Nitric Oxide Production and Tolerance Differ Among Symbiodinium Types Exposed to Heat Stress

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Nitric oxide (NO) is a ubiquitous molecule and its involvement in metazoan–microbe symbiosis is well known. Evidence suggests that it plays a role in the temperature-induced breakdown ('bleaching') of the ecologically important cnidarian–dinoflagellate association, and this can often lead to widespread mortality of affected hosts. This study confirms that dinoflagellates of the genus Symbiodinium can produce NO and that production of the compound is differentially regulated in different types when exposed to elevated temperature. Temperature-sensitive type B1 cells under heat stress (8°C above ambient) exhibited significant increases in NO synthesis, which occurred alongside pronounced photo inhibition and cell mortality. Tolerant type A1 cells also displayed increases in NO production, yet maintained photosynthetic yields at levels similar to those of untreated cells and displayed less dramatic increases in cell death. Type C1 cells displayed a down-regulation of NO synthesis at high temperature, and no significant mortality increases were observed in this type. Temperature-induced mortality in types A1 and B1 was affected by the prevailing level of NO and, furthermore, photosynthetic yields of these temperature-tolerant and -sensitive types appeared differentially susceptible to NO donated by pharmacological agents. Taken together, these differences in NO synthesis and tolerance could potentially influence the varying bleaching responses seen among hosts harboring different Symbiodinium types.

Keywords: Cell death • Cnidarian–algal symbiosis • Coral bleaching • Thermal stress • Nitric oxide • Zoanthellae.

Abbreviations: cPTIO, 2−(4-carboxyphenyl)-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxo-3-oxide; DAF-FM-DA, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; FCM, flow cytometry; FSW, filtered seawater; GSNO, S-nitrosothiol; ITS, internal transcribed spacer; LED, light-emitting diode; NA, numerical aperture; NO, nitric oxide; NOS, nitric oxide synthase; NPQ, non-photochemical quenching; PCD, programmed cell death; PI, propidium iodide; RM-ANOVA, repeated measures analysis of variance; ROS, reactive oxygen species; S-NONO, spermine NONOate; SNP, sodium nitroprusside.

Introduction

A symbiotic association between scleractinian corals and photosynthetic dinoflagellates of the genus Symbiodinium (Freudenthal) underpins the existence of coral reefs (Muller-Parker and D’Eliia 1997). This relationship is highly sensitive to environmental change, however, and thermal anomalies associated with global warming are now driving its collapse throughout tropical oceans (Hoegh-Guldberg and Bruno 2010). The loss of Symbiodinium cells from their cnidarian host (a major component of ‘coral bleaching’) can result in colony mortality (Jones 2008) and the decline of entire reef systems (Graham et al. 2008). However, despite its increasing frequency, little is known of bleaching’s underlying physiological basis (Weis 2008, Davy et al. 2012). Given the time scales of projected reef decline (Pandolfi et al. 2011), it is vital that this is addressed.

The genus Symbiodinium is highly diverse (Stat et al. 2006), with nine distinct clades [identified using the internal transcribed spacer 2 (ITS2) region of the nuclear ribosomal DNA] and numerous subclades (herein referred to as ‘types’) currently recognized (Pochon et al. 2006, Pochon and Gates 2010). This diversity is reflected in the sensitivities of different types to environmental stress (Robison and Warner 2006, Suggett et al. 2008, Takahashi et al. 2009, Ragni et al. 2010) and this often correlates with the differential bleaching of reef corals (e.g. Berkelmans and van Oppen 2006, Sampayo et al. 2008). The initial event in the bleaching process appears to be the chronic photoinhibition of these Symbiodinium cells (Warner et al. 1999, Smith et al. 2005), which is associated with the overproduction of reactive oxygen species (ROS) (Suggett et al. 2008) and the onset of oxidative stress (e.g. Lesser 2006). If left unchecked, this can result in mortality (Dunn et al. 2004, Pernice et al. 2011) and/or the activation of immune-like responses that result in the symbionts’ ejection (Perez and Weis 2006, Weis 2008). Programmed cell death (PCD) in particular has been proposed as a crucial step in coral bleaching (Dunn et al. 2007) and this process has also been reported in symbiotic dinoflagellates (Dunn et al. 2004). Linking the oxidative component of coral...
bleaching with observed patterns of cellular mortality is now a major area of research, and it is here that nitric oxide (NO) could play a significant role (Brune et al. 1999, Chung et al. 2001, Almeida et al. 2007).

