Transitional Waters Bulletin
TWB, Transit. Waters Bull. 1(2008), 31-38
ISSN 1825-229X, DOI 10.1285/i1825229Xv2n1p31
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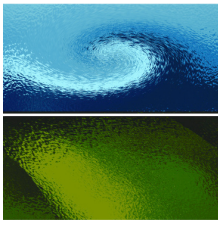

RESEARCH ARTICLE

Effect of salinity on growth, cell size and silicification of an euryhaline freshwater diatom: *Cyclotella meneghiniana* Kütz.

Vincent Roubeix and Christiane Lancelot

Université Libre de Bruxelles, Ecologie des Systèmes Aquatiques, CP-221 Boulevard du Triomphe, B-1050 Bruxelles, Belgium;
Vincent Roubeix, ESA, Université Libre de Bruxelles, Boulevard du Triomphe, CP 221, B-1050 Bruxelles, Belgium;
tel : +32 2 650 57 15, fax : +32 2 650 59 93,
e-mail : vincent.roubeix@bordeaux.cemagref.fr

Abstract

1 - The rivers transport plankton seawards which finally enters estuaries and often experiences lethal osmotic stress along an increasing salinity gradient. The tolerance of river phytoplankton to salinity increase can modify the nutrient cycles in estuaries. In this experimental study based on two culture experiments, the effect of salinity on growth, cell size and silicification of the euryhaline freshwater diatom *Cyclotella meneghiniana* was studied.

2 - Diatoms were first grown in batch cultures at 8 different salinities ranging from 0 to 33. The maximal growth rate (0.92 d^{-1} ±0.04) occured at salinity 18 and decreased continuously from this optimum toward higher or lower salinities reaching 0.44 (±0.06) and 0.64 d^{-1} (±0.13) at salinities 0 and 33 respectively.

3 - In a second experiment two continuous cultures of the diatom were studied at salinities of 1 and 30. At equilibrium, the difference in the amount of Si per cell between the two cultures was due to a decreased cell height at salinity 30 and did not reflect any change in cell silicification on a surface basis.

4 - This work reveals the importance of accounting for the effect of salinity on freshwater euryhaline diatoms in the study of the Si cycle in the river-sea transition zone.

Keywords: diatoms; estuaries; experimental culture, salinity gradient; silica

Introduction

Freshwater phytoplankton undergoes drastic salinity change when transported down rivers into estuaries. Stenohaline river species lyse at the beginning of the salinity gradient where their specific limit of salinity tolerance is exceeded and results in the high phytoplankton mortality reported in the oligohaline regions (salinity < 5) of estuaries (Morris *et al.*, 1978; Ahel *et al.*, 1996; Heiskanen and Keck, 1996; Ragueneau *et al.*, 2002). On the contrary some euryhaline species are able to regulate their intracellular osmotic pressure and grow at higher salinities further seaward in estuaries (Flameling and Kromkamp, 1994; Orive *et al.*, 1998; Erdmann and Hagemann, 2001; Roubeix *et al.*, 2008).

The centric diatom *Cyclotella meneghiniana*, a cosmopolitan planktonic species recorded in many rivers and estuaries all over the world (Finlay *et al.*, 2002) is reported as euryhaline but its domain of salinity tolerance varies among studies (Tuchman *et al.*, 1984). The presence of freshwater euryhaline diatoms able to grow in an estuarine salinity gradient might be an important factor controlling land to sea Si transfer and its consequences for coastal eutrophication (Officer and Ryther, 1980).

Cell wall silicification and cell size are two integrative parameters of diatom physiology

whose variations in a population have been extensively studied. Diatom cell-wall silicification was shown to change in response to various environmental factors like the presence of aluminium in the growth medium (Gensemer, 1990), the grazing pressure (Pondaven *et al*., 2007) or as a consequence of growth reduction by nitrogen, phosphorus, iron or light limitation (Claquin *et al*., 2002; Leynaert *et al*., 2004). As regards to salinity, a lower cellular silica content of euryhaline diatoms was reported at higher salinity (Tuchman *et al*., 1984; Olsen and Paasche, 1986). Diatom cell size decreases slightly after each vegetative cell division because the frustule valves of the two daughter cells are formed inside the mother valves (Jewson, 1992). However cell size might also be reduced by the elevation of Al concentration in the environment (Lynn *et al*., 2000) or by nutrient limitation (Leynaert *et al*., 2004). As far as we know, no study demonstrated an effect of salinity on cell size. Nevertheless, Hildebrandt *et al*. (2006) observed a lower size of *Thalassiosira pseudonana* when grown at higher NaCl concentration.

