J. Fish. Sci. Tech. 2(1), 58~65, 1999

JOURNAL OF
FISHERIES SCIENCE
AND TECHNOLOGY

The Comparison of Two Strains of *Fibrocapsa japonica* (Raphidophyceae) in New Zealand and Japan

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(Received February 1999, Accepted May 1999)

Fibrocapsa japonica (Raphidophyceae) is regarded as a harmful algal bloom organism in Japanese waters, where it has been linked to fish kills. *Fibrocapsa* is a common species in New Zealand coastal waters, particularly in the Hauraki Gulf, where it has regularly bloomed in the spring under El Niño climate conditions for the past six years. The New Zealand isolate had 1.4 times more total polyunsaturated acids than the Japanese isolate under the same growth conditions, suggesting that eicosapentaenoic acid in particular could be used as a discriminating chemotaxonomic marker. The molecular probes tested showed no differential binding of the raphidophytes to lectins, but oligonucleotide probes targeted *F. japonica* ribosomal RNA bound specifically to both isolates. Neither strain was toxic in mouse or neuroblastoma bioassays. There is no evidence that the New Zealand *F. japonica* isolates investigated to date produce ichthyotoxins.

Key words: *Fibrocapsa japonica*, raphidophytes, micro-algae, blooms, El Niño

Introduction

Fibrocapsa japonica Toriumi et Takano (Raphidophyceae) was first described following an ichthyotoxic bloom in Japan, in 1972 (Toriumi and Takano 1973; Yoshimatsu 1987; Iwasaki 1989). The cell morphology was identical to an ichthyotoxic bloom recorded a year earlier, and tentatively called *Botryococcus* sp. (Okaichi 1972). *Fibrocapsa* blooms caused massive mortalities of young caged fish at that time, and *F. japonica* is consequently regarded as a HAB (harmful algal bloom) species (Fukuyo et al., 1990; Hallegraeff and Hara 1995). It has since bloomed annually in Japanese coastal waters (Yoshimatsu 1987; Iwasaki 1989; Montaini et al., 1995), but was not observed elsewhere for nearly two decades. Then, in 1991, it was observed in France, Holland (Billard 1992; Vrieling et al., 1995), and New Zealand (Rhodes et al., 1993). No associated fish mortalities were recorded. In spring 1992 *F. japonica* bloomed ($>10\times10^4$ cells $\cdot$ l^{-1}) extensively along the north-east coastline of the North Island of New Zealand, and the event was linked to El Niño climatic conditions (Rhodes et al., 1993). A *Gymnodinium mikimotoi* bloom which succeeded it in early January 1993 was held responsible for mortalities of shellfish in Northland, the death of cats fed shellfish viscera (Bates et al., 1993), and neurotoxic shellfish poisoning (NSP) symptoms in humans, in particular respiratory distress due to aerosols from the *Gymnodinium* bloom (Jasperse 1993).

Fibrocapsa blooms recurred in the Hauraki Gulf in 1994 (max. 2.4×10^5 cells $\cdot$ ℓ^{-1}) and 1997 (max. 1.7×10^5; Public Health and Coromandel Aquaculture Association phytoplankton data), again in conjunction with El Niño events. Massive *Noctiluca scintillans* and *Myrionectra rubrum* blooms coloured extensive areas prior to the *Fibrocapsa* bloom of 1994 and succeeding that of 1997. As in 1992-93, the 1997 *Fibrocapsa* blooms occurred in spring and early summer, and in mid-summer high numbers of *Gymnodinium* cf. *mikimotoi* ($\approx1.2\times10^5$ cells $\cdot$ ℓ^{-1}) with associated human respiratory distress reactions were reported further south, first along the Wairarapa Coast (early 1998), then

