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Effect of zeolites on cultures of the marine micro-algae *Emiliania huxleyi*

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Abstract

The yield of cultures of *Emiliania huxleyi*, a coccolithophore marine micro-alga species, in natural seawater and in seawater previously exposed to elutriates of ZEBEN-06 and ZESTEC-56, two zeolitic-nature products (ZNPs) (supernatant of 2.0 g l^{-1} of ZNP in natural seawater, stirred for 30 min) was evaluated. Total concentrations of dissolved trace metals and organic ligands (and respective conditional stability constants) in the initial media and at 7th day cultures were determined, by anodic and cathodic striping voltammetry. The changes introduced by the ZNPs in the media significantly enhanced *E. huxleyi* growth yield, the effect being more extent in the ZESTEC-56 elutriate. It was observed that both the ZNPs enriched the seawater with trace and not toxic quantities of Mn. In addiction, ZEBEN-06 removed small fractions of Cu and Zn from the media. The ZEBEN-06 elutriate stimulated the cell exudation but that of ZESTEC-56 inhibited exudation. Algae were also incubated in a culture medium with 0.05 g l^{-1} ZESTEC-56 in situ, and the growth yield was similar to that of the control culture. ZEBEN-06 was not studied in situ because it was hard to distinguish its particles from the cells, during cells counting. The ZESTEC-56 in situ enriched the medium in Mn, Cu, Pb and Cd, but impoverished it in Zn. The cell exudation was about four times higher in the presence of the ZNP in situ. Liberation/adsorption of micro-nutrients at the surface of the zeolites seems to be the cause of the observed changes in the biological response of the algae. The yield of the algal growth has economic relevance in aquaculture. On the other hand, ZNP are cheap, only small amounts (few mg l^{-1}) are required and the addition of some nicro-nutrients may be omitted. Therefore, the inclusion of zeolites in algal cultures in aquaculture may be economically advantageous. However, it is recommendable

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an previous investigation, in order to select the zeolitic characteristics and concentration that will maximise the algal yield in each particular case (alga nature and seawater trace metal contents).

Keywords: Zeolites; Marine algae; Exudates; Trace metals; Algal yield; Stripping voltammetry

1. Introduction

Zeolites, which are crystalline hydrated aluminosilicates of alkaline and earth-alkaline elements (particularly of sodium and calcium), are abundant and readily accessible (in the surface and near to mono-mineral deposits) and, therefore, normally inexpensive. Instead of being taken out from the nature, where they are generally extracted in the form of fragile solid rocks, zeolites can also be artificially produced. In the last case they are designated as zeolitic-nature products (ZNP). The major advantage of using produced ZNP is the possibility of size selection and the standardisation of particles, pores and channels from zeolites crystalline structure, which allow obtaining zeolites with very specific and selective characteristics, including ionic-exchange properties (López-Ruiz, 1999).

In the last few years, zeolites have been tested successfully for the treatment of wastewater and soils (Leppert, 1990; Misaelidis and Godelitsas, 1995; Shanableh and Kharabsheh, 1996; Reyes et al., 1997; Huttenloch et al., 2001; Samkutty and Gough, 2002). Their remarkably high cation-exchange capacity of up to 6 $mmol_{(eq.)}\ g^{-1}$ exceeds, by far, that of, for example, smectite clays, and thus permits an efficient removal of heavy metal cations (Bailey et al., 1999). The pre-treatment of zeolite surface with cationic surfactants additionally facilitates the retention of non-polar contaminants, e.g. chlorinated hydrocarbons, of anionic contaminants, e.g. chromate and arsenate (Haggerty and Bowman, 1994; Li and Bowman, 1997; Li et al., 1999) and benzene and its ionizable analogues (Li et al., 2000). The regeneration of modified surface zeolites was also investigated (Li and Bowman, 2001). Hence, it is possible to modify a natural zeolite in a way that it becomes a powerful sorptive agent for nearly all kinds of contaminants in aquatic systems (Jacobs and Förstner, 1999; Zhang et al., 2002; Bowman et al., 2001).

Zeolites have been used in fish farming in order to stimulate the bacterium mediated decomposition of proteins of non-consumed fish feed with production of nitrite and nitrate, instead of ammonia (López-Ruiz and Gómez, 1994). In addition, the presence of ZNPs, namely ZECER-56 (López-Ruiz et al., 1999) and ZESTEC-56 (López-Ruiz et al., 1995), stimulated the growth of some diatom species.

As the phytoplankton constitutes the basis of the marine food web, the understanding of the reciprocal interactions between the aquatic environment and these micro-organisms, as well as the factors that conditions such interaction, particularly those that stimulate the yield of cultures, have very much scientific and economic interest, therefore deserving research. It is known that massive production of micro-algae for feeding larval marine species in commercial laboratories makes out to be a considerable fraction of the total costs of operation of these labs, owing to the requirements in terms of qualified workers, additional infrastructure and chemicals.

