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Roscoff

Effect of iron and manganese on the growth of the Diatom *Haslea ostrearia* in batch culture

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Abstract : The importance of iron and manganese for the growth of the "blue diatom" *Haslea ostrearia* Simonsen was studied using an enrichment bioassay method. The bioassays were set up using a culture medium chemically similar to the oyster-pond waters supporting the diatom. A mixture of iron and manganese provided the optimum conditions for the algal growth : the number of cells produced during the exponential growth phase was higher than in the presence of either individual metal ; the daily growth rate was also greater when metals were present in combination. However, the production of the blue pigment "marennine" was unaffected by any of the factors mentioned previously.

Résumé : Effet du fer et du manganèse sur la croissance de la diatomée *Haslea ostrearia* en cultures non renouvelées.

L'effet du fer et du manganèse sur la croissance de la diatomée bleue *Haslea ostrearia* Simonsen est recherché, en présence d'un chélateur, par application de tests biologiques. Ceux-ci sont réalisés à partir d'un milieu de base de composition proche de celle du milieu naturel. Le mélange de fer et de manganèse entraîne la plus forte stimulation de croissance de l'algue : le nombre de cellules produites en fin de phase exponentielle de croissance et le taux journalier de division sont augmentés en comparaison avec les résultats obtenus avec chacun des métaux. En revanche, aucun de ces éléments, qu'il soit apporté seul ou en association, ne semble agir sur la synthèse du pigment bleu caractéristique, la "marennine".

INTRODUCTION

The seawater contains varying concentrations of different metals which are traceable in unpolluted areas. Despite their low quantities, they are essential for marine plankton metabolism in combination with nutrients (Hunstman and Sunda, 1980). The effects of the metals may be beneficial or toxic, depending on their concentration and their physico-chemical form more or less available for the organism (Florence, 1982 ; Morel and Hudson, 1985 ; Wells, 1991 ; Wells *et al.*, 1991). Among all the trace metals, iron and manganese are known to be the most important for microalgae (Huntsman and Sunda, 1980). Thus, Brand *et al.* (1983) and de Baar *et al.* (1989) described the stimulation of a natural phytoplanktonic population developed in the presence of these two elements.

The effect of trace metals on the growth of phytoplankton can be estimated by using bioassays with unicellular algae strains belonging to international collections or isolated from the studied natural medium. These assays are carried out either in artificial seawater with added chemicals (Morel *et al.*, 1979 ; Harrison *et al.*, 1980 ; Keller *et al.*, 1987 ; Price *et al.*, 1991) or using natural seawater where the algae are incubated in trophic conditions as close as possible to their natural environment (Maestrini *et al.*, 1984). Using the latter method,

the present work studies the effects of iron and manganese on the growth and development of the pennate diatom *Haslea ostrearia* Simonsen or "Navicule bleue". With this species, the synthesis of the supplementary blue pigment "marennine" is in combination with cytological modifications determining a particular development of cells defined by four main pigmentation stages (Robert *et al.*, 1975). Since Ranson's work (1927), *H. ostrearia* is known to be responsible for the greening of the oysters and consequently to be used by the oyster-culturists in the maturing process. This is practiced on the south-west atlantic coast of France in seawater ponds called "claires", where environmental conditions are favourable to the diatom blooms : the hydrosoluble blue pigment is liberated by the cells and concentrated on the gills and labial palps of oysters immersed in the ponds.

The conditions of "Navicule bleue" blooms in the natural medium and the factors acting on the "marennine" synthesis are still unknown. Neuville and Daste (1978) found that the synthesis of "marennine" is strongly activated in a medium when nitrate, silicate and iron are deficient and that stress conditions induce the supplementary pigmentation. The influence of trace metals on the growth and development of *H. ostrearia* have never been studied in more detail. Nevertheless, Robert and Rouillard (unpublished) observed an active growth of populations cultivated on underground salt waters, naturally rich in metallic trace elements, especially iron and manganese. Thus, this first approach try to determine the effects of these two metals on the growth of the alga and on the "marennine" production by the cells.

