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PRIMARY RESEARCH PAPER

Evidence for inhibition of bacterial luminescence by allelochemicals from *Fibrocapsa japonica* (Raphidophyceae), and the role of light and microalgal growth rate

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Abstract The marine microalga *Fibrocapsa japonica* Toriumi and Takano (Raphidophyceae) produces haemolysins, neurotoxins and reactive oxygen species (ROS). To quantify potential effects of such bioactive compounds on surrounding organisms the marine bacterium *Vibrio fischeri* was exposed to *F. japonica* culture samples. Inhibition of *V. fischeri* 's natural luminescence, indicative of impaired metabolism, was related to the number of *F. japonica* cells added. The effect was fast, within 15 min. It was caused by one, possibly several, excreted substances that were less active after heating. Freezing of culture supernatant partly inactivated these substances, but ROS-scavenging enzymes had no effect. Light enhanced the *V. fischeri* luminescence inhibition in two ways. The direct effect of light on the action of *F. japonica* luminescence inhibiter(s) could be described by a saturation curve with maximum effect above 20 μmol photons m^{-2} s^{-1}. Light also had an indirect effect: biomass production, dependent on light availability, was closely related to the amount of inhibiting compound(s) produced by the alga. Algal growth rate, rather than its cell density, determined the bacterial luminescence inhibition per *F. japonica* cell, resulting in a 5-fold stronger inhibition at maximum growth rates compared to cells that barely grew during the stationary growth phase. The bioassay with *F. japonica* and *V. fischeri* has allowed quantification of the negative effects on bacteria in the microalgal microenvironment. The results presented here suggest that at favourable growth conditions *F. japonica* releases bioactive compounds that improve its competitive abilities.

Keywords Allelopathy · Raphidophytes · *Vibrio fischeri* · Bioassay · Bio-Tox

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Introduction

Fibrocapsa japonica Toriumi and Takano (1973), is a microalga of the class Raphidophyceae with a worldwide distribution in temperate regions (de Boer et al., 2005). *Fibrocapsa japonica* blooms have been related to massive mortality of economically important fish stocks in the Seto Inland Sea of Japan (Iwasaki, 1971; Okaichi, 1972, 1989; Toriumi & Takano, 1973; Cho et al., 1999). Toxic events have also been reported since 1990 along the German coast and in the Mediterranean Sea (European Commission, 2003).

The reason why fish die in the presence of *F. japonica* has been ascribed to toxic and/or mechanical 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), haemolytic compounds (Fu, 2003; Fu et al., 2004a, b), mucocyst ejection (de Boer et al., 2005) and possibly combinations of these (de Boer et al., 2004; Marshall et al., 2005b). The production of bioactive compounds by plants is often associated with chemical deterrence of competitors and/or predators and this could be the case for *F. japonica* as well. When phytoplankton interact, grazer deterrence is distinguished from allelopathy defined as "the chemical interaction among competing microalgae and bacteria in which one or more allelochemicals inhibit the growth of competing algae or bacteria" (Legrand et al., 2003).

A number of studies on raphidophytes is in support of survival mechanisms involving allelopathy. Oda et al. (1992) found that *Chattonella marina* affected growth and viability of the bacterium *Vibrio alginolyticus*, an effect they attributed to the production of ROS by the algae. In short term measurements superoxide and peroxide were shown to be produced. Addition of superoxide dismutase and catalase scavenged the radicals and alleviated growth inhibition of *V. alginolyticus* during incubation with *C. marina* (Oda et al., 1997). *Olisthodiscus luteus* affected *V. alginolyticus* in the same way (Kim et al., 1999). *Fibrocapsa japonica*, like the other raphidophytes, produces O_2^- and H_2O_2 during growth and also inhibits growth of *V. alginolyticus* (Oda et al., 1997; Marshall et al., 2005a). Live *H. akashiwo* as well as spent medium inhibited growth of *Skeletonema costatum*, which may explain why these species are never abundant simultaneously (Pratt, 1966; Honjo et al., 1978; Yamasaki et al., 2007).

Here we study the possibility of allelopathy as a competitive mechanism in *F. japonica*. Allelopathy is likely to be operative when bioactive compounds involved are (1) excreted from live cells, (2) effective at natural concentrations and (3) regulated in such a way that there is a trade-off between the effect on the one hand and the effort to produce them on the other. To study this possibility we used a bioassay based on the luminescence response of *Vibrio fischeri*.

More than 1,500 substances have been described that adversely affect bioluminescence of *V. fischeri* in tests commercially available for assessing the toxicity of water, soil or sediment samples (Parvez et al., 2006 and references therein). The mechanisms by which toxic compounds reduce the bioluminescence of the bacterium are still poorly understood. Light is emitted upon oxidation of reduced riboflavin phosphate by the enzyme luciferase. This bioluminescence is directly proportional to the metabolic activity of the bacterial population and any inhibition of enzymatic activity causes a corresponding decrease in bioluminescence. A substance that is toxic for *V. fischeri* often demonstrates similar toxic effects on other organisms, despite the existence of different toxicity mechanisms for various organisms of different species (Kaiser, 1998; Botsford, 2002; Parvez et al., 2006).

