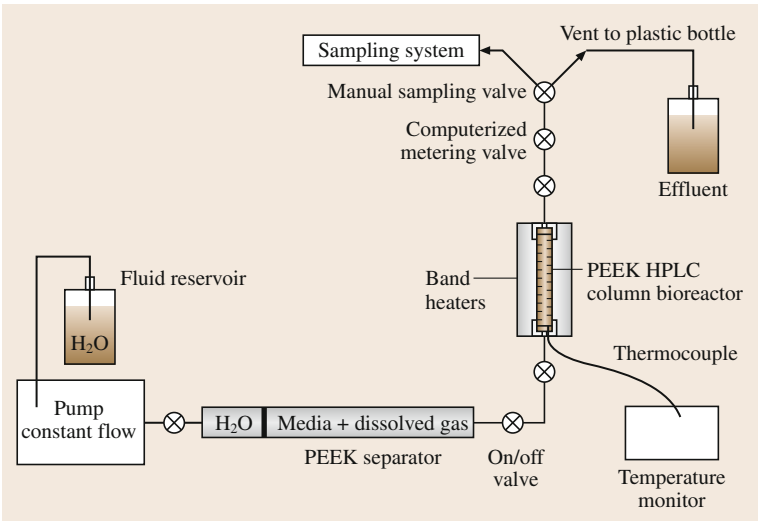

Fig. 12.43 Schematic drawing of the flow-through apparatus designed for this study. All parts were constructed of PEEK plastic, with the exception of the control valves that were composed of stainless steel. The bioreactor in line with the fluid-delivery system contains the sulfide-chimney material used for the experiments. Two thermocouples were attached to the reactor, which in conjunction with a fully automated control system, maintained temperature and pressure within very narrow limits during the 100–180 h experiments (after *Houghton* et al. [12.81])

in situ deep-sea hydrostatic pressures, in order to investigate microbial activity at conditions similar to seafloor hydrothermal vents. The experimental setup (Fig. 12.43) was designed to promote the growth of microbial populations at temperatures $< 100\,°C$ and pressures of ≈ 250 bar while permitting dynamic monitoring of changes in fluid chemistry and microbial diversity.

An HPLC pump and linked *separator* continuously provided fluid reactants and dissolved gases at high pressure to bioreactor columns made of PEEK (I.D. 0.75 cm, length 10 cm, volume 4.41 cm^3), which were maintained at a constant temperature by a series of band heaters external to the reactor and enclosed in an insulated tube furnace. Time proportioning controllers provided temperature control in response to input from a series of thermocouples directly attached to the reactors in the furnace. Except flow control valves, made from S.S. 316, all wetted parts of the fluid delivery and control system were made of PEEK, although buna O-rings were used as pressure seals throughout. Moreover, all tubing, PEEK reactors, and stainless valves were replaced between experiments to minimize contamination effects. Fluid samples could be taken throughout experiments by activating manual and computer-controlled valves, which not only diverted sample fluid into gas-tight containers for processing, but also maintained the system at the desired pressure. The media, mainly artificial seawater was prepared under anaerobic conditions, which were also maintained by bubbling CO_2 and H_2 through the media while filling the separator, which, by piston action, was used to achieve high-pressure conditions. The separator permitted appropriate amounts of gas to be dissolved in the media by filling a headspace with gas before fully pressurizing the system. The media used for all experiments was thus saturated with dissolved CO_2 and contained ≈ 2.2 mM H_2. Water delivered at high pressure to the input side of the piston in the separator ensured complete dissolution of the gas in the media as well as complete pressurization to the operational condition of 250 bar.

The study achieved growth and culture enrichment of thermophiles under conditions of temperature and pressure in a flow-through bioreactor that provided an effective means for investigating hydrothermal systems at temperatures, pressures, and chemical conditions similar to seafloor chimney (vent) deposits and their associated microbial communities.

