

Growth, death, and photobiology of dinoflagellates (Dinophyceae) under bacterial-algicide control

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Abstract Naturally occurring allelopathic compounds, specific to some phytoplankton, may be a good source of bio-control agents against microalgae responsible for harmful algal blooms (HABs). Global expansion of HABs has invigorated research into different approaches to control these algae, including the search for naturally derived algicidal compounds. Here, we investigated the effects of a filtrate from the algicidal marine bacterium *Shewanella* sp. IRI-160 on photochemical function of four cultured dinoflagellates, *Karlodinium veneficum*, *Gyrodinium instriatum*, *Prorocentrum minimum*, and *Alexandrium tamarense*. The filtrate (designated IRI-160AA) contains bioactive compound(s), which were recently shown to inhibit growth of several dinoflagellate species. Results of this study show that all dinoflagellates but *P. minimum* exhibited photosystem II (PSII) inhibition, loss of photosynthetic electron transport, and varying degrees of cellular mortality. Exposure assays over 24 h showed that PSII inhibition and loss of cell membrane integrity occurred simultaneously in *G. instriatum*, but not in *K. veneficum*, where PSII activity declined prior to losing outer-membrane integrity. In addition, PSII inhibition and population growth inhibition were dose-dependent in *K. veneficum*, with an average EC-50 of 7.9 % (v/v) IRI-160AA. Application of IRI-160AA induced significantly higher PSII inhibition and cell mortality in *K. veneficum* subjected to continuous darkness as compared to cells maintained with 12:12 h light/dark cycles, while no such dark effect was noted for *G. instriatum*. The marked differences in the rate and impact of this algicide suggest that multiple cellular targets and different cascades of cellular dysfunction occur across these dinoflagellates.

Keywords Bacterial algicide · Dinoflagellates · Photobiology · PSII · HAB · Membrane permeability

Introduction

It is widely accepted that harmful algal blooms (HABs) are increasing (Hallegraeff 1993; Van Dolah 2000; Anderson et al. 2002), and that certain HAB species are expanding their biogeographic ranges (e.g., Mcleod et al. 2012). Anthropogenic eutrophication (Anderson et al. 2002, 2008; Heisler et al. 2008), ballast water transportation (Hallegraeff 1993; Bolch and de Salas 2007; Smayda 2007) and potentially climate change (Moore et al. 2008; Hallegraeff 2010; Mcleod et al. 2012) are all thought to contribute to a continued global expansion of HABs. Irrespective of the proximal causes, these blooms will continue to increase environmental, and economic costs which are currently estimated at US\$82 million and US\$813 million annually in the US and European Union, respectively (Hoagland and Scatista 2006), thus highlighting the need for effective management solutions. HAB management is broadly segregated by whether methods target solutions for the present or the future, and then by whether the actions are direct or indirect. Actions that aim to prevent or reduce HAB occurrences in the *future* are termed “prevention” approaches. The most common form of prevention aims to restrict nutrient loads to aquatic systems to reduce anthropogenic eutrophication. However, in estuarine and marine waters, successful prevention of HABs by nutrient management may be less straightforward than for freshwater HABs because of a wider variety of HAB species and environmental conditions (Smith and Schindler 2009). Furthermore, Heisler et al. (2008) note that enacting effective policies for nutrient restrictions is quite challenging and costly and would require adaptive management to account for imperfect understanding of HAB dynamics (see also Zingone and

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Enevoldsen 2000). Likewise, some HABs are not linked to anthropogenic eutrophication and therefore would not respond to restricted nutrient releases (e.g., Bachmann et al. 2003; Anderson et al. 2008). Recognizing these difficulties, it is imperative to pursue more immediate strategies as well. In this regard, actions that aim to minimize the *impacts* of HABs (e.g., aquaculture losses, human illnesses) in the *present* or *immediate future*, are termed “mitigation” approaches, and are not mutually exclusive from yielding “prevention”. Mitigation approaches are further sub-divided into either direct actions that reduce or contain a HAB *population*, termed “control”, or indirect/passive actions to ameliorate HAB *impacts*, which we term “non-control mitigation”. Non-control mitigation, such as moving finfish cages to avoid fish kills (Rensel and Whyte 2003), and monitoring to prevent the harvesting of toxic organisms (Zingone and Enevoldsen 2000; Trainer and Suddleson 2005) has proved successful. However, non-control mitigation is sometimes impractical and costly, or too passive to ameliorate certain impacts while a bloom still exists (e.g., aerosolized toxins or toxicity to wild animals). Consequently, under these circumstances, control mitigation may be more effective.

Control mitigation encompasses many different chemical, physical and biological strategies (Kim 2006; Sengco 2009). Physical controls, such as mixing (accomplished by bubbling) alleviated *Microcystis* sp. blooms in a lake (Jungo et al. 2001), and clay flocculation has been successful at removing a wide variety of HAB species (Sengco and Anderson 2004). Chemical controls, such as copper sulfate additions (Rounsefell and Evans 1958), and hydrogen peroxide (Matthijs et al. 2012) have also been effective. Often however, broad toxicity is cited as an obstacle to real-world use (Boylan and Morris 2003; HAB RDDTT 2008). Recent discoveries of less broadly toxic chemicals have shown promise, such as L-lysine which has been used against *Microcystis* spp. (Takamura et al. 2004) and thiazolidinediones (Kim et al. 2010). In addition, a number of chemicals effective against HAB species have been derived from biological origins, such as unknown compounds in barley straw extracts (Terlizzi et al. 2002), polyphenols from tea leaf extracts (Lu et al. 2013), rhamnolipid biosurfactants from *Pseudomonas aeruginosa* (Wang et al. 2005) and prodigiosin from the bacterium *Hahella chejuensis* (Jeong et al. 2005). Lastly, biological control techniques have been successful in controlling certain HAB species in controlled incubations, primarily via algicidal bacteria (Kim et al. 2008; Kang et al. 2011; Paul and Pohnert 2012) and micrograzers (Jeong et al. 2008). To date, no single approach has been truly championed, and the unique advantages of these many strategies have warranted continued examination.

Hare et al. (2005) described a bacterium, *Shewanella* sp. IRI-160, that had a growth inhibiting effect on three dinoflagellates (*Prorocentrum minimum*, *Pfiesteria piscicida*, and *Gyrodinium uncatenum*), but had no growth inhibiting effects

on four non-dinoflagellate species. A recent investigation by Pokrzywinski et al. (2012) found that the algicidal activity of *Shewanella* sp. IRI 160 was due to a thermally stable, polar and water soluble compound or compounds secreted by the bacterium. The agent(s) accumulated in the medium, such that cell-free bacterial filtrates induced algicidal and growth-inhibitory effects, thereby negating the need to apply live bacteria. Furthermore, algicidal activity was significantly greater when applied to dinoflagellates in logarithmic growth, as compared to stationary or lag-phase cultures. The specific cellular targets, and mode of action of this secreted algicide is currently under investigation.

Testing of naturally derived algicides and allelochemicals *in vitro* has revealed that many act on specific components of the photosynthetic apparatus of susceptible algal cells. For example, free fatty acids disrupt membranes, resulting in the dissociation of phycobilins (but not integral chlorophylls) from the thylakoid membranes of cyanobacteria (Wu et al. 2006). Polyphenolic algicides produced by a macrophyte inhibit photosystem II (PSII) in *Anabaena* sp. by interfering with electron transport between the primary and secondary quinones Q_A and Q_B in the PSII reaction center (Leu et al. 2002). Likewise, the compounds Fischerellin A and Fischerellin B from the cyanobacterium *Fischerella muscicola*, target multiple sites on the electron transport chain surrounding PSII (Smith and Doan 1999), and Cyanobacterin derived from *Scytonema hofmannii* likely targets Q_A on the acceptor side of PSII (Gleason and Case 1986). Lastly, rhamnolipid biosurfactants from the bacterium *Pseudomonas aeruginosa* inactivated PSII in a range of HAB taxa (Gustafsson et al. 2009). Furthermore, Shi et al. (2009) found that algicidal agents produced *in vivo* were also capable of inhibiting the photosynthetic apparatus, showing that the algicidal bacterium *Pseudomonas mendocina* caused PSII inactivation in *Aphanizomenon flos-aquae*. In the current study, we explored whether the algicide IRI-160AA affects PSII function in a range of cultured dinoflagellates and report on the timing of membrane permeability in affected cells.