As part of a general study of processes affecting the transfer of continental Si to the coastal sea, we explored under laboratory conditions the salinity tolerance of a pure culture of *Cyclotella meneghiniana*. Batch cultures were first conducted to assess the variations of *C. meneghiniana* 's growth rate in the salinity range 0-33. Next, two steady-state continuous cultures of *C. meneghiniana* grown at two extreme salinities were carried out in order to isolate the effect of salinity on cell size and silicification which are both growth dependant.

Materials and methods

Experiments

A pure culture of *C. meneghiniana* isolated by K. Muylaert (Katolieke Universiteit Leuven) from the freshwater reaches of the Schelde estuary was maintained in freshwater WH medium (Guillard and Lorenzen, 1972) at 20°C and at an irradiance of 100 µmol quanta m^{-2} s^{-1}. This strain was used to inoculate both batch and continuous cultures.

Batch cultures

The growth rate of *C. meneghiniana* was measured in batch cultures at salinities 0, 2, 5, 10, 18, 25, 30 and 33 under optimal light and nutrient conditions. The growth media at different salinities were obtained by the dilution of artificial seawater (Harrison *et al*., 1980) with Milli-Q water and the addition of the nutrients of the WH growth medium. The cultures placed on a platform shaker were incubated at 20°C under a continuous irradiance of 130 µmol quanta m^{-2} s^{-1}. After a first growth phase of acclimation, the cultures were diluted with new medium at the same salinity and two other series of cultures were carried out for growth rate measurements.

Continuous cultures

Two continuous cultures of *C. meneghiniana* were grown at 20°C in 2 L polycarbonate bottles at salinities 1 and 30 and under continuous light of about 40 µmol quanta m^{-2} s^{-1}. They received a continuous input of new growth medium (dilution rate = 0.28 d^{-1}) from two 10 L bottles containing diluted artificial seawater at the corresponding salinity enriched with WH nutrients to reach a final silicic acid concentration > 50 µmol L^{-1}. Cell sedimentation and carbon limitation were prevented through filtered air bubbling. After 2 weeks, the cultures had reached an equilibrium with residual fluctuating variations in cell density <10% during the last 4 days before sampling. At steady state, pH (Eutech PC 300) and irradiance (Li-250 Li-Cor) were measured in the bottles and a 200 mL sample was collected for cell counts and chemical measurements.

Biological measurements

For the batch cultures, cell density in each culture was measured by spectrophotometry (Kontron 930) at 750 nm wave length (Sorokin, 1973; Shafik, 1997) on sub-samples taken regularly. The affine relation between absorbance at 750 nm (A_{750}) and cell density was verified by diluting a concentrated culture (2.6 10^7 cell L^{-1}) at stationary phase with the culture medium and by reading absorbance for each dilution (Sorokin, 1973):

$$A_{750} = 0.0119 \times dilution - 0.0002 \quad (R^2 = 0.9995)$$

The exponential growth rate μ was derived from absorbance measurements by fitting the following equation during the exponential growth phase starting at t_0:

$$\mu = \frac{\ln A_{750}(t) - \ln A_{750}(t_0)}{t - t_0}$$

For the continuous cultures, sub-samples (3 mL) for cell counts and cell size measurements were preserved by lugol addition (1% final concentration) and storage in the dark at 4°C. A minimum of 1000 diatom cells was counted in each sample with an inverted microscope (Leitz Fluovert) at 320× magnification. Diatom dimensions were determined by image analysis (Lucia 4.6 software). Cell diameter and height were measured on different cells appearing on the sedimentation slide in valve or girdle view respectively. Cell surface and volume were calculated considering the cells as cylinders.

