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The Morphological and Growth Characteristics of Two Strains of *Fibrocapsa japonica* Isolated from New Zealand and Japan

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The blooms caused by *Fibrocapsa japonica* have occurred regularly in New Zealandean coastal waters, and no fish kills and economic impacts have been reported. However, *Fibrocapsa japonica* in Japan killed caged juvenile fish and has been regarded as a harmful microalgae. In this comparative study a New Zealand isolate was found to be morphologically similar to a Japanese isolate, although slightly larger on average than. Optimal temperatures for growth differed, with fastest growth rates occurring at 22~24℃ for the New Zealand strain and 16~22℃ for the Japanese strain, with a decrease in growth rate exhibited by the latter at 24℃. Both isolates had low salinity optima of 20~25 PSU, although they grew between 15 and 30 PSU. Growth declined significantly for both strains above 30 PSU.

Key words: *Fibrocapsa japonica*, mucocysts, fish kills, chloroplast, growth, El Niño

Introduction

The outbreak of *Fibrocapsa japonica* (Toriumi and Takano 1973) bloom occurred in 1972, with associated massive death of young caged fish. Since then, blooms caused by *F. japonica* have occurred continuously (Khan et al., 1996). This species has been regarded as a harmful microalga by some researchers (Fukuyo et al., 1990). *Fibrocapsa japonica* has a similar morphology to *Chattonella* under the light microscope, but differs significantly in that the mucocysts were assigned to the posterior section of the cell compared with *Chattonella*, and thus it has been suggested that this microalga should be an independent species (Hara and Chihara 1985). In addition, from recent phylogentic analysis on the basis of large sub-unit rRNA gene sequences, *F. japonica* and *Chattonella* were morphologically similar, but were clearly separated from the other raphiophyte genera (John Tyrrell, Auckland University, personal communication). However, in Korea, located near to Japan, no *Fibrocapsa japonica* blooms have been associated with fish mortality (unpublished data). Since 1990 European countries including New Zealand have reported *Fibrocapsa japonica* (Billard 1992, Rhodes et al., 1993; Vrieling et al., 1995) and in New Zealand the blooms were associated with the unusual cold sea water temperature associated with El Niño climate conditions (Rhodes et al., 1993). In none of these cases have fish mortalities been recorded. The species is regarded as a toxic microalga in Japan, but non toxic in New Zealand and European countries.

The blooms caused by *Heterosigma akashiwo* has occurred annually in June and July in Korean coastal waters since 1980 with no fish kill (Kim et al. 1996), whereas in New Zealand in 1989, dense blooms of *Heterosigma akashiwo* resulted in the death of cultured young salmon worth nearly $ 20 million (Chang et al., 1990; Mackenzie 1991). Thus, although the same species was reported from both countries, differences in non-toxic and toxic microalgae were clear. It is desirable to discriminate non-toxic from toxic phytoplankton for harmful algae monitoring systems.

In the present study, we compare the morphology of New Zealand and Japanese strains of

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Fibrocapsa japonica using light microscopy, transmission electron microscopy (TEM) and the growth responses at different conditions of temperature, salinity, pH and nutrient addition (nitrogen, phosphorus, metal and selenium).

Materials and Methods

Microalgae

Fibrocapsa japonica (CAWR02) for this study was isolated from Leigh in New Zealand, and was collected by picking cells out individually under the light microscope with a micropipette or by serial dilution (Rhodes et al., 1993). After a unialgal culture was established, it has been maintained and cultured in GP medium (Loeblich and Smith 1968). The other *Fibrocapsa japonica* (CAW03) obtained from CSIRO Culture Collection of Microalgae, Hobart, Tasmania, Australia (Japanese strain) has been cultured as above. New Zealand and Japanese strains of cultures were subcultured weekly until growth was established. For the differentiation of the two strains, we selected 50 cells randomly without fixation in late exponential to the early stationary phase (fixation caused changes in cell form) from Multiwell tissue culture plates (Becton Dickinson) and measured the cell length (from flagella to posterior portion) and the cell width of vegetative cells. Significant differences in measurements were determined statistically by one-way ANOVA.

