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Diatom artificial medium (DAM): a new artificial medium for the diatom *Haslea ostrearia* and other marine microalgae

Sindy Gagneux-Moreaux[a*], Christophe Moreau[a],
Jean-Louis Gonzalez[b], Richard P. Cosson[a]

[a] ISOMer, EA-2663, Université de Nantes, BP 92208, 44322 Nantes cedex 3, France
[b] IFREMER, Biogéochimie et Ecotoxicologie, BP 330, 83507 la Seyne sur Mer, France

*Author for correspondence:
phone +(33)251125691
fax +(33)251125668
e-mail: sindy.gagneux@univ-nantes.fr

Abstract:
Artificial media are used in physiological studies of microalgae to maintain consistent conditions from one experiment to another and these media must be adapted to the needs of the organism studied. The artificial medium, in this case named diatom artificial medium (DAM), was designed to maintain long-term cultures of Haslea ostrearia and 19 other planktonic microalgae, and to allow physiological studies related to metal metabolism. The biomass and biochemical composition of H. ostrearia grown in the DAM and in a modified Provasoli medium were compared to assess the suitability of this new artificial medium for the culture of this diatom. The DAM provided sufficient nutrients to allow H. ostrearia to grow as efficiently as in the enriched seawater medium, without negative impact on metabolism. The DAM was tested with 19 other microalgae in order to widen its potential use, and 18 of the 19 showed a good adaptation to this medium. The chemical speciation of metals (Cd, Cu, Pb, Zn) was assessed using a speciation mathematical model. The presence of EDTA resulted in the total complexation of the trace metals implying that they were present in a sole chemical species in the DAM.

Keywords: Artificial culture medium - Microalgae - Diatom - Biochemical parameters - Metal speciation

Introduction

Natural seawater enriched with nutrients, trace metals and vitamins is commonly used to maintain microalgal strains. The composition of seawater may be different for several sampling sites and periods, thereby leading to less reproducible and reliable experiments. Since 1890, media composed of seawater or seawater-like artificial solutions have been developed to keep axenic microalgal cultures under controlled laboratory conditions (Provasoli et al., 1957). Some artificial media were developed for the study of marine microalgal physiology (Berland et al., 1973; Carvalho and Malcata, 2000; Lane and Morel, 2000; Reinfelder et al., 2000). Moreover, the bioaccumulation of trace metals by microalgae was studied by using specific artificial media (Jensen et al., 1982; Price and Morel, 1990; Ahner et al., 1994; Yee and Morel, 1996; Yan and Pan, 2002). The advantages of such media are an accurate control of the concentration and speciation of metals in experimental conditions, and the opportunity to reproduce further experiments under identical conditions.

Many studies about microalgae have focused on species largely used in aquaculture. The diatom *Haslea ostrearia* (Simonsen) is involved in the oyster greening, which consists in acquiring a blue green color on the gills and the labial palps (Ranson, 1937), increasing value of oyster products. Robert (1983) obtained long term productive cultures with this diatom after modifying the enriched seawater medium ES (Provasoli, 1968). In order to study some physiological aspects of this diatom, cultures were grown using artificial media. The artificial medium ESAW (Harrison et al., 1980; Berges et al., 2001) is based on an artificial seawater medium similar to that of Kester et al. (1967) enriched with Provasoli's ES solution. Cultures of *H. ostrearia* in ESAW showed cell deformation after several transfers (Mouget, pers. comm.). Moreau (1996) used the artificial Aquil medium (Morel et al., 1979; Price et al., 1988) and the f/50 medium derived from the enriched seawater f/2 medium (Guillard, 1982) for experiments involving cultures of *H. ostrearia*. Cultures appeared to be more productive in the f/50 seawater medium than in the artificial medium, which implies specific nutritional requirements for elements found in seawater but not in the artificial medium. Actually, the medium Aquil designed was tailored to study metal metabolism in microalgae, rather than a phytoplankton culturing medium in the first place (Morel et al., 1979). Neuville and Daste (1971) used a synthetic medium for *H. ostrearia* in order to observe

production by this diatom of a specific blue-green pigment, called “marennine”, with short term cultures. This medium was very rich in nutrients and trace elements compared to other media (enriched natural water or artificial seawater).

