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Cell death in three marine diatom species in response to different irradiance levels, silicate, or iron concentrations

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ABSTRACT: The effects of light, silicate, or iron limitation on cell death rates and the accompanying effects on growth rates and photosynthetic efficiencies were investigated for 3 species of marine diatoms, *Chaetoceros brevis*, *Chaetoceros calcitrans* and *Thalassiosira antarctica*. Cell death rates were based on changes in time of the percentage dead cells using the SYTOX Green membrane permeability assay. The death rates increased with decreasing irradiance levels and silicate concentrations for *T. antarctica*. In contrast, the cell death rates were hardly affected by low irradiances or silicate for *C. brevis* and *C. calcitrans*. Iron limitation experiments did not affect algal death rates significantly in the species tested. Growth rates under limiting conditions decreased from 0.6–0.4 to 0.2–0.1 d^{-1}, and only under low light conditions did growth halt completely. Photosynthetic efficiencies did not always co-vary with cell death rates and were shown to be a sensitive indicator of light and iron limitation, but not of silicate limitation. This is the first detailed study providing data on diatom cell death under different growth-limiting conditions. The differential response in cell death rates indicates irradiance as a determining factor in diatom species succession and distribution.

KEY WORDS: Cell death · Growth · Marine diatoms · Iron · Irradiance · Silicate · SYTOX Green

INTRODUCTION

So far, most efforts to understand the diatoms' success have been focussed on growth-related processes. The effects of light and variable irradiance levels on marine diatom growth have been the subject of many studies (Geider et al. 1986, Berges & Falkowski 1998, Timmermans et al. 2001a, Boyd et al. 2002, MacIntyre et al. 2002). In a similar manner, the effects of silicate on diatom growth are also well documented (see review by Martin-Jézéquel et al. 2000, and references therein). More recently, the effects of iron as a growth-controlling factor on diatoms were studied (Sunda & Huntsman 1995, Timmermans et al. 2001b). However, it is clear that for a complete understanding of diatom biomass and species distribution, the investigation of growth in relation to environmental conditions and changes is not sufficient. Loss parameters have to be taken into account as well. Traditionally, grazing and/or sedimentation have been considered the major loss factors for phytoplankton cells in oceans. It is evident by now that we cannot balance the growth and losses of phytoplankton in many ecosystems based on these 2 loss terms only (Walsh 1983, Van Boekel et al. 1992, Brussaard et al. 1995, Bidle & Falkowski 2004). Increasing evidence points to cell death as a third major algal loss factor (Kirchman 1999, Agustí & Duarte 2000, Brussaard 2004, Franklin et al. 2006). Due to the development of sensitive fluorescent stains in combination with single cell analysis, an awareness of the importance of viruses as mortality agents, and the discovery of programmed cell death in algae, the research on algal cell death has gone through a remarkable evolution (Brussaard et al. 1996, Berges & Falkowski 1998, Agustí & Sánchez 2002, Bidle & Falkowski 2004, Franklin & Berges 2004, Franklin et al. 2006).

Still, the number of laboratory studies on physiological cell death in diatoms is very limited. There are the

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studies by Lee & Rhee (1997, 1999) under N or P limitation and light limitation, respectively, but these were performed on freshwater cyanobacteria. The marine diatom *Ditylum brightwellii* was also studied under N and P limitation, showing that nutrient-stress-induced cell lysis was a significant loss factor (Brussaard et al. 1997). Peters & Thomas (1996) and Peters (1996) have studied diatom cell death responses in relation to prolonged darkness. The diatom *Thalassiosira weissflogii* hardly showed any cell death after 6 d of light deprivation, whereas significant death was found in response to nitrogen stress; in contrast, the chlorophyte *Dunaliella tertectiola* showed massive cell death in connection with light deprivation (Berges & Falkowski 1998).

The specific objectives of the present study were to quantify cell death rates, together with growth rates and photosynthetic efficiencies, of diatoms in response to light, silicate, or iron limitation. The fluorochrome SYTOX Green was used to discriminate between live and dead cells. Two Antarctic diatoms (*Chaetoceros brevis*, *Thalassiosira antarctica*) and 1 temperate diatom (*Chaetoceros calcitrans*) were tested. We relate variations to the possible differences in autoecology between the different diatom species.

MATERIALS AND METHODS

Algal cultures and general culture conditions. Unialgal, xenic cultures of *Chaetoceros brevis*, *Thalassiosira antarctica* and *Chaetoceros calcitrans* originating from the Royal NIOZ culture collection were used. These diatoms are typically small species with diameters of 3 to 6 µm and cell volumes in the range of 30 to 60 μm^3. The diatoms were incubated in 125 ml polycarbonate bottles at 4°C (*C. brevis*, *T. antarctica*) or 15°C (*C. calcitrans*). The diatoms were grown under semi-continuous conditions at constant growth rates. When cell abundances became too high (>100 000 cells ml^{-1}), the diatoms were diluted with fresh growth medium to approximately 100 cells ml^{-1}, as to prevent nutrient depletion and effects of self-shading. The cultures growing at the highest rates (i.e. grown at the highest irradiance levels, silicate, or iron concentrations) determined the time of dilution for all cultures. These dilution steps were repeated at least twice, in order to acclimate the diatoms to the experimental conditions of nutrient or low light stress. The growth media consisted of either natural, filtered Southern Ocean seawater for the irradiance and iron experiments, or natural, filtered, nutrient-poor surface water from the Canary Basin for the silicate experiments. All filtrations were done using Sartobran 0.2 µm pore size filters. The Southern Ocean water had background concentrations of 26.1 µM nitrate, 24.5 µM reactive silicate, 1.82 µM orthophosphate and 0.3 nM dissolved iron. The Canary Basin water had background concentrations of nitrate, reactive silicate and orthophosphate concentrations of 0.8, 0.7 and <0.05 µM, respectively. The culture bottles were cleaned with 1 M HCl prior to use, followed by rinses with Milli-Q water (3 times, of which the last was with boiling Milli-Q). Finally, the bottles were dried in a laminar flow bench and stored sealed till used. This treatment rendered trace-metal-clean and sterile bottles. All handling of the incubations was done in a laminar flow bench to prevent contamination by microorganisms and/or trace metals. A 16:8 h light:dark photoregime, with cool white fluorescence tubes (Phillips TLD 36W/54) as a light source, was maintained throughout all experiments. Light intensities of 100 µmol photons m^{-2} s^{-1} were maintained (except for the irradiance experiments, see below).

