Harmful Algae 17 (2012) 92–101

Contents lists available at SciVerse ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

The toxic effect of the marine raphidophyte *Fibrocapsa japonica* on larvae of the common flatfish sole (*Solea solea*)

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ARTICLE INFO

Article history:
Received 21 November 2011
Received in revised form 12 March 2012
Accepted 12 March 2012
Available online 20 March 2012

Keywords:
Brevetoxin
Fish test
Haemolysin
Ichthyotoxicity
Poly-unsaturated fatty acids
Raphidophyceae

ABSTRACT

Blooms of the marine microalga *Fibrocapsa japonica* (Raphidophyceae) are suggested to lead to the diminution of fish stocks, but the exact mechanism(s) involved in ichthyotoxicity is still unclear. In the present study fish tests were performed, using larvae of the common flat fish sole (*Solea solea*), to further investigate properties and variability of *F. japonica* toxicity. Sole larvae were directly and indirectly exposed to a density range of a Dutch Wadden Sea *F. japonica* strain, as well as *F. japonica* cell extracts, for a maximum of 7 days, during which larval mortality was monitored. Most experiments were done at 16 °C, however some experiments were performed at 20 °C. Furthermore, larvae were exposed to concentration ranges of the alleged toxic compounds, e.g. the brevetoxins PbTx-2 and PbTx-3, and three poly-unsaturated fatty acids (PUFAs): AA, EPA and OTA.

The larvae showed highly variable responses to the *F. japonica* treatments. At the late exponential growth phase of *F. japonica* highest sole mortality was found: 100% mortality after 7 days of direct exposure. Indirect and cell extract exposures as well as direct exposure to other growth phases caused lower larval mortality (up to 40%) after the same incubation period. Temperature had a distinct effect on sole mortality, showing significantly enhanced toxicity at 20 °C, compared with 16 °C. Exposure to PbTx-2 showed an LC_{50} of 28.7 ng mL^{-1} after 48 h, while exposure to PbTx-3 caused no 100% mortality up to 10 ng mL^{-1}. Exposure to pure PUFAs showed the fastest mortality observed in all experiments, with an LC_{50} of <5 μg mL^{-1} of AA, EPA or OTA after 24 h of exposure.

Our research suggests pronounced ichthyotoxicity in late exponential *F. japonica* cells, related primarily with the combination of endo- and exotoxins, with most probably haemolytic PUFAs as the main endotoxins, and other haemolysins and/or ROS as the main exotoxins. Implications are given for present and future risks in the already warming Dutch coastal waters.

1. Introduction

Marine raphidophytes can cause harmful algal blooms (HABs) and most representatives of this class have so far been reported to be potentially ichthyotoxic (Landsberg, 2002 and references therein). *Fibrocapsa japonica* Toriumi and Takano can be distinguished from other marine raphidophyte species by the presence of a typical mucocyst and a reticulate organization of the chloroplasts (Toriumi and Takano, 1973). *F. japonica* has a global distribution but it is found primarily in temperate regions (de Boer et al., 2005). To survive adverse conditions, *F. japonica* has the ability to form cysts (Yoshimatsu, 1987; de Boer et al., 2004a; Cucchiari et al., 2010). This cyst formation may facilitate the transport in ballast water tanks. In support of this, a phylogenetic study of *F. japonica* strains from different global regions indicated recent habitat expansion, possibly due to anthropogenic influences (Kooistra et al., 2001). The first observation of this potentially toxic alga in Dutch coastal waters was in 1991 (Vrieling et al., 1995) and since then *F. japonica* has occurred yearly, reaching cell densities up to 1.6×10^2 cells mL^{-1} (de Boer, 2006).

The ichthyotoxicity of *F. japonica* has so far been ascribed to the production of brevetoxins (Khan et al., 1996b; Bridgers et al., 2004), reactive oxygen species (ROS) (Marshall et al., 2005a; Oda

Abbreviations: PUFA, poly-unsaturated fatty acids; AA, 5,8,11,14-eicosatetraenoic acid; EPA, 5,8,11,14,17-eicosapentaenoic acid; OTA, 6,9,12,15-octadecatetraenoic acid; ROS, reactive oxygen species; ELS, early life stage; ELISA, enzyme-linked immunosorbent assay.

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doi:10.1016/j.hal.2012.03.005

Table 1
Overview of *Fibrocapsa japonica* culture conditions used in the present experiments, compared with previously described fish bioassay experiments.

Fibrocapsa japonica strain	Temperature (°C)	Irradiance (μmol photons $m^{-2} s^{-1}$)	Salinity	L:D (h)	Medium	Reference
W420, the Netherlands	16	40	25	16:8	f/2-Si	This study
W420, the Netherlands	20	40	25	16:8	f/2-Si	This study
W420, the Netherlands	25	60	30	12:12	Provasoli ES media	Khan et al. (1996a,b)
New Zealand	24	100	20	[a]	Erdschreiber	Bridgers et al. (2004)
Hilton Head, USA	24	100	20	[a]	Erdschreiber	Bridgers et al. (2004)
FJAP0603, Italy	20	100	35	16:8	f/2-Si	Pezzolesi et al. (2010)

[a] Short experiment <24 h with continuous light.

et al., 1997), haemolysins (Fu et al., 2004a,b; de Boer et al., 2004b), haemoglutinating compounds (Onoue and Nozawa, 1989; Guidi-Rontani et al., 2010) mucocyst threads (de Boer et al., 2005), or a combination of these vectors (Marshall et al., 2003; de Boer et al., 2009; Pezzolesi et al., 2010). The suggested mechanisms may affect the fish in different ways. For example the mucus threads may clog the gills and subsequent gill damage may be enhanced if *F. japonica* cells are disrupted or lysed leading to endotoxin exposure (de Boer, 2006; Pezzolesi et al., 2010). Apart from the production of the neurotoxin brevetoxin certain poly-unsaturated fatty acids (PUFAs) may be involved in ichthyotoxicity (Marshall et al., 2003). The three main PUFAs in *F. japonica* are 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 or AA); Mostaert et al., 1998; Cho et al., 1999; Fu et al., 2004b; Marshall et al., 2005a; Pezzolesi et al., 2010). These PUFAs isolated from *F. japonica* cells showed strong haemolytic activity to rat, human and fish blood cells (Fu et al., 2004a; de Boer et al., 2009; Pezzolesi et al., 2010) and were not released into the extracellular medium by living cells (Pezzolesi et al., 2010). So far only the individual PUFA EPA was checked for its ichthyotoxicity and was found to be toxic toward damselfish *Acanthochromis polycanthus* with an LC_{50} of 2.7 μg mL^{-1} (155 min; Marshall et al., 2003). These three characterized PUFAs could not explain the total intra- and extracellular haemolytic activity of *F. japonica* (de Boer et al., 2009). Other alleged ichthyotoxins could be the unknown haemolytic compounds that were suggested in different studies (Fu, 2003; van Rijssel et al., 2008; de Boer et al., 2009, in press). Furthermore, as shown recently, *F. japonica* exotoxins express the highest haemolytic activity at the beginning of the exponential growth phase, at approximately 600 cells mL^{-1} while at higher cell densities endotoxins caused higher haemolytic activity (de Boer et al., in press). Additionally, a synergistic effect was observed when the PUFA's were exposed to fish together with ROS (Marshall et al., 2003), though ROS production alone could not be the cause of ichthyotoxicity (Twiner et al., 2001; Woo et al., 2006; Pezzolesi et al., 2010). If the different ichthyotoxic mechanisms of *F. japonica* are summarized it can be assumed that neurotoxic (brevetoxins), suffocation (gill blocking by mucus threads), gill epithelium damage, reduction of pO_2 and/or osmoregulatory dysfunction (haemolysins alone or in combination with ROS) all can lead to fish mortality (de Boer, 2006; based on *Chattonella* scheme of Marshall et al., 2003).