Electron microscopy

For transmission electron microscopy (TEM), the filter containing collected particles was subsequently postfixed in 1% osmium tetroxide for 1 hr at room temperature, washed, and then dehydrated by a graded ethanol series to 100% ethanol. The filter was then placed in two changes of propylene oxide and then into a 1:1 mixture of Epon resin and propylene oxide overnight, then in fresh Epon resin for 8 hours. The filter was again placed in fresh resin and polymerised for 24 hours. Selections were cut with a glass knife approaching the particles on the membrane filter parallel to the membrance surface. Ultrathin sections (70 nm) were cut with a diamond knife, stained with uranyl acetate followed by lead citrate, and examined at 80 kV with Philips 2000 TEM.

Growth

Experimental culture conditions: preliminary growth curves obtained at 18℃ and 20℃ under standard conditions (GP medium; 100 μmol m^{-2} s^{-1} ; 14:10 h light:dark regime) indicated that stationary phase was reached on the days 10th and 14th of growth respectively. Culture conditions to determine optimal growth, and to induce plano-hypnozygotes, included: a temperatures from 16℃ to 24℃ (interval 2℃), salinities from 15 PSU to 35 PSU (interval 5 PSU), and pH from 6.5 to 8.5 (interval 0.5), respectively. Nutrient modifications of synthetic seawater-based GP medium included: potassium nitrate (as nitrogen source). For the study of nutrient requirements, potassium nitrate additions as nitrogen source were 0 $\mu g/\ell$, 10 $\mu g/\ell$, 100 $\mu g/\ell$, 500 $\mu g/\ell$; di-potassium hydrogen orthophosphate addition as phosphorus source were 0 $\mu g/\ell$, 1 $\mu g/\ell$, 10 $\mu g/\ell$, 100 $\mu g/\ell$ and finally GP medium was prepared without metal (ferric chloride, manganese chloride, cobaltous chloride) and without selenite. Growth was measured by directly counting cells under an inverted Olympus B2002 light microscope in Multiwell tissue culture plates (Becton Dickinson) in quadruplicate. The tissue chambers were sealed to reduce evaporation of the cell medium. Initial cell density was ca. 10~30 cells/2 $m\ell$, inoculated into filtered medium (0.2 μm AS 020).

Specific growth rate (division rate/day) was computated as follows: growth rate $= 1/t \cdot \ln(N_t/N_o) \cdot (1/\ln 2)$, where t means inoculation days, N_t means cell density after t days and N_o means initial cell density.

Data analysis and determination of statistical significance were carried out using Student t-test (microsoft excel ver. 7.0) to compare specific growth rates.

Results

Morphology

Vegetative cells of the New Zealand and Japanese isolates of *Fibrocapsa japonica* used for comparison in the study (FjNZ and FjJ respectively), conformed to previous light microscope descriptions of the species (Toriumi and Takano 1973; Billard 1992), and were difficult to differentiate (Fig. 1a, b, c, d). However, the relationship between cell length and cell width was different; FjNZ (New Zeland isolates of *Fibrocapsa japonica*) measured 28.3~44.5 μm (mean 39.7 μm) by 25.6~47.2 μm (mean 37.7 μm), whereas FjJ (Japanese isolates of Fibrocapsa japonica) measures 18.9~44.5 μm (mean 32.1 μm) by 18.9~48.6 μm (mean 28.5 μm). Overall, FjNZ