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further north in Hawke Bay, and by March in Wellington Harbour (Chang et al., 1998; Public Health data). The *Gymnodinium* bloom was associated with wild fish kills, and with mortalities of cultured seahorses, cockle larvae, brine shrimp and turbot at the National Institute of Water and Atmosphere's Mahanga Bay Hatchery, Wellington (Tong 1998). Raphidophyte blooms in the Hauraki Gulf could thus provide an early warning of NSP risk during El Niño climatic conditions. No fish kills recorded in New Zealand have been attributed to *Fibrocapsa*, although the related *Heterosigma akashiwo* was responsible for caged salmon deaths in 1989 (Mackenzie 1991), and the ichthyotoxic *Chattonella marina* has been identified in Northland waters (Rhodes and Thomas 1997). *Fibrocapsa japonica* is the only species in its genus (Fukuyo et al., 1990). When it was first described by light microscopy (LM), morphological similarities to *Chattonella* were noted. However, it differed in the posterior positioning of the mucocysts and was considered to be a separate species (Hara and Chihara 1985). The view has been supported by recent DNA sequencing data, which clearly separates *Fibrocapsa* from the other raphidophyte genera on the basis of large sub-unit ribosomal RNA gene sequences (John Tyrrell, Auckland University, personal communication). A neurotoxic fraction has been reported in an extract from a Dutch isolate of *Fibrocapsa*, although the toxicity was considered weak compared with the ichthyotoxicity of *Chattonella* (Khan et al., 1996a), and toxins resembling brevetoxin derivatives of Pbtx 2 and 3 were detected by HPLC and radioimmune assays in *Fibrocapsa* and *Chattonella* from Florida (Tomas 1998). The New Zealand *Fibrocapsa* strain was also tested for neurotoxicity, by mouse bioassay and neuroblastoma assay. The differential effects of New Zealand and Japanese isolates on *Artemia salina* were described previously (Rhodes et al., 19 93), the New Zealand isolate being non-toxic, and the Japanese isolate causing acute distress in brine shrimps.

Fatty acid (FA) composition was assessed as a chemotaxonomic tool. Polyunsaturated FAs, such as arachidonic, eicohapentaenoic, and docosapentaenoic acid, necessary for the functioning of the thylakoid membranes, are derived from the precursors linoleic acid (n-6) and linolenic acid (n-3) (Ackman et al., 1968). FA concentration differs between microalgal species, and FA analysis therefore has taxonomic potential (Ahlgren et al., 1992; Hallegraeff et al., 1991). Highly unsaturated FAs can also be haemolytic, causing damage to the epithelial cells of fish gills (Suzuki and Matsuyama 1995), and might be a cause of the ichthyotoxicity associated with *Fibrocapsa* blooms in Japanese waters. In this study, biochemical characteristics of *F. japonica* from New Zealand waters were compared with those of a Japanese isolate, using molecular probes, including FITC-conjugated lectins. Species-specific DNA probe assays, targeted at rRNA and developed for discriminating between ichthyotoxic raphidophyte species in New Zealand (Tyrrell et al., 1997; John Tyrrell, Auckland University, personal communication), were also tested against the *Fibrocapsa* isolates.

Materials and Methods

Microalgal culture

Fibrocapsa japonica (CAWR02), isolated from the Hauraki Gulf, New Zealand, and *F. japonica* (CAWR03), isolated in Japan, but obtained from the CSIRO Culture Collection of Microalgae, Hobart, Tasmania, were used in this study. Both isolates were grown and maintained in GP medium (Loeblich and Smith 1968), under standard conditions (Rhodes et al., 1993), in the Cawthron Culture Collection of Micro-algae.

Lectins

Fresh solutions of FITC-conjugated lectins (100 $\mu g \cdot m\ell^{-1}$; Sigma Chemical Co.) were made with filtered synthetic sea water (0.4 μm Nuclepore; Sigma Chemical Co.), salinity 28 ppt, and pH 8.0. Exponentially growing microalgae cells were collected by centrifugation (1828g; 15 min, 16℃) and ten different fluorescein isothiocyanate FITC-labeled lectins (Table 1) were added to aliquots of $\cong 10^5$ cells for 15 min at 16℃. Unbound lectin was removed by washing in filtered seawater (Nucleopore 0.4 μm). The treated cells were mounted on siliconised glass slides and binding/agglutination observed under epifluorescence microscopy. *Chattonella antiqua, C. marina* and *C. subsalsa* used in the lectin study were obtained from Tokyo University of Fisheries, Japan. In addition, this study used *Heterosigma akashiwo* isolated from New Zealand coastal waters and cultured in the Cawthron Culture Collection, New Zealand. Microalgae were examined following