The aim of this study was to develop an artificial medium suitable to maintain long term productive cultures of *H. ostrearia*, and allowing physiological studies related to trace metal metabolism. For this reason, the artificial medium DAM (Diatom Artificial Medium) was developed after the Aquil model. Modifications were introduced according to literature data about *H. ostrearia* and artificial media, and assays based on the variation of the abundance of several elements (presence/absence, ratio). The ability of the DAM to sustain *H. ostrearia* was tested with a culture that lasted for two and a half months. For each growth cycle, the biomass and the total carbohydrate and protein contents were compared with those obtained with a strain grown in enriched seawater medium of ES type (Provasoli, 1968; Robert, 1983) usually used for *H. ostrearia* cultures (Robert, 1983; Lebeau et al., 2000; Tremblin et al., 2000; Robert et al., 2002). The DAM was also tested using nineteen different species of microalgae, essentially diatoms, through growth cycles, in order to widen its potential use.

To understand metal/algal interactions, knowledge of speciation in the medium is essential. The chemical speciation of essential (copper and zinc) and non essential (cadmium and lead) metals was assessed using a speciation mathematical model.

Methods

Composition of the media, DAM and ES1/3

The composition of nutrient stock solutions and the DAM preparation protocol are given in Table 1. The trace element solution (III) was acidified in order to keep metals in solution and to prevent adsorption onto the flask. EDTA was added to the trace element solution before $FeCl_3$ incorporation to avoid precipitation of ferric hydroxide (Morel et al., 1979). The pH of the medium was adjusted at 7.8 using a 1M hydrochloric acid solution. The filtered vitamin solution (IV) was added to the medium after being sterilized by filtration. This vitamin solution can be prepared in advance, fractioned and stored at –18°C.

Table 1

The composition of the modified enriched seawater medium ES1/3, derived from the ES medium (Provasoli, 1968; Robert, 1983), is presented in Table 2 and is compared to the final concentration of elements in DAM and in the ES medium described by Provasoli in 1968. The ES1/3 enrichment solution results in the third of the ES enrichment for a main part of elements and the vitamin solution is that described by Guillard and Ryther (1962). The optimal biomass attained in ES and ES1/3 was within the same range; but ES1/3 appeared to be more suitable for *H. ostrearia.* Whereas Robert (1978) showed a depletion of the culture maintained in ES medium with a decrease of the biomass and an increase of the number of deformed cells, this author did not mention any depletion in cultures of *H. ostrearia* performed in ES1/3.

Table 2

Culture conditions

All the algal strains used in these experiments were provided by the Nantes Culture Collection (WDCM 856, ISOMer, Université de Nantes, France). All the experiments were carried out at 17°C under 80 µmol photons.m^{-2}.s^{-1} irradiance with a 14:10 hour photoperiod. The cultures were grown either in 250 mL flasks with 150 mL of sterilised medium (*Haslea crucigera* (NCC 32), *Haslea ostrearia* (NCC 143), *Odontella aurita* (NCC 87), *Phaeodactylum tricornutum* (NCC 45), *Rhizosolenia setigera* (NCC 81), *Skeletonema costatum* (NCC 52), *Thalassionema sp*. (NCC 69) and *Tetraselmis suecica* (NCC 62)) or in 100 mL tubes with 25 mL of DAM (all the other species tested in this study). Each flask/tube was inoculated with the parent culture maintained in enriched seawater at an initial concentration of 5000 cells.mL^{-1} (cycle n). When the cultures reached the stationary phase, the cells were transferred into a new flask containing fresh medium at 5000 cells.mL^{-1} (cycle n+1).

In order to test the capacity of the new medium to maintain the diatom *H. ostrearia*, a strain was grown in the DAM and in the ES1/3 during five consecutive cycles. For each culture condition, five replicates were realised.

