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Haemolytic activity within the species *Fibrocapsa japonica* (Raphidophyceae)

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ABSTRACT

Strong haemolytic activity was observed for extracts of 15 *Fibrocapsa japonica* strains collected from different global regions. The EC_{50} values ranged between 0.4×10^4 and 1.9×10^4 *F. japonica* cells ml^{-1}. The relationship between the haemolytic activity observed in the cell extracts and the amount and nature of polyunsaturated fatty acids (PUFAs) was investigated. Between 7% and 89% of the haemolytic activity could be attributed to the amount and toxicity of the PUFAs C18:4n3 (OTA), C20:5n3 (EPA), and C20:4n6 (AA). However, the higher the haemolytic activity of a strain the more it appeared to be caused by haemolytic compounds other than the PUFA's. The EC_{50} of the extracts with the highest haemolytic activity strongly depended on light intensity and was in line with observations made on *Chattonella marina*.

In the cell extracts of the strains studied brevetoxins were always below detection limits. For strains where 20–35% of the haemolytic activity was not caused by PUFAs, the concentration of these compounds was close to LC_{50} values reported for fish. Overall the haemolytic activity of the *F. japonica* strains was in the range of known harmful microalgae. Therefore, PUFAs in combination with potent light dependent haemolytic compounds could be a major cause of ichthyotoxicity observed during *F. japonica* blooms.

1. Introduction

The unicellular golden brown flagellate *Fibrocapsa japonica* Toriumi and Takano is one of the marine harmful algal bloom (HAB) species of the class Raphidophyceae (Hara and Chihara, 1985). *F. japonica* has a world wide distribution in temperate regions (de Boer et al., 2005), but in recent years this species was observed in tropical regions as well (Del Carmen Cortès Lara et al., 2003; Band-Schmidt et al., 2004; Ø. Moestrup personal communication). A phylogenetic study of *F. japonica* strains from different global regions indicated that its habitat expanded, possibly due to anthropogenic influences (Kooistra et al., 2001). There is no historical record of *F. japonica* observations because cells are hard to identify in seawater samples. Similar to other raphidophytes, cells of *F. japonica* lack a rigid cell wall and are enveloped by the plasma membrane. Cells are very sensitive to environmental stress triggering the discharge of mucocysts and eventually lysis of the cells (Tillmann and Reckermann, 2002; de Boer et al., 2005).

F. japonica and other marine raphidophyte species such as *Chattonella* spp. and *Heterosigma akashiwo* were documented as ichthyotoxic (Landsberg, 2002 and references therein). *F. japonica* caused massive mortality events of economically important fish stocks in the Seto Inland Sea of Japan (Iwasaki, 1971; Okaichi, 1972; Toriumi and Takano, 1973; Okaichi, 1989). Since 1990 toxic events related to blooms of *F. japonica* were reported off the German coast and the Mediterranean Sea (Rademaker et al., 1998; European Commission, 2003). Fish kills caused by *F. japonica* were ascribed to mechanical and/or toxic damage due to the production of brevetoxins (Khan et al., 1996; Bridgers et al., 2004), reactive oxygen species (ROS; Oda et al., 1997; Marshall et al., 2005a), haemolysins (Fu et al., 2004a,b), and even mechanical clogging of fish gills due to mucocyst discharge (de Boer et al., 2005). It is possible that the overall toxicity of *F. japonica* results from the combination of several (if not all) of these different factors (Marshall et al., 2003, 2005b; de Boer et al., 2004).

Kooistra et al. (2001) analysed the genetic variation among nuclear ribosomal DNA internal transcribed spacer sequences (ITS) of 16 *F. japonica* strains. There was not much genotypic differentiation among the strains and the ambiguities observed were similar across the global collection. Such a pattern could be explained by recent range expansion and hybridisation events, resulting in a cosmopolitan population. Highest ITS polymorphism was observed in the Wadden Sea area where hybridisation may have resulted in an increased diversity in toxicity in that region.

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The EC_{50} for haemolytic activity of a *F. japonica* strain from the Wadden Sea area (strain Cl 3) was high compared to other harmful algal bloom species. Toxicity was even higher at low salinities but toxicity was not affected by nutrient limitation because values were the same for exponentially growing cells compared to cells that were P-limited in the stationary phase of growth in batch cultures (de Boer et al., 2004).

Bioassay guided purification of toxic fractions from *F. japonica* strain Cl 3 yielded haemolytic components identified by Fu et al. (2004b) as 6,9,12,15-octadecatetraenoic acid (C18:4n3; OTA or stearidonic acid), 5,8,11,14,17-eicosapentaenoic acid (20:5n3; EPA), and 5,8,11,14-eicosatetraenoic acid (C20:4n6; arachidonic acid (AA)). These polyunsaturated fatty acids (PUFAs) were identified earlier in fatty acid composition studies on raphidophyte species (Mostaert et al., 1998; Marshall et al., 2002). For the two *F. japonica* strains (NIES136 and 560) analysed in both studies, about 80% of the total PUFAs was present as the three PUFAs, with AA the least dominant.