Irradiance experiments. Nitrate, reactive silicate and orthophosphate were added to the filtered seawater to final concentrations of 80, 80 and 5 µM, respectively. With respect to micro-nutrients, only 10 nM Fe and 10 nM Mn (final concentrations) were added. Effects of nutrient depletion on cell death can be excluded in our study, since nutrients were still present at sufficiently high concentrations at the time of the experiments (data not shown, >30 µM N and Si, >3 µM P) and low biomass cultures were maintained throughout. The diatoms were incubated under 7 (*Chaetoceros brevis*, *Thalassiosira antarctica*) or 6 (*Chaetoceros calcitrans*) different irradiance levels from light-replete to light-limited intensities (Table 1). A gradient of irradiances was created by fixing neutral screens over the incubation bottles, in combination with placing the incubation bottles at different distances to the light source. The neutral screens ensured an unaltered photosynthetically active radiation (PAR) light spectrum inside the incubation bottles. The irradiance level inside each individual incubation bottle was measured with a QSL-100 irradiance meter (Biospherical Instruments). The intensity of the cool white fluorescence tubes was checked monthly in a calibration bottle, to determine the change in light intensity over time. Less than 10% change of the initial intensity was measured.

Silicate experiments. As the background silicate concentration (0.7 µM) in the growth medium was not low enough to induce silicate limitation in any of the 3 species tested, diatoms were used to further deplete silicate from seawater. After the diatoms ceased growing in these cultures, the water was gently filtered using 0.2 µm pore size polycarbonate filters, resulting in background silicate concentrations of 0.18 µM. This preconditioned water was used as a basis for the preparation of new growth medium. Macro- and micronutrients (including iron), standard for *f*/2 medium, were routinely added, except silicate, which was

Table 1. Average (±SE) photosynthetic efficiencies (F_v/F_m, n = 5) in cultures of *Chaetoceros brevis*, *Thalassiosira antarctica* and *Chaetoceros calcitrans* in response to different irradiances (I), silicate, or iron (Fe′) concentrations. n.d.: not determined; a.u.: arbitrary units

I (µmol photons m^{-2} s^{-1})	F_v/F_m (a.u.)	Silicate (µM)	F_v/F_m (a.u.)	Fe′ (M)	F_v/F_m (a.u.)
Chaetoceros brevis					
15	0.26 ± 0.05	0.18	0.51 ± 0.01	4.92×10^{-15}	0.16 ± 0.10
18	0.37 ± 0.06	0.26	0.55 ± 0.08	6.13×10^{-15}	0.10 ± 0.06
38	0.45 ± 0.06	0.35	0.54 ± 0.11	8.15×10^{-15}	0.08 ± 0.04
45	n.d.	0.45	0.51 ± 0.09	1.21×10^{-14}	0.14 ± 0.08
65	0.52 ± 0.02	0.73	0.52 ± 0.09	2.38×10^{-14}	0.19 ± 0.13
78	0.47 ± 0.05	1.07	0.57 ± 0.04	4.86×10^{-14}	0.31 ± 0.10
100	0.45 ± 0.05	1.48	0.58 ± 0.04	8.58×10^{-14}	0.30 ± 0.05
		3.26	0.58 ± 0.06	1.04×10^{-13}	0.34 ± 0.07
		4.15	0.57 ± 0.11	1.25×10^{-13}	0.36 ± 0.08
		9.14	0.57 ± 0.08	1.32×10^{-13}	0.32 ± 0.02
				1.83×10^{-13}	0.39 ± 0.06
				3.01×10^{-13}	0.41 ± 0.11
				5.32×10^{-13}	0.35 ± 0.10
				1.18×10^{-12}	0.43 ± 0.16
				8.00×10^{-10}	0.53 ± 0.09
				1.80×10^{-9}	0.54 ± 0.10
Thalassiosira antarctica					
12	0.35 ± 0.06	0.17	0.33 ± 0.08	4.92×10^{-15}	0.19 ± 0.07
15	0.51 ± 0.03	0.27	0.54 ± 0.08	6.13×10^{-15}	0.12 ± 0.05
30	0.55 ± 0.03	0.32	0.57 ± 0.10	8.15×10^{-15}	0.13 ± 0.06
40	0.56 ± 0.02	0.41	0.57 ± 0.11	1.21×10^{-14}	0.17 ± 0.08
50	n.d.	0.70	0.54 ± 0.06	2.38×10^{-14}	0.28 ± 0.10
70	0.58 ± 0.02	1.06	0.62 ± 0.02	4.86×10^{-14}	0.24 ± 0.06
103	0.54 ± 0.01	1.17	0.62 ± 0.01	8.58×10^{-14}	0.23 ± 0.10
		2.20	0.63 ± 0.02	1.04×10^{-13}	0.38 ± 0.08
		3.98	0.61 ± 0.03	1.25×10^{-13}	0.36 ± 0.10
		10.82	0.62 ± 0.03	1.32×10^{-13}	0.38 ± 0.05
				1.83×10^{-13}	0.43 ± 0.08
				3.01×10^{-13}	0.44 ± 0.08
				5.32×10^{-13}	0.50 ± 0.13
				1.18×10^{-12}	0.52 ± 0.11
				8.00×10^{-10}	0.58 ± 0.03
				1.80×10^{-9}	0.57 ± 0.04
Chaetoceros calcitrans					
15	0.33 ± 0.08	0.18	n.d.		
17	0.38 ± 0.06	0.41	n.d.		
18	0.43 ± 0.07	0.61	n.d.		
23	0.48 ± 0.06	0.65	n.d.		
58	0.49 ± 0.06	0.85	0.40 ± 0.08		
95	0.57 ± 0.02	0.95	0.40 ± 0.08		
		2.93	0.41 ± 0.04		
		4.20	0.52 ± 0.07		
		7.70	0.52 ± 0.09		

