Effects of salinity and nutrient conditions on growth and haemolytic activity of *Fibrocapsa japonica* (Raphidophyceae)

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ABSTRACT: The influence of salinity and a number of nitrogen (N) and phosphorus (P) levels on growth and haemolytic activity of the harmful algal bloom (HAB) species *Fibrocapsa japonica* were studied simultaneously. The *F. japonica* strain studied had a euryhaline growth profile with an optimum growth rate at 26 psu. Measurements of the minimum cell quota of N and P revealed an optimum N:P ratio of 24.5 and a relatively high requirement of nutrients (q_0N = 7.1 pmol cell^{-1}; q_0P = 0.29 pmol cell^{-1}). These characteristics fit the habitat of this species, among which is the P-controlled eutrophied Dutch coastal waters. Salinity had a significant effect on the growth rate of *F. japonica* and on its haemolytic activity, even on the basis of equal cell volume. The highest haemolytic activity was found at 16 psu, the lowest salinity condition tested. The EC$_{50}$ values that were expressed on a per cell basis revealed that *F. japonica* had a high haemolytic activity when compared with other HAB species. Under N limitation, cells of *F. japonica* were less haemolytic than cells not limited for N, but this could be caused by a slight decrease in cell volume only. Remarkably, cells not limited for N appeared to form aggregates, which in combination with the elevated levels of haemolytic compounds per cell may result in obstruction of gills, causing fish kills during *F. japonica* blooms. So, despite the absence of brevetoxin in our strain of *F. japonica*, this strain may be lethal to fish due to its haemolytic activity.

KEY WORDS: Cell quota · N:P ratio · Brevetoxins · Toxicity · Harmful algal blooms · Phytoplankton

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INTRODUCTION

Proposed explanations for the global increase in harmful algal blooms (HABs) are the stimulation of plankton blooms by eutrophication and/or unusual climatological conditions (Hallegraeff et al. 2003). There is a considerable concern (Ryther & Dunstan 1971, Officer & Ryther 1980, Smayda 1990) that coastal waters such as the North Sea with high anthropogenic nitrogen input may favour blooms of nuisance species. The altered nutrient ratios are beneficial for flagellates that replace the normal spring and autumn blooms of siliceous diatoms (Colijn & Cadée 2003, Hallegraeff et al. 2003). In the North Sea, along the Dutch and German coast, an increase in phytoplankton abundance and shifts in species composition were observed

over the past decades, which have been attributed to anthropogenically altered nutrient conditions (Radach & Berg 1986, Riegman et al. 1992, Cadée & Hegeman 1993, Philippart et al. 2000, Cadée & Hegeman 2002). Since the 1990s, one of the frequently observed HAB species in these coastal waters is the marine raphidophyte *Fibrocapsa japonica* Toriumi and Takano (Vrieling et al. 1995, Rademaker et al. 1998). Possibly, this species also thrives due to changing environmental conditions.

Fibrocapsa japonica and other marine raphidophyte species, *Chattonella* spp., *Heterosigma akashiwo* and *Olistodiscus luteus*, have been documented to be ichthyotoxic (Landsberg 2002 and references therein). *F. japonica* has caused massive fish mortality events in the Seto Inland Sea of Japan (Iwasaki 1971, Okaichi

1972, 1989, Toriumi & Takano 1973); since 1990 ichthyotoxicity of *F. japonica* has been reported from the German coast and the Mediterranean Sea (European Commission 2003). The fish kills caused by *F. japonica* have been ascribed to the production of brevetoxins (Khan et al. 1996, Bridgers et al. 2004), reactive oxygen species (ROS; Oda et al. 1997), haemolytic compounds (Fu et al. 2004a,b), mucocyst ejection (de Boer et al. 2004b), and possibly combinations of these. Raphidophyte species are not always toxic to fish, as was shown for *H. akashiwo* and *Chattonella* spp. (Ishimatsu et al. 1996, Twiner & Trick 2000). Therefore, it is likely that different strains produce different toxic compounds or sometimes no toxins at all; this may be the case for *F. japonica* as well. The production of toxic compounds by raphidophytes could also be affected by environmental conditions such as light (Ono et al. 2000, Khan et al. 2001, Marshall et al. 2002), temperature (Twiner & Trick 2000, Khan et al. 2001), salinity (Haque & Onoue 2002a,b) and the presence of bacteria (Carrasquero-Verde 1999). For other HAB algae, it is known that algal species react differently to altered nutrient conditions with respect to toxin production (Granéli et al. 1998).

So far, toxin production by raphidophytes has not been studied in relation to nutrient conditions. In this study, we concentrated on the role of salinity and nutrient conditions both on growth performance and on the production of toxins. We used a European strain of *Fibrocapsa japonica*, which is known to give results in toxicity tests with bioassays using the brine shrimp *Artemia salina* or luminescent bacteria (Fu et al. 2004b) and that contains haemolytic compounds identified as polyunsaturated fatty acids (PUFAs; Fu et al. 2004a,b). This study is part of a larger project on the competitive advantage of *F. japonica* necessary for establishment in new areas. Therefore, we investigated the physiological niche of this species, which is defined by several aspects of growth performance. We studied growth in relation to temperature (de Boer et al. 2004b) and life cycle phenomena related to survival (de Boer et al. 2004a).

