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Pranali Deore, Anbarasu Karthikaichamy, John Beardall & Santosh Noronha

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Non-photochemical quenching, a non-invasive probe for monitoring microalgal grazing: an early indicator of predation by Oxyrrhis marina and Euplotes sp.

Pranali Deoreab,c, Anbarasu Karthikaichamyab,d, John Beardall© and Santosh Noronhaa

©IITB-Monash Research Academy, Mumbai, India; bDepartment of Chemical Engineering, Indian Institute of Technology Bombay, Mumbai, India; cSchool of Biological Sciences, Monash University, Melbourne, Australia; dDepartment of Microbiology, Monash University, Melbourne, Australia

ABSTRACT
Microalgae are major primary producers in aquatic environments, and many synthesize an array of industrially important biomolecules such as pigments, lipids and proteins. As a result, there is considerable interest in and effort into growing algae for industrial purposes. An economical method to produce a large amount of biomass is the use of the open-raceway pond cultivation platform. However, the nature of the cultivation and the nutrient-rich profile of such cultures attract contaminants such as zooplankton predators, which can result in a sudden culture crash. For effective pond management, an early indicator of grazer presence is needed to implement timely interventions. Currently available tools are offline, time-consuming and dependent on predator concentration. This study explores grazing-mediated changes in photosynthetic parameters during infestation of Dunaliella tertiolecta cultures by the heterotrophic dinoflagellate Oxyrrhis marina and the ciliate Euplotes sp. A significant reduction in non-photochemical quenching levels 24–48 h prior to the crash was observed in both bulk and single-cell prey samples. An increasing rate of grazer ingestion correlated with decreasing non-photochemical quenching levels as the culture progressed towards the crash. The reductions in the non-photochemical quenching levels were consistent in grazing cultures at different prey cell concentrations. Although the maximum photosynthetic yield remained unaltered, maximum relative electron transport rates were enhanced and the light-harvesting efficiency, alpha, reduced in comparison to controls. We suggest that, along with traditional methods, non-photochemical quenching monitoring could be used as a part of integrative pest management to minimize predator outbreaks.

Introduction
Microalgae are major primary producers in marine and freshwater environments. These organisms synthesize an array of biomolecules such as proteins, lipids and carbohydrates, which in turn provide the nutrients in the pelagic food web ecosystem used by the primary consumers, namely zooplankton and mesoplankton (Falkowski & Knoll, 2007). Some of the metabolites produced by microalgae have biotechnological importance and, in recent years, microalgae have been exploited as potential sources for mass-scale production of feed, cosmetics and biofuel. To date, open-raceway ponds are the most economical of methods for marine microalgae cultivation, using abundantly available seawater (Pienkos & Darzins, 2009). However, the nature of open-pond cultivation and the nutrient-rich properties of algal cells make the occurrence of invading microbes such as primary consumers an almost inevitable event (Carney et al., 2014).

For large-scale cultivation platforms, high-pigment-accumulating algae such as Dunaliella sp. (Moreno-Garrido & Caña, 2001), and triglyceride synthesizing species such as Nannochloropsis and Chlorella (Guccione et al., 2014) are preferred. However, outdoor cultivation ponds of Dunaliella salina have been reported to be infested by ciliates (Moreno-Garrido & Caña, 2001) and amoebae (Post, Borowitzka, Borowitzka, Mackay, & Moulton, 1983). For instance, an outbreak of the protozoan Poterioochromonas malhamensis in a Chlorella sorokiniana cultivation pond has been reported as occurring consistently for 5 years (Ma et al., 2017); this protozoan also invaded cultures of Synechocystis under laboratory-controlled conditions (Touloupakis, Cicci, Benavides, & Torzillo, 2016). In high-rate algal ponds, seasonal variation in the density of grazer species, which resulted in a reduction of ~35–90% in algal productivity across a period of fourteen months, has been reported (Montemuzzani, Duggan, Hogg, & Craggs, 2016). Often, invading grazers...
are a seasonal infestation and algal-grazer interactions are poorly characterized; hence, the timely detection of invasion is important.