Methods

Bacterial filtrate preparation

Bacterial filtrates were prepared following Hare et al. (2005) and modifications detailed in Pokrzywinski et al. (2012). Briefly, *Shewanella* sp. IRI-160 was plated onto LM medium agar without antibiotics. Plates were incubated at room temperature until colony formation, whereupon a colony was transferred to 100 mL of liquid LM medium without antibiotics and incubated at 25 °C on an orbital shaker at 100 rpm for 18 h. Cultures were split into two, centrifuged, and washed twice in *f/2* seawater algal medium (Guillard and Rytner

1962). The bacteria were resuspended in 80 mL fresh *f/2*, and incubated at 30 °C for 1 week. Finally, the culture was centrifuged, and the supernatant was filtered (0.2 µm) and stored at –80 °C until later use. The filtrate was thawed prior to use and then stored at 4 °C during subsequent applications. Following Pokrzywinski et al. (2012), we refer to this filtrate as IRI-160AA.

Algae species

Five algae species were tested for photochemical inhibition with the algicide: four dinoflagellates [*Karlodinium veneficum* (CCMP 2936), *Gyrodinium instriatum* (CCMP 2935), *Prorocentrum minimum* (CCMP 2233), *Alexandrium tamarense* (CCMP 1493)], and one cryptophyte, [*Rhodomonas* sp. (CCMP 757)]. Non-axenic batch cultures were grown in autoclaved 20 PSU *f/2* medium at 24 °C, under 150 µmol photons m⁻² s⁻¹ on a 12:12 light/dark cycle provided by cool-white fluorescent lights. For all experiments, algae cultures were sampled during logarithmic growth, as this growth phase shows the greatest susceptibility to the algicidal and growth-inhibitory effects of IRI-160AA (Pokrzywinski et al. 2012).

Species testing

Tests comparing five species were carried out aseptically in autoclaved 15-mL glass test tubes, with independent replication. Treatments and controls were run in triplicate. Treatment cultures included addition of 4 % (v/v) IRI-160AA while controls included 4 % (v/v) *f/2* medium. Logarithmic-stage cultures were inoculated mid light-cycle (of a 12:12 L/D) and were sampled for both photochemistry (see below) and cell density after 18 h of exposure and then in three subsequent 24 h intervals (=42, 66, 90 h). The 18-h sampling point was chosen because this was previously noted as a time of peak inhibition in other physiological variables (Pokrzywinski et al. submitted). Cells were fixed with Lugols Iodine solution, and later enumerated by light microscopy in a Neubauer Haemocytometer or Sedgwick-Rafter cell. Percent cell loss was calculated as: (number of cells lost/initial abundance) × 100.

Algal photochemistry

PSII function was assessed by fast repetition rate fluorometry (Kolber et al. 1998; FASTtrack II and FASTact system, Chelsea Instruments, UK). A series of 100 subsaturating LED “flashlets” at ~1 µs intervals were applied to generate a single-turnover fluorescence transient. Fluorescence transients were used to evaluate three primary parameters: (1) the maximum quantum yield of PSII ($F_v/F_m = F_m - F_o/F_m$), (2) the rate of primary quinone re-oxidation and electron transport out of

PSII (τ , $Q_{A^-} \rightarrow Q_A$), and (3) the photochemical connectivity between PSII reaction centers (ρ) (Kolber et al. 1998; Cosgrove and Borowitzka 2010). The F_v/F_m ratio describes the maximum efficiency of PSII for converting absorbed light into a photochemical charge separation, and lower F_v/F_m values can be used to infer PSII inactivation (i.e., damage) or down-regulation of PSII reaction centers. Tau (τ) describes the time constant of electron transfer on the acceptor side of PSII, and specifically describes the time required to re-oxidize the reduced primary quinone (Q_{A^-}) measured in µs. PSII connectivity (ρ) describes the relative capacity to re-direct excitation energy from closed reaction centers, to nearby open PSII reaction centers.

Prior to fluorescence measurement, samples were dark-acclimated at room temperature for ≥20 min to re-open PSII reaction centers and relax non-photochemical quenching. The FRR sample chamber was maintained at 24 °C. The fluorescence measurement protocol consisted of three consecutive acquisitions, with each acquisition consisting of 40 repetitions of the following sequence: 100 flashlets at 1-µs intervals to reach saturation, and 50 flashlets at 49-µs intervals to record relaxation kinetics. Curve-fitting software provided with the instrument (FASTpro v3.0, Chelsea Instruments, UK) was used to derive F_v/F_m , τ , and ρ . All curve fits and fluorescence transients were manually inspected in real time.

Dose responses

The dose response of *K. veneficum* to IRI-160AA was assessed in triplicate at final concentrations of 2, 4, 6, 8, 12, and 16 % (v/v), alongside an 8 % (v/v) *f/2* control. F_v/F_m and cell density were measured after 18 h of incubation. The control was limited to an 8 % concentration because a dilution series of *f/2* concentrations was expected to yield identical results. F_v/F_m and cell density were converted to percent inhibition of the *f/2* control ((treatment/average–control) × 100). Prior to the conversion of cell density to percent inhibition, the average control cell densities were corrected to account for the differing dilutions used relative to the control using:

Average 8% control cell density

$$-\left(\frac{\% \text{ treatment concentration} - 8}{100} \times \text{control cell density}\right)$$

The F_v/F_m ratio is unaffected by cell density and does not require this dilution correction.

In order to test whether IRI-160AA activity was affected by dinoflagellate cell density, a second experiment was conducted which compared IRI-160AA activity against dilutions of *K. veneficum*. IRI-160AA was added at a 4 % (v/v) final

concentration, in triplicate to three dilutions of *K. veneficum* culture (no dilution, 1:3 dilution and 2:3 dilution in *f/2* medium). Control cultures included addition of 4 % (v/v) *f/2* medium to undiluted culture. Measurements of cell density and F_v/F_m were made after 18 h.

Membrane permeability

Outer-membrane permeability in relation to IRI-160AA activity was investigated in *K. veneficum* and *G. instriatum* with the fluorescent dye, SYTOX Green (Invitrogen, USA; SYTOX). SYTOX fluoresces brightly when bound to DNA but cannot pass through intact membranes, and can therefore be used to indicate the presence of dead cells in which the membrane has been compromised. Treatments included 8 % (v/v) IRI-160AA additions, and controls included 8 % (v/v) *f/2*. Subsamples were collected every 3 h for 24 h. SYTOX was added to subsamples in duplicate at a 1 μM final concentration and incubated for 90 min in the dark, and fluorescence was measured on a microplate reader with excitation and emission at 485 and 520 nm, respectively (FLUOstar Omega, BMG Labtech). The cellular origin of SYTOX fluorescence was verified using an epifluorescence microscope (Microphot-FXA, Nikon, Japan). Total SYTOX fluorescence values were blank corrected (*f/2* medium+1 μM SYTOX) and converted to SYTOX fluorescence per cell. The SYTOX fluorescence per cell of IRI-160AA treatments were converted to a percent of the average *f/2* control fluorescence per cell (which did not vary significantly over 24 h, one-way ANOVA, $n=3$, $P>0.05$).

