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Effects of light and food availability on toxin production, growth and photosynthesis in *Dinophysis acuminata*

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ABSTRACT: Diarrhetic shellfish poisoning (DSP) toxins constitute a severe health risk to shellfish consumers and a serious economic risk to the shellfish industry. The most prominent producers of DSP toxins are the mixotrophic dinoflagellates *Dinophysis* spp., which rely on chloroplasts from their prey, the ciliate *Mesodinium rubrum*; i.e. they are kleptoplastidic. Studies based on field collected material have indicated that cellular toxin contents in *Dinophysis* spp. vary intraspecifically by several orders of magnitude, but the regulating factors of cellular toxin contents are still poorly understood. We isolated 7 strains of *D. acuminata*, all of which produced pectenotoxin-2 (PTX-2) in the range of 12.7 to 35.6 pg cell^{-1} when supplied with abundant nutrients, light and food. One strain was selected for detailed studies on the effects of irradiance and food availability on photoacclimation, photosynthesis, growth and toxin production. Photoacclimation was detected for the first time in this genus, indicating some genetic control of sequestered chloroplasts. Growth and photosynthesis increased 4-fold with irradiances from 7 to 130 µmol photons m^{-2} s^{-1} after a minimum of 2 wk under ample food conditions. Despite this, cellular PTX-2 content increased only slightly from (mean ± SE) 4.6 ± 0.3 to 7.8 ± 1.1 pg PTX-2 cell^{-1}. Toxin production continued for up to 30 d after food was depleted, leading to a build-up of toxin in *D. acuminata* cells to 24.7 ± 1.7 pg PTX-2 cell^{-1}. Thus, we demonstrate that PTX-2 content is not directly coupled to irradiance and that PTX-2 production is not directly associated with ingestion of ciliate prey. Intraspecific variations in toxicity are instead most likely governed primarily by growth phase and genetic differences between different populations of *Dinophysis* spp.

KEY WORDS: *Dinophysis acuminata* · Diarrhetic shellfish poisoning · DSP · Mixotrophy · Pectenotoxin · PTX · Food availability · Light

INTRODUCTION

Diarrhetic shellfish poisoning (DSP) outbreaks have been reported frequently ever since the first documented accounts from Japan in 1976 and 1977 (Yasumoto et al. 1978). They appear more or less worldwide, with reported incidents from Asia, Australia, New Zealand, Africa, Europe and South, Central and North America (Reguera et al. 2012). Clinical symptoms include diarrhea, vomiting and abdominal pain, but human fatalities have never been linked to DSP incidents. However, DSP toxins are tumor promoting and thus constitute a severe, chronic threat to shellfish consumers (Suganuma et al. 1988).

A few years after its first discovery, DSP was linked to the dinoflagellate *Dinophysis fortii* (Yasumoto et al. 1980), and several species of *Dinophysis* have now been shown to contain DSP toxins (Reguera et al. 2012). Some benthic species of *Prorocentrum*, another dinoflagellate genus, are also capable of producing DSP toxins, but *Dinophysis* spp. are generally considered the primary causative organism of DSP

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(Murakami et al. 1982, Pan et al. 1999, Reguera et al. 2012). More than 200 species have been ascribed to the genus *Dinophysis* (Hallegraeff & Lucas 1988). Ten of these, as well as 2 species of the closely related genus *Phalacroma*, have been unambiguously reported to contain DSP toxins, but only 7 have been linked to DSP incidents (Reguera et al. 2012).

Two groups of toxins are produced by *Dinophysis*: (1) okadaic acid (OA) and its derivates, the dinophysistoxins (DTXs), and (2) the pectenotoxins (PTXs) (Tong et al. 2011). The toxicity of PTXs has, however, been questioned within the last decade since they do not appear to be toxic when ingested orally (Miles et al. 2004a). Yet, PTX is still subject to a regulatory threshold of 160 µg kg^{-1} mussel meat and thus retains the potential to put mussel harvesting on hold (European Council 2004).

Occurrence of causative species above a certain threshold often provides the grounds for closures of mussel harvesting, but this may not be a particularly well-suited method since *in situ* toxin contents of *Dinophysis* spp. are highly intra- and interspecifically variable. For example, *Dinophysis acuta* varied in OA content from 0.8 to 9.5 pg $cell^{-1}$ during ~1 mo of sampling at the same site off the coast of Western Iberia. Similarly, DTX-2 and PTX-2 varied from 1.0 to 6.6 and 0.4 to 3.1 pg $cell^{-1}$ respectively (Pizarro et al. 2009). In another recent study from off the SW coast of Ireland, *D. acuta* contained up to 16, 28 and 39 pg $cell^{-1}$ of OA, DTX-2 and PTX-2 respectively (Fux et al. 2010), and in Sweden, the same species has been found with no or very low levels of OA (Lindahl et al. 2007). Likewise, *in situ* populations of *D. acuminata* have also been found with highly different toxin contents. Blanco et al. (2007) found *D. acuminata* with high amounts of PTX-2 (180 pg $cell^{-1}$) as the sole toxin, Jørgensen & Andersen (2007) found it with varying, high amounts of OA (19 to 72 pg $cell^{-1}$) and no DTX (PTXs were not measured), and Kim et al. (2010) found it with varying contents of OA (0.4 to 0.9 pg $cell^{-1}$), DTX-1 (0 to 0.8 pg $cell^{-1}$) and PTX-2 (0 to 18.5 pg $cell^{-1}$) as did MacKenzie et al. (2005) (OA: 0.1 to 1.2 pg $cell^{-1}$, DTX-1: 0.1 to 2.4 pg $cell^{-1}$, PTX-2: 2.4 to 25.8 pg $cell^{-1}$). Additionally, cells of both *D. acuta* and *D. acuminata* may contain a suite of OA diol-esters and DTX and PTX isomers, often in unique combinations and/or quantities for every new sample or isolate (Vale & Sampayo 2000, Miles et al. 2006, Fux et al. 2011).

Dinophysis species (sensu stricto) all seem to contain chloroplasts of cryptophyte origin, and it was a mystery for a long time whether these chloroplasts were fully incorporated or whether they were sequestered through ingestion of cryptophyte chloroplast-containing prey. A breakthrough came in 2006, when Park et al. succeeded in culturing *D. acuminata* using the mixotrophic ciliate *Mesodinium rubrum* as prey (Park et al. 2006). *M. rubrum* itself preys on cryptophytes within the *Teleaulax/Plagioselmis/Geminigera* clade (Hansen et al. 2012). At present, 6 species are in culture worldwide (*D. acuminata*, *D. acuta*, *D. caudata*, *D. infundibulus*, *D. forti* and *D. tripos*). All data published so far suggest that *Dinophysis* species are obligate mixotrophs, relying on both light and food uptake for growth in the long term (Riisgaard & Hansen 2009, Kim et al. 2010). Not much is known about the growth of *Dinophysis* species at low irradiances or to what extent food uptake can substitute photosynthesis. However, it is known that as much as 80% of the carbon uptake comes from ingestion of prey at high irradiance and high prey concentration (Riisgaard & Hansen 2009). It is also now evident that prey uptake supplies *Dinophysis* cells with functional chloroplasts (Kim et al. 2012). To what extent *Dinophysis* species can photoacclimate is presently unknown. This raises a number of questions concerning the cellular toxin content and production, e.g. to what extent is cellular toxin content and production coupled to photosynthesis, food uptake and growth?

The first attempts to study toxin production of *Dinophysis* spp. revealed huge differences in cellular toxin contents, and values ranging from 0 to 59, 0 to 10 and 0 to 170 pg $cell^{-1}$ were found for OA, DTX-1 and PTX-2 respectively (Hackett et al. 2009, Kamiyama & Suzuki 2009, Kamiyama et al. 2010, Fux et al. 2011, Nagai et al. 2011, Tong et al. 2011). Thus, toxins found in *Dinophysis* vary considerably in cellular quotas — all of them even to the extent that they can be completely absent. Despite recent studies, it is still largely unknown what causes these variations in toxin content among species of *Dinophysis*. Among the few available studies, Kamiyama et al. (2010) showed that during the exponential growth phase, temperature (10 to 22°C) affected cellular contents of PTX-2, whereas it had no significant effect on OA and DTX-1. Tong et al. (2011) similarly found that irradiances from 65 to 300 µmol photons m^{-2} s^{-1} (all levels were growth saturating) did not affect cellular toxin contents or production rates (OA, DTX-1, PTX-2 and OA-D8). They did find, however, that toxin production continued long into the stationary growth phase, and *D. acuminata* thus accumulated toxins intracellularly when not dividing. However, the variations in toxin contents were within 1 order of magnitude, and cellular toxin quotas of OA and DTX-1

were in the very low end of the spectrum found for *Dinophysis* cultures (OA: <50 fg cell^{-1} and DTX-1: <330 fg cell^{-1}). No environmental factors have so far been found to control toxin contents of any *Dinophysis* species to the level that toxins appear or disappear, indicating that *Dinophysis* toxicity is subject first and foremost to strong genetic control.

