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Review Article

Chronicle of Marine Diatom Culturing Techniques

Supriya G and *Ramachandra TV
Energy & Wetlands Research Group, Centre for Ecological Sciences, Indian institute of Science
Bangalore 560 012, India
**Author for Correspondence*

ABSTRACT

Diatoms are regarded as useful neutral lipid sources, as liquid fuel precursors, as foods for marine culture of zooplankters, larval and post-larval shrimp, copepods, juvenile oysters and as micromachines in nanotechnology. Combining microscopic observation with *in situ* culturing has been useful in areas of taxonomy, ecology, biomonitoring, biotechnology, etc. This communication reviews various culturing techniques of marine diatoms with the relative merits.

Key words: Diatoms, isolation, culture media, marine, evolution

INTRODUCTION

Diatoms (Greek = "cut in half") are the major group of unicellular, photosynthetic and eukaryotic algae. They constitute the most speciose group of organisms (worldwide distribution ~ 200,000 species, Bentley *et al.*, 2005) and are found inhabiting a range of habitats from oceans to freshwater systems like rivers, lakes and ponds (Armbrust *et al.*, 2004). Importance of these unique intricate cell patterned organisms, since then has increased manifold in areas of taxonomy, ecology, biomonitoring, biotechnology, etc combining microscopic observation with *in situ* culturing. It has taken a long time to recognize the significance of the ubiquity of the microscopic life, revealed by Robert Hooke through his compound microscope, despite of the reliance on microorganisms (Ash *et al.*, 2002). Microscope since time immemorial has been used to understand many biological functions in prokaryotes and eukaryotes. Among all the organisms, study of diatoms was started off with microscopic observations i.e., taxonomy (Müller, 1786). Diatom taxonomy is based either on the identification of ribosomal sequences (Medlin *et al.*, 1996) or more classically on the morphology and the shape of frustules, the extracellular silica cell walls (Karthick *et al.*, 2010). Culturing of diatoms is followed in morphometry and phylogeny (Mann, *et al.*, 2008) and to understand the teratological structures in diatoms (Falasco *et al.*, 2009, Håkansson and Chepurnov, 1999) by herbicidal effects (Debenest *et al.*, 2008), etc., which can be applied in biomonitoring practices (Debenest *et al.*, 2009). Toxicological studies for metal contamination and bioaccumulation of trace metals is also done for biomonitoring applications (Wang and Dei, 2001; Price and Morel, 1990).The community structure (deJong and Admiraal, 1984, Debenest *et al.*, 2009) of diatoms could be understood to unravel ecological intricacies by culturing them in an artificial media, which mimic the natural condition of diatoms.

Culturing got impetus with Cohn (1850) cultivating unicellular flagellate *Haematococcus* (Chlorophyceae) *in situ*. However, these attempts had setback due to the absence of suitable culture media or maintenance (Preisig and Andersen, 2005). Later, Famintzin (1871) cultured algae (*Chloroccum infusionum* (Schrank) Meneghini and *Protococcus viridis* Agardh) using a media with a few inorganic salts that was adopted from Knop (1865) used for vascular plants (Preisig and Andersen, 2005).

In situ culturing helps to decipher physiological and biological processes including enzymatic behavior, genetics, etc. affecting growth of an organism in an *in vitro* environment (except when cultured in outdoor ponds). This requires appropriate culture medium or an agar medium containing essential nutrients (macronutrients, micronutrients, vitamins) and chelator elements, etc., required for the sustained growth of cells. This is being customized considering the requirement of microorganism (Pelczar *et al.*, 1993).

Culture media can be broadly grouped as marine or freshwater culture media based on the ecology of the diatom species. Although culturing of algae has a very long history of as old as 1871 (Famintzin 1871), researchers were intrigued with diatom culturing for various reasons. The various fields in which diatom culturing is done to unravel its mystery are illustrated in Figure 1. Many facets of diatom biology like sexual behavior, chloroplast and protoplast dynamics have been

