MaCuMBA

Marine Microorganisms: Cultivation Methods for Improving their Biotechnological Applications

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Summary

In this deliverable we present experiments aimed at improving the growth of autotrophic cultures.

Partner(s) involved in Deliverable production: NIOZ (1), CNRS (16).

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1. Growth medium

Comparison of existing media

Traditionally the Roscoff Culture Collection (RCC) use the K medium (Keller et al. 1987) for all photosynthetic eukaryotes. However recently we tested the systematic use of the L1 medium (Guillard and Hargraves 1993) which is derived of the K and f/2 medium (Appendix 1). The use of L1 resulted in higher biomass (Fig. 1) for a set of representative cultures (Table 1). As a result we have begun to switch RCC strains from K to L1 medium.

Fig. 1 : Comparison of RCC3052 (*Micromonas*) growth in L1 vs. K/2.

Table 1 : Qualitative evaluation of growth for cultures from the RCC collection transferred in parallel in K and L1 media. +, ++ and +++ correspond respectively to low, medium and high densities (e.g. in Fig. 1, left would correspond to +++ and right to +).

RCC	Class	Genus	L1	K
3072	Cryptophyceae	Proteomonas	+++	+
3052	Mamiellophyceae	Micromonas	+++	+
2339	Prasinophyceae	Clade VII	+++	+
2337	Prasinophyceae	Clade VII	+++	+
2335	Prasinophyceae	Clade VII	+++	++
2684	Prasinophyceae	Prasinococcus	+++	+
2687	Prasinophyceae	Prasinococcus	+++	++
2693	Prasinophyceae	Prasinococcus	+++	++
3066	Prasinophyceae	Prasinoderma	+++	+
2686	Prasinophyceae	Prasinoderma	++	++
2336	Prasinophyceae	Pycnococcus	+++	+
3064	Nephroselmidophyceae	Nephroselmis	+++	++
3073	Nephroselmidophyceae	Nephroselmis	+++	+
3069	Pelagophyceae	Pelagococcus	++	+
2696	Pelagophyceae	Unknown	++	++
2690	Prymnesophyceae	Ochrosphaera	+++	++
2691	Prymnesophyceae	Ochrosphaera	+++	++
2692	Prymnesophyceae	Unknown	+++	++
2340	Trebouxiophcyeae	Chlorella	+++	+++
3065	Trebouxiophcyeae	Pichlorum	+++	++
2697	Ulovphyceae	Unknown	++	++
2678	Bacilliarophyceae	Cocconeis	+	+
2675	Bacilliarophyceae	Minutocellus	+	+
3057	Bacilliarophyceae	Minutocellus	++	+
3058	Bacilliarophyceae	Minutocellus	++	+

Newly designed medium

Traditionally, the NIOZ used different types of medium for specific groups of phytoplankton, i.e. f/2 (Guillard 1975), K (Keller et al. 1987), ESAW (Harrison et al. 1980)), L1 (Guillard and Hargraves 1993), and DINO medium (Hansen 1989).

Recurrent crashes in growth for some of the species in culture (particularly during autumn) stimulated us to develop a medium that allows better performance (higher biomass and/or faster growth). This MIXtx medium (Appendix 2) is derived from f/2 medium and ESAW medium (ES enriched artificial seawater), the latter enriched with Tris-HCl and selenium (Cottrell and Suttle 1991). The f/2 medium is based on aged, nutrient-poor natural seawater that is 0.2 µm filtered before use. Sterile stocks of major nutrients (N, P, Si), trace elements and vitamins are added after autoclaving of the seawater. ESAW is prepared artificially, all stocks are added (vitamin stock at double concentration) with the exception of Tris-HCL and stock 1 (containing KBr, NaF, KCl, $NaHCO_3$ and H_3BO_3) that are added as sterile stocks to the remaining autoclaved medium. Note that stock solution containing Fe and EDTA should be allowed to equilibrate for more than 24h in the medium before the phytoplankton is inoculated to the medium.
The f/2 and modified ESAW are prepared and autoclaved (20 min 120°C) separately, each with 500 mL medium per 1 L glass bottle, whereby the bottles are autoclaved with lids closed. The latter prevents adaptation of pH afterwards and by keeping half of the bottle volume air, no damage to the glass bottles occurs. After finishing the modified ESAW, the MIXtx medium is then prepared by adding together equal volumes of f/2 and ESAW.
We tested MIXtx vs all the above-mentioned media for phytoplankton species from main phytoplankton groups and 7 different classes (see Table 2), including species/strains that performed relatively poorly under the standard medium used thus far. Under batch as well as semi-continuous culturing, MIXtx gave similar results or increased the algal biomass and/or growth rates. This was particularly the case for the more sensitive species tested, e.g. *Phaeocystis globosa*.