Dark incubation

To test how light might influence the susceptibility of *K. veneficum* and *G. instriatum* to IRI-160AA, we compared the effect of IRI-160AA incubations in the dark to IRI-160AA incubations in light/dark (L/D) cycles. Using cultures of *K. veneficum* and *G. instriatum* that were grown under 12:12 L/D cycles, two 4 % IRI-160AA treatments were tested. One of these treatments was transferred to continuous (24 h) darkness, and the other treatment was kept in the regular 12:12 light/dark cycle. Controls included addition of 4 % *f/2*, with one control transferred to continuous darkness, and the other kept in 12:12 light/dark cycles. Both treatments and both controls were performed in triplicate and transferred to their respective light/dark treatments immediately after IRI-160AA or *f/2* addition, at approximately mid light-cycle. Cell density and photochemistry were measured at 0, 18, 42, 66, and 90 h after addition of IRI-160AA.

Statistical analyses

Statistical analyses were performed in Prism 5 (GraphPad Software Inc., USA). All experimental treatments tested were independent with small balanced sample sizes ($n=3$). Different treatments were analyzed by two-way ANOVA with time and treatment as independent variables, and were followed by Bonferroni post hoc tests. The culture dilution experiment was tested by one-way ANOVA and the Tukey's HSD post hoc test.

Results

Species comparison

All four species of dinoflagellates showed significant growth inhibition with IRI-160AA, whereas the cryptophyte, *Rhodomonas* sp., responded with an increased growth rate and a higher maximal abundance relative to *f/2* controls (Table 1 and Fig. 1a–e). Despite all dinoflagellates showing susceptibility to IRI-160AA by growth inhibition, there were species-specific responses in the relative magnitude and timing of cell mortality, growth inhibition and recovery (Fig. 1a–d, Table 1), and photochemical inhibition and recovery (Figs. 1f–i and 2). *G. instriatum* and *K. veneficum* showed similar levels of susceptibility to the algicide (54 ± 19 % and 50 ± 20 % cell loss, respectively), with some recovery or stabilization in cell density occurring after 42 and 66 h respectively (Fig. 1, Table 1). *Alexandrium tamarense* exhibited a continuous decline in cell density before reaching a maximum cell loss of 7 % (± 10 %), at 90 h (Fig. 1c, Table 1). *P. minimum* did not decline in abundance during the experiment, but did exhibit growth inhibition relative to the control (Fig. 1d, Table 1).

Table 1 Specific growth rates from Fig. 1 (algal species tested with 4 % IRI-160AA or 4 % *f/2* medium). IRI-160AA growth rates and percent cell loss were calculated from T0 to the time point with the lowest cell number, except for *P. minimum* and *Rhodomonas* sp. which did not show a drop in cell density (N/a) and were calculated over 90 h. Percent cell loss was rounded to the nearest whole percentage with ± 1 SD given in parentheses

Growth rates	μ (day^{-1})		Cell loss (%)
	Control	IRI-160AA	
<i>G. instriatum</i>	0.27	−0.26	54 (± 19)
<i>K. veneficum</i>	0.39	−0.40	50 (± 20)
<i>A. tamarense</i>	0.27	−0.01	7 (± 10)
<i>P. minimum</i>	0.21	0.08	N/a
<i>Rhodomonas</i> sp.	1.00	1.06	N/a

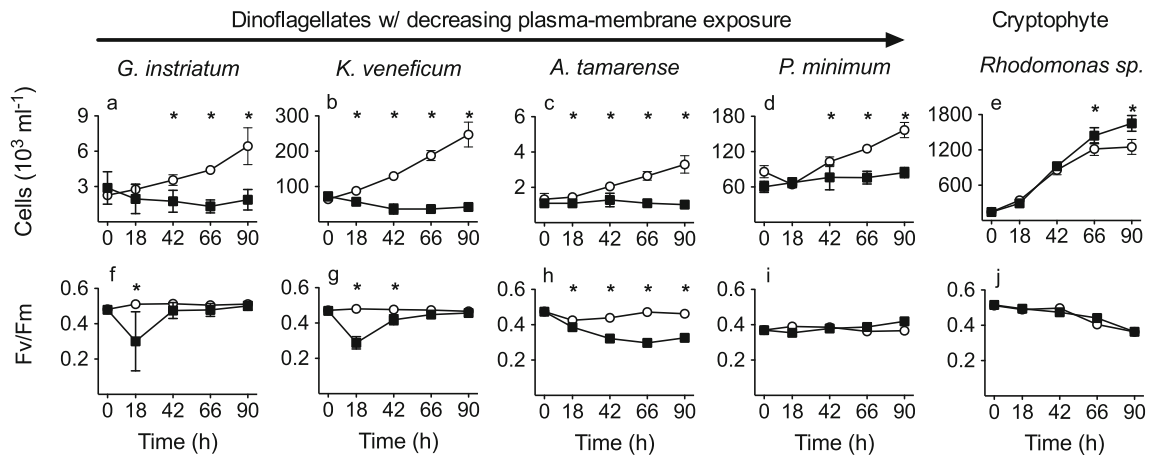


Fig. 1 Species-specific responses in cell density (a–e) and F_v/F_m (f–j) among four dinoflagellates: *G. instriatum* (a, f), *K. veneficum* (b, g), *A. tamarensis* (c, h), and *P. minimum* (d, i), and 1 cryptophyte, *Rhodomonas* sp. (e, j), after addition of 4 % IRI-160AA concentration (filled squares) compared to control cultures (open circles) after addition of 4 % *f/2* medium. The species are ordered from highest to lowest

response (left to right) and by relative plasma-membrane exposure, which reflects the change from naked to thecate dinoflagellates and then also by number of plates/valves (see “Discussion”). Asterisks indicate a significant difference as determined by two-way ANOVA and Bonferroni post tests ($n=3$, $P<0.05$). Error bars are ± 1 SD of the mean

G. instriatum, *K. veneficum*, and *A. tamarensis* exhibited similar levels of PSII inactivation after exposure to IRI-160AA. Maximal inhibition occurred at 18 h for algicide-treated cultures of both *K. veneficum* and *G. instriatum*, and at 66 h in *A. tamarensis* (Fig. 1f, g, h). Recovery from PSII inactivation in remaining cells in the treatment cultures occurred by 42 h in *G. instriatum* and by 66 h in *K. veneficum* (Fig. 1f, g), while *A. tamarensis* showed negligible recovery between 66 and 90 h (Fig. 1h). *Prorocentrum minimum* showed no significant PSII inactivation (two-way ANOVA, $P>0.05$, Fig. 1i) after exposure to IRI-160AA. *Rhodomonas* sp. treated with IRI-160AA exhibited no significant difference in F_v/F_m compared to the control (Fig. 1j).

Following the species’ responses in F_v/F_m , PSII re-oxidation (τ) in the algicide-treated samples took significantly longer than the *f/2* controls in *G. instriatum*, *K. veneficum*, and *A. tamarensis* (two-way ANOVA, $P<0.05$) and was also observed in *P. minimum* (Fig. 2a). Temporally, τ was altered in *G. instriatum* and *K. veneficum* only after 18 h of exposure, and recovered to control levels (0 % horizontal line) by 42 h. In *A. tamarensis*, τ remained significantly slower as compared to the controls until the end of the experiment (Fig. 2a), and in *P. minimum* τ was inhibited after 90 h only. Connectivity (ρ) was significantly lower only in *K. veneficum* and *A. tamarensis* (two-way ANOVA, $P<0.05$), and recovered by 42 h in *K. veneficum*, and did not recover in *A. tamarensis* (Fig. 2b). Conversely, ρ was significantly higher relative to the control in *P. minimum* after 90 h when exposed to the algicide (two-way ANOVA, $P<0.05$, Fig. 2b). In *Rhodomonas* sp. treated with IRI-160AA, τ was significantly faster at 42 and 90 h, and ρ was significantly lower than the control at 90 h.