was the larger in all morphological biometries (slope 0.95; FjJ, 0.88); the strains differed significantly ($p<0.05$) in the relationship between length and width (Fig. 2). Mucocysts were posteriously situated in both strains, and cells of stationary phase were more spherical in shape with many mucocysts (Fig. 1a, c), whereas those of exponential phase were ellipsoid with few mucocysts (Fig. 1b, d). Cells discharged numerous mucous filaments under high light intensity (data not shown). *Fibrocapsa japonica* underwent normal binary fission, with the onset of division correlated with the appearance of two extra flagella in the anterior portion. The two newly formed cells twisted around the remaining central strand to separate, with only one daughter cell accumulating mucocysts (data not shown). Different sized daughter cells, and sometimes three daughter cells, were observed (Fig. 1e, f). A day after separation they regained the usual vegetative form (Fig. 1a, b, c, d). Transverse sectioning and TEM confirmed that stationary phase cells of both strains had numerously accumulated posteriorly (Fig. 3a, b), each with a single membrane layer (Fig. 3c). Mucous filaments spread out radially (Fig. 3d), and in some instances mucocysts and nucleus appeared fused (Fig. 3h). The nuclear membrane (Fig. 3e), mitochondri and tubular cristae (Fig. 3f), thylakoid lamellae and chloroplast membranes (Fig. 3g) were all observed under TEM, and were similar in both strains. They were not zygotes, and carried out binary fission when fresh medium was added. No resting cysts were formed, despite growing cultures

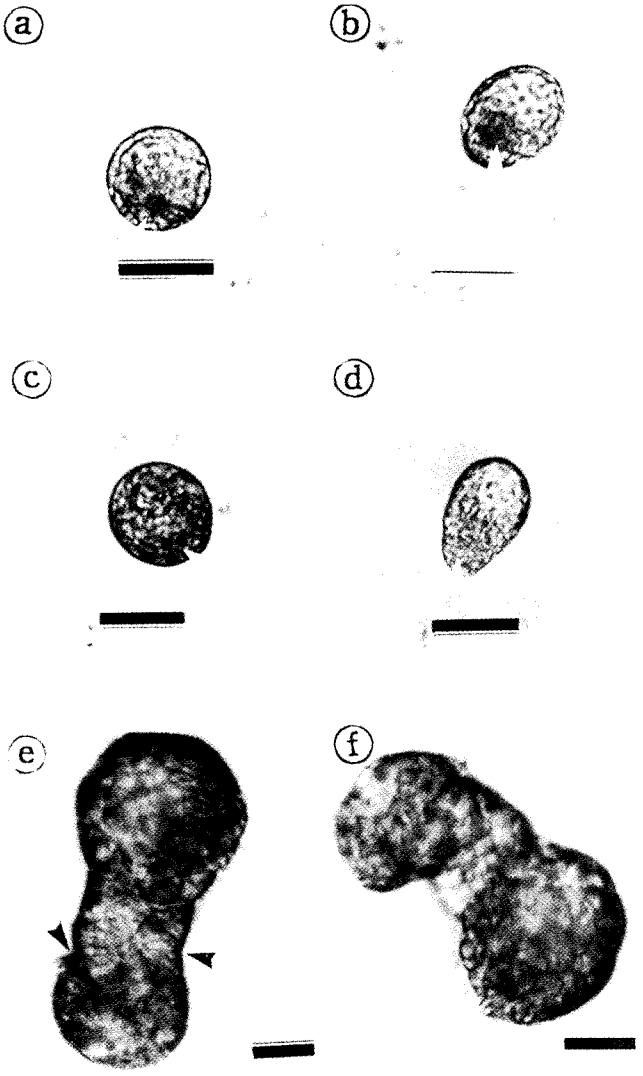


Fig. 1. Two strains of *Fibrocapsa japonica* observed by light microscope. A: Spherical form of *Fibrocapsa japonica* isolated from Leigh in New Zealand. B: Ovoid form of *.Fibrocapsa japonica* isolated from Leigh in New Zealand. C: Sperical formation of *Fibrocapsa japonica* obtained from CSIRO (Japanese strain), Australia. D: Ovoid formation of *Fibrocapsa japonica* obtained from CSIRO (Japanese strain), Australia. The white arrowhead represents the deposition of mucocysts (scale bar is 20 μm). E-F: Triple-division of *Fibrocapsa japonica* obtained from CSIRO (Japanese strain), Australia. The black arrowhead shows the beginning of third cell division point. (scale bar is 10 μm)