The very limited number of direct fish bioassays with *F. japonica* performed so far all showed an ichthyotoxic effect (Khan et al., 1996b; Bridgers et al., 2004; Pezzolesi et al., 2010). In 2 studies brevetoxins were suggested to be responsible (Khan et al., 1996b; Bridgers et al., 2004). Khan et al. (1996b) described a rapid (<30 min) toxic effect of cells of the same Dutch *F. japonica* strain as used in the present study toward juvenile red sea bream (*Pagrus major*) when a cell density of 1.7×10^4 cells mL^{-1} was reached. Also, toxicity varied with the growth phase, increasing with the age of the culture and decreasing when the culture entered the early stationary phase. Bridgers et al. (2004) performed a toxicity experiment with adult mosquito fish (*Gambusia affinis*). Here, the fish died in a short time period ranging from less than two hours up to six hours when exposed to living *F. japonica* cells in the stationary phase; however cells in the exponential phase were not tested. Also Pezzolesi et al. (2010) only investigated ichthyotoxicity of living *F. japonica* cells in the stationary phase. Moreover differences in culture conditions (composition of the medium, light conditions, temperature) between these studies could explain the differences in toxicity (Table 1). Next to differences in abiotic conditions also strain specific differences could explain the variability in toxicity (Table 1) even when using the same bioassay (Rhodes et al., 1993; Cho et al., 1999; de Boer et al., 2009; Guidi-Rontani et al., 2010).

One of the Dutch fish species that may be affected by *F. japonica* is sole (*Solea solea*, Linnaeus 1758) since both *F. japonica* and sole are known to occur in the coastal areas of the North Sea including the Wadden Sea. This area is one of the important recruitment areas of the Northeast Atlantic sole stocks (van Beek et al., 1989; Rijnsdorp et al., 1992). Sole spawns around April and May, which is triggered by a sea water temperature between 8 and 12 °C. After around 2 weeks the pelagic sole larvae hatch and settlement occurs from May onwards (van der Veer et al., 2001). Since, 1995 cells of *F. japonica* are found yearly in these coastal areas with most observations from March to October (de Boer, 2006). Sole is a commercially important fish species in the Netherlands and therefore ichthyotoxicity of *F. japonica* toward this species would be harmful to local economy.

In the present work, we aimed to generate insight in fish mortality caused by a Dutch Wadden Sea *F. japonica* strain that was grown at temperature and light conditions similar to the field conditions when sole fish larvae are present. In different experiments sole larvae were exposed to *F. japonica* cell extracts (providing endotoxins) or to living cells, distinguishing between direct (providing endo- and exotoxins) and indirect (providing exotoxins) exposure. Furthermore, in the fish bio assays with direct and indirect exposure the impact of *F. japonica* cell density and temperature was examined. Finally, the hypothesized ichthyotoxins of the *F. japonica* cells (brevetoxins and PUFAs) were tested individually on sole larvae.

2. Materials and methods

2.1. Species and their maintenance

F. japonica strain W420 was obtained from the algal culture collection of the Department of Ocean Ecosystems of the University of Groningen, The Netherlands. This strain was isolated from the Wadden Sea (the Netherlands) in 1992 and is a monoclonal and non-axenic strain. This strain was used before by Khan et al. (1996a,b) where it was demonstrated to be highly ichthyotoxic, but this could be changed over time. The algae were pre-cultured in freshly prepared f/2-Si medium (Guillard, 1975) based on sterile natural seawater that was diluted with MilliQ to a salinity of 25. Cultures were maintained in a climate room at 16 ± 1 °C, a light intensity of 40 ± 5 μmol photons $m^{-2} s^{-1}$ (provided by fluorescent

tubes L36 W/19 light, Osram) and a light–dark cycle of 16:8 h. Precultures were diluted regularly to keep the cells in the exponential growth phase.

The sole larvae were obtained from Solea B.V. IJmuiden, the Netherlands, where sole is cultivated continuously under controlled (±12 °C) conditions. During experimentation, the larvae had grown out of the yolk sac stage; however they had not entered metamorphosis yet. Throughout the experimental period, three batches of larvae were used, and a slightly different appearance was observed over time as the experiments progressed. One to two days prior to the experiments the larvae were collected from Solea B.V. where they had been fed with nauplii of *Artemia salina* and then transported to the University of Groningen in a temperature controlled (±12 °C) cool box. The larvae were acclimated to the experimental conditions for at least one day prior to the start of the experiments. The larvae were not fed during experimentation in order to reduce the risk of contamination; since it had been shown earlier that food deprivation during incubation with the algae (7 days) did not significantly affect the natural sole mortality (results not shown).

2.2. Experiments performed

Most experiments were performed under identical environmental conditions as the culture maintenance conditions (16 ± 1 °C, light–dark cycle 16:8 h and growth irradiance of 40 ± 5 μmol photons m^{-2} s^{-1}). In total three series of experiments were performed under these conditions.

2.2.1. Identification of F. japonica growth phases

The first series (n = 3 experiments) was meant to establish *F. japonica* growth curves, in order to determine the cell densities at which the cultures were in the exponential, late exponential and stationary growth phases. In total ten times 1 L Erlenmeyer flasks were incubated for the experiments, with an initial cell density of $1 \times 10^3 \pm 0.4$ cells mL^{-1}.The cultures were sampled every day over a period of 2 weeks and after that samples were taken regularly until day 24. After sampling cell numbers and biovolume were immediately determined. Based on the obtained growth curve (Fig. 1), cell densities in exponential, late exponential and stationary growth phases were determined as follows: up to 1×10^4 cells mL^{-1} the cells were considered in the exponential growth phase; between 1×10^4 and 3×10^4 cells mL^{-1} the cells were considered in the late exponential growth phase, while cell densities above 3×10^4 cells mL^{-1} were in the stationary phase (Fig. 1).