The DAM was developed to grow *H. ostrearia* but its suitability for growing other species of microalgae was tested to widen its potential use for physiological experiments. Cultures in tubes or in flasks were run in a single example through 10 or 6 transfers depending on species. The experiment duration (4 months) allowed 10 cycles to take place for cultures carried out in tubes while only 6 cycles were achieved by

cultures carried out in flasks. The experiments included several repeated microscopic observations to identify shape modifications and to estimate number of cells per mL.

Culture monitoring

In order to compare the strain grown in the DAM with those grown in the modified ES medium (ES1/3), growth rate and biochemical composition were determined for *H. ostrearia* cultures. The cells were counted daily from the beginning of the exponential phase to the stationary phase using a Nageotte hematocymeter. At this step, each culture was filtered on Whatman GF-F membrane for analysis of a few biochemical parameters. The physiological status of the microalgae was estimated using carbohydrate, pigment (carotenoids and chlorophyll *a*) and protein concentrations determined by the spectrophotometric methods of Dubois et al. (1956), Lorenzen (1967) and Lowry et al. (1951) respectively.

Metal speciation in the DAM

A speciation mathematical model (MOCO) developed by IFREMER was used to calculate essential metals (Cu and Zn) speciation in the DAM. Development and applications of this speciation model has already been described (Gonzalez et al., 2001a; 2001b; Laurier et al., 2003). The complexation constants used for the calculation of the chemical species taken into account were compiled by Morel and Hering (1993). Calculations were also performed with the addition of non-essential metals (Cd and Pb) to determine their speciation in the DAM. Simulations were run with the initial element concentrations at pH 7.8, either with or without EDTA. Speciation of EDTA was determined and the competition of metals with Ca^{2+} and Mg^{2+} was taken into account.

Statistical analysis

The normality and the homogeneity of variances being checked, the biomass and biochemical parameters obtained for cultures grown in DAM and in enriched seawater ES1/3 were compared using Student's t-test and one way analysis of variance (ANOVA) with a significance level at $\alpha<0.05$ using Sigma Stat 2.0 (Jandel Scientific) software. *A posteriori* tests (Tukey tests) were run with ANOVA data.

Results

Growth experiments

Fig. 1 shows the growth curves of *H. ostrearia* cultures carried out in the DAM and in the ES1/3. For the five cycles presented in Fig. 1, the strain seems to grow better in the DAM than in the modified ES medium, but the statistical analysis did not put forward any significant difference with the exception of the third ($p < 0.001$) and the fifth transfers ($p < 0.001$). The total carbohydrate and protein contents of *H. ostrearia* (Fig. 2) were stable with time for each tested medium (DAM and ES1/3). No significant difference was shown between these contents at each sampling date. The carotenoid and chlorophyll *a* cell concentrations (Fig. 3) were significantly higher ($p < 0.05$) in cells grown in the DAM than those in cells grown in the ES1/3 since the second transfer. The repeated microscopic observations did not reveal a significant proportion of deformed cells in DAM compared to ES1/3.

Fig. 1

Fig. 2

Fig. 3

Growth tests using various microalgae

The results of the cultures carried out with 19 other microalgal species in DAM are summarised in Table 3, which lists the species'names, the NCC strain codes and the number of successful cycles.

Table 3

All species kept in tubes grew successfully over ten cycles. Among the strains kept in flasks, only *Rhizosolenia setigera* showed difficulties to grow in the DAM. Neither shape modification nor growth decrease were noticed for the five other species kept in flasks after 6 cycles achieved. Microscopic observations did not put forward any modification of algal cells.

Chemical speciation of Cd, Cu, Pb and Zn

Calculations of Cd, Cu, Pb and Zn speciation are presented in Table 4. Without EDTA, each considered metal had specific affinities for inorganic ligands. The major amount of copper was associated to carbonates (97%) whereas zinc was mainly associated to phosphates (90%). The cadmium was mainly (90%) associated to chlorides. Lead was present as two major species, phosphates and chlorides for 50% and 40 % of the total lead respectively.