Dose response

K. veneficum exhibited a typical dose response in photochemical inhibition and cell density with increasing IRI-160AA

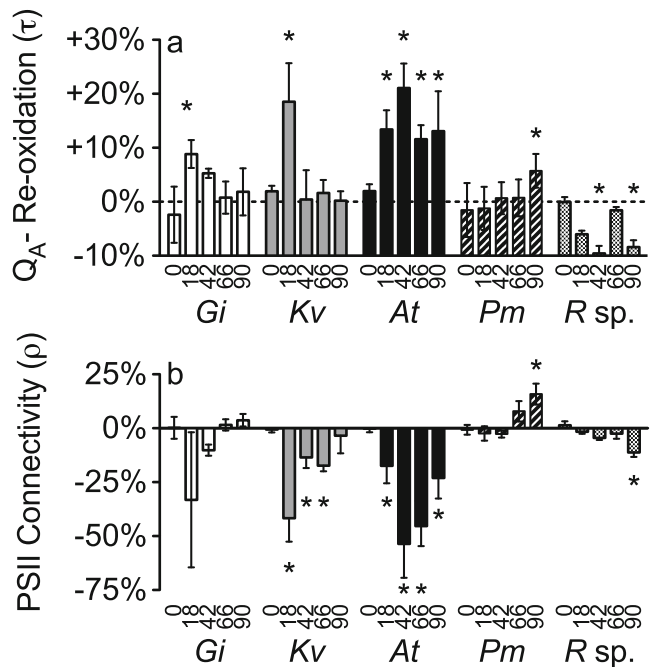


Fig. 2 Species-specific responses in the Q_A - re-oxidation rate τ (a), and in PSII connectivity ρ (b), both expressed as relative change from the *f/2* control, so that the 0 % horizontal lines represent the average *f/2* control. *Gi* *G. instriatum* (open bars); *Kv* *K. veneficum* (gray bars); *At* *A. tamarensis* (black bars); *Pm* *P. minimum* (striped bars); *R sp.* *Rhodomonas* sp. (fine checkered). Data are from the respective experiments shown in Fig. 1. Asterisks indicate a significant difference ($n=3$, $P<0.05$) determined by two-way ANOVA, and Bonferroni post tests. Error bars are ± 1 SD of the mean

concentrations from 2 to 16 % (Fig. 3). The percent inhibition using F_v/F_m values was significantly lower than for cell density only at a 4 % concentration (two-way ANOVA, $n=3$, $P<0.05$). The average EC-50 calculated from the fitted dose response curves was 7.0 and 8.7 % (v/v) IRI-160AA when calculated using cell density and F_v/F_m respectively. Final IRI-160AA concentrations of 12 to 16 % resulted in near to 100 % photochemical and cellular inhibition relative to the control after 18 h. The time required for recovery in growth and F_v/F_m was also dose-dependent (i.e. longer at higher doses), as was relative membrane permeability as determined by SYTOX fluorescence (higher SYTOX fluorescence was noted at higher doses; data not shown). In contrast to the effects of IRI-160AA concentration, modifying algal cell density by dilution had no effect on the relative inhibition in photochemistry or cell density after 18 h exposure to IRI-160AA at 4 % (v/v) concentration (Fig. 3 inset). Cell densities and F_v/F_m in all IRI-160AA treatments were, however, significantly lower than the control in the algal dilution experiment ($n=3$, one-way ANOVA and Tukey test, $P<0.05$).

Membrane permeability

When photochemistry was followed with greater temporal resolution, *K. veneticum* exhibited moderate PSII inactivation after 18 h exposure to the algicide (Fig. 4a), whereas *G. instriatum* showed large and immediate (<3 h) PSII

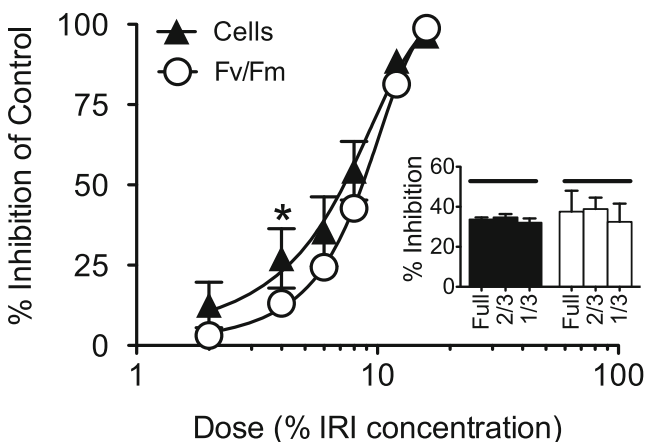


Fig. 3 Dose response based on cell density (filled triangles) and F_v/F_m (open circles) in *K. veneticum* expressed as % inhibition relative to the *f/2* control, after 18 h. Variable slope dose response curves are fitted to the data. Error bars are ± 1 SD. Percent inhibition based on cell density and F_v/F_m are significantly different at the 4 % concentration (two-way ANOVA and Bonferroni post tests, $n=3$, $P<0.05$). The inset figure shows relative inhibition in F_v/F_m (filled bars) relative to the *f/2* control and cell density (open bars) relative to initial densities in culture dilutions treated with 4 % IRI-160AA. Full no dilution, 2/3 33 % dilution, 1/3 66 % dilution. No significant differences were observed among dilutions for either cellular inhibition, or photochemical inhibition (one-way ANOVA and Tukey test). However, IRI-160AA treatments all differed significantly from *f/2* controls ($n=3$, $P<0.05$)

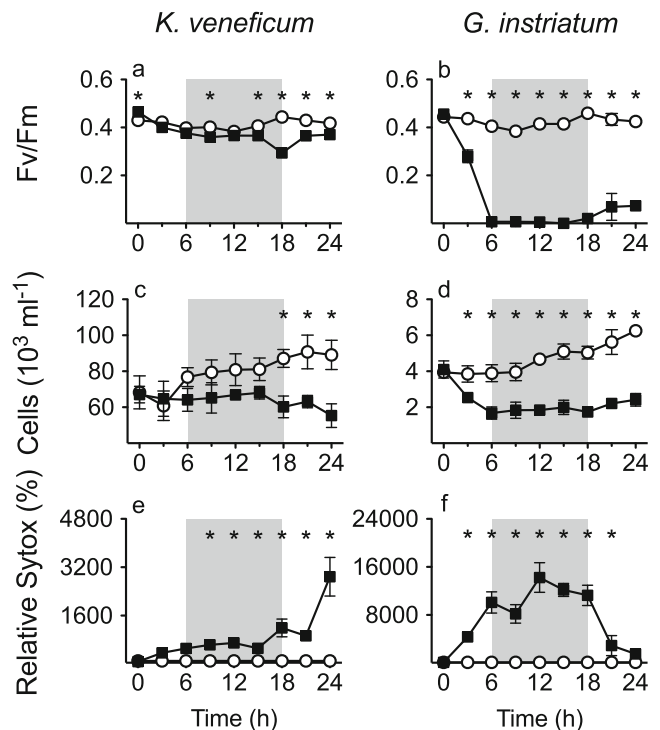


Fig. 4 Twenty-four-hour incubations with *K. veneticum* (left panes) and *G. instriatum* (right panes). Cultures were incubated with either 8 %v/v IRI-160AA (filled squares) or 8 %v/v *f/2* medium (open circles). F_v/F_m (a, b), cell density (c, d) and SYTOX fluorescence (e, f, relative to the *f/2* control) are presented. Grayed areas behind the data represent the dark period. Asterisks indicate time points with a significant difference, as determined by two-way ANOVA and Bonferroni post tests ($P<0.05$, $n=3$). Error bars represent ± 1 SD

