Cercetări marine	I.R.C.M.	Nr. 4	25-39	1972

MONOSPECIFIC CULTURE ISOLATION OF SOME PHYTOPLANKTONIC ALGAE OF NAPLES GULF

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ABSTRACT

Taking into consideration the ecological and physyological requirements of planktonic and bentonic unicellular algae, a new method was established for isolation in monospecific cultures. For those organisms, that developed well on the solid media, Mulvany's microbiological method (1969) was adapted.

There has passed at least a century since the first attempts to cultivate the unicellular algae with a view to understanding their behaviour and, later, their practical significance (COMBS, 1952; DE, 1939).

Specialized literature mentions several isolation techniques (DUKER and WILLOUGHBY, 1964; LEWIN, 1959; PINTNER and PROVASOLI, 1958; SOLI, 1963; SOLI, 1964; TASSIGNY, LAPORTE and POURIOT, 1969; WURTZ, 1948, ZSUSZA and FELFODDY, 1959) but our attempts to apply them were not successfull. We suppose that these lame

attempts are due to the fact that respective methods are not adequate for the species being the subject of our experiences. Besides they need either a longer period to be obtained, or a manipulation technique of great accuracy. For instance, to isolate some species from the Black Sea, we tried the method of micropipette washing and the isolation of only one cell; in most of our attempts obtaining of the culture lasted for a long time and there was possible only the isolation of frequent species from the Black Sea phytoplankton.

The purpose of our paper is to describe a new and very easy method, which ensures the possibility of obtaining in the culture the vegetable microorganisms less frequent or even very scarce.

All information presented hereinafter were obtained under the auspices of Dohrn Foundation. We express our gratitude to Dr. Dohrn Piedro for his generous assistance. We also wish to thank Dr. Dohrn Antoanetta for her aid during the carrying out of this work.

Method and working technique

I. Culture media

The present method is based upon the following ecological - physyological observations :

- the interdependence between the electrolytic dissociation degree of the salts of the medium and the self feeding capacity of the organisms (FOGG, 1947 ; PROVASOLI, Mc LAUGHLIN and PINTNER, 1953 ; PROVASOLI, 1960) ;

- the ability of unicellular algae to feed autotrophically or heterotrophically, depending on their ecological niche (PROVASOLI and McLAUGHLIN, 1963; GRIFFITS, 1967).

Taking into account the above mentioned there were elaborated forty five medium variants belonging to two categories :

1. Liquid media

Two types of liquid medium were used: "PM", exclussively with mineral composition and "AA" with both mineral and organic

composition. For the "PM" type there were made ten succesive concentrations (table 1). The "AA" type medium, in each of its five variants, had mineral salts in the same concentrations, the difference being only in additional organis substances, both quantitatively and qualitatively (table 2).

2. Solid media

The solid media were obtained from all the variants of liquid media (table 1 and 2) by adding 0.5 and 1% of agar-agar. These rates of agar-agar addition were sugested by earlier observations which showed that not all the algae are able to develop on a very solid medium ; on the other hand, on a strongly solidified medium, the algae lose their characteristic shape, their identification becoming difficult.

II. Obtaining of raw cultures

All the media, both liquid and solid, were inseminated, from twelve water samples, of Naples Gulf, taken during a sampling cruise in the 20th of February 1970.

The inseminations were made as follows :

- for liquid media, we inseminated 0.05 - 5.0 ml of sea water, in each bottle ; the bottles were shaken to ensure the dissemination of the cells within the liquid ;

- for solid media, the inseminations were carried out in two ways :

a) direct inseminations of sea water on agar-agar, by micropipetting.

b) 15-1000 ml of sea water were filtered on HA Millipore filters and then the filters were placed on the apartmess surface.

Both the bottles and Petri dishes were exposed in a thermostable room, at 19^{o}C, and were illuminated for eight hours onday with fluorescent light, of 3000 lx intensity. The inseminated media remain motionless until the emergence of the raw cultures.