Chemical measurements

Si concentrations

Ten mL sub-samples were filtered on 47 mm diameter polycarbonate membrane filters (0.6 μm pore size, Nucleopore) for biogenic silica ($bSiO_2$) and silicic acid (DSi) determinations. Filters ($bSiO_2$) were stored at room temperature in sealed petri dishes and filtrates were acidified with 0.2% HCl suprapur and kept at 4°C until analysis. DSi concentration was determined spectrophotometrically (Kontron 930) at 810 nm using the silicomolybdate method (Grasshoff *et al*., 1983). Standards were prepared for each tested salinity to avoid salinity interference on coloration. The $bSiO_2$ collected on filters was first solubilized in 0.1 M NaOH at 100°C during 1 hour, then neutralized with 1N HCl and finally measured in the same way as DSi (Paasche, 1980).

Chlorophyll a

Sub-samples (5 mL) were filtered on Whatman GF/F filters and stored in the freezer (-20°C) until further processing. The collected chlorophyll a (Chl *a*) was extracted overnight in 10 mL 90% acetone in the dark at 4°C. Chl *a* concentration was determined by fluorescence (Kontron SFM 25) at 460 and 672 nm for excitation and emission respectively.

Particulate organic carbon (POC)

Sub-samples (5 mL) were filtered through pre-combusted (4 h at 450°C) fibreglass Whatman GF/F filters. Organic carbon retained on the filters was determined with a Dorhmann Apollo 9000 Total Carbon Analyser. Only one sample was analysed for each culture. The relative standard deviation of POC analysis according to this procedure and with the same device was estimated in a previous study at 10% (Servais *et al*., 1999).

Results and discussion

Effect of salinity on growth

Fig. 1 shows a parabola-like salinity dependence of *C. meneghiniana's* specific growth rate at 20°C. Mean growth rates (n=2) ranged between 0.44 ±0.06 and 0.92 ±0.04 d^{-1}. Surprisingly, the minimal value was obtained at salinity 0 while the maximum was at the intermediate salinity 18, twice as high as at salinity 0. At the highest salinity (33) the mean growth rate was 0.64 d^{-1} (±0.13) i.e

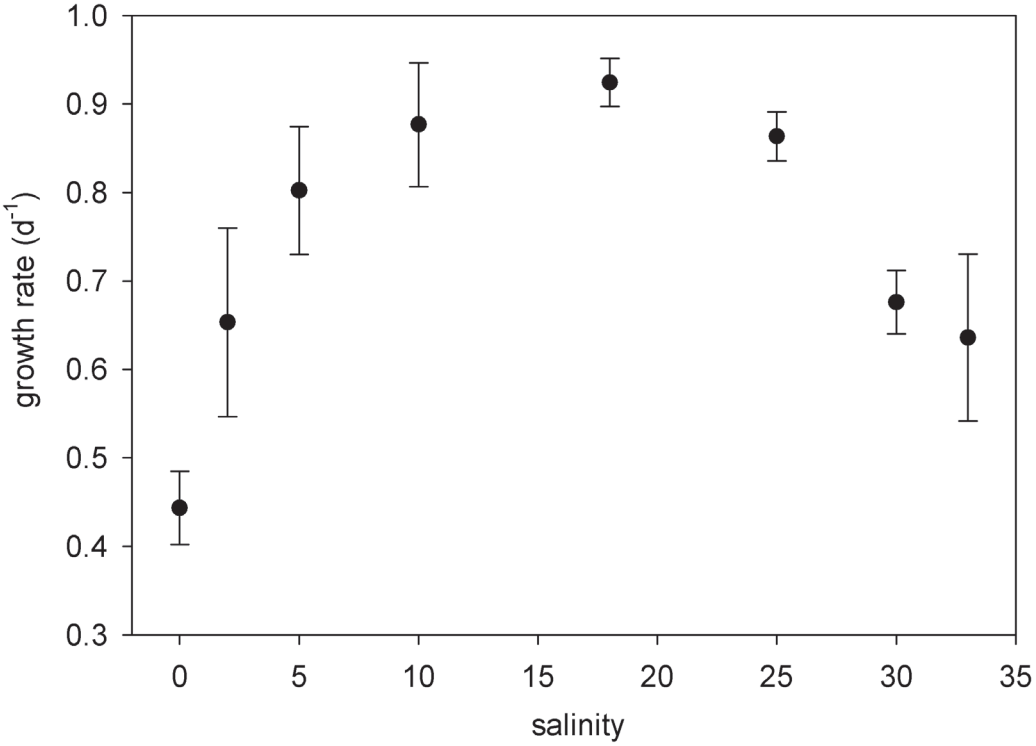

Figure 1. Mean optimal specific growth rates of *Cyclotella meneghiniana* for different salinities at 20°C and 130 µmol quanta m^{-2} s^{-1} irradiance. The error bar indicates the values of the duplicate measurements.

significantly higher than at salinity 0.