A bioassay with *V. fischeri* seemed ideal to quantify potential effects of bioactive compounds excreted by live *F. japonica* on surrounding organisms. We needed a sensitive assay that could be used in seawater in a pH range between 7 and 9, and at a temperature optimal for growth of the alga, between 16.5°C and 24.5°C (de Boer et al., 2005). Standard assays with the marine bacterium *V. fischeri* are usually run at 15°C (Perez et al., 2006) and pH has no effect on light emission by *V. fischeri* within the range 5–9 (Fulladosa et al., 2004). Polyunsaturated fatty acids (PUFAs) and ROS which are produced by raphidophytes including *F. japonica* (Oda et al., 1992) inhibit *V. fischeri* luminescence (Arzul et al., 1995; Marshall et al., 2005b). Moreover, these raphidophytes inhibit growth of another *Vibrio* species, *V. alginolyticus* (Oda et al., 1997). Thus, the bioassay with *V. fischeri* was adjusted to quantify effects of bioactive compounds excreted by *F. japonica* to provide ecologically relevant data on the possibility of allelopathy as a competitive mechanism in *F. japonica*.

Materials and methods

Algal culturing and sampling

Strain CCRuG-Cl3 (culture collection University of Groningen, the Netherlands) is a non-axenic clonal *Fibrocapsa japonica* culture started in 1997 by isolating one cell from a monospecific bloom in the harbour of Büsum, Germany, in 1995 (courtesy U. Tillmann). The culture medium f/2-Si consists of autoclaved seawater enriched with nutrients (Guillard, 1975). Cultures were routinely maintained in a climate chamber at 17 ± 1°C under long-day (16:8 h

LD) conditions, at a salinity of 25 practical salinity units (psu) and a photon flux density of 35 ± 5 µmol photons m^{-2} s^{-1} provided by fluorescent tubes (L36W/19 light, Osram). The irradiance was measured with a Li-Cor quantum meter equipped with a cosine collector. Stock cultures were transferred every month.

Culture samples were taken after careful swirling of the culture flasks to avoid shear stress that tends to result in the extrusion of mucocysts that initiate aggregate formation. To obtain cell free culture fluid normal filtration procedures could not be used due to the fragility of the cells. Therefore, reverse filtration was applied by carefully lowering a cylinder that was covered at the bottom with a polycarbonate 2.0 µm filter (47 mm diameter, Osmonics Inc.) into the culture. The difference in fluid levels caused the cylinder to fill slowly with medium containing components < 2.0 µm, keeping intact cells out. Samples were then taken from the inside of the cylinder. They are referred to as supernatant in this publication.

To assess cell density and total biovolume, subsamples (5 ml) were taken after careful swirling of the cultures (1 h after the dark period). Analyses were performed with a particle counter (Coulter Counter ZM) equipped with the 'channelyser 256' of Coulter and a 100 µm aperture (Coulter Electronics Ltd.). When the cell density was too high for accurate estimates, samples were diluted in 0.2 µm filtered artificial seawater of 25 psu. Per sample four counts of 500 µl were made within 1 h after sampling. Particle size was calibrated with latex spheres 8.7 µm in diameter (Coulter Electronics Ltd.) and regularly checked by microscopic measurements.

Bioluminescence bioassay

The bioassays were carried out with the marine bacterium *Vibrio fischeri* (NRRL B-11177) that is commercially available in the Aboatox BO 1243–500 Bio-Tox™ kit. This kit is designed to determine toxicity of water samples. The lyophilised bacteria from one vial of the kit were reconstituted at 4°C for 30 min in 6.25 ml f/2-Si medium followed by 30 min incubation at 17 ± 0.5°C prior to use. We used medium instead of the salt solution (diluent) supplied in the kit because the bacterium had a higher luminescence in the medium that was used as a control in the experiments (Fig. 1). The tests were carried out in 96-well plates (Greiner, flat bottom polystyrene microtiter plates). In each well 25 µl of the bacterial suspension was injected using a combitip plus pipette (Eppendorf). After 15 min at 17°C 275 µl of the sample to be tested was added (275 µl of medium was added in the controls, $n = 4$ for test samples and controls). No salt was added to the test samples since they already had the same salinity as the revived bacterial suspension. Due to the improved luminescence in f/2-Si (2.5 times higher than the value at the manual conditions) and the use of a small incubation volume (300 µl instead of 1.0 ml) about 250 tests could be run using one vial of lyophilized *V. fischeri*. Luminescence was measured with a Wallac 1420 Multilable Counter Victor™ using the manufacturer's luminescence protocol. The microtiter plates were incubated for 15 min at the experimental conditions agreeable for both organisms (17°C and a photon flux density of 35 ± 5 µmol photons m^{-2} s^{-1} unless stated otherwise, see experiments below) and measured again. The inhibition effect (% of control; INH%) was calculated on the basis of luminescence intensity after 15 min contact time corrected for the luminescence decay in the control samples which contained f/2 medium:

$$INH\% = 100 - \frac{IS_{15}}{(IC_{15}/IC_0) * IS_0} * 100$$

where INH% is the inhibition of luminescence of the tested sample compared to the control; IC_{15} the luminescence intensity of control in f/2 medium after contact time (15 min) in relative luminescence units