Thus, the aim of the present study was to examine the regulating factors for toxin production in *Dinophysis acuminata*, a key DSP-producing harmful algal bloom species. By sampling different populations of *D. acuminata* and performing controlled laboratory experiments, we assessed genetic variability, and effects of irradiance, food availability and/or growth phase on the cellular toxin content and rate of production.

MATERIALS AND METHODS

Cultures and growth conditions

Cultures of *Teleaulax amphioxeia* and *Mesodinium rubrum* were isolated from Helsingør Harbor during summer 2009. Every 3 to 4 d, *M. rubrum* (MBL-DK2009) was fed *T. amphioxeia* (K-0434, Scandinavian Culture Collection of Algae and Protozoa) at a ratio of 1:10 to enable mixotrophic growth. During 2009, 7 non-clonal (based on ~10 cells each) cultures of *Dinophysis acuminata* were isolated from 7 different locations in Denmark, including Limfjorden, Mariager Fjord, Lillebelt and Oresund. These were grown in 24-well multi-dish trays and fed *M. rubrum* once or twice a week at a ratio of ~1:10. All cultures were grown in f/2 autoclaved seawater medium with 2.3 ± 0.1 mmol l^{-1} dissolved inorganic carbon (DIC), a pH of 8.0 ± 0.02 and a salinity of 32 ± 1 (Guillard & Ryther 1962). They were kept in a temperature-controlled room at 15°C and provided ~130 µmol photons m^{-2} s^{-1} PAR on a 16 h light:8 h dark cycle. Irradiance was delivered by Osram cool white 58W/640 fluorescent tubes. All cultures were non-axenic. All established cultures of *D. acuminata* were analyzed for DSP toxin contents using 0.5 ml centrifugal filters as described below.

Toxin sampling tests

Initial experiments were conducted with one of the 7 *Dinophysis acuminata* cultures, 'FR101009' (isolated from Lillebelt, October 2009), to test the DSP toxin sampling method. *D. acuminata* cells were harvested from a 200 ml culture using Eppendorf tubes with 0.5 ml centrifugal filter inserts (VWR) with a pore size of 0.45 µm. The aims were to establish the optimal centrifugal force and time and to show that there was a constant relationship between the number of *D. acuminata* cells added and the amount of toxin found per filter. For the first test, 0.5 ml of *D. acuminata* culture was added to 9 centrifugal filters, and these were centrifuged at different accelerations (ranging from 50 to 12 800 × *g*) for 1 min. For the second test, 4 filters with 0.5 ml of *D. acuminata* culture were centrifuged at 800 × *g* for 30 to 240 s. For the third test, 5 different cell concentrations of *D. acuminata* were produced (200 to 3200 cells ml^{-1}) by diluting sub-samples of another *D. acuminata* culture with f/2 medium. Each of the cell concentrations was added to triplicate centrifugal filters, and the filters were centrifuged at 800 × *g* for 1 min. The filtrate was then removed from all Eppendorf tubes, and the tubes, including filter inserts, were stored at −18°C. Later, after 1 wk of storage, samples were extracted for 1 h using 100 µl 100% methanol, and after centrifugation, the extract was transferred to a 2 ml HPLC vial with a 250 µl glass insert.

Effects of irradiance, food availability and growth phase on toxin production

Feeding experiments were set up in triplicate 65 ml polystyrene flasks at 4 different irradiances (7, 15, 30 and 130 µmol photons m^{-2} s^{-1}, henceforth termed I_7, I_{15}, I_{30} and I_{130} respectively) to test the effects of irradiance, food availability and growth phase on the toxin production of *Dinophysis acuminata*. The same non-clonal culture, FR101009, was used also for this experiment. Two different *D. acuminata* stock cultures (both FR101009) were used for the experiment setup, one for the I_7 and I_{30} treatments and the other for the I_{15} and I_{130} treatments. The stock culture used for the I_7 and I_{30} treatments was starved for ~1 mo before the experiment was initiated, whereas the stock culture used for the I_{15} and I_{130} treatments was relatively well fed and in exponential growth. Initial concentrations were setup at ~200 *D. acuminata* ml^{-1} and ~2000 *Mesodinium rubrum* ml^{-1}. At subsequent samplings (every 3 to 4 d), cell concentrations were adjusted to these target concentrations (after counting) by diluting with f/2 medium and adding *M. rubrum* culture that had been grown at, and fed with *T. amphioxeia* that also had been grown at the corresponding treatment irradiances. *D. acuminata* was grown this way for 5 to 7 samplings, corresponding to

15 to 25 d, and after this, cellular gross photosynthetic rates were measured, and 2 (I_7 and I_{30}) of the 4 irradiance level treatments were terminated. The other 2 (I_{15} and I_{130}) were allowed to continue, but with no additional dilutions or food additions. The experiments were conducted in a 15°C temperature-controlled room, using the same seawater medium as for culturing. Irradiance was provided by a cool white light source providing 130 µmol photons m^{-2} s^{-1} on a 16 h light:8 h dark cycle, and neutral density filters were applied to achieve the reduced irradiance levels.

At each sampling, 3 ml subsamples were removed from each flask, and cells were fixed using acidic Lugol's solution in a final concentration of 1 to 2%. Cell counts were performed on an Olympus CK2 inverted microscope at 40 to 200× using 1 ml Sedgewick-Rafter sedimentation chambers. A minimum of 200 cells was counted when possible. When cell concentrations were <200 ml^{-1}, a minimum volume of 1 ml was inspected. Based on these microscopy cell enumerations, exponential growth rates (µ) of *Dinophysis acuminata* and *Mesodinium rubrum* were calculated from the following equation:

$$\mu = \frac{\ln\left(\frac{N_t}{N_0}\right)}{t} \quad (1)$$

where t is the time (in days) between 2 consecutive samplings, and N_0 and N_t are the cell concentrations at the beginning and at the end of this sampling interval.

On the final day of feeding, micrographs were taken of well-fed *D. acuminata* to document possible chloroplast acclimation. Normal light micrographs were taken using an Olympus BX50 microscope equipped with an Olympus DP71 color camera using the software Cell^F (Olympus). For epifluorescence microscopy, 5 ml subsamples were fixed with glutaraldehyde (final solution 2%) and gently filtered onto 25 mm black 2 µm nuclepore filters that were mounted in non-fluorescent immersion oil. Micrographs were taken with a monochrome digital camera (Soft Imaging System FViewII) mounted on an Olympus IX81 inverted microscope equipped with a spinning-disk unit (DSU) and a green filter cube (U-MWIG3 green: excitation 530 to 550 nm; emission filter 575 nm).

Cell dimensions of *Dinophysis acuminata* were measured on Lugol's preserved samples from the final day of feeding at 400× using an Olympus CK2 inverted microscope. Lengths and widths of 10 cells from each replicate were measured. Cell thicknesses were measured on the first 10 cells found in a position that allowed widths to be measured, and the 10 values were assigned randomly to the 10 length/width pairs. The biovolume of *D. acuminata* was calculated as a rotational ellipsoid with an elliptic cross-sectional area, and cellular carbon contents were calculated from the biovolumes following the equation from Menden-Deuer & Lessard (2000).