The grazer-detection tools that are currently in use with mass cultures involve microscopy (Day, Gong, & Qiang, 2017), continuous flow-cytometer monitoring (Day, Thomas, Achilles-Day, & Leakey, 2012; Wang, Castillo-Keller, Eustance, & Sommerfeld, 2017), molecular tools such as qPCR (Fulbright, Dean, Wardle, Lammers, & Stephen, 2014) and microbiome analysis by sequencing methods (Carney et al., 2016). Effective grazer-detection tools need to be rapid and online with the cultures. However, currently deployed methods such as microscopy are time-consuming and dependent on cell concentration (Day et al., 2017, 2012). Molecular tools which deploy grazer-specific primers can detect very low levels of contaminants, but fail to detect uncharacterized grazer species (McBride et al., 2014). Although pond metagenomic analysis can yield more insights into the microbial population at a given point, it is time-intensive and depends on the quality of genomic material preparation (Carney et al., 2016).

Overall, effective algal pond management needs real-time monitoring for early detection of various grazer types to implement preventive treatment regimes.

In open-pond microalgae cultivation, various abiotic and biotic stresses are inevitable, and photosynthesis, an energy harvesting process, is primarily affected under abiotic stresses such as high light (Kok, 1956), salinity (Karthikaichamy et al., 2018) and metal toxicity (Dao & Beardall, 2016). Photosynthesis is a multistep redox reaction process that captures and generates energy for the other metabolic processes within microalgal cells; therefore, monitoring photosynthesis parameters can provide insights into the overall health of algal cultures (Malapascua et al. 2014). However, studies on photosynthesis alterations under a biotic stress such as algal grazing are limited. Importantly, photosynthesis is a unique property of algal (the prey) cells unlike the prey’s heterotrophic grazers (the predator), which consume algae. Understanding prey cell photosynthesis in the presence of the grazers can provide valuable insights that may help in devising better grazer-detection methods.

Chlorophyll a fluorescence is a commonly used technique to probe various parameters of photosynthesis and photophysiology in algae and higher plants. Commonly measured parameters are maximum quantum yield (Fv/Fm) in the dark-adapted state, often used as an indicator of stress, and effective quantum yield in the light-adapted state (Fv’/Fm’). Both measurements indicate the efficiency of photon absorption by chlorophyll and accessory pigments, which is further translated into electron flow in the electron transport chain. Therefore, effective quantum yield can help in an estimation of the rate of electron transport, which is highly dependent on absorbance of the cells, though relative electron transport rates (rETRmax) can be determined in cases where absorbance is unknown and is often excluded (Figueroa, Conde-Alvarez, & Iván, 2003; Johnsen & Sakshaug, 2007; Kalaji et al., 2017). In-depth information about light-harvesting efficiency (alpha) and electron transport rates can be obtained by constructing photosynthesis irradiance curves. Non-photochemical quenching (NPQ) is a measure of the ability of photosynthetic cells to dissipate excess absorbed energy in the form of heat. NPQ formation involves activation of the violaxanthin cycle which leads to synthesis of zeaxanthin, a pigment that assists in heat dissipation. Failure of NPQ activation can lead to transfer of excess energy to oxygen, and as a result reactive oxygen species (ROS) are generated, which can potentially damage various cellular processes (Müller, Xiao-Ping, & Niyogi, 2001).

According to a recent report, NPQ levels were affected in green algae, cyanobacteria and a diatom culture following ciliate and copepod infection, as a function of seawater chemistry (Ratti, Knoll, Giordano, & Waller, 2013). However, grazer-induced changes in the NPQ levels have not yet been established as a useful early indicator of predation. This study aimed to explore the possibility of developing photophysiology-based measurements, particularly NPQ, as an early indicator of grazer infection of algal cultures and subsequent culture crash. We used the green alga Dunaliella tertiolecta as prey for Oxyrrhis marina, a protozoan dinoflagellate, and the ciliate Euplotes sp. as an alternative predator in some experiments.