inactivation (Fig. 4b). Growth for *K. veneticum* appeared to slow by 6 h after addition of IRI-160AA and cell density was significantly lower than the controls between 18 and 24 h (Fig. 4c), whereas a large immediate decline in cell density occurred within the first 6 h of exposure to the algicide for *G. instriatum* (Fig. 4d). By 9 h, there was a significant increase in cell membrane permeability (detected by SYTOX green) in algicide-treated *K. veneticum*, which peaked abruptly after 24 h (Fig. 4e). In comparison, significant cell permeability was detected in *G. instriatum* immediately after IRI-160AA addition, which then peaked earlier near 12 h (Fig. 4f). For *G. instriatum*, the timing and relative changes in SYTOX fluorescence correlated well with the relative changes in F_v/F_m and cell density. In contrast, the main decline in F_v/F_m in *K. veneticum* at 18 h preceded the maximum permeability by 6 h, but did coincide with the commencing decline in cell density between 15 and 24 h.

Darkness

K. veneticum and *G. instriatum* showed different responses to darkness both in the presence and absence of IRI-160AA. Compared to light controls, dark *K. veneticum* controls

showed photochemical inhibition and stunted growth by 42 h, and precipitous declines in both photochemistry and cell number by 90 h (Fig. 5a, b). Contrarily, dark *G. instriatum* controls showed a small but consistent down-regulation of PSII photochemistry after 18 h and showed inhibited growth from 42 h, but neither photochemistry nor cell density showed the precipitous declines by 90 h as in *K. veneficum*. Dark IRI-160AA treatment of *K. veneficum* induced greater PSII inactivation after 18 h (Fig. 5a), and a larger loss in cell density after 42 h (Fig. 5b) as compared to light IRI-160AA treatments. Neither cell density nor PSII inactivation recovered in *K. veneficum* treated with IRI-160AA and kept in continuous darkness. Contrary to the dark response of *K. veneficum*, *G. instriatum* showed no significant increase in cellular mortality in dark algicide applications relative to those kept in light/dark cycles (Fig. 5d). However, *G. instriatum* did show significantly more PSII inactivation by 42 h in dark IRI-160AA treatments, and like *K. veneficum* failed to fully recover from the initial PSII inactivation (Fig. 5c) in the dark IRI-160AA treatments.

Discussion

A large proportion of algicides and allelochemicals derived from marine and freshwater microalgae appear to target competitor phytoplankton cell membranes, often simultaneously inactivating PSII and photosynthetic electron transport, and inhibiting growth (Smith and Doan 1999; Legrande et al. 2003

and references therein). Here, we have employed analysis of single-turnover chlorophyll fluorescence to evaluate PSII inhibition in dinoflagellates under algicidal treatment, using a previously described filtrate from the bacterium *Shewanella* sp. IRI-160 (Hare et al. 2005; Pokrzywinski et al. 2012).

General dynamics of photobiology and cell number

With the exception of *P. minimum*, we found a significant decline in the maximum quantum yield of PSII (F_v/F_m) during algicide incubations, and observed declines in cell density and inhibition of growth in all dinoflagellates tested. Significant loss in PSII photochemistry was accompanied by inhibition in τ (Q_A - re-oxidation) and ρ (PSII connectivity). Broadly, these results describe the disruption of photosynthetic electron transport (PET) through PSII. While we cannot ascertain temporal loss in carbon fixation from this data, such reduction in PET would most likely lead to a substantial loss in ATP and NADPH necessary for carboxylation and other cellular processes. Although the relative magnitude of PSII inactivation showed some variability among experiments, we are confident in our species-specific differences because repeated experiments returned the same trends on average (data not shown). We believe some of this variability may stem from natural variations in the production and accumulation of the bioactive compound(s) in each batch of *Shewanella* sp. IRI-160.

The recovery of F_v/F_m back to control levels after incubation with 4 % IRI-160AA occurred within 90 h in all but *A. tamarensis*, and was considered to originate from a small population of less effected cells, while the majority were lysed and were no longer contributing to the active chlorophyll *a* fluorescence signal. In addition to the photochemical recovery seen in the residual population that avoided mortality at lower concentration algicide treatments, these cells were also capable of cell division. Clearly, the dynamics of any population re-growth would be complicated further if these experiments were carried out in natural parcels of water that contained other competing phytoplankton as well as micro and mesograzers.

Species specificity and membrane exposure

Pokrzywinski et al. (2012) concluded that thecate dinoflagellates exhibited reduced levels of algicidal activity compared with naked dinoflagellates. The results presented here corroborate this pattern, with *K. veneficum* and *G. instriatum* exhibiting similar declines in cell density and photochemistry, whereas the thecate dinoflagellate *A. tamarensis* exhibited reduced susceptibility to IRI-160AA with a lag in the photochemical response. However, the lack of any photochemical response with algicide treatment yet significant reduction in cell growth noted for *P. minimum* provides evidence that the algicidal mode of action across these different dinoflagellates

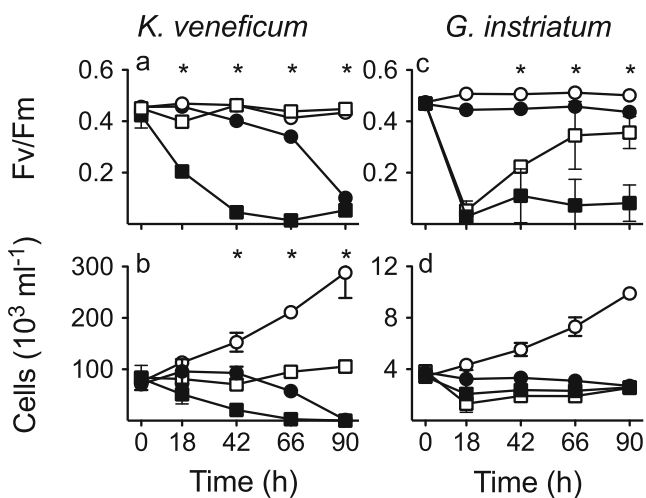


Fig. 5 Effects of shifting treated cultures into continuous darkness immediately upon addition of 4 % (v/v) IRI-160AA in *K. veneficum* (a, b) and *G. instriatum* (c, d). Light treatments are indicated by open symbols, dark treatments are indicated by filled symbols, circles indicate controls and squares are IRI-160AA treatments. Asterisks indicate a significant difference only between IRI-160AA light and IRI-160AA dark treatments (two-way ANOVA and Bonferroni post tests, $P < 0.05$, $n = 3$). Error bars are ± 1 SD of the mean

is not ubiquitous. Furthermore, the thecal morphology of *A. tamarensis* and *P. minimum* are very different, with *A. tamarensis* having many similarly sized thecal plates (Fukuyo 1985), and *P. minimum* comprised primarily of two large valves (Faust et al. 1999). It is possible that plasma-membrane access is required for the algicidal compound(s) to be effective against chloroplast function or that *P. minimum* is only susceptible to the algicide by an as yet unknown cellular pathway. Several examples in the literature of algicide resistance in organisms with physical barriers lend support to the thecae protection hypothesis. For example, reduced cytotoxicity from fatty acids in *Anabaena* P-9 heterocysts compared to vegetative cells implicated a protection mechanism of the heterocyst's thick cell wall (Wu et al. 2006). Secondly, the cellulose plates of the dinoflagellates, *Cochlodinium polykrikoides*, *P. minimum*, and *Prorocentrum micans*, were thought to reduce the speed and magnitude of algicidal effects by peptides, compared with the high activity on the relatively exposed plasma membranes of raphidophytes (Park et al. 2011). Thirdly, gram-positive bacteria were susceptible to a rhamnolipid biosurfactant, but a gram-negative bacterium was not, leading Sotirova et al. (2008) to suggest that lipopolysaccharides in the outer membrane formed a barrier to surfactant activity. Further, the results of Sotirova et al. (2008) led Gustafsson et al. (2009) to propose that the complex cell wall of *Microcystis aeruginosa* (a gram-negative harmful cyanobacteria) may contribute to this species' resistance to a rhamnolipid biosurfactant as well.