MATERIALS AND METHODS

Culture conditions. The western European *Fibrocapsa japonica* strain CCRuG-Cl3 (Culture Collection University of Groningen, The Netherlands) was used for this study. Strain CCRuG-Cl3 is a clonal *F. japonica* culture established in 1997 from a culture of a monospecific bloom that occurred in 1995 in Büsum harbour, Germany (U. Tillmann pers. comm.). This non-axenic strain was grown in batch culture using autoclaved natural seawater (Doggersbank, North Sea) adjusted to a salinity of 25 psu with Milli-Q water and enriched with

f/2 minus Si (Guillard 1975). The cultures were routinely maintained at 17 ± 1°C under long-day (16:8 h light : dark, L:D) conditions and a photon flux density of 50 ± 5 µmol photons m^{-2} s^{-1} (provided by fluorescent tubes L36W/19 light, Osram). The irradiance was measured with a Li-Cor quantum meter equipped with a cosine collector. A stock culture was diluted every second day with new f/2 medium, keeping the cells in an exponential growth phase for at least 4 generations. The last generation was used as inoculum for the experiments. In pilot experiments, a direct shift from 25 psu to 15 or 35 psu did not show cell stress, which is observed as the absence of mucus ejection, nor did it induce a lag phase.

Experimental set-up. The *Fibrocapsa japonica* strain was cultured at different salinities (16, 21, 26, 31, 36 psu). The initial N:P ratios (4 nutrient treatments) were varied too; abbreviations for nitrogen (N) and phosphorus (P) are: N_H = high [N]; N_L = low [N]; P_H = high [P]; and P_L = low [P] (Table 1). All possible combinations (n = 20) between salinity (n = 5) and nutrient treatment (n = 4) were tested.

When the experiment was started, the stock culture was not nutrient limited, the cells were in exponential phase and all the cells had the same nutrient history. The inoculum used was small (50 into 750 ml) to minimise influence on the initial salinity and nutrient concentration of the fresh medium. The experiment was done in 20 1 l Erlenmeyer flasks with different salinity/nutrient treatments, and each flask had an initial cell density of 800 ± 200 cells ml^{-1}.

Every second day, each culture was sampled for cell density, N and P analysis, erythrocyte lysis assay and brevetoxin analysis. Before sampling, each culture was checked by visual inspection for motile and non-motile cells and for cell aggregation, and cultures were gently swirled and placed back randomly after sampling. Salinity and pH in each flask were checked regularly, but no changes were recorded during the experiment.

Cell density measurements. Phytoplankton samples (5 ml) were kept in 20 ml brown glass flasks and preserved with 75 µl acid Lugol's solution (Veldhuis et al.

Table 1. The 4 nutrient conditions at the start of the experiment. N_H = high [N]; N_L = low [N]; P_H = high [P]; and P_L = low [P] . Each nutrient treatment was carried out for all 5 salinities (16, 21, 26, 31, 36 psu). Nutrient conditions with initial N:P ratio of 24 were the enriched seawater media f/20 and f/2 (Guillard 1975)

Initial N:P ratio	µM N	µM P	Abbreviation
2.4	88.3	36.3	$N_L P_H$
24 (f/20)	88.3	3.63	$N_L P_L$
24 (f/2)	883	36.3	$N_H P_H$
240	883	3.63	$N_H P_L$

1986). The samples were stored in the dark at 4°C and counted within 4 d after sampling in a Sedgwick Rafter counting chamber under a Zeiss microscope using 125 magnification (n = 6; each count with a minimum of 100 cells and all counts of 1 sample had a CV < 0.25, where CV = standard deviation of all counts divided by average of all counts). Growth rates (μ) were calculated from a curve fit on at least 4 data points in the exponential phase of growth using the following equation:

$$N_t = N_0 \cdot e^{(\mu t)} \tag{1}$$

where N_t and N_0 are cell concentrations at time t and 0 (in days).

Nutrient measurements. A phytoplankton sample of 20 ml was centrifuged for 15 min at $1360 \times g$. The cell-free supernatant was used for the analysis of phosphorus (P) and nitrogen (N) concentration of the medium. P concentration was determined colorimetrically at 882 nm (Pye Unicam PU 8600 UV/VIS spectrophotometer, Philips) in 4 cm quartz cuvets according to Harrison (1988). N concentration [NO_x] was determined with the Total Nitric Oxide Assay kit (DE 1600, R & D systems). The analysis was carried out in 96 well plates (Greiner, PS microplate, F bottom) and absorbance was measured with a Wallac 1420 Multilable Counter Victor^{TM} at 545 nm.

The cell quota (q) of N or P (pmol cell^{-1}) for each nutrient treatment was determined by the differences of the nutrient concentration (S) of the medium between time t and 0 divided by the difference in cell density (N) between time t and 0, using the following equation:

$$q = (S_0 - S_t)/(N_t - N_0) \tag{2}$$

Extraction of toxic substances. The *Fibrocapsa japonica* cells of each phytoplankton sample were harvested by filtration of 10 ml culture on GF/F, 25 mm ∅, Whatman filters, and the filters were immediately stored at –80°C. The filters were cut into pieces and extracted with 2 ml MeOH (HPLC grade). After 2 h sonication on ice, the filter material was centrifuged to the bottom of the vial. The extract was harvested and stored at –18°C until analysis. For toxicity analysis, the MeOH was evaporated in a vacuum centrifuge (DNA 110, Speed Vac®, Savant) at room temperature and the algal extract was re-dissolved in 1 ml assay buffer (ELA buffer; Eschbach et al. 2001) and partly used for the erythrocyte lysis assay (ELA). The remaining part of the extracts was used for brevetoxin analysis. As a control, f/2 medium was filtered over a GF/F, 25 mm ∅ filter. The control was treated identically as the filtered culture samples and was tested for haemolytic activity and brevetoxins (n = 4 for both toxicity test); none were positive.