Subsamples (1 ml) were removed from each flask for photosynthesis measurements at the end of the 'well-fed' period, and for I_{15} and I_{130}, subsamples were also collected thrice during the starved period. A total of 80 *D. acuminata* cells were picked from the 1 ml subsamples and washed once in f/2 medium by micromanipulation using a stereoscope. A total of 40 cells were transferred to each of two 20 ml glass scintillation vials containing 2 ml f/2 medium, and 20 µl of $NaH_{14}CO_3$ stock solution (specific activity = 100 µCi ml^{-1}, Carbon-14 Centralen) was added. One vial was incubated for ~3 h at treatment irradiance, while the other was kept in complete darkness (by wrapping in several layers of aluminum foil). After incubation, a 100 µl sub-sample was transferred to a new vial containing 200 µl phenylethylamine for determination of specific activity. The remaining 1.9 ml were acidified with 2 ml 10% glacial acetic acid in methanol and evaporated overnight at 60°C to remove all inorganic carbon. The residue was then re-dissolved in 2 ml Milli-Q water. All vials were treated with 10 ml Packard Insta-Gel Plus scintillation cocktail, and disintegrations per minute were measured using a Packard 1500 Tri-Carb liquid scintillation analyzer with automatic quench correction. The photosynthetic activity (PA, pg C $cell^{-1}$ h^{-1}) per cell was calculated as follows:

$$PA = \frac{DPM \times [DIC]}{^{14}C_{added} \times h \times cells} \quad (2)$$

where DPM is disintegrations min^{-1}, DIC is the concentration of dissolved inorganic carbon (pg C ml^{-1}), $^{14}C_{added}$ is the specific activity (disintegrations min^{-1} ml^{-1}), h is the incubation time, and cells is the number of cells in the vial.

DIC concentrations were measured on 1 ml subsamples using an ADC 225 Mk3 infrared gas analyzer as described elsewhere (Nielsen et al. 2007). Glass vials with screw caps were used for DIC samples allowing no headspace, and the samples were analyzed within a few hours.

DSP toxins were quantified at each sampling by transferring a 0.5 ml subsample from each flask to 0.5 ml centrifugal filters (pore size = 0.45 µm, VWR). Based on results from the centrifugal filter tests, the

samples were centrifuged at 400 × *g* for 2 min. The filtrate was removed, and Eppendorf tubes with filter inserts were stored at −18°C until later extraction and analysis. Samples were extracted as described for the first experiment.

Additional experiments at 15 and 130 µmol photons m^{-2} s^{-1}

An additional, similar set of experiments were conducted at 15 and 130 µmol photons m^{-2} s^{-1} after the completion of the first set (henceforth termed $I_{15\text{-}2}$ and $I_{130\text{-}2}$). The setup was the same as in the previous experiments, but 4 times during the experiments, in addition to the previously described method, DSP toxins were also sampled by picking 300 *Dinophysis acuminata* cells under a stereoscope using glass Pasteur pipettes. The cells were washed once in f/2 growth medium and transferred to an Eppendorf spin filter. From there, they were centrifuged and processed like the other toxin samples. Additionally, pH was recorded at each sampling using a WTW 3210 pH meter equipped with a WTW Sentix 41 combination electrode, calibrated before each use with standard NBS pH 7 and 10 buffers. The aim of these additional experiments was to establish how much of the observed toxicity was actually found intracellularly during the different growth phases.

Toxin measurements

Measurements were done on an ABI-SCIEX-4000 Q Trap (Applied Biosystems) triple quadrupole mass spectrometer equipped with a TurboSpray® interface coupled to an Agilent model 1100 LC. The LC equipment included a solvent reservoir, in-line degasser (G1379A), binary pump (G1311A), refrigerated autosampler (G1329A/G1330B) and temperature-controlled column oven (G1316A).

After injection of 5 µl of sample, separation of lipophilic toxins was performed by reverse-phase chromatography on a C8 column (50 × 2 mm) packed with 3 µm Hypersil BDS 120 Å (Phenomenex) and maintained at 20°C. The flow rate was 0.2 ml min^{-1}, and gradient elution was performed with 2 eluents, where eluent A was water and eluent B was acetonitrile/water (95:5 v/v), both containing 2.0 mmol l^{-1} ammonium formate and 50 mmol l^{-1} formic acid. Initial conditions were 12 min column equilibration with 5% B followed by a linear gradient to 100% B within 10 min and isocratic elution until 15 min with 100% B. The program was then returned to the initial conditions for 18 min (total run time: 30 min).

The chromatographic run was divided into 3 periods: (1) 0 to 8.75 min for domoic acid, (2) 8.75 to 11.20 min for gymnodimine and spirolides and (3) 11.20 to 18 min for OA, DTXs, PTXs, yessotoxin and azaspiracid-1 (curtain gas: 10 psi, CAD gas: medium, ion spray voltage: 5500 V, temperature: ambient, nebulizer gas: 10 psi, auxiliary gas: off, interface heater: on, declustering potential: 50 V, entrance potential: 10 V, exit potential: 15 V). Selected reaction monitoring experiments were carried out in positive ion mode by selecting the following transitions (precursor ion > fragment ion) for period 3: specific mass to charge ratio (m/z) 822 > 223 (collision energy, CE: 55 V) for OA and DTX-2, m/z 836 > 237 (CE: 55 V) for DTX-1, m/z 946 > 223 (CE: 55 V) for OA diol ester, m/z 874 > 213 (CE: 55 V) for pectenotoxin-12 (Miles et al. 2004b) and PTX-14, m/z 876 > 213 (CE: 55 V) for PTX-2, m/z 892 > 213 (CE: 55 V) for PTX-1, PTX-4, PTX-8, PTX-11 and PTX-13, m/z 894 > 213 (CE: 55 V) for PTX-2 seco acid, m/z 842 > 824 (CE: 55 V) for azaspiracid-1 and m/z 1160 > 965 (CE: 55 V) for yessotoxin. Dwell times of 100 to 200 ms were used for each transition.

Calculations and statistical analyses

The generation time (τ) for each irradiance treatment during the well-fed period was calculated as $\tau = \ln(2)/\mu$. Carbon contributions from photosynthetic activity (C_{PA}) during the well-fed period were calculated as follows:

$$C_{PA} = \frac{PA \times h_{light} \times \tau}{C_{cell}} \quad (3)$$

where h_{light} is the daily hours of light (= 16), τ is generation time in days, and C_{cell} is the carbon content per *Dinophysis acuminata* cell.

Acclimated growth rate and PA were both fitted to treatment irradiances according to the model of Harrison & Platt (1986) using the 'Regression Wizard' of SigmaPlot 12.0:

$$PA = P_{max}\left(1 - e^{-\left(\frac{\alpha \cdot E_d}{P_{max}}\right)}\right) \quad (4)$$

where P_{max} is the photosynthetic capacity at saturating light, α is the initial slope, and E_d is the downwelling irradiance (PAR).

Daily toxin production rates (R_{tox}) were calculated as follows:

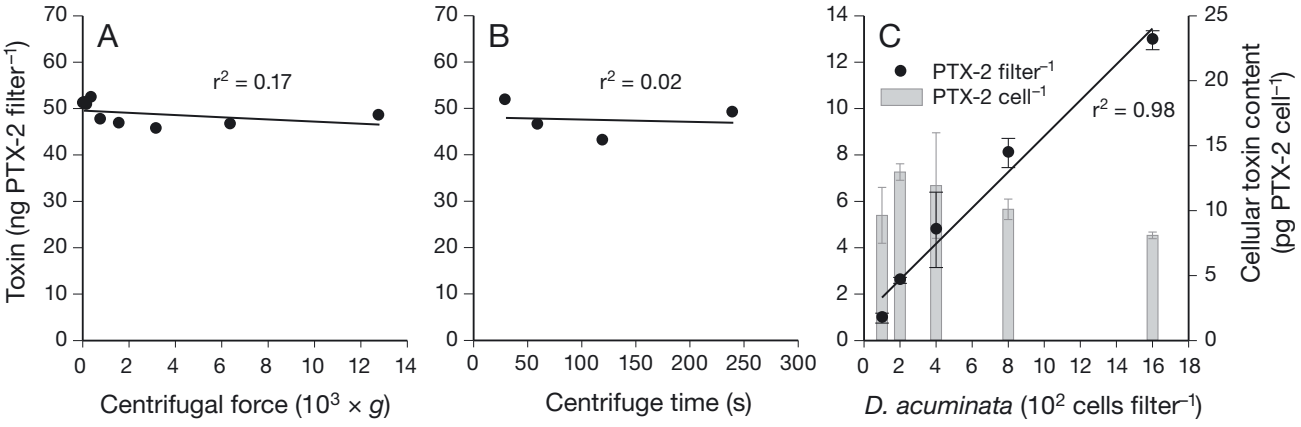

Fig. 1. *Dinophysis acuminata.* Initial toxin sampling tests with centrifugal filters. Pectenotoxin-2 (PTX-2) collected per filter as a function of (A) the centrifugal force applied (time = 1 min), (B) centrifuge time (force = 800 × *g*) and (C) (dots and line) number of *D. acuminata* cells per filter (1 min at 800 × *g*) and (bars) cellular PTX-2 quotas calculated from the same data. Symbols and error bars represent mean ± SD (n = 3)

$$R_{tox} = \frac{T_2 - T_1}{\overline{N} \times (t_2 - t_1)} \quad (5)$$

where T_1 and T_2 are toxin contents per ml of culture at 2 subsequent samplings taken on days t_1 and t_2, and $\overline{N}$ is the ln-average of the cell concentration per ml calculated as follows:

$$\overline{N} = \frac{N_2 - N_1}{\ln(N_2 / N_1)} \quad (6)$$

All statistical comparisons between the 4 irradiance treatments were made in SigmaStat 3.5 using 1-way ANOVA with a predefined α of 0.05 and a Tukey test for pairwise comparisons. All results are presented as mean ± SD.