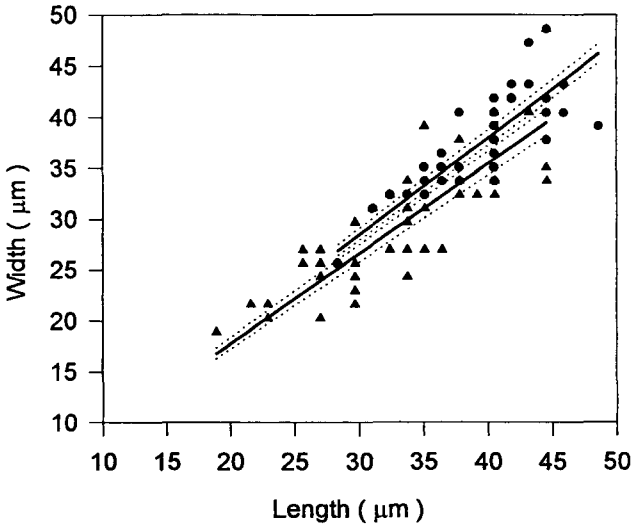


Fig. 2. Differences in width versus length between New Zealand (●) and Japanese (▲) isolates of *Fibrocapsa japonica*. Linear regressions bold (correlation coefficient 0.05), confidence intervals dotted, lines (95%).

in a range of media and under different environmental conditions (see Methods)

Growth characteristics

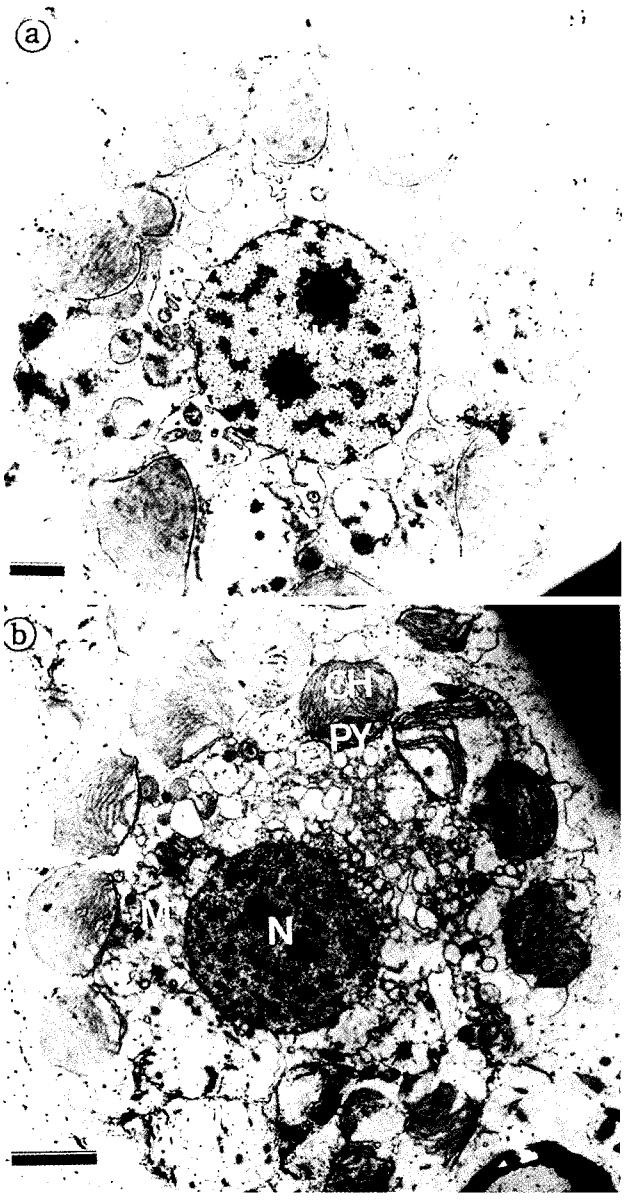
The growth rate (GR) increased in FjNZ, but decreased in FjJ, when the temperature was raised from 20℃ to 24℃. The optimal specific GR for FjNZ, 0.22, was achieved between 22~24℃, and this was significantly faster than at 16~20℃ ($p<0.05$). The optimal GR for FjJ, 0.26, was 16~22℃, with a significantly slower GR at 24℃ ($p<0.05$; Table 1). The optimal GR of both strains of *F. japonica* occurred between 20~25 PSU, and was significantly slower at salinities of 15 and ≥30 PSU ($p<0.05$). Differences in GR between the two strains were significant at both 15 PSU and 30 PSU (Table 1-1). Both strains of *Fibrocapsa* grew equally well from pH 6.5~8.0; GR was slower at pH 8.5 ($p<0.05$; Table 1-2). The fastest GR was achieved with 10 μg/ℓ, 100 μg/ℓ of nitrate addition. The greater concentrations, the slower GR. However, differences in GR were significant for FjNZ, but not for FjJ ($p<0.05$, Table 1-3). GR was also similar for both strains with different phosphorus concentrations. However, FjJ has a faster GR than FjNZ ($p<0.05$) when no nitrate or no phosphate was added (Table 1-4). There was no difference in GR within or between strains when different concentrations of selenite were added (Table 1-5).