added to final concentrations in a range of from 0.18 to about 10.0 µM (Table 1).

Iron experiments. Only *Chaetoceros brevis* and *Thalassiosira antarctica* were tested in these experiments. As the diatoms could not be grown into iron limitation in this medium, due to high background concentrations of dissolved Fe (0.3 nM dissolved iron), desferrioxamine B (DFB), a natural siderophore was added to the medium in increasing concentrations of up to 120 nM. Tests to exclude toxicity of high concentrations of DFB on the diatoms were routinely performed in all experiments by adding a surplus of iron, after which growth resumed rates representative of non(-iron)-limiting conditions (data not shown). Knowing the background Fe concentration, the concentration (0.5 nM) and the conditional stability constant of natural iron ligands (K'_{Fe3+}: 22.1; L. J. A. Gerringa, Royal NIOZ, pers. comm.) in the medium, and the concentration and the conditional stability constant of DFB (K'_{Fe3+}: 21.6; Witter et al. 2000), the concentration of Fe′ (all inorganic Fe species; *C. brevis* and *C. calcitrans*, Table 1) was calculated (for details see Timmermans et al. 2001b).

Analyses. The cultures were checked daily using flow cytometry (Beckman Coulter Epics XL-MCL). The excitation wavelength was 488 nm, with the trigger on chlorophyll autofluorescence of the cells. At least 1000 cells were counted in each culture. In between the dilution of the cultures to keep them exponentially growing, we determined the net growth rates (µ, based on total cell numbers), photosynthetic efficiencies (F_v/F_m) and cell death rates (δ, see below) over 3 to 5 consecutive days. The results were averaged and plotted against irradiance, silicate ($Si(OH)_4$) and inorganic iron (Fe′) concentrations. In order to exclude potential effects of the light regime, samples were always taken at the same time in the light:dark cycle. Growth rates were fitted using a non-linear Monod fit (Monod 1950), rendering maximum growth rates (μ_{max}) and the half-saturation value (K_m) with respect to irradiance, $Si(OH)_4$, or Fe′ concentration.

A PAM fluorometer (Pulse Amplitude Modulated-CONTROL Universal Control Unit, WATER-mode, Walz) was used to determine F_0 (autofluorescence), F_m (maximum fluorescence) and F_v/F_m (photosynthetic efficiency, where $F_v = F_m - F_0$) of the diatom samples in relation to the different experimental conditions.

The presence and number of dead diatom cells were determined using the fluorochrome SYTOX Green (Invitrogen), a cyanine stain with high affinity for nucleic acids. SYTOX Green can only penetrate cells with permeabilised plasma membranes, here defined as dead cells (Veldhuis et al. 2001). Prior to the cell death assays, tests were carried out on live and preserved (dead) cells to determine the optimal time of incubation and optimal concentration of SYTOX

Green. Preservation was done overnight at room temperature, using different fixatives: either formaldehyde (1% final concentration, *Chaetoceros brevis*, *Thalassiosira antarctica*) or glutaraldehyde (0.5% final concentration, *Chaetoceros calcitrans*). For all 3 species an incubation with 0.5 µM final concentration SYTOX Green (freshly diluted from a 50 µM working stock in Milli-Q water stored at –20°C) for 10 min in the dark, at room temperature was chosen (Figs. 1 & 2). Samples were analysed using flow cytometry, with the trigger on chlorophyll autofluorescence. Cells were only considered dead if the DNA/green fluorescence signal exceeded the auto-green fluorescence by at least 5-fold (emission wavelength at 525 ± 20 nm). Live cells showed only a minimum 3-fold increase of autofluorescence. Although some species-specific differences in staining results were observed in the refinement of the SYTOX staining system, most notably in *C. brevis* (Fig. 2, staining as a function of the SYTOX concentration), the SYTOX concentration and staining time as specified above were considered to be optimal. Cell death rates were calculated according to Eq. (1), suitable for the batch phase of the cultures (Brussaard et al. 1997), such that:

$$\delta = \frac{\ln x_t - \ln x_0}{t \cdot \left\{ \left[\frac{(x+y)_t - (x+y)_0}{y_t - y_0} \right] - 1 \right\}} \quad (1)$$

where δ is the specific cell death rate (d^{-1}), x is the number of living cells and y is the number of SYTOX Green stained (i.e. dead) cells at time t and time 0, respectively. It was assumed that only SYTOX Green-stained cells were dead. No signs of viral infection were found in the cultures of the 3 diatom species used (C. P. D. Brussaard unpubl. data).