RESULTS

DSP toxin contents of 7 Danish strains of *Dinophysis acuminata*

Under the standard laboratory conditions given in the previous section, all 7 analyzed strains of *D. acuminata* contained PTX-2 in the range of 12.7 to 35.6 pg cell^{-1}, whereas none of them produced OA or DTX.

Toxin sampling tests

Results from the centrifugal filter tests showed that the amount of PTX-2 retrieved per filter was independent of the centrifugal force (Fig. 1A) and centrifuge time (Fig. 1B). Also, there was a good correlation (r^2 = 0.98) between the number of *Dinophysis acuminata* cells per filter and the amount of retrieved PTX-2 (Fig. 1C). Cellular PTX-2 content was 9.6 ± 2.2 and 8.1 ± 0.3 pg PTX-2 cell^{-1} at 100 and 1600 *D. acuminata* cells per filter respectively, and there were no statistically significant differences between cellular PTX-2 contents at the different *D. acuminata* concentrations assessed (p = 0.11, 1-way ANOVA, n = 3) (Fig. 1C).

Effects of light on photosynthesis, growth and cellular toxin content and production

Dinophysis acuminata showed positive growth under all 4 irradiance treatments (Fig. 2). During the well-fed period, *D. acuminata* increased between samplings from 200 to 314 ± 36, 662 ± 69, 687 ± 305 and 1268 ± 242 cells ml^{-1} for the 4 treatments I_7, I_{15}, I_{30} and I_{130} respectively. At I_7, I_{15} and I_{30}, *Mesodinium rubrum* almost always declined in numbers between samplings due to grazing exceeding new growth (Fig. 2). At I_{130}, the growth of *M. rubrum* often counterbalanced the rate of grazing. For all treatments, however, cell concentrations of *M. rubrum* were >1000 ml^{-1} most of the time during the well-fed period, and only on a single occasion did the concentration decrease below 500 ml^{-1} (I_7, Day 22). After 18 d, when dilutions and feeding were terminated, *D. acuminata* in the 2 treatments I_{15} and I_{130} kept growing exponentially for 8 and 4 d, respectively, after which growth was impeded (Fig. 2B,D). On Day 26, cell concentrations of *D. acuminata* were 4817 ± 279 and 7333 ± 471 cells ml^{-1} in treatments I_{15} and I_{130}, respectively. Growth continued slowly in I_{15} until Day 47, at which point the cell concentration was

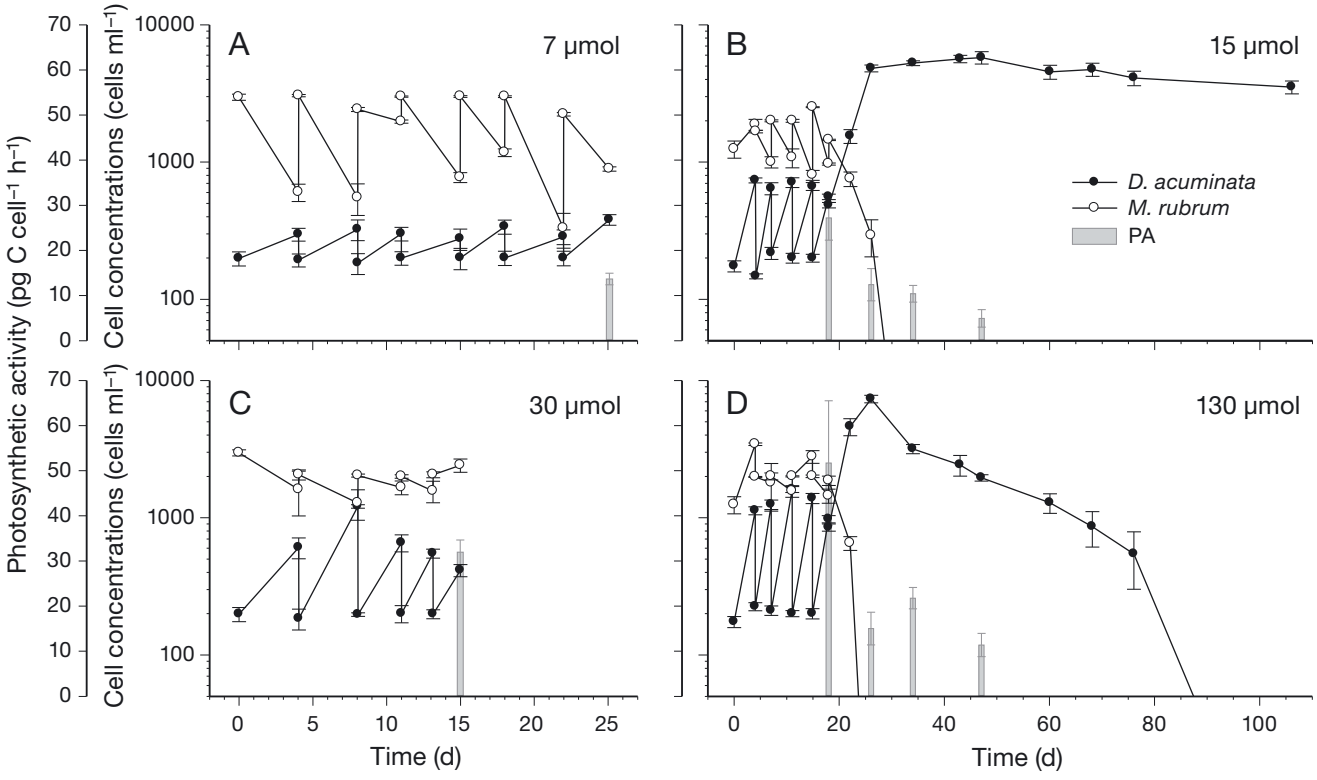

Fig. 2. *Dinophysis acuminata* and *Mesodinium rubrum*. Cell concentrations of (●) *D. acuminata* and (○) *M. rubrum* and (bars) rates of photosynthetic activity in the first set of experiments of the 4 treatments at 7, 15, 30 and 130 µmol photons m^{-2} s^{-1} (I_7, I_{15}, I_{30} and I_{130} respectively). Data are mean ± SD (n = 3)

5775 ± 602 cells ml^{-1}. After this no further growth was seen, and on Day 106 the cell concentration had declined to 3517 ± 377 cells ml^{-1}. I_{130}, in contrast, showed net cell death immediately after the cell concentration peaked on Day 26. On Day 76, the cell concentration had declined to 545 ± 244 cells ml^{-1}, and no cells were alive on Day 106. *M. rubrum* was completely gone after 8 to 12 and 4 to 8 d in I_{15} and I_{130}, respectively.

Dinophysis acuminata µ-values during the well-fed period were 0.136 ± 0.008, 0.340 ± 0.006, 0.408 ± 0.003 and 0.512 ± 0.008 d^{-1} for the 4 irradiances I_7, I_{15}, I_{30} and I_{130}, respectively (Fig. 3A). All of these were statistically significantly different ($p < 0.001$, 1-way ANOVA, n = 3). The well-fed period spanned 5 to 7 samplings (15 to 25 d), and, given the achieved growth rates, this corresponds to 5 to 13 divisions. Based on the model of Harrison & Platt (1986), the derived maximum µ was 0.51 d^{-1}.