MATERIAL AND METHODS

The alga

The study was carried out with an axenic strain of *H. ostrearia* isolated from greening-pond waters of the Bouin district (Vendée-France). The clone cells used were characterized by a modal length of 75 µm (Fig. 1), greater than 60 µm which is the critical size below which the algal growth is perturbated (Robert, 1978). The algal cultures were maintained by weekly inoculations on Guillard's f/2 medium (1982). The cells used for the assays were taken from cultures in growth exponential phase and exhausted of their cellular reserves before been used : the cells were cultivated over two days in seawater deficient of nutrients (Maestrini & Robert, 1981). In all cases, 300 ml of each medium to be tested were divided equally in two 250 ml flasks ; the algae were inoculated in order to obtain an initial cell density of 1000 cells. ml^{-1}. The flasks were placed in a culture room at 15 °C and a light intensity of about 3.10^{16} quanta. cm^{-2}. s^{-1} with a 14^{h} light/10^{h} dark cycle.

Definition of the basic medium

Preliminary, in order to characterize a basic medium used for metallic enrichments, and giving to the algal cells a nutrient quality similar to the natural environment conditions of their multiplication in oyster-ponds, a first experiment was carried out cultivating the algae

on different enriched media using natural waters. The characteristics of the basic medium were fixed indirectly, by estimation of produced cells biochemical composition : biomass = cell number ; development = the cellular amount of chlorophyll a and "marennine" ; nitrogen utilization efficiency = the quantity of mineral nitrogen taken up with regard to the produced biomass. In fact, these characteristics must be similar to those which characterize the results of bioassays already practiced on natural waters with the same species and in identical incubation conditions (Maestrini et Robert, 1981). In this way, seven different media were tested = ES Provasoli (1968) modified by Robert (1983) = ES 1/3 ; f/2 Guillard (1982) and f/10, f/20, f/30, f/40, f/50 (= f/2 diluted 5 to 25 times). All media were adjusted to a salinity of 28 and enriched with silicium (100 µm) in order to avoid a limitation of production by this element.

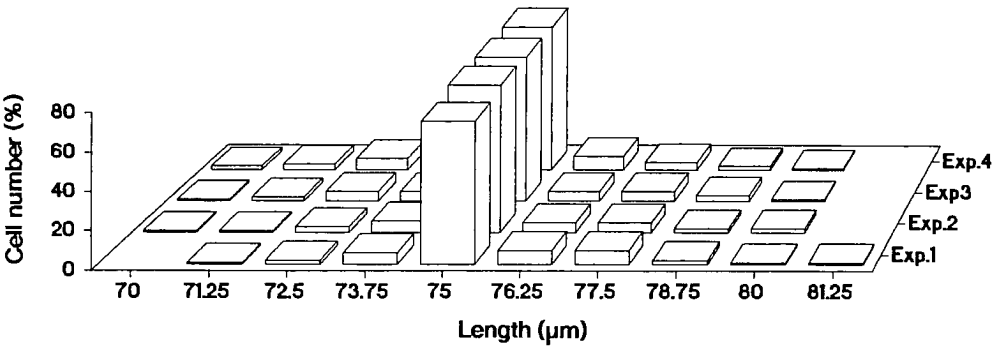

Fig. 1 : Distribution of cell lengths of the *Haslea ostrearia* clone used for bioassays, repeated four times.

The seawater used for the preparation of media was collected at the end of the "greening" (= blooming of *H. ostrearia*) of an oyster-pond. It was preliminary passed through a 0,45 µm filter ; the amounts of nutrients and trace metals of this water are reported in table I.

The amounts of N- NH_4-NO_2-NO_3, DON, P-PO_4 and Si-SiO_3 were estimated by the protocol of Strickland and Parsons (1972) adapted to a Skalar auto-analyser. The amounts of chlorophyll a were determined by the Lorenzen's method (1967). The trace-metals were determined according to the protocol described by Amiard *et al.* (1991).

Metallic enrichments and tests

Solutions of iron and manganese were added to the basic medium chosen, at concentrations recommended by Guillard for the preparation of the f/2 medium : Fe Cl_3-$6H_2O$ (12.10^{-6}M) and Mn Cl_2 - $4H_2O$ (10^{-6}M), with Na_2 -EDTA (13.10^{-6}M). The metals are added either alone or in association, and each type of metallic enrichment was tested eight times : four series of bioassays with each enrichment tested two times.