To evaluate if haemolytic activity of this cosmopolitan species is especially high in the Wadden Sea area extracts of 15 *F. japonica* strains were compared for their haemolytic activity. Half of the *F. japonica* strains originated from the Wadden Sea area, the others from various coastal regions around the world. To identify the role of PUFAs in haemolysis by *F. japonica*, the concentration of the three PUFAs (AA, EPA, OTA) in the extracts were measured. In addition to this extracts were assayed for brevetoxins as well.

2. Material and methods

2.1. Culture conditions and experimental set-up

For our study we used 15 strains of *Fibrocapsa japonica* isolated from various locations around the world (Table 1). At the University of Groningen the strains were grown under environmental conditions typical for the Wadden Sea area. All strains (Table 1) were genetically and/or morphologically identified as *F. japonica* (Kooistra et al., 2001). Each non-axenic strain was grown in batch cultures using autoclaved natural seawater (Doggersbank, North Sea), adjusted to a salinity of 25 with Milli-Q water and enriched with f/2 nutrients minus Si (Guillard, 1975). The cultures were maintained in a climate controlled chamber at 17 ± 1 °C under long-day conditions (16:8 h light:dark) and with a photon flux density of 35 ± 5 μmol photons m^{-2} s^{-1} (provided by fluorescent L36W/19 tubes, Osram). The irradiance was measured with a Li-Cor quantum meter equipped with a cosine collector. Cultures were inoculated from growing cultures at a cell density of about 5000 cells ml^{-1} for each strain tested. Throughout the experiments cell number and biovolume per cell were determined, using a Coulter model ZM particle counter equipped with a channelyzer 256 and a 100 μm aperture tube (de Boer et al., 2005). Maximum growth rates (μ_{max}) were calculated from a curve fit on at least 4 data points in the exponential growth phase, using the following equation:

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

where N_t and N_0 are cell concentrations at day t and 0 (Guillard, 1973). The growth rate at harvesting was based on counts of the last two samples only.

At the onset of the stationary phase (where $\mu < \mu_{max}$), *F. japonica* cells of each strain were harvested on two GF/F filters, 47 mm Ø (Whatman), to an amount of 2×10^6 cells on each filter. Both filters were immediately stored at −80 °C. One filter was used for PUFA and haemolysis analysis, the other for brevetoxin analysis. In a separate experiment two cultures of strain Md 0913 I were harvested filtering an amount of 1×10^6 cells on filters which were stored at −80 °C. Filtrate was assayed to evaluate losses of haemolytic components during the filtration procedure.

2.2. PUFA, brevetoxin and haemolytic activity analysis

To prepare a cell extract a frozen filter was submerged in 4 ml MeOH (HPLC grade) in a glass vial. Vials were sonified for 2 h on ice in an ultrasonic bath and the supernatants (further denoted as extracts) were stored −20 °C until HPLC analysis at Terramare Research Centre (Fu, 2003; Fu et al., 2004b). The characterized PUFAs; OTA, AA and EPA isolated from *F. japonica* strain Cl 3 by Fu (2003) and AA and EPA from Sigma–Aldrich were used as standards (Fu, 2003; Fu et al., 2004b).

For the analysis of haemolytic activity MeOH extracts were dried at room temperature in a Savant Speed Vac® DNA 110 vacuum centrifuge and dissolved in 1 ml erythrocyte lysis assay (ELA) buffer. (Eschbach et al., 2001). The ELA was performed as described previously (de Boer et al., 2004). The 24 h incubation of blood cells with samples was at the same environmental

Table 1
Origin and isolation dates of *F. japonica* strains.

Strain	Locality or mother strain	Date of isolation	Culture collection	Used name in this study next to strain number
LB 2162[a]	Point Loma, California	1970	UTEX	American strain
CS 332	NIES-136	1993	CSIRO	Japanese strain
	Tsuda Bay, Seto Inland Sea, Japan	1978	NIES	
CS 220[b]	Hobsons Bay, Victoria, Australia	1988	CSIRO	Australian strain
CCMP 1661	Hobsons Bay, Victoria, Australia	1988	CCMP	Australian strain
CAWR 02	Leigh, New Zealand	1992	Cawthron	New Zealand strain
W 420	Wadden Sea, the Netherlands	1992	CCRuG[c]	Dutch strain
K 0542	Sea of Büsum, Germany	1995	SCCAP	German strain
Büsum Or[d]	Büsum Harbour, Germany	1995	Büsum	German strain
Cl 3[e]	Büsum OR	1997	CCRUG[c]	German strain
Cl 4[e]	Büsum OR	1997	CCRUG[c]	German strain
Cl 5[e]	Büsum OR	1997	CCRUG[c]	German strain
Cl 6[e]	Büsum OR	1997	CCRUG[c]	German strain
CAWR 19	Wellington Harbour, New Zealand	1999	Cawthron	New Zealand strain
Md 09 13 I	Marsdiep, the Netherlands	2000	CCRuG[c]	Dutch strain
Md 09 13 III	Marsdiep, the Netherlands	2000	CCRuG[c]	Dutch strain