The aim of this work was to investigate, for the first time, the influence of ZNPs on both the chemical composition of the medium and the growth of a marine micro-alga. To pursue after this aim, the growth of *Emiliania huxleyi* (a coccolithophore alga, widely spread in the oceans) in natural seawater was compared with those obtained in natural seawater that had been previously exposed to ZEBEN-06 and to ZESTEC-56 (elutriates of those ZNPs). Cultures in seawater with ZNPs in situ were also carried out, but only for ZESTEC-56, as the authors were not able to distinguish ZEBEN-06 from the algae particles, which prevented cells counting and, therefore, the estimation of the algal growth. The initial and final chemical composition of the culture media, in terms of trace elements and organic ligands, were determined and considered for the interpretation of the results. The chemical speciation is extremely important because the concentrations of trace inorganic or organic species may condition the biological response.

2. Materials and methods

2.1. Decontamination of materials and culture media

All materials including filters, erlenmeyers, polycarbonate bottles and plastics (pipette tips, filtration system, etc.) were acid cleaned and microwave-sterilised as described elsewhere (Leal et al., 1999). Culture media were sterilised by filtration (0.1 μm pore-size filter). All sample manipulations were carried out using gloves in a Class 100 laminar flow hood in a clean room with HEPA filtered air. The absence of bacteria in the cultures was tested periodically using standard bacteriological non-selective growth medium, and it was found that the cultures remained axenic during the entire growth period. The absence of bacteria was confirmed independently using a microscope with an oil immersion lens.

2.2. Reagents

Natural seawater was collected from 0.4 to 3.9 m depth, depending on the tide, at 150 m from Póvoa do Varzim coast (Northwestern Portugal) in November 1999 and in July 2001. Seawater was pumped continuously from the ocean through a PVC pipe at a rate of 100 l s^{-1}, and collected at the end of the discharge pipe directly to decontaminated 25 l HDPE containers. At the laboratory, seawater (35‰ salinity) was immediately filtered (0.1 μm polycarbonate membrane, Millipore) and stored in 50 l HDPE containers. Seawater was sub-sampled in 500 ml HDPE bottles, stored in the dark at 4 °C and used for experiments within 1 week.

ZNPs were supplied by the Zeolites Research Group of Cadiz University and were prepared as described elsewhere (López-Ruiz, 1999). Composition of the main constituents of each ZNP is shown in Table 1. Reagents were AnalaR grade unless indicated otherwise.

The metal standard solutions used in the voltammetric determinations were prepared by dilution of the atomic absorption spectrometry standard solutions (BDH, Spectrosol grade) in 0.01 M HCl. Salicylaldoxime (SA, Aldrich) 0.01 M was prepared monthly in 0.1 M HCl. 8-hydroxyquinoline (oxine, Merck) 0.1 M was prepared in 0.15 M HCl; a solution of 0.01 M oxine was prepared monthly by dilution. Dimethylglyoxime (DMG, Sigma) 0.1 M was prepared in NaHO. Diethylenetriaminepentacetic acid (DTPA, Sigma) 0.25 M was

Table 1
Major composition (% dry weight) of ZESTEC 56 and ZEBEN 06

	SiO_2	Al_2O_3	Fe_2O_3	CaO	MgO	Na_2O	K_2O	TiO_2	V_2O_5	$C_{organic}$
ZESTEC 56	55.7	25.5	6.9	1.2	1.2	–	3.5	1.2	0.2	4.6
ZEBEN 06	68.3	12.2	3.7	1.2	12.2	1.2	1.2	–	–	–

prepared in 0.35 M NH_3. $NaNO_3$ 5 M was prepared in water. Boric acid (Aldrich) 1 M and ammonia 0.35 M (or 0.67 M) (borate buffer) was used in the voltammetric determinations; 100 μl of this buffer in 10 ml seawater (0.01 M) gave a pH of 8.3 (or 9.3) (NBS pH scale). Acetate (Merck) 1 M was also used in the voltammetric determinations; 100 μl of this buffer in 10 ml seawater (0.01 M) gave a pH of 5.2 (NBS pH scale). All the metal, ligands and buffer solutions were stored at 4 °C. The water used for reagent preparation and rinsing was deionised (conductivity $<0.1\ \mu S\ cm^{-1}$).

2.3. Elutriates of ZNP

The elutriate solutions used as culture media as described below were prepared as follows: 2 g of each ZNP (ZEBEN-06 and ZESTEC-56) were added to 1 l of natural seawater and the solution was vigorous mechanically stirred for 30 min, with manual stirring at each 10 min. Suspension was then filtered (0.45 μm cellulose nitrate membrane, Millipore).