TABLE I

Concentrations of macronutrients (mineral nitrogen : $N\text{-}NO_3$, $N\text{-}NO_2$, $N\text{-}NH_4$; inorganic phosphorus : $P\text{-}PO_4$; silicium : $Si\text{-}SiO_3$), of organic matter (dissolved organic nitrogen : DON) and of trace metals (Cu, Fe, Mn, Zn) in the oyster-pond water used for the enriched culture media.

Macronutrients (µM)					Organic matter (µM)	Trace metals ($\mu g.l^{-1}$)			
$N\text{-}NO_3$	$N\text{-}NO_2$	$N\text{-}NH_4$	$P\text{-}PO_4$	$Si\text{-}SiO_3$	NOD	Cu	Fe	Mn	Zn
3,5	0,8	1,6	0,6	6,5	16,3	0,4	0,5	0,5	1,4

Parameters of growth and development

The results of the bioassays were expressed in maximum quantity of cells produced at the end of the growth exponential phase (estimated by counting the cells using a Nageotte slide) and by the quantity of total produced "marennine", determined at the end of the culture according to the spectrophotometric method described by Robert et Hallet (1981). In fact, during preceding experiments realized on enriched seawater media (ES 1/3 and f/2), it was shown that there is a significant linear regression between the concentration of total "marennine" produced by the algae and the cell density (Fig. 2). But it is important in the present case to study the effect of the metal enrichments on the preservation or not of this linear correlation.

The effects of metals were calculated by an average index of biomass - or production of blue pigment - expressing the ratio : 100 x biomass produced on enriched medium/biomass produced on the basic medium. The calculations of average indexes and their standard deviation used the formule of Cochran (1977) ; the significant level of the observed differences was evaluated by an analysis of variance (Lison, 1968).

RESULTS AND DISCUSSION

Choice of the basic medium used for metallic enrichments

With the seven tested media, the maximum number of produced cells changes between $55{,}5.10^3$ cells. ml^{-1} with f/50 and $117{,}5.10^3$ cells. ml^{-1} with f/2 (Table II). Moreover, during the growth of the cells cultivated on the richest media f/2 and ES 1/3 show long chloroplasts with smooth contours and a slight blue pigmentation ; on f/50, plasts are short and indented, the apical pigmented areas are strongly coloured in blue resembling cells from the transitory and benthic stages of the natural greening (Robert *et al.*, 1975 ; Robert, 1984).

With the f/50 medium, the biomass parameters of the alga and the nitrogen uptake efficiency present values near those found by Maestrini and Robert (1981) carying out bioassays on natural oyster-pond waters : $1\ 724.10^3$ cells. $\mu M^{-1}N$ on f/50 and $1\ 720.10^3$ cells. $\mu M^{-1}N$ on oyster-pond waters. Moreover, the concentrations of nutrients are similar with those estimated *in situ* by Robert *et al.* (1979), during the pre-spring period. So the f/50