[a] In UTEX referred to as *Chattonella japonica*.
[b] In CSIRO referred to as *Fibrocapsa* spp. In this study the strain was called *F. japonica*, because Kooistra et al., 2001 did not find any genetic difference between this strain and other *F. japonica* strains.
[c] Culture Collection of the University of Groningen.
[d] Original Büsum culture; this culture was established from a monospecific bloom (U. Tillmann, personal communication).
[e] The clonal strains were established in 1997 by taking a single cell from the original Büsum culture (Vrieling and Tjallingii, unpublished data).

conditions as applied for the batch cultures, unless stated otherwise. Dilution series of the cell extracts as well as the standards OTA, AA and EPA (Sigma–Aldrich) were tested for haemolytic activity. For each measurement seawater with nutrients was used as blank.

Two additional extracts of different cultures of the *F. japonica* strain Md 0913 I were incubated in duplicate dilution series at 0, 5, and 30 μmol m^{-2} s^{-1} to investigate the effect of light intensity on the haemolysins in this strain. The four *F. japonica* cultures were harvested at a cell concentration of about 3.1×10^4 cells ml^{-1}. Dilution series of saponin were used as a control at all different light intensities.

Brevetoxins were measured at the Center for Marine Science (USA), using a competitive ELISA (Naar et al., 2002; de Boer et al., 2004). Brevetoxin standards PbTx-2 and PbTx-3 (both Calbiochem) were used as standards. The assay was tested for false positives using the PUFA standards: OTA, EPA and AA (Sigma–Aldrich).

2.3. Data analysis

To calculate the haemolytic EC_{50} value for *F. japonica* cell extracts or PUFAs, the % erythrocyte lysis was plotted against the cell density corresponding with the dilution of the sample (4 densities in triplicate). From the equation of the sigmoidal curve fit (Sigmaplot) the EC_{50} value for each strain was determined as the amount of *F. japonica* cells ml^{-1} necessary for 50% lysis of the erythrocytes. To compare our data with data from other haemolytic assays, saponin (Sigma–Aldrich) was used as a reference; the EC_{50} for saponin in our assay was 4.5 μg ml^{-1} (de Boer et al., 2004).

As a measure of haemolytic activity per cell, the corresponding cell density of the extracts used for HPLC (5×10^5 cells ml^{-1}) was divided by the amount of cells per ml at EC_{50}, for each strain involved. Assuming no synergy or antagonism between the PUFAs the following equation was applied to predict haemolytic activity based on the amounts of PUFAs present in a sample: Haemolytic activity (arbitrary units $cell^{-1}$) = ([AA]/EC_{50} of AA) + ([EPA]/EC_{50} of EPA) + ([OTA]/EC_{50} of OTA).

Maximal growth rates during exponential phase and growth rates just before harvesting were correlated with the haemolytic activity of the cell extract using linear regression analysis. Also the predicted haemolytic activity versus measured activity was tested using regression analysis. Comparison between groups of data was done by ANOVA using the data analysis tool of Excel at significance level 0.05.

3. Results

Batch cultures of 15 *F. japonica* strains with maximum growth rates (μ_{max}) ranging from 0.13 to 0.32 day^{-1} in the exponential growth phase, were harvested at the onset of the stationary growth phase; the cell densities ranged between 1.2 and 3.5×10^4 cells ml^{-1} (data not shown). The polyunsaturated fatty acids OTA, EPA and AA were found in all cell extracts of the *F. japonica* strains (Fig. 1). Clear differences between strains were observed in the amounts of these PUFAs per cell. Cells of strain Cl 4 contained most PUFAs (450 pg $cell^{-1}$). For the other strains the total amount of PUFAs varied between 150 and 350 pg $cell^{-1}$, with the exception of the Wadden Sea strain Md 0913 I that contained only 40 pg $cell^{-1}$. The OTA fraction was the most abundant PUFA for all strains except Md 0913 I, the latter had AA as the dominant PUFA. For strain Md 0913 I and the Japanese strain (CS 332) EPA was the least dominant PUFA while AA was the minor PUFA in the other strains.

From their dose–response curves for haemolytic activity the EC_{50} values for OTA, EPA and AA standards were calculated (Fig. 2). AA was the most haemolytic compound with an EC_{50} value of 1.8 μg ml^{-1}, which was half the value of EPA (EC_{50} = 4.0 μg ml^{-1}) and more than ten times less compared to the EC_{50} for OTA (25.0 μg ml^{-1}).