III. Isolation of monocultures from raw cultures

At the starting time of algae division it is noticed the appearance of different colour spots both on bottle bottoms and on agar-agar or on filters upon the agar-agar. The spots represent the development zones of inseminated species, each spot being the colory of a single alga (fig. 1, 2, 3, 4). This is the convenient moment to pick out some cells from the middle of a spot. The operation is carried out with the help of a very fine Pasteur pipette, wich permits by capillarity the ascension of a reduced number of cells without being necessary to clean the algae colony. The picked cells will be reinseminated in a fresh, sterile medium.

On picking out, the stirring of the culture flask and the sudeen movements of the Pasteur pipette must be avoided both in liquid medium and on agarised surface or on filters. The stirring leads to the penetration of several species in pipette capillar. So, it is possible to obtain a monospecific culture, after only one reinsemination, only when the operation is carried out cautiously. When the optimal time of isolation is exceeded, the colonies grow much, often interfering (fig. 5) and the picking out leads to the picking of two or more species. Successive dilutions and reinseminations will be resorted to until the desired organism are obtained in culture.

The direct insemination on agar-agar, by micropipetting did not yield satisfactory results : after three or four days after the insemination the whole surface of agar-agar plate was infected by bacteria, and after six days amoebas appeared; so, the samples being compromised.

Good results were obtained by using inseminations upon filters (method adapted from the bacteria isolation technique of MULVANY, 1969). After the appearance of the algae spots, the filter is removed on a slant and observed with a magnifying glass. The areas with developed algae of interest shall be fixed and then pickings shall be made with the help of a Pasteur pipette or of an adequate tool. The cells will be inseminated in an adequate sterile medium like in the first case.

When colonies of ephifite algae have developed over large areas

Table 1

The concentrations of different culture media

Chemical compounds	Media	PMa	PMb	PMc	PM1	PM2	PM3	PM4	PM5	PM6	PM7
$NaNO_3$	mg	43.74	87.50	175.00	350.00	700.00	1050.00	1400.00	1750.00	2100.00	2450.00
Na_2glycerophosph	"	6.24	12.50	25.00	50.00	100.00	150.00	200.00	250.00	300.00	350.00
$Fe(NH_4)_2(SO_4)_2 6H_2O$	"	0.36	4.34	8.70	17.40	34.80	52.20	69.60	87.00	104.40	121.00
Na_2EDTA	"	2.06	4.12	8.25	16.50	33.00	49.50	66.00	82.50	99.00	115,50
H_3BO_3	"	3.60	7.40	15.00	30.00	60.00	92.00	120.00	150.00	180.00	210.00
$FeCl_3 \cdot 6H_2O$	"	0,18	0.36	0.75	1.50	3.00	4.50	6.00	7.50	9.00	10.50
$SO_4Mn.4H_2O$	"	0.50	1.00	2.00	5.00	8.00	10.20	16.00	20.00	24.00	28.00
$ZnSO_4 \cdot 7H_2O$	"	0.70	1.40	0.03	0.60	1.20	1.80	2.40	3.00	3.60	4.20
$CoSO_4 \cdot 7H_2O$	"	0.12	1.24	0.50	0.10	0.20	0.30	0.40	0.50	0.60	0.70
Vitamina B_1	"	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamina B_2	"	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Vitamina B_{12}	µg	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00

Sterilized redistrilled water 250 ml

Sterilized sea water 750 ml

Table 2

The composition of "organic" media

Chemical compounds	Media	AA^{10}	AA^{11}	AA^{12}	AA^{13}	AA^{14}
KNO_3	mg	350	350	350	350	350
Na_2HPO_4	"	100	100	100	100	100
Na_2EDTA	"	50	50	50	50	50
Proline	"	12	-	-	-	-
Asparagine	"	-	12	-	-	20
Arginine	"	-	-	12	-	-
Alanine	"	-	-	-	20	-
Glicocol	"	-	-	-	-	20
Histidine	"	-	-	-	-	20

Sterilized redistilled water 250 ml
Sterilized sea water 750 ml.

on the filters, it is possible to cut up those filter areas, to wash them carefully in sterile culture medium and place them in flasks with liquid medium, ensuring both the monospecificity and the removal of bacteria.