Deusner et al. [12.82] developed a novel two-stage high-pressure bioreactor system for gas-phase free continuous incubation of enriched, highly active marine microbial consortia (prepared from microbial mats obtained from the Black Sea at a depth of 213 m) performing anaerobic oxidation of methane (AOM). The systems developed, viz., High-pressure continuous incubation system (HP-CI) and high-pressure manifold incubation system (HP-MI) were used to demonstrate the feasibility of investigating bioprocesses in high-pressure fed-batch or continuous incubation systems with gas-enriched medium without free gas in the incubation stage. In continuous operation in the HP-CI system, initial methane-dependent sulfide production increased 10–15 times on increasing methane par-

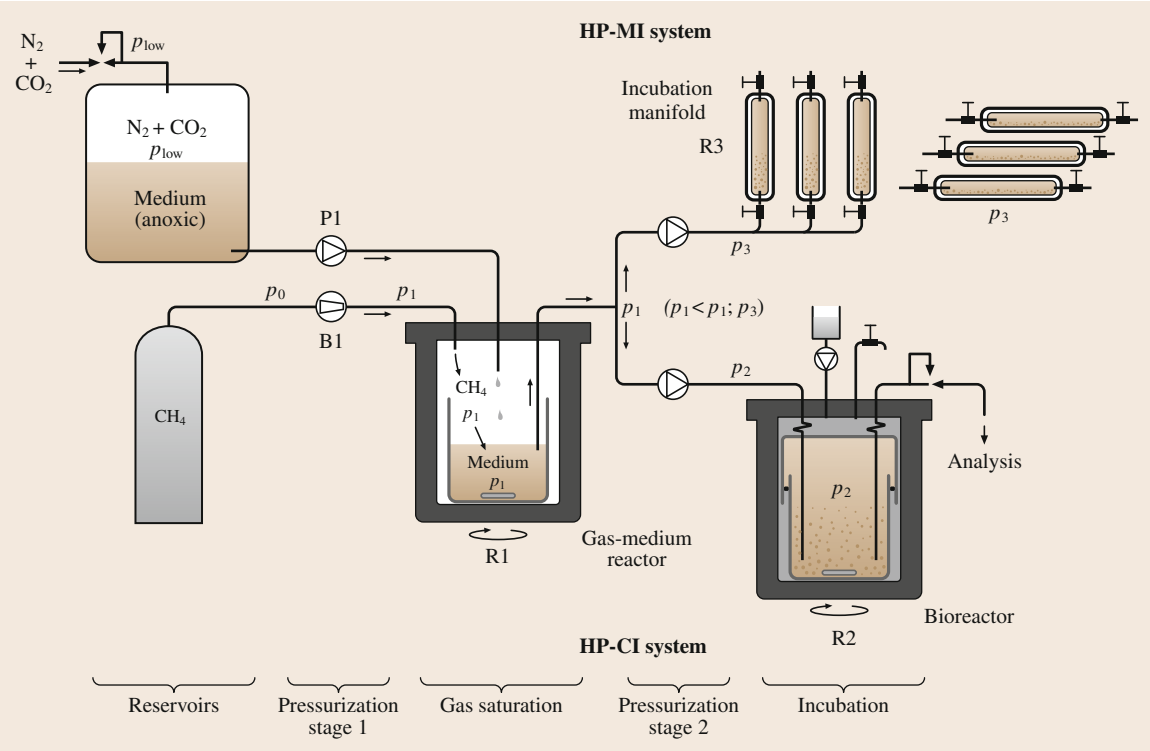

Fig. 12.44 Incubation principle. In two-stage operation, medium is enriched with methane under high-pressure conditions in the continuously stirred gas-medium reactor R1. The gas-enriched medium is further transferred to the bioreactor R2 or R3. Pressure p2 and p3 within R2 and R3, respectively, is increased compared to p1 in R1. In R1, R2, and R3 medium and culture fluid are contained in internal vessels to avoid contact to the metal surfaces. Incubation is carried out without free gas in R2 and R3. To compensate pressure/volume changes in R2 the internal vessel is adjustable in volume. Pressure vessels in R3 can be detached and manipulated separately. Sensors for electronic regulation are not indicated (after *Deusner* et al. [12.82])

tial pressure from near ambient pressure (0.2 MPa) to 10 MPa at a hydrostatic pressure of 16 MPa in the incubation stage. A methane partial pressure of 6 MPa and a hydrostatic pressure of 12 MPa in manifold fed-batch incubation in the HP-MI system gave a sixfold enhancement in the volumetric AOM rate.

The HP-CI system (Fig. 12.44) consists of a two stage high-pressure reactor, viz., a gas-medium reactor (R1) and a bioreactor (R2), along with two high-pressure pumps (P1, P2), and a gas compressor station (B1). Continuous enrichment of medium with methane in R1 occurs at pressures up to 20 MPa. In R1, while gas transfer from the headspace into the medium is expedited by agitation using a magnetic stirrer, liquid remains confined within an internal vessel (made of inert polymeric materials e.g., poly-ether-ether-ketone (PEEK) or poly-oxy-methylene (POM)) that prevents corrosion of the pressure vessel as well as contamination of the biomass. In R1 the fluid flow and the liquid level are automatically adjusted with constant – flow inflow and outflow pumps (P1 and P2) and is also monitored gravimetrically. The pressure P1 is adjusted with the gas compressor unit B1, operated in constant pressure mode, which is linked to R1 through a gas-distribution system comprising of a shut-off valve, a pressure-regulating valve, and a metering valve for fine regulation of gas flow. Pressurized gas can be supplied either batchwise or continuously, which facilitates continuous exchange of the gas phase in R1 and may be utilized for supply of low-concentration gaseous substrates during continuous operation.