2.2.2. Endotoxin only exposure

During the growth curve experiments duplicate-samples were taken from 6 different cultures. At a cell density of approximately 2×10^4 cells mL^{-1} from each culture 1×10^6 cells were collected on a polycarbonate filter (2 μm, Ø 25 mm) using mild vacuum (standard filtration). From these filters cell extracts were obtained as described in de Boer et al. (2009) (preparation cell extract haemolytic activity), and were dissolved in 1 mL f/2-Si media. All dissolved filter extracts were stored at −20 °C until exposure to the larvae (Table 2A, cell extracts, endotoxin only exposure). Sole larvae were exposed in 16 ml wells (six well plate Greiner Bio One, Ø 35 ± 1 mm, depth 16.5 mm, total working volume of 11 mL) to the concentrated cell extracts (9×10^4 cells mL^{-1} of the late exponential growth phase). The experiment was performed with 5 larvae per well for the duration of 7 days with n = 12. The results of fish mortality by endotoxins show the mortality of the endotoxins from a 4.5 times concentrated cell extract. As control, instead of *F. japonica* culture extract, 50 mL f/2-Si medium was used.

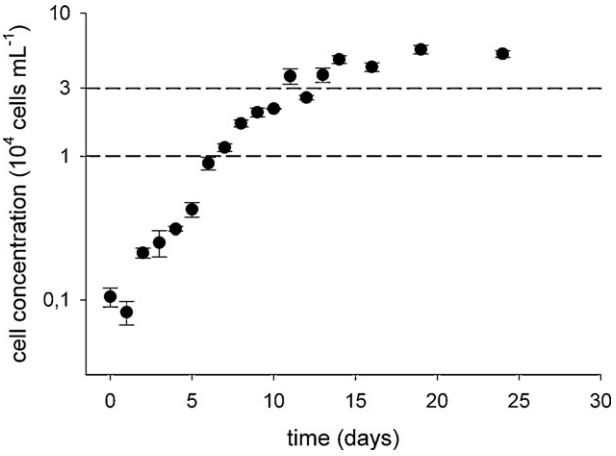

Fig. 1. Growth curve of the Dutch *Fibrocapsa japonica* strain W420 when grown at standard culture conditions (f/2-Si medium; salinity of 25; temperature of 16 ± 1 °C, a light intensity of 40 ± 5 μmol photons m^{-2} s^{-1} and a light–dark cycle of 16:8 h). Data are based on 3 independent experiments using 10 different cultures. The dotted lines show the range of the different growth phases; exponential growth phase, $<1 \times 10^4$ cells mL^{-1}; late exponential growth phase, $>1 \times 10^4$ cells mL^{-1} and $<3 \times 10^4$ cells mL^{-1}, stationary growth phase $>3 \times 10^4$ cells mL^{-1}. The error bars show the standard errors.

2.2.3. Direct and indirect exposure to living F. japonica cells

In the second series of experiments sole larvae were exposed to living *F. japonica* cells, directly or indirectly. Direct exposure experiments were performed in six well plates, each well containing 7 ml of *F japonica* culture and 3 mL of f/2-Si medium. For the indirect exposures, 7 ml of *F. japonica* culture was added to the wells, after which filter inserts (1 μm, Greiner Bio One) were applied. These filter inserts were filled with 3 mL of f/2-Si medium to prevent floating. *F. japonica* cell concentration was followed as much as possible during the whole experiment. To each well, 5 larvae were added, and for each condition different amounts of wells were screened (Table 2A). The choice of 5 larvae per well is based on a capacity test where 3, 5, 7 and 10 larvae per well were tested (n = 3). After 3 days no mortality was found for all larval densities, but in case of possible longer exposure time we used 5 larvae to keep natural mortality as low as possible. For all experiments in this series, mortality was determined daily and corrected for the blank, expressed by larval mortality in algae-free f/2-Si medium ($n \geq 3$ or dependent on the n of the experiment, Table 2A). The plates were covered with a lid to prevent evaporation. During the experiments of maximal 7 days, the plates were moved randomly every day to obtain homogeneous light distribution. The lids were removed for about one to two hours per day to prevent oxygen depletion. Experiments within this series were performed at the three different growth phases of *F. japonica*, predetermined in the first experimental series and referred to as exponential, late exponential and stationary growth phases (Table 2A). In one experiment the fish test was carried out at 2 temperatures: 16 °C and 20 °C. Here, an initial *F. japonica* concentration of 2.4×10^4 cells mL^{-1} was used (Table 2A), because mortality rates were expected to be higher at the elevated temperature, possibly leading to shorter exposure times. To test the possible interference of mucocyst ejection in the cultures mucocyst ejection was monitored by eye and stereomicroscopy.

2.2.4. Single compound exposures

In the last experimental series sole larvae were exposed to the alleged ichthyotoxins of *F. japonica*; the individual brevetoxins and PUFAs. The tested brevetoxins were PbTx-2 and PbTx-3 (Calbiochem), both postulated to be produced by *F. japonica* cells (Khan et al., 1996b), at three to four different concentrations (Table 2B).

Table 2
Overview of all bioassay experiments where *Solea solea* larvae were exposed to (A) living cells or cell extracts of *Fibrocapsa japonica* and (B) the single compound exposures of the two brevetoxins; PbTx-2 and PbTx-3, and of the poly-unsaturated fatty acids; 6,9,12,15-octadecatetraenoic acid (C18:4n3; OTA), 5,8,11,14,17-eicosapentaenoic acid (20:5n3; EPA), and 5,8,11,14-eicosatetraenoic acid (C20:4n6; AA). Each *n* expresses one well containing 5 sole larvae.

Temperature (°C)	Start cell density (10^4 cells mL^{-1})	Fraction	Type of exposure	Mucocysts	Growth phase	*n*
(A)						
16	0.05	Living cells	Direct contact	No	Exponential	4
16	0.13	Living cells	Direct contact	No	Exponential	2
16	1.07	Living cells	Direct contact	Yes	late Exponential	4
16	1.24	Living cells	Direct contact	No	late Exponential	2
16	1.33	Living cells	Direct contact	Yes	late Exponential	4
16	0.88	Living cells	Direct contact	No	late Exponential	2
16	2.80	Living cells	Direct contact	No	stationary	12
20	2.44	Living cells	Direct contact	No	stationary	12
16	1.94[a]	Cell extract	Endotoxins	Both	late Exponential	12
16	0.05	Living cells	Indirect contact	No	Exponential	4
16	0.13	Living cells	Indirect contact	No	Exponential	2
16	1.07	Living cells	Indirect contact	Yes	late Exponential	4
16	1.24	Living cells	Indirect contact	No	late Exponential	2
16	1.33	Living cells	Indirect contact	Yes	late Exponential	4
16	0.88	Living cells	Indirect contact	No	late Exponential	2
16	2.80	Living cells	Indirect contact	No	Stationary	3

Temperature (°C)	Toxin ($ng\ mL^{-1}$ or [b] $\mu g mL^{-1}$)	Concentration	*n*
(B)			
16	PbTx-2 and PbTx-3	1.5	6 and 6
16	PbTx-2 and PbTx-3	5	6 and 6
16	PbTx-2 and PbTx-3	10	6 and 6
16	PbTx-2	50	3
16	PbTx-2	100–50–25–12.5–6.25–3.1–1.6–0	1
16	OTA, EPA and AA	5[b]	3 and 3 and 3
16	OTA, EPA and AA	100[b]	3 and 3 and 3
16	EPA and AA	500–125–31.3–15.6–0[b]	1 and 1
16	OTA	500[b]	1

[a] Cell density in culture at time of harvesting.
[b] $\mu g\,mL^{-1}$ instead of $ng\,mL^{-1}$.