Table 1. FITC-conjugated lectins used as probes

Lectin	Source	Specificity
ConA	*Canavalia ensiformis*	Methyl α-D-mannopyranoside; D-mannose; D-glucose
ECA	*Erythrina cristagalli* (coral tree)	α-lactose; N-acetyl-D-galactosamine; D-galactose
HPA	*Helix pomatia* (snail)	N-acetyl-D-glucosamine; N-acetyl-D-galactosamine; D-galactose
PEA	*Pisum sativum* (pea)	Methyl α-D-mannopyranoside; D-mannose; D-glucose
PHA	*Phaseolus limensis* (lima bean)	N-acetyl-D-galactosamine
PNA	*Arachis hypogaea* (peanut)	α-lactose; D-galactose
PWM	*Phytolacca americana* (pokeweed)	N-acetyl-D-glucosamine
SBA	*Glycine maxima* (soy bean)	N-acetyl-D-galactosamine; D-galactose; methyl α-D-galactopyranoside
UEA	*Ulex europaeus* (gorse)	L-fucose
WGA	*Triticum vulgaris* (wheat germ)	N-triacetylchitotriose; N-diacetylchitobiose; sialic acid

treatment with FITC-conjugated lectins with Vanox Olympus photomicrographic epifluoresence microscope using UV (excitation, 375 nm; emission, > 420 nm) and blue light (excitation 490 nm, emission 520 nm). Binding activity was determined by Rhodes et al. (1995).

DNA probes

The whole cell format assays were carried out by John Tyrrell as previously described (Tyrrell et al., 1997; Scholin et al., 1996; Rhodes et al., 1998a). Raphidophyte cells were relatively delicate so they had to be treated carefully so that their cell integrity was maintained. This study combined saline/EtOH fixative into a 50 mℓ plastic tube and then added 5~10 mℓ of culture. It leave to stand- do not vortex or mix for at least 15 minutes otherwise mucocysts will discharge causing cells to clump, making quantifiable results impossible. Therefore, we are responsible for leaving it in order to fix for 1~2 hours. Considering on the number of cells fixed, try to capture at least 2,000 cells on the 13 mm, 1.2 μm pore size isopore polycarbonate membranes (Millipore) which have been placed in the custom filter tubes. Once the cells have been captured on filter, rinse briefly with 1 mℓ of hybridisation buffer. Filter and then add 500 μℓ of hybridisation buffer to each tube to resuspend cells. Add 12 μℓ of the appropriate probe, cap tubes and then place manifold into a waterbath or dry incubator at 50℃ for 15 to 30 minutes to hybridise. After hybridisation, filter and then rinse briefly with 1 mℓ of hybridisation buffer to remove excess unbound probe. After filtration, place membranes onto glass slides, add 20 μℓ SlowFade Light (Molecular probes) to each membrane, mount the cover slip, then view under Olympus Venox microscope with a FITC optical filter set. This study used 5 kinds of probes; Fib 1.25F (specific probe for *F. japonica*), Het 1.25$_a$F (specific probe for *H. akashiwo*), UniC (positive control for all eukaryotes), UniR (negative control to test for non-specific retention of probe) and No probe (negative control to compare against UniR for autofluorescence versus non-specific retention of probe), and provided by John Tyrrell, Auckland University.

Fatty acid analysis

Bulk cultures of the two strains of *Fibrocapsa* were grown under standard conditions for one month in 10 L plastic culture tanks. Cells were harvested by filtration (Whatman GF/C, 47 mm) and frozen (−20℃) until analysed. The cold solvent extraction method (Bligh and Dyer 1959; Hanson and Olley 1963) was modified for this study. Filters were suspended in 32 mℓ deionised water (10 min.), ground, and suspension ultrasonicated (400 mℓ beaker; 5 min.; cell disrupter, Watson Victor Ltd.). Chloroform (80 mℓ) and methanol (40 mℓ) were added prior to further ultrasonication in a bath sonicator (10 min.). Ultrasonication (5 min.; 40 mℓ added chloroform) was repeated three times, with water replacing chloroform the third time. The resultant slurry was centrifuged (10 min., 2000 rpm.) and the chloroform fraction transferred to the filter paper containing anhydrous sodium sulphate, and rotary evaporated to dryness. For the derivatisation, methanolic 0.5 M sodium hydroxide (4 mℓ) was added to the extracts and refluxed (5 min.), boron trichloride in methanol added (5 mℓ) and boiled (10 min.), then pentane (5 mℓ) added and boiled (1 min.). After cooling saturated sodium chloride (3 mℓ) was added, mixed by flask rotation, and further sodium chloride added. The pentane layer was removed, dried with anhydrous sodium sulphate, and resuspended in pentane and volume reduced under nitrogen before injection into a Hewlett-Packard 5890A gas chromatograph with flame