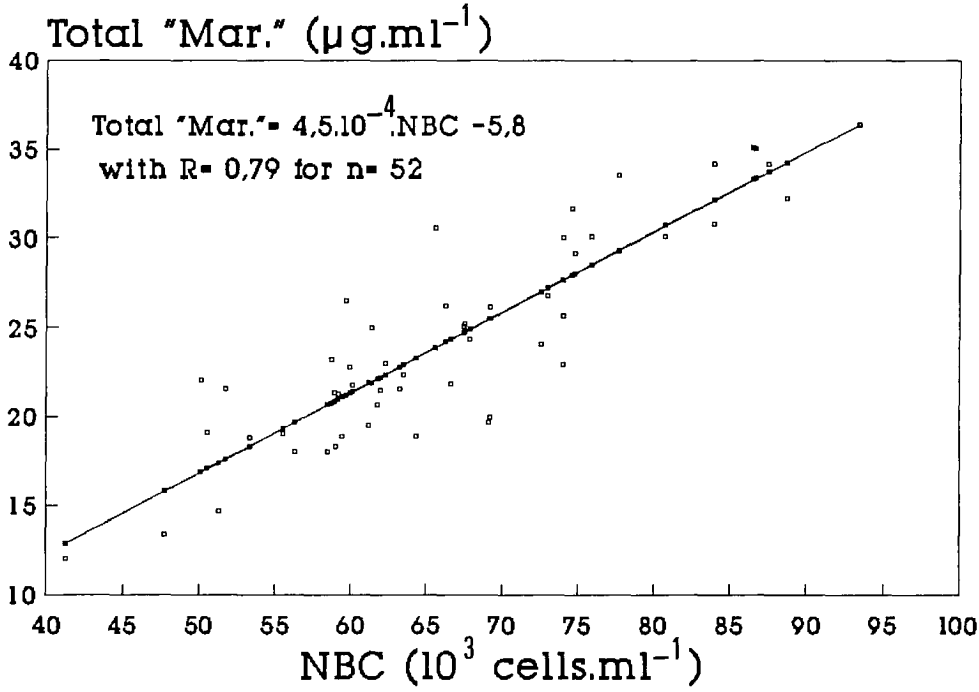

Fig. 2 : Dispersion diagram and correlation of total "marennine" contents (Total "Mar.") versus the produced algal biomass (NBC) in enriched seawater media (ES 1/3 and f/2). The correlation coefficient is significant at a level lower than 1%.

TABLE II

Maximum number of cells produced (NBC), chlorophyll a (Chl a) and total marennine (Total "Mar.") cellular contents and yield indexes of nitrogen (NBC/dN and Chl a/dN) of *Haslea ostrearia*. The algae are cultivated in controlled conditions on different media (ES 1/3, f/2 and f/2 after dilutions 1/5 to 1/25) and on natural oyster-pond waters.

ENRICHMENT	NBC (cells.ml^{-1})	Chl a (µg.10^{-6} cells)	Total "Mar." (µg.10^{-3} cells)	NBC/dN (10^3 cells.µM^{-1}N)	Chl a/dN (µg.µM^{-1} N)
ES 1/3	99 080	4.74	1.84	363.64	1.72
f/2	117 504	3.40	3.15	231.48	0.79
f/10	100 038	3.75	3.53	473.93	1.78
f/20	71 336	2.78	4.40	909.09	2.52
f/30	67 980	2.72	4.52	1369.86	3.73
f/40	63 384	2.05	4.02	1785.71	3.66
f/50	55 520	2.28	3.97	1724.14	3.93
Natural waters of oysterponds (from Maestrini and Robert, 1981)	-	2.37	-	1720.00	3.95

medium may be considered as the basic medium, because it facilitates trophic conditions similar to the natural cell environment found in "claires". The maximum biomass is thus achieved in the 9th day : after a four days lag phase stage, the growth rate is constant up to the stationary stage ; the daily average rate of division is then 0,49 divisions. day^{-1}.

Moreover the metallic enrichments are added in combination with Na_2-EDTA. Consequently, the basic medium can be defined by the medium f/50 enriched with this chelator at the concentration already fixed, in order to estimate the effect of iron and manganese enrichments on the growth and development of the alga.

Effect of Fe and Mn on the algal growth

Maximum cell numbers obtained with the two metals and their mixture are mentioned in Table III. Neither of these elements alone involves a significant statistical increase in algal growth. In this study, the iron and manganese concentrations in the basic medium which is similar to an oyster-pond water before a spring bloom (respectively 5.10^{-7} and 5.10^{-8}M), are higher than the growth limiting treshold values of these metals, which were found by Brand *et al.* (1983) ; so the results obtained with the tychopelagic diatom *H. ostrearia* confirm those obtained with the planktonic neritic diatoms studied by these authors. On the other hand, the association Fe+Mn induces a growth increase with regard to the control culture, with a significant level less than 1%. In fact there is a complementary interaction between iron and manganese causing a significant increase in biomass and in the algal growth rate (Table IV).

TABLE III

Effect of iron, manganese and their association on the growth of *Haslea ostrearia* on the marennine production by cultures, and also on the cellular blue pigment concentrations. This effect is expressed by the mean index of biomass (estimated by cell numbers) or of "marennine" production (expressed by ml of culture or by 10^3 cells). The standard deviation and the F variance ratio (F factor) express the significant levels of the results : F (5 %) = 4 (*) ; F (1 %) = 7,08 (**).