These concentrations are within the range of the PbTx-2 LC_{50} value of 13 $ng\ mL^{-1}$ (24 h) found for adult mosquito fish (Rein et al., 1994). For every treatment, including a control, 30 larvae (5 per well, $n = 6$) were observed daily for a maximal duration of 7 days. To collect more detailed information on PbTx-2 an additional test was performed using a dilution series from 100 to 0 $ng\ mL^{-1}$ (Table 2B).

Furthermore, larvae were exposed to PUFAs, known to be haemolytic and produced by *F. japonica* cells (Fu et al., 2004a,b; de Boer et al., 2009): AA, EPA and OTA (all from Sigma Aldrich). Individual PUFAs were applied at two concentrations (5 $\mu g\ mL^{-1}$ and 100 $\mu g\ mL^{-1}$ final concentration both $n = 3$), and mortality was determined at regular intervals. To collect more detailed information on the most toxic PUFAs AA and EPA additional tests were performed, based on PUFA dilution series from 500 to 0 $\mu g\ mL^{-1}$ (Table 2B).

For all experiments in this series, mortality was corrected for the corresponding blank, expressed by larval mortality in alga-free f/2-Si medium. Since the PUFA standards were dissolved in methanol (MeOH) or ethanol (EtOH), the possible effect of these compounds on larval mortality was assessed prior to the experiments. The highest MeOH or EtOH concentration in the PUFA exposures was 1% and therefore larvae were exposed to a dilution series of 50, 10, 5, 1% and 0.1% in seawater in a separate experiment ($n = 10$, 50 larvae,) for 6 days. Since no effect of $\leq$1% was found ($\geq$5% showed mortality), the effect of MeOH or EtOH was not further considered (results not shown). An overview of all larvae exposure experiments is given in Table 2B.

2.3. Analytical procedures

During all experiments *F. japonica* cell concentrations and cell volume were determined on a coulter counter (Coulter Counter ZM) equipped with a channelyzer 256 at 100 μm aperture (Coulter Electronics Ltd.). Cultures were diluted in sterile artificial seawater and two mL sample was counted in four steps of 500 μL. A duplicate measurement was done from the same sample.

The larvae were observed by eye or using a binocular/stereomicroscope. Larvae were recorded as dead if the fish was shriveled. This was done because the lack of movement was not always an indication of mortality. Additionally, the heart beat was not always visible under the binocular. The concentrations of the pure toxin from the different experiments were plotted against the % fish larvae mortality and were fitted with a sigmoidal equation, or alternatively, by linear regression. From this, the LC_{50} was calculated for each treatment as the concentration responsible for 50% fish mortality.

Prior statistical analysis data were tested for normality and homogeneity of variances. Effects of the factors growth phase and exposure mode and their interaction on the larval mortality and joint effects of temperature and exposure time on larval mortality were tested by a two-way factorial general linear model (GLM). Comparison between groups of data was done by one-way ANOVA. All statistical analyses were performed with Statistica 10 or Excel 2010 at a significance level $p < 0.05$.

3. Results

3.1. Larval mortality as a function of F. japonica growth phase end exposure mode

Significant effect on larval mortality was found related with *F. japonica* growth phase and exposure mode ($F = 3.7$ $\hat{}p = 0.034$; Fig. 2). In the experiments with living algae no effect of *F. japonica* growth rate during experimentation on larval mortality was found (results not shown). No significant difference of mucocyst ejection (Table 2A) on sole larvae mortality was found. The highest mean mortality of 80 ± 10% was found in experiments when sole larvae

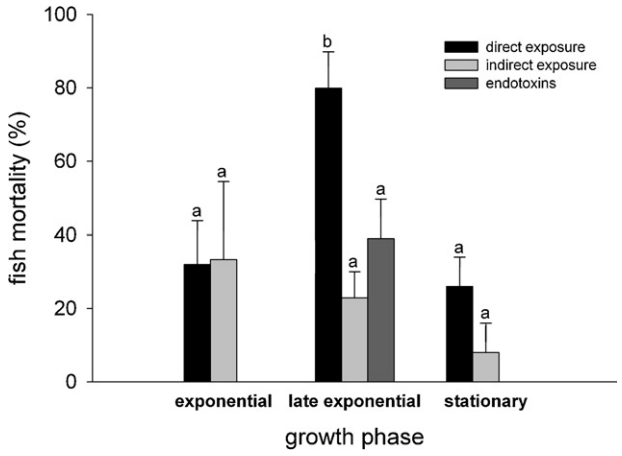

Fig. 2. The effect of *Fibrocapsa japonica* cells during different growth phases on *Solea solea* larval mortality after 7 days of exposure. Black bars indicate direct exposure; light gray bars indicate indirect exposure and gray bars are the endotoxin only exposures. The error bars show the standard errors. Significant differences are indicated with *a* and *b*.

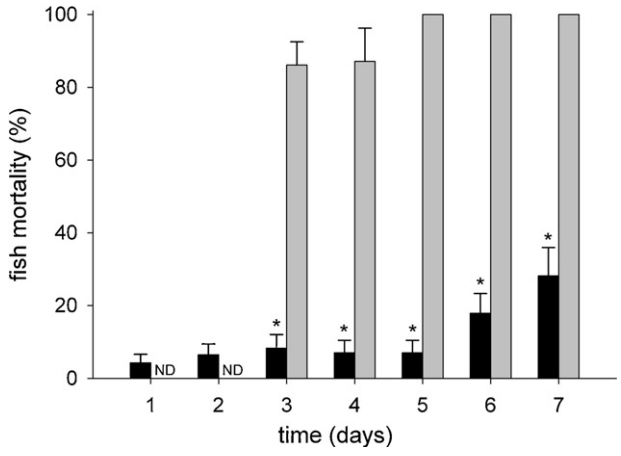

Fig. 3. The effect of temperature on *Solea solea* larval mortality when the fish larvae are directly exposed to *Fibrocapsa japonica* cells in stationary growth phase grown at 16 °C (black bars) and 20 °C (gray bars). The error bars show the standard errors. ND indicates that there is no data of 20 °C experiment. * shows a significant difference between the two temperature treatments at that time of exposure.