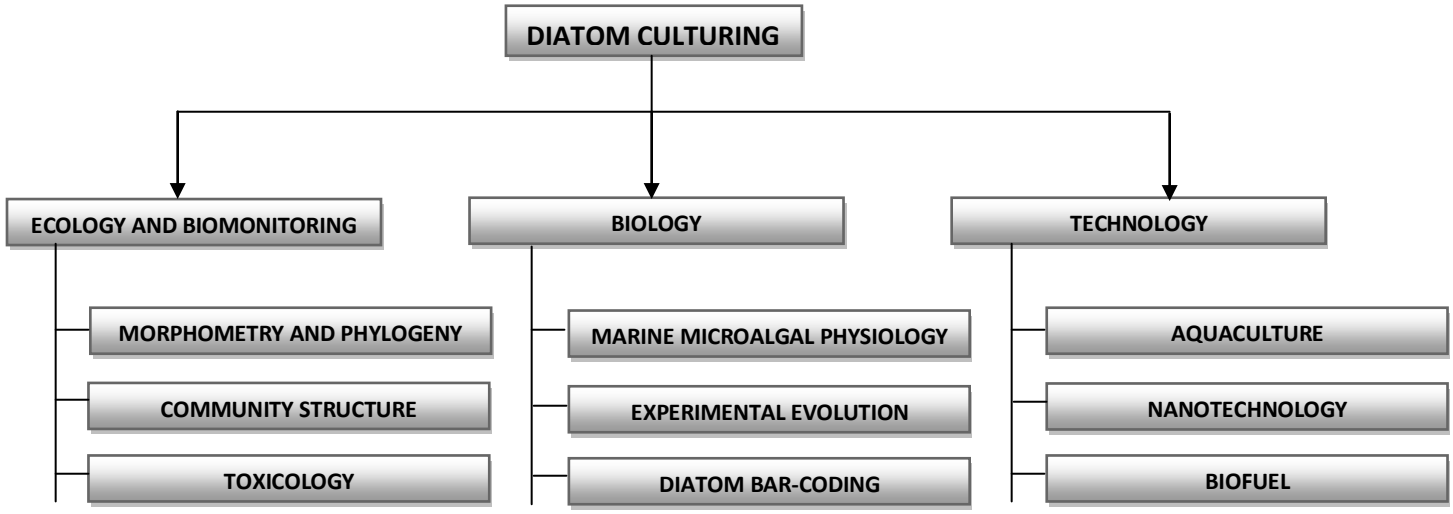

Figure 1: Applications of diatom culturing

understood with the help of in *situ* culturing (Edlund and Stoermer, 1991, Mann *et al.,* 1999, Davidovich and Bates, 1998, Chepurnov *et al.,* 2002, Sabbe *et al.,* 2004, Chepurnov *et al.,* 2004). Various physiological activities (Berland *et al.,* 1973; Lane and Morel, 2000; Reinfelder *et al.,* 2000) and evolution related questions have also been understood by culturing (Armbrust *et al.,* 2004 and Connolly *et al.,* 2006). The concept of bar-coding was introduced to diatom taxonomy (Evans *et al.,* 2007; Kaczmarska *et al.,* 2007) on the premise that the divergence of a small DNA fragment coincides with biological separation of species. This DNA fragment becomes a DNA barcode for species which can be used to flag new species, select optimal taxa for phylogenetic studies, or to signal the geographical extent of divergences in a population (Hajibabaei *et al.,* 2007). DNA bar-coding is used as an initial approach for diverse applications, followed by larger in-depth studies in the respective fields. Different DNA regions within the nuclear, mitochondrial and chloroplast genomes have been considered for testing as a universal DNA barcode for diatoms (Moinz and Kaczmarska, 2009). Culturing helps to isolate the specific diatom and also isolating nuclear, mitochondrial and chloroplast genomes for DNA barcode of a species (Moinz and Kaczmarska, 2009)

Diatoms, in particular, were regarded as useful neutral lipid sources, as liquid fuel precursors, as foods for marine culture of zooplankters (Ahlgren *et al.,* 1990), larval and postlarval shrimp (Chu, 1989), copepods (Bourdier and Amblard, 1989), juvenile oysters (Tsitsa-Tzardis *et al.,* 1993) and as micromachines in nanotechnology (Drum and Gordon, 2003). Many diatoms (*Chaetoceros muelleri* Schütt, McGinnis *et al.,* 1997; *Thalassiosira pseudonana* Hasle & Hemidal, *Pheodactylum tricornutum* Bohlin., Yu *et al.,* 2009; *Melosira varians* Agardh., *Stephanodiscus binderanus* (Kütz.) Krieger, *Cyclotella meneghiniana* Kütz., Sicko-Goad and Andresen, 1991) have been screened through culturing to assess its relevance as prospective biofuel feedstock. Gordon *et al.,* 2005 suggest the need for standardizing and scaling up of diatom *in situ* culturing to track and prevent diatom malformations associated with culturing. Silica being the component of diatom cell wall, understanding its silicification process through genetic transformation experiments, is essential in the field of diatom nanotechnology.

In the preceding sections, we explain the evolution of the successive marine diatom media, since Miquel (1892-93)'s work. As a result, this deals with primitive to a modernized isolation techniques as it forms a defining step for any species-specific experiments. We then focus on the significance of recipe compositions from 19th to 21st century.

Isolation techniques:

Diatom culturing was initially done with the natural light as the source of illumination (Miquel, 1892-93; Allen and Nelson, 1910). Later, it was Warburg (1919) and Hartmann (1921) who contributed significantly to use of electric lights as a source of illumination. Use of a screen of cold water between the lights and the cultures to avoid heating was also contributed by them (https://ccmp.bigelow.org/ accessed on 20th June 2011,

19:00 hrs). To provide light which nearly matches the natural light full spectrum, fluorescent bulbs are used (Andersen and Kawachi, 2005).

The maintenance of sterile technique was first adapted from microbial research (Beijerinck, 1890, 1891, 1893; Miquel, 1890/92a-e). These were then replaced by the Laminar Air Flow (Price *et al.,* 1989) and sophisticated microwave sterilization (Keller *et al.,* 1988). "Isolation" of an organism (or multiple organisms at a time) describes the process by which individual cells are physically separated from each other and/or from matrix material, such as water, air, soil particles, or eukaryotic tissues. Isolation therefore represents the most crucial step during the process of obtaining pure cultures (Zengler, 2009). Isolation based culturing gained impetus with Pasture's work on bacteria and fungi. A pure culture consists of one species whose identity is known and contains progeny of that species alone. Attempts of Beijerinck, a Dutch microbiologist in obtaining axenic ("pure") culture from cyanobacteria (Beijerinck, 1901) and diatoms (Beijerinck, 1904) were allegedly fruitful. Miquel (1893d) was however the first one to obtain axenic cultures of diatoms followed by Lockwood, Karsten, Stenft, (Eppley *et al.*, 1977), Richter (1903) and Chodat (1904). Invariably to acquire pure culture of diatoms, isolating techniques are very important. Isolating specific freshwater and marine diatoms into culture was primarily done by Miquel (1893a-e) with contribution of Macchiati (1892a, b, c) for obtaining axenic cultures of diatoms. The isolation techniques are broadly grouped into:

i. Manual isolation technique.

ii. Automated isolation technique.

i. Manual isolation technique:

a. Algal cells were isolated using micropipette (Miquel, 1893a-e; Preisig and Andersen, 2005). However this method required refinement as it gave bacteria-infected diatom cultures (Allen and Nelson, 1910; Peach and Drummond, 1924), although of reduced population, a detrimental factor for any pure culture. Use of Pasteur pipettes in the isolation of specific diatoms was later implemented by (Price *et al.,* 1989, Allen and Nelson, 1910; Peach and Drummond, 1924). This technique was subsequently refined to avoid bacterial contamination by picking up single cells of filaments with a capillary pipette (Preisig and Andersen, 2005). An exhaustive description of the Pasteur pipette technique is given in Algal culturing techniques (Andersen, 2005). Micropipette method gave rise to bacterized culture of diatoms although of reduced population (through Pringsheim's technique), which is detrimental to any pure culture.