ionisation detection (column: SGE BPX 70, 12 m× 0.53 mm; 130℃ initial temperature, 2℃ $min.^{-1}$ gradient to 210℃ (5 min.); column flow: 3 mℓ nitrogen $min.^{-1}$; injector 220℃, detector 230℃).

Toxin bioassays

Fibrocapsa isolates were grown under standard conditions in 10 litre plastic vessels (Nalgene). Cells were harvested by filtration (Whatman GF/C, 0.47 μm; total cell number for each isolate ≈4.0×10^7) and frozen until required. Samples were extracted by an acetone/dichloromethane method (Hannah et al., 1995), resuspended in Tween/saline (3.5 mℓ), and injected intraperitoneally (1 mℓ) into each of 3 mice (body weight: 19~20 g; bioassay carried out at the Institute of Environmental Science and Research (ESR), Wellington, NZ). A neuroblastoma assay was also carried out at Communicable Diseases Centre ESR, Kenepuru Drive, Porirua, New Zealand by Dr Penny Truman (Truman and Lake 1996).

Results

The saturated and monounsaturated FA content for the 2 strains grown under the same conditions were similar. However, the total polyunsaturated fatty acid content of FjJ was 29.8% of the total FA content, whereas that of FjNZ was 41.4% (1.4 times greater). Both strains produced major fatty acids (palmitic acid, oleic acid, eicosapentanoic acid), but neither produced long chain carbon FAs (above C_{22}). Essential linolenic acid, 18:3 (n-3), a precursor of eicosapentaenoic acid, 20:5 (n-3) and docosapentaenoic acid, 22:6 (n-3), and essential linoleic acid, 18:2 (n-6), a precursor of arachidonic acid, 22:4 (n-6), were present (1.5%) in both strains. A series of n-6 polyunsaturated fatty acids were present in similar amounts (1.9%), but n-3 polyunsaturated fatty acids differed (FjNZ, 39.5%; FjJ, 27.9%; Table 2).

Table 2. Fatty acid composition of New Zealand (NZFj) and Japanese (JFj) isolates of *Fibrocapsa japonica*. Fatty acid composition (% of total fatty acids) calculated from area of peak divide (total area-solvent peak area). The fatty acids are described by x:y (n-z), where x is the number of carbon atoms, y is the number of double bonds, and z is the position of the first double bond counting from the methyl end of the molecule; nd=not detected

Fatty acid	NZFj (%)	JFj (%)
Saturates		
16:0	30.4	30.7
17:0	nd	nd
18:0	4.3	6.1
20:0	nd	nd
Monounsaturates		
16:1	4.7	5.8
18:1	16.3	24.0
20:1	nd	nd
22:1	nd	nd
Polyunsaturates		
16:4 (n-3)	0.4	0.1
18:2 (n-6)	1.3	1.7
18:3 (n-3)	1.6	1.5
18:4 (n-3)	7.7	6.5
18:5 (n-3)	0.5	0.2
20:2 (n-6)	0.2	0.2
20:4 (n-3)	7.4	5.4
20:5 (n-3)	20.9	12.3
22:2 (n-6)	nd	nd
22.5 (n-3)	nd	nd
22:6 (n-3)	1.4	1.9
Others	2.9	3.6
Total	100.0	100.0

Table 3 showed the binding response of ten kinds of lectins added to microalgae pellets for this study under fluorescence microscope. *Chattonella* spp., *Fibrocapsa japonica* and *Heterosigma akashiwo* showed no binding with lectins and also two strains of *Fibrocapsa japonica* (New Zealand and Australia strains) did not bind them. But the dinoflagellate *Coolia monotis* (CAWD39) was responsible for binding activity of ConA and HPA that appeared as weak fluorescence. ConA bound to *Coolia monotis* (CAWD39) and *Prorocentrum lima* (CAWD32), whereas PNA and WGA presented strong fluorescence with *C. monotis*. Additionally, PNA also had a strong binding acitivity to *Alexandrium ostenfeldii* (CAWD16). On the other hand, *Gymnodinium mikimotoi* were very difficult to differentiate between New Zealand and Japan strains because the two strains seemed to have the same binding and fluorescing intensity with ConA, PHA, PEA (Table 3). Therefore, it is very difficult to identify between the two strains of *Gymnodinium mikmotoi* using lectins, and also between the two strains of *Fibrocapsa japonica*.