Table 4

The addition of EDTA resulted in a total complexation (100%) of the considered metals with this molecule. This complexation was assessed with concentration values from 10^{-8} mol.L^{-1} to 10^{-6} mol.L^{-1} of the different metals.

Discussion

The Diatom Artificial Medium DAM allows long term and productive culturing *H. ostrearia* in controlled conditions. The cell shape and the global biochemical composition appeared to be maintained after several transfers, which implies that DAM contains the various elements in sufficient amounts for the optimal development of this diatom. Requirement in silicon and copper appeared to be a main factor in the development of the diatom. The slight increase in cell density observed from a transfer to another could be explained by an acclimation of the strain to the medium supplies, since carbohydrate and protein contents are similar for all transfers. Cultures of *H. ostrearia* in larger volumes (10 L, 25 L and 200 L) have been attempted at our laboratory and the biomass achieved was as high as that obtained in enriched seawater medium (cultures realised in ES1/3 and F/2 media, unpublished data). Consequently, DAM has to be considered as a well-adapted artificial medium for *H. ostrearia* culture. Furthermore, DAM allowed the culture of 18 species of microalgae through several transfers, other than *H. ostrearia*, even though the artificial medium did not seem to be convenient for the strain of *Rhizosolenia setigera* used in this experiment. It is noteworthy that this diatom did not grow either in Aquil beyond 3 transfers (Morel et al., 1979).

The speciation model showed that, in the absence of EDTA in DAM, the speciation of the considered metals was similar to that reported for seawater (Florence, 1982; Fernando, 1995, Stumm and Morgan, 1996). The presence of EDTA resulted in the complexation of metals from low concentrations (encountered in natural ecosystems) to higher concentrations (used in experimental studies). A low bioavailability of metals decreases their direct toxicity. Using an adapted ratio of EDTA/metals, it is possible to control the amount of metals bioavailable to microalgae and to mimic the low concentrations found in natural environments. Moreover, when EDTA was not added to DAM, the four tested metals were presented as various chemical species, whereas, in EDTA-added DAM, these metals were essentially in a sole species. The interaction

metals/microalgae is, thus, dependant on the dissociation constants of the complexes EDTA-metals.

In conclusion, the medium DAM can be used for growing various microalgae in medium term and for studying metal impact on the physiology of these microalgae. Using this artificial medium would allow to study, in controlled conditions, 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.

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Figure legends

Fig. 1 Growth curves of *Haslea ostrearia* maintained for two and a half months in the Diatom Artificial Medium (◆) and in the enriched seawater medium ES1/3 (×). Mean ± standard deviation.

Fig. 2 Concentrations in carbohydrates and proteins per cell for culture of *Haslea ostrearia* maintained for two and a half months in the Diatom Artificial Medium and in the enriched seawater medium ES1/3. Mean ± standard deviation.

Fig. 3 Concentrations of carotenoids and chlorophyll *a* per cell for cultures of *Haslea ostrearia* maintained for two and a half months in the Diatom Artificial Medium and in the enriched seawater medium ES1/3. Mean ± standard deviation.