The Pasteur pipette technique could be a viable method due to its narrow mouth and fine sized nozzle which is useful for the passage of most of the diatoms. However, the laborious technique has limitation in its inability to be used for the sample which has less of bacillariophyceae members as other members might pass through the opening. The above three mentioned pipettes (micropipette, capillary pipette and Pasteur pipette) have their own role to play in eliminating bacteria or other algal forms (except diatoms) to a certain extent. Depending on the opening of the pipettes they can be used for the sample ranging from a higher diatom population to a lower one.

Therefore, use of all the three techniques in complementary to each other could give an axenic culture, although, automated microinjectors could be a viable replacement of these three techniques.

b. *Agar plate method combined with antibiotic treatment:* Agar plating method is used for the isolation of diatoms infested with bacteria, algae, etc thereby acquiring axenic culture of diatoms. Generally, higher concentrations of antibiotics combined with short-term incubations were more efficient than using low concentrations for longer periods.

Algal contamination: Diatoms are first concentrated by continuous centrifugation or sonication to avoid clumping and then isolated by a micropipette onto an agar plate containing the required media. This is then followed by repeated subculturing and streaking the colonies onto agar plates (Knuckey *et al.,* 2002). Streaking of smaller fast-growing diatoms (1–5 µm) on agar plates is followed to separate the organisms without the need of antibiotics (Bruckner and Kroth, 2009).

Bacterial contamination: Microscopic observation of the larger benthic diatoms during exponential growth phase is suggested due to low population of bacteria (Bruckner and Kroth, 2009). Spreading the diatoms after ultrasound treatment (for 10 s, at an amplitude of 40 % at 0.5 s intervals) or by vortexing (10 mins) on agar plates containing high concentration of antibiotics (Penicillin G, Streptomycin and Chloramphenicol) followed by removal of single cells by a suitable micropipette is recommended.

Co-culture with *E. coli* is also recommended since many diatoms in coculture with bacteria grew denser and faster than while being axenic (Bruckner *et al.,* 2008). Often, such bacterial effects on diatom growth were inducible by E. coli. This was followed by antibiotic treatment (Penicillin G, Streptomycin and

chloramphenicol) at higher concentration. Three diatom cultures (*Achnanthes linearis* (W.Sm.), *Gomphonema clavatum* Ehr., *Navicula cincta* (Ehr.) Ralfs.) were purified by substituting the associated bacteria with E. *coli*. Purification of diatoms from unialgal cultures usually was more difficult and less successful than from biofilm samples (Knuckey *et al.*, 2002, Bruckner and Kroth, 2009).

These methods use combinations of most of the techniques and eliminate bacteria assuring high susceptibility of acquiring axenic cultures.

c. *Serial dilution method:* Serial dilution technique was developed in late 19th Century as an isolation technique to obtain axenic cultures of diatoms (Miquel, 1890/92d, e; Allen and Nelson, 1910), which later led to its exhaustive description (Kufferath, 1930; Droop, 1969 and Throndsen, 1978). However, axenic isolates are not often obtained with this dilution technique, because bacteria are usually more abundant than algae (Andersen and Kawachi, 2005).

A centrifugation technique to isolate algae was introduced by Mainx (1927). Centrifugation was done at 1000 revolutions per minute (rpm) for 10 minutes, (Price *et al.*, 1978) to separate mixed cultures of diatom species like *Thalassiosira pseudonana*, *Skeletonema costatum* (Grev.) Cl., *Cyclotella cryptic* Reimann, Lewin & Guillard, *Pheodactylum tricornutum* and *Nitzschia* species with the help of density gradients (Peroll, silica solution) (Price *et al.*, 1978). Gentle centrifugation for a short duration can be implemented for the isolation of dinoflagellates and diatoms (Andersen and Kawachi, 2005). Centrifugation technique with minimal speed ranging from 1000 – 1500 rpm for 10 minutes is apt as high speed would lead to clumping of diatom cellular mass.

ii An automated isolation technique like flow sorting was also attempted successfully to isolate diatoms (Reckermann and Colijn, 2000). Production of cultures of *Thalassiosira*, unidentified diatoms and pico-eukaryotes from mixed natural assemblages has also been done (Reckermann, 2000). The main advantage of the flow cytometric sorting is the simultaneous use of multiple cell characteristics to identify the cells enhancing much needed accuracy and speed in analysis (Ueckert *et al.*, 1995). If the sorting is done carefully, purity of the sorted cells could be as high as 98% (Hoffman and Houck, 1998). However, the disadvantages are the relative complexity, cost of the instrumentation and requirement of relatively longer time to obtain large numbers (millions) of sorted cells (Hoffman and Houck, 1998). This is not a serious concern in recent time due to the applications of the isolated diatoms in various fields.

Sophisticated instrumental techniques for the analysis and characterization of microorganisms are becoming more common. Although these newer, often experimental approaches will not replace traditional methods involving cultures, microscopy, etc. in the immediate future, their development will continue to grow (Isolation, purification, techniques, etc).

Combining techniques like flow sorting, Pasteur pipette and agar plating methods, would improve the possibility of pure isolated cultures. The former technique aids in primary isolation to quantify the diatom population from an algal sample and the latter isolates the required diatom species from the concentrated mass. Combinations of various isolation techniques are responsible for the establishment of many axenic cultures of diatoms in collections like The Provasali-Guillard National Center for Culture of Marine Phytoplankton (https://ccmp.bigelow.org/), UTEX The culture collection of Algae (http://web.biosci.utexas.edu/utex/), etc.