RESULTS

Cell death rates in relation to irradiances

The calculated cell death rates of *Chaetoceros brevis*, *Thalassiosira antarctica* and *Chaetoceros calcitrans* (Fig. 3) ranged from 0 to 0.26 d^{-1} and were only related to the irradiance levels for *T. antarctica* (Fig. 3b). For this species we found, at the lowest irradiances (<15 µmol photons m^{-2} s^{-1}), strongly enhanced cell death rates of about 30% of the μ_{max} growth rate. In *C. brevis* and *C. calcitrans*, cell death rates were low (<0.05 d^{-1}; Fig. 3a,c) and increased only slightly between 20 and 40 µmol photons m^{-2} s^{-1}. The growth rates of all 3 species tested decreased with decreasing irradiances (Fig. 3). For *C. brevis* and *T. antarctica*, growth halted at the lowest tested irradiance level of 15 µmol photons m^{-2} s^{-1} (Fig. 3a,b). *C. calcitrans* maintained growth even at the lowest irradiance level (Fig. 3c). Calculated μ_{max} were similar for the 3 species tested (0.72 to 0.75 d^{-1}), matching the published growth rates for species of the *Thalassiosira* and *Chaetoceros* genera under light-replete conditions

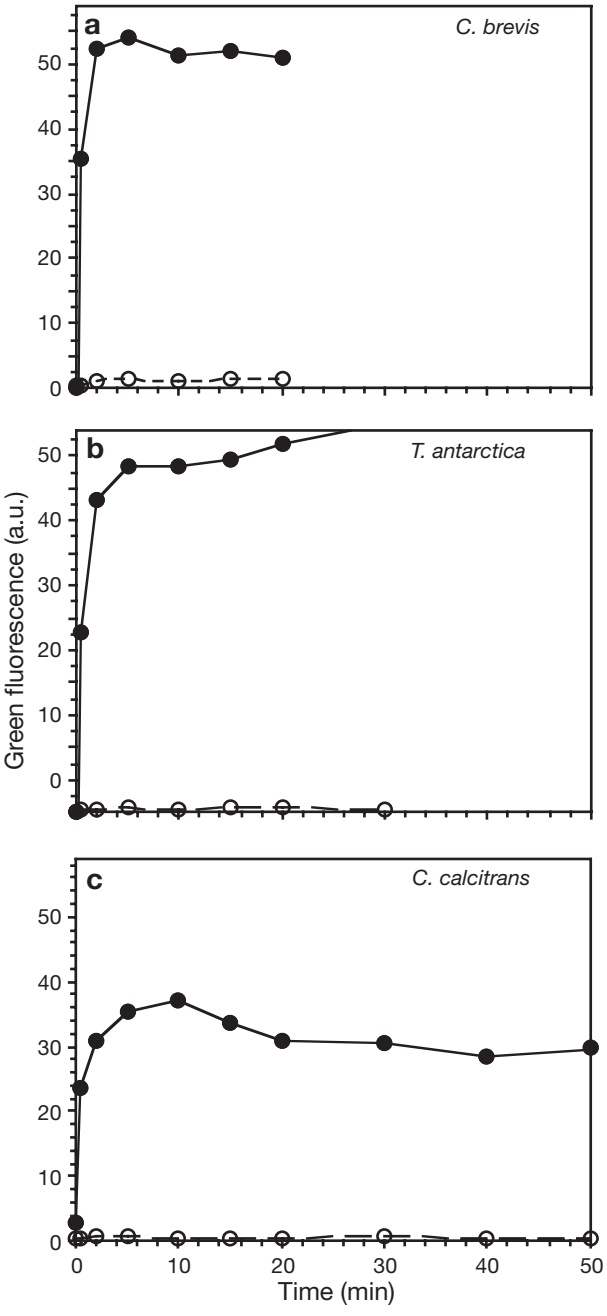

Fig. 1. Green fluorescence intensity (a.u.: arbitrary units) in cultures of (a) *Chaetoceros brevis*, (b) *Thalassiosira antarctica* and (c) *Chaetoceros calcitrans* after staining with SYTOX Green as a function of time (min^{-1}). ●: fixed (dead) cells (formaldehyde or glutaraldehyde, depending on the diatom species, see 'Materials and methods'); O: live cells. An incubation time of 10 min was taken as standard to be used for the experiments