Materials and methods
Cultivation conditions of prey and predator strains

The Dunaliella tertiolecta (CS-175) culture was purchased from the CSIRO Culture Collection, Hobart, Tasmania, Australia and maintained axenically in F/2 seawater-based medium at pH 8. The predator strains, Oxyrrhis marina and Euplotes sp. (see figures 1 and 2 in the supplementary information), were isolated from seawater collected from the west coast of Gujarat, India and maintained in the same F/2 medium as used for the prey alga, D. tertiolecta. Grazing cultures were established using prey cells which were harvested during late exponential phase, centrifuged at 1500 g for 10 min and washed with fresh F/2 media to remove cell
grazing cultures were cultivated in 500 ml Erlenmeyer flasks (Borosil) and incubated at 25°C under 30–50 µmol photons m⁻² s⁻¹ cool white fluorescent light (Philips, Eindhoven, Netherlands) fixed at ~35 cm above the culture flasks. The crash assay conditions, cell density and other related parameters were optimized in preliminary experiments. Light intensity was measured using a light sensor (LI-210R, LI-COR, USA). Morphological features of all the strains were observed under a phase-contrast microscope (Leica DM2000, Germany). Prey and predator cells were fixed in Lugol’s iodine for cell counts using an Improved Neubauer haemocytometer and Sedgwick Rafter chamber, respectively. The ingestion rates were calculated by using Equation (1) (refer to the supplementary information for detailed methods and equations) described by Jin et al. (2001).

\[ I = C' \times F \]

where \( I \) is ingestion rate (prey cells predator⁻¹ d⁻¹), \( C' \) is the mean prey cell concentration and \( F \) is the clearance rate.

**Chlorophyll a fluorescence measurements**

The grazing cultures of *O. marina* feeding on *D. tertiolecta* were operated in batch mode. Ungrazed cultures of *D. tertiolecta* served as controls. The prey-predator cell concentration was monitored daily by manual cell counting, and cells from the control and grazing flasks were harvested for measurements of chlorophyll *a* fluorescence using a PhytoPAM II (Walz, Germany). A preliminary method optimization process was performed to minimize the background signal using the auto-gain and zero-offset controls of the PAM. Additionally, the settings of the saturation pulse were optimized to obtain maximum fluorescence rise and a stable plateau. Other parameters were set as per the manufacturer’s instructions (Walz, 2003). For \( F_i/F_m \) and NPQ measurements, 3 ml of algal samples were dark-adapted for 15 min. Photosynthetic measurements were then performed using the PhytoPAM II with a measuring light intensity of 2 µmol photons m⁻² s⁻¹, which was followed by a saturation pulse (SP) that had an intensity of 8,000 µmol photons m⁻² s⁻¹ and width of 350 ms. The minimum fluorescence (\( F_o \)) in the measuring light and maximum fluorescence (\( F_m \)) from the saturating pulse were determined, allowing calculation of the variable fluorescence following dark adaptation (\( F_v \)) and maximum photosynthetic yield (\( F_i/F_m \)) using Equations (2) and (3), respectively. Subsequently, actinic light (AL) of 480 µmol photons m⁻² s⁻¹ was switched on after the first SP, and SPs were then delivered at intervals of 20 s until steady values for light-adapted maximum fluorescence (\( F_m^\text{AL} \)) were
attained. The NPQ values were computed using the 'Stern–Volmer' formula, Equation (4). For \( \text{rETR}_{\text{max}} \) and alpha estimation, the RLCs were constructed using the built-in PhytoWin software by applying incremental actinic illumination (16–610 µmol photons m\(^{-2}\) s\(^{-1}\)) in 20 steps, with each intensity applied for 20 s. The derived parameters’ alpha was computed by the in-built PAM, PhytoWin, software which uses the model reported by Peeters and Eilers (1978) to fit the RLC using relative rate of electron transport (rETR) values calculated by Equation (5) (Figueroa et al., 2003; Johnsen & Sakshaug, 2007) in which \( Y(II) \) is effective quantum yield of photosystem II and \( E_{\text{PAR}} \) is irradiance intensity. For a representative RLC refer to the supplementary data. All the assays were performed with three replicate experiments, each involving three technical replicates.

\[
F_v = (F_m - F_0) 
\]

\[
F_v/F_m = F_m - F_0/F_m 
\]

\[
NPQ = (F_m/F_m^{'}) - 1 
\]

\[
\text{rETR} = Y(II) \times E_{\text{PAR}} 
\]