Diatom Media: Chronology of Evolution

"For microbes everything is everywhere, but the environment selects" (Patterson, 2009) and the environment being either natural or artificial. The preceding section, explains the artificial selectable environment. For a better understanding of the contributions during previous years, the historical development towards revolutionizing the diatom marine culture media is divided into three centuries (19th, 20th and 21st Centuries).

Miquel (1892) in 19th century suggested media recipe which is a stepping stone towards the success in further developments in diatom seawater media. Table 1 provides media recipes which showed evolution in the true sense in chronological order.

Miquel (1892) observed that the water samples (of lakes, ponds and sea) could not sustain luxuriant growth of algae in controlled conditions of the laboratory environment. Analysis showed that, natural water requires artificial enrichment of mineral salts like nitrogen, phosphorous, sulphur, potassium, calcium, magnesium, iron, silicon, sodium, bromine and iodine (Miquel, 1892). This led to the in situ culture of diatoms (freshwater and marine) with nutrient elements (Peach and Drummond, 1924). Miquel formulated a nutrient media (Miquel, 1890-93) for freshwater diatoms which subsequently tried for marine benthic diatoms (Allen and Nelson, 1910). Miquel also distinguished between "ordinary cultivations" in which one or more species are

Table 1: Molar concentrations of the nutrients found in different marine diatom medium

NUTRIENTS	18TH CENTURY		19TH CENTURY									20TH CENTURY	
	1892-93 (1)	1893-96 (2)	1910 (3)	1938 (4)	1942 (5)	1948 (6)	1957 (7)	1968 (8)	1964, 1978 (9)	1993 (10)	1987 (11)	2001 (12)	2007 (13)
$MgSO_4.7H_2O$	$8.30x10^{-2}$	$3.32x10^{-2}$	-	-	-	$1.01x10^{-3}$	$2.03x10^{-3}$	-	$2.00x10^{-2}$	-	-	-	-
$MgCl_2.6H_2O$	-	-	-	-	-	-	-	-	-	-	-	$4.72x10^{-2}$	$5.46\ x10^{-2}$
NaCl	$1.70x10^{-1}$	$1.37x10^{-1}$	-	-	-	$3.42x10^{-3}$	$3.08x10^{-2}$	-	$4.00x10^{-1}$	-	-	$3.63x10^{-1}$	$3.52x10^{-1}$
Na_2SO_4	$3.52x10^{-2}$	$2.82x10^{-2}$	-	-	-	-	-	-	-	-	-	$2.49x10^{-2}$	$2.16x10^{-2}$
$NaNO_3$	$2.35x10^{-2}$	-	-	-	$2.35x10^{-2}$	-	$5.88x10^{-5}$	$4.11x10^{-2}$	$1.01x10^{-3}$	$8.82x10^{-4}$	$8.82x10^{-4}$	$5.49x10^{-4}$	$3.00x10^{-4}$
Na_3PO_4	-	$1.20x10^{-2}$	-	-	-	-	-	-	-	-	-	-	-
anhy. Na_2HPO_4. 12 H_2O	$1.12x10^{-2}$	-	$1.12x10^{-2}$	$1.12x10^{-2}$	-	-	-	-	-	-	-	-	-
$Na_2SiO_3.9H_2O$	-	-	-	-	$3.50x10^{-3}$	$1.76x10^{-5}$	$5.28x10^{-5}$	-	$1.99x10^{-4}$	$1.06x10^{-4}$	$5.40x10^{-5}$	$1.06x10^{-4}$	$2.00x10^{-4}$
$Na_2EDTA.2H_2O$	-	-	-	-	-	-	$8.05x10^{-6}$	$2.26x10^{-3}$	-	$1.19x10^{-2}$	$1.11x10^{-1}$	$6.55x10^{-3}$	$2.34x10^{-5}$
$NaHCO_3$	-	-	-	-	-	-	-	-	$2.00x10^{-3}$	-	-	$2.07x10^{-3}$	$1.79x10^{-3}$
$NaH_2PO_4.H_2O$	-	-	-	-	-	-	-	-	$1.00x10^{-4}$	$3.62x10^{-5}$	-	$2.24x10^{-5}$	$2.00x10^{-5}$
Na_3citrate.$2H_2O$	-	-	-	-	-	$3.40x10^{-4}$	-	-	-	-	-	-	-
Na_2 b-glycerophosphate H_2O	-	-	-	-	-	-	-	$2.31x10^{-3}$	-	-	$9.99x10^{-6}$	-	-
$NaMoO_4.2H_2O$	-	-	-	-	-	$5.2x10^{-7}$	-	-	$5.00x10^{-3}$	$4.63x10^{-5}$	$1.47x10^{-5}$	$3.44x10^{-6}$	$5.21x10^{-8}$
NaF	-	-	-	-	-	-	-	-	-	-	-	$6.67x10^{-5}$	$5.36x10^{-5}$
$Na_2SeO_3.5H_2O$	-	-	-	-	-	-	-	-	-	-	-	$1.00x10^{-6}$	$6.46x10^{-9}$
Na_2CO_3	-	$3.77x10^{-2}$	-	-	-	-	-	-	-	-	-	-	-
Na_3VO_4	-	-	-	-	-	-	-	-	-	$1.00x10^{-5}$	-	-	-
NH_4NO_3	$1.24x10^{-2}$	-	-	-	$1.24x10^{-2}$	$6.25x10^{-4}$	-	-	-	-	-	-	-
NH_4Cl	-	-	-	-	-	-	-	-	-	-	$4.99x10^{-5}$	-	-
KNO_3	$1.98x10^{-2}$	$3.96x10^{-2}$	$1.99x10^{-1}$	$1.99x10^{-1}$	$1.98x10^{-2}$	-	-	-	-	-	-	-	-
KBr	$1.68x10^{-3}$	-	-	-	$1.68x10^{-3}$	-	-	-	$4.32x10^{-1}$	-	-	$7.25x10^{-4}$	$6.3x10^{-4}$
KCl	-	-	-	-	-	-	$8.04x10^{-4}$	-	$1.01x10^{-2}$	-	-	$8.03x10^{-3}$	$7.04x10^{-}3$
KI	$1.20x10^{-3}$	-	-	-	$1.20x10^{-3}$	-	-	-	-	-	-	-	-
K_2HPO_4	-	-	-	-	-	$2.29x10^{-4}$	$2.87x10^{-6}$	-	-	-	-	-	-
K_2CrO_4	-	-	-	-	-	-	-	-	-	$9.99x10^{-6}$	-	-	-
$CaCl_2.6H_2O$	$1.83x10^{-2}$	$3.60x10^{-2}$	$1.83x10^{-2}$	$1.83x10^{-2}$	-	-	$9.01x10^{-5}$	$1.01x10^{-2}$	-	-	-	$9.14x10^{-3}$	$7.82x10^{-3}$
Ca_2O_4Si	-	$1.45x10^{-1}$	-	-	-	-	-	-	-	-	-	-	-
$CaCO_3$	-	-	-	-	-	$1.39x10^{-4}$	-	-	-	-	-	-	-
Capantothenate	-	-	-	-	-	-	$2.09x10^{-8}$	-	$5.00x10^{-5}$	-	-	-	-