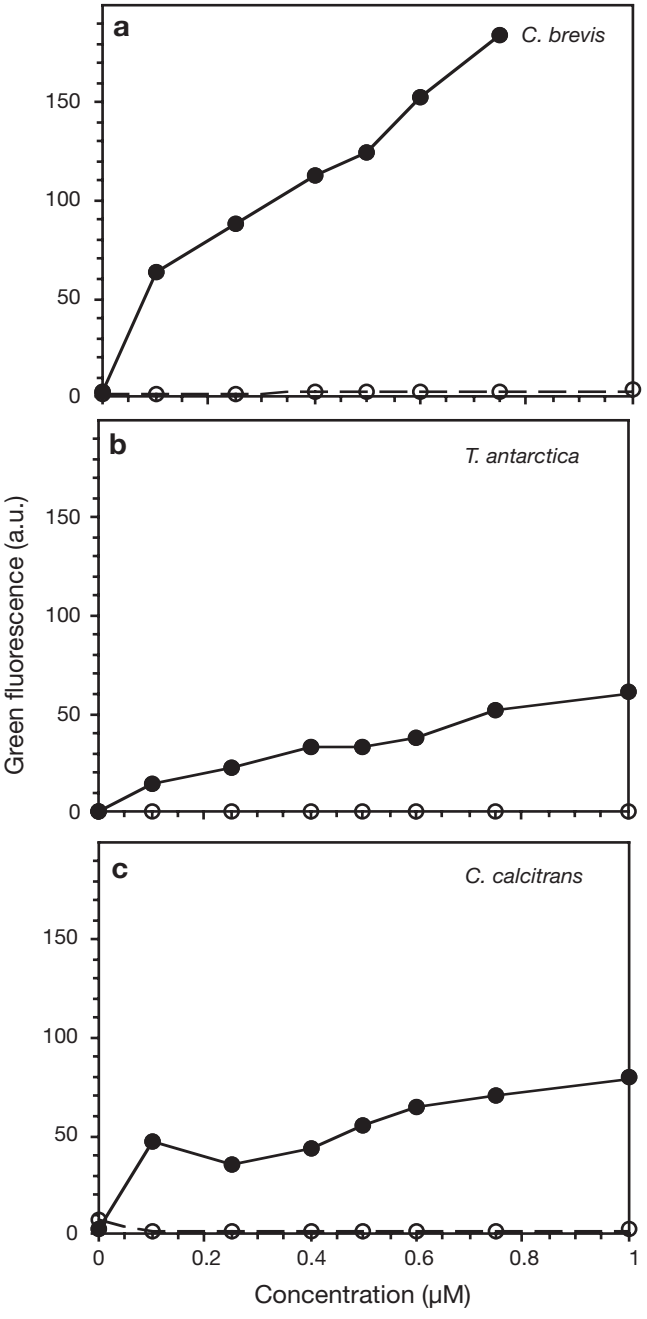

Fig. 2. Green fluorescence intensity (a.u.: arbitrary units) in cultures of (a) *Chaetoceros brevis*, (b) *Thalassiosira antarctica* and (c) *Chaetoceros calcitrans* as a function of SYTOX Green final concentration. ●: fixed (dead) cells (formaldehyde or glutaraldehyde, depending on the diatom species, see 'Materials and methods'); ○: live cells. A final concentration of 0.5 µM SYTOX Green was used for the experiments

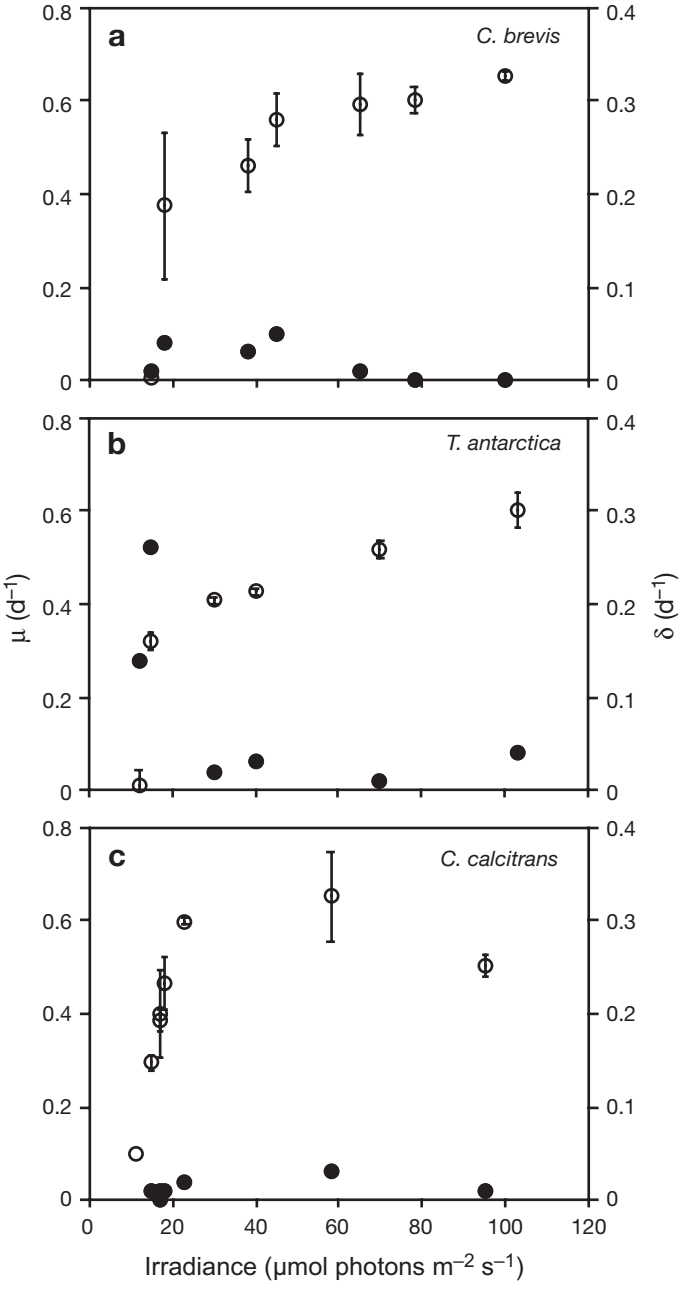

Fig. 3. Cell death rates (δ, ●) and growth rates (µ, ○) versus irradiance in cultures of (a) *Chaetoceros brevis*, (b) *Thalassiosira antarctica* and (c) *Chaetoceros calcitrans*. δ were calculated according to Eq. (1); µ were calculated based on changes in cell abundances over time. Note the difference in scale for δ compared to Figs. 4 & 5. For µ, data points indicate average (±SD, n = 3). When no error bars are visible, they are smaller than the symbol

(Timmermans et al. 2001a, Claquin et al. 2002). The decline in growth rates of the 3 diatom species tested with reducing irradiance is comparable to that found for other marine diatoms (Laws & Banister 1980, Geider et al. 1986). Calculated K_m values with respect to irradiance levels varied between 12 (*C. calcitrans*), 20 (*C. brevis*) and 22 (*T. antarctica*) µmol photons m^{-2} s^{-1}. In all 3 species tested, F_v/F_m values increased from 0.3 to 0.5–0.6 with increasing irradiance levels (Table 1).