IV. The removal of colourless flagellates

Colourleass flagellates appear frequently in unicellular algae cultures; we could not elimnate them by dilution. They were destroyed under microscope with coffeine (BOWNE, 1959). From the algal suspension drop treated and examined under the microscope, there were carried out inseminations immediatly after the death of the flagellates.

Discussions

By using these methods we have obtained in culture thirteen species of algae belonging to the following groups : diatomae, chlophyceae and cocolitophoridae (table 3). Following table 3, in which are given the isolated species and the media on which they preferably developed, we

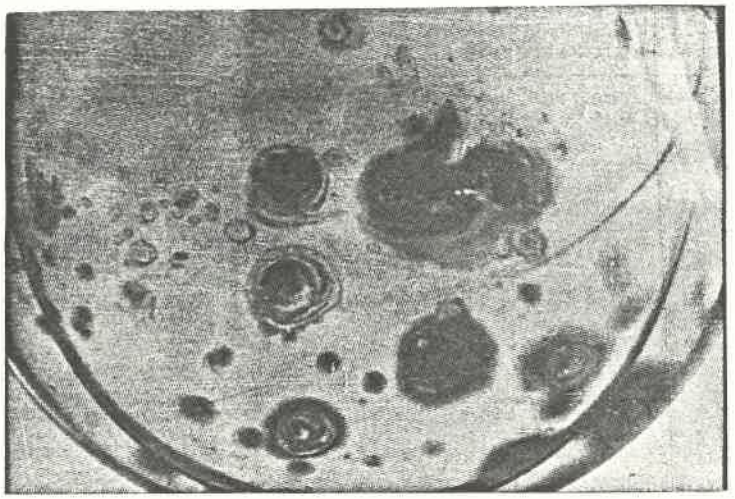

Fig. 1.

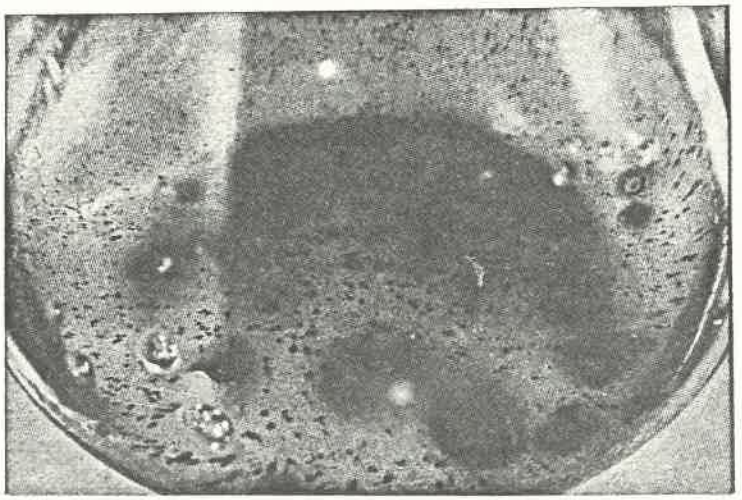

Fig. 2.

Fig. 1 and 2 - Incipient stages in the development algae colonies in raw culture, in liquid media