1 Table 1 Preparation of the Diatom Artificial Medium (DAM).

2

Composition of stock solutions	
Solution I: Ion solution	1 liter
H_3BO_3	1.50 g
KBr	5.00 g
KCl	35.00 g
NaF	0.15 g
$NaHCO_3$	10.00 g
$SrCl_2$-$6H_2O$	0.85 g
Solution II :Nutrients (Three different solutions are prepared and stored at 6°C)	1 liter of each solution
NaH_2PO_4-H_2O	1.38 g
$NaNO_3$	25.50 g
Na_2SiO_3-$9H_2O$	28.40 g
Solution III :Trace Elements (in HNO_3 0.01M)	1 liter
$CoCl_2$-$6H_2O$	1mL of a 10 $g.L^{-1}$ solution
$CuSO_4$-$5H_2O$	1mL of a 9.80 $g.L^{-1}$ solution
$MnCl_2$.$4H_2O$	1mL of a 180 $g.L^{-1}$ solution
Na_2MoO_4.$2H_2O$	1mL of a 6.30 $g.L^{-1}$ solution
Na_2SeO_3-$5H_2O$	1mL of a 0.85 $g.L^{-1}$ solution
$NiCl_2$-$6H_2O$	1mL of a 0.74 $g.L^{-1}$ solution
$ZnSO_4$-$7H_2O$	1mL of a 22 $g.L^{-1}$ solution
$FeCl_3$-$6H_2O$	3.15 g
Na_2EDTA-$2H_2O$	4.36 g
Solution IV: Vitamin solution (After sterilization)	1 liter
Biotin	10mL of a 0.1$g.L^{-1}$ solution
Cyanocobalamin	1mL of a 1 $g.L^{-1}$ solution
Thiamine-HCl	0,20 g
Composition of the DAM	
Salts	
NaCl	20.570 g
Na_2SO_4	3.067 g
$CaCl_2$-$2H_2O$	1.150 g
$MgCl_2$-$6H_2O$	11.100 g
Solution I: Ion solution	15 mL
Solution II: Nutrients	
NaH_2PO_4-H_2O	2 mL
$NaNO_3$	1 mL
Na_2SiO_3-$9H_2O$	2 mL
Solution III: Trace Elements	2 mL
Solution IV: Vitamin solution (After sterilization)	1 mL
Deionised water	***Fill up to 1 liter***

3

1 Table 2 Final concentrations of elements in DAM, ES and ES1/3 media.

2

	DAM Final concentration ($mol.L^{-1}$)	ES (Provasoli, 1968) Final concentration ($mol.L^{-1}$)	ES1/3 (Robert, 1983) Final concentration ($mol.L^{-1}$)
Salts			
NaCl	3.52×10^{-1}		
Na_2SO_4	2.16×10^{-2}		
$CaCl_2$-$2H_2O$	7.82×10^{-3}		
$MgCl_2$-$6H_2O$	5.46×10^{-2}		
Solution I :Ion solution			
H_3BO_3	3.64×10^{-4}	9.22×10^{-5}	3.07×10^{-5}
KBr	6.30×10^{-4}		
KCl	7.04×10^{-3}		
NaF	5.36×10^{-5}		
$NaHCO_3$	1.79×10^{-3}	9.52×10^{-4}	9.52×10^{-4}
$SrCl_2$-$6H_2O$	4.61×10^{-5}		
Solution II :Nutrients			
NaH_2PO_4-H_2O	2.00×10^{-5}		
Na_2 Glycerophosphate-$6H_2O$		4.63×10^{-5}	1.54×10^{-5}
$NaNO_3$	3.00×10^{-4}	8.24×10^{-4}	2.75×10^{-4}
Na_2SiO_3-$9H_2O$	2.00×10^{-4}	5.00×10^{-5}	5.00×10^{-5}
Solution III :Trace			
$CoCl_2$-$6H_2O$	8.41×10^{-8}		
$CoSO_4$-$5H_2O$		8.54×10^{-8}	2.85×10^{-4}
$CuSO_4$-$5H_2O$	7.85×10^{-8}		
$FeCl_3$-$6H_2O$	2.33×10^{-5}	9.06×10^{-7}	3.02×10^{-7}
$Fe(NH_4)_2(SO_4)_2$-$6H_2O$		8.95×10^{-6}	2.98×10^{-6}
$MnCl_2$-$4H_2O$	1.82×10^{-6}		
$MnSo_4$-$4H_2O$		3.26×10^{-6}	1.09×10^{-6}
Na_2MoO_4.$2H_2O$	5.21×10^{-8}		
Na_2SeO_3-$5H_2O$	6.46×10^{-9}		
$NiCl_2$-$6H_2O$	6.30×10^{-9}		
$ZnSO_4$-$7H_2O$	1.53×10^{-7}	3.83×10^{-7}	1.28×10^{-7}
Na_2EDTA-$2H_2O$	2.34×10^{-5}	2.23×10^{-4}	7.43×10^{-5}
Solution IV: Vitamin			
Biotin	4.09×10^{-9}	4.09×10^{-9}	8.19×10^{-8}
Cyanocobalamin	7.38×10^{-10}	1.48×10^{-9}	2.95×10^{-9}
Thiamine-HCl	5.93×10^{-7}	2.96×10^{-7}	2.96×10^{-6}
Optimal Biomass Achieved (10^3 $Cells.mL^{-1}$)	Present study	230-250 at long term, decrease of the dividion rate of the culture and increase of the deformed cell ratio	200-250 (Robert, 1983, Present study)