**Single-cell chlorophyll a fluorescence measurement**

To investigate grazing-mediated effects on NPQ levels at the single-cell level, starved cells of *O. marina* were fed on *D. tertiolecta* to establish the grazing culture that was compared with the control (containing *D. tertiolecta* cells only). Chlorophyll a fluorescence measurements were made daily using a Walz microscopy-PAM on a) the control, that is *D. tertiolecta* cells alone; b) prey cells that were ingested, i.e. inside the predator and c) prey cells outside the predator (uneaten prey cells in grazed cultures). For \( F_v/F_m \) and NPQ determination, 10 µl sample aliquots from control and grazing cultures were mounted on poly L-lysine-coated slides (Thermoscientific-Menzel-Glaser, Germany) for 10–15 min dark acclimatization. Single-cell photosynthetic measurements were performed with a PAM-CONTROL unit using a Hundwetzlar (Germany) fluorescence microscope under a 15x objective lens. Measuring light of 2 µmol photons m\(^{-2}\) s\(^{-1}\) followed by a 0.8 s long SP of 8,000 µmol photons m\(^{-2}\) s\(^{-1}\) intensity was applied to a sample. Actinic light (AL) of 570 µmol photons m\(^{-2}\) s\(^{-1}\) was switched on for 8 min and, in the presence of AL, repeated SPs were given with an interval of 20 s. NPQ was calculated using Equation (4), once \( F_m^{'}, \) values had stabilized. A prior optimization was done to determine the ideal incubation time (20 min) for cells to adhere to poly L-lysine-coated slides and emit a signal between 300 and 3500 fluorescence intensity. A longer incubation time affected the cell viability and hence yielded poor fluorescence values below 300 units. Higher signals, above 3500 units, were non-linear and overestimated photosynthetic parameters. Artefactual light effects and the background signal in the absence of cells were corrected by the F-offset function using the poly L-lysine slide alone. The current of the photomultiplier detector and photomultiplier gain were kept minimal to improve signal-to-noise ratio and ensure no ambient light contributed to the signal. At every saturation pulse, the fluorescence-induction kinetics was observed for the fluorescence rise and stable plateau formation. All the other settings were set as per the instruction manual (Walz, 2000).

**Effect of prey-cell concentration on NPQ levels**

Grazing cultures were established using different prey, *D. tertiolecta*, and predator, *O. marina*, concentrations to fall into the categories of: high-level infestation 1:1, moderate level 100:1, low level 1000:1 and very low-level infestation 10000:1. A further grazing experiment was set up, in which additional prey cells were added to grazing cultures on day 2 (pulse-prey treatment). The NPQ profiles of the grazing cultures, along with the control flasks, were monitored daily until complete removal of prey cells was observed.

**Effect of elevated growth light and low salinity on NPQ profiles under the influence of *O. marina* grazing**

Conditions such as elevated light for growth (150 µmol photons m\(^{-2}\) s\(^{-1}\)) and low salinity (16.5 practical salinity units, PSU) were assessed for their effect on the NPQ profiles to determine if suboptimal growth conditions affected the responses of NPQ to grazing. The elevated light intensity was chosen as a supra-saturating intensity based on rapid-light curves. The NPQ levels were recorded daily as described above until the culture crash occurred. Light intensity and salinity were monitored using a light sensor (LI-210R, LI-COR) and salinity refractometer (Ade advance optics), respectively.

**Effect of *Euplotes* sp. grazing on NPQ profiles of prey cells**

To validate that changing NPQ levels were due to grazing pressure, a different species of predator, *Euplotes* sp., was fed on *D. tertiolecta* cells. The NPQ levels and ingestion rates were estimated at intervals of 24 h using the techniques outlined above.
Statistical analysis

The statistical significance of the difference between the control and the grazer-infested group across different time-points was analysed using GraphPad software 7.02 (GraphPad Software Inc, CA, USA) for carrying out an unpaired two-sample t-test. The level of statistical significance for all the analyses was P < 0.05.

Results

Prey and predator numbers

In the ‘crash’ experiments, O. marina grazed at an average rate of ~9.47 x 10^{11} prey cells predator^{-1} d^{-1}, and 92% (P < 0.0001) prey depletion (Figure 1) was observed by day 2. From the third day onwards, the population of O. marina significantly (P < 0.0001) exceeded the concentration of prey cells, which resulted in a culture crash, and prey cells were completely cleared on day 4 (Figure 2). The ingestion rate of O. marina increased across 3 days but halted after the crash. Euplotes sp. grazed at a rate of ~1.76 x 10^{14} prey cells predator^{-1} d^{-1} and on day 2, 57% (P = 0.035) prey depletion was observed. The maximum clearance, 94%, was observed on day 3 (P < 0.0001), leading to a culture crash (Figures 3, 4). The ingestion ability of O. marina and Euplotes sp. (Figures 2, 4) peaked on the third day and halted thereafter. Henceforth, day 3 will be referred to as a crash day.