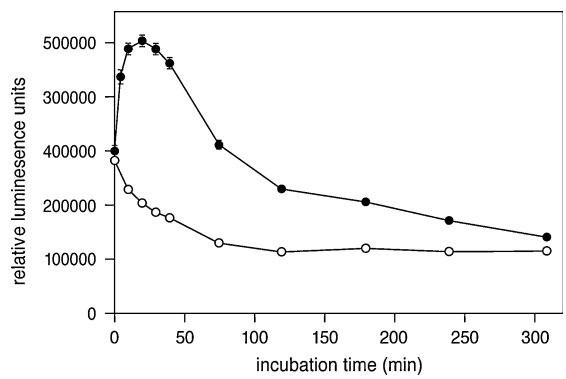

Fig. 1 Relative luminescence measured after adding f/2-Si medium (•) or diluent supplied by the manufacturer of the Bio-Tox™ kit (○) to lyophilised *Vibrio fischeri* bacteria (error bars represent standard deviation, $n = 2$)

(RLU); IC_0 the initial luminescence intensity of bacterial suspension in RLU; IS_{15} the luminescence intensity of test sample after contact time (15 min) in RLU; IS_0 the initial luminescence intensity of the bacterial suspension in RLU.

When *F. japonica* cells were present in the test sample another correction for IS_{15} was necessary to compensate for light scattering. Luminescence in the bioassay is generated by the bacteria throughout the well and the average light path is half the path of the absorbance measurement with the light source at the bottom of the well. Therefore, the extinction at 490 nm (average wavelength of the emitted luminescence) was measured against f/2 medium blanks and divided by two. Transmission (T) was calculated using the equation: $E_{490} = -\log T$ and T could be used in the overall equation:

$$INH\% = 100 - \frac{IS_{15}/T}{(IC_{15}/IC_0) * IS_0} * 100$$

To determine the sensitivity of this bioassay to brevetoxins a brevetoxin standard PbTx 2 (Calbiochem) up to a concentration of 10 mg ml^{-1} was assayed. Also a sample of a growing culture of *Thallassiosira weisflogii* was assayed at standard conditions. The diatom (courtesy S. Hazelaar) was cultured under similar conditions, but with the addition of 10.7 mM Si in the f/2 medium.

Experiments

Dilution series and initial characterization

The response of *V. fischeri* to increasing amounts of *F. japonica* was studied with a dilution series of an exponentially growing culture of the raphidophyte (cell density 1.8×10^4 cells ml^{-1}). Dilution of samples with medium was done in 1.5 ml cups prior to the addition of 275 µl to the 25-µl bacteria suspension in the test. Dilution series were also made of the cell-free supernatant. Another culture that was close to the stationary phase of growth (cell density 4.0×10^4 cells ml^{-1}) was used to characterize the bioactive compounds. Cell-free supernatant was obtained by reverse filtration (2 µm filter) and aliquots of 2 ml (in 2.5 ml Eppendorf cups) were treated in different ways. Two were put next to the culture sample in the culture cabinet, two were heated for 10 min at 100°C in a water bath, and two were frozen (−20°C). Samples from culture, supernatant and boiled supernatant were assayed simultaneously 1 h after the samples were taken from the culture; 5 days later the frozen supernatants were thawed and assayed.

Role of reactive oxygen species

The enzymes catalase and superoxide dismutase SOD (both from Sigma) were used to investigate involvement of ROS in luminescence inhibition. One millilitre enzyme diluted with medium was added to 1 ml *F. japonica* culture (cell density at sampling 5×10^4 cells ml^{-1}), resulting in a catalase activity of 1, and 0 up to 100 U ml^{-1} in steps of 10. SOD was added to have an activity of 1, 10 and 100 U ml^{-1}. Series were incubated at room temperature for 15 min before taking subsamples of 275 µl that were added to the standard 25 µl *V. fischeri* suspension in the bioassay. A series of solely catalase with medium instead of *F. japonica* culture was used as a blank to correct for the colour of the catalase solution.

Density-dependent effects

To evaluate the *V. fischeri* luminescence response on *F. japonica* cultures at different cell densities and differences in growth phase, a total of 25 *F. japonica* culture samples were assayed using undiluted samples (i.e. 275 µl from the culture was directly added to 25 µl bacterial suspension). Eight of these cultures (500 ml) were inoculated at the same time from the same stock culture and harvested at different moments in time.