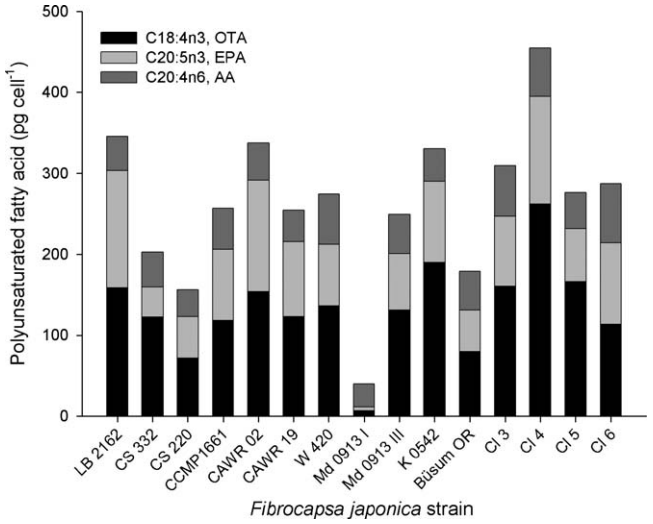

Fig. 1. The amount of the polyunsaturated fatty acids (PUFAs): 6,9,12,15-octadecatetraenoic acid (C18:4n3; OTA), 5,8,11,14,17-eicosapentaenoic acid (C20:5n3; EPA) and 5,8,11,14 eicosatetraenoic acid (C20:4n6; AA) in cell extracts of the different *F. japonica* strains.

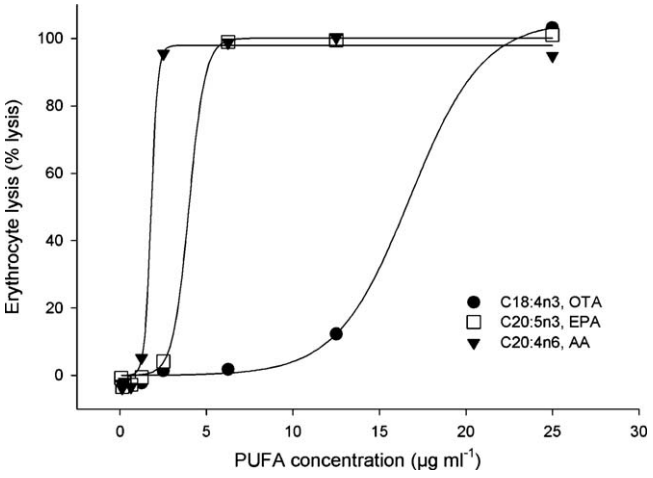

Fig. 2. Dose–response curves for three polyunsaturated fatty acids (OTA, EPA, AA), and erythrocyte lysis. The equation of the sigmoidal curves was used to calculate the compound specific EC_{50}; the amount of PUFA necessary for 50% lysis of erythrocytes, in all cases standard deviation <1%.

EC_{50} values expressed as the corresponding cell density of the cell extracts (Table 2), varied among strains and was neither related to the maximum growth rate of each strain (n = 15, r^2 = 0.09, p = 0.28) nor to the growth rate over the last two sampling points prior to harvesting of each strain (n = 15, r^2 = 0.04, p = 0.46). The Wadden Sea strain Md 0913 I was the most haemolytic strain having an EC_{50} corresponding to 0.4×10^4 *F. japonica* cells ml^{-1}. This EC_{50} value was five times lower than that of the least haemolytic strain Cl 5 ($EC_{50} = 1.9 \times 10^4$ *F. japonica* cells ml^{-1}). Cell sizes differed between the examined strains, with cell volumes varying between 4800 and 8000 μm^3 (Table 2). The haemolytic activity of strains isolated from originally the same bloom displayed a twofold difference in EC_{50} values. The clonal strains isolated from Büsum OR; Cl 3, Cl 4, Cl 5, Cl 6 had EC_{50} values of 1.1, 0.9, 1.9, and 0.9×10^4 cells ml^{-1}. Similarly, the EC_{50} of two

Table 2
Haemolytic activity found for each *F. japonica* strain and ranked by EC_{50} values on biovolume. The EC_{50} for saponin was 4.5 μg ml^{-1} (de Boer et al., 2004). The EC_{50} value expressed as equivalent biovolume per ml was calculated using the cell volume determined for each strain.

Strain	EC_{50} ± standard error (10^4 cells ml^{-1})	Cell volume (μm^3)	EC_{50} ± standard error (10^6 μm^3 ml^{-1})
Fibrocapsa japonica Md 0913 I	0.4 ± 0.02	7300	30 ± 1.3
Fibrocapsa japonica CS 332	0.7 ± 0.11	6100	41 ± 6.7
Fibrocapsa japonica CCMP 1661	1.0 ± 0.08	4800	49 ± 3.7
Fibrocapsa japonica Md 0913 III	0.9 ± 0.05	6600	62 ± 3.5
Fibrocapsa japonica W420	0.9 ± 0.05	6800	63 ± 3.2
Fibrocapsa japonica Cl 6	0.9 ± 0.06	7100	66 ± 4.5
Fibrocapsa japonica Cl 4	0.9 ± 0.05	7500	68 ± 3.8
Fibrocapsa japonica CS 220	1.2 ± 0.04	6000	74 ± 2.7
Fibrocapsa japonica Cl 3	1.1 ± 0.06	6600	75 ± 4.2
Fibrocapsa japonica CAWR 02	1.0 ± 0.16	7300	76 ± 11.6
Fibrocapsa japonica CAWR 19	1.5 ± 0.05	5500	80 ± 3.0
Fibrocapsa japonica Büsum OR	1.3 ± 0.08	6200	83 ± 4.7
Fibrocapsa japonica K 0542	1.5 ± 0.07	6000	89 ± 4.0
Fibrocapsa japonica LB 2162	1.2 ± 0.05	8000	93 ± 4.4
Fibrocapsa japonica Cl 5	1.9 ± 0.06	6400	120 ± 3.7