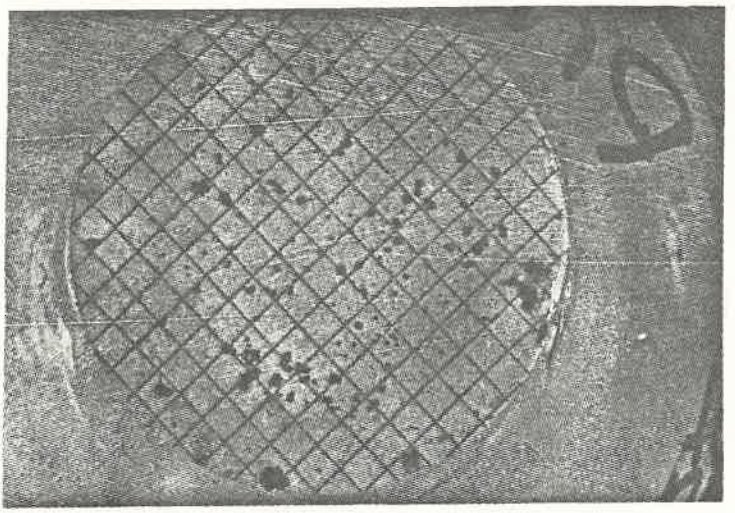

Fig. 3 - Appearence of microphyte algae colonies on HA Millipore filter. A suitable moment for picking out

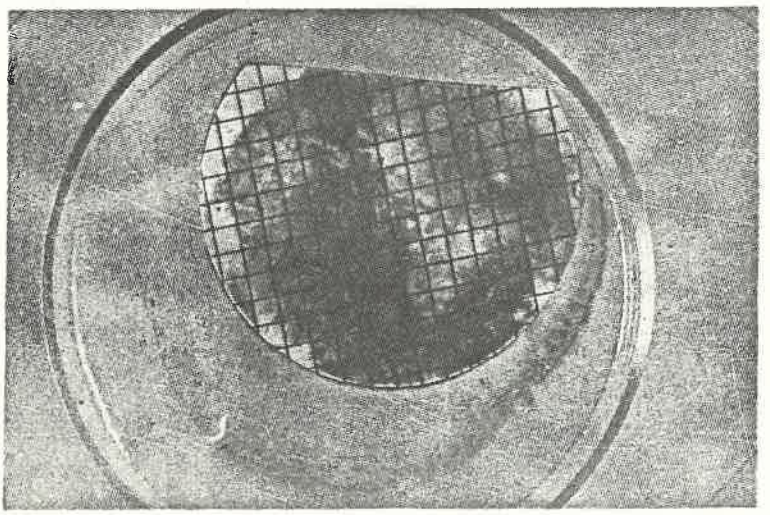

Fig. 4 - Monospecific culture obtained by picking-out the colonies appeared in raw culture

noticed that all liquid media of "AA" type permitted a good development of cocolitophoryde, pennate diatoms and two of centric diatoms : Cyclotella sp. and Sceletonema sp.

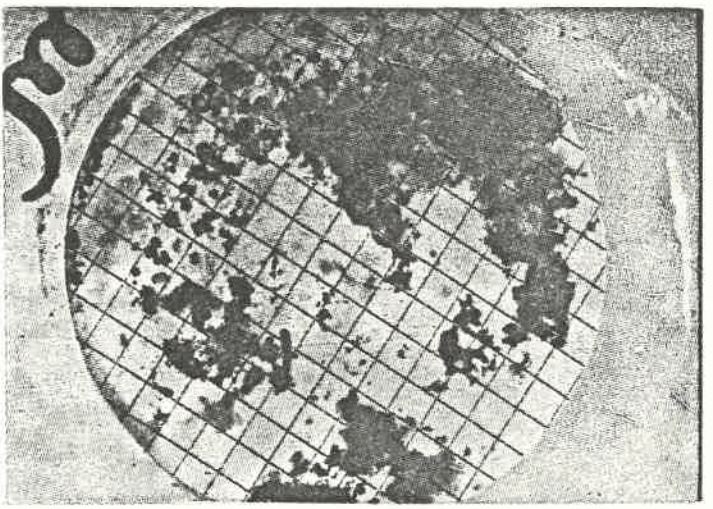

Fig. 5 - Colonies, belonging to different species, which after two weeks from insemination, have interferred

The agarised media, both "PM" and "AA" type, permit the development of the diatoms, both centric and pennate, and also of isolated clorophycea (nonidentified species for the time being).

The versatility of some vegetable unicellular organisms, as regards the feeding, is well known. The tendency of passing from autotrophy to heterotrophy, under medium conditions, was suggested in many papers (SAMEJIMA & MYERS, 1958; LEWIN, 1963; NORTH & STEPHENS 1966).