Light experiments

The influence of light on the effect of *F. japonica* on *V. fischeri* bioluminescence during the 15 min bioassay was studied for a range of irradiance levels (0–100 µmol photons m^{-2} s^{-1}) in undiluted samples of a growing *F. japonica* culture (cell density 3.6×10^4 cells ml^{-1}; Fig. 2, experiment 1). To investigate the effect of light on the production of bioluminescence inhibiting components excreted by *F. japonica* cells (Fig. 2, experiment 2), the culture was divided into six subcultures of 50 ml that were incubated for 24 h at a the same range of irradiance levels during the light periods: 15 h light, 8 h dark, 1 h light. After the 24 h

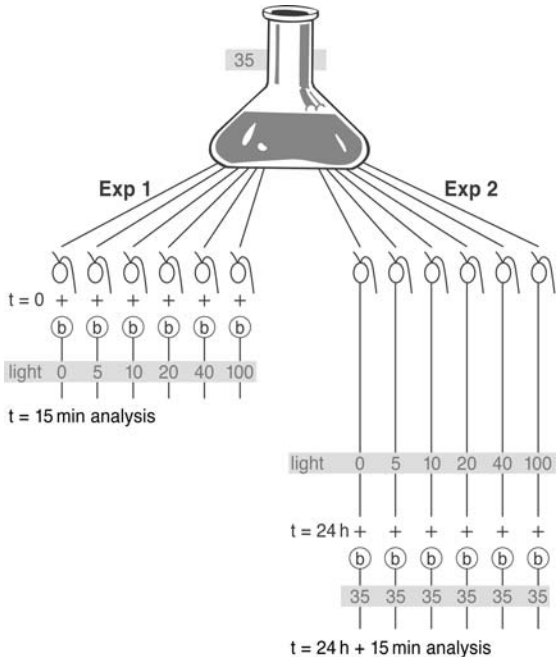

Fig. 2 Experimental set up of experiment 1: a test to investigate the effect of light upon the *Fibrocapsa japonica* (flagellate symbol) induced luminescence inhibition of *Vibrio fischeri* (symbol b) in the bioassay performed on samples from a growing culture; experiment 2: a test to investigate the influence on the production of *Vibrio fischeri* luminescence inhibitors by *F. japonica* subcultures growing for 24 h at different light intensities (assayed at standard light conditions)

incubation of the *F. japonica* subcultures, samples were taken for cell density measurements and total biovolume, as well as for bioassays performed at the irradiance level of the bioassay incubation (35 µmol photons m^{-2} s^{-1}; Fig. 2).

Statistical analysis

Regression analysis was performed on the data using SPSS 13 for Windows. In case of the culture density for undiluted culture samples a log transformation on the data was performed to study possible non-linearity. A dummy variable was used for comparing of subsets of data.

Results

Bioassay development

Luminescence of *V. fischeri* was stimulated by the medium in which we cultured the alga *F. japonica* compared to the luminescence in the sodium chloride solution provided in the kit (diluent; Fig. 1). This resulted in negative inhibition values compared to the blank, unless we used a proper control, i.e. f/2-Si medium instead of the diluent supplied by the manufacturer. The adjustment had another advantage: the sensitivity of the assay was enhanced 2-fold.

To be able to correct for loss of luminescence compared to the control caused by absorbing and scattering by the algal cells added, we measured the absorbance at 490 nm (average wavelength of the emitted light). From these measurements we could calculate transmission values needed to correct for light losses due to the addition of the cells in the incubation mixture. In Fig. 3 a number of dilution series (cultures growing at different light levels to get the full range of possible cell sizes that may influence scattering in a different way) were used to make a transmission calibration curve to correct for absorbed and scattered light by *F. japonica* cells. The formula is Transmission = -1.84×10^{-6} * culture density (cells ml^{-1}) + 1 ($r^2 = 0.812$, $n = 55$, $P < 0.00001$).

Experiments

Dilution series and fractionation

Inhibition of *V. fischeri* luminescence by an exponentially growing *F. japonica* culture was proportional to the amount of algal culture added to the bioassay (Fig. 4). The same was true for the amount of cell-free supernatant present in the assay (Fig. 4).