strains from a Dutch coastal bloom (the Md strains) had EC_{50} values of 0.4 and 0.9 × 10^4 cells ml^{-1} (Table 2). There was no significant difference in EC_{50}-values of clonal strains originating from the Wadden Sea area (Md 0913 I, Md 0913 III, K 0524, Cl 3, Cl 4, Cl 5, Cl 6) compared to those from strains from different global regions (LB 2162, CS 332, CS 220, CCMP 1661, CAWR 02, CAWR 19). For the strains used in this comparison, however, there was a difference between the average amounts of ITS ambiguities reported (data from Kooistra et al., 2001), being 7.8 for the Wadden Sea strains compared to 4.8 for the other strains.

The haemolytic activity measured for each strain (Fig. 3) was compared with the predicted haemolytic activity that was calculated from the amount and activity of the PUFAs measured (Figs. 1 and 2). For 11 of the 15 strains 50–89% of the haemolytic activity could be explained by the presence of the three PUFAs. For the four strains Md 0913 I, CS 332, CS 220, and Md 0913 III, respectively 7, 26, 42, and 47% of the measured activity was expected to be caused by the presence of the PUFAs. It seemed that the higher the haemolytic activity, the higher is the contribution of haemolysins other than the three PUFAs. To further investigate this observation the percentage unexplained haemolytic activity (HU) was plotted against the total haemolytic activity measured (HT; Fig. 4); this exercise revealed a linear correlation with HU = 0.803 (HT) × −0.117 (n = 15, r^2 = 0.713, $p < 0.0001$). Apparently, the increase in total activity was mainly due to an increase in the unexplained haemolytic activity, because there was no (negative) trend in the total haemolytic activity measured versus predicted activity on basis of PUFAs present (n = 15, r^2 = 0.253, p = 0.06, data not shown). The average haemolytic activity caused by the three PUFAs was 26 units per cell, equivalent to an EC_{50} of 1.9 × 10^4 cells ml^{-1}.

The *F. japonica* strain with the highest haemolytic activity and the lowest amount of PUFAs (Md 0913 I), was cultured again and the extracts of two cultures were tested for the effect of light on the haemolytic activity. EC_{50} values at 30 μmol photons m^{-2} s^{-1} were slightly higher compared to the one at about 35 μmol photons m^{-2} s^{-1}; i.e. the applied growth and assay conditions in the comparison of all strains (Fig. 5, Table 2). At zero light only 7% of the haemolytic activity observed at high light was measured. At 5 μmol photons m^{-2} s^{-1} the activity was 21% of the high light value. In contrast to *F. japonica* extracts, light did not affect the EC_{50} of saponine (data not shown).

Despite extensive efforts none of the cell extracts obtained in this study were found to contain brevetoxins when the sensitive

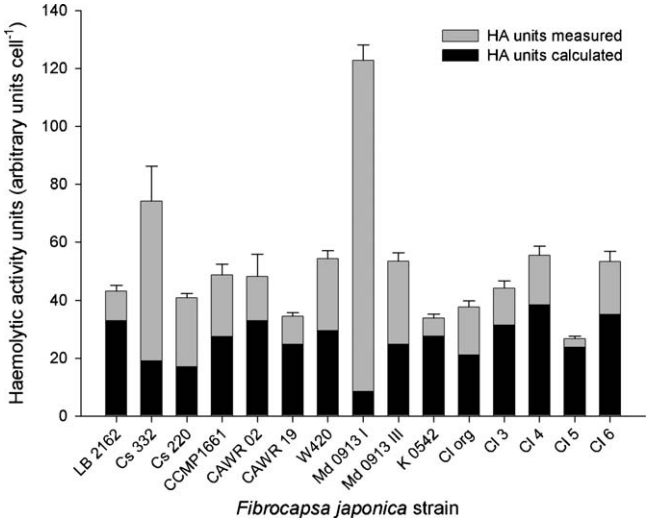

Fig. 3. The calculated (black) haemolytic activity and the measured total haemolytic activity (grey + black) in extracts of different *F. japonica* strains. The calculated haemolytic activity was obtained by combining the EC_{50} values of the individual polyunsaturated fatty acid standards (OTA, EPA, AA) with their measured amounts in the cell extracts of each strain. The measured total haemolytic activities were obtained by performing haemolytic assays on dilution series of each *F. japonica* strain extract.