Another consideration is the different plastid origins of the dinoflagellates tested in this study, with a view to identifying possible links driving the species-specific susceptibilities and photosynthetic responses observed. *G. instriatum*, *P. minimum* and *A. tamarensis* share the putatively ancestral type plastid, with the photosynthetic pigment peridinin, a form II RuBisCo, and a highly reduced mini-circle genome, while *K. veneficum* has a the more derived plastid with fucoxanthin, form I RuBisCo, and a full circular plastid genome (Daugbjerg et al. 2000; Yoon et al. 2005; Wisecaver and Hackett 2011). In consequence, the fewer plastid encoded genes, and possibly less redox regulation of remaining plastid genes in peridinin containing dinoflagellates, require a higher degree of nuclear-encoded plastid-bound protein translocation and are therefore possibly more susceptible to plastid damage if inter-organelle signaling and plastid protein targeting is impaired by IRI-160AA. However, this relationship between plastid genome reduction and algicide susceptibility is likely complex, as evidence suggests that *K. veneficum* has a chimeric proteome derived from nuclear-encoded secondary (peridinin-based) and tertiary (fucoxanthin-based) endosymbioses (Patron et al. 2006). If there is an influence of plastid origin, then the importance of the plastid would likely depend upon the extent of endosymbiotic gene transfer, and in particular, how many and which genes have been transferred to the nucleus.

Dose responses

The small but significantly greater inhibition based on cell density as compared to F_v/F_m at 4 % (Fig. 3) is difficult to interpret, but may be due to highly susceptible cells lysing quickly, with little to no time spent in an inhibited-state, such that photoinhibitory signals are not readily observed. The broad similarity between the dose response curves based on photochemistry and cell density however suggests that the increased inhibition based on F_v/F_m values is likely due to a larger number of affected cells, rather than from increased inhibition in a subset of affected cells. In further support of this, we observed that under a low IRI-160AA concentration (4 %), a proportion of the population (found at the surface of the culture medium) retains a significantly higher photosynthetic competency than the integrated population as a whole (data not shown). Likewise, this also explains why photochemistry appears to recover by 42 h following treatment with 4 % IRI-160AA (Fig. 1). It is important to stress here, however, that this recovery represents a substantially smaller population of cells than at the start of the experiment, and the number of residual photochemically active cells is dose-dependent (Fig. 3). Further testing is needed in order to discern if multiple applications of a low concentration of IRI-160AA may eliminate these residual cells as well.

In a second dose response experiment, serial dilutions of algal culture were treated with 4 % IRI-160AA to examine the possibility that the algicidal activity is dependent on cell density. Here, the degree of mortality and the decline in F_v/F_m did not vary between culture dilutions (Fig. 3 inset). This response differs from the results described in Mu et al. (2009), in which enhanced algicidal activity was observed by the algicidal bacterium, *Ochrobactrum* sp. FDT5, when inoculated into cultures with lower chlorophyll *a* concentrations (Mu et al. 2009). Given the dose response elicited by increasing IRI-160AA concentration as described above, where higher concentrations induced higher mortalities and larger decreases in PSII efficiency, we conclude that IRI-160AA activity is independent of cell density within the range tested here.

Membrane permeability and F_v/F_m

When photochemistry and membrane permeability were compared in tandem, the two dinoflagellate species tested responded differently to IRI-160AA addition. *G. instriatum* had a simultaneous decline in F_v/F_m and cell viability, while *K. veneficum* lost chloroplast function before plasma membrane integrity, with peak PSII inactivation 6 h prior to the peak loss in cell viability. Two hypotheses could explain this discrepancy between the species. Firstly, that the location, concentration, and susceptibility of algicide target sites differ between the two dinoflagellates (Pokrzywinski et al. submitted). Alternatively, Pokrzywinski et al. (2012) identified three

mass ions within the bacterial filtrate (with masses of 239.2, 301.2, and 470.1 Da), which may each contribute to algal mortality and/or growth inhibition. Given that multiple targets and multiple compounds are a common feature of allelopathy (Einhellig 1999), an alternative hypothesis is that differing susceptibility to each component may explain the differences observed between the two dinoflagellates. Confirmation of such hypotheses cannot be resolved without the isolation and identification of the bioactive compound(s) and subsequent identification of the specific cellular target sites.

Darkness

There is a range in dark tolerance across phytoplankton taxa, including species known to cause harmful algal blooms (Smayda and Mitchell-Innes 1974; Peters and Thomas 1996; Furusato et al. 2004; Popels et al. 2007), thus it is not surprising that *G. instriatum* displayed a much higher degree of general dark tolerance than *K. veneficum*. However, it is important to note the significant difference between the responses of these two species when treated with the algicide under prolonged darkness, and how their response correlates to what appears to be their basal tolerance to prolonged dark exposure. While this area of research is limited, Mayali et al. (2007) have noted that the bacterial exposure while in prolonged darkness increased the rate of ecdysis in the dinoflagellate *Lingulodinium polyedrum*. Mayali et al. (2007) suggested that darkness may reduce the ability of *L. polyedrum* to produce antibiotics, and although not directly applicable here since live bacteria were not used, an analogous mechanism involving antagonistic/protective molecule production could be a possibility for *K. veneficum* as well. The more rapid loss of PSII photochemistry noted for IRI-160AA-treated *K. veneficum* in the dark could be due to either a direct amplification of chloroplast dysfunction or may point to an accelerated loss of cellular function at another location. In this regard, disruption in mitochondrial activity and/or synthesis of chloroplast-targeted proteins in the cytoplasm could result in a similar decline in PSII activity as noted here. It is well known that homeostatic chloroplast protein repair requires some light and complete darkness can lead to greater inactivation (Bergo et al. 2003), however, our data may point to indirect disruption of chloroplast function as a better explanation. Notably, Pokrzywinski et al. (submitted) have shown that IRI-160AA exposure in dinoflagellates induces several cellular markers indicative of programmed cell death (PCD), including extracellular release of hydrogen peroxide, caspase-like activity, and phosphatidylserine inversion of cellular membranes. Likewise, Segovia and Berges (2005) have noted that PCD in the chlorophyte *Dunaliella tertiolecta* was accelerated when cells were placed in prolonged darkness in the presence of the cytoplasmic protein synthesis inhibitor cycloheximide. Given that maintenance respiration in dinoflagellates can

represent over half of the total dark respiration rate in some species, due in part to high protein turnover (Jauzein et al. 2011), and that disruption in mitochondrial activity may lead to down-stream loss in photochemistry (Saradadevi and Raghavendra 1992), it appears that IRI-160AA may affect *K. veneficum* mitochondrial activity to a greater degree than *G. instriatum*, and this leads to an even faster disruption in chloroplast electron transport activity in *K. veneficum*.