Incubation in R2, may be performed up to a hydrostatic pressure of 35 MPa. The gas-enriched medium is transferred to R2 via pump P2 which is set to increase

the hydrostatic pressure relative to the pressure in R1 from P1 to P2 and allows incubation at increased hydrostatic pressure compared to the methane partial pressure in R1. The fluid flow in R2 is also automatically adjusted with pump P2 working in constant flow mode. R2 is also equipped with an internal vessel (made of PEEK or POM) to prevent potential chemical contamination of the biomass. Due to the mode of headspace-free incubation, the internal vessel is designed as a closed vessel. For compensating pressure or volume changes it is has a top and a bottom part and its volume is adjustable. The bottom part is equipped with an O-ring sealing that permits axial movement of the two parts relative to each other. The internal vessel is housed inside the pressure vessel surrounded by a hydraulic fluid that can be pressurized independently. Under standard operating conditions, the free volume for incubation is 420 mL within R2. Medium transport upstream and downstream of R2 is carried out through a tubing that is directly connected to the internal vessel through flexible lead-through systems. The internal vessel can be heat-sterilized and inoculated. The culture broth inside R2 is agitated with a magnetic stirrer. Liquid samples were taken from downstream section of both R1 and R2 at the sampling ports, directly into glass vials closed with butyl stoppers or into glass syringes after opening the shut-off needle valves in the sampling lines.

The HP-MI system (R3), originally developed (*Kallmeyer* et al. [12.71]) for high-pressure/high-temperature (HPHT) batch incubation, was modified and adapted in this study to be operated for manifold fed-batch incubation. Gas enrichment of liquid medium and delivery is attained in a similar method as in the HP-CI system. Each pressure vessel is equipped with shut-off valves at the inlet and outlet, respectively, and can be manipulated independently. Gas-enriched medium is transferred separately to each of the high-pressure vessels and each vessel is filled in upstream mode. Like R2, pressure vessels in R3 are operated without free gas and are equipped with internal vessels (free volume for incubation, 7 mL) (made of PEEK or POM), which are constructed as open vessels that seal against the steel by a polytetrafluoroethylene (PTFE) plate that presses into the cap of the steel vessel. While the medium is delivered into an internal vessel via lead-through tubings (similar to R2 in the HP-CI system), the upper outlet of an internal vessel has a built-in glass filter plate which allows permeation by both gas and liquid medium. Before sampling the biomass was allowed to settle to the bottom of the internal vessel; subsequently, an aliquot of the supernatant was collected through the bottom capillary while the sampled liquid volume was replaced with gaseous methane through the top capillary at the pressure corresponding to the saturation methane partial pressure applied during incubation. On commencing the fed-batch mode of operation, the design of the internal vessels ensured that the gas introduced during sampling was completely removed during filling of the vessels under high-pressure conditions.

This study demonstrated that biomass-specific activity increases substantially as a function of methane partial pressure in both continuous and fed-batch incubation. The systems could be used to control incubation parameters and to simulate environmental conditions with regard to constant substrate supply and product removal in continuous incubation. Stability with respect to sulfide production in continuous operation and reproducibility of results from manifold fed-batch incubation indicate that both the system components and the experimental procedure could be successfully applied for the study and incubation of AOM-performing marine microbial consortia.