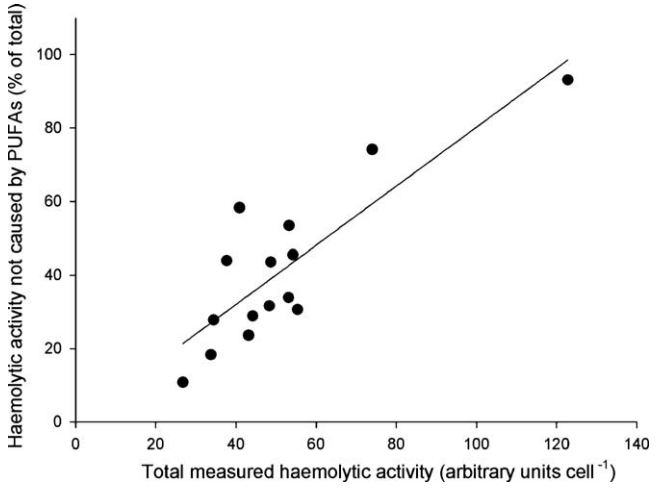

Fig. 4. Relationship between the total haemolytic activity measured in the extracts of different *F. japonica* strains (HT) and the percentage of the haemolytic activity that was not explained by the presence of three polyunsaturated fatty acids OTA, EPA and AA (HU).

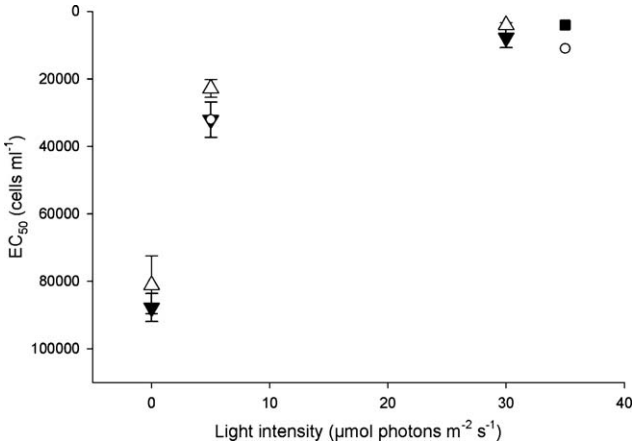

Fig. 5. Effect of light intensity on the haemolytic activity (EC_{50}) of the extracts of *F. japonica* cells. Triangles (open and filled) represent data for the two different light experiments using the *F. japonica* strain Md 9013 I (error bars represent standard error, $n = 2$), and the filled square represents the value measured at 35 μmol photons m^{-2} s^{-1} in the strain comparison. The open circles represent the *F. japonica* strain Cl 3 measured at 5 μmol photons m^{-2} s^{-1} in the study of de Boer et al. (2004) and at 35 photons m^{-2} s^{-1} in the strain comparison.

ELISA with a detection limit of 0.2 ng PbTx ml^{-1} was used. Control studies did show that haemolytic activity was not observed for the PbTx-2 standard in the concentration range from 20 ng up to 10 μg ml^{-1}. Haemolytic activity was below detection limits in the filtrate indicating that less than 1% was lost during the filtration procedure.

4. Discussion

Cell extracts of the fifteen *F. japonica* strains studied all displayed haemolytic activity. Among these strains a fivefold difference in EC_{50} values was observed ranging between 0.4 and 1.9×10^4 cells ml^{-1}. Comparing our results with EC_{50} values found in studies with other haemolytic algae (Table 3), *F. japonica* extracts seems to be even more haemolytic than observed for *Chrysochromulina polylepis*, a species known for its devastating blooms off the coasts of Norway, Sweden and Denmark (Simonsen and Moestrup, 1997). High and low values were found for strains originating from even the same bloom. The phenomenon of hybrids vigour (Kooistra et al., 2001), where hybridisation events in the Wadden Sea area potentially may have resulted in more or extremely toxic varieties, could not be demonstrated here in view of haemolytic activity. In fact, haemolytic values found for strains originating from the Wadden Sea area – where ITS polymorphism was highest – were not different from those measured for strains from other regions.

The applied environmental conditions were the same for all simultaneously growing *F. japonica* strains in the experiment. Therefore, the determined haemolytic response of each strain may reflect its characteristic toxicity for these conditions, whereas the haemolytic responses could vary, depending on strain specific growth characteristics. The incubation temperature of 17 ± 1 °C used in this study represents the water temperature when blooms of *F. japonica* occur in Dutch coastal waters (Peperzak, 2003). Three strains from very different climates (CS 332, Japan; CAWR 19, New Zealand;Cl 3, Wadden Sea) were previously studied for their growth performance related to temperature. It appeared that although the optimal growth rate was measured at 25 °C the net production a measure for biomass production was much higher at lower temperatures. At 17 °C net production was 75%, 98% and 100% of the maximal net production of the strains CS 332, CAWR 19, and Cl 3 (de Boer et al., 2005). This indicates that favourable growth conditions were applied in this study. In an earlier study on *F. japonica* strain Cl 3 no difference in haemolytic activity was observed for extracts from cells harvested in either the exponential or stationary growth phase (de Boer et al., 2004). Here, cells of all strains were harvested at the onset of the stationary phase and could not have led to growth phase induced differences. Consequently, it could be concluded that temperature and growth phase did not hamper to analyse the haemolytic characteristics of the examined *F. japonica* strains.