2.4. Cultures of E. huxleyi

E. huxleyi was grown in three very distinct culture media: elutriates of ZNP, natural seawater containing $0.050\ g\ l^{-1}$ ZESTEC-56 in situ and natural seawater (control medium). The experiments with ZNP elutriates and those with ZNP in situ were carried out in different date using seawater with different trace metal concentrations. Therefore, two series of control experiments were also performed.

In all cases, culture media were enriched only with nitrate (from sodium nitrate, Merck) and phosphate (from sodium hydrogenophosphate, Merck) added to final concentrations of 176 and 7.26 μM, respectively (equivalent to f/10 concentrations). After being sterilised, culture media were inoculated, in 1 l polycarbonate bottle, with 1ml of the f/2 (Stein, 1973) stock unialgal, axenic, *E. huxleyi* culture (Lohmann PCC No 92 and 92d, isolated by Hay and Muller, Plymouth Marine Laboratory) on Day 7 of growth (exponential phase). The initial concentration of algae was approximately 0.5×10^6 cells l^{-1}. For culture media containing ZNP in situ, ZESTEC-56 was microwave-sterilised (5 min at 700 W) prior to addition to the sterilised culture media. Control experiments containing just ZESTEC-56 ($0.05\ g\ l^{-1}$) in natural seawater, without algae, were also carried out in the same conditions as described above, in order to permit comparison of the medium composition with the growing of the algae in the medium containing ZESTEC-56 in situ. ZEBEN-06 was not used for in situ experiments, once this ZNP was practically undistinguished from *E. huxleyi* when observed on microscope, which prevented the cells counting.

Cultures were incubated with continuous illumination (24 h) at 18 °C, until the stationary phase was reached. A continuous light source does not mimic the natural conditions where

these algae would grow in normally. Nevertheless, it has been observed that the cultures are able to display exponential growth, and all incubations were maintained in identical conditions, to enable valid conclusions. All experiments were carried out in triplicate.

2.5. Cells counting

Cells number was counted using a standard microscope (Nikon, Eclipse E400) according to established methods (Guilard, 1978). The algae culture was placed directly in a hemacytometer, 0.1 mm deep, with an Improved Neubauer ruling.

The observation of the cells with an optical microscope, as well as with a scanning electronic microscopy, revealed that this algae species did not produce coccoliths in these experimental conditions. Cells' counting was carried out in quadruplicate.

2.6. Determination of trace metal contents in the culture media

The total dissolved concentrations of the different metals ($[M]_d$) in the culture media were determined after removing the algae by filtration (0.45 μm pore-size filter) in a vacuum filtration system (Millipore) at 0.6 bar. Microscopic analysis of the algae in the filter revealed that they did not suffer from rupture or breaking during filtration.

Acidified aliquots (pH 2.2) of the media were UV-digested (Ultramed 1000-W mercury vapour lamp, from Osram) for 1 h. Acidification at a lower pH value (pH 1.5) and/or higher UV-irradiation times originated statistically identical results.

Metal concentrations were determined by anodic stripping voltammetry (ASV) (Zn, Cu, Pb and Cd) or cathodic stripping voltammetry (CSV) (Mn, Ni, Co and Cr) after sample pH neutralisation with ammonia (6 M). The voltammetric equipment consisted of an Autolab voltammeter (Ecochimie) connected to a Metrohm 663-VA eletrode stand provided with a hanging mercury drop electrode (HMDE). Operational details have been described previously (Leal et al., 1999). The determination of Cr was carried out by square wave CSV at pH 5.2 (0.01 M acetate buffer, 0.5 M $NaNO_3$) using 2.5 mM DTPA as the competitive ligand and a deposition potential, E_d, of −1.0 V. The scanning parameters were: frequency of 50 Hz, modulation amplitude of 25 mV and step potential of 2.44 mV. Using the same procedure, the $[M]_d$ was also determined in a seawater reference material for trace metals (NASS-5) with the experimental and certified values being statistically identical ('t' test, $P = 0.05$). The concentration of Ca and Mg in the filtered culture media was determined by atomic absorption spectrometry with flame atomisation (AAS-F) (Philips PU 9200X), after 1000 times (Ca) and 5000 times (Mg) dilution of the samples, in the presence of lanthanum (0.2% for Ca, and 0.1% for Mg).

Total metal concentrations in the algae ($[M]_{algae}$) (extracellular adsorption plus intracellular uptake) were determined after microwave-digestion (Milestone MLS-1200 Mega) of the filters with 1 ml of suprapure, concentrated nitric acid (Merck) in high pressure Teflon vessels and then were diluted with water and neutralised with ammonia. The metal concentrations were then determined as in the culture media. The metal fixed per algal cell ($[M]_{cell}$) was calculated from the $[M]_{algae}$ and cells counting. Control filters were processed using the same procedure, but the metal contamination was found to be insignificant (<1%). $[M]_{algae}$ approximately balanced the metal lost from seawater (with an error <5%) confirming that

metal adsorption onto the polycarbonate culture bottles was negligible. The pH was estimated potentiometrically.