Discussion

Taxonomic description

Fibrocapsa japonica from New Zealand agreed closely with descriptions of other geographic isolates, but as the cell membrane was flexible and affected by environmental conditions (Vrieling et al. 1995), it was not surprising that some unusual morphological forms were observed in this study. According to Fukuyo et al. (1990), *Fibrocapsa japonica* isolated from Japanese waters measured 20~30 μm in length and 15~20 μm in width, but both the New Zealand (FjNZ) and Japanese (FjJ) strains in this study were longer and wider with a greater size range (Fig. 2). The relationship between cell length and cell width differed between the two strains (Fig. 2), and apart from the occasional appearance of "giant" cells in FjJ cultures, the vegetative cells of FjNZ were, on average, larger. Anderson (1980) suggested that planozygotes or hypnozygotes were larger than vegetative cells, but the "giant" cells appeared to be a temporary phenomenon formed in responsible to nutrient depletion.

According to Fukuyo et al. (1990), *Fibrocapsa japonica* underwent asexual reproduction, but not planozygotic or hypnozygotic sexual reproduction. The life history *Fibrocapsa japonica* has not been studied extensively, but Yoshimatsu (1987) has described spherical (15~20 μm diameter), brown cysts of *Fibrocapsa* in bottom sediments in Harima-Nada in Japan. Cysts have also been formed in culture under artificially formulated environmental conditions, in particular nitrogen (Imai 1989) and phosphorus depletion (Nakamura et al., 1990). However, in this study, the induction of planozygotes or hypnozygotic resting cysts from