Cell death rates in relation to silicate concentrations

For *Thalassiosira antarctica* a clear relation between δ and silicate concentrations was observed (Fig. 4b), increasing from 0.01 to 0.08 d^{-1} with decreasing silicate concentrations. In cultures of *Chaetoceros calcitrans*, a similar but weaker response was observed (<0.02 to 0.04 d^{-1}; Fig. 4c). *Chaetoceros brevis* showed minimal variation in δ in response to varying silicate concentrations (Fig. 4a). Growth rates of the 3 diatom species decreased with decreasing silicate concentrations (Fig. 4), in agreement with previous studies on marine diatoms (see review by Martin-Jézéquel et al. 2000). In cultures of *C. brevis* and *T. antarctica* a sudden drop in growth rates was observed (silicate concentrations <1 µM), whereas *C. calcitrans* showed a more gradual decline in growth rate, starting also at higher silicate concentrations (<4 µM). Even at the lowest silicate concentrations tested (0.18 µM), growth of the 3 species never halted. Calculated μ_{max} were comparable to those reported for the species in the irradiance experiments: 0.65 d^{-1} for *T. antarctica*, 0.69 d^{-1} for *C. brevis* and 0.76 d^{-1} for *C. calcitrans*. Calculated K_m values with respect to silicate were 0.16 µM for *T. antarctica*, 0.25 µM for *C. brevis* and 0.85 µM for *C. calcitrans*. For *C. brevis* and *C. calcitrans*, F_v/F_m values were always high and indicated good photosynthetic efficiency, with values >0.5 for all Si concentrations tested (Table 1). F_v/F_m for *T. antarctica* was >0.54, except for the lowest silicate concentration of 0.17 µM (0.33; Table 1). For *C. calcitrans*, F_v/F_m decreased slightly from 0.52 to 0.40 with decreasing silicate concentrations (Table 1).

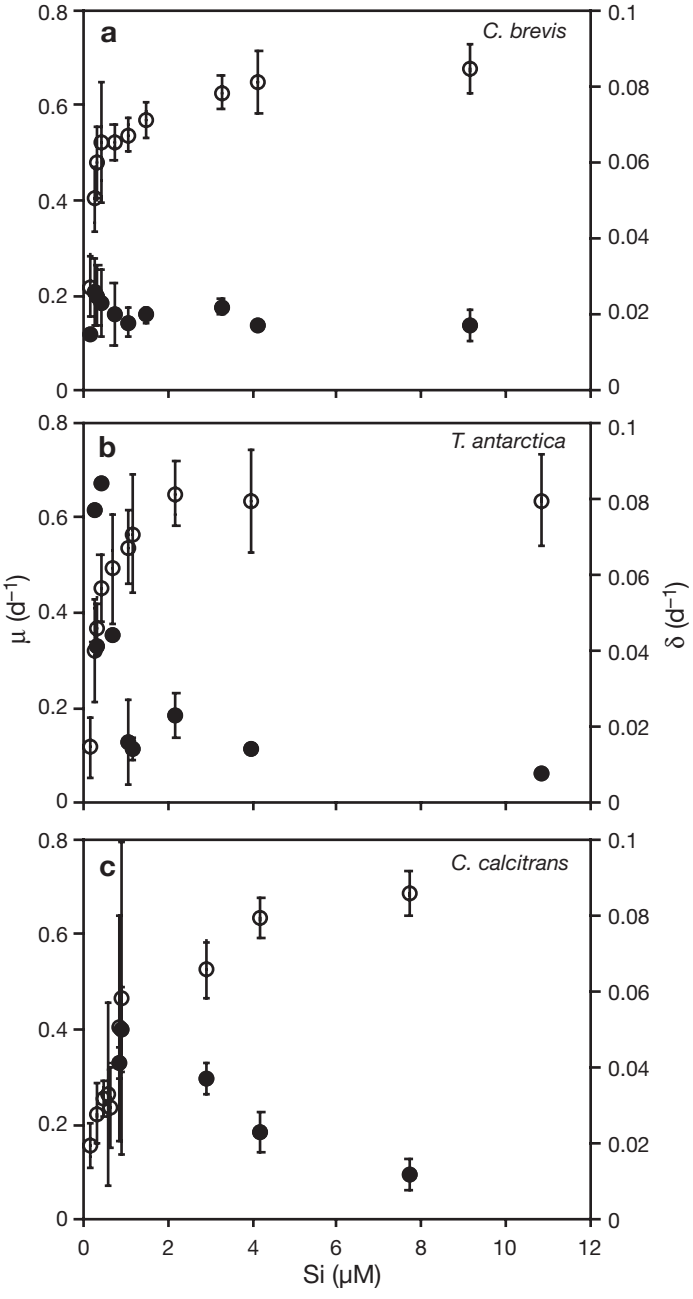

Fig. 4. Cell death rates (δ, ●) and growth rates (μ, ○) versus silicate concentrations in cultures of (a) *Chaetoceros brevis*, (b) *Thalassiosira antarctica* and (c) *Chaetoceros calcitrans*. Calculations of δ and μ as indicated for Fig. 3. Note the difference in scale for δ compared to Figs. 3 & 5. For μ, data points indicate average (±SD, n = 3). When no error bars are visible, they are smaller than the symbol