Table 2. Overview of phytoplankton used for developing new medium.

Class	Genus	Species	Official code
Bacillariophyceae	*Cylindrotheca*	*closterium*	RCC-1713
Bacillariophyceae	*Chaetoceros*	*tenuissimus*	strain 2-10
Bacillariophyceae	*Chaetoceros*	*calcitrans*	CCMP-1315
Bacillariophyceae	*Thalassiosira*	*rotula*	CCMP-1018
Prymnesiophyceae	*Phaeocystis*	*globosa*	Pg G(A)
Prasinophyceae	*Micromonas*	*pusilla*	LAC 38
Dinophyceae	*Gymnodinium*	*simplex*	
Dinophyceae	*Prorocentrum*	*balticum*	CCMP-1260
Dinophyceae	*Prorocentrum*	*minimum*	Helgoland E 66
Dinophyceae	*Prorocentrum*	*micans*	CCMP-1589
Cryptophyceae	*Rhodomonas*	*maculta*	CCY-0234
Raphidophyceae	*Heterosigma*	*akashiwo*	H93616
Cyanophyceae	*Synechococcus*	sp.	CCMP-835

When working with specific model species, we recommend testing the type of buffer. MIXtx contains Tris-HCl that works well normally. However, we noted that after testing different buffers (Tris, HEPES, EPPS, and EPPS - Fig. 2) that the yield of the diatom *Chaetoceros calcitrans* (thus not for other diatoms tested) was higher using HEPES (20 mM) or EPPS (8 mM). Bubbling with air or CO_2 (750 µatm) either had no additional positive effect or even a negative effect on the yield (as compared to gentle mixing once a day), independent of the type of buffer (Tris-HCl or HEPES).

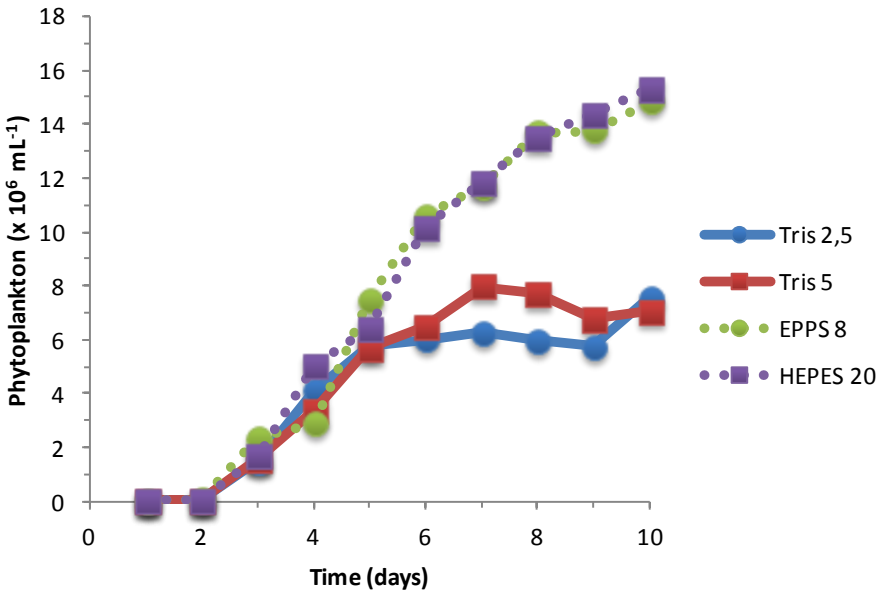

Fig. 2 : Phytoplankton cell abundances (mL^{-1}) of *Chaetoceros calcitrans*, grown in f/2:ESAW mix medium with different buffers. The buffers tested were : Tris-HCl 2.5 mM (blue: solid line, circles), Tris-HCl 5 mM (red: solid line, squares), EPPS 8mM (green: dotted line, circles), HEPES 20 mM (purple: dotted line, squares).