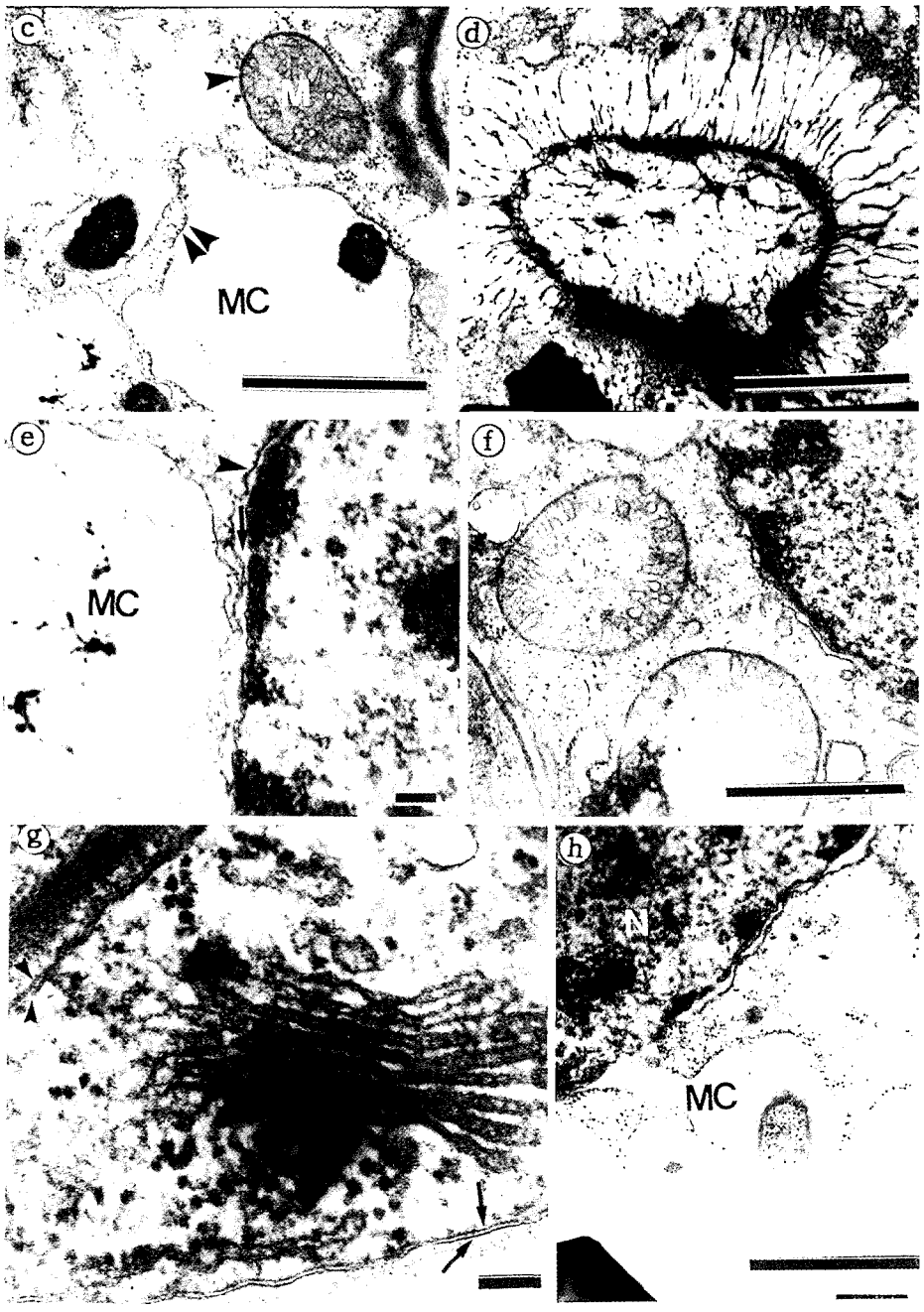


Fig. 3. The observation of two strains of *Fibrocapsa japonica* using Transmission Electron Microscope (TEM). Major cellular components and arrangement are as follows: nucleus (N), chloroplast (CH), pyrenoid (PY) and mitochondria (M). A: Cross section in whole cell from New Zealand strain. This cell shows an early exponental stage as indicated by lack of mucocysts (MC) and branched fibrils (scale bar is 2 μm). B: Cross section in whole cell from Japanese strain. This shows accumulation of mucocysts (MC) in posterior of cell and radially branching fibrils. Mucocysts (MC) are positioned facing to outside of cell (scale bar is 5 μm). C: Mitochodria (M) has double membrane (black single arrowhead), whereas mucocysts (MC) have single membrane (black double arrowhead) and appear dense (scale baris 1 μm). D: Many filaments spread out in all directions (scale bar is 1 μm). E: Nucleus (N) has double membrane (black arrowhead) and mucocyst (MC) membrane lies close to, but separates from nuclear membrane (indicated by black arrow). (scale bar is 100 nm) F: Mitochondria (M) possess tubular cristae (scale bar is 1 μm). G: Two thylakoid lamella (black arrowhead) and chloroplast are surrounded by two membranes (black arrow). (scale bar is 100 nm). H: Mucocyst (MC) and nucleus (N) fused (scale bar is 1 μm).