2.7. Determination of Cu-complexing ligands

The Cu-complexing concentrations of organic ligands (C_L) and the respective conditional stability constants (K'_{CuL}) were determined by titration of the filtered culture media with Cu(II) and detection of labile Cu by CSV (Campos and Van Den Berg, 1994; Leal et al., 1999). Cu (II) was selected for estimating of the organic ligands concentration in the media, owing to its ability in forming the most stable complexes with mainly of the organic ligands of the Irving-William's series. A batch mode was used. The pH was buffered at 8.3 (with 0.01 M borate buffer, pH 8.3) for the control culture media (Days 0 and 7), ZNP elutriates media (Day 0) and culture medium containing ZESTEC 56 in situ (Day 0). For the other culture media (elutriates of ZNP and ZESTEC 56 in situ at Day 7) pH was buffered at 9.3 (with 0.01 M borate buffer, pH 9.3).

Salicylaldoxime (SA) (5 μM final concentration) was added as a competition ligand to 100 ml of filtered culture medium. Aliquots of 10 ml were pipetted into ten polystyrene tubes also containing Cu ions, previously added, in the required range. Solutions were equilibrated overnight (12–15 h) prior to the determinations.

2.8. Calculations

The Cu-complexing ligand concentration (C_L) and the conditional stability constant of Cu complexes (K'_{CuL}) were calculated as described before (Campos and Van Den Berg, 1994; Leal et al., 1999), using the following relationship (van den Berg, 1982).

$$\frac{[Cu]_{labile}}{[CuL]} = \frac{[Cu]_{labile}}{C_L} + \frac{\alpha}{(K'_{CuL} C_L)} \quad (1)$$

where α is the overall labile side-reactions coefficient for the metal ion:

$$\alpha = \alpha_{Cu} + \alpha_{CuSA} \quad (2)$$

Values of 36 and 57 for α_{Cu}, pH 8.3 and 9.3, respectively, in seawater (35‰ of salinity and 25 °C), were calculated using an ion-pairing model and metal stability constants from Turner et al. (1981). The temperature assumed was 18 °C (present study), but there is a lack of values for stability constants at this temperature. Therefore, the α_{CuSA} value is an approximation of the actual value. The calculated values of α_{CuSA} were 36×10^3 (pH 8.3) and 47×10^3 (pH 9.3).

[CuL] is calculated as follows:

$$[CuL] = [Cu]_d - [Cu]_{labile} \quad (3)$$

Linearity of the $[Cu]_{labile}/[CuL]$ versus $[Cu]_{labile}$ in a certain range of the plot was interpreted to indicate complexion of the metal by a single ligand in the covered range.

3. Results and discussion

3.1. Effects of the ZNP elutriates

The curves of algae growth observed for the seawater culture (control) and, in parallel, for the cultures of ZEBEN-06 and ZESTEC-56 elutriates are shown in Fig. 1. Both ZNP elutriates significantly enhanced the *E. huxleyi* growth yield and the growth rate (calculated for the exponential portion of the growth chart, see Table 2) the effect being more extensive in the case of ZESTEC-56, where the cells number after 7 day growth was 2.6 times higher than that observed in the control culture. Fig. 1 also shows that, particularly in the elutriates, the maximum culture growth occurred after 7 days of incubation, the number of cells decreasing from Days 7 to 9, indicating cellular death. This suggests that after 7 days of growth, the medium became too poor in some nutrients, preventing the cultures development.

The total concentrations of dissolved trace metals and organic ligands were determined in the initial culture media and again after algae filtration at Day 7.

Table 3 shows that both ZNPs released significant amounts of Mn to the culture media. In fact, the Mn concentration in elutriate was three times higher than that in the control medium. In addition, ZEBEN-06 removed from the culture medium about 30% of Cu and 37% of Zn initially present in the seawater, whereas ZESTEC-56 only removed 2% of Pb. Statistically significant variations in the concentration of the other studied metals did not occurred, probably because the ionic-exchange sites were mainly occupied by ions of alkaline (particularly Na) and earth-alkaline metals which exist at very high concentrations in seawater, and the ionic-exchange with trace elements is slow and, therefore, did not take place in significant extent during the elutriate preparation (30 min).

In all culture media, a decrease in the dissolved micro-nutrient concentrations was observed after 7 days of growth, as it was expected. The uptake percentage was 60–80% for

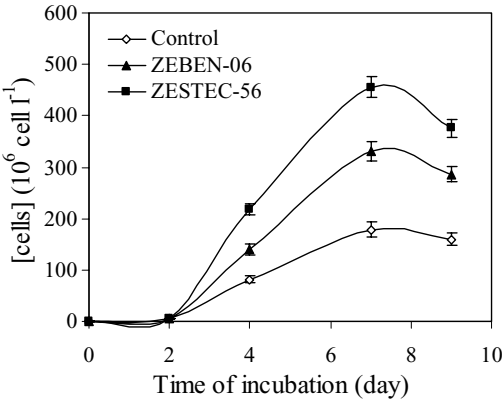

Fig. 1. Mean values of *E. huxleyi* growth in natural seawater (control) and in elutriates of ZEBEN-06 and ZESTEC-56 (2 g l^{-1} in seawater with constant stirring for 30 min, followed of rejection of ZNPs). Standard deviations ($n = 3$) are also given.