Erythrocyte lysis assay. An ELA was carried out to test the haemolytic activity of the samples. The method was essentially from Eschbach et al. (2001), but was adjusted to rat erythrocytes instead of using fish erythrocytes. From a rat that was chosen randomly, a 0.25 ml blood sample was taken and immediately diluted in 20 ml ELA buffer. The erythrocytes were used for the ELA within an hour after blood sampling. A dilution series was made in duplicate for each extract. The ELA test started when the washed erythrocyte solution with proper density was added to the samples. After 24 h incubation at 16°C, the absorption of the supernatant was measured at 405 nm using the Wallac 1420 Multilable Counter Victor^{TM}.

The cell densities of *Fibrocapsa japonica* from the dilution series were plotted against the % erythrocyte lysis and were fitted with a sigmoidal equation. From this exercise, the EC$_{50}$ was calculated for each treatment as the concentration of *F. japonica* (cells ml^{-1}) necessary for 50% lysis of the erythrocytes. Saponin (Sigma) was used as a reference.

Khan et al. (1996) suggested that the brevetoxin PbTx 2 is one of the main components of the neurotoxin fraction produced by *Fibrocapsa japonica*. Therefore, PbTx 2 (Calbiochem) was used as brevetoxin control. Dilution series of PbTx 2 were made up to a maximum concentration of 10 μg ml^{-1}, and these were tested for haemolytic activity as well.

Competitive ELISA for brevetoxin analysis. Filter extracts in ELA buffer were used for the brevetoxin analysis using competitive ELISA methodology. The brevetoxin method developed by J. Naar and coworkers, is a multistep competitive biotin-strepavidin coupled immunoperoxidase technique described previously (Bourdelais et al. 2002, Naar et al. 2002). This analysis of samples was performed in collaboration with J. Naar at the Centre for Marine Science (University of North Carolina at Wilmington, USA). Before brevetoxin analysis of the samples the ELA buffer was tested for interference with either brevetoxin or one of the antibodies used in the ELISA. No interference was found. To make sure that the amount of cells tested was sufficient regarding the detection limits of ±1.3 ng ml^{-1}, we used 1 to 2×10^5 cells ml^{-1}, which is 3 to 6 times the concentration used by Bridgers et al. (2004), who used 3×10^4 cells ml^{-1} of *Fibrocapsa japonica* cells and detected brevetoxins.

Statistical analysis. Differences in N or P cell quota were tested with a single factor ANOVA using the statistics tools of Excel 97 at a significance level p < 0.05. Growth rates and haemolytic activity in relation to salinity, nutrient treatment and growth phase were analysed by regression models using SPSS 11.0. Models were constructed using a stepwise procedure including both linear and quadratic terms for the independent variable (salinity) to investigate possible non-linearity. Nutrient treatment and growth phase were compared using 'dummy' variables. Tests for inclusion of variables

were based on F-distribution of the change in deviance per degree of freedom (when the variable was dropped from the full model) divided by the deviance per degree freedom of the full model (Crawley 1993).

RESULTS

Growth at different environmental conditions

At each salinity a similar picture for the nutrient treatments was observed. Fig. 1 shows the typical curves for these observations at a salinity of 26 psu. In the expo-

nential growth phase (Fig. 1A) all low P treatments became P depleted (depleted at [P] < 0.7 µM) at Day 4 (Fig. 1B). N depletion (depleted at [N] < 5 µM) for the low N treatments was reached at Day 6 (Fig. 1C).

In the f/2 treatment of each salinity, N_HP_H, P depletion was observed later (from Days 10 to 18). At Days 18 to 26, the [N] in the N_HP_H treatment levelled off at concentrations between 7 and 54 µM, for the whole salinity range. In the treatments N_LP_H and N_HP_L, with depletion of only 1 nutrient, the non-limiting nutrient remained constant at much higher levels; at N_LP_H the [P] > 10 µM (e.g. Fig. 1B) and at N_HP_L the [N] > 200 µM (e.g. Fig. 1C).

During the exponential growth phase the relationship between the growth rate (µ) and salinity could be described with a quadratic function (Fig. 2; salinity: $F_{1,17}$ = 10.63, p = 0.005, salinity^2: $F_{1,17}$ = 12.90, p = 0.002). Optimal growth was determined at 26 psu (0.43 d^{-1}), which agreed well with the regression model with a calculated optimum at 23.6 psu. Nutrient treatments did not differ from each other ($F_{3,14}$ = 1.054, p = 0.400). In other words, at each applied salinity the growth rate of *Fibrocapsa japonica* cells in exponential phase did not vary for the applied nutrient treatments. Consequently, in all treatments, *F. japonica* cells had an exponential growth phase (Figs. 1A & 2), even when at low P treatments the nutrients were already depleted in P for at least 2 d (Fig. 1B). Obviously, cells in these cultures were using P from storage pools in their cells.