In this case the species of pennate diatoms and cocolitophorides are reprezentative of benthic microflora ; so that their development, both in liquid media with organic substance addition and on solidified media, proves that these organisms are adapted to saprophytic or heterotrophyc feeding because of the nature of their existence conditions.

The development of planktonic diatoms Sceletonema sp. and Cyclotella sp. and also of a chlorophycea species (nonidentified speciec C),

Table 3

The species isolated in monoculture, the sampling place and the culture medium on which they preferably developed

Isolated species	The depth from which the sample was taken	The culture medium on which it preferably develops
Diatoms		
Pennatae		
Achanthes brevipes Ag.	0 m	AA^{11} liquid PM^{3} liquid
Asterionella japonica Hass.	0 m	AA^{14} agarized millipore filter
Navicula sp.	10 m	PM^{c} agarized millipore filter
Synedra sp.	0 m	PM^{3} liquid AA^{1} liquid AA^{1} agarized millipore filter
Nonidentified species A	0 m	PM^{a} liquid
Nonidentified species B	0 m	PM^{a} liquid
Centrics		
Chaetoceros socialis Lauder	0 m	PM^{5} agarized PM^{5} liquid A11 "AA" liquid media
Chaetoceros curvisetus C1	0 m	Idem
Sceletonema costatum Grev.	50 m	A11 "AA" agarized media filter
Cyclotella sp.	10 m	A11 liquid and agarized media filter
Chlorophycee		
Polythoma sp.	0 m	PM^{3} liquid
Nonidentified species C	50 m	PM^{3} agarized filter
Cocolithophorida		
Nonidentified species D	100 m	AA^{16} liquid

on a substratum with organic addition, supports the idea regarding the part played by the organic substances in the feeding both benthic and planktonic unicellular organisms, sugessted, by PROVASOLI (1963). It is possible that the organic substance addition might play the part of a chelator, of part nutrient substratum, activator or inhibiter of metabolic processes,

The media exclusive mineral, with low concentrations, like PM^a, PM^b, PM^c, PM^1 permitted the development of all the species obtained by us, with the specification that the nonidentified penate diatoms A and B, as well as the Cyclotella sp. have an obvious better development than other species, expressed by the shorting of latent phase and also by an increased rate of division.

PM^5, PM^5 and PM^7 variants permitted the development of neritic diatoms : Thalassiosira sp.[x] Asterionella japonica, Chaetoceros socialis and Chaetoceros curvisetus ; these species appeared after one week after the insemination and reached the maximum density in five days after the starting of the exponential phase.

PM^3 and PM^4 variants, therefore those with a mean concentration of mineral addition, proved to be suitable for the development of the chlorophicsa; for instance, Polythoma sp. was obtained in PM^3 medium. The development rate of this algae in other media was lower and the latent phase longer.

So the preference for a certain electrolytic ratio of medium variants, PM, is closely connected with the natural environment in which the algae develop. If the samples are collected from littoral zone, the phytoplanktonic elements develop most favourably at low or mean concentrations of PM variants.

Neritic zone species prefer solutions with greater concentration (PM^5, PM^6, PM^7).

We do not deny the great algae plasticity, which explains their existence everywhere in the water mass, but we emphasize that, for

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[x] At the time of data collecting, this alga was not in monospecific culture yet