A second medium was designed specifically for culturing under low dissolved iron conditions. The MIXtx medium was modified such that the concentration of all potentially contaminating nutrients were lowered and use of typical metal chelators such as EDTA was avoided. The medium is based on natural seawater with low background dissolved iron concentrations (0.2 nM). This **Low Trace (LT) medium** (Appendix 2) is prepared under trace metal clean conditions. PC bottles/flasks were acid rinsed with 1 M HCL for 24 h, followed microwave sterilization with MilliQ water. The trace stocks were autoclaved and the non-trace stocks were equilibrated with MnO_2 twice (to remove potential iron) and filtered through 0.2µm PC filters.
Using this medium, we were able to grow *Phaeocystis globosa* at similar maximum rates as under iron-replete conditions using 1 nM dissolved iron (final concentration). *Micromonas pusilla* needed 3 nM before good growth was obtained.

Role of specific nutrients

When culturing dinoflagellates, we recommend reducing the final concentration of $FeCl_3.2H_2O$ in the MIXtx medium from 6.1 (MIXtx) to around 2 µM, as we found strongly improved yields for *Prorocentrum minimum* (Fig. 3). Additionally raising the EDTA concentration from 13 to 48 µM furthermore improved the growth rate and yield of the otherwise rather poorly growing *P. balticum*.

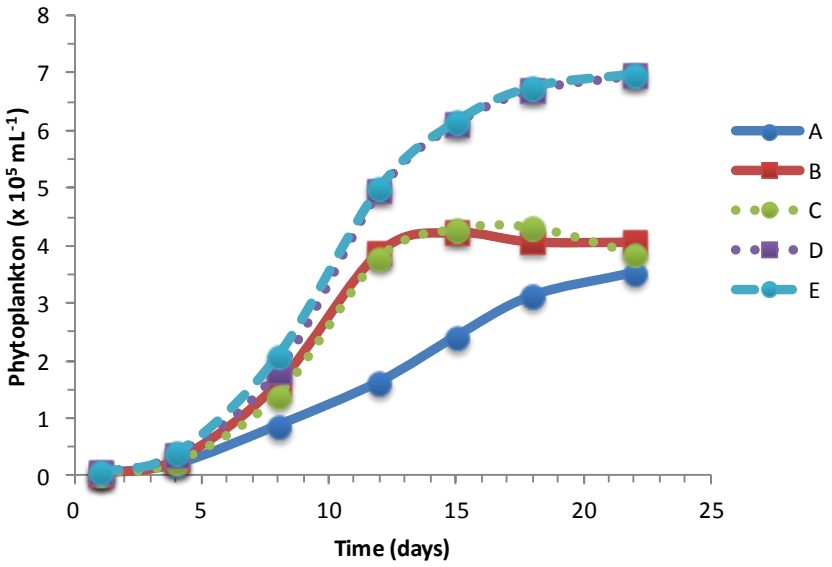

Fig. 3: Cell abundances (mL^{-1}) of *Prorocentrum minimum*, grown in media with varying trace metal and EDTA concentrations. See Table 3 for description.

Table 3. Media with different EDTA and Fe concentrations.

Code	Medium	EDTA (µM)	Fe (µM)
A	Dino	48	1.9
B	MIXtx	13	6.1
C	MIXtx	48	6.1
D	MIXtx	48	1.9
E	MIXtx	13	1.9

2. Growth conditions

Temperature

We investigated the growth capacities over a range of temperatures of a panel of six strains of marine *Synechococcus*, isolated along a latitudinal gradient of the North Atlantic Ocean (Table 4).

Table 4: Isolation site Information regarding the *Synechococcus* strains used in the study (Pittera et al. 2014).

Strain name	**A15-37**	**M16.1**	**WH7803**	**ROS8604**	**MVIR-16-2**	**MVIR-18-1**
RCC #	2526	791	752	2380	1594	2385
Isolation site	Offshore Mauritania	Gulf of Mexico	Sargasso Sea	English Channel	Southern Norwegian Sea	Southern Norwegian Sea
Isolation latitude	23°33′ N	27°42′ N	33° 45′ N	48° 43′ N	60° 19′ N	61° 00′ N
Isolation longitude	19°59′ W	91°18′ W	67° 30′ W	3° 59′ W	3° 29′ W	1° 59′ E
Isolation date	29/09/2004	09/02/2004	03/07/1978	24/11/1986	21/07/2007	23/07/07
Isolation Depth	10 m	275 m	25 m	1 m	10 m	25 m
Isolation temperature (°C)	24.53	24.15	25.85	12.81	11.99	13.98

At the lab routine temperature growth (*i.e.* 20-22°C), the 6 strains exhibited quite similar growth rates (Fig. 4). However, at higher and lower temperatures, they differed considerably in their growth optima and limits, which were related to the latitude of their isolation site. Low latitude strains showed optimal growth at about 33°C and could not grow at temperature lower than 16°C. Their high growth temperature limit appeared to be close to 35-36°C. By contrast, high latitude strains could not grow at temperature higher than ca 25-28°C and showed optimal growth around 22-25°C, depending on the isolation latitude. Although the maximal growth rates displayed by these strains were markedly lower than low latitude strains, they were however capable of growing at temperatures lower than 10°C.