Rates of PA on the final day of the well-fed period were 13.7 ± 1.3, 27.2 ± 4.9, 31.9 ± 2.7 and 51.7 ± 13.8 pg C $cell^{-1}$ h^{-1} in I_7, I_{15}, I_{30} and I_{130} respectively (Fig. 3B). Micrographs of *Dinophysis acuminata* from I_{15} and I_{130} showed increased amounts of chlorophyll at the low irradiance treatment, I_{15}, compared to the high irradiance treatment I_{130} (Fig. 4). The modeled P_{max} was 51.0 pg C $cell^{-1}$ h^{-1}. Given the observed growth rates of the 4 treatments I_7, I_{15}, I_{30} and I_{130}, photosynthetic carbon assimilation contributed 68 ± 9, 49 ± 10, 51 ± 4 and 61 ± 17 % of the carbon content of *D. acuminata* between each cell division. With an assumed growth efficiency of 50 % for photoautotrophically derived carbon, this corresponds to 34 ± 4, 25 ± 5, 25 ± 2 and 30 ± 9 % of the required carbon for each division. After feeding was terminated on Day 18 and *D. acuminata* growth was impeded, rates of PA quickly decreased (Fig. 2B,D). For I_{15}, the rate decreased from 27.2 ± 4.9 to 12.4 ± 3.6, 10.4 ± 1.9 and 4.9 ± 1.9 pg C $cell^{-1}$ h^{-1} on Day 18, 26, 34 and 47, respectively. For I_{130}, the rate decreased from 51.7 ± 13.8 to 15.0 ± 3.6, 21.8 ± 2.4 and 11.4 ± 2.6 pg C $cell^{-1}$ h^{-1} on Day 18, 26, 34 and 47, respectively. For both treatments, the first (well-fed) rate was statistically significantly higher than the subsequent (starved) value, whereas any further decreases as starvation continued were not statistically significant ($p < 0.001$, 1-way ANOVA, n = 3).

Cell dimensions of *Dinophysis acuminata* from all treatments were: length: 48 ± 2 µm, width: 32 ± 2 µm and thickness: 16 ± 2 µm. The calculated carbon content of *D. acuminata* in the 4 treatments I_7, I_{15}, I_{30} and I_{130} was 1654 ± 200, 1804 ± 185, 1711 ± 117 and 1856

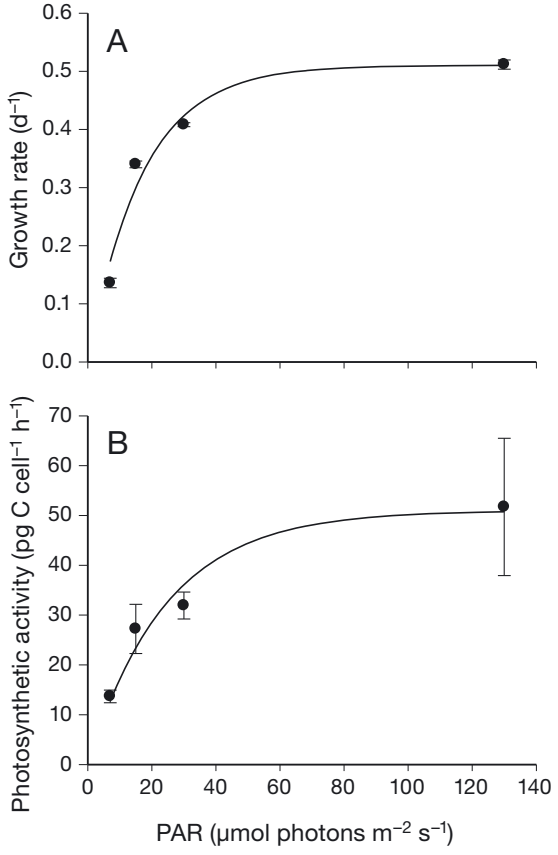

Fig. 3. *Dinophysis acuminata*. First set of experiments. (A) Growth rate and (B) photosynthetic activity vs. irradiance. The fitted line is after Harrison & Platt (1986). Data are mean ± SD (n = 3)

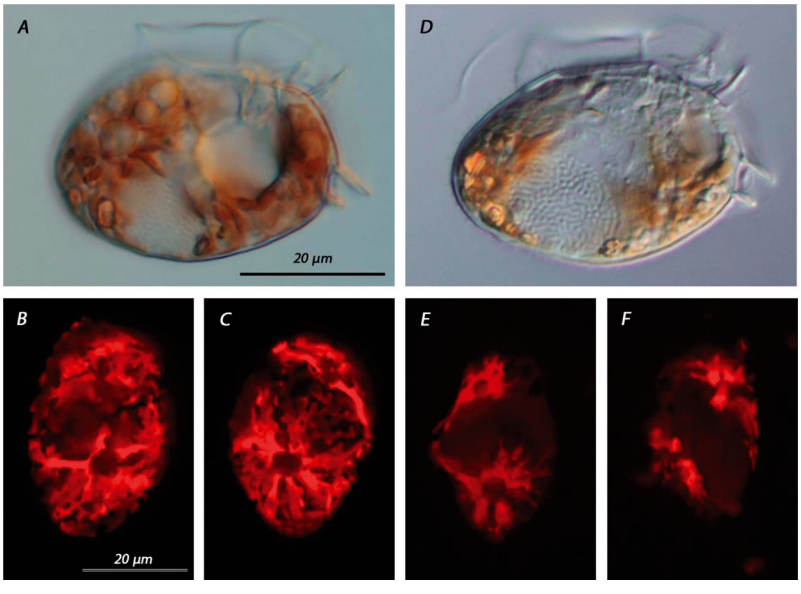

Fig. 4. *Dinophysis acuminata*. Representative micrographs of well-fed cells grown at (A–C) 15 and (D–F) 130 µmol photons m^{-2} s^{-1}. Epifluorescence micrographs show that *D. acuminata* had more chlorophyll when grown at (B,C) 15 µmol photons m^{-2} s^{-1} than at (E,F) 130 µmol photons m^{-2} s^{-1}

± 215 pg C cell^{-1}, respectively. Carbon contents of the 4 treatments were not statistically significantly different ($p = 0.56$, 1-way ANOVA, $n = 3$).

PTX-2 was the primary toxin observed in all samples. Apart from this, PTX-2-sa was also observed, but always in a constant, small amount (1 to 2%) compared to PTX-2. No OA or DTX was observed at any time. Cellular PTX-2 contents in the 4 treatments I_7, I_{15}, I_{30} and I_{130} were initially 29.5 ± 3.7, 8.8 ± 2.5, 29.5 ± 3.3 and 8.6 ± 2.5 pg PTX-2 cell^{-1}, respectively (Fig. 5). The cellular PTX-2 contents in I_7 and I_{30} decreased within the first 8 to 11 d of feeding to an average during the remaining well-fed period of 4.4 ± 1.2 and 5.2 ± 0.9 pg cell^{-1}, respectively. Cellular PTX-2 contents of the I_{15} and I_{130} treatments were relatively stable during all of the well-fed period, averaging 7.3 ± 2.3 and 9.0 ± 2.9 pg cell^{-1}, respectively. For irradiance-acclimated values of toxin content, only data from the last 3 samplings of the well-fed period were used, and thus well-fed, acclimated toxin contents were 4.6 ± 0.3, 6.2 ±t 1.1, 5.1 ± 0.5 and 7.8 ± 1.1 pg PTX-2 cell^{-1} in the 4 treatments I_7, I_{15}, I_{30} and I_{130}, respectively (Fig. 6). The I_{130} treatment had statistically significantly more PTX-2 per cell than the I_7 and I_{15} treatments, whereas none of the PTX-2 contents in the other treatments differed ($p = 0.007$, 1-way ANOVA, $n = 3$). Calculated daily PTX-2 production rates during the well-fed period were highly variable, ranging from –2.7 to 3.8, 0.5 to 5.2, 1.3 to 4.4 and 1.6 to 8.0 pg cell^{-1} d^{-1} for I_7, I_{15}, I_{30} and I_{130}, respectively. However, when averaged over the irradiance-acclimated, well-fed period for each replicate, all treatment combinations except one (I_{15} vs. I_{30}) were statistically significantly different from each other in daily PTX-2 production rates ($p < 0.005$, 1-way ANOVA, $n = 3$). The corresponding daily rates of PTX-2 production were 0.7 ± 0.04, 2.0 ± 0.4, 2.1 ± 0.2 and 3.9 ± 0.5 pg cell^{-1} d^{-1} (Fig. 6). After dilution and feeding ceased, the I_{15} treatment showed continued positive rates of PTX-2 production for another 42 d (Day 60) even though cell division had stopped (Fig. 5B). Thus, toxin accumulated inside *Dinophysis acuminata* cells, resulting in a PTX-2 content of 24.7 ± 1.7 pg cell^{-1} compared to the average content of 6.2 ± 1.1 pg cell^{-1} found for that irradiance during the well-fed period. For the I_{130} treatment, an apparently similar increase in the