Zhang et al. [12.83] developed a novel, continuous high-pressure bioreactor (HPBR) system to mimic a diverse methane-rich deep-sea ecological niche, to stimulate in vitro sulfate reduction (SR)-coupled- anaerobic oxidation of methane (AOM) activity; and to examine the kinetics of the SR-AOM process. The HPBR system (Fig. 12.45) comprises three main parts: (a) the conditioning vessel, (b) the high-pressure pump, and (c) the incubation vessel. The conditioning vessel (volume 1 L) made of S.S. 316 is directly connected to two pipettes – a liquid pipette (to charge liquid medium at high-pressure) and a gas pipette (to charge methane gas at high-pressure) both of which are pressurized by the methane gas bottle. Artificial seawater medium is saturated with methane at a determined pressure (up to 8 MPa) in the conditioning vessel and transferred to the incubation vessel by a high-pressure pump. In the conditioning vessel, built-up gas pressure determines the amount of methane saturated into the medium and available as substrate. A motor-mixer (40 rpm) is used to achieve a homogeneous dissolved methane concentration in the whole vessel. The high-pressure pump is piston based with a separated chamber to rinse the piston while it is moving – this self-flushing mechanism allows feed of saline water without damage to the piston and its seals. The incubation vessel (volume 0.6 L), made of titanium to prevent potential microbial corrosion, is placed inside a thermal regulated incubator (5–55 °C) to control the incubation temperature. The pressure inside this incubation vessel could be in-

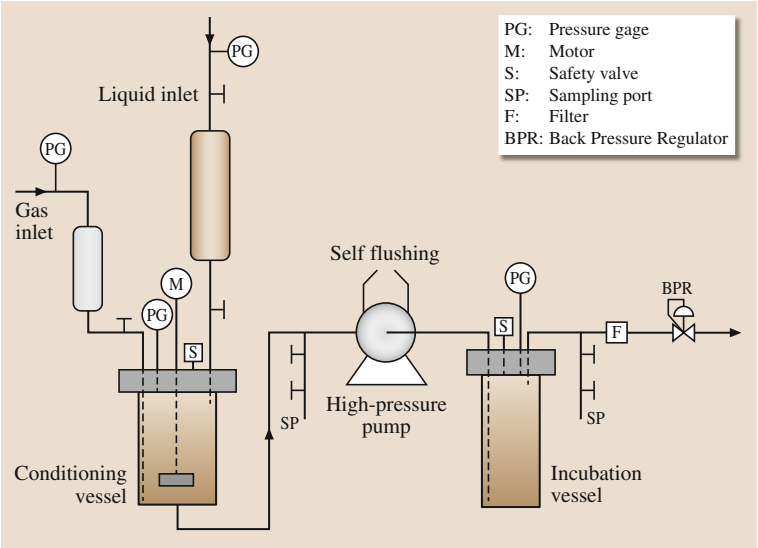

Fig. 12.45 The continuous high-pressure bioreactor system. PG – pressure gage; M – motor; S – safety valve; SP – sampling port; F – filter; BPR: back pressure regulator (after *Zhang* et al. [12.83])

creased to 16 MPa, and is regulated by the pump flow and a backpressure regulator. Two sampling ports were provided – one between the conditioning vessel and the high-pressure pump to take influent samples; the other between the incubation vessel and the backpressure regulator to take effluent samples.

Marteinsson et al. [12.84] examined the growth and survival of the deep-sea hyperthermophilic archaeon *Pyrococcus abyssi* GE5, isolated from hydrothermal vents in the North Fiji Basin at a depth of 2000 m, in sterile gas-tight glass syringes with cut pistons incubated in a high-pressure high-temperature (HPHT) cultivation system (Fig. 12.46) consisting of four S.S. pressure vessel incubators heated in four vertically positioned ovens (300 °C maximum). Pressure was generated with a hydraulic pump (with cold tap water as the hydraulic fluid) and monitored by Bourdon gauges (100 MPa) (maximum working pressure 60 MPa). Thermocouples, one internal and one external, were used for each pressure vessel, independently connected to a microprocessor in a temperature controlling unit, and the vessels could be heated from room temperature to 100 °C in < 30 min. The authors concluded that the effect of growth temperatures and pressure on the deep sea hyperthermophilic archaeon *P. abyssi* was associated with changes in the level of phospholipids more than the level of core lipids or proteins. The resistance shown by *P. abyssi* to lethal temperatures under in situ pressure was considered as the principle reason for its surviving tremendous spatial variability in temperature.

Marteinsson et al. [12.85] studied the effect of pressure, in pressurized syringes, at the temperature range for the growth of *Thermococcus barophilus* sp. nov. (Strain MP^T), a barophilic and hyperthermophilic, anaerobic, sulfur-metabolizing archaeon, isolated under high hydrostatic pressure (40 MPa) and temperature (95 °C) from a deep-sea hydrothermal vent (depth 3550 m). All culturing procedures before the high-pressure experiments were carried out under anaerobic conditions. Samples, stored at 4 °C under 40 MPa hydrostatic pressure, were depressurized and 0–5 mL suspension/fluids were transferred into 10 mL syringes which were sealed (by plunging needles into rubber stoppers), before media/samples were dispensed in them. Finally, the pistons were positioned and the gas phase was purged out before fastening the seal on each syringe. The syringes were then transferred into the HPHT incubation system (*Marteinsson* et al. [12.84]) pressurized to 40 MPa and heated to 95 °C. The archeaon was found to be barophilic at 75, 80, 85, 90, 95 and 98 °C, and was identified as an obligate barophile between 95 and 100 °C. For growth > 95 °C, a pressure of 15.0–17.5 MPa was necessary. It grew at 48–95 °C under atmospheric pressure. The optimal temperature for growth was 85 °C at both high (40 MPa) and low (0.3 MPa) pressures. The growth rate at 85 °C under in situ hydrostatic pressure was double that noted at low pressure.