Cell quota for N and P

Since similar trends (Fig. 1) for nutrient depletions at different salinities were observed, we calculated the cellular N and P quota (qN and qP) at t_1, when
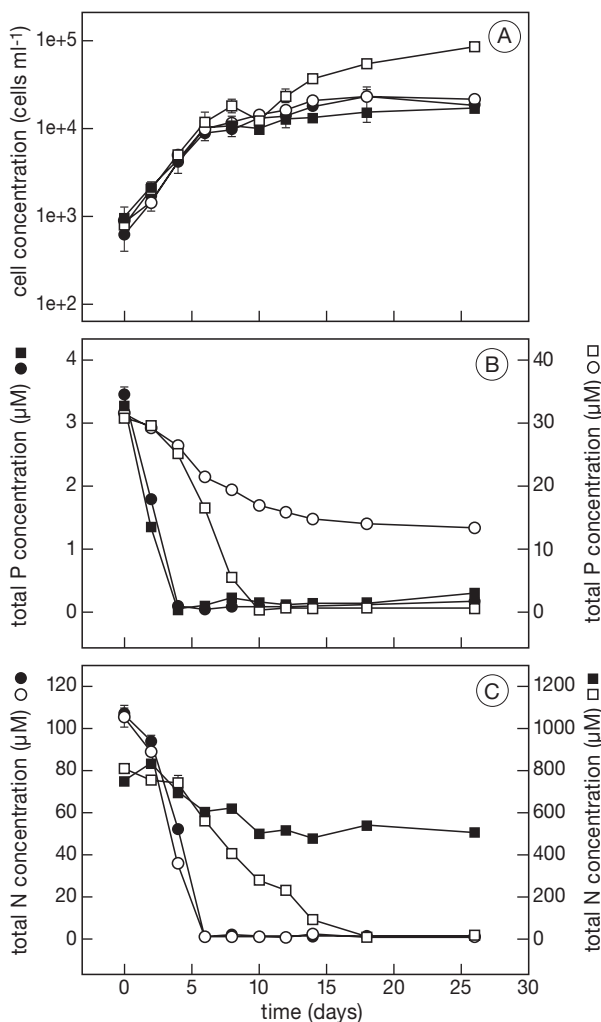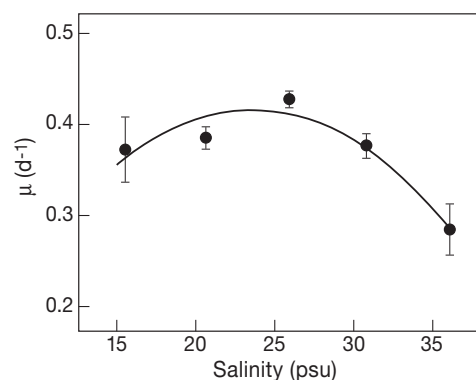

Fig. 1. *Fibrocapsa japonica*. Typical curves for (A) growth, (B) phosphorus and (C) nitrogen uptake in the cultures at 26 psu for all treatments. Symbols refer to the distinct applied nutrient treatments; they are also used to show the accompanying *y*-axes; ● = N_LP_L; ■ = N_HP_L; ○ = N_LP_H; □ = N_HP_H (N = nitrogen; P = phosphorus; L = low concentration; H = high concentration)

Fig. 2. Relationship between growth rate (µ, d^{-1}) and salinity (model outcome: µ = –2.34 × 10^{-2} + 1.96 × 10^{-2} salinity – 4.14 × 10^{-4} salinity^2, with salinity in psu. Averaged values of 4 nutrient treatments with standard errors are shown

cells were in the exponential phase (for all treatments before depletion occurred), and at t_2 in the stationary phase at Day 14, when the depleted cells had used their internal P storage (Fig. 3). At time t_1 (remember, no differences in μ between nutrient treatments), there were no differences in qN or qP either between the treatments or between salinities; qNt_1 was 15.3 ± 1.4 pmol cell^{-1} and qPt_1 was 1.25 ± 0.06 pmol cell^{-1}. In comparison with cell quota at times t_1 and t_2 with no N or P depletion the cell quota for low N and low P treatments (N_LP_L for both nutrients; N_LP_H for N; N_HP_L for P) at time t_2 showed significant differences. Therefore, at these conditions the nutrients N or P were limited and qNt_2 was 7.1 ± 0.7 pmol cell^{-1} and qPt_2 was 0.29 ± 0.05 pmol cell^{-1}; both so-called minimum cell quota are denoted as q_0N and q_0P. The optimum N:P ratio for *Fibrocapsa japonica*, determined as the ratio of minimum cell quotas $q_0N:q_0P$ (Oh & Rhee 1991), was 24.5. At t_2, cultures with the treatment N_LP_L had an N:P ratio of 31; apparently these cultures became P limited first. The maximum storage capacity under nutrient-sufficient conditions (N:P ratio 12.3) was 2.2 times higher for N and 4.3 times higher for P than at the optimum N:P ratio of 24.5. For the N_HP_H treatment there was no difference in cell quota at t_1 and t_2, indicating that neither N nor P was limited at Day 14 for this treatment.