Table 2
Growth of *E. huxleyi* and releasing of exudates in the first series of experiments[a]

Time (day)	Growth (10^6 cell l^{-1})	Growth rate[b] (day^{-1})	C_L (nM)	C_L released[c] (10^{-18} mol $cell^{-1}$)	log K'_{CuL}
Control (natural seawater)					
0	0.52 ± 0.05		58 ± 4		12.7 ± 0.1
2	5.8 ± 0.5				
4	82 ± 8				
7	178 ± 15	0.85	76 ± 6	101	12.6 ± 0.2
9	160 ± 11				
Elutriate of ZEBEN-06 in natural seawater					
0	0.54 ± 0.05		58 ± 5		12.6 ± 0.1
2	5.0 ± 0.5				
4	139 ± 11				
7	331 ± 19	0.95	95 ± 6	112	12.6 ± 0.2
9	286 ± 15				
Elutriate of ZESTEC-56 in natural seawater					
0	0.52 ± 0.05		56 ± 3		12.5 ± 0.3
2	5.0 ± 0.4				
4	218 ± 11				
7	456 ± 20	1.0	94 ± 5	83	12.4 ± 0.1
9	376 ± 17				
Natural seawater enriched with 10 nM Mn					
0	0.50 ± 0.06		58 ± 4		12.7 ± 0.1
2	11 ± 1				
4	298 ± 13				
7	470 ± 20	0.99	87 ± 1	62	12.9 ± 0.1
9	420 ± 19				

[a] See in Table 3 the trace metal concentrations of the media.
[b] Calculated for the exponential phase of growth, from an exponential equation.
[c] Calculated as follows: $(C_{L(7\,day)} - C_{L(0\,day)})/([cells]_{(7\,day)} - [cells]_{(0\,day)})$.

Cd, Zn and Mn; 20–40% for Cu, Pb and Ni and less than 10% for Cr. As Cd is normally considered to be a non-essential or toxic metal, an uptake similar to that observed for the Zn and Mn micro-nutrients is an interesting result that corroborates some previous recent information. Lee et al. (1995) demonstrated that Cd can nutritionally substitute Zn at least in some phytoplankton species. High Cd uptake percentages were also obtained in a previous study in *E. huxleyi* cultures (Vasconcelos and Leal, 2001).

Table 3 also includes the metal cellular levels determined in 7-day cultures. The cellular metallic levels in the ZNP elutriate cultures were lower than those in the control culture, except for Mn, to which an opposite behaviour was observed. Lower levels of metal per cell maybe due to the higher number of cells in the cultures in elutriates, which implicated less metal available per cell. The maximum observed level of Mn per cell was found for ZEBEN-06 elutriate, 23×10^{-18} mol per cell, and the minimum for the control, 12×10^{-18} mol per cell. It is also interesting to note that the final dissolved levels of Mn were similar in the three culture media, ca. 1–2 nM, in spite of the initial elutriates being much richer in Mn than the control (ca. 9 nM versus 3 nM in the control). These

Table 3
Metal concentrations in the *E. huxleyi* cultures, in the first series of experiments[a]

Medium	Cu	Pb	Cd	Zn	Mn	Ni	Co	Cr
Control								
Just after inoculation[b]: $[M]_d$[c](nM)	9.3 ± 0.5	0.16 ± 0.01	2.5 ± 0.1	16 ± 1	2.9 ± 0.3	24 ± 2	n.d.[d]	37 ± 2
After 7 days growth								
$[M]_d$[c] (nM)	7.1 ± 0.3	0.10 ± 0.01	0.51 ± 0.02	4.3 ± 0.2	0.8 ± 0.1	18 ± 1	n.d.[d]	35 ± 1
Uptake[e] (%)	24	38	80	73	72	25		5.4
$[M]_{cell}$ (10^{-18} mol cell^{-1})[f]	12	0.34	11	66	12	34		11
ZEBEN-06 elutriate								
Just after inoculation[g]: $[M]_d$[c](nM)	6.2 ± 0.6	0.18 ± 0.02	2.3 ± 0.1	10.5 ± 0.4	9 ± 2	27 ± 2	n.d.[d]	38 ± 1
After 7 days growth								
$[M]_d$[c] (nM)	5.0 ± 0.2	0.12 ± 0.01	0.36 ± 0.02	3.8 ± 0.2	1.47 ± 0.04	19 ± 1	n.d.[d]	35 ± 2
Uptake[e] (%)	20	33	84	64	84	30		7.9
$[M]_{cell}$ (10^{-18} mol cell^{-1})[h]	3.6	0.18	5.9	20	23	24		9.1
ZESTEC-56 elutriate								
Just after inoculation[i]: $[M]_d$[c](nM)	8.6 ± 0.5	0.13 ± 0.01	2.4 ± 0.2	18.9 ± 0.7	9.1 ± 0.5	25 ± 2	n.d.[d]	35 ± 1
After 7 days growth: $[M]_d$[c] (nM)	6.9 ± 0.3	0.09 ± 0.01	0.48 ± 0.04	5.6 ± 0.5	1.7 ± 0.1	17 ± 1	n.d.[d]	34 ± 1
Uptake[e] (%)	20	31	80	70	81	32		2.9
$[M]_{cell}$ (10^{-18} mol cell^{-1})[j]	3.7	0.09	4.2	29	16	18		2.2