Conclusion

Dinoflagellate responses to IRI-160AA were found to be species-specific, but included PSII inactivation, loss in membrane integrity, inhibition of growth, and mortality. In agreement with Pokrzywinski et al. (2012), thecae appeared to provide protection from algicidal attack, and also appeared to limit PSII inactivation. Due to differences in susceptibility between *A. tamarense* and *P. minimum*, we suggest that the thecae-armoring hypothesis could be extended to include relative plasma-membrane exposure. In comparing the effects of IRI-160AA on *K. veneficum* and *G. instriatum* we found that peak PSII inactivation was decoupled from peak SYTOX permeability in *K. veneficum* but not in *G. instriatum*, and that darkness affected *K. veneficum* substantially more than *G. instriatum*. These discrepancies led us to speculate that physiological differences can substantially alter susceptibility to IRI-160AA. Ultimately, the specificity of this algicide for certain dinoflagellates remains promising from an applied standpoint and further work on the mode of action, the efficacy of the algicide in situ, and identification of the bioactive compounds are currently underway.

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References

- Anderson DM, Glibert PM, Burkholder JM (2002) Harmful algal blooms and eutrophication: nutrient sources, composition, and consequences. *Estuaries* 25:562–584
- Anderson DM, Burkholder JM, Cochlan W, Glibert PM, Gobler CJ, Heil CA, Kudela RM, Parsons ML, Rensel JEJ, Townsend DW, Trainer VL, Vargo GA (2008) Harmful algal blooms and eutrophication: examining linkages from selected coastal regions of the United States. *Harmful Algae* 8:39–53
- Bachmann RW, Hoyer MV, Fernandez C, Canfield DE Jr (2003) An alternative to proposed phosphorus TMDLs for the management of Lake Okeechobee. *Lake Reserv Manage* 19:251–264
- Bergo E, Segalla A, Giacometti GM, Tarantino D, Soave C, Andreucci F, Barbato R (2003) Role of visible light in the recovery of photosystem II structure and function from ultraviolet-B stress in higher plants. *J Exp Bot* 54:1665–1673

- Bolch CJS, de Salas MF (2007) A review of the molecular evidence for ballast water introduction of the toxic dinoflagellates *Gymnodinium catenatum* and the *Alexandrium* “tamarensis complex” to Australasia. *Harmful Algae* 6:465–485
- Boylan JD, Morris JE (2003) Limited effects of barley straw on algae and zooplankton in a midwestern pond. *Lake Reserv Manage* 19:265–271
- Cosgrove J, Borowitzka MA (2010) Chlorophyll fluorescence terminology: an introduction. In: Suggett DJ, Prasil OJ, Borowitzka MA (eds) *Chlorophyll a fluorescence in aquatic sciences: methods and applications*. Springer, Dordrecht, pp 1–18
- Daugbjerg N, Hansen G, Larsen J, Moestrup Ø (2000) Phylogeny of some of the major genera of dinoflagellates based on ultrastructure and partial LSU rDNA sequence data, including the erection of three new genera of unarmoured dinoflagellates. *Phycologia* 39:302–317
- Einhellig FA (1999) An integrated view of allelochemicals amid multiple stresses. In: Inderjit, Dakshini KMM, Foy CL (eds) *Principles and practices in plant ecology: allelochemical interactions*. CRC, Boca Raton, pp 479–494
- Faust MA, Larsen J, Moestrup Ø (1999) Potentially toxic phytoplankton. 3. Genus *Prorocentrum* (Dinophyceae). In: Lindley JA (ed) *ICES identification leaflets for plankton*. Leaflet no. 184. Copenhagen, pp 1–24
- Fukuyo Y (1985) Morphology of *Protogonyaulax tamarensis* (Lebour) Taylor and *Protogonyaulax catenella* (Whedon and Kofoid) Taylor from Japanese coastal waters. *Bull Mar Sci* 37:529–537
- Furusato E, Asaeda T, Manatunge J (2004) Tolerance for prolonged darkness of three phytoplankton species, *Microcystis aeruginosa* (Cyanophyceae), *Scenedesmus quadricauda* (Chlorophyceae), and *Melosira ambigua* (Bacillariophyceae). *Hydrobiologia* 527:153–162
- Gleason FK, Case DE (1986) Activity of the natural algicide, cyanobacterin, on angiosperms. *Plant Physiol* 80:834–837
- Guillard RRL, Ryther JH (1962) Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* Cleve. *Can J Microbiol* 8:229–239
- Gustafsson S, Hultberg M, Figueroa RI, Rengefors K (2009) On the control of HAB species using low biosurfactant concentrations. *Harmful Algae* 8:857–863
- HAB RDDTT (2008) In: Dortch Q, Anderson DM, Ayres DL, Glibert PM (eds) *Harmful algal bloom research, development, demonstration, and technology transfer national workshop report*. Woods Hole, Massachusetts, USA
- Hallegraeff GM (1993) A review of harmful algal blooms and their apparent global increase. *Phycologia* 32:79–99
- Hallegraeff GM (2010) Ocean climate change, phytoplankton community responses, and harmful algal blooms: a formidable predictive challenge. *J Phycol* 46:220–235
- Hare CE, Demir ED, Coyne KJ, Cary SC, Kirchman DL, Hutchins D (2005) A bacterium that inhibits the growth of *Pfiesteria piscicida* and other dinoflagellates. *Harmful Algae* 4:221–234
- Heisler J, Glibert P, Burkholder J, Anderson D, Cochlan W, Dennison W, Dortch Q, Gobler C, Heil C, Humphries E, Lewitus A, Magnien R, Marshall HG, Sellner K, Stockwell D, Stoecker DK, Suddleson M (2008) Eutrophication and harmful algal blooms: a scientific consensus. *Harmful Algae* 8:3–13
- Hoagland P, Scatasta S (2006) The economic effects of harmful algal blooms. In: Granéli E, Turner JT (eds) *Ecology of harmful algae*. Springer, Berlin, pp 391–402
- Jauzein C, Collos Y, Laabir M, Vaquer A (2011) Dark metabolism and carbon-nitrogen uncoupling in the toxic dinoflagellate *Alexandrium catenella* (Dinophyceae). *Harmful Algae* 11:73–80
- Jeong H, Yim JH, Lee C, Choi SH, Park YK, Yoon SH, Hur CG, Kang HY, Kim D, Lee HH, Park KH, Park SH, Park HS, Lee HK, Oh TK, Kim JF (2005) Genomic blueprint of *Hahella chejuensis*, a marine microbe producing an algicidal agent. *Nucleic Acids Res* 33:7066–7073
- Jeong HJ, Kim JS, Yoo YD, Kim ST, Song JY, Kim TH, Seong KA, Kang NS, Kim MS, Kim JH, Kim S, Ryu J, Lee HM, Yih WH (2008) Control of the harmful alga *Cochlodinium polykrikoides* by the naked ciliate *Strombidinopsis jeokjo* in mesocosm enclosures. *Harmful Algae* 7:368–377
- Jungo E, Visser PM, Stroom J, Mur LR (2001) Artificial mixing to reduce growth of the blue-green alga *Microcystis* in Lake Nieuwe Meer, Amsterdam: an evaluation of 7 years of experience. *Water Sci Technol Water Supply* 1:17–23
- Kang Y-H, Jung SW, Jo S-H, Han M-S (2011) Field assessment of the potential of algicidal bacteria against diatom blooms. *Biocontrol Sci Technol* 21:969–984
- Kim HG (2006) Mitigation and controls of HABs. In: Granéli E, Turner JT (eds) *Ecology of harmful algae*. Springer, Berlin, pp 327–338
- Kim B-H, Sang M, Hwang S-J, Han M-S (2008) *In situ* bacterial mitigation of the toxic cyanobacterium *Microcystis aeruginosa*: implications for biological bloom control. *Limnol Oceanog-Meth* 6:513–522
- Kim Y-M, Wu Y, Duong TU, Ghodake GS, Kim SW, Jin ES, Cho H (2010) Thiazolidinediones as a novel class of algicides against red tide harmful algal species. *Appl Biochem Biotechnol* 162:2273–2283
- Kolber ZS, Prasil O, Falkowski PG (1998) Measurements of variable chlorophyll fluorescence using fast repetition rate techniques: defining methodology and experimental protocols. *Biochim Biophys Acta* 1367:88–106
- Legrande C, Rengefors K, Fistarol GO, Granéli E (2003) Allelopathy in phytoplankton—biochemical, ecological and evolutionary aspects. *Phycologia* 42:406–419
- Leu E, Krieger-Liszka A, Goussias C, Gross EM (2002) Polyphenolic allelochemicals from the aquatic angiosperm *Myriophyllum spicatum* inhibit photosystem II. *Plant Physiol* 130:2011–2018
- Lu Y, Wang J, Yu Y, Su W, Kong F (2013) Inhibition of *Camellia sinensis* (L.) O. Kuntze on *Microcystis aeruginosa* and isolation of the inhibition factors. *Biotechnol Lett*. doi:10.1007/s10529-013-1188-4
- Matthijs HCP, Visser PM, Reeze B, Meeuse J, Slot PC, Wijn G, Talens R, Huisman J (2012) Selective suppression of harmful cyanobacteria in an entire lake with hydrogen peroxide. *Water Res* 46:1460–1472
- Mayali X, Franks PJS, Azam F (2007) Bacterial induction of temporary cyst formation by the dinoflagellate *Lingulodinium polyedrum*. *Aquat Microb Ecol* 50:51–62
- McLeod DJ, Hallegraeff GM, Hosie GW, Richardson AJ (2012) Climate-driven range expansion of the red-tide dinoflagellate *Noctiluca scintillans* into the Southern Ocean. *J Plankton Res* 34:332–337
- Moore SK, Trainer VL, Mantua NJ, Parker MS, Laws EA, Backer LC, Fleming LE (2008) Impacts of climate variability and future climate change on harmful algal blooms and human health. *Environ Health*. doi:10.1186/1476-069X-7-S2-S4
- Mu R, He Y, Liu S, Wang X, Fan Z (2009) The algicidal characteristics of one algae-lysing FDT5 bacterium on *Microcystis aeruginosa*. *Geomicrobiol J* 26:516–521
- Park SC, Lee JK, Kim SW, Park Y (2011) Selective algicidal action of peptides against harmful algal bloom species. *PLoS ONE* 6(10): e26733
- Patron NJ, Waller RF, Keeling PJ (2006) A tertiary plastid uses genes from two endosymbionts. *J Mol Biol* 357:1373–1382
- Paul C, Pohnert G (2012) Interactions of the algicidal bacterium *Kordia algicida* with diatoms: regulated protease excretion for specific algal lysis. *PLoS ONE* 6(6):e21032
- Peters E, Thomas DN (1996) Prolonged darkness and diatom mortality: I. Marine Antarctic species. *J Exp Mar Biol Ecol* 207:25–41
- Pokrzywinski KL, Place AR, Warner ME, Coyne KJ (2012) Investigation of the algicidal exudate produced by *Shewanella* sp. IRI-160 and its effect on dinoflagellates. *Harmful Algae* 19:23–29