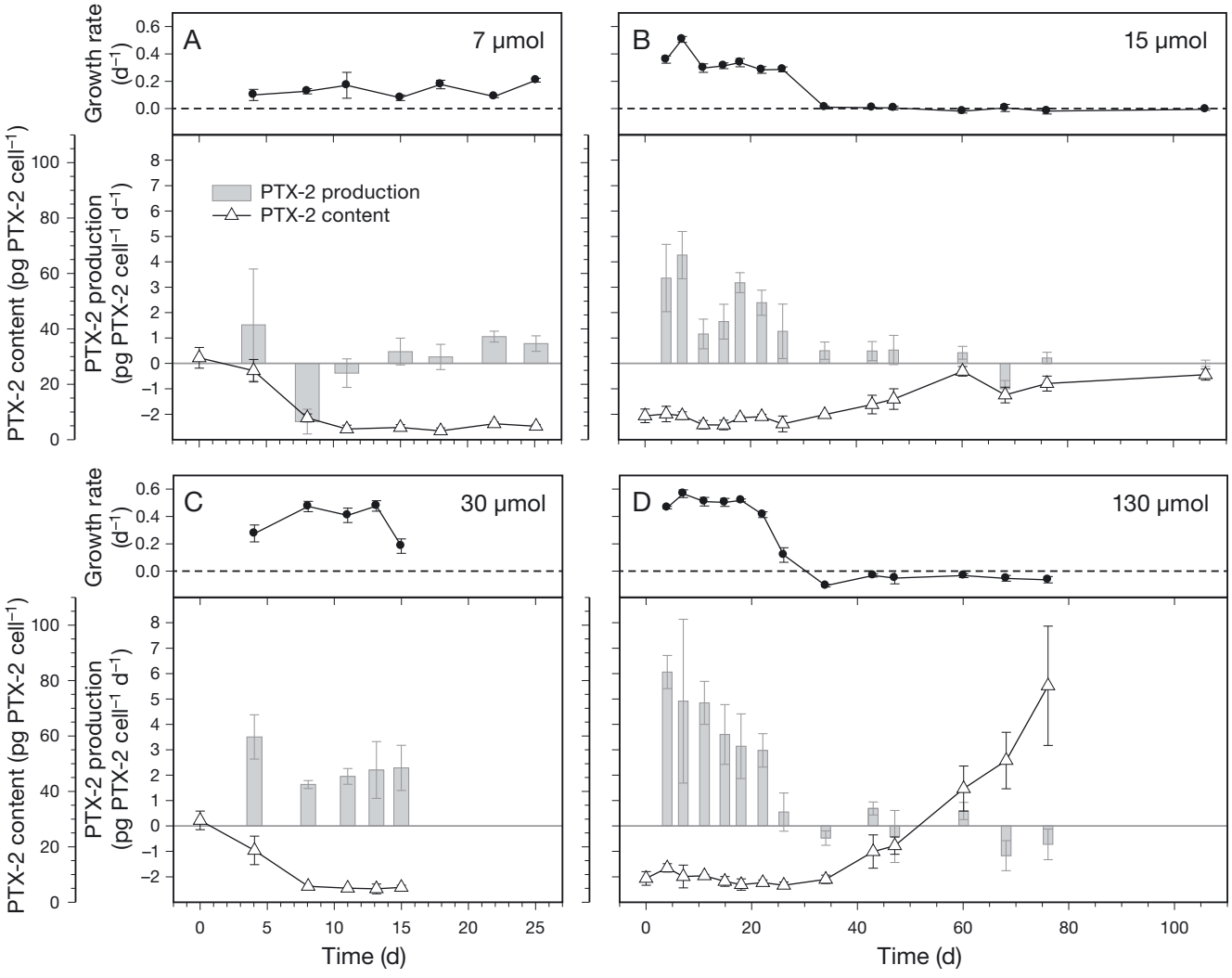

Fig. 5. *Dinophysis acuminata*. Toxin contents and toxin production rates in the 4 treatments during the first set of experiments (see Fig. 2). Top panels show (●) specific growth rates of *D. acuminata*, whereas bottom panels show (△) cellular toxin contents (note the second *y*-axis) and (bars) toxin production rates. Data are mean ± SD (n = 3)

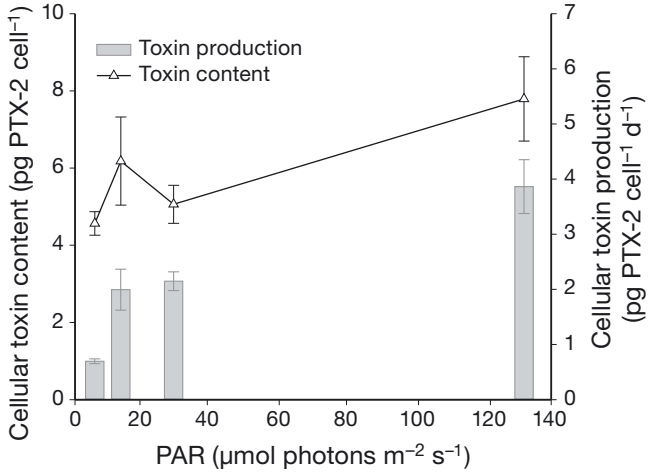

Fig. 6. *Dinophysis acuminata*. Toxin content and production in the first set of experiments as a function of treatment irradiance. Data are mean ± SD (n = 3)

cellular toxin content was found, but it continued all the way to the end of the experiment, finally ending with a cellular toxin quota of 78.1 ± 21.5 pg PTX-2 cell^{-1} on Day 76 (Fig. 5D).

Additional experiments at 15 and 130 µmol photons m^{-2} s^{-1}

Both additional experiments progressed similarly to their respective counterparts in the first set of experi-