[a] The initial *E. huxleyi* cells concentration was about 0.5×10^6 cell l^{-1}.
[b] pH: 7.8; [Ca]/[Mg] (mg l^{-1}): 392/1170.
[c] Average±standard deviation ($n = 3$).
[d] Not detected. Limit of detection=0.010 nM.
[e] Percentage of the initially soluble metal that was taken up by the cells (adsorbed and absorbed).
[f] [cells] (10^6 cell l^{-1}): 178 ± 15 pH: 8.4.
[g] pH: 8.0; [Ca]/[Mg] (mg l^{-1}): 416/1220.
[h] [cells] (10^6 cell l^{-1}): 331 ± 19 pH: 9.3.
[i] pH: 7.7; [Ca]/[Mg] (mg l^{-1}): 400/1160.
[j] [cells] (10^6 cell l^{-1}): 456 ± 20 pH: 9.3.

results indicated that in the used seawater Mn was a micro-nutrient limitant for *E. huxleyi* growth.

As Mg and Ca are macronutrients, the respective total dissolved concentrations were also determined in the culture media just after the algae inoculation (see Table 3). Compared with the control, the changes in the concentration of these earth-alkaline cations caused by the contact with the ZNP were very small and probably were not the cause of the higher yield of the cultures carried out in the elutriates.

The pH of the media was measured in all the *E. huxleyi* cultures. It was observed (Table 3) an increase in pH after 7 days growth, the pH variation being higher in the cultures with higher growth yield. A pH enhancement in the algae cultures is understandable since the algae were grown in the presence of constant (24 h) light and photosynthesis used up $CO_{2(aq.)}$, increasing the final pH. Thus, whilst it is reasonable to consider that the control medium was buffered (the change in pH was negligible), the buffer strength of natural seawater was not enough for stabilising the pH in the ZNP elutriate cultures, where the final number of cells was much higher.

3.1.1. Effect of Mn addition

As both ZNPs introduced significant amounts of the micro-nutrient Mn into the initial culture media and the *E. huxleyi* growth was much higher in those cultures, the Mn seemed to be a limiting agent for the algae growth in the natural seawater used. In order to test this hypothesis, seawater enriched with 10 nM Mn (similar to the concentration introduced by both ZNPs in the media) was used as *E. huxleyi* culture medium and the growth was followed for 9 days. It was observed that the enrichment of the medium in Mn favoured both *E. huxleyi* growth yield and rate (Table 2). The cellular growth yield at Day 7 was statistically identical to that observed in the ZESTEC-56 elutriate culture, suggesting that the increase of Mn level in the medium may be a major cause of the observed phenomenon in elutriates. However, other factors contributed for the results obtained in the different media, since both ZNPs have introduced similar amounts of Mn in the initial media and the cultures showed different growth yields as well as different exudation extent (see below). As mentioned before, zeolites have been considered capable of stimulating the microbial oxidation of N-ammoniacal to nitrite or nitrate (López-Ruiz et al., 1999). As nitrate is a macro-nutrient of the algae a promotion of nitrate levels might be the cause of enhancement of algal yield in aquaculture. However, in the present study the enhancing effect can not be attributed to that phenomenon because the zeolites were not in situ during the cell growth and, in addition, the cultures were axenic. Therefore, the present data demonstrated that zeolites can play in the cultures other role beyond that pointed out before (López-Ruiz et al., 1999).

3.1.2. Total organic ligands concentration

The total concentration of organic ligands that are strong complexing agents of Cu(II) were also determined in the culture media. For this purpose, aliquots of each culture medium were titrated with Cu(II) at pH 8.3 or at pH 9.3 (accordingly to the pH of the medium under study). The results obtained are included in Table 2.