Cell behaviour

For the 4 nutrient treatments, changes in cell behaviour were observed, which depended on the growth phase and nutrients, not on salinity. We observed 2 distinct changes in cell behaviour: impaired motility and aggregation of *Fibrocapsa japonica* cells (Fig. 3). Non-motile *F. japonica* cells and cell aggregates at the bottom of the flask were observed in the stationary phase at P limitation (N_LP_L and N_HP_L). In the N_HP_H treatment, cells formed aggregates at the surface of the culture in the stationary phase. Only in cultures under N limitation (only N_LP_H) did cells not aggregate in the stationary phase, but non-motile cells were observed.

Toxin analysis

For toxicity analysis, at each salinity the samples of the incubations at N_LP_L, N_LP_H and N_HP_L at t_2 were chosen to make sure that cells were either N or P limited and the cell densities were in the same range. The filters of the fourth treatment (N_HP_H) were selected for cells in exponential growth phase not suffering from nutrient limitation. From the dilution series for each treatment (fitted with a sigmoidal curve, r^2 > 0.99) the

EC_{50} was calculated as the concentration of *Fibrocapsa japonica* (cells ml^{-1}) necessary for 50% lysis of the erythrocytes. The relationship between this EC_{50} and salinity could be described with a quadratic function (Fig. 4; salinity: $F_{1,16} = 15.68$, p = 0.001, salinity^2: $F_{1,16} = 12.06$, p = 0.003). Among all the nutrient conditions examined there were significant differences in EC_{50} data sets ($F_{3,14} = 11.06$, p = 0.001). This was due to the difference between treatment N_LP_H (N limited) and the others (non-N limited), since values from the non-N limited treatments were not different from each other ($F_{2,10} = 0.181$, p = 0.837). In the final model, the EC_{50} values for the N_LP_H cultures were higher than for the other cultures ($F_{1,16} = 36.95$, p < 0.001), indicating less haemolytic activity when cells are N limited. The effect of the growth phase in the 2 sets of data (exponential growth phase vs cells in stationary growth phase) did not differ (i.e. there were no interactions $F_{2,14} = 4.73$, p = 0.112). The highest haemolytic activity was found
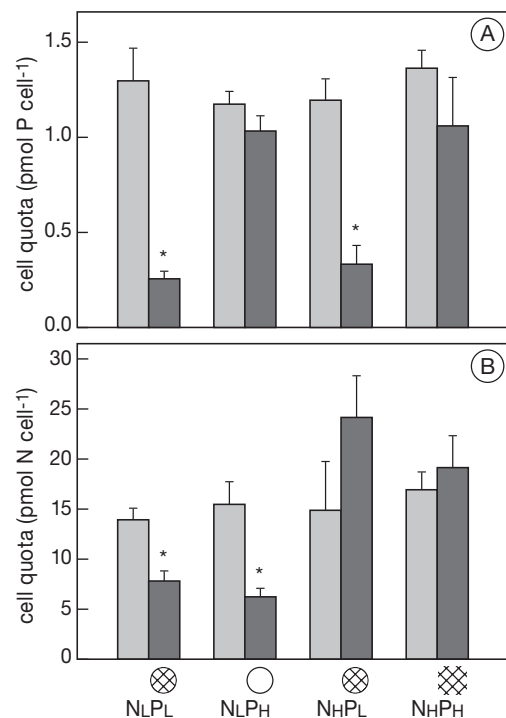

Fig. 3. *Fibrocapsa japonica*. Cell quota for (A) P and (B) N of cell for each nutrient treatment at t_1, exponential phase (light-grey bars), and t_2, stationary phase (dark-grey bars). Average values of 5 salinity treatments with their standard errors were used, because there were no significant differences between the salinities. *: differences between t_1 and t_2 are significant at the 0.05 level. Changes in cell behaviour at t_2 are indicated as: circle = non-motile cells in culture observed at the bottom of the flask; crosshatched = cell aggregation in culture; crosshatched circle = non-motile cells and cell aggregates at the bottom of the flask
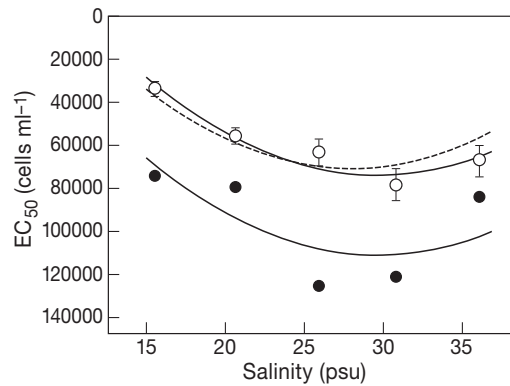
Fig. 4. Relationship between the *Fibrocapsa japonica* cell density (cells ml^{-1}) causing 50% haemolysis (EC$_{50}$) and salinity (●) N-limited cells measured at the beginning of the stationary phase of growth (model outcome: EC$_{50}$ = 3.66 × 10^4 + 6216 salinity – 105 salinity^2). (○) Average values of 3 non-N-limited treatments with standard error (model outcome: EC$_{50}$ = –5.5 × 10^4 + 6216 salinity – 105 salinity^2). Dotted line: relationship between the EC$_{50}$ (cells ml^{-1}) for non-N-limited treatments corrected on basis of mean equal cell volume (model outcome: EC$_{50}$ = –5.1 × 10^4 + 6177 salinity – 111 salinity^2), with salinity in psu

at 16 psu (mean EC$_{50}$ = 1.7 × 10^4 cells ml^{-1}) and was at least 2.3 times more haemolytic than at 30 psu.