Contd…

$FeCl_3.6H_2O$	-	$3.08x10^{-3}$	-	-	$6.17x10^{-5}$	$8.95x10^{-6}$	$4.93x10^{-7}$	$3.69x10^{-3}$	$1.99x10^{-3}$	$1.17x10^{-2}$	$1.17x10^{-2}$	$6.55x10^{-6}$	$1.53x10^{-7}$
Fe EDTA	-	-	-	-	-	-	-	-	$2.29x10^{-5}$	-	-	-	-
Fe $(NH_4)_2(SO_4)_2.6H_2O$	-	-	-	-	-	-	-	$4.08x10^{-4}$	$2.43x10^{-2}$	-	-	-	-
$MnCl_2.4H_2O$	-	-	-	-	$3.18x10^{-6}$	$9.1x10^{-9}$	$9.53x10^{-7}$	-	$9.99x10^{-3}$	-	$8.99x10^{-4}$	-	$1.82x10^{-6}$
$MnSO_4.4H_2O$	-	-	-	-	-	-	-	$7.28x10^{-3}$	-	-	-	$2.32x10^{-3}$	-
H_3BO_3	-	-	-	-	$6.47x10^{-6}$	-	$9.70x10^{-6}$	$1.85x10^{-1}$	$3.99x10^{-1}$	-	-	$3.72x10^{-4}$	$3.64x10^{-4}$
H_2SeO_3	-	-	-	-	-	-	-	-	-	$1.00x10^{-5}$	$1.00x10^{-8}$	-	-
$CuSO_4.5H_2O$	-	-	-	-	$1.25x10^{-7}$	$7.87x10^{-7}$	$8.93x10^{-10}$	-	$3.00x10^{-4}$	$1.00x10^{-5}$	$1.00x10^{-5}$	-	$7.85x10^{-8}$
$ZnCl_2$	-	-	-	-	-	$7.65x10^{-7}$	$1.10x10^{-7}$	-	-	-	-	-	-
$ZnSO_4.7H_2O$	-	-	-	-	-	-	-	$7.65x10^{-4}$	$3.5x10^{-2}$	$7.99x10^{-5}$	$7.99x10^{-5}$	$2.54x10^{-4}$	$7.65x10^{-2}$
B	-	-	-	-	-	$4.62x10^{-6}$	-	-	-	-	-	-	-
$NiSO_4.6H_2O$	-	-	-	-	-	-	-	-	-	$1.00x10^{-5}$	-	-	-
$NiCl_2.6H_2O$	-	-	-	-	-	-	-	-	-	-	-	$6.27x10^{-6}$	$6.30x10^{-9}$
$CoCl_2.6H_2O$	-	-	-	-	-	-	$2.31x10^{-9}$	-	$2.98x10^{-4}$	$5.00x10^{-5}$	$4.20x10^{-5}$	-	$8.41x10^{-8}$
$CoSO_4.7H_2O$	-	-	-	-	-	-	-	$1.71x10^{-4}$	-	-	-	$5.69x10^{-5}$	-
TRIS	-	-	-	-	-	-	-	$4.12\ x10^{-2}$	$5.00x10^{-3}$	-	$9.99x10^{-4}$	-	-
EDTA	-	-	-	-	-	-	-	-	$3.76x10^{-2}$	-	-	-	-
$SrCl_2.6H_2O$	-	-	-	-	-	-	-	-	$1.68x10^{-1}$	-	-	$2.25x10^{-5}$	$4.61x10^{-5}$
Thiamine.HCl	-	-	-	-	-	-	$1.48x10^{-7}$	$1.48x10^{-6}$	$5.00x10^{-4}$	$5.93x10^{-7}$	$5.93x10^{-7}$	$2.96x10^{-4}$	$5.93x10^{-7}$
Nicotinic acid	-	-	-	-	-	-	$8.12x10^{-8}$	-	$9.99x10^{-5}$	-	-	-	-
p-aminobenzoic acid	-	-	-	-	-	-	$7.29x10^{-10}$	-	-	-	-	-	-
Biotin	-	-	-	-	-	-	$4.09x10^{-13}$	$2.22x10^{-8}$	-	$4.09x10^{-6}$	$4.09x10^{-6}$	$4.09x10^{-6}$	$4.09x10^{-9}$
Inositol	-	-	-	-	-	-	$2.78x10^{-6}$	-	$4.99x10^{-3}$	-	-	-	-
Folic acid	-	-	-	-	-	-	$4.53x10^{-11}$	-	-	-	-	-	-
Thymine	-	-	-	-	-	-	$2.67x10^{-6}$	-	-	-	-	-	-
Cyanacobalomin	-	-	-	-	-	-	$1.48x10^{-7}$	$7.37x10^{-9}$	-	$7.37x10^{-7}$	$7.37x10^{-7}$	$1.48x10^{-6}$	$7.38x10^{-10}$
Glycylglycine	-	-	-	-	-	-	-	-	$4.99x10^{-3}$	-	-	-	-
Ru	-	-	-	-	-	-	-	-	$2.39x10^{-3}$	-	-	-	-
Li	-	-	-	-	-	-	-	-	$6.10x10^{-2}$	-	-	-	-
I	-	-	-	-	-	-	-	-	$2.36x10^{-0}$	-	-	-	-