Chlorophyll a fluorescence measurements

The NPQ profiles were found to be strongly affected in predated cultures. As shown in Figure 2, algal cultures infested with O. marina showed a statistically significant ~55% and 74% reduction (P < 0.0001) in NPQ on days 1 and 2, respectively. Additionally, a significant increase in the rate of O. marina ingestion was observed over this time. The NPQ levels continued to decline moderately after day 2 in comparison to the initial time points until the culture crash occurred. Similar to grazing by O. marina, reductions of ~35% and 60% in NPQ levels in Euplotes-infested cultures were observed on days 1 and 2, respectively (Figure 4). O. marina-mediated grazing did not alter the F_{r}/F_{m} (Figure 5) of D. tertiolecta cells across all time points. An increasing rETR_{max} was observed in the grazing group in which an increment (P = 0.0011) of 50% was seen on day 3 (Figure 6). In contrast, alpha, the initial slope of the rapid-light curve, was reduced by 20% (P < 0.0001) and 11% (P = 0.0009) on days 2 and 3 (Figure 6). From day 1 to 3, a moderate negative correlation between the ingestion rate and the NPQ level was found (see table 1, figures 3 and 4 in the supplementary information) in the grazing cultures of both O. marina (R^2 = 0.703) and Euplotes sp. (R^2 = 0.725).

Single-cell chlorophyll a fluorescence measurement

In Figure 7, no significant difference was observed in NPQ levels of control and prey cells (outside predator)
until day 1. However, prey cells engulfed by predators showed significantly lower (P = 0.0002) NPQ levels. From the second day onwards, un-engulfed prey cells that remained outside the predators, showed a decline of 54% in NPQ levels (P = 0.0004). The trend of declining NPQ was further observed in both uneaten prey (outside predator) and engulfed prey (inside predator) cells until the culture crash. No significant differences (Figure 8) in Fv/Fm levels were found between control and prey cells (outside predator) until the prey cells were completely cleared in the grazing culture on day 3, whereas Fv/Fm levels of prey cells (inside predator) were consistently low after day 0.

**Effect of prey-cell concentration on NPQ levels**

The culture that was highly infested (1:1) crashed on day 2 (Figure 9) and showed 69% lower NPQ levels (P = 0.0001), in comparison to the control, 24 h prior to the crash. The moderately infested culture (100:1) showed a 52% (P = 0.0004) to 72% (P < 0.0001) decline in NPQ levels from day 1 onwards, and complete prey cell displacement occurred on day 3. In low grazer density (1000:1) cultures, a reduction of 56% (P = 0.0025) in NPQ levels was observed on day 2, with a delayed culture crash on day 4. Cultures with very low grazer (10,000:1) infestation crashed on day 5 and showed a declining NPQ trend ~43% (P = 0.0194) from day 3 onwards. Overall, reductions in the NPQ levels were observed in all grazer-infested groups at least 24–48 h, prior to the crash (see table 2 in the supplementary information).

In a ‘prey-pulsing experiment’ (Figure 10), additional prey cells were provided on day 2, which resulted in an increased (P = 0.0017) prey population, and 69% increase (P = 0.0073) in NPQ levels in prey cells in the pulsed group in comparison with the non-prey-pulsed group on day 3. However, a declining NPQ trend was observed in the pulsed group after day 3 until the crash, which was similar to the non-pulsed group.
Overall, from day 2 onwards, NPQ levels of both the pulsed and the non-pulsed group were significantly ($P < 0.0001$) lower than those of the control group.

**Effect of elevated growth light and low salinity on NPQ profiles under the influence of Oxyrrhis marina grazing**

On day 0, NPQ levels in both elevated growth light (150 $\mu$mol photons m$^{-2}$ s$^{-1}$) and low salinity (16.5 PSU) were higher compared with other days. On day 2, the grazing group showed a significant reduction in both conditions. The elevated light (Figure 11) and low salinity (Figure 12) resulted in a decline of 47% ($P = 0.0057$) and 40% ($P = 0.0266$), respectively, in the NPQ levels. The culture crash in both groups occurred on day 3, with declining NPQ profiles from day 1 until the day of the crash (Figures 11, 12). The growth rate of *D. tertiolecta* cells in the control group was found to be low in both elevated light and low salinity conditions (see table 3 in the supplementary information) compared with under optimal growth conditions (see Section 2.1).