No haemolytic activity was observed for PbTx 2 and no brevetoxins were found in the samples.

DISCUSSION

For the first time, cell quotas have been determined for nutrient-limited (q_0N and q_0P) and non-limited (qN and qP) cells of *Fibrocapsa japonica*. In comparison with the minimum cell quota for N and P of *Emiliania huxleyi* and various HAB species, the minimum cell quotas of *F. japonica* were usually higher; they were similar to the values for the dinoflagellate *Alexandrium catenella*, and lower than those of the raphidophyte *Chattonella antiqua* (Table 2). The cell volumes of *A. catenella* and *C. antiqua*, however, are 3 and 8 times larger than we have determined for *F. japonica*. Other raphidophyte species (*Chattonella verruculosa* and *Heterosigma akashiwo*) have half the cell volume of *F. japonica* combined with much lower minimum cell quotas (Watanabe et al. 1982, Yamaguchi et al. 2001). This indicates that (under similar nutrient conditions) these raphidophytes are expected to grow to higher cell densities than *F. japonica*. In proportion to its cell volume, *F. japonica* needs substantially more N and P compared to other species (Nakamura 1985, Matsuda et al. 1999). When minimum cell quotas are expressed per cell volume unit, the coccolithophorid *E. huxleyi* has, so far, the same high nutrient requirements (N and P in pmol μm^{-3}). In addition to *F. japonica*, *E. huxleyi* and the HAB species *C. verruculosa* and *A. catenella* also had an optimum N:P ratio higher than the Redfield ratio of 16 (Riegman et al. 1998, Matsuda et al. 1999, Yamaguchi et al. 2001). A high optimum nutrient ratio (the ratio at which transition from one nutrient limitation to another occurs; Rhee & Gotham 1980) indicates that at Redfield ratio these HAB species are N limited. When sufficient nutrients were available, *F. japonica* cells appeared to have a higher maximum storage capacity for P than for N. *F. japonica* cells entering stationary phase with sufficient nutrients (N$_H$P$_H$ treatments) were most likely rate limited by diffusion of CO$_2$.

Our results show that *Fibrocapsa japonica* is very suitable to grow in eutrophied coastal waters and is able to compete with species with a low optimum N:P ratio; *F. japonica* requires less phosphate. Since 1987 a shift was observed in the nutrient-rich eutrophied

Table 2. Optimum N:P ratios and minimum cell quotas (q_0N and q_0P) of a selection of marine harmful algal bloom species and *Emiliania huxleyi* (derived from Yamaguchi et al. 2001). The volume of the cell is from the accompanying reference or calculated using the morphological parameters of the species (Fukuyo et al. 1990) and the species-specific formulas from Hillebrand et al. (1999)

Species	Optimum N:P ratio	Minimum cell quota		Cell volume[a] (μm^3)	Source
		q_0N (fmol cell^{-1})	q_0P (fmol cell^{-1})		
Heterocapsa circularisquama	12.4	1100	89	2000	Yamaguchi et al. (2001)
Gymnodinium mikimotoi	12.5	3130	250	6000[b]	Yamaguchi & Itakura (1999)
Chattonella antiqua	12.6	7800	620	24000[c]	Nakamura (1985)
Heterosigma akashiwo	15.2	1440	95	2100	Watanabe et al. (1982)
Emiliania huxleyi	17.9	71.4	4	50	Riegman et al. (1998)
Alexandrium catenella	22.8	7300	320	11100[b]	Matsuda et al. (1999)
Fibrocapsa japonica	24.5	7100	290	4400	Present study
Chattonella verruculosa	33.6	84	2.5	1700[b]	Yamaguchi et al. (2001)

[a]Given cell volume, irrespective of environmental conditions
[b]Calculated from cell dimensions
[c]Nakamura & Watanabe (1983)

Dutch coastal waters towards a phosphorus-controlled system due to reduction of phosphate discharge (Peeters et al. 1999, Philippart et al. 2000). Such eutrophied phosphorus-controlled systems may favour large phytoplankton species with relatively high optimum N:P ratios (>Redfield ratio), such as *F. japonica.* Notably, this species has been observed in Dutch coastal waters since 1991 (Vrieling et al. 1995).

Its relatively large minimum cell quota suggests that *Fibrocapsa japonica* is not very efficient with nutrients, and therefore probably needs other ecological advantages to enable it to become a dominant species in the natural environment. A good example is the decreased vulnerability to grazers as demonstrated for the raphidophyte *Heterosigma akashiwo* (Tomas 1980). For this species the dual strategy of grazer avoidance and inhibition of autotrophic competitors partially compensates for its inferior N uptake capability (French & Smayda 1995). Motility of *F. japonica* appears to be quite an effective defence mechanism against grazers such as pallium-feeding dinoflagellates (Tillmann & Reckermann 2002). Our *F. japonica* cells, however, showed less motility when growth entered the stationary phase and either N or P became limiting. We conclude that under nutrient limitation, cell motility of *F. japonica* does not seem to be the strategy for grazer avoidance.