This growth experiment confirmed the euryhalinity of *Cyclotella meneghiniana*. According to the classification of Carpelan (1978), *C. meneghiniana* is part of the holeuryhaline group encompassing species able to grow from freshwater to marine conditions. The term halophilous (Tuchman *et al*., 1984; Muylaert and Sabbe, 1999) appears also well suited to this diatom as its growth rate increases when salinity rises up to an optimum around 18. Such a mid-salinity optimum was already observed by Shoberi (1974) for the same species (around 15). However other studies reported a much lower tolerance to salinity of *C. meneghiniana* (Kopczynska, 1979; Tuchman, 1984). Such a difference could be explained by genetic variations among clones of the species isolated in different environments (Tuchman, 1984). Recently Beszteri *et al*. (2005) showed indeed that the wide ecological range of *C. meneghiniana* could result from the existence of cryptic sexual species, possibly more stenotopic. The clone used in this study shows a particularly good adaptation to estuarine conditions, the optimal salinity for its growth being at the middle of the salinity range from river to sea as already noticed for some salt marsh diatoms (Williams, 1964).

Effect of salinity on cell size and stoichiometry

Steady-state parameters obtained for the continuous cultures of *C. meneghiniana* at salinities 1 (culture 1) and 30 (culture 2)

are summarized in Table 1. A single pH unit difference between the two cultures was measured, seawater being usually more basic than fresh or slightly brackish waters. Cell concentration was more than two fold higher at salinity 30 creating slightly lower irradiance in this culture by self shading. Similarly Chl *a*, $bSiO_2$ and POC had higher concentrations in culture 2 (Table 1). DSi concentrations were low in the two cultures suggesting an almost complete use of nutrients by diatoms. The ratio POC:Chl *a* was the same in both cultures (44 µg μg^{-1}, Table 2) indicating that the cells were in the same physiological state at both salinity levels. The mean cell diameter and cell height were significantly lower at salinity 30 ($P<0.001$). This size reduction is due to a much larger decrease of cell height (-45%) than of cell diameter (-6%).

Such a decrease of diatom cell dimensions has been already observed in conditions of nutrient limitation (Lynn *et al.*, 2000; Leynaert *et al.*, 2004). Leynaert *et al.* (2004) interpreted this as an adaptation to a shortage in nutrients by increasing the surface to volume ratio which facilitates solute diffusion into the cells. In our experiment however, the two continuous cultures at equilibrium were both nutrient-limited. Thus it is surprising that cell size reduction was more intense at high salinity. It is possible that salinity has a direct effect on cell morphogenesis. Hildebrandt et al. (2006) observed that only the height of the centric diatom *Thalassiosira pseudonana* was reduced at increased salinity, the diameter remaining unchanged. The elongation of diatom cells during the interphase preceding division is driven by turgor pressure which makes the siliceous components of the cell walls slide apart (Pickett-Heaps *et al.*, 1990; Harold, 2002). At high salinity the freshwater diatom might not be able to produce the intracellular osmolarity needed to generate the same turgor pressure as at low salinity. Thus if cell elongation is less efficient before each cell division, cell height might decrease faster in high salinity water.

The mean cellular Si content was 5.1 and 3.1 pmol $cell^{-1}$ at salinity 1 and 30 respectively, indicating a 38% lower value at high salinity (Table 2). However a similar difference in the mean frustule surface resulted in an unchanged Si amount per unit of frustule surface (4.8 fmol μm^{-2}; Table 2). This suggests that cell silicification was not affected by salinity. The ratio Si:C was equal to 0.08 at salinity 1 and was 10% lower at salinity 30. The same difference in the ratio Si:Chl *a* was measured between the two cultures. However, since POC and Chl *a* are more related to cell volume (Mullin *et al.*, 1966; Montagnes *et al.*, 1994), the higher surface:volume ratio

Table 1. Steady state parameters in the two continuous cultures of *Cyclotella meneghiniana* at salinities 1 and 30 (standard deviation in brackets; n= number of samples).