**Discussion**

Algal predation is a process of sequential prey capture, processing and digestion that manifests in the degradation of the algal pigments and in the storage of digested products in food vacuoles. Upon predation, the algal prey cells also undergo cellular and metabolic responses. For instance, both prey capture and predation-specific stimuli alter the photosynthetic pigment content of prey (Feinstein, Traslavina, Sun, & Lin, 2002). In this study, we investigated possible photosynthetic alterations in *D. tertiolecta* cultures under grazing pressure which can provide insights into potential predator outbreaks so that preventative measures can be deployed.

Photosynthetic parameters such as $F_{v}/F_{m}$, $\text{ETR}_{\text{max}}$ and NPQ are measured in order to monitor the overall physiological state of algal cultures. $F_{v}/F_{m}$ is a measure of the maximum quantum yield of photosystem II, which reflects the ability to perform photochemistry. The maximum photosynthetic yield has been reported to be negatively affected by the presence of ‘weed’ (contaminating) algae (Winckelmann, Bleeke, Thomas, Elle, & Gerd, 2015). However, in the present study, no significant alterations were observed in $F_{v}/F_{m}$ under grazing pressure, which correlates with the experiments on grazing of diatom and cyanobacterial cultures reported by Ratti et al. (2013). This can occur due to the relatively higher proportion of healthy and ungrazed prey cells at initial time points in the PAM measurement of the bulk sample. The low photosynthetic yield after day 3 in the control and grazing cultures may be attributed to decreased photosynthetic function during the late log and stationary phases, mostly due to nutrient exhaustion (Fogg, 1957).
as experiments were operated in a batch mode. Similarly, Young and Beardall (2003) reported a drop in $F_v/F_m$ values in *Dunaliella tertiolecta* culture under nitrogen depletion, and recovery on restoration of nitrogen replete conditions. $rETR_{\text{max}}$ is a measure of the capacity for electron transport rate between reaction centre and electron carriers of PSII; it is reported to be negatively affected under abiotic stress conditions such as nutrient depletion (José et al., 2014; Zhao et al., 2017) and other factors such as salinity stress (Karthikaichamy et al., 2018). In contrast, exposure of *D. tertiolecta* cells to biotic stress caused by *O. marina* grazing resulted in an overall increase in $rETR_{\text{max}}$ from day 1 until the culture crash on day 3, although alpha, i.e. the light-harvesting efficiency, was reduced in the *O. marina*-infested culture in comparison with the control. Another photosynthetic parameter, which reflects the photo-protective ability of an algal cell, is NPQ. Hence, NPQ is an important measurement for monitoring the state of accessory pigments and stress responses of algae. Under the grazing pressure from *O. marina* and *Euplotes* sp., NPQ levels dropped in comparison with the control culture. A sequential NPQ reduction was observed from day 1 to day 3 as the predator population increased exponentially, with a reduction in the prey concentration. Similarly, an alteration of the NPQ levels was reported in grazed cultures of a...
diatom and cyanobacterium as a function of seawater chemistry, in which *Euplotes* sp.-mediated grazing reduced NPQ levels in *Tetraselmis suecica* and increased NPQ in *Synechococcus* sp. cultures (Winckelmann et al., 2015). A negative correlation between the light-harvesting efficiency and rETR$_{\text{max}}$ levels, coupled with increasing NPQ levels, is reported in various algal strains upon exposure to various abiotic stress conditions (Nitschke, Connan, & Stengel, 2012; Serodio, Vieira, Cruz, & Coelho, 2006). The exposure to biotic stress (grazing) in this study resulted in a similar negative correlation between the light-harvesting efficiency and rETR$_{\text{max}}$ but in contrast to previous studies (Nitschke et al., 2012; Serodio et al., 2006), NPQ levels were instead significantly reduced.