were directly exposed for at least 7 days at cell densities typical for the late exponential growth phase ($>1 \times 10^4$ and $< 3 \times 10^4$ cells mL^{-1}). Exponentially growing cells as well as stationary phase cells caused significantly lower mortality ($F = 8.5$ $\hat{}p = 0.01$; $F = 12.7$ $\hat{}p = 0.003$), with a maximum fish mortality of $32 \pm 12\%$ after 7 days. On average exponential cultures showed slightly higher mortality than the stationary cultures, however this difference was not statistically significant. The indirect exposures did not cause substantial larval mortality after 7 days. Maximum mortality levels were found to be $33 \pm 21\%$ caused by cells from the exponential growth phase ($<1 \times 10^4$ cells mL^{-1}). At the indirect exposure a slight trend was observed of decreasing mortality at increasing cell densities, however these differences were not statistically significant (Fig. 2). Direct exposure of stationary phase cells seemed to cause a higher mortality than indirect exposure, but again this was not statistically supported (Fig. 2). For the stationary and exponential phases, mortality numbers were not significant between direct and indirect exposures, but for the late exponential growth phase, direct and indirect exposures were significantly different ($F = 21.8$ $\hat{}p = 0.0001$). Exposure to the concentrated extracts obtained from cells in the late exponential phase, resulted in mortality numbers in between the direct and indirect exposures, but was only found to be significantly different ($F = 7.9$ $\hat{}p = 0.01$) from the direct exposures, with an average mortality of $39 \pm 11\%$.

3.2. Larval mortality as a function of temperature

Larval mortality due to exposure to *F. japonica* cells was found to be affected by temperature. At the 16 °C condition mortality was $28 \pm 8\%$ after 7 days of exposure, comparable with the former series (3.1; Fig. 2, stationary phase cells). However, cultures grown and exposed at 20 °C showed a significantly higher mortality, with 100% mortality already observed after 4 days (temperature: $F = 655.9$ $\hat{}p < 0.000$; exposure time: $F = 4.4$ $\hat{}p = 0.002$; Fig. 3).

3.3. Brevetoxin and PUFA exposures

Exposure to PbTx-2 resulted in higher larval mortality when compared with PbTx-3 (Fig. 4, Table 3A). Highest mortality was found for 50 ng mL^{-1} PbTx-2 after 48 h. Only the LC_{50} of PbTx-2

Table 3
LC_{50} values of the single compounds of (A) the two brevetoxins; PbTx-2 and PbTx-3, and of (B) the poly-unsaturated fatty acids; 6,9,12,15-octadecatetraenoic acid (C18:4n3; OTA), 5,8,11,14,17-eicosapentaenoic acid (20:5n3; EPA), and 5,8,11,14-eicosatetraenoic acid (C20:4n6; AA), on *Solea solea* larvae at different exposure times. For comparison, earlier published LC_{50} values of these compounds are included. The used fishes in these publications were *Acanthochromis polycanthus* (Marshall et al., 2003) and *Gambusia affinis* (Lewis, 1992; Rein et al., 1994).

Compound	Exposure time (h)	LC_{50} (ng mL^{-1})	R^2	Reference
(A)				
PbTx-2	24	50.9	0.6	This study
PbTx-2	48	28.7	0.93	This study
PbTx-3	>166	Not detected		This study
PbTx-2	24	12.8		Rein et al. (1994)
PbTx-2	48	9		Lewis (1992)
PbTx-3	24	14.2		Rein et al. (1994)
Compound	Exposure time (h)	LC_{50} (μg mL^{-1})	R^2	Reference
(B)				
OTA	2	250.2	0.89	This study
OTA	4	92.3	0.92	This study
OTA	24	<5		This study
EPA	2	100.6	0.85	This study
EPA	4	28.1	0.76	This study
EPA	24	<5		This study
AA	2	50.8	0.55	This study
AA	4	<5		This study
AA	24	<5		This study
EPA	2.5	2.7	0.94	Marshall et al. (2003)

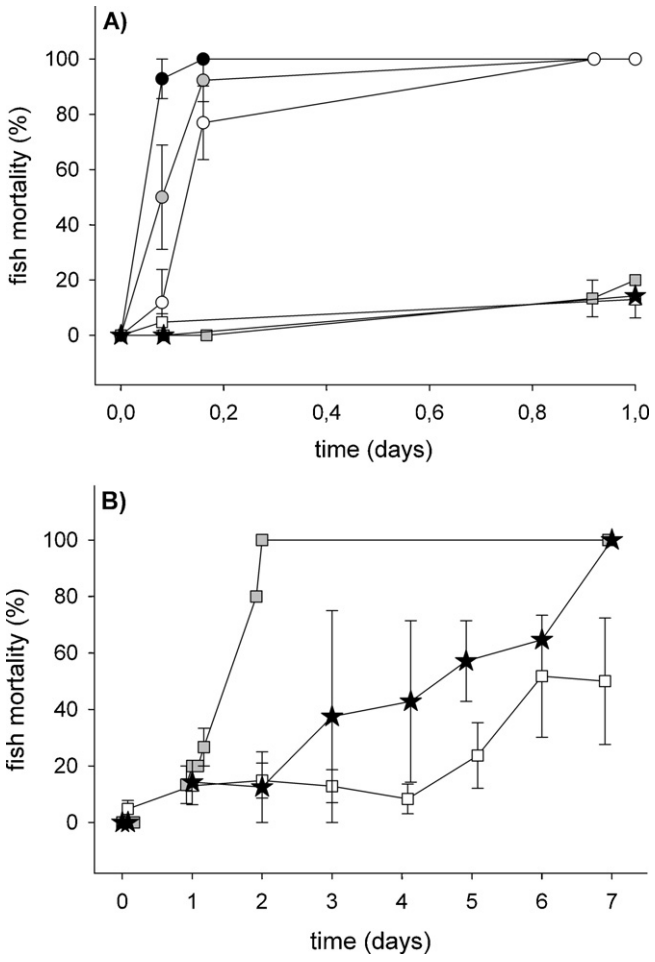

Fig. 4. The effect of the different single compounds of the poly-unsaturated fatty acids; 6,9,12,15-octadecatetraenoic acid (C18:4n3; OTA), 5,8,11,14,17-eicosapentaenoic acid (20:5n3; EPA), and 5,8,11,14-eicosatetraenoic acid (C20:4n6; AA), and of the brevetoxin PbTx-2 as well as the living *Fibrocapsa japonica* cells (late exponential growth phase) on *Solea solea* larval mortality in direct fish tests over an exposure time of (A) one day and (B) 7 days. Black circles indicate 100 μg mL^{-1} of AA; gray circles indicate 100 μg mL^{-1} of EPA; white circles indicate 100 μg mL^{-1} of OTA; black star indicate living *F. japonica* cells in late exponential growth phase; gray squares indicate 50 μg mL^{-1} of PbTx-2; white squares indicate 10 μg mL^{-1} of PbTx-2. The error bars show the standard errors.

could be calculated (Table 3A). The fish mortality at 10 ng mL^{-1} PbTx-2 after long exposure of 7 days showed lower ichthyotoxicity than the exposure of sole larvae to *F. japonica* cells in the late exponential growth phase (Fig. 4B). Exposure of sole larvae to high concentrations of PUFAs (100 μg mL^{-1}) caused a very high mortality rate within one hour (Fig. 4A). There was a difference in mortality when the sole larvae were exposed to the different PUFAs. The PUFA AA was more toxic than EPA and OTA (AA > EPA > OTA). Also the LC_{50}'s of these compounds after different exposure times showed the order AA > EPA > OTA, with a range between 50.8 and 250 μg mL^{-1} after 2 h of exposure time (Table 3B).