	culture 1		culture 2	
salinity	1		30	
pH	7.4	(n=1)	8.4	(n=1)
Cell density (10^6 cell L^{-1})	10.93 (0.18)	(n=2)	26.31 (0.52)***	(n=2)
Irradiance (µmol quanta m^{-2} s^{-1})	46	(n=1)	35	(n=1)
$bSiO_2$ (µmol L^{-1})	55 (12)	(n=5)	82 (17)*	(n=5)
Chl *a* (µg L^{-1})	188 (11)	(n=2)	308 (18)*	(n=2)
POC (mgC L^{-1})	8.2	(n=1)	13.6	(n=1)
DSi (µmol L^{-1})	4.81 (0.09)	(n=2)	3.44 (0.24)*	(n=2)
cell diameter (µm)	13.3 (1.3)	(n=44)	12.6 (0.7)***	(n=61)
cell height (µm)	18.3 (1.7)	(n=100)	10.1 (0.7)***	(n=45)

Significant difference compared to culture 1 (t-test): * P<0.05, ** P<0.01, *** P<0.001

Table 2. Mean calculated variables from steady-state measured parameters and relative variations in culture 2 compared to culture 1.

	culture 1	culture 2	%variation
diatom surface (μm^2)	1045	646	- 38
diatom volume (μm^3)	2550	1250	- 51
surface:volume (μm^{-1})	0.41	0.52	+26
Si:cell (pmol $cell^{-1}$)	5.1	3.1	- 38
Si:surf (fmol μm^{-2})	4.8	4.8	0
Si:C (mol mol^{-1})	0.081	0.073	- 10
Si:Chl *a* (μg μg^{-1})	8.3	7.5	- 10
POC:Chl *a* (μg μg^{-1})	44	44	0

at salinity 30 should induce higher Si:C and Si:Chl *a* ratios. The slightly lower values of these ratios at salinity 30 reflects higher concentrations of POC and Chl a in the cells at this salinity, which may be due to the relatively fast size reduction without regulation of cellular content.

The silica content of *C. meneghiniana* cells was shown to decrease with increasing salinity (Tuchman *et al*., 1984). Similarly, an experiment on another euryhaline species, *Thalassiosira pseudonana*, revealed a higher cellular Si content at salinity 0 than at 24 whatever the concentration of DSi in the medium was (Olsen and Paasche, 1986). However in these two experiments, the possible changes in cell size were not explored. Moreover growth rate was not the same at the tested salinities which gives a bias on the results since cell silicification generally changes with growth rate (Martin-Jézéquel *et al*., 2000; Claquin *et al*., 2002). In our experiment, the growth rate was the same at the two chosen salinities and cell silicification was best assessed by the ratio between the biogenic silica per cell and the mean surface of the frustules. In this way, no direct effect of salinity on silicification was detected. Marine diatoms are in average less silicified than freshwater ones (Conley *et al*., 1989). From our experiment on an euryhaline species, it seems that this difference can not be attributed to a direct effect of salinity.

Conclusion

Cyclotella meneghiniana is clearly an euryhaline diatom which is able to grow from freshwater to marine conditions. However its growth rate changes with salinity being maximal at an intermediate salinity level between river and sea. At high salinity, cell height seems to be reduced faster, but cell silicification apparently does not change on a surface basis suggesting that the thickness of the frustules remains constant. The silicification of *C. meneghiniana* appears to be quite high compared to values reported from other diatoms (1-3 fmol Si μm^{-2}; Martin-Jézéquel *et al*., 2000; Claquin *et al*., 2002).

The survival of some diatom species like *C. meneghiniana* in an estuarine salinity gradient can lead to a larger export of $bSiO_2$ to the sea as noticed by Orive et al. (1998) for the Urdaibai estuary. Euryhaline freshwater diatoms like *C. meneghiniana* have an advantage in their ability to take up new or remineralised nutrients of river origin after the death of stenohaline species at low salinity in an estuary. Consequently, when sinking to the sediment, they contribute to increase river nutrient retention in estuaries.

Acknowledgements

The present work has been performed in the

scope of the EU Research Training Network Si-WEBS (contract N° EU/CT-2002-00218). We are grateful to Koenraad Muylaert for providing us with algal cultures. Thanks to the anonymous reviewers for their interesting comments.

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