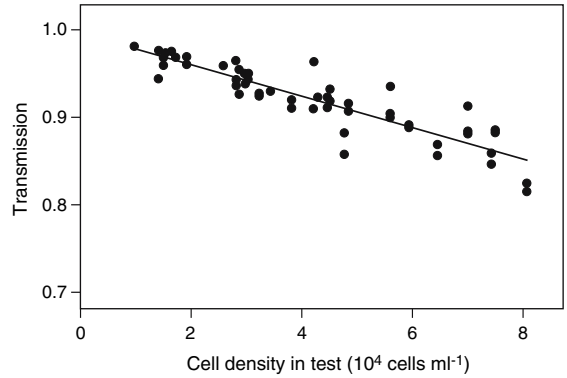

Fig. 3 Light-transmission at 490 nm versus the cell density of *Fibrocapsa japonica* samples

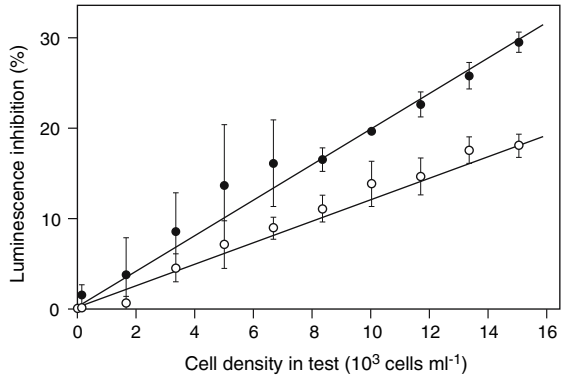

Fig. 4 *Vibrio fischeri* luminescence inhibition after addition of *Fibrocapsa japonica* cultures from a dilution series of an exponentially growing batch culture (● Culture inhibition (%) = 0.0019 * cell density, r^2 = 0.977), ○ supernatant inhibition (%) = 0.0012 * cell density, r^2 = 0.950, error bars represent standard deviation for n = 2)

The contribution of the supernatant luminescence inhibition to the inhibition measured for the culture was 64% (calculated from the slopes of the regression lines depicted in Fig. 4). Doubling of the contact time between samples (culture or supernatant) and bacteria gave the same inhibitory response, indicating that the effect was already complete within the standard incubation time (15 min). Culture samples of the diatom *Thallassiosira weisflogii* did not inhibit *V. fischeri* luminescence.

Density-dependent effects

None of the 25 *F. japonica* cultures growing under standard conditions caused more than 50% inhibition of *V. fischeri* luminescence (Fig. 5A), yet some of these cultures were at more than twice the cell density of the culture in the exponential phase used for the dilution series (Fig. 4). Luminescence inhibition induced by undiluted *F. japonica* cultures increased with the log of the density of the cultures (n = 25, r^2 = 0.300, P = 0.01; regression line of log transformed culture densities in Fig. 5A). There was a large variation in luminescence inhibition by high-density cultures causing a rather poor fit of the data to the regression line. However, a subset of the data (circled points in Fig. 5A) that was from a series of *F. japonica* cultures (n = 8) that were initiated with the same inoculum and harvested at different points in time followed the general trend in Fig. 5A indicating

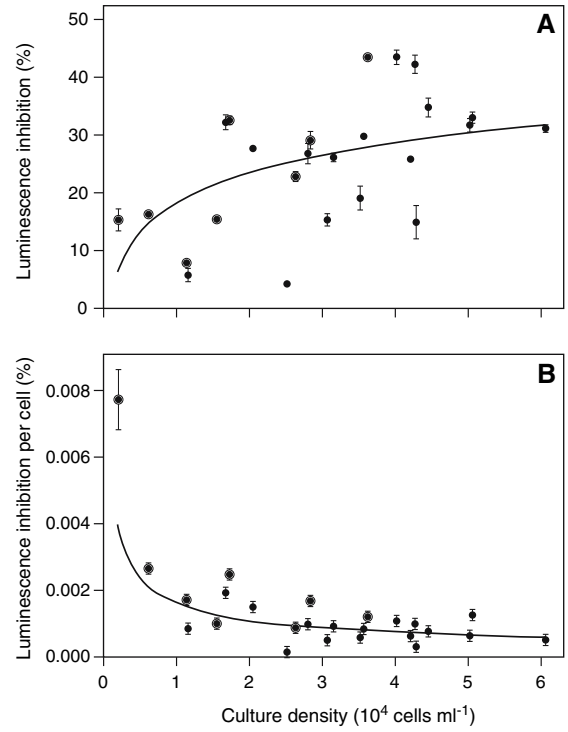

Fig. 5 Relationship between the cell density of *Fibrocapsa japonica* batch cultures and their inhibition of *Vibrio fischeri* luminescence (**A**) response per culture, (**B**) response per *F. japonica* cell. Circled dots are from one data set of eight *F. japonica* cultures that were initiated with the same inoculum and sampled at different points in time, the trend line in (**B**) is based on the regression line in (**A**) Inhibition (%) = 16.99 * Log (cell density) − 49.6, error bars represent standard error, $n \geq 4$

that a growing batch culture has an increasing capacity to inhibit bacterial luminescence (one regression model for the sets of the 8 and 17 cultures revealed no significant difference between the data sets, $F_{1,22}$ = 1.06, P = 0.315). In the exponential phase of growth at low densities the cells had a much higher luminescence inhibition compared to high density cultures; a 15-fold difference between the highest and lowest value measured and a 5-fold difference based on the trend line in Fig. 5B that was derived from the regression line in Fig. 5A.