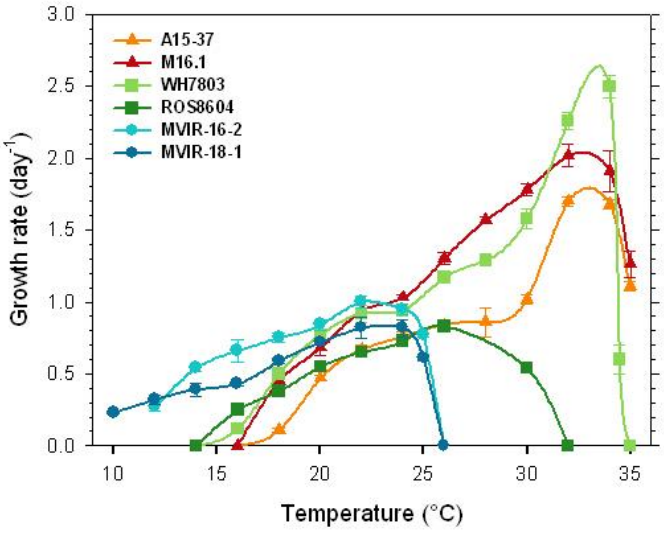

Fig. 4: Growth rate as a function of temperature in the tropical (triangles), mid-latitude (squares) and high latitude (circles) marine *Synechococcus* strains. Errors bars are standard deviation from the average based on 4 replicates (Pittera et al. 2014).

These results highlight the fact that, even if most marine *Synechococcus* strains display similar growth in the temperature conditions routinely used in laboratories, this does not reflect the real capacities of each strain, which rather seem to be related to their thermal niche. In addition, the growth rate curves of the northern-most strains indicate that they are not psychrophilic organisms.

3. Choice of culture vessels

We tested 3 types of culture vessels that are regularly used for isolating or maintaining cultures:

- Flasks: 50 mL
- Tubes: glass (5 and 15 mL) or plastic (15 mL)
- Multi-well plates: 48 and 24-wells

With *Isochrysis*, growth (Fig. 5) and final yield are lowest in multi-well plates and appears best either in 50 mL flasks or glass tubes. Similar results were obtained with *Micromonas* and *Synechococcus*.