	Metal enrichment	Mean index %	Standard deviation	F Factor
Biomass	Control (f/50 + EDTA)	100	1,91	
	Fe	109,39	12,98	1,19
	Mn	104,42	34,20	0,26
	Fe+Mn	131,00	12,87	13,04 **
Total "marennine" production.ml^{-1}	Control (f/50 + EDTA)	100,00	18,10	
	Fe	76,75	28,34	2,64
	Mn	85,09	28,48	1,08
	Fe+Mn	92,79	33,99	0,25
Total "marennine" production.10^{-3} cells	Control (f/50 + EDTA)	100,00	6,85	
	Fe	93,19	41,80	0,26
	Mn	82,97	9,13	1,55
	Fe+%Mn	94,62	6,63	0,17

TABLE IV

Interactions between iron and manganese according to their effect on biomass. The F factor expresses the significance levels of the results : F (5 %) = 4 (*) ; F (1 %) = 7,08 (**).

Metal enrichment	Basic medium	Added metal	Effect	F factor
Te+Fe+Mn	Te+Fe	Mn	+	6,34 *
Te+Fe+Mn	Te+Mn	Fe	+	9,58 **

During the first 6 days (Fig. 2), the mean daily division rate was the same after enrichments with Fe and with Fe+Mn (in the order of 0,96), but slight higher than with the Mn enrichment (0,90). By stimulating the nitrate reductase activity (Lobban *et al.*, 1985 ; Doucette and Harrison, 1991 ; Price *et al.*, 1991), Fe would activate and control the algal growth rate until the inorganic nitrogen of the medium was exhausted (6th day). After the 7th day, without nitrate, the growth takes place only with the Fe+Mn mixture : Mn would participate indirectly to the taken up and assimilation of nitrogen in an inorganic form, since the final biomass is multiplied by 1,25 with Fe+Mn in comparison with the biomass produced by each metal alone. This other nitrogen form could correspond with the 16,3 µM of dissolved organic nitrogen (DON) initially estimated in the oyster-pond water used for the medium preparation. In fact, Maestrini and Robert (1987) showed that *H. ostrearia* is able to take up some organic nitrogen forms by photoheterotrophic path.

So the Fe+Mn association allows an increase in the biomass of *H. ostrearia* under controlled conditions. A growth stimulation of a natural phytoplanktonic population in the presence of each of these two elements was described previously by Brand *et al.*, 1983 and de Baar *et al.*, 1989.

Individually these two elements are essential for photosynthesis : Fe is a constituent of many enzymes and a catalyst in redox processes (Hipkins, 1983), particulary in the photosystem II with the cytochrome-b559 and in the photosystem I with the cytochrome-b6, the cytochrome-f (electron donor to plastocyanin) and with also the iron-sulphur centers (ferredoxin for example) ; it is moreover an essential cofactor in the synthesis of chlorophyll, with the ferredoxin and the Rieske iron-sulphur protein (Bienfait and Van der Mark, 1983 ; Hoober, 1987 ; Martin *et al.*, 1991). Greene *et al.* (1991, 1992) showed also an effect of iron limitation on the photosynthesis of the diatom *Phaeodactylum tricornutum* with a reduction in cellular chlorophyll concentration and an alteration of the efficiency of excitation energy transfer from the antenna to the reaction centers in the photosystem II. Like Fe, Mn is an important constituent of many catalysts of oxydoreduction reactions (Raven, 1990) ; it is also involved in the O_2 release during the photosynthesis by Mn-protein complexes in the photosystem II (Cheniae, 1970 ; Hipkins, 1983) and in the maintenance of the chloroplasts membrane structure (Lobban *et al.*, 1985).