Initial characterization

To investigate the nature of compounds responsible for the inhibitory effect we tested the cell-free supernatant of a dense culture close to the stationary

phase of growth. Supernatant contributed 75% of the culture inhibitory effect which was higher than the 64% measured for the culture in Fig. 4. Boiling for 10 min inactivated the supernatant compound(s) for 80% (Fig. 6). Aliquots of the supernatant were frozen for 5 days at −20°C to see if the compound(s) remained active during storage in the freezer. Thawed samples inhibited *V. fischeri* luminescence at 71% of the original activity. Increasing amounts of catalase or superoxide dismutase added to bacteria and algae with the purpose to scavenge peroxide/superoxide radicals did not change the inhibitory effect of the culture ($n = 12$, $P = 0.125$, resp. $n = 3$, $P = 0.61$ data not shown). Brevetoxin standard (PbTx 2) up to a concentration of 10 mg ml^{-1} did not inhibit *V. fischeri* luminescence.

Light experiments

The influence of light on inhibition by *F. japonica* on bacterial luminescence was examined in two ways. The direct effects of light (Fig. 2, experiment 1) were studied on samples of an exponentially growing *F. japonica* culture for a range of irradiance levels. Part of the inhibitory effect appeared to be affected by light: a culture at 20 µmol photons m^{-2} s^{-1} caused a luminescence inhibition of 30.2 % (Fig. 7, equivalent to 8.6×10^{-4}% $cell^{-1}$), almost twice the value compared to the dark incubation (17.3%, equivalent to 5.1×10^{-4}% $cell^{-1}$). More light, however, did not result in higher values (Fig. 7).

To study the effect of light on the production of inhibitory substances (Fig. 2, experiment 2) six *F. japonica* subcultures of light experiment 1 (direct light effects) were allowed to grow for 24 h at the same range of irradiances but assayed at saturating light conditions for the direct effect (35 µmol photons m^{-2} s^{-1}, the standard light condition). In this case the amount of light received during the 24 h incubation stimulated inhibition up to a maximum level of 25.5% (6.6×10^{-4}% $cell^{-1}$) at 40 µmol photons m^{-2} s^{-1}. Maximum levels were slightly lower compared to those of experiment 1, the previous day (28.8% corresponds to 8.2×10^{-4}% $cell^{-1}$), but luminescence inhibition was completely absent during the dark incubation. Cells of the *F. japonica* cultures receiving more light during the 24 h incubation were bigger (biovolume per cell from 3050 μm^3 to 4600 μm^3), but there was no effect of the light treatment on the cell density of the six subcultures after the 24-h growth period (cell density = 42×10^3 cells ml^{-1}, $P = 0.72$, data not shown); the division rate was the same for each subculture (0.18 d^{-1}). Potentially the observed effect of light on

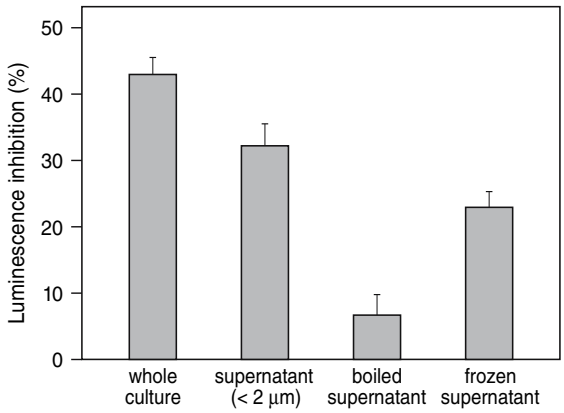

Fig. 6 Characterization of the nature of the compounds that inhibit *Vibrio fischeri* luminescence. *Fibrocapsa japonica* samples of a batch culture close to the stationary phase of growth were treated in different ways and tested for their inhibitory effect (error bars represent standard deviation for $n = 4$)

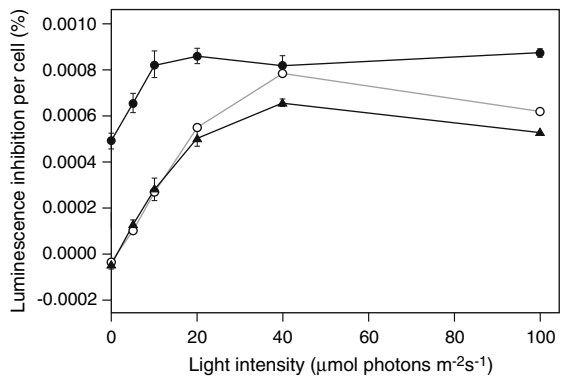

Fig. 7 Effect of light on the action and production of compounds made by *Fibrocapsa japonica* that inhibit *Vibrio fischeri* luminescence (see experimental set-up in Fig. 2). Black dots represent inhibition by samples from a *F. japonica* culture incubated at different light intensities during the bioassay (exp. 1). Triangles represent *V. fischeri* luminescence inhibition of samples taken from six subcultures of the *F. japonica* culture that were incubated for 24 h at the different light intensities and measured at saturating light conditions (exp. 2, error bars represent standard deviation for $n = 4$). Open circles are values for the latter data set calculated for hypothetical cells with the same average volume, for assumptions see text