When entering stationary phase, cells under P and/or C limitation produce sticky aggregates that could play a role in their palatability. Extrusion of mucocysts by *Fibrocapsa japonica* acts mechanically as a grazer deterrent (Tillmann & Reckermann 2002). Cells of their strain tore up the pallia of the dinoflagellate *Oblea rotunda* (Tillmann & Reckermann 2002) and cells of a Japanese strain induced stress behaviour in *Artemia salina* (Rhodes et al. 1993, Cho et al. 1999).

Another explanation for the ability of *Fibrocapsa japonica* to produce blooms is toxicity of the cells. Fish kills related to *F. japonica* blooms have been explained by the production of brevetoxins, ROS, haemolytic compounds or mucocyst threads (Khan et al. 1996, Oda et al. 1997, Bridgers et al. 2004, Fu et al. 2004a,b, de Boer et al. 2004b). From some raphidophyte species (*Heterosigma akashiwo*, *Chattonella antiqua* and *Chattonella marina*), it is known that salinity affects the production of brevetoxins (Haque & Onoue 2002a,b). The highest brevetoxin concentration and ichthyotoxicity was found at low salinity (20 psu) in the late exponential phase for *H. akashiwo* (Haque & Onoue 2002a). For *C. marina* and *C. antiqua*, toxicity dropped drastically when salinity exceeded 30 psu (Haque & Onoue 2002b). For some *F. japonica* strains, brevetoxins were found (Khan et al. 1996, Bridgers et al. 2004). Using the sensitive ELISA assay, however, we could not demonstrate the presence of brevetoxins in our strain.

Our *Fibrocapsa japonica* is capable of producing haemolytic compounds, 3 of which have been identified as polyunsaturated fatty acids (PUFAs; Fu et al. 2004a,b). We demonstrated the haemolytic activity of *F. japonica* cell compounds in all 20 nutrient/salinity treatments (Fig. 4). Between exponential and stationary phase, no significant difference was found in the haemolytic activity of *F. japonica.* Production of these haemolytic compounds could have ecological advantages like inhibitory effects on growth of nutrient competitors (algae, bacteria) and act as grazer deterrent.

Salinity had a significant effect on the maximum growth rates and on the haemolytic activity (Figs. 2 & 4). If the differences in haemolytic activity coupled to salinity are due to osmotic effects, these do not couple with growth rates. To investigate the effect of salinity on cell size we did additional growth experiments (data not shown) that revealed bigger cells with increasing salinity (cell volume = 2908 + 57.07 salinity, n = 7; r^2 = 0.973; p < 1×10^{-4}). This finding has consequences for the interpretation of toxicity expressed on a per cell basis. Assuming a constant haemolytic activity per cell volume unit an approximation on the basis of equal biovolume for each activity was made for conditions without N limitation (Fig. 4). After correction for cell volume there is still an effect of salinity on the EC_{50} of *Fibrocapsa japonica* indicating a physiological basis for the observed differences related to salinity.

For other algae it is shown that nutrient limitation affects cell volume as well (Boyer et al. 1987, Simonsen & Moestrup 1997, Riegman et al. 1998), although for *Alexandrium tamarense* an increase in cell volume at P limitation did not entirely explain the increased toxin production (Boyer et al. 1987, Simonsen & Moestrup 1997). In our case, a slight decrease in cell volume of *Fibrocapsa japonica* under N limited conditions (from 4300 µm³, ⌀ 20.2 µm, to 2800 µm³, ⌀ 17.5 µm at 25 psu) is enough to explain the significant difference in haemolytic activity observed between N and non-N limited conditions. Therefore, volume changes rather than physiological adaptation seem to be the reason for the observed differences.

The effects of different environmental conditions on the production of toxins can vary substantially and are likely to be species specific (Johansson & Granéli 1999b). Here *Fibrocapsa japonica* cells always produced haemolytic compounds in contrast to the nutrient-limiting conditions that were needed for prymnesiophyte toxicity (Johansson & Granéli 1999a,b). For *Alexandrium tamarense*, both the haemolytic activity and the production of PSP toxins were highest in the exponential growth phase (Simonsen et al. 1995). Salinity rather than growth phase had a significant effect on the haemolytic activity of *F. japonica*.

To compare our results with other haemolytic species, studies were selected where the EC_{50} of saponin as reference compound was also determined (Table 3). Except for *Chrysochromulina simplex* all species are HAB species. Note that increasing EC_{50} values relate to decreasing haemolytic activities. The highest haemolytic activity of *Fibrocapsa japonica* was found at low salinity (16 psu), which was in the same range as *Prymnesium parvum* under N- or P-limited conditions (Johansson & Granéli 1999b). However, if for the EC_{50} the differences in cell volume are taken into account, the haemolytic activity of *P. parvum* on a per volume basis is about 14 times higher than *F. japonica* and equal to *Alexandrium tamarense*, which had a high haemolytic activity per cell (Eschbach et al. 2001). Cell volume based haemolytic activity of *F. japonica* is about 8 times higher than that of *Chrysochromulina polylepis* (Simonsen & Moestrup 1997). The haemolytic activity of phytoplankton cell extracts does not

always translate into toxicity for aquatic organisms (Yasumoto et al. 1987, Simonsen & Moestrup 1997). This toxicity is also dependent on the amount and type of haemolytic compounds, which differ between species (Yasumoto et al. 1987, Simonsen & Moestrup 1997) and environmental conditions (Johansson & Granéli 1999b).