3

Table 3 Transfers of different microalgae in DAM (Grown in 25 mL tubes, except species noted (*) grown in 250 mL flasks)

Species	Clone	Successful Transfers
Bacillariophyceae		
Amphora hyalina	NCC 1 D-Am.hy.	10
Bacillaria paradoxa	NCC 6 D-Ba.pa.	10
Chaetoceros sp.	NCC 8 D-Ch.sp.	10
Coscinodiscus granii	NCC 11 D-Co.gr.	10
Entomoneis alata	NCC 17 D-En.al.	10
*Haslea crucigera**	NCC 32 D-Ha.cr.	6
Heliotheca thamesis	NCC 59 D-He.th.	10
Navicula ramosissima	NCC 73 D-Na.ra.	10
Nitzschia compressa	NCC 38 D-Ni.co.	10
*Odontella aurita**	NCC 87 D-Od.au.	6
*Phaeodactylum tricornutum**	NCC 45 D-Ph.tr.	6
Pleurosigma intermedium	NCC 78 D-Ple.in.	10
*Rhizosolenia setigera**	NCC 81 D-Rh.se.	2-†
*Skeletonema costatum**	NCC 52 D-Sk.co.B2	6
*Thalassionema sp.**	NCC 69 D-Thn.sp.	6
Other microalgae		
Dunaliella sp.	NCC 14 C-Du.sp.	10
Isochrysis sp.	NCC 24 Pry-Is.sp.	10
Porphyridium cruentum	NCC 49 R-Po.cr.	10
*Tetraselmis suecica**	NCC 62 Pra-Te.Su.	10

*cultures performed in flasks.
†no algal growth after transfer

Table 4 Calculations of metal speciation (Cu, Zn, Cd, Pb) in the artificial medium DAM with and without presence of EDTA (in percentage of the total concentration; nc: non calculated)

Association metal-ligand (%)	Cu[a]	Zn[a]	Cd[b]	Pb[b]
Without EDTA				
Free metal (M^{n+})	0.030	6.412	3.723	5.515
Chloride (4.8×10^{-1} mol.L^{-1})	0.011	2.340	95.233	39.177
Hydroxyde	0.014	0.210	0.006	2.367
Phosphate (2×10^{-5} mol.L^{-1})	2.643	89.889	n.c.	48.869
Carbonate (1.8×10^{-3} mol.L^{-1})	97.293	n.c.	n.c.	n.c.
Sulfate (2.2×10^{-2} mol.L^{-1})	0.009	1.149	1.037	4.072
With EDTA[c]				
EDTA	100	100	100	100
Other species	$< 1\times10^{-8}$	$< 1\times10^{-8}$	$< 1\times10^{-8}$	$< 1\times10^{-8}$

[a] with concentrations of the artificial medium DAM: [Cu] 7.85×10^{-8} mol.L^{-1} and [Zn] 1.53×10^{-7} mol.L^{-1}

[b] with an addition of 10^{-8} mol.L^{-1} of total cadmium and lead to the DAM

[c] major species of EDTA(Y): HY^{3-} (97.5%), H_2Y^{2-} (2.5%)