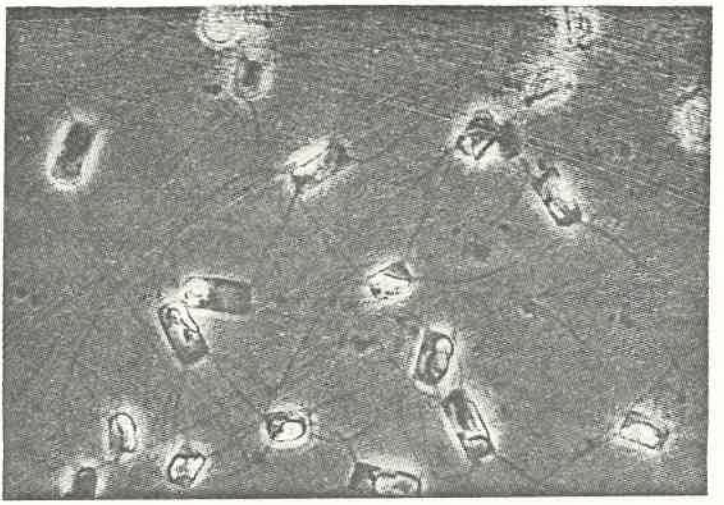

Fig. 6 - Monospecific culture of Chaetoceros socialis Laud.

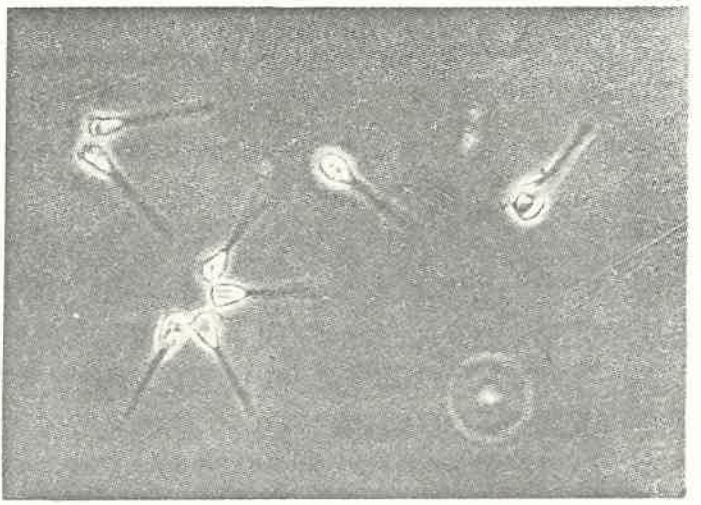

Fig. 7 - Monospecific culture of Asterionella japonica Hass

cultivation practice, the ecological and physiological requirements of these organisms must be taken into account. From the known data about the influence of medium factors upon unicellular algae it was inferred that it was possible to force a selective development in cultures.

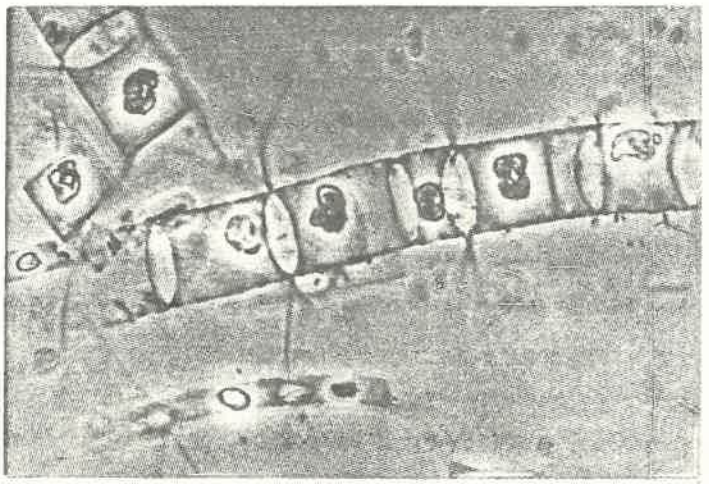

Fig. 8 - Monospecific culture of Chaetoceros curvisetus C1.

The use of liquid media is recommended for obtaining the algae frequently meet in phytoplankton.

The obtaining of rate species is possible by filtering of adequate water quantities (depending on cell number per liter) on HA Millipore filters and their atachament on agarized substratum. In this case, after the appearence of the culture it is recommended to resort to repeated dilutions and reinsemination, until the obtaining of monoculture of desired species.

The described method proves that the use of the knowledge about ecology and physiology of planktonic and benthic organisms is the best way to obtain a great number of monoalgal cultures in a short time.

The technique is adequate both for algae and marine fungs. isolation.

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