- Popels LC, MacIntyre HL, Warner ME, Zhang YH, Hutchins DA (2007) Physiological responses during dark survival and recovery in *Aureococcus anophagefferens* (Pelagophyceae). *J Phycol* 43:32–42
- Rensel JE, Whyte JNC (2003) Finfish mariculture and harmful algal blooms. In: Hallegraeff, GM, Anderson DM, Cembella AD (eds) *Manual on harmful marine algae*, revised edn. IOC UNESCO, pp 693–722
- Rounsefell GA, Evans JE (1958) Large-scale experimental test of copper sulfate as a control for the Florida red tide. *US Fish Wld S Spec Sci Rep* 270
- Saradadevi K, Raghavendra S (1992) Dark respiration protects photosynthesis against photoinhibition in mesophyll protoplasts of pea (*Pisum sativum*). *Plant Physiol* 99:1232–1237
- Segovia M, Berges JA (2005) Effect of inhibitors of protein synthesis and DNA replication on the induction of proteolytic activities, caspase-like activities and cell death in the unicellular chlorophyte *Dunaliella tertiolecta*. *Eur J Phycol* 40:21–30
- Sengco MR (2009) Prevention and control of *Karenia brevis* blooms. *Harmful Algae* 8:623–628
- Sengco MR, Anderson DM (2004) Controlling harmful algal blooms through clay flocculation. *J Eukaryot Microbiol* 51:169–172
- Shi S, Tang D, Liu Y (2009) Effects of an algicidal bacterium *Pseudomonas mendocina* on the growth and antioxidant system of *Aphanizomenon flos-aquae*. *Curr Microbiol* 59:107–112
- Smayda TJ (2007) Reflections on the ballast water dispersal—harmful algal bloom paradigm. *Harmful Algae* 6:601–622
- Smayda TJ, Mitchell-Innes B (1974) Dark survival of autotrophic, planktonic marine diatoms. *Mar Biol* 25:195–202
- Smith GD, Doan NT (1999) Cyanobacterial metabolites with bioactivity against photosynthesis in cyanobacteria, algae and higher plants. *J Appl Phycol* 11:337–344
- Smith VH, Schindler DW (2009) Eutrophication science: where do we go from here? *Trends Ecol Evol* 24:201–207
- Sotirova AV, Spasova DI, Galabova DN, Karpenko E, Shulga A (2008) Rhamnolipid-biosurfactant permeabilizing effects on gram-positive and gram-negative bacterial strains. *Curr Microbiol* 56:639–647
- Takamura Y, Yamada T, Kimoto A, Kanehama N, Tanaka T, Nakadaira S, Yagi O (2004) Growth inhibition of *Microcystis* cyanobacteria by L-lysine and disappearance of natural *Microcystis* blooms with spraying. *Microbes Environ* 19:31–39
- Terlizzi DE, Ferrier MD, Armbruster EA, Anlauf KA (2002) Inhibition of dinoflagellate growth by extracts of barley straw (*Hordeum vulgare*). *J Appl Phycol* 14:275–280
- Trainer VM, Suddleson M (2005) Monitoring approaches for early warning of domoic acid events in Washington State. *Oceanography* 18:228–237
- Van Dolah FV (2000) Marine algal toxins: origins, health effects, and their increased occurrence. *Environ Health Persp* 108:133–141
- Wang X, Gong L, Liang S, Han X, Zhu C, Li Y (2005) Algicidal activity of rhamnolipid biosurfactants produced by *Pseudomonas aeruginosa*. *Harmful Algae* 4:433–443
- Wisecaver JH, Hackett JD (2011) Dinoflagellate genome evolution. *Annu Rev Microbiol* 65:369–387
- Wu J-T, Chiang Y-R, Huang W-Y, Jane W-N (2006) Cytotoxic effects of free fatty acids on phytoplankton algae and cyanobacteria. *Aquat Toxicol* 80:338–345
- Yoon HS, Hackett JD, Van Dolah FM, Nosenko T, Lidie KL, Bhattacharya D (2005) Tertiary endosymbiosis driven genome evolution in dinoflagellate algae. *Mol Biol Evol* 22:1299–1308
- Zingone A, Enevoldsen HO (2000) The diversity of harmful algal blooms: a challenge for science and management. *Ocean Coast Manage* 43:725–748