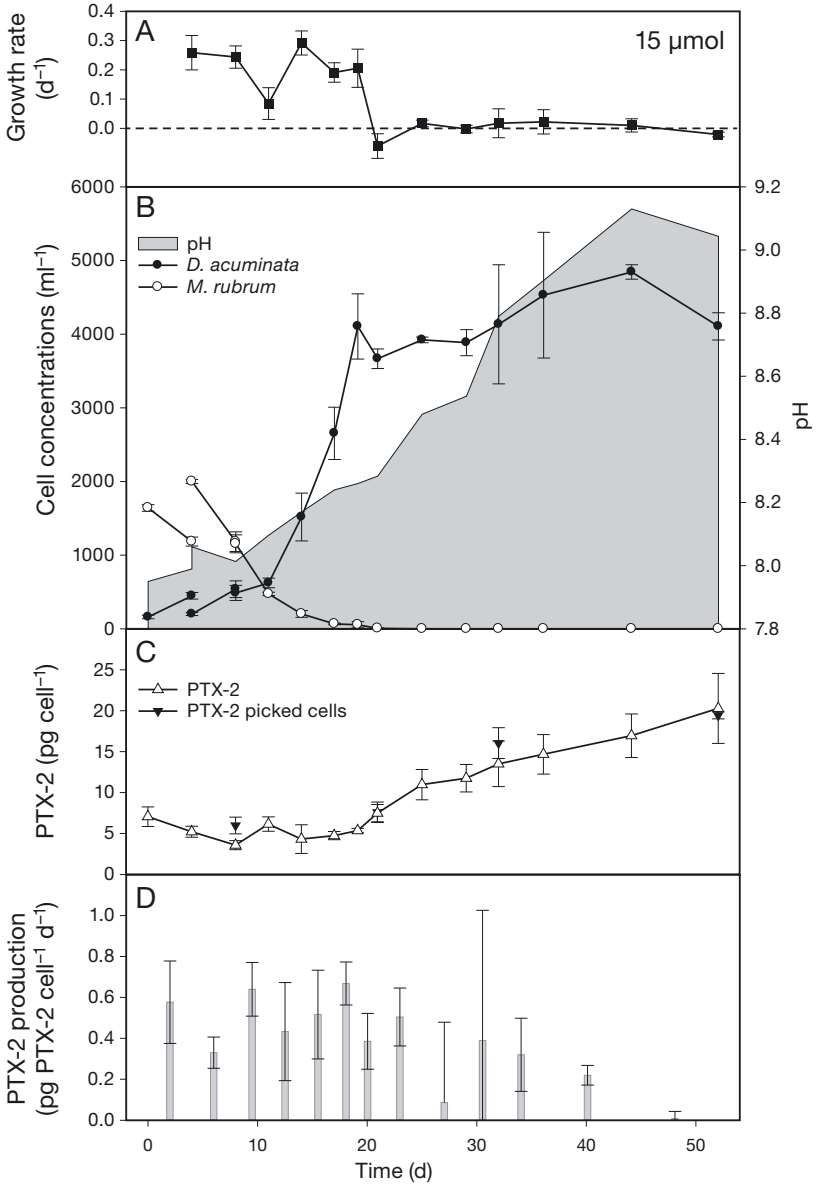

Fig. 7. *Dinophysis acuminata* and *Mesodinium rubrum*. Second set of experiments at 15 µmol photons m^{-2} s^{-1} ($I_{15\text{-}2}$). (A) Specific growth rate. (B) Cell concentrations of *D. acuminata* and *M. rubrum* as well as average pH. (C) Cellular toxin contents from (Δ) spin filter samples and (▼) picked cells. (D) Cellular PTX-2 production rates during the 52 d experiment. Data are mean ± SD (n = 3)

ments (Figs. 7 & 8). One exception was the slightly shorter acclimation period with high food availability; in the second set of experiments, food (*Mesodinium rubrum*) was only added the first week and was almost completely gone on Day 17 and 14 in $I_{15\text{-}2}$ and $I_{130\text{-}2}$, respectively. After the final feeding and dilution on Day 8, *Dinophysis acuminata* in $I_{15\text{-}2}$ grew exponentially until a level of 4100 cells ml^{-1} was reached on Day 19. From here, only very limited growth was seen until the termination of the experiment on Day 52. During the well-fed, diluted period, the pH in this treatment was 8.02 ± 0.05, but after the last dilution, it increased steadily until Day 44, when it peaked at 9.13 ± 0.09 (Fig. 7B). The initial PTX-2 content was 7.06 ± 1.20 pg $cell^{-1}$, but this decreased slightly to an average of 4.90 ± 0.89 pg $cell^{-1}$ in the well-fed, exponential growth phase (Fig. 7C). During this period, the average rate of PTX-2 production was 0.53 ± 0.19 pg $cell^{-1}$ d^{-1} (Fig. 7D). After growth ceased, PTX-2 production continued, resulting in a steady increase in the cellular PTX-2 content toward the 20.3 ± 4.3 pg $cell^{-1}$ found on Day 52. On Day 8, the cellular PTX-2 content was statistically significantly higher in the sample with picked and washed *D. acuminata* cells compared to the 0.5 ml bulk culture samples (1-way ANOVA, p = 0.02, n = 3), but no other differences between the 2 toxin sampling methods were found in this treatment (Fig. 7C). The $I_{130\text{-}2}$ treatment reached a higher maximum *D. acuminata* concentration of 9483 ± 579 cells ml^{-1} on Day 20 compared to the maximum cell concentration achieved in $I_{15\text{-}2}$ (Fig. 8B). From Day 20 to Day 50, the cell concentration decreased slowly to 6080 ± 656 cells ml^{-1} and from there dropped quickly to 2894 ± 1001 cells ml^{-1} on the final day, Day 58. In this high irradiance treatment, pH fluctuated more during the well-fed period, ranging from 7.88 to 8.41, and a maximum value of 9.48 ± 0.02 was recorded on Day 20. From here, pH decreased slightly until Day 58, when a pH of 9.14 ± 0.03 was registered. The initial PTX-2 content of 11.6 ± 1.9 pg $cell^{-1}$ was somewhat higher than in the $I_{15\text{-}2}$ treatment. However, this decreased within 8 d to an average of 3.65 ± 0.88 pg $cell^{-1}$ during the acclimated, well-fed period. During the well-fed period, the PTX-2 production rate was 1.03 ± 0.61 pg $cell^{-1}$ d^{-1}, and PTX-2 production continued after cell division stalled, thus increasing the cellular toxin content to 13.8 ± 2.0 pg $cell^{-1}$ on Day 27. On the final day, Day 58, the cellular PTX-2 content increased even further

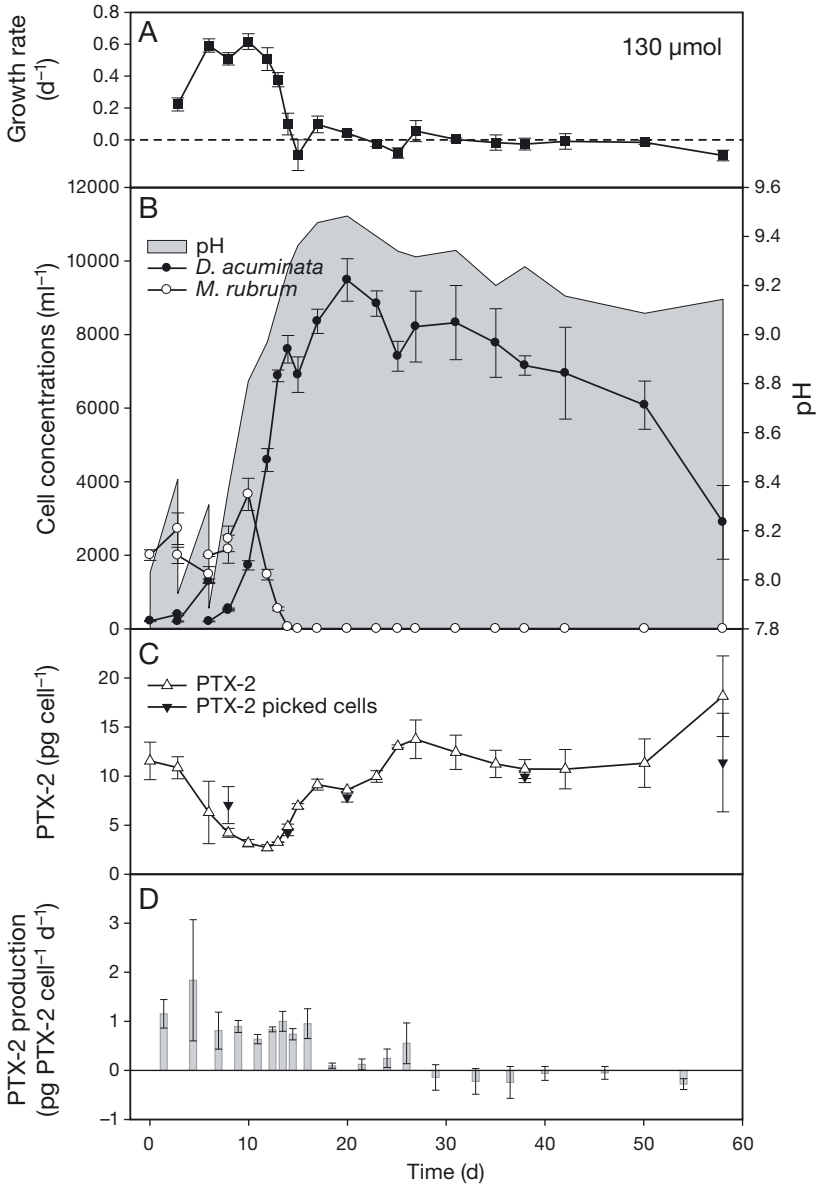

Fig. 8. *Dinophysis acuminata* and *Mesodinium rubrum*. Second set of experiments at 130 µmol photons m^{-2} s^{-1} ($I_{130\text{-}2}$). (A) Specific growth rate. (B) cell concentrations of *D. acuminata* and *M. rubrum* as well as average pH. (C) Cellular toxin contents from (Δ) spin filter samples and (▼) picked cells. (D) Cellular PTX-2 production rates during the 58 d experiment. Data are mean ± SD (n = 3)

to 18.1 ± 4.1 pg $cell^{-1}$. On the 5 d when toxins were also sampled by picking and washing *D. acuminata* cells, results from the 2 methods generally mirrored each other. However, on Day 58, after the rapid cell death, the average PTX-2 content was 11.4 ± 5.0 pg $cell^{-1}$ in picked cells compared to 18.1 ± 4.1 pg $cell^{-1}$ derived from spin-filter samples. Variation was, however, too large to allow for a statistically significant difference (p = 0.15, 1-way ANOVA, n = 3).