Cell death rates in relation to iron concentrations

Cell death rates of *Chaetoceros brevis* and *Thalassiosira antarctica* were virtually zero (<0.01 d^{-1}) in the iron limitation experiments and appeared non-related to the iron concentration (Fig. 5). Both diatom species showed reduced growth rates (<0.2 d^{-1}) only at extremely low iron concentrations (below 10^{-13} M Fe′), but growth in both species never halted. The apparent μ_{max} were similar for *T. antarctica* and *C. brevis* (0.40 d^{-1}), which was about 40% lower than the μ_{max} in the light and silicate experiments. The calculated μ_{max} in all 3 experiments (light, silicate, iron limitation) should, in principle, be the same under replete conditions. This was the case for the light- and silicate-controlled conditions. The medium for the Fe-controlled cultures differed from the light- and silicate-controlled one, as a different type of natural seawater was used as the basis (see 'Materials and methods'). The lower apparent μ_{max} in the Fe-limitation experiments indicated that the culture conditions were not replete in the most optimal case. However, this does not alter our general findings on the effects of Fe limitation on cell death. The K_m values with

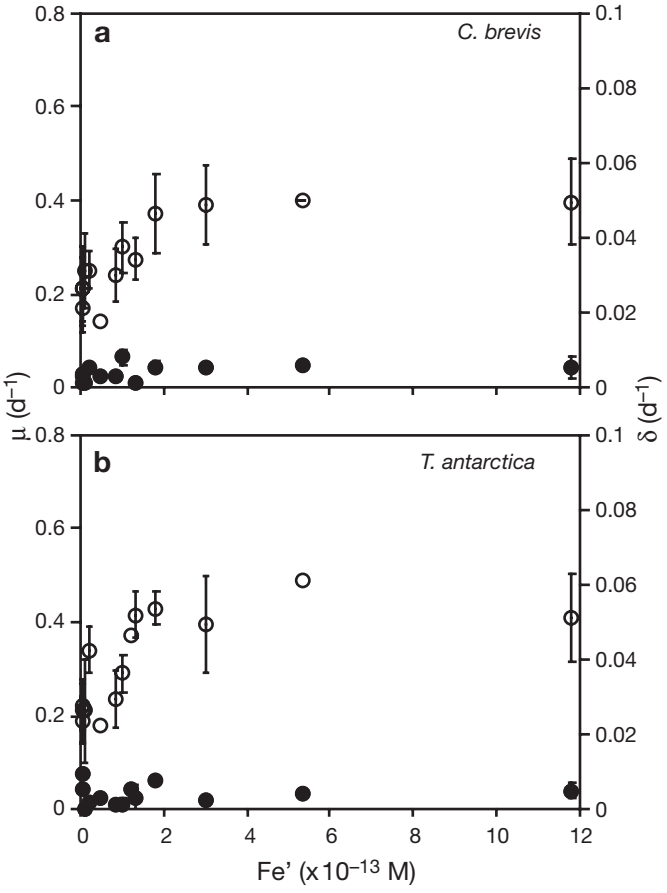

Fig. 5. Cell death rates (δ, ●) and growth rates (μ, ○) versus Fe′ concentrations in cultures of (a) *Chaetoceros brevis*, and (b) *Thalassiosira antarctica*. Calculations of δ and μ as indicated for Fig. 3. Note the difference in scale for δ compared to Figs. 3 & 4. For μ, data points indicate average (±SD, n = 3). When no error bars are visible, they are smaller than the symbol. The Fe′ concentrations were calculated as explained in the 'Materials and methods'

respect to Fe′ varied between 1.0×10^{-14} M for *T. antarctica* and 2.1×10^{-14} M for *C. brevis*. These K_m(Fe′) values were much lower than those reported for 4 large Antarctic diatom species (Timmermans et al. 2004) or the average K_m(Fe′) values for 12 diatoms as reported by Sarthou et al. (2005), indicating a K_m(Fe′) range from 0.59×10^{-12} to 1.12×10^{-9} M. Besides temperature (Eppley 1972) and the size of the cells (Sunda & Huntsman 1995, Timmermans et al. 2001b) as K_m-regulating factors, the conditional stability constants for artificial or natural organic complexes used to calculate Fe′ concentrations may also cause considerable variance in K_m values (Witter et al. 2000, Timmermans et al. 2001b). The index for photosynthetic efficiency (F_v/F_m) decreased strongly with decreasing iron concentrations from 0.6 to 0.1 at Fe′ concentrations from 8×10^{-10} to 2×10^{-14} M (Table 1, *C. brevis* and *T. antarctica*).

DISCUSSION

In this study we present cell death rates (δ) based on single cell analyses for marine diatoms under various growth-limiting conditions, combined with results on growth rates and photosynthetic efficiencies. Clear species-specific differences were recorded. The highest death rates were observed for *Thalassiosira antarctica*, with a maximum δ of 0.26 d^{-1} under light limitation. Our results imply that light, and to some extent also silicate, is a principal factor controlling diatom biomass, species succession and distribution. Despite generally low cell death rates, irrespective of the controlling factor, it was mainly the growth rate that declined with increasing limitation. It should be noted that in this study the cells were acclimated to the specific limiting conditions. Under certain (meso)eutrophic conditions, nutrient depletion may be a fast process and therefore the effect on cell death rates may be more pronounced.