(1) Miquel, 1892-93(2) van Heurck, 1893-96(3) Allen and Nelson, 1910 (4) Ketchum and Redfield, 1938 (5) Matudaira, 1942 (6) Hunter, 1948 (7) Provosaliet al., 1957 (8) Provasoli, 1968 (9) McLachlan 1964, Goldman and McCarthy, 1978 (10) Guillard and Hargraves, 1993 (11) Keller et al., 1987 (12) Bergeset al., 2001 (13) Gagneux-Moreauxet al., 2007

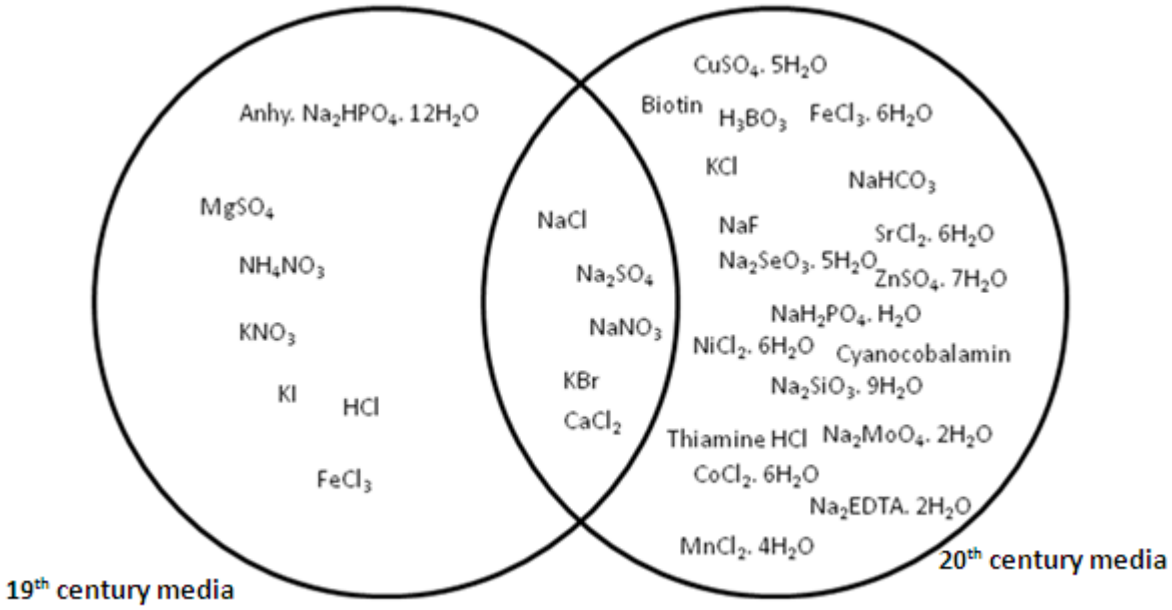

Figure 2: The presence and absence of nutrients in the 19th century (Miquel, 1892-93) and 20th century media (Gagneux-Moreaux *et al.*, 2007)

cultivated together and "pure cultivations" where a single species is made to pass through all the phases of its existence in order to follow every modification. Pure cultivations were found viable for artificial culture of diatoms and also for a number of microscopic observations (van Heurck, 1893-96). Macchiati (1892a, b, c) published theoretical data based on the experiments with the cultivation of diatoms. Further, Gill H. (van Heurck, 1893-96), also designed a media for the growth of diatoms where the salts were added into the sterilized seawater. Miquel points out the harmful effects in exposure of diatom cultivation to direct light (van Heurck, 1886). Flasks were exposed to the direct sunlight on a board, close to some glass windows which were situated facing north direction, at the same time care was taken to place between the glass and the flasks a plate of pale green glass of the height of the flask and a wooden board slightly higher than the liquid (van Heurck, 1893-96). Diatoms cultured were *Pleurosigma angulatum* W.Sm., *Cymatopleura solea* (Brèb) W.Sm. various *Nitzschia*, *Cymbella* and *Navicula* species (van Heurck, 1893-96). Subsequent contributions by Allen EJ, Nelson EW, Guillard RRL, Provasali L and coworkers paved way for the success in seawater media. The major contribution in the artificial seawater media by Allen and Nelson (1910) were done with the intention of having a suitable and a stable food in the form of diatoms to rear marine larvae. Grave (1902) cultivated diatoms as food for larvae of marine origin. He obtained diatoms by placing sand collected from the sea bottom, in aquaria. This was the first attempt to try artificial sea water for diatoms, which was a solution based on the molecular concentrations of sea water (van't Hoff, 1905). After some preliminary experiments on Miquel's media, Allen and Nelson (1910) found that; potassium nitrate, sodium nitrate and ammonium nitrate are the most important elements, resulting in the omission of potassium bromide and potassium iodide which did not affect much. They realized that silica was important for diatoms, and found potassium silicate was not a satisfactory source of silica (https://ccmp.bigelow.org/ accessed on 20th June 2011). They persistently grew many of the ecologically important diatoms *Asterionella japonica* Cl., *Biddulphia mobiliensis* (Bail.) Grun., *Biddulphia regia* (Schultze), *Chaetoceros densum* Cl., *Chaetoceros decipiens* Cl., *Chaetoceros constrictum* Grun, *Cocconeis scutellum* Ehr. var. *minutissima* Grun, *Coscinodiscus excentricus* Ehr, *Coscinodiscus granii* Gough, *Ditylium brightwellii* (West) Grun., *Lauderia borealis* Grun., *Nitzschia closterium* W.Sm., *Phaeodactylum tricornutum*, *Nitzschia seriata* Cl., *Rhizosolenia stolterfothii* Perag, *Skeletonema costatum*, *Streptotheca thamensis* Shrubs., *Thalassiosira decipiens* Grun) but were contaminated