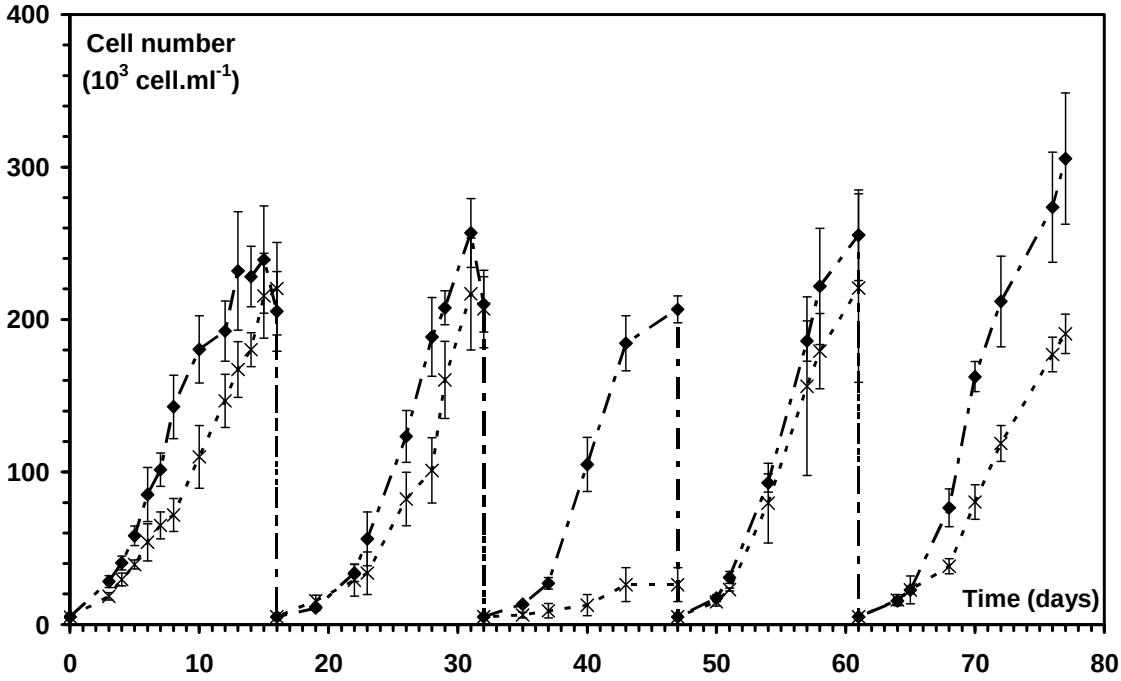
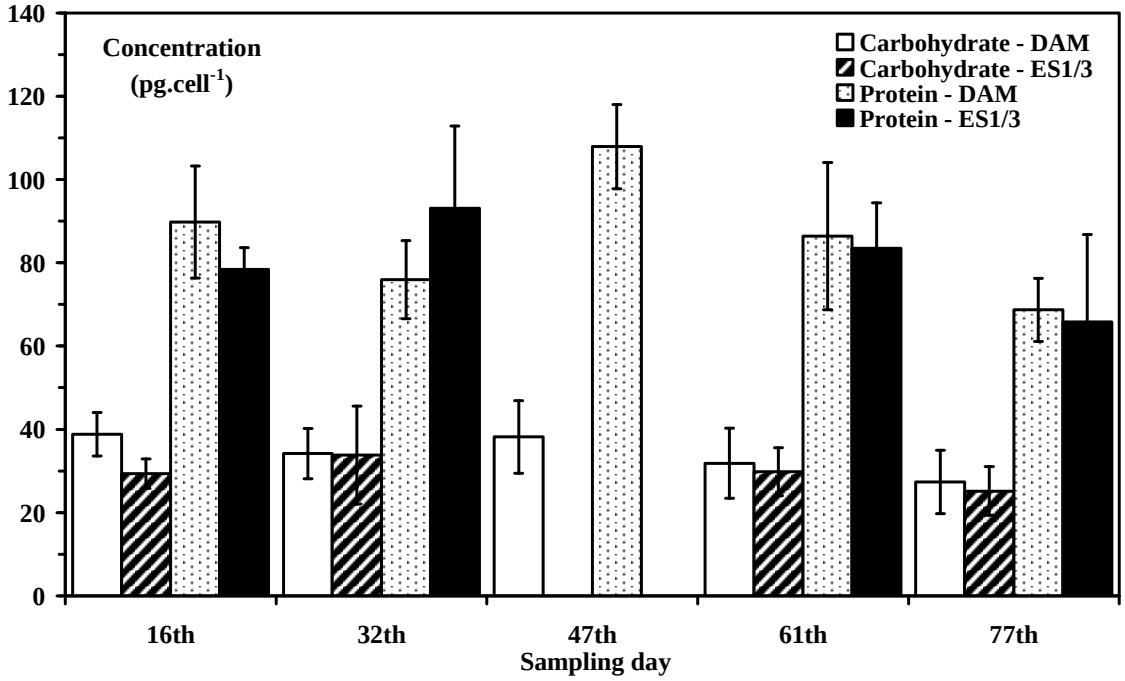