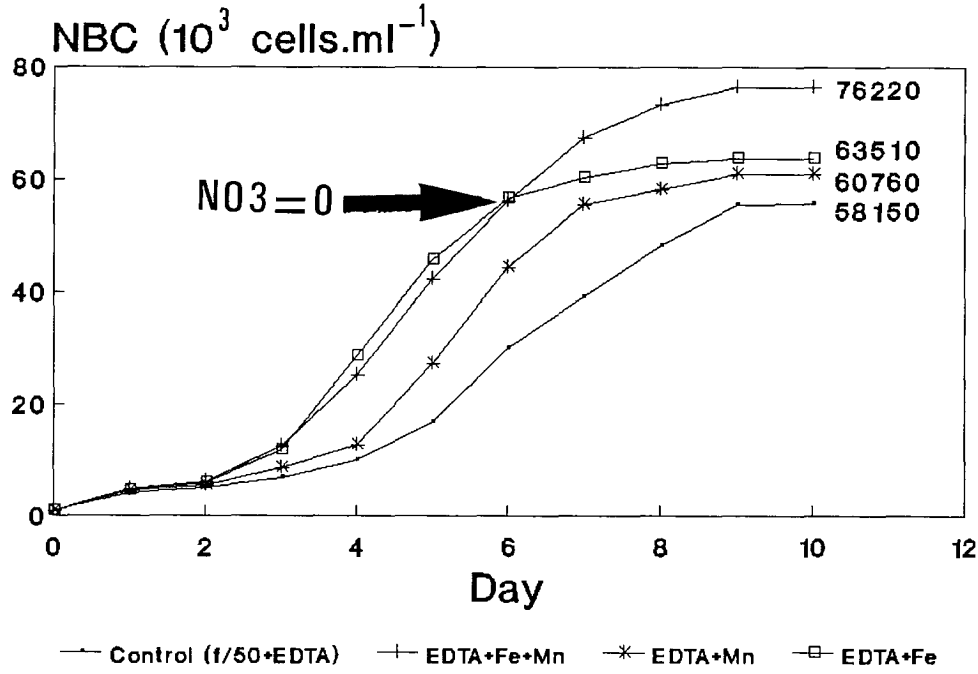

Fig. 3 : Growth of *Haslea ostrearia* cultivated on the f/50 medium (control) and after iron (Fe), manganese (Mn) and Fe+Mn enrichments of the same medium. The growth is estimated by the cell number increase (NBC) ; the mineral nitrogen exhaustion is illustrated by $NO_3 = 0$, for Fe and Fe+Mn enrichments.

3 - Influence of Fe and Mn on the production of "marennine".

The total amount of marennine produced by the cultures depends on the number of cells and simultaneously the amount of "marennine" synthesized by cell. The association Fe+Mn induces an increase in the algal biomass but in parallel, there is no increase in the blue pigment production (Table III). Consequently, the linear correlation between cell numbers and "marennine" concentrations is not preserved.

The produced "marennine" quantity at the cellular scale is not significantly related to the type of the metallic enrichment. Thus neither of these two metals seems to act, favourably or unfavourably, in the blue pigment synthesis, in association or alone. Neuville and Daste (1978) observed an increase of "marennine" concentration of cells cultivated in Fe deficiency conditions. Nevertheless in the present experiment, the "marennine" content of cells produced on the f/50 medium is not increased in comparaison with cells produced on the same medium enriched with Fe.

CONCLUSION

In an oyster-pond water enriched with macronutrients at concentrations similar to those in the natural medium, it is recommendable to add iron and manganese at concentrations defined in the usual culture media (ES Provasoli, f/2 etc...) to achieve an optimal production of *H. ostrearia* in batch culture. Under experimental conditions the addition of the metals is easy but in the natural environment of the "claires" it is not so straight forward because the sandy-slimy bottom complicates the nutrient exchanges between the sediment and the water.

By extending these observations to the oyster-pond ecosystem conditions, we can conclude that iron participates in the increase of algal growth rate, and that manganese would permit the use of the dissolved organic nitrogen when the inorganic forms of the medium are exhausted. The concentrations of these two trace-metals in the oyster-pond waters may be considered as determining factors in the greening process of the oyster-ponds.

Refering to empirical observations, some oyster-culturists already added iron in the Fe^{3+} form, by immersing metallic objects in the "claires". The effect of iron and manganese will be verified experimentally by differential enrichment bioassays carried out in *in situ* immersed containers, taking into account phenomenous of algal competition between *H. ostrearia* and other diatoms species. In parallel the effect of these same metals will be studied *in vitro* by cultivating several axenic dominant diatom strains on artificial media enriched according to a preliminary defined experiment plan.

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