Wright et al. [12.86] demonstrated the potential of high pressure as a stressing agent for marine bioprocess intensification by evaluating and confirming the

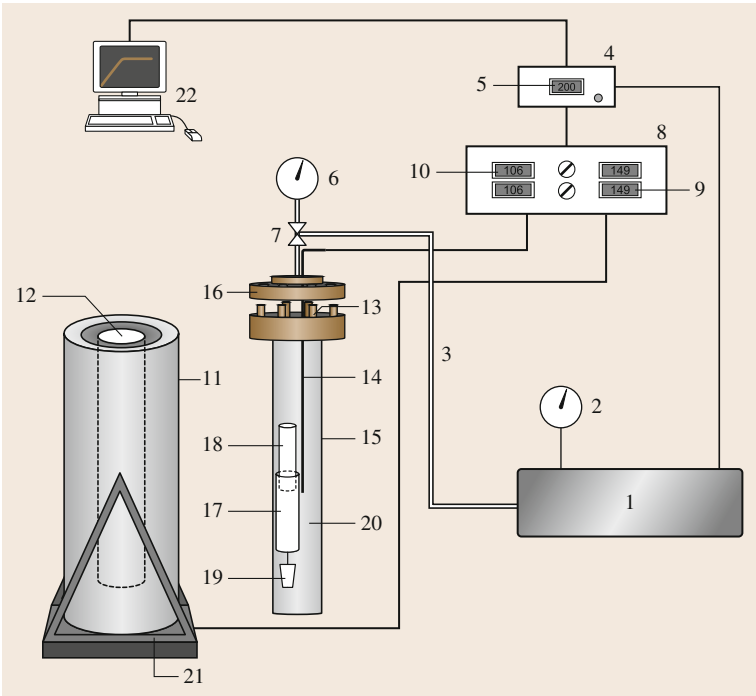

Fig. 12.46 Schematic representation of the high-pressure apparatus *hot bucket* for cultivating microorganisms at high temperature and pressure. One unit of four is shown. Each can be loaded or unloaded separately while the others are kept under constant temperature and pressure conditions. Key: 1 – hydraulic pressure generator; 2 – Bourdon gauge (100 MPa); 3 – water tube (inox); 4 – pressure indicator; 5 – digital pressure indicator; 6 – Bourdon gauge; 7 – valve; 8 – computerized independent thermoregulators; 9 – digital temperature indicator in oven; 10 – digital temperature indicator inside the pressure vessel; 11 – heating element (oven); 12 – bucket for the pressure vessel; 13 – O-ring; 14 – thermocouple in jacket; 15 – stainless steel pressure vessel; 16 – the head for the pressure vessel; 17 – culture syringe; 18 – cut piston; 19 – needle embedded in rubber stopper; 20 – water; 21 – thermocouple for heating unit; 22 – computer (after *Marteinsson* et al. [12.84])

barotolerance (at 12 MPa) of several marine bacteria isolated from shallow surface waters (< 1.5 m depth, pressure < 50 Pa) rather than from the deep sea. For this purpose, they used a high-pressure batch bioreactor (HPBBR) to create a maximum hydrostatic pressure of 120 MPa and to survey the pressure tolerance of the marine isolates. The HPBBR (maximum working volume 3.9 mL) was made of stainless steel and hydrostatic pressure was applied to the culture broth using an electrically driven piston. High-pressure cycling was employed since it is known to lower the tolerance of the microorganisms to high pressure. Interestingly, it was observed that barotolerance was much higher in bacteria obtained from biofilms anchored to the surface of seaweed than those isolated from open waters. It was noted that barotolerant rather than barophilic microorganisms is much more preferable for most bioprocess engineering operations as isolation and culture enrichment of barophiles is technologically more challenging as the barophilic strains must be continuously maintained at the remarkably high-pressure levels (> 50 MPa) for viability.

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