Fig. 1 Gagneux-Moreaux, Moreau, Gonzalez, Cosson

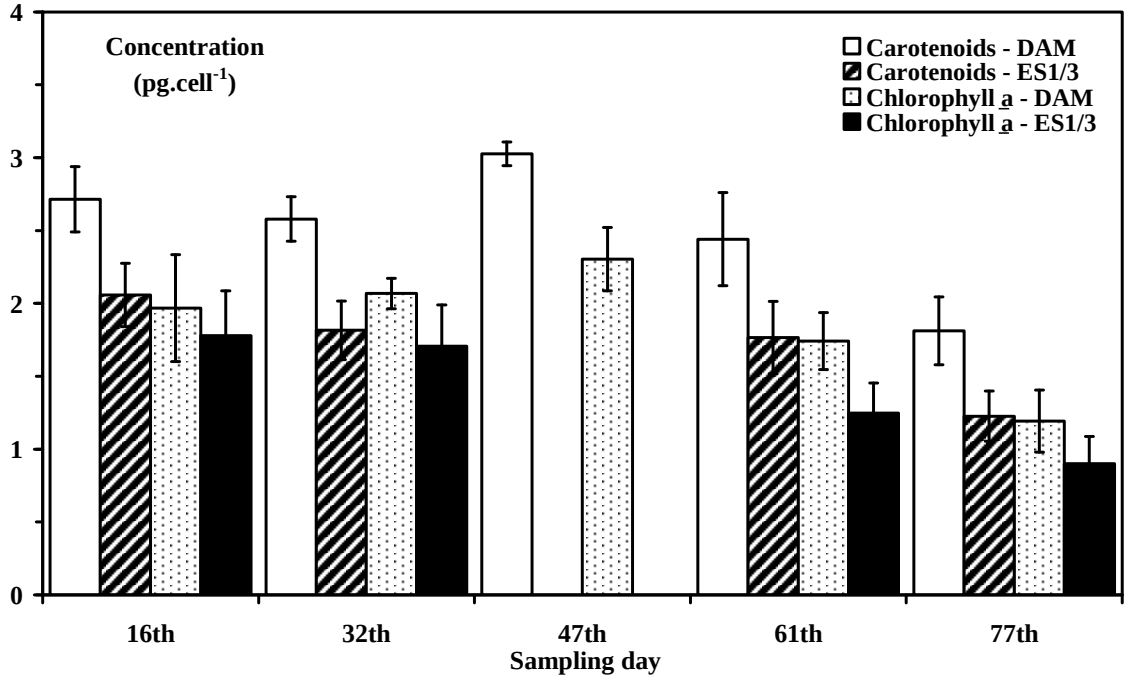
1



2

3 Fig. 2 Gagneux-Moreaux, Moreau, Gonzalez, Cosson

1



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3 Fig. 3 Gagneux-Moreaux, Moreau, Gonzalez, Cosson