Irradiance experiments

It is clear that low light levels result in enhanced cell death in phytoplankton. In our study only *Thalassiosira antarctica* experienced enhanced cell death (to 0.26 d^{-1}) at low irradiance. Related to this, growth rate and photosynthetic efficiency were also reduced at the lower irradiance levels. Both signals are indicative of serious physiological stress (Geider & LaRoche 1994). These findings indicate that *T. antarctica* can endure prolonged stress of low irradiance (30 to 40 μmol photons m^{-2} s^{-1}) well. The *Chaetoceros* species tested in this study, *C. brevis* as model species for cold water and *C. calcitrans* as a typical species of temperate waters, did not show enhanced cell death rates despite the fact that both μ and F_v/F_m did decrease. Previously, light stress as a factor inducing cell death in diatoms has only been investigated by Berges & Falkowski (1998), Peters & Thomas (1996) and Peters (1996), but these studies were under relatively extreme culture conditions, since the algae were kept in the dark for a long period. A field study measuring total cell lysis in the Mediterranean reported different death rates for the cyanobacteria *Synechococcus* and *Prochlorococcus* related to the light regime (Agustí 2004). It was suggested that the difference in cell death rates between these 2 species was an underlying process structuring the phytoplankton community. Our results suggest that the 2 *Chaetoceros* species may survive periods of reduced light better than the *Thalassiosira* species. This observation seems to contradict the general idea that polar phytoplankton species may have evolved special adaptations for survival under the variable and low light intensities that are commonly experienced by

these species (Peters 1996, Peters & Thomas 1996). Our study shows, however, that species-specific differences in response to low light periods do occur in diatoms, and that one should therefore not generalise too much. For a more conclusive statement, more polar versus temperate species should be studied.

Silicate experiments

To our knowledge, our study provides the first data on Si-controlled cell death rates for marine diatoms. As for light stress, *Thalassiosira antarctica* was most sensitive, as shown by the enhanced cell death rates at low silicate concentrations (0.26 d^{-1} at 0.2 µM). The 2 *Chaetoceros* species showed only very low cell death rates (<0.04 d^{-1}). μ_{max} were generally lower than those of other diatoms studied thusfar (Martin-Jézéquel et al. 2000), possibly related to the relatively low temperatures at which the diatoms were grown in the present study (Eppley 1972). In our experiments, remarkably low K_m(Si) values (0.16 to 0.85 µM) were calculated, most likely the consequence of working with acclimated, small diatom cells, known to have lower K_m values (Eppley et al. 1969). Generally, higher K_m(Si) are reported for marine diatoms. For example, Sarthou et al. (2005) reported a range in K_m(Si) values of 0.2 to 22 µM (average 3.9 ± 5.0 µM). For natural diatom communities in the Monterey upwelling system a K_m for silicate uptake of 4.2 µM (White & Dugdale 1997) and limitation of biogenic silicate production at 5 µM (Brzezinski et al. 1997) were reported. The photosynthetic efficiency showed only a minimal response to changes in silicate concentrations. The most likely explanation for this is that the silicate metabolism is regarded as an energy-cheap process (Raven 1983), without direct involvement of photosynthetic energy (Martin-Jézéquel et al. 2000).

In temperate regions, high-nutrient conditions are commonly found in spring and early summer (Ragueneau et al. 2000). Under these conditions, blooms of diatoms like *Chaetoceros calcitrans* can develop rapidly. After the depletion of silicate to concentrations well below 1 µM, other phytoplankton species take over. Based on our results and assuming that we can extrapolate the work we did with acclimated cells to the natural system, population declines of temperate diatoms like *C. calcitrans* will be caused by reduced growth rates rather than by increased cell death rates. Alternatively, in the Southern Ocean, the habitat of *Chaetoceros brevis* and *Thalassiosira antarctica*, silicate concentrations in the surface waters are commonly well above 10 µM (Levitus et al. 1993) and usually remain high. This silicate concentration is sufficient to maintain high growth rates, accompanied by low cell death rates.

Iron experiments

Obviously, low iron concentrations lead to physiological stress in the diatom cells, as exemplified in the reduced growth rates (0.2 d^{-1}, a 50% reduction of the apparent μ_{max}), as well as in a low photosynthetic efficiency (F_v/F_m < 0.2), confirming previous work in which suboptimal values of F_v/F_m were regarded as strong evidence for physiological stress (Geider & LaRoche 1994, Behrenfeld & Kolber 1999). Remarkably, these photosynthetically unhealthy diatoms did not show increased cell death rates. On the contrary, cell death rates were extremely low (<0.01 d^{-1}) and appeared to be independent of the Fe′ concentrations. The ability to maintain growth even under low Fe′ concentrations confirms the low iron requirements of small diatoms, either by a favourable surface to volume ratio, and/or by efficient use of the available iron (Sunda & Huntsman 1995, Timmermans et al. 2001b). These results provide experimental evidence for the so-called ecumenical iron hypothesis (Cullen 1995): small cells would not be growth limited by low Fe concentrations, but rather controlled in numerical abundance by other loss factors like grazing. The low cell death rates as observed in this study indicate that natural mortality is not an important loss factor, at least for fully acclimated diatoms. An earlier study using *Chaetoceros brevis* as the model species did, however, show enhanced cell death rates upon lifting the iron (and light) limitation (Timmermans et al. 2001a). This may indicate that cell death rates can vary under different experimental (and natural) conditions. Obviously, more research is needed on the effects of co-limitation (iron and light), as well as on the effects of the sudden removal of a limitation on cell death rates of phytoplankton.

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