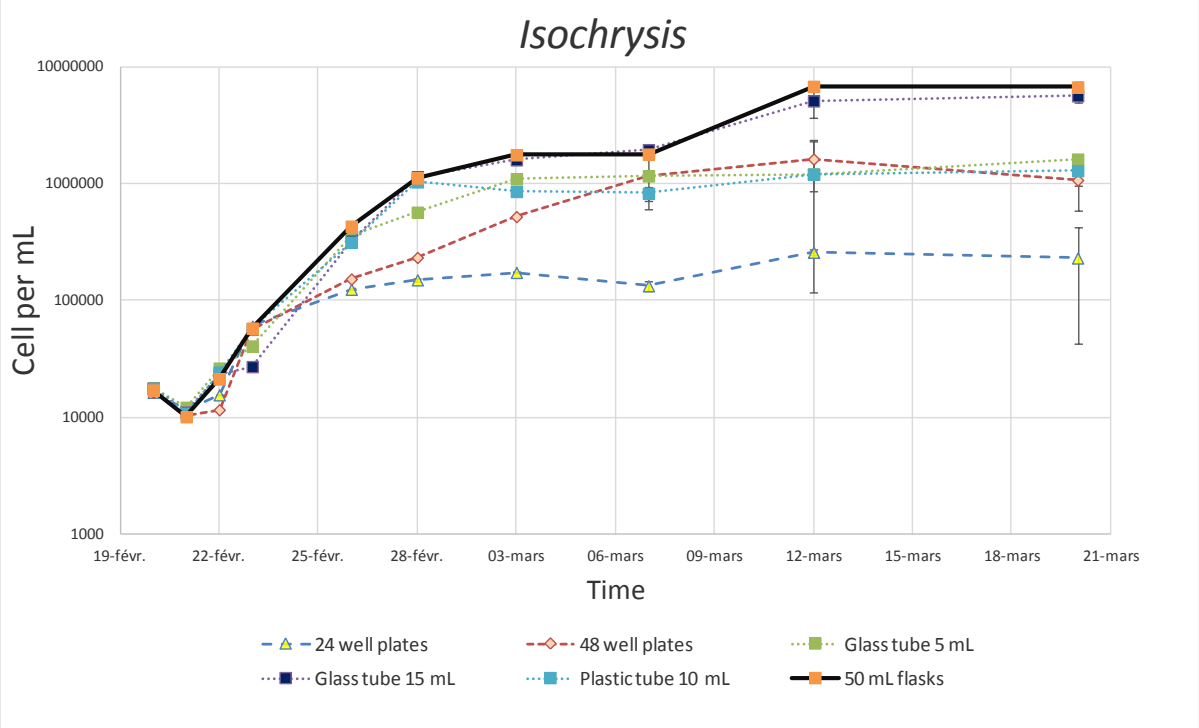

Fig. 5 : Growth of *Isochrysis* (RCC90) in different containers.

Growth of *Phaeocystis globosa* and *Micromonas pusilla* was tested with MIXtx medium using different types of culture vessels (glass and plastic culture flasks, glass tubes, large volume continuous culture vessels). No difference in growth yield was detected for the different flasks types (data not shown).

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Appendix 1 – Comparison of three culture media for photosynthetic plankton

All concentrations are M	f/2	L1	K
NaNO3	8.82E-04	8.82E-04	8.82E-04
NH4Cl			5.00E-05
NaH2PO4·H2O	3.62E-05	3.62E-05	
Na2b b-Glycerophosphate			1.00E-05
Na2SiO3·9H2O	1.06E-04	1.06E-04	5.04E-04
Tris-Base (pH 7.2)			1.00E-03
Na2EDTA·2H2O	1.17E-05	1.17E-05	1.11E-04
FeCl3·6H2O	1.17E-05	1.17E-05	1.17E-05
MnCl2·4H2O	9.10E-07	9.09E-07	9.00E-07
ZnSO4·7H2O	7.65E-08	8.00E-08	8.00E-08
CoCl2·6H2O	4.20E-08	5.00E-08	5.00E-08
CuSO4·5H2O	3.93E-08	1.00E-08	1.00E-08
Na2MoO4·2H2O	2.60E-08	8.22E-08	2.60E-08
H2SeO3		1.00E-08	1.00E-08
NiSO4·6H2O		1.00E-08	
Na3VO4		1.00E-08	
K2CrO4		1.00E-08	
thiamine·HCl(vit.B1)	2.96E-07	2.96E-07	2.96E-07
biotin(vit.H)	2.05E-09	2.05E-09	2.05E-09
cyanocobalamin (vit. B12)	3.69E-10	3.69E-10	3.69E-10

Appendix 2 – Composition MIXtx and LT medium.

MEDIUM	**MIXtx**	**LT**
All concentrations are M		
NaNO3	7.15E-04	1.28E-04
NH4HCl		
Na2HPO4.12H2O	1.80E-05	8.00E-06
Na2-glycerol phosphate	1.09E-05	
Na2SiO3.9H2O	1.50E-04	
Tris HCl	2.50E-03	2.50E-03
Na2EDTA	1.33E-05	
FeCl3.2H2O	6.12E-06	1 *to* 3E-09
(NH4)2(FeSO4)2.6H2O	2.98E-06	
MnCl2.4H2O	4.55E-07	9.00E-09
MnSO4.H2O	1.21E-06	
ZnSO4.7H2O	1.65E-07	4.00E-09
CoCl2.6H2O	2.10E-08	5.00E-09
CoSO4.7H2O	2.85E-08	
CuSO4.5H2O	2.00E-08	1.00E-09
Na2MoO4.2H2O	9.92E-09	4.00E-09
H3BO3	3.07E-05	3.75E-06
H2SeO3		
Na2SeO3	4.92E-09	1.10E-08
NiSO4.6H2O		1.00E-09
Na3VO4		1.00E-09
K2CrO4		1.00E-09
Cyanocobalamine (B12)	3.69E-10	3.69E-10
Thiamine (B1)	2.96E-07	2.96E-07
Biotine (H)	2.05E-09	2.05E-09
Artificial seawater		
NaCl	1.78E-01	
Na2SO4	1.23E-02	
CaCl2.2H2O	4.48E-03	
SrCl2.6H2O	4.01E-05	
MgCl2.6H2O	2.31E-02	
KCl	3.94E-03	
NaHCO3	1.01E-03	
KBr	3.55E-04	
H3BO3	1.82E-04	
NaF	3.22E-05	