Table 1. The results of specific growth rate and significance test of New Zealand (NZFj) and Japanese (JFj) isolates of *Fibrocapsa japonica* at different temperatures after 14 days

Temperature (℃)	NZFj					JFj				
	16[a]	18[b]	20[c]	22	24[d]	16[a]	18[b]	20[c]	22	24[d]
	0.165	0.228	0.162	0.212	0.231	0.255	0.247	0.284	0.229	0.161
	0.119	0.162	0.182	0.160	0.160	0.268	0.228	0.229	0.235	0.150
	0.167	0.142	0.183	0.221	0.237	0.209	0.240	0.284	0.211	0.124
	0.081	0.143	0.153	0.175	0.231	0.255	0.196	0.244	0.225	0.163
Average	0.133[ab]	0.169	0.170	0.192[a]	0.215[b]	0.247[c]	0.228[d]	0.260[e]	0.225[f]	0.150[cdef]

Figures in the same row having the same superscripts are significantly different (95% level).

Table 1-1. Salinities

Salinity (PSU)	NZFj					JFj				
	15[a]	20	25	30[b]	35	15[a]	20	25	30[b]	35
	0.094	0.165	0.185	0.092	−0.035	−0.004	0.128	0.196	0.052	−0.011
	0.058	0.148	0.143	0.125	0.053	0.024	0.119	0.162	0.088	0.014
	0.097	0.155	0.200	0.106	0.049	−0.001	0.162	0.198	0.047	0.030
	0.034	0.114	0.119	0.062	−0.012	0	0.200	0.149	0.032	0.026
Average	0.071[ab]	0.146[acd]	0.162[blm]	0.096[cln]	0.013[dmn]	0.004[efg]	0.152[ehi]	0.176[fjk]	0.055[ghj]	0.015[ik]

Figures in the same row having the same superscripts are significantly different (95% level).

Table 1-2. pH

pH	NZFj					JFj				
	6.5	7.0	7.5	8.0	8.5	6.5	7.0	7.5	8.0	8.5
	0.139	0.144	0.144	0.136	0.088	0.121	0.127	0.161	0.189	0.153
	0.125	0.185	0.176	0.143	0.068	0.093	0.183	0.204	0.118	0.121
	0.157	0.188	0.163	0.134	0.130	0.106	0.173	0.197	0.146	0.105
	0.216	0.125	0.203	0.086	0.076	0.217	0.166	0.150	0.164	0.092
Average	0.159	0.160[ab]	0.171[c]	0.125[a]	0.090[bc]	0.134	0.162	0.178[d]	0.154	0.117[d]

Figures in the same row having the same superscripts are significantly different (95% level).

Table 1-3. Nutrient requirements (KNO_3)

KNO_3 ($\mu g/\ell$)	NZFj				JFj			
	0[a]	10	100	500	0[a]	10	100	500
	0.135	0.204	0.226	0.113	0.230	0.194	0.233	0.200
	0.093	0.202	0.158	0.103	0.196	0.166	0.204	0.192
	0.155	0.151	0.172	0.091	0.206	0.204	0.234	0.133
	0.093	0.228	0.177	0.155	0.147	0.245	0.176	0.135
Average	0.119[a]	0.196[b]	0.183[ac]	0.116[bc]	0.195	0.202	0.212	0.165

Figures in the same row having the same superscripts are significantly different (95% level).