DISCUSSION

Effects of irradiance on growth, photosynthesis and toxin content

The 4 treatments represented 4 different levels of light limitation in *Dinophysis acuminata*, from severely limited (27%) to fully saturated (100%) both in terms of growth and photosynthesis. Despite these clear-cut differences in both growth rate and photosynthesis, effects on cellular toxin content were very limited. First, the only toxin observed in the experiment was PTX-2 (except 1 to 2% PTX-2-sa), so changing irradiance did not trigger the production of OA, DTX or other PTX isomers. Second, while the high irradiance treatment, I_{130}, gave a higher PTX-2 content than the 2 lowest irradiances, the differences were <2-fold and thus represent only a fraction of the PTX-2 variation reported in the literature (0 to 180 pg PTX-2 $cell^{-1}$) (Blanco et al. 2007, Kamiyama & Suzuki 2009). Finally, the PTX-2 content found at 130 µmol photons m^{-2} s^{-1} in the second experiment was markedly lower than the values found for any of the irradiances in the first experiment. Although the 2 sets of experiments were separated by half a year of culturing and are thus not directly comparable, this certainly questions the robustness of the irradiance effect found in the first experiment.

Our findings support the only previous laboratory study of light effects on cellular toxin content and production in *Dinophysis* spp. (Tong et al. 2011). The authors concluded that irradiances from 65 to 300 µmol photons m^{-2} s^{-1} had no influence on cellular toxin contents of *D. acuminata*. Also, field populations of *D. acuta* have been shown to produce toxin (OA, DTX-2 and PTX-2) below the euphotic zone, thus demonstrating that toxin production in this species is not directly coupled to photosynthetic light harvesting (Fux et al. 2010). A similar conclusion was drawn from another *in situ* study, based on the fact that the highest cellular toxin content (OA, DTX-2, PTX-2 and OA-D8) of *D. acuta* was found 8 h after sunset, at

01:00 h (Pizarro et al. 2008). Thus, both field and controlled laboratory studies now indicate that toxin production in *Dinophysis* spp. is not directly dependent on light.

However, in the present experiment, growth and photosynthesis were equally limited by irradiance, and this could explain why irradiance did not alter toxin content much in *Dinophysis acuminata*. Photosynthetic carbon assimilation in each generation accounted for roughly the same proportion (49 to 68%) of the carbon content of a *D. acuminata* cell under all irradiances. This was mediated by 2 important facts: (1) chloroplasts were clearly able to photoacclimate by increasing their cellular pigment concentration under low light (Fig. 4), and (2) ingestion rates of *D. acuminata* are known to decrease with decreasing irradiance (Kim et al. 2008). If toxin production is based on photosynthetic surplus, the 4 irradiance treatments in fact had quite similar conditions since photosynthesis constituted similar proportions of the carbon demand. We expected the contribution of the photosynthetic assimilation to vary more among the 4 treatments, but since it did not, we cannot rule out the idea that *Dinophysis* spp. toxin production depends on photosynthetic surplus. To further investigate this, culture conditions that increase the proportion of photosynthetically derived carbon must be employed, e.g. by reducing food availability to the point where it limits growth. This should create a scenario of slow growth but a high photosynthetic input. Further, the fact that autotrophic carbon assimilation accounted for a minimum of 25% of the carbon demand under all 4 irradiances suggests that heterotrophic carbon uptake cannot completely substitute for photosynthetically derived carbon.

Effects of food availability and growth phase on photosynthesis and toxin content

The I_{15} and I_{130} treatments were tracked after the well-fed period without further diluting or adding food. Within ~1 wk, this resulted in a peak *Dinophysis acuminata* concentration and a major reduction in the PA per cell. When prey was abundant, the photosynthetic rate at 130 µmol photons m^{-2} s^{-1} (51.7 ± 13.8 pg C $cell^{-1}$ h^{-1}) was similar to the only previously published rate from a laboratory culture of *D. acuminata* (Riisgaard & Hansen 2009). A much higher rate (≤925 pg $cell^{-1}$ h^{-1}) was found in a field study, but at high irradiances (≤500 µmol photons m^{-2} s^{-1}) using low-light adapted cells from deep waters (14 to 80 m), so this rate probably reflects an artificially high value (Setala et al. 2005). The major drop in PA, found just 1 wk after the final feeding, strongly suggests that photosynthesis in *D. acuminata* is tightly coupled to food uptake. The increasing pH found at this stage of the experiments could potentially also have caused the photosynthetic decrease, but a similar decrease found in a pH-controlled experiment with the same species suggests that this was not the case (Riisgaard & Hansen 2009). Also, one should note that although pH was high in the experimental flasks, photosynthesis measurements were done in fresh medium, where pH would have been close to 8.0. The idea that photosynthesis in *Dinophysis* spp. depends on food uptake aligns well with several recent publications that all argue that these species rely on kleptoplastids, obtained from their ciliate prey, for PA. (Janson & Graneli 2003, Minnhagen & Janson 2006, Wisecaver & Hackett 2010, Kim et al. 2012). However, little is known about the prevalence and dynamics of this kleptoplasty.

The cellular PTX-2 quota increased in the present experiments when food was depleted and growth stopped. In I_{15} and $I_{15\text{-}2}$ as well as in the first half of $I_{130\text{-}2}$ (Days 12 to 17), the increase was a result of accumulation of PTX-2 in non-dividing cells, i.e. the PTX-2 content per volume of culture increased. This is very much in line with the conclusion from an earlier laboratory study with the same *Dinophysis* species (Tong et al. 2011). It is also in concordance with field observations from Galician waters, reporting higher toxin quotas in a senescent population of *D. acuta* compared to an exponentially growing one (Pizarro et al. 2008, Pizarro et al. 2009). The effect of growth phase was also evident at the beginning of the present experiment. Here, there was a clear-cut difference between the I_7 and I_{30} treatments on one hand and the I_{15} and I_{130} treatments on the other hand. This difference was almost certainly caused by the fact that the treatments were set up in pairs, with the I_7 and I_{30} treatments originating from one (starved) stock culture and the I_{15} and I_{130} treatments originating from another (well-fed) culture. Thus, it is now evident that toxin production continues at least for some weeks after growth has ceased. The fact that toxin was produced in the absence of prey demonstrates thoroughly that toxin production in *D. acuminata* does not rely directly upon food uptake in the short run (Tong et al. 2011, present study).

In I_{130} and in the second half of $I_{130\text{-}2}$ (Days 17 to 27 and 50 to 58), the increases in the cellular content of PTX-2 were not caused by additional toxin production but rather by cell death and a slow rate of breakdown of the residual toxin. This resulted in an

increasing ratio between PTX-2 and the number of viable *Dinophysis acuminata* cells. That implies, though, that residual toxin from dead cells binds to objects (organic matter) larger than the pore size of the spin filters (0.45 µm), since free (<0.45 µm) toxin was not included in our samples. If toxin leaks from *D. acuminata* and binds to particles, cellular toxin quotas could potentially have been overestimated during the first experiment. In the second experiment, however, the toxin contents from picked cells closely resembled those obtained from spin filter samples, indicating that this was not the case. Instead, *D. acuminata* cells accounted for all of the PTX-2 found at all times in the second experiment, except perhaps on the final day of the $I_{130\text{-}2}$ treatment. We assume this was not the case in I_{130}, where cell death was more pronounced, and the PTX-2 quota increased by a factor of 10 towards the final day of the experiment. Here, much of the toxin probably originated from dead cells instead. A slow rate of decomposition and a tendency of toxins to stick to cell debris could explain the sometimes weak coupling between *Dinophysis* spp. densities and toxin contents from net hauls (Pizarro et al. 2009).

Conclusions and perspectives

Cellular toxin contents varied only slightly as a function of irradiance despite the marked effects on growth rates and photosynthetic activities. Toxin production clearly decreased when food was depleted and growth stopped, but some toxin was still produced long after the last prey cells were gone, indicating that toxin production is not coupled directly to food uptake. In other words, PTX-2 is not a breakdown product directly derived from ingested food. Instead, growth phase and genetic differences between populations seem to be the cause of intraspecific differences in toxin contents within species of *Dinophysis*. We also showed that *Dinophysis acuminata* is able to photoacclimate despite the kleptoplastid nature of its chloroplasts, and that this, combined with reduced food uptake at low light, causes photosynthetic carbon assimilation to comprise a relatively stable proportion of the carbon demand of *D. acuminata*.

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