In addition to NPQ levels, the ETR/NPQ ratio is reported to decrease in macroalgae with incremental exposure to UVB irradiance (Figueroa, Domínguez-González, & Korbee, 2014). In contrast, ETR/NPQ ratio significantly increased (refer to figure 8 in supplementary material) under grazing pressure from *O. marina*. Although this ratio can potentially indicate the presence of a predator in this study, currently deployed photosynthesis measurement devices do not capture the ratio in a relatively straightforward manner as compared to the 'Stern-Volmer' equation-based NPQ calculation. This underlines the need for further investigation of changes in the ETR/NPQ ratio in a variety of algal prey-predator combinations and possible improvements in the device algorithm for the estimation of the ETR/NPQ ratio.

A decreased capacity to utilize electrons in carbon assimilation (manifest as a decreased rETR$_{\text{max}}$) is reported to activate NPQ, driven by proton (pH) gradient formation and xanthophyll cycle activation. An increased proton gradient across the thylakoid membrane leads to activation of an enzyme, violaxanthin de-epoxidase, which triggers conversion of violaxanthin to zeaxanthin. Zeaxanthin is reported to assist in dissipation of excess photos as heat, a process reflected in NPQ activation (Genty, Harbinson, Briantais, & Baker, 1990; Serodio et al., 2006). Increased alpha and rETR$_{\text{max}}$ bring NPQ relaxation as electrons are efficiently carried through the electron transport chain and utilized (Serodio et al., 2006). In contrast, the moderately reduced alpha, elevated rETR$_{\text{max}}$ and lower NPQ values in the grazing culture compared with the control may indicate possible alterations in proton gradient formation across the thylakoid membrane. A similar observation was made upon addition of proton gradient uncouplers in algal cultures and isolated chloroplasts (Cao et al., 2013; Markou, Depraetere, & Muylaert, 2016). This suggests the possibility of a similar effect being caused by the invasion of *O. marina* and *Euplotes* sp. However, experimental validation is required to deduce the exact cause of the NPQ decrease in the grazing cultures of *D. tertiolecta*. The observations of the present study suggest, however, that the monitoring of NPQ levels can provide an early indication of an outbreak of *O. marina* and *Euplotes* sp.

Single-cell microscopy PAM was deployed to investigate further the reduction in NPQ levels as an outcome of the grazing-specific response of prey cells. Both uneaten (outside predator) and grazed (inside predator) prey cells from the experimental grazing cultures showed significantly lower NPQ levels than the control. Interestingly, the NPQ levels of uneaten (outside predator) prey cells started to decline from day 2 and followed the same trend until the crash. This suggests that NPQ reduction can be a strong indicator of grazing of *D. tertiolecta* cells and that the declining trend is related to the exponential increase of the population of *O. marina*. Microalgal prey cells are reported to exert metabolic responses against grazing stimuli (Amato et al., 2018), and the trend of declining NPQ in *D. tertiolecta* cells outside the predator could possibly indicate predator-mediated secretion of extracellular cues which may directly or indirectly alter NPQ levels. Our preliminary hypothesis is that ammonia excreted by grazers (Dolan, 1997) may act as a photosynthetic uncoupler (Crofts, 1967), thereby reducing NPQ levels (Deore et al., personal communication). The predator, *O. marina* is heterotrophic and non-photosynthetic in nature, and therefore fails to show chlorophyll a fluorescence (Slamovits & Keeling, 2008). Therefore, the possibility of NPQ reduction due to the mere presence of grazers in a prey culture can be overruled. On day 0, predator cells are starved or have limited prey cells consumed, therefore NPQ levels were low. Although freshly consumed prey cells continue to emit chlorophyll fluorescence, degradation of pigments due to digestion of prey inside the predator (Feinstein et al., 2002) can reduce the chlorophyll a fluorescence response. Pigment or prey degradation inside the predator is reflected in reduced F$_{\text{v}}$/F$_{\text{m}}$ values from day 1 and continued to drop until the culture crash.