Table 1-4. Nutrient requirements (K_2HPO_4)

K_2HPO_4($\mu g/\ell$)	NZFj				JFj			
	0[a]	1	10	100	0[a]	1	10	100
	0.177	0.163	0.199	0.186	0.212	0.236	0.188	0.161
	0.210	0.199	0.172	0.181	0.220	0.217	0.202	0.203
	0.153	0.148	0.192	0.195	0.183	0.204	0.174	0.259
	0.140	0.221	0.170	0.166	0.180	0.188	0.212	0.208
Average	0.170	0.183	0.183	0.182	0.199	0.211	0.194	0.208

Figures in the same row having the same superscripts are significantly different (95% level).

Table 1-5. Metal and sodium selenite

	NZFj		JFj	
	non metal	non selenium	non metal	non selenium
	0.183	0.184	0.210	0.171
	0.108	0.170	0.175	0.181
	0.184	0.163	0.159	0.157
	0.170	0.101	0.238	0.190
Average	0.161	0.154	0.195	0.175

exual fusion was not achieved, despite applying a range of environmental and nutrient conditons.

Electron microscope (EM) differentiation of the two strains was achieved on the basis of chloroplast morphology. The FjJ strain had discoid chloroplasts, unlike the irregular forms observed in FjNZ (data not shown). According to Vrieling et al. (1995), *Fibrocapsa japonica* isolated in Holland also had numerous yellowish-brown discoid chloroplasts. The FjNZ chloroplasts occurred independently, with irregular interconnections, whereas FjJ exhibited a regular "diamond soccer ball" effect, with connections between each chloroplast (data not shown). Both strains have pores distributed over the whole cell surface, probably relating to mucocyst ejection. In this study numerous mucous fibrils were ejected under strong lighting, and with the fixing agents (osmium tetroxide and glutaraldehyde) used for EM examination, and it was useful to find that discharge could be minimized by using 3% glutaraldehyde+1% osmium tetroxide, 0.5% glutaraldehyde+0.2% osmium tetroxide, or 0.2% osmium tetroxide). Toriumi and Takano (1973) observed under the light microscope that trichocysts were suddently discharged before the death of a cell and that the first trichocyst was ejected from the posterior end of the cell, and this was supported by EM observation. In this study TEM showed no strain differences in the arrangement of nucleus, mitochondria and mucocysts (Fig. 3a-h). Mucocysts in particular were distributed mainly towards posterior of the cells, and were densely accumulated in older cells compared with young cells (Fig. 3a, b), although young cells discharged their mucocysts more readily, and were possibly more sensitive than older cells because of the lack of a rigid cell membrane (Vrieling et al., 1995). Hara and Chihara (1985) noted that each chloroplast were not studied in detail, but FjNZ appeared to have a thinner membrane than FjJ.

Growth characteristics

The optimal temperature for growth was 22~24℃ for FjNZ, whereas optimal growth for FjJ was at 16~22℃, with an abrupt and significant decline in growth at 24℃ (Table 1). This suggests that New Zealand strains could be more tolerant of shallow embayments, where seawater temperatures can reach ambient summer temperatures, and it fits the geographic spread of *Fibrocapsa* in New Zealand, where it is mainly concentrated in the sub-tropical north. Only rare occurrences have been recorded in the southern North Island and northern South Island waters (unpublished data). Salinity optima and tolerance ranges for both strains strongly supported the contention that *Fibrocapsa* was suited to estuarine and coastal conditions, not oceanic waters. Higher than normal rainfall, with resultant run-off into coastal waters would favour microalgal species with low salinity optima, as occurred when *Fibrocapsa* bloomed under El Niño climate conditions in the latter half of 1992 (Rhodes et al., 1993). It was assumed that the lack of rigidity of the cell membrane was comparable in these organisms, leading to similar physiological responses. However, the New Zealand strain did exhibit a significantly higher specific growth rate at 15 and 30 PSU than the Japanese strain (Table 1-1), suggesting that there might be slightly different physiological characteristics between the two strains. Furuki and Kitamura (1981) demonstrated that *Chattonella antiqua* changed its cell morphology to spindle or globular shape with nutrient deficiency. No morphological changes due to nutrient depletion were observed in this study, although some shrinkage was noted in salinity and pH stress (personal observation).

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