with bacteria(Allen and Nelson, 1910). They also reported that "Miquel's Sea-water" in addition to the growth of diatoms also supports several other unidentified species of Rhodophyceae, Myxophyceae, filamentous Chlorophyceae (*Enteromorpha*, *Vaucheria*, etc.) and even young plants of Laminaria (Provasali *et al.*, 1957).

Foyn's Erd-Schreiber medium (1934) is a combination of Schreiber's medium (Schreiber, 1927) and soil extract. Gross in the early 1930's used the modified media (Foyn's) to cultivate pure cultures of marine diatoms - *Biddulphia mobiliensis* (Bail.) Grun., *Chaetoceros didymus* Ehr., *Chaetoceros pseudocritinus* Ostenfeld, *Coscinodiscus excentricus*, *Coscinodiscus granii* Gough, *Coscinodiscus radiatus* Ehr., *Coscinodiscus sub-bulliens* Jörgensen, *Coscinodiscus obscurus* (?), *Coscinodiscus* sp., *Ditylium brightwelli* (West), *Melosira borreri* Grev., *Rhiziosolenia alata* Brightw. f. *indica* (Pérag.), *Skeletonema costatum*, *Streptotheca thamensis* and *Thalassiosira* sp.

Ketchum and Redfield's media a modified variant of Allen and Nelson's media with $MgSO_4$ to enrich media to culture *Navicula closterium* and produced a continuous supply of axenic culture. This media was subsequently used to culture other unicellular organisms which require physiological research (Ketchum and Redfield, 1938). Matudaira in 1942 modified Miquel's solution with compounds like sodium bicarbonate, sodium silicate, manganese chloride, boric acid and copper sulphate to obtain the effects of inorganic sulphides on *Skeletonema costatum*.

Comprehensive review of Provasali *et al.*, (1957) on the development of marine media to culture marine diatoms gave a host of new recipes. This also showed that sea water substitutes based on analyses of sea water retain the defects of the former and are unsuitable for most species even when enriched with essential trace elements. Small additions of extracts of natural substances improve the media. The ASP – 2 Medium, an artificial seawater medium designed by Provasali *et al.*, (1957), was to serve both for bacterized and pure cultures of photosynthetic marine algae. The media had a lower value of nitrate and phosphate to suppress excessive bacterial growth. As the isolated marine algae are being strict phototrophs, no carbon sources were added. Due to these, there was no bacterial growth in the media. The most prominent element in their S3 vitamin mixture was Cyanacobalamin (B_{12}) followed by thiamine and biotin. Other elements like Nicotinic acid, Thymine, Inositol, Ca pantothenate, p-Aminobenzoic acid and Folic acid were added as a precautionary measure. It allowed the growth of several diatoms, chrysomonads, cryptomonads, dinoflagellates, blue-green algae and chlorophytes and was a very good medium for *Phormidium persicinum* Gomont., *Gyrodinium californicum* Bursa.and two other species of *Gyrodinium*, *Amphidinium klebsii* Carter, *Prymnesium parvum* Carter, *Rhodomonas lens* Pascher & Ruttner, *Stephanopyxis turris* (Grev.) Ralfs. and *Pilinia* sp. It has been found, however, that some organisms may require more trace metals or more metal chelators or both. Their further modification of parent media by the addition of Nitrilotriacetic acid (ASP- 2 NTA) (Provasali *et al.*, 1957) was found to be useful for growing diatoms like *Chaetoceros ceratosporus* Ostenfeld (Yamaguchi *et al.*, 2005). The media has separate component for vitamins, while Tris and Nitrilotriacetic acid played a role as buffers.

ASP-M media (McLachlan 1964, Goldman and McCarthy 1978) an artificial enriched sea water medium was derived from the Provasoli's earlier ASP Medium series for culturing marine macro and micro algae. The trace metal solutions (TMS II) were derived from the S1 metal solution Provasali and Pintner (1953) alongwith a complex vitamin solution.

The ES1/3 enrichment solution results in a third of the ES enrichment (Provasoli, 1968) for a main part of elements and the vitamin solution is that described by Guillard and Ryther (1962). ES1/3 appeared to be more suitable for H. *ostrearia*. Robert (1983) obtained long-term productive cultures of H. *ostrearia* after modifying the enriched seawater medium ES (Provasoli, 1968).

The original artificial Aquil medium (Morel *et al.*, 1979) was modified by Price *et al.*, (1989). The modifications are as follows:

Major nutrient solutions:

- The major nutrients PO_4^{3-}, NO_3^- and SiO_3^{2-} are prepared as concentrated stock solutions.
- The concentration of $SiO3^{2\Pi}$ and NO_3^- is increased to 10^{-4} and 3 X 10^{-4} respectively.
- The nutrient solutions are diluted with Q-H_2O to a get a final concentration twice that of Aquil and the chelaxed together.