4. Discussion

The present study has demonstrated that *F. japonica* cells are toxic to sole larvae after a maximum exposure time of 7 days, despite the sometimes high variability in the data (Figs. 2–4). This variability was mainly due to experimental constraints, in particular differences in blank mortality (larvae maintained in f/2-Si medium without algae). Occasional high mortality in the blanks could be related with fitness differences between larval batches, while mortality was not significantly affected by feeding (see Section 2.1).

The highest sole larvae mortality (100%) was found after seven days of exposure to a final *F. japonica* cell concentration of 1.8×10^4 cell mL^{-1}, e.g. in the late exponential phase (Table 2A, start concentration 1.07 and 1.24×10^4 cells mL^{-1}). Thus, the toxicity varied with growth phase as described by Khan et al. (1996b). In addition, our most effective cell concentration was similar to their study where a cell concentration of 1.7×10^4 cell mL^{-1} induced maximum mortality of the Red Sea bream, *P. major* (Khan et al., 1996b). However, in the latter study, mortality was shown after 30 min and was performed with 4–5-month-old juveniles and not with larvae (Table 4). In contrast to juveniles and adults who use their branchial area (mouth and gills), larvae perform mainly gas exchange through their skin and/or cutaneous area (Hiroi et al., 1998; Rombough, 1999). This is a slower process and therefore it is expected that sole larvae could be less sensitive to toxic compounds, or could be affected only after a prolonged exposure period. The latter was found for sole in the early life stage (ELS) assay with field relevant levels of PCB (Foekema et al., 2008). Own incidental histological investigation showed that the gill area of the sole larvae was poorly developed (data not shown). In addition, no damage on the (developing) gills was found either in the control or in the larvae that had been exposed to *F. japonica* cells (data not shown). Damaged gill tissue of *Dicentrarchus labrax* due to exposure to *F. japonica* cells was found by Pezzolesi et al. (2010), who suggested that the mucus substances might have interfered with O_2 transfer. In our study mucocysts threads were observed (Table 2A), but this mucocyst ejection did not significantly affect sole mortality. The reason of incidental mucocyst ejection by *F. japonica* in this study remains unclear, but in general most mucocyst ejection was found when the algal cells were grown in f/2-Si medium at the onset of the stationary growth phase under phosphate and/or C limiting conditions (de Boer et al., 2004b) and at temperatures higher than 16 °C (de Boer et al., 2005). Stereoscopic observations showed that the cultures with mucocyst ejection showed higher concentration of *F. japonica* cells around the sole larvae and clogging of the cells in the area of gills and pectoral fin. Nevertheless the mortality results indicate that sole larvae were not sensitive to clogging of

Table 4
Overview of *Fibrocapsa japonica* ichthyotoxicity based on present and earlier published experiments. The cell density of the *F. japonica* culture (10^4 cells mL^{-1}) is given when for the first time100% mortality was observed per individual experiment.

Fish species	Development state (fish)	Mortality (time)	Temperature (°C)	Cell density algae (10^4 cells mL^{-1})	Growth phase (algae)	Reference
Solea solea	Larvae	6–7 days	16	1.8	Late exponential	This study
Solea solea	Larvae	2–3 days	20	2.4[a]	Late exponential	This study
Pagrus major	Juvenile	30 min	25	1.7	Late exponential	Khan et al. (1996b)
Gambusia affinis	Adult	2–4 h	24	7$^{(New\ Zealand)}$	Stationary	Bridgers et al. (2004)
Gambusia affinis	Adult	2–4 h	24	10.4$^{(USA)}$	Stationary	Bridgers et al. (2004)
Dicentrarchus labrax	Juvenile	11 days	20	5[a]	Stationary	Pezzolesi et al. (2010)

[a] Start concentration; exact concentration at time of 100% mortality is not known.

cells and mucus in the gill area, probably because of the poorly developed gills. In other words, in this study we can exclude fish mortality by *F. japonica* cells due to mechanical stress (de Boer, 2006). Sole larvae died because of intoxication due to toxic compounds of *F. japonica* cells and not by suffocation in the branchial area. Since the last few years toxicity tests with sole larvae have become a valuable tool for example used in the ELS test in PCB research (Foekema et al., 2008). Tests with embryos or early development states of fishes are often used because of their sensitivity and practical advantages, requiring less test volume and space, thus facilitating the statistical power of test results (Wedekind et al., 2007). However, results of the mortality rate obtained using ELS with sole larvae could not be representative for older life stages due to the necessary prolonged exposure period.

Only direct exposure of the fish larvae to *F. japonica* cells induced 100% mortality while exposure to exotoxins or endotoxins alone was much less pronounced (Fig. 2). Bridgers et al. (2004) found opposite results: cell pellets (endotoxins) or, in one case, supernatant (exotoxins) alone caused faster fish mortality than direct exposure to *G. affinis* (Table 4; Bridgers et al., 2004). In addition Bridgers et al. (2004) observed strain specific differences, with the USA strain causing 100% mortality within 2 h, and the New Zealand strain not causing any fish mortality. Next to strain specific difference this difference in toxicity could be explained by cell damage during the centrifuge step to separate cells and supernatant. Furthermore, the tested strains seemed to be both in their stationary phase so leakage of endotoxins by old or lysed cells cannot be excluded. In our study we found excretion of exotoxins during the whole growth phase, despite the absence of a centrifuge step. In the late exponential and stationary growth phases mortality was lower after indirect exposure than after direct exposure, while this was not found in the exponential phase. This is in agreement with a recent study by de Boer et al. (in press) on haemolysins, which showed enhanced haemolytic activity of the endotoxins at increasing cell numbers, while the opposite was found for the exotoxins. The maximum sole larvae mortality in the indirect fish tests was not higher than 33%. Therefore the high levels of haemolytic active compounds excreted at the start of exponential phase ($<0.5 \times 10^4$ cells mL^{-1}; de Boer et al., in press) could not be coupled to high sole larvae mortality and were therefore not considered highly ichthyotoxic. High levels of haemolytic exotoxins produced by *F. japonica* cells at the beginning of the exponential phase seemed to be derived from other compounds than the haemolytic endotoxins. Consequently these exotoxins probably have another function than grazer defense and indicate an allelopathic mechanism as suggested earlier (van Rijssel et al., 2008). Possibly, in the late exponential and stationary phases endotoxins are released by *F. japonica* cell damage or lysis, which could occur to a much more limited extent during the exponential phase. Pezzolesi et al. (2010) showed that PUFAs were not released (as exotoxins) into the extracellular medium by living *F. japonica* cells in the stationary growth phase. It has to be noted that in our study *F. japonica* cells were neither ingested by the larvae nor could *F. japonica* cells have affected their gills, as explained above. As a result, endotoxins were likely to affect the larvae only after algal damage or lysis. The concentrated fraction of endotoxins alone harvested at the cell density of highest fish mortality in direct fish tests (1.9×10^4 cells mL^{-1}), showed very high variability in fish mortality (after 7 days from 20 up to 100%; mean of all tested filters 40%). The impact of endotoxins can be much higher during direct contact between algae and fish since cells can accumulate and subsequently be disrupted in the surrounding of gills. The ichthyotoxicity of the cell pellets of the USA and New Zealand *F. japonica* strains were high and induced 100% fish mortality of *G. affinis* within 2 h (Bridgers et al., 2004). While cell pellet and extracted cells from filters both should concern only the endotoxins, differences in toxicity between these sampling methods were found. The chance that the toxicity came from the combination of endo- and exotoxin could be higher in the cell pellet procedure. Instead of filtration, freezing and extraction steps that ruptured whole cells the cells were centrifuged to a pellet, sonificated and analyzed immediately after collection (Bridgers et al., 2004). On one hand the toxicity of the cell pellet could be higher due to direct analysis where damage to the cells could lead to more ROS production and therefore also to different and more toxic compounds due to lipid peroxidation of PUFAs and ROS (Jüttner, 2001; Pohnert, 2005). On the other hand the rupturing of *Chattonella marina* cells by sonification did not show any toxic effect to bacteria probably due to decreased ROS production (Oda et al., 1992). It is also possible that rupturing of the cells by freezing caused the elimination of the ichthyotoxic effect as was found for the raphidophyte *C. marina* (Ishimatsu et al., 1996).