The dominant PUFAs in *F. japonica* strains were OTA, EPA and AA, consistent with the fatty acid profiles reported in chemotaxonomic studies on marine raphidophytes (Mostaert et al., 1998; Cho et al., 1999; Marshall et al., 2002). The amount of PUFAs in our study was much higher than measured in the chemotaxonomic studies where the algae grew at higher temperatures and irradiances (40–450 pg $cell^{-1}$ versus 14–50 pg $cell^{-1}$ at 20 °C, 150 μmol photons m^{-2} s^{-1}). The size of the cells may have influenced the cellular PUFA content as well as the temperature. At low temperatures the contribution of PUFAs may be higher to maintain fluidity of the cell membrane (Renaud et al., 1995).

The contribution of the PUFAs to the overall haemolytic activity in cell extracts could be assessed using the EC_{50} values determined for each individual PUFA in the applied assay. The order of toxicity of the PUFA standards in the haemolytic assay was: AA > EPA > OTA and was slightly different from the observation of Arzul et al. (1995) in another haemolytic test, where AA = EPA > OTA. For strain Cl 3 that was used to characterize the nature of its main haemolytic compounds (Fu et al., 2004b), 70% of the activity was explained by presence of OTA, EPA and AA. The other 30% haemolytic activity thus must have been caused by (an)other haemolytic compound(s). The contribution of haemolytic activity not accounted for by the three PUFAs was different for

Table 3
Comparison between haemolytic studies on microalgae to the results obtained for strains of *Fibrocapsa japonica* in this study. Differences in experimental conditions were normalized to values comparable to our conditions using the reported assay-specific saponin EC_{50} values (de Boer et al., 2004). Volume of cells is from Simonsen and 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	Microalgae EC_{50} density [10^4 cells ml^{-1}]	Cell volume [μm^3]	Microalgae EC_{50} biovolume [10^6 μm^3 ml^{-1}]	Source
Alexandrium tamarense	0.0152	18700[a]	2.8	Eschbach et al., 2001
Prymnesium parvum	0.3–24.7	280[b]	1–69	Simonsen and Moestrup, 1997 Johansson and Granéli, 1999 Eschbach et al., 2001 Stolte et al., 2002 Fistarol et al., 2003
Fibrocapsa japonica	0.4–1.9	4800–8000	30–120	This study
Chrysochromulina polylepis	116–170	270[b]	310–460	Simonsen and Moestrup, 1997

[a] Calculated from cell dimensions.
[b] Given cell volume irrespective of environmental conditions (Simonsen and Moestrup, 1997).

Table 4
An overview of the toxicity studies on the effect of polyunsaturated fatty acids (PUFAs) 6,9,12,15-octadecatetraenoic acid (C18:4n3; OTA), 5,8,11,14,17-eicosapentaenoic acid (C20:5n3; EPA) and 5,8,11,14 eicosatetraenoic acid (C20:4n6; AA), to different target species. The equivalent *F. japonica* density was calculated using the concentration of the specific PUFA divided by the PUFA concentration of the *F. japonica* cell (pg ml^{-1}) from the strain with the highest cell concentration of that specific PUFA at the applied harvest time of this study.

Effect	Concentration [$\mu g\ ml^{-1}$]	Target species	Equivalent *Fibrocapsa japonica* density	Source
EPA			LB 2162 [10^4 cells ml^{-1}]	
EC_{50}[a]	0.012	Microtox bacteria *Photobacterium phosphoreum*	0.01	Arzul et al., 1995
IC_{50}[b]	0.1	Sea urchin *Paracentrotus lividus* eggs cleavage	0.07	Sellem et al., 2002
50% growth inhibition	0.9	Diatom *Chaetoceros gracile*	0.6	Arzul et al., 1995
100% growth inhibition	1.5	Diatom *Chaetoceros gracile*	1.0	Arzul et al., 1995
LC_{50}[c]	2.7	Damselfish *Acanthochromis polycanthus*	1.9	Marshall et al., 2003
EC_{50}	4	Erythrocytes	2.8	this study
LC_{50}[d]	10.3	Zooplankton *Thamnocephalus platyurus*	7.1	Jüttner, 2001
AA			Cl 6 [10^4 cells ml^{-1}]	
EC_{50}[a]	0.4	Microtox bacteria *Photobacterium phosphoreum*	0.5	Arzul et al., 1995
EC_{50}	1.8	Erythrocytes	2.5	This study
OTA			Cl 4 [10^4 cells ml^{-1}]	
IC_{50}[b]	0.043	Sea urchin *Paracentrotus lividus* eggs cleavage	0.02	Sellem et al., 2002
EC_{50}[a]	1.6	microtox bacteria *Photobacterium phosphoreum*	0.6	Arzul et al., 1995
EC_{50}	25	Erythrocytes	9.5	This study