the inhibitory effect could be the result of differences in the amount of biovolume (directly related to biomass) present and the effect would disappear when correcting for these biovolume differences. Assuming a linear correlation between biovolume and fluorescence inhibition per cell allowed calculation of inhibition by hypothetical cells of the same size. The average size of cells in the six cultures (3900 μm^3) that was the same as the cells had the day before in the direct light experiment was used for the calculation. The result of this exercise is presented in Fig. 7. Biovolume by itself is apparently not the cause of the differences observed in luminescence inhibition of the cultures that grew at different light intensities, because the saturating effect of light on inhibition is still there.

Growth rates based on total biovolume of the cultures were increasing up to 40 µmol photons m^{-2} s^{-1} and there was no indication of growth inhibition at 100 µmol photons m^{-2} s^{-1}. Growth rates were closely related to the luminescence inhibition on a per algal cell basis (Fig. 8, $n = 6$, $r^2 = 0.806$, $P = 0.0154$).

There was no effect of light on the performance of *V. fischeri* in the bioassay: IC_{15}/IC_0, the luminescence loss of the control with medium, was constant for the whole light range ($IC_{15}/IC_0 = 0.72$, $n = 12$, $P = 0.196$, data not shown).

Discussion

Vibrio fischeri from the Bio-Tox™ kit was used for the quantitative measurement of negative effects imposed on bacteria by live *Fibrocapsa japonica* cells but the standard assay had to be adjusted for this application. The diluent to revive the dormant bacteria was replaced by the seawater based algal medium to have a proper control. The seawater stimulated the bacterial luminescence, a phenomenon reported earlier with the use of seawater in a Microtox analyser (Derby et al., 2003; Onorati & Mecozzi, 2004). To correct for absorbance and scattering of luminescence by the algal cells added to the bacterial suspension the extinction of an algal control had to be measured at 490 nm, the wave length of the emitted light produced by *V. fischeri*.

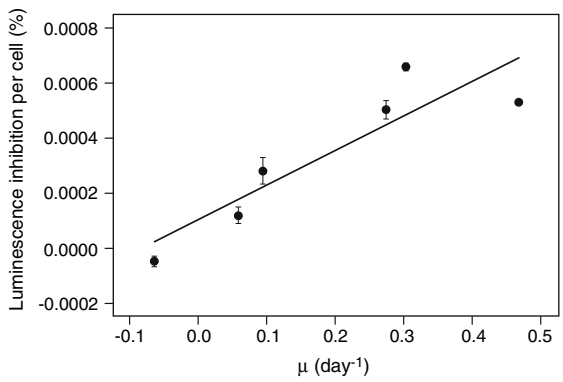

Fig. 8 The effect of the growth rate based on biomass production of *Fibrocapsa japonica* cells on *Vibrio fischeri* luminescence inhibition per *F. japonica* cell (error bars represent standard deviation for $n = 4$)

More than half of the luminescence inhibition imposed by *F. japonica* culture was present with supernatant alone. Apparently, the active compound(s) is (are) excreted but part of it remains associated with the *F. japonica* cells. Continued excretion of active compounds by the cells during the incubation is less likely because inhibition was rapid (within 15 min) and doubling of the bioassay time did not enhance luminescence inhibition. Culture samples of the diatom *Thallassiosira weisflogii* cultured under the same conditions did not inhibit *V. fischeri* luminescence.

Since *V. fischeri* luminescence was not inhibited by the addition of the brevetoxin PbTx 2, it seems unlikely that the inhibition of *V. fischeri* luminescence was caused by this main neurotoxin produced by *F. japonica* (Khan et al., 1996). Derby et al. (2003) reported 95% inhibition by adding partly purified PbTx 3 from *Karenia brevis* cell extract in a similar bioassay. However, the brevetoxin fraction used could have been contaminated by bioactive compounds other than brevetoxins that have been described for this species (Kubanek et al., 2005; D.F. Martin, pers. comm.).