Given the relatively long period before high mortality was reached, the exposure to living algae, both directly (involving endotoxins and exotoxins) and indirectly (involving exotoxins), seems a relatively slow process when considering sole larvae. A similar long response period was found by Pezzolesi et al. (2010) who studied the effect of an Italian *F. japonica* strain on juvenile sea bream (Table 4). These results were not in agreement with the results of Khan et al. (1996b) and Bridgers et al. (2004) (Table 4). Pezzolesi et al. (2010) suggested that this difference could be strain specific, but also the effect of different fish species and fish age and/or phase should be taken into account. Finally the fish tests with *F. japonica* done so far differed not only in fish species, fish age (Table 4) and *F. japonica* strains but also in experimental set up (Table 1). The comparison between our and other studies suggests that differences in ichthyotoxicity cannot be explained by differences in strains (Khan et al., 1996b; this study), fish age and/or phase (Pezzolesi et al., 2010; this study), light or salinity (Bridgers et al., 2004; Pezzolesi et al., 2010). Strain dependent differences were found for haemolytic activity and haemaglutination activity (de Boer et al., 2009; Guidi-Rontani et al., 2010). Unfortunately no fish tests were carried out in these latter studies. Fish species, temperature and the use of different media could explain the differences in ichthyotoxicity. Our temperature experiment (Fig. 3) suggests that temperature could be an important factor determining ichthyotoxicity. In general, the results of all fish tests so far imply increasing toxicity of *F. japonica* with increasing temperature up to 20 °C (Tables 1 and 4), as was also found for *Heterosigma akashiwo* (Ono et al., 2000). Studies on ichthyotoxicity of other marine raphidophyte species showed species specific differences due to temperature effects (Khan et al., 2001; Ono et al., 2000).

The highest total brevetoxin production per cell of *C. antiqua* and *H. akashiwo* was found at the lowest temperatures tested (15 and 10 °C), but changes in cell volume were not considered (Ono et al., 2000; Khan et al., 2001). This suggests that the production of brevetoxin per biovolume may not be affected by temperature. On the other hand, instantaneous hydrogen peroxide (H_2O_2) production by *H. akashiwo* was found to be positively correlated with temperature (Twiner and Trick, 2000). A respiratory and/or membrane bound process was suggested as the source of ROS formation (Palenik et al., 1987; Twiner and Trick, 2000). Yet, H_2O_2 was not produced in sufficient amounts by *H. akashiwo* to affect vertebrate cell lines but extracellular organic components did (Twiner et al., 2004, 2005). The ROS compounds superoxide (O_2^-) and H_2O_2 are produced by *F. japonica* strains as well (Oda et al., 1997; Marshall et al., 2005a). *F. japonica* produced intra- and extracellular H_2O_2 (at 26 °C) and showed higher H_2O_2 concentration in cell free filtrate than *H. akashiwo* (Oda et al., 1997). The temperature related differences in *F. japonica* toxicity, as observed in our study, should be further addressed in future studies, since this phenomenon is highly relevant when considering present

warming trends, as observed in Dutch coastal waters (see also below).

Ichthyotoxicity induced by *C. marina* seems to be the result of a combined effect of O_2^- and PUFAs produced by exponentially growing algae especially at densities between 10^3 and 10^4 cells mL^{-1} (Marshall et al., 2005b). Highest fish mortality due to exposure to *C. marina* cells was found at a cell concentration of 10^3 cells mL^{-1} (Marshall et al., 2003). The rate of production of both O_2^- and H_2O_2 by *C. marina* and *H. akashiwo* seemed to increase with cell density but at cell densities >1–2×10^4 cells mL^{-1} the production decreased again (Marshall et al., 2005b; Twiner and Trick, 2000). The ichthyotoxicity of *F. japonica* of the Dutch strain W420 was highest at a cell density of 1.8×10^4 cells mL^{-1}. It is tempting to assume that the ichthyotoxicity observed in our study was caused by a combined action of PUFAs and ROS. However, Khan et al. (1996b) found in the same strain at the same cell density also the highest cellular brevetoxin production, with PbTx-2 being the most important brevetoxin (Khan et al., 1996b).

After an exposure time of seven days PbTx-2 was more toxic to sole larvae than PbTx-3 (Table 3A, Fig. 4B). In addition, it was not possible to calculate an LC_{50} of PbTx-3 due to the fact that the tested concentrations were too low (Table 3A). Only a small difference in toxicity between these two toxins was described by Rein et al. (1994). Comparison between the studies showed that female mosquito fishes, *G. affinis*, were four times more sensitive to PbTx-2 than the sole larvae in this study (Table 3A). Own incidental brevetoxin analysis, using enzyme-linked immunosorbent assay (ELISA), revealed a maximum total brevetoxin concentration of only 11 ng mL^{-1} in the late exponential phase at 16 °C, and $\pm$ 5.5 ng mL^{-1} during the stationary phase cultured at 25 °C. This concentration is lower than reported at the same temperature in stationary phase by Khan et al. (1996b) and Bridgers et al. (2004). Nevertheless the used methods do not characterize the PbTx compounds and so HPLC traces (Khan et al., 1996b) and ELISA methods (Bridgers et al., 2004; this study) are not definitive and they cannot exclude that the results involve artifacts of another compound. Other techniques like LC–MS should be used to characterize the brevetoxins but with this technique nor Fu (2003) neither Pezzolesi et al. (2010) could characterize brevetoxins in *F. japonica* cells. Furthermore, differences in brevetoxin production of the Dutch strain could be explained when higher temperatures would stimulate brevetoxin production by *F. japonica* cells (Table 1), but this is not supported by our data and by experiments conducted with other species (Ono et al., 2000; Khan et al., 2001).