The Fib 1.25F and UniC DNA probes bound (observed as fluorescence) to both *Fibrocapsa* strains tested, but not to a New Zealand isolate of the related raphidophyte, *Heterosigma akashiwo* (CAWR07). A *Heterosigma* probe (Het 1.25$_a$F) was negative for *F. japonica*, although *H. akashiwo* (CAWR07) bound specifically (personal

Table 3. Binding response at different lectins used as fluorescent probes. + (bright fluorescence), +*p* (pale but clear fluorescent outline of the cell), − (no fluorescence)

	ConA	PHA	PEA	ECA	SBA	PWM	PNA	HPA	WGA	UEA
Rapidophyceae										
Chattonela antiqua	−	−	−	−	−	−	−	−	−	−
C. marina	−	−	−	−	−	−	−	−	−	−
C. subsalsa	−	−	−	−	−	−	−	−	−	−
Fibrocapsa japonica (CAWR02)	−	−	−	−	−	−	−	−	−	−
Fibrocapsa japonica (CAWR03)	−	−	−	−	−	−	−	−	−	−
Heterosigma akashiwo (CAWR07)	−	−	−	−	−	−	−	−	−	−
*Coolia monotis** (CAWD39)	+*p*	−	−	−	−	−	+	+*p*	+	−
*Prorocentrum lima** (CAWD32)	+*p*	−	−	−	−	−	−	−	−	−
*Alexandrium ostenfeldii** (CAWD16)	−	−	−	−	−	−	+	+	−	−
*Gymnodinium mikimotoi** (Japan)	+	+*p*	+	−	−	−	−	−	−	−
*G. cf mikimotoi** (CAWD37)	+	+*p*	+	−	−	−	−	−	−	−

Note: * Species was reported in Rhodes et al. 1995 as FITC-conjugated lectins.

communication, John Tyrrell, University of Auckland, Table 4).

Toxicity bioassays

The mouse bioassays for NSP and DSP, and the neuroblastoma assay for NSP were negative for both the New Zealand and Japanese *Fibrocapsa* isolate extracts.

Discussion

It was difficult to differentiate between FjNZ and FjJ using standard brightfield microscopy, and FITC-conjugated lectin binding profiles were obtained to determine differences in surface sugars of the two isolates. However, the lack of lectin binding in any of the raphidophyte tested in this study excluded this assay as a useful tool for discrimination of isolates, or even of raphidophyte genera. It was possible that sugars were present on the cell surface, but were not accessible to the lectins tested. ConA, PEA and UEA binding has recently been observed in a Korean strain of *Heterosigma akashiwo* (Cho et al., 1998), suggesting the presence of D-mannose, D-glucose and/ or L-fucose related moieties at the cell surface of this non-toxic isolate, but not in the ichthyotoxic New Zealand isolate of this species. DNA sequence data has previously been obtained from members of the raphidophyceae, and the data analysis suggested that *F. japonica* was the most basal taxon of the group (John Tyrrell, Auckland University, personal communication). Fluorescent-tagged species-specific oligonucleotide-derived probes, recently developed on the basis of this sequence data, bound to the targeted rRNA of both *Fibrocapsa* strains. Much effort has gone into the development of these probes, particularly as the undesirable disruption of mucocysts by *F. japonica* can confound quantification (Tyrrell et al., 1997). Collapse of raphidophyte cells on addition of fixatives is a problem in microscope based phytoplankton monitoring, and DNA probes have great potential for refining monitoring programmes in New Zealand. The probes are already being used in shellfish biotoxin management programmes for the rapid identification and quantification of some toxic and noxious microalgae genera (Rhodes et al., 1998 b, c). The raphiophyte probes, including probes for *Chattonella*, should prove extremely useful to fish farmers to improve early detection of cells and prodiction of bloom development, and thus enhance farm management.