Chattonella marina, another raphidophyte, affects growth, viability and luminescence of *Vibrio* species. This effect has been attributed to ROS production by *C. marina*, perhaps in combination with polyunsaturated fatty acids (PUFAs; Oda et al., 1992; Marshall et al., 2005b). Luminescence inhibition of *V. fischeri* by *C. marina* was comparable to *F. japonica* inhibition (28% at 2×10^4 cells ml^{-1}, Marshall et al., 2005b). Viability of *V. alginolyticus* was apparently protected when superoxide dismutase and/or catalase were added, a phenomenon also observed for effects

of the raphidophyte *Olisthodiscus luteus* on *V. alginolyticus* (Kim et al., 1999). Rupturing of *C. marina* cells enhanced the viability of the bacteria (Oda et al., 1992), which made them conclude that only intact cells produced the ROS. Rupturing of cells also eliminated the toxic effect of *C. marina* on fish (Ishimatsu et al., 1996).

O_2^- and H_2O_2 are produced by *F. japonica* strains (Oda et al., 1997; Marshall et al., 2005a). Scavenging of these radicals completely removed the inhibition of *V. alginolyticus* in one strain (Kagoshima Prefectural Fisheries Station, Japan), but these radicals do not seem to play an important role in luminescence inhibition in our *F. japonica*: scavenging of ROS by catalase or SOD had no effect. Bioactive substance(s) were still active after heating and freezing (20% and 70% of original activity), characteristics that argue against a role for ROS that are chemically unstable. Twiner et al. (2001) concluded that peroxide was not produced in sufficient amounts by the raphidophyte *Heterosigma akashiwo* to affect vertebrate cell lines but extracellular organic components did (Twiner et al., 2004, 2005).

Harmful PUFAs have been found in many raphidophyte extracts, including the *F. japonica* used here (Marshall et al., 2003; Fu et al., 2004a). It is tempting to speculate that these compounds are involved in the effect on bacteria in the present study. Several PUFAs are known to be haemolytic, and inhibit bacterial luminescence (Arzul et al., 1995; Marshall et al., 2005b). Excretion of PUFAs by live *F. japonica* would require active release of these compounds after conversion from precursors that are structural parts of the cell membrane (Jüttner, 2001), which is until now only observed as a result of cell damage. Passive leakage of PUFAs during cell division is not a likely mechanism because the magnitude of the inhibitory effect per *F. japonica* cell was proportional to the rate of biomass increase (Fig. 8), not to division rate.

Light is known to affect both the production and the toxicity of some bioactive compounds (Aanesen et al., 1998; Marshall et al., 2001; Wiegman et al., 2001), including the haemolysins present in methanol extracts of the raphidophytes *C. marina*, *H. akashiwo* and *F. japonica* (Kuroda et al., 2005). The purified haemolysin of *C. marina* lacked the characteristic PUFA light-absorption peaks; it may be a derivative of chlorophyll c pigment (Kuroda et al. 2005). Supernatant of *C. marina* cultures were not haemolytic. Excretion of such a cytotoxic component, however, would explain the direct effect of light on the inhibition reaction that we observed in our sensitive bioassays (Fig. 7). Light is also involved in the ichthyotoxicity of *C. marina* (Ishimatsu et al., 1996; Khan et al., 2001; Marshall et al., 2001).

Growth rate-dependent production of bacterial growth inhibitors was observed for the microalga *Fragilaria crotonensis* (Nakano, 1996) and for algal inhibitors excreted during growth of the producing microalga (Perez et al., 1999; Suikkanen et al., 2004; Nagasoe et al., 2006; Yamasaki et al., 2007). The inhibiting capacity per *F. japonica* cell was highest at low-cell densities in batch culture (Fig. 5B), when growth rates are highest. This observation fits favourably with the measured coupling between growth rate and luminescence inhibition per *F. japonica* cell (Fig. 8).

The results indicate that the production of inhibiting compounds is growth-rate regulated rather than density dependent. Such a regulation is different from the one often observed in the stationary phase of growth that may be triggered by nutrient stress (Gross, 2003; Legrand et al., 2003; Granéli, 2006). Ichthytoxicity induced by *C. marina* seems to be the result of a combined effect of PUFAs and superoxide caused by exponentially growing algae especially at densities below 1×10^{-4} cells per ml^{-1} (Marshall et al., 2005b). Remarkably, the inhibiton of *V. alginolyticus* growth by *C. marina* and *Olisthodiscus luteus* is also highest at low cell densities (Oda et al., 1992, 1997; Kim et al., 1999).

Conclusion

F. japonica produces one or more bioactive compounds that have a light-dependent inhibitory effect on *V. fischeri*'s luminescence. The nature of the compound(s) is unknown but brevetoxins and ROS are unlikely candidates. A light-dependent haemolysin, PUFAs or a combination of these could inhibit bacterial luminescence. The compounds are excreted into the medium and their production appeared to be growth regulated. These characteristics (active at natural concentrations, excreted by live cells, regulated production) indicate an allelopathic mechanism operative in *F. japonica.* If these compounds do harm

competing neighbours (bacteria and maybe other microorganisms) in the natural environment, this could be an additional advantage for *F. japonica* at favourable growth conditions to outgrow these microorganisms and develop a *F. japonica* bloom.

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