[a] EC_{50} is the effective concentration producing 50% light attenuation of the bioluminescence of *Photobacterium phosphoreum* after 5 min incubation.
[b] IC_{50} is the concentration for 50% inhibition of eggs cleavage.
[c] Toxicity of EPA in this experiment was calculated from the survival time of the fish and expressed as LT_{50}, the time it takes for 50% of fish to die at the prescribed concentration of toxin or algal cells of *Chattonella marina* (Marshall et al., 2003).
[d] EPA derived from epilithic diatom film.

each strain. The haemolytic activity of strain Cl 5 was predominantly caused by PUFAs (89%), but up to 93% of the activity of Md 0913 I was due to (an)other haemolysin(s). In fact, it appeared that the higher the toxicity of the extracts, the higher the contribution of the other haemolysin(s). Kuroda et al. (2005) reported the presence of a light-dependent haemolytic chlorophyll *c* derivative in raphidophytes, including *F. japonica*. The activity of extracts as well as a purified fraction was dependent on the light conditions used in the assay. The haemolytic activity of extracts of *F. japonica* strain Md 0913 I was also affected by light revealing saturation with maximal values at an irradiance above 30 μmol photons $m^{-2}\ s^{-1}$. The haemolytic activity in the dark was only 7% of the activity at high light conditions. This value is the same as the contribution of PUFAs to the total activity at standard conditions. The effect of light on the haemolytic activity of the extracts explains that a higher EC_{50} of strain Cl3 (3.2×10^4 cells ml^{-1})was determined at 5 μmol photons $m^{-2}\ s^{-1}$ (de Boer et al., 2004) instead of the value of 1.1×10^4 cells ml^{-1} found in this study (Fig. 5).

Light is a factor more often reported in toxicity studies with raphidophytes. A light saturation curve was also found for the inhibiting effect of exudates of strain Cl3 on bacterial fluorescence, indicating saturation between 20 and 40 μmol photons $m^{-2}\ s^{-1}$ (van Rijssel et al., 2008). The production of these compounds was indirectly affected by light through growth rate. The growth rates at the stage just prior to harvesting cells of the 15 strains, however, were not related to the haemolytic activity that was measured. Whether there is a connection between the light dependent intracellular haemolysin(s) and the light dependent extracellular compound(s) remains to be investigated. The ichthyotoxicity of *Chattonella marina* cultures also depends on light intensity (Ishimatsu et al., 1996; Marshall et al., 2001) and light is also involved in the production of ROS that is produced by raphidophytes, including *F. japonica* (Oda et al., 1997; Marshall et al., 2001, 2005a). Noteworthy is that in diatoms ROS are involved in a lipoxygenase-mediated transformation of fatty acids – triggered upon cell damage – which lead to a cascade reaction generating toxic aldehydes (Jüttner, 2001; Pohnert, 2002; Pohnert et al., 2002). A relation between ROS and PUFAs may well occur in *F. japonica*; in fact, a synergistic effect of superoxide and PUFAs on both bacterial luminescence and ichthyotoxicity caused by *C. marina* on *Vibrio fischeri* and damselfish already has been demonstrated (Marshall et al., 2005b).

So far, three *F. japonica* cultures have been incubated with fish. Strain W 420 that was also used in our strain comparison induced death after 30 min (Khan et al., 1996). Other *F. japonica* strains from New Zealand and North America induced death in less than 2 h (Bridgers et al., 2004). In both studies these effects were attributed to the brevetoxins measured. In the cell extracts of the strains in our study, however, presence of brevetoxins could not be confirmed. Nevertheless, when PUFAs are actively released or liberated by cell damage, they could be lethal to fish and in particular this is expected to occur when the fragile raphidophyte cells accumulate in fish gills and damage the epithelial tissue (Suzuki and Matsuyama, 1995; Marshall et al., 2003). In order to evaluate whether PUFAs were produced in sufficient amounts to suspect a role in ichthyotoxicity we calculated the equivalent density of *F. japonica* strains needed to reach LC_{50} values for different target species (Table 4). Such an exercise, based on EPA content in the *F. japonica* strain LB 2162, showed that lethal amounts can occur in blooms with cell densities at 1.9×10^4 cells ml^{-1}. Bearing in mind that strain LB 2162 contains a similar amount of OTA and AA, half of the density would be sufficient for lethal concentrations of PUFAs. Such cell densities of *F. japonica* actually have been measured during blooms in the field (Landsberg, 2002; Del Carmen Cortès Lara et al., 2003).

The average amount of PUFAs in *F. japonica* strains was equivalent with an EC_{50} of 1.9×10^4 cells ml^{-1}, the same value that was found for the least haemolytic strain and also the density calculated to be lethal to damselfish on basis of EPA content alone. On top of this *F. japonica* cells may produce one or more haemolysin(s) that can even elevate its toxicity in fivefold. Therefore, PUFAs in combination with the potent light dependent haemolytic compound could readily account for toxic events involving fish as well as other organisms.

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