Currently practiced grazer monitoring methods, such as microscopy and continuous flow-cytometry, fail to detect very low predator concentrations; such methods, moreover, are not deployed on-site (Day et al., 2012). Cultures with different prey and predator intensities crash on different days, which could be due to the severity of the *O. marina* infestation. The very high (1:1) predator concentration, which is caused by the higher grazing rate that results in a crash on day 2, can be traced to 69% reduction in NPQ 24 h prior to the crash. Similarly, moderate (100:1), low (1000:1), and very low (10,000:1) levels of infestation showed 72%, 43% and 56% reduction in NPQ levels, respectively, 24 h prior to the crash.
Experiments with different prey-predator intensities indicate that relative reduction in NPQ level can be detected 24–48 h prior to a crash for as few as $10^4$ grazer cells ml$^{-1}$ of algal culture.

NPQ levels have been found to be elevated in algal strains in response to various abiotic stress conditions (Harker et al., 1999; Serodio et al., 2006). This study reports decreased NPQ levels under biotic stresses such as predator invasion in D. tertiolecta cultures. In outdoor mass cultures, both biotic and abiotic stresses are inevitable and NPQ levels can be modulated under cumulative stress conditions. Under elevated growth light exposure conditions, NPQ levels increase by ~10 fold on day 0 in both control and grazing cultures. This reflects the activation of the NPQ mechanism as a part of the protective response. Further, this NPQ elevation relaxes from day 1 onwards. Similar NPQ relaxation after stress exposure has been reported in a study that describes the multiple photoprotective mechanisms of D. tertiolecta (Ihnken, Kromkamp, & Beardall, 2011; Segovia et al., 2015). The reduction in NPQ levels in the grazing culture under abiotic stress conditions is consistent with the observations from the grazing experiments alone.

This study demonstrates a consistent reduction of NPQ in D. tertiolecta culture, in both bulk and single-cell PAM measurements, 24–48 h prior to culture crash following introduction of predators. At the large-scale microalgal cultivation platform, there are various environmental conditions which can induce changes in photosynthetic parameters such as NPQ. As a result, grazer-induced changes in NPQ profiles of the prey cells may go unnoticed due to the dynamic nature of the photosynthetic process. Therefore, development of an array of potential diagnostic measures in addition to NPQ profiling could provide a more accurate indication of grazing. Other energy dissipation measures of photosynthesis that have been demonstrated to be affected under stress, in addition to NPQ, are regulated (YNPQ) and passive (YNO) heat loss (Blain & Shears, 2019). Therefore, the effects of biotic stress on YNPQ and YNO could be investigated and further explored as potential photosynthesis-based tools for investigating algal predation. An integrated approach using NPQ assessment along with traditional microscopy and molecular methods would enable the deployment of preventive strategies to avoid biomass loss in mass cultures. Different algal species have different acclimation potential and can respond to stress conditions differently (Segovia et al., 2015). Therefore, the development of NPQ as an indicator of grazing requires systematic testing across different prey and predator species.

In conclusion, the current study suggests the possibility of detection of grazer invasion based on the photosynthetic signature, NPQ, which can be recorded in combination with currently used grazer-detection methods. Online and real-time monitoring of the photosynthetic signature would be an ideal approach to monitor contamination at large-scale microalgal cultivation platforms. Currently, only a few handheld devices, such as AquaPen, FluroPen, etc., are available to record photosynthetic measurements, including NPQ. However, to obtain real-time data with high-quality temporal resolution requires better guided sensors, probes and software integration. There is a possibility to leverage image-based and remote sensing-based chlorophyll a fluorescence measurements that can reflect NPQ levels of cultures in real-time. The decline in NPQ levels of algal prey under grazing pressure also indicates possible underlying changes at cellular level. The potential role of ammonia as a driver for the observed changes in NPQ in grazing cultures is investigated further in the companion paper (Deore et al., personal communication).

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ORCID

John Beardall http://orcid.org/0000-0001-7684-446X

Supplementary information

E-supplementary data of this work can be found in the online version of the paper. The e-supplementary data contain equations that are used for estimation of the ingestion rate of predators, microscopic photographs, tables and graphs indicating absolute and relative values of photosynthetic parameters.

Author contributions

Pranali Deore conceptualized the research hypothesis, performed experiments, data analysis and prepared the
manuscript. Anbarasu Karthikaichamy recorded non-photochemical quenching and light-curve measurements. John Beardall conceptualized research hypothesis, critically reviewed the data and manuscript. Santosh Noronha conceptualized research hypothesis, provided statistical data analysis support and reviewed the manuscript.

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Author/s: Deore, P; Karthikaichamy, A; Beardall, J; Noronha, S

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