Trace metal enrichment:

- The concentration of Molybdenum (Mo) is increased from 1.5 to 100 nM.
- $NaMoO_4$ is used in place of $(NH_4)_6.Mo_7O_{24}.4H_2O$.
- Na_2SeO_3 is included in the composition at a concentration of 10nM.

- The concentration of EDTA is increased from 5µM to 10 or 100µM to minimize the effects of contaminating metals.

Thalassiosira pseudonana (clone CCMP 1335), *Thalassiosira weissflogii* (Grunow) Fryxell & Hasle (clone ACTIN, CCMP 1336) (Roberts *et al.,* 2007), *Emiliania huxleyi* (Lohmann) Hay & Mohler (Dupont *et al.,* 2004), *Thalassiosira oceanica* Hasle (Granger *et al.,* 2004) were cultured in this media. Aquil media is best suited to study the physiological studies related to trace metal metabolism (Gagneux-Moreaux *et al.,* 2007). Moreau (1996) used the artificial Aquil medium (Morel *et al.,* 1979; Price *et al.,* 1989) and f/50 medium derived from the enriched seawater f/2 medium (Guillard, 1982) for experiments involving cultures of *Haslea ostrearia* (Gallion) Simonsen.

The L1 medium (Guillard and Hargraves, 1993) a natural seawater enriched media is a modification of the f/2 medium. . The difference is a broader trace metal composition in L1. L1-trace metal solution is used also in many other media like the *Ostreococcus* Medium Brian Palenik (https://ccmp.bigelow.org/ accessed on 20th June 2011). Culture collection like Scandinavian Culture Collection of Algae and Protozoa use this media as a standard medium for marine diatoms (http://www.sccap.dk/media/marine/2.asp accessed on 20th June 2011). *Thalassiosira pseudonana*, *Phaeodactylum tricornutum* (Ast *et al.,* 2009). *Chaetoceros elmorei* Boyer, *Cyclotella quillensis* Bailey, *Cymbella pusilla* Grun. and *Anomoeoneis costata* (Kütz.) Hust. Trace elements, vitamins and silica were added according to the `L1' medium (Saros and Fritz, 2002).

K medium (Keller *et al.,* 1987) was developed for oligotrophic marine phytoplanktons. The prominent feature of this medium is that it uses 10-fold higher EDTA chelation than most common marine media, and hence availability of trace metals, thereby reducing the possibility of metal toxicity. Drawback lies in the high macronutrient for some ocean organisms and the precipitation of silica. Algal culturing book prescribes of using natural oligotrophic ocean water rather than coastal seawater for the base. Diatoms used were *Pseudonitzschia pungens* (Grun. ex Cl.) Hasle., *Pseudonitzschia fraudulenta* (Cl.) Hasle., *Pseudonitzschia pungens* v. *pungens* and *Pseudonitzschia pungens* v. *multiseries* Hasle (Hargraves *et al.,* 1993).

The artificial medium ESAW (Berges *et al.,* 2001) is a modified media of the Harrison *et al.,* (1980). The artificial medium, ESAW (Harrison *et al.,* 1980) based on artificial seawater medium was similar to the ionic composition of sea water (Kester *et al.,* 1967) enriched with Provasoli's ES solution to balance the macronutrient and chelate concentrations. After the proposed ESAW medium by Harrison *et al.,* 1980, numerous minor changes led to a modified ESAW medium (Berges *et al.,* 2001). Berges *et al.,* 2001 found that the modified media has improved the older one significantly. The only modifications lie in the:

- Addition of borate in the salt solution (Original: Addition of borate in trace metals)
- Inorganic phosphate (Original: Glycerophosphate)
- Preparing silicate stock solution at half strength without acidification.
- Additional trace elements like Na_2MoO_4. $2H_2O$, Na_2SeO_3 and $NiCl_2$. $6H_2O$.
- Iron added as chloride (to remove ammonium) from a separate stock with equimolar EDTA.
- Filter sterilization (Berges *et al.,* 2001)

Diatom artificial medium (DAM) was developed based on the Aquil model (Gagneux-Moreaux *et al.,* 2007). The diatom artificial medium DAM allows long-term and productive culturing of *Haslea ostrearia* in controlled conditions. DAM contains the various elements in sufficient amounts for the optimal development of this diatom. This medium would allow the study of the potential bioaccumulation of metals in H. *ostrearia* (absorption and adsorption of metals, kinetics) and to evaluate their impact on the growth and the culture quality under controlled conditions. Consequently, DAM was considered as a well-adapted artificial medium for H. *ostrearia* culture. *Amphora hyaline* Kütz., *Bacillaria paradoxa* Gmelin., *Chaetoceros* sp. *Coscinodiscus granii, Haslea crucigera* (W.Sm.) Simonsen, *Navicula ramosissima* (Ag.) Cl., *Nitzschia compressa* (Bailey) Boyer, *Odontella aurita* (Lyngbye) Ag., *Phaeodactylum tricornutum*, *Pleurosigma intermedium* W.Sm. and *Skeletonema costatum*, *Thalassionema* sp.

CONCLUSION

The micropipette, capillary tube methods still lack in obtaining bacteria free cultures. Although sophisticated instrumental techniques for the analysis and characterization are newer, often these experimental approaches will not replace traditional methods involving culturing, microscopy and extraction. Similar to the variety in the isolation methods, culture media recipes are enormous. The advancement in the

techniques over a century has provided several advanced culture media with diverse composition. The evolution in the media lies in the addition of more nutrients which mimics the natural environment. Miquel's (1892-93) recipe contained fewer amounts of nutrients but included all the important nutrients; it lacked the inclusion of the trace elements, vitamins and some of the macro nutrients mentioned in the complex media diatom artificial media (Figure 2). The most "evolved" diatom culture media is important for growing various microalgae and also to understand the metal impact on the physiology (Gagneux-Moreaux *et al.*, 2007).

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