The concentration of Cu-complexing exudates released in the media (difference between initial and final concentration of C_L) was higher in the ZNP elutriate cultures than in that in

natural seawater culture. This was already expected since the cells number was also higher in the ZNP elutriate cultures. However, exudates concentration released per cell was not similar in the different cultures. Compared to that in the control culture, 101×10^{-18} mol per cell, the exudation was just slightly higher in the ZEBEN-06 elutriate culture, 112×10^{-18} mol per cell, lower in the ZESTEC-56 elutriate culture, 83×10^{-18} mol per cell, and much lower in the natural seawater enriched with Mn, 62×10^{-18} mol per cell. Differences in the media composition in terms of micro-nutrients (those determined and eventually other not identified) may be the cause for those results.

The conditional stability constants of the Cu-complexes, K'_{CuL}, were similar for all the studied culture media, either at the beginning (just after the algae inoculation) or at 7th day growth.

3.2. Effects of the ZNP in situ

Growth yield and rate results obtained in the culture containing $0.050\,g\,l^{-1}$ ZESTEC-56 in situ and in the respective control are shown in Table 4 (see also Fig. 2). Experiments were carried out only until Day 7, since results obtained for the ZNP elutriates showed that cells started to die after this period. Table 4 shows that the presence of ZESTEC-56 in the culture medium did not influence significantly the algal yield and rate.

The total dissolved trace metals concentrations observed in the culture media and in 7-day cultures are shown in Table 5. In a comparison of the composition of the natural seawater with that where $0.050\,g.l^{-1}$ ZESTEC-56 had been in situ for 7 days in the absence of algae, one can see that the ZNP enriched the water in Cu (80% higher), Pb (86%), Cd (32%) and Mn (161%). The Cr concentration did not change. In contrast, the ZNP removed from the seawater Zn (33% of the initial level in seawater), Ni (16%) and Co (11%). Therefore, the seawater composition in terms of trace elements was much more modified by $0.050\,g.l^{-1}$ ZESTEC-56 in situ than during the preparation of the elutriates ($2\,g\,l^{-1}$ of ZESTEC-56 or

Table 4
Growth of *E. huxleyi* and releasing of exudates in the cultures in the second series of experiments[a]

Time (day)	Growth (10^6 cell l^{-1})	Growth rate[b] (day^{-1})	C_L (nM)	C_L released[c] (10^{-18} mol $cell^{-1}$)	log K'_{CuL}
Natural seawater					
0	0.55 ± 0.03		81 ± 3		12.15 ± 0.02
2	11.7 ± 2.8				
4	152 ± 16				
7	370 ± 25	0.89	89 ± 3	23	12.70 ± 0.08
Natural seawater with $0.050\,g\,l^{-1}$ ZESTEC-56 in situ					
0	0.55 ± 0.03		81 ± 3		12.15 ± 0.02
2	15.0 ± 5				
4	138 ± 2.5				
7	351 ± 19	0.91	111 ± 3	87	12.44 ± 0.03

[a] See in Table 5 the trace metal concentrations of the media.
[b] Calculated for the exponential phase of growth, from an exponential equation.
[c] Calculated as follows: $(C_{L(7\,day)} - C_{L(0\,day)})/([cells]_{(7\,day)} - [cells]_{(0\,day)})$.

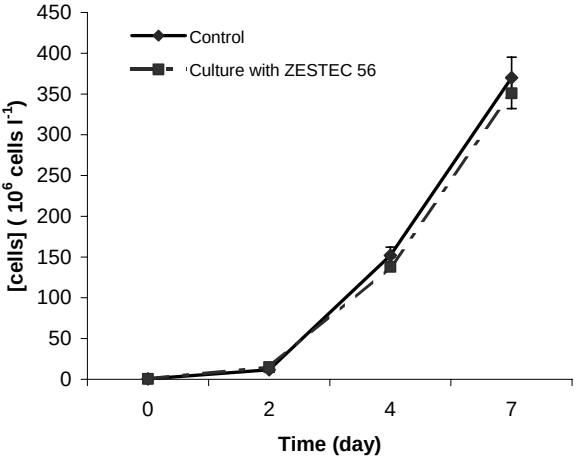

Fig. 2. Mean values of *E. huxleyi* cell concentration in natural seawater (control) and in the culture medium containing 0.050 g l^{-1} ZESTEC-56 in situ. Standard deviations ($n = 3$) are also shown.

ZEBEN-06, for 30 min, Table 5 versus Table 3 data). Very probably, this resulted of slow rates of metal ion-exchange at the zeolite surface and cavities. In all cases, the most extent change in the media composition occurred for Mn, which increased from 8 nM in initial seawater to 21 nM in the presence of the ZESTEC-56 in situ. In the first set of experiments it was observed an accentuated increase of the growth yield when the seawater was enriched with 10 nM of Mn (total level in initial culture medium of 12.9 nM). However, in the present case, the growth yield was similar in the absence and in the presence of ZNP. This resulted of the complex and varied combination of events (release and adsorption of several metals beyond Mn) that simultaneously occurred in the presence of the ZNP in situ, whose the particular biological effects very probably antagonised each other.