We expect that most of the observed haemolytic activity of *Fibrocapsa japonica* was due to the haemolytic PUFAs; 6,9,12,15-octadecatetraenoic acid (OTA or stearidonic acid; C18:4n3), 5,8,11,14,17-eicosapentaenoic acid (EPA; C20:5n3) and 5,8,11,14-eicosatetraenoic acid (arachidonic acid, AA; C20:4n6), that were recently identified in this strain (Fu et al. 2004a). These may be involved in a lipoxycascade starting with the hydrolysis of lipids and rapid formation of PUFA when a cell is disrupted (Jüttner 2001). Mass aggregation of *F. japonica* cells could indicate such a cell damage, causing leakage of allelopathic, toxic

Table 3. An overview of the haemolytic studies on microalgae compared to the results obtained for our strain of *Fibrocapsa japonica*. For comparison, microalgae EC_{50} values (cell density [cells ml^{-1}] causing 50% haemolysis) were corrected for differences in experimental conditions using the reported assay-specific saponin EC_{50} values and normalising for the EC_{50} for saponin of this experiment. The volume of the cell is from Simonsen & Moestrup (1997) or calculated using the morphological parameters of the species (Fukuyo et al. 1990) and the species specific formulas from Hillebrand et al. (1999)

Species	Nutrient conditions	EC_{50} saponin (mg ml^{-1})	EC_{50} microalgae (10^4 cells ml^{-1})	Cell volume[a] (μm^3)	Source
Fibrocapsa japonica	N limited	0.0045	6.3	4400	At 26 psu; this study
Fibrocapsa japonica	Non-N limited	0.0045	3.2	4400	At 26 psu; this study
Fibrocapsa japonica	Non-N limited	0.0045	1.7	3800	At 16 psu; this study
Alexandrium tamarense CCMP115		0.0045	0.0152	18700[b]	Eschbach et al. (2001)
Chrysochromulina polylepis K-0259	P limited	0.1430	170.3	270	Simonsen & Moestrup (1997)
Chrysochromulina polylepis K-0259	P limited	0.1430	154.8	270	Simonsen & Moestrup (1997)
Chrysochromulina polylepis K-0259	P limited	0.1430	155.7	270	Simonsen & Moestrup (1997)
Chrysochromulina polylepis K-0259	P limited	0.1430	116.2	270	Simonsen & Moestrup (1997)
Chrysochromulina simplex K-0272	P limited	0.1430	1575.6	60	Simonsen & Moestrup (1997)
Prymnesium parvum K-0081	P limited	0.1430	0.6	280	Simonsen & Moestrup (1997)
Prymnesium parvum K-0081	(Day 2; log phase)	0.1430	18.8	280	Simonsen & Moestrup (1997)
Prymnesium parvum K-0081	(Day 6; log phase)	0.1430	11.0	280	Simonsen & Moestrup (1997)
Prymnesium parvum K-0081	(Day 10; late log phase)	0.1430	7.2	280	Simonsen & Moestrup (1997)
Prymnesium parvum K-0081	(Day 16; stat phase)	0.1430	17.0	280	Simonsen & Moestrup (1997)
Prymnesium parvum K-0081	(Day 21; stat phase)	0.1430	24.7	280	Simonsen & Moestrup (1997)
Prymnesium parvum	N limited	0.2270	1.0	280	Johansson & Granéli (1999b)
Prymnesium parvum	P limited	0.2270	1.2	280	Johansson & Granéli (1999b)
Prymnesium parvum	Not limited	0.2270	6.8	280	Johansson & Granéli (1999b)
Prymnesium parvum RL10		0.0045	1.0	280	Eschbach et al. (2001)
Prymnesium parvum KAC 39[c]	0% DOM 100% NO_3	0.1610	0.4	280	Stolte et al. (2002)
Prymnesium parvum KAC 39[c]	25% DOM 75% NO_3	0.1610	0.3	280	Stolte et al. (2002)
Prymnesium parvum KAC 39[c]	50% DOM 50% NO_3	0.1610	0.3	280	Stolte et al. (2002)
Prymnesium parvum KAC 39[c]	Cell-free filtrate, $t = 0$	0.1610	1.0	280	Fistarol et al. (2003)
Prymnesium parvum KAC 39[c]	Cell-free filtrate, $t = 48$ h	0.1610	No haemolysis	280	Fistarol et al. (2003)

[a]Given cell volume, irrespective of environmental conditions
[b]Calculated from cell dimensions
[c]*Prymnesium parvum* f. *patelliferum* KAC 39

and/or haemolytic substances into the environment. Such leakage of haemolytic compounds into the medium was demonstrated when cells of *Prymnesium parvum* shifted from exponential growth phase into stationary growth phase (Simonsen & Moestrup 1997).

In some studies, EPA was shown to provide a defensive role against grazers (zooplankton; Jüttner 2001), allelopathic protection towards phytoplankton species (Arzul et al. 1998) and a toxic effect for fish (Marshall et al. 2003). Enhanced ichthyotoxicity of EPA in the presence of superoxide seems to account for the high potential of *Chattonella marina* to kill fish (Marshall et al. 2003). It is possible that the same type of mechanism is involved in *Fibrocapsa japonica* ichthyotoxicity, where haemolytic compound production is combined with the generation of ROS (Oda et al. 1997).

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