Given the low mortality by a PbTx-2 concentration of 10 ng mL^{-1} as observed in this study (Fig. 4B) and the low total brevetoxin concentration in our strain, it is not likely that the ichthyotoxic effect of our strain is caused by brevetoxin action alone. This contrasts with the studies by Bridgers et al. (2004) and Khan et al. (1996b) who proposed that brevetoxins was the major vector of *F. japonica* ichthyotoxicity. Behavioral studies of the sole larvae underline our suggestions and showed differences between control and exposure to *F. japonica* and PbTx-2 exposure. PbTx-2 exposure showed the very specific behavior of circling that was not seen in all other tests (data not shown). Also Pezzolesi et al. (2010) found 100% mortality within 1 day when fish were exposed to the cells of the brevetoxin producing alga *Karenia brevis* (maximum concentration of 1.17×10^3 cells mL^{-1}), much faster than the effect observed for *F. japonica*.

The toxic effect of the three known PUFAs; AA, EPA and OTA on sole larvae was much faster than observed in the other fish tests of this study (Fig. 4A). A large difference in mortality was found between 4 and 22 h and therefore in future research mortality should be determined on an hourly basis using a larger dilution series, in order to obtain more accurate LC_{50} values. The order of toxicity of these 3 compounds as was found in this study, AA > EPA > OTA, was the same as described earlier (Takagi et al., 1984; de Boer, 2006). Arzul et al. (1995) found the same toxicity for AA and EPA, which were both found to be more toxic that OTA. Nevertheless the LC_{50} found for *A. polycanthus*, damselfish, exposed to EPA showed that damselfish are 19 times more sensitive than sole larvae (Table 3B). Here, the difference in sensitivity was even higher than found for brevetoxins, possibly due to the poor development of the gills of sole larvae. It was suggested before that the first effects of PUFAs on fish, together with ROS, was gill epithelium damage (Marshall et al., 2003). The 100% mortality due to *F. japonica* was much slower than the found effect of the three pure PUFAs but could be due to a lower concentration of these PUFAs. Our Dutch *F. japonica* strain W420 produces 1.12 μg mL^{-1} AA; 1.37 μg mL^{-1} EPA and 2.45 μg mL^{-1} OTA at a cell density of 2.2×10^4 cells mL^{-1} (de Boer et al., 2009). These concentrations are realistic toward ichthyotoxicity of sole larvae, especially in combination with ROS production (Marshall et al., 2005a; Pezzolesi et al., 2010). In addition, *F. japonica* produces more haemolysins than the three tested PUFAs (de Boer et al., 2009), however these haemolysins are not characterized yet and their ichthyotoxicity remains unknown. Overall the observed trends of the total haemolytic activity (de Boer et al., in press) and ichthyotoxicity in the direct exposure over the different growth phases are not similar. In case of *F. japonica*, haemolytic tests, using rat, human or fish red blood cells are not good alternatives to replace the fish tests since the haemolytic activity did not coincide with fish mortality. The trend in the direct fish tests over the different growth phases found in this study compares more to the produced toxins analyzed (so not characterized) as brevetoxin by Khan et al. (1996b). Assuming that PbTx-2 is the most produced brevetoxin by *F. japonica* we postulate that brevetoxin alone was not responsible for 100% fish mortality, as the amount of total brevetoxin was too low in the *F. japonica* culture. In contrast, *F. japonica* produces enough PUFAs to cause fish death in 7 days, especially in combination with ROS production. Not the change in brevetoxin (Khan et al., 1996b) but the decrease in production of peroxide could also explain the decrease in toxicity after the late exponential phase. In summary, *F. japonica* seems most ichthyotoxic when residing in close proximity of the fish. This implies that future experiments including toxin analysis should be executed based on fish presence.

In our study sole larvae were used due to its presence in Dutch coastal waters. In these waters, the highest recorded *F. japonica* concentration is 1.2×10^2 cells mL^{-1} but monitoring data show an increasing trend (de Boer, 2006). However, the present concentrations are ten times lower than the concentrations causing any larval mortality in our study. Furthermore, the *F. japonica* cell concentration needed to generate the lethal ichthyotoxin concentrations like PUFAs (de Boer et al., 2009) are not reached in Dutch coastal waters. Yet, Peperzak (2003) predicted that by the year of 2100 conditions in the Dutch coastal zone would become more favorable for *F. japonica*, leading to a doubling in growth rates. Mesocosm experiments showed that not only *F. japonica* but also other marine raphidophytes became the most dominant species at the predicted changing environmental conditions, increasing water temperature and decreasing salinity (Peperzak, 2003). In addition to this, *F. japonica* may have an increased chance of surviving during winter, since the lower temperature limit for survival now match the winter temperatures in the warming North Sea and Wadden Sea (de Boer, 2006). In other regions, like the Adriatic Sea much higher cell *F. japonica* concentrations (4.6×10^4 cells mL^{-1}) have been observed (ARPAM, 2001). Here, *F. japonica* cell densities are high enough (>1.8×10^4 cells mL^{-1}) to generate lethal toxin concentrations *in situ*.

In conclusion, our research suggests first of all pronounced ichthyotoxicity at direct exposure in late exponential *F. japonica* cells, related primarily with the combination of endo- and exotoxins, with most probably not brevetoxins but, haemolytic PUFAs as the main endotoxins, and other haemolysins and ROS as the main exotoxins. It also shows that the combination of endo- and exotoxins showed 100% mortality while endo- or exotoxins alone did not. Secondly, *F. japonica* may become problematic in Dutch coastal waters, since the already observed temperature increase may not only enhance *F. japonica* survival during winter and its growth during spring and summer, but may also increase its toxicity. Higher temperatures will decrease the exposure time necessary to cause fish mortality. Moreover the important fish nursery grounds, including the sole nursery grounds, of the Dutch coastal waters become more sensitive to harmful algal outbreaks by short winters, and a faster increase in water temperature during spring up to temperatures >20 °C, when fish larvae are still in the area.

Acknowledgments

Thanks are due to Solea BV especially Ewout Blom and Jan van der Heul for maintaining fish larval batches of sole at IMARES and Solea BV in IJmuiden. We gratefully acknowledge Dr. Hans van de Vis for his support with animal ethics; Gemma Kulk for valuable lab support; Prof. Dr. Jerome Naar (UNCW, North Carolina, USA) for providing ELISA test and Dr. ir. Marc Engelsma, (Central Veterinary Institute, department of fish diseases, Wageningen University, The Netherlands) for his observations of the histological samples. The authors thank the anonymous reviewers for the valuable comments on the manuscript. This research was funded by the Dutch National Science Foundation; Earth and Life Science (NWO-ALW) project 815.01.004.[SS]

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