Comparing now the decrease of the trace metal concentrations after 7 days of algal growth in the absence and in the presence of the ZNP in situ, very interesting results can be found. The extent of consumptions of the different metals significantly and markedly differed between the culture with ZNP in situ and that in the natural seawater. It is not possible to secure that the metal consumption was wholly due to metal fixed by the algal cells (adorbed and absorbed), because the cellular metal was not determined by technical limitations, as mentioned above. But that is assumed here for the interpretation of the data. The initial and final numbers of cells were statistically identical in the absence and in the presence of ZNP, which facilitate the discussion. The cellular consumption of Mn was much higher in the presence of the ZNP, whereas those of Zn and Cd and Ni were not detected. These results suggest that Mn can partially replace Zn (and Cd) in some *E. huxleyi* cellular functions. These results are compatible with those found by Sunda and Huntsman (2000) for cultures of oceanic diatoms. Those authors have observed that the cellular Cd:C ratios increased with decreasing of Zn and/or Mn free concentrations. The effects of Zn and Mn apparently were related to the uptake of Cd (an element of the group of Zn in the periodic table) by the cells' Mn and Cd/Co transport systems, which are under negative feedback regulation by cellular

Table 5
Metal concentrations in the *E. huxleyi* cultures[a] in natural seawater in the absence and in the presence of 0.050 g l^{-1} ZESTEC-56 (second series of experiments)

Medium	Cu	Pb	Cd	Zn	Mn	Ni	Co	Cr
Seawater								
Just after inoculation: $[M]_d$[b](nM), pH: 8.3	4.7 ± 0.4	0.22 ± 0.07	0.34 ± 0.09	9.4 ± 0.1	8 ± 1	21.5 ± 2.0	0.53 ± 0.04	15.6 ± 1.2
Culture after 7 day growth: $[M]_d$[b] (nM)	4.1 ± 0.2	0.28 ± 0.02	0.16 ± 0.01	8.1 ± 1.2	6.8 ± 0.8	20.0 ± 0.3	0.33 ± 0.09	14.2 ± 0.9
[cells] (10^6 cell l^{-1}): 489 ± 51 pH: 9.2								
ZESTEC 56 in situ								
After 7 day without cell inoculation: $[M]_d$[b](nM) pH: 8.4	8.5 ± 0.3	0.41 ± 0.06	0.45 ± 0.02	6.3 ± 0.6	20.9 ± 0.6	18 ± 1	0.47 ± 0.04	16 ± 1
Culture after 7 day growth: $[M]_d$[b] (nM)	7.1 ± 0.5	0.8 ± 0.1	0.6 ± 0.3	7.3 ± 0.1	15 ± 1	21 ± 2	0.35 ± 0.03	14 ± 1
[cells] (10^6 cell l^{-1}): 457 ± 25 pH: 9.3								

[a] The initial *E. huxleyi* cells concentration was about 0.5×10^6 cell l^{-1}.
[b] Average±standard deviation ($n = 3$).

Mn and Zn (Sunda et al., 2000). In the present study, the consumption of Cd was much higher (50% or higher) in the natural seawater cultures where the dissolved concentration of Mn was very little than in the media richer in Mn (see Tables 3 and 5).

Differences in the cellular uptake of different metals were probably the cause of the difference in cellular exudation observed between the culture with ZNP in situ and the respective control (see Table 4).

4. Conclusions

This study demonstrated that zeolites were able to significantly enhance the *E. huxleyi* growth. On the other hand, they changed the chemical composition of seawater, at least in relation to total dissolved trace metal concentrations. Zeolites can enrich the solution with trace quantities of some metallic micro-nutrients, which initially are present in the medium at limitant concentrations and are present in the zeolites as impurities. On the other hand, zeolites can reduce the dissolved concentration of other elements by adsorbing them. Such changes in the medium composition may result in variations of the culture yield, promoting or inhibiting the algal growth, according to the changes have positive or negative effects on the optimum composition of the medium.

The yield of the algal growth has high economic relevance in aquaculture, as massive production of micro-algae, for feeding larval marine species, makes out to be a considerable fraction of the total costs of operation of commercial laboratories. Zeolites are cheap (15 € per kg), only small amounts (few $mg\,l^{-1}$) are required and the addition of some micro-nutrients may be omitted. Therefore, the inclusion of zeolites in algal cultures in aquaculture may be economically advantageous.

However, it is recommendable a previous investigation, in order to select the zeolitic characteristics and concentrations that will maximise the algal yield in each particular case. In fact, it is expected that several factors condition the role of zeolite in the yield of a culture, namely as follows: (a) composition of the seawater used in terms of trace elements; (b) characteristics and concentration of the zeolite used; (c) nature of the algae; (d) pH of the medium.

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