Table 4. Response of different microalgae to DNA probes

Species	Fib 1.25F[1]	Het 1.25$_a$F[2]	UniC[3]	UniR[4]	No probe[5]
Fibrocapsa japonica (CAWR02)	+	−	++	−	−
F. japonica (CAWR03)	+	−	++	−	−
Heterosigma akashiwo (CAWR07)	−	+	++	−	−

++: strong binding, +: weak binding, −: no binding

[1] specific probe for *F. japonica*

[2] specific probe for *H. akashiwo*

[3] positive control for all eukaryotes

[4] negative control to test for non-specific retention of probe

[5] negative control to compare against UniR for autofluorescence versus non-specific retention of probe

Fatty acid (FA) and sterol data can provide useful chemotaxonomic information on microalgal relationships (Hallegraeff et al., 1991; Suzuki et al., 1997), and as the abundance of eicosapentaenoic acid differed between the two strains of *F. japonica*, FAs might be useful as comparative chemotaxonomic tools for the raphidophyte group. Recently, Suzuki and Matsuyama (1995) determined the content of free FAs in the raphidophytes *Chattonella antiqua* and *Heterosigma akashiwo*, and found a high abundance (≈60%) of polyunsaturated FAs, with $C_{20:5}$ (n-3) being 31.0% of FAs in *C. antiqua* and 59.6% in *H. akashiwo*. It was suggested that highly unsaturated FAs could act as toxic substances, damaging the epithelial tissues of fish gills. The FA composition of the two *Fibrocapsa* strains was tested in this study, and particularly the lack of unsaturated FAs, seemed to be decrease the likelihood of FAs from *Fibrocapsa* being responsible for haemolytic damage to fish gills.

The raphidophytes include known fish killers, and *Heterosigma akashiwo* was responsible for a massive farmed salmon kill in New Zealand in 1989 (Mackenzie 1991). *Chattonella marina*, also a known ichthyotoxin producer in Japan (Onoue et al., 1990; Khan et al., 1995), was implicated in farmed bluefin tuna (*Thunnus maccoyi*) kills in Australia in 1996 (Hallegraeff et al., 1998), and was identified at that same time in Northland waters in New Zealand (Rhodes and Thomas 1997). Red tides of *F. japonica* have caused massive mortalities of caged young fish in Japan (Khan et al. 1996b), although no toxic component was detected. Conversely, five neurotoxic components have been identified in a Dutch strain of *F. japonica*, which were similar to brevetoxin components (Khan et al., 1996a), although no damage to cultured fish has been recorded (Vrieling et al., 1995). In this study, both FjNZ and FjJ were non-toxic to mice, and no NSP/DSP fraction was found. According to Rhodes et al., (1993), FjJ killed *Artemia salina*, but FjNZ did not. However, the deaths could have been due to entanglement in discharged mucocysts. Some researchers have suggested that ciguatoxin and brevetoxins cause neurotoxic shellfish poisoning by activating sodium channels, and this sodium channel activity has been adapted for use in a neuroblastoma cell culture assay for NSP toxin (Truman and Lake 1996). Neuroblastoma assays for the two strains of *F. japonica* were negative. *Fibrocapsa* continued to be a major bloom former in New Zealand waters, but has not been shown to be ichthyotoxic to date. However, its regular appearance in recent years in early summer prior to *Gymnodinium* blooms, suggests that it could prove to be a useful indicator species. The related genera, *Chattonella* and *Heterosigma*, continue to be of concern to fish farmers world wide.

Acknowledgments

The assistance of David White (fatty acid analyses), Annabel Thomas, Lincoln MacKenzie, Kirsten Todd and the phytoplankton monitoring team, Cawthron Institute, Nelson, is appreciated. Also Penny Truman, ESR, Porirua (toxin bioassays), John Tyrrell, Auckland University (DNA probes and sequencing data) and Hoe Chang, NIWA, Wellington. Access to data from the Ministry of Health and Coromandel Aquaculture Association phytoplankton monitoring programmes has been appreciated. The work was funded by the Foundation for Research, Science and Technology (contract CAW601), and the Korean Government.

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