NO is the smallest signaling molecule in biology (Smith, 1998), and its involvement in cell death (e.g. Brune et al. 1999), ROS-based signaling (Crawford and Guo 2005), innate immunity (Nurnberger et al. 2004) and symbiosis (Trapido-Rosenthal et al. 2001, Davidson et al. 2004) has long been recognized. Perez and Weis (2006) proposed NO as an ‘eviction notice’ in the bleaching of the sea anemone Aiptasia pallida during heat shock, and initial work suggested that symbiotic dinoflagellates possess their own nitric oxide synthase (NOS) (Bhagooli et al. 2001, Buxton et al. 2002, Trapido-Rosenthal et al. 2005) although this has since been contradicted (Safavi-Hemami et al. 2010). Plants and green algae employ NOS-independent NO pathways (Yamasaki et al. 1999, Sakihama et al. 2002, Delledonne 2005), however, and these appear to be present in symbiotic dinoflagellates isolated from the giant clam Tridacna crocea (Bouchard and Yamasaki, 2008). Furthermore, temperature-induced NO synthesis correlated with increasing caspase-like activity (enzymes involved in the execution of PCD), leading the authors to propose NO as a mediator of PCD (Bouchard and Yamasaki 2009). While NO has been shown to mediate mortality in some diatom species (Vardi et al. 2006, Chung et al. 2008), Bouchard and Yamasaki (2009) did not apply an NO scavenger to the cells, and thus any link between NO and cell death in Symbiodinium remains correlative at best. As is the case in other photosynthetic organisms (Beligni and Lamattina 1999), heightened NO synthesis may be a protective strategy that is simply overwhelmed during prolonged stress.

Whether protective or harmful, if temperature-induced synthesis of NO is widespread in the genus Symbiodinium then its production might also vary with the differing thermal sensitivities of individual ITS2 types (Robison and Warner 2006, Suggett et al. 2008, Ragni et al. 2010, Fisher et al. 2012). As a small lipophilic molecule, NO could potentially diffuse across symbiont membranes into the host cells. With the cnidarian–Symbiodinium association apparently sensitive to NO (Perez and Weis 2006), in hospite leakage of the compound may have profound implications (Weis 2008) and perhaps contribute to the differential bleaching of hosts containing different symbiont types (Sampayo et al. 2008, Weis 2010).

This study sought to determine the role of NO in the stress responses of symbiotic dinoflagellates by examining its production in three different Symbiodinium ITS2 types exposed to elevated temperature, as well as its effects on the photophysiology and viability of these algae. Further elucidation of the mechanisms underpinning the declining health of thermally stressed Symbiodinium will greatly improve our understanding of the cellular basis of coral bleaching, and the role that symbiont diversity may play in this phenomenon.

**Results**

**PSII fluorescence at elevated temperature**

Responses differed significantly between Symbiodinium types exposed to elevated temperatures (**Table 1, Fig. 1**). Types A1 and C1 were tolerant of moderate temperature stress (32°C) and A1 maintained dark-adapted yields at control levels even after 48 h at 34°C (P = 0.606). The maximum fluorescence yield of PSII (Fmax/Fm) of type C1 declined slightly at 34°C (P < 0.001), and type B1 exhibited significant and dramatic reductions in Fmax/Fm declining to near zero within 36 h at both 32°C and 34°C (P < 0.001).

**Nitric oxide synthesis in Symbiodinium cells**

All cultures were capable of generating NO (**Fig. 2**), and pre-treatment levels differed significantly among types (**Table 1**). Responses to temperature stress also differed significantly (**Table 1**), with two distinct trends apparent. Types A1 and B1 up-regulated NO synthesis over 24 h at 32°C (A1, P = 0.002; B1, P = 0.026) and, while B1 cells sustained this increase over 48 h (P < 0.001), the response was transient in type A1 wherein NO levels declined to control levels after 48 h (P = 0.458). After 48 h at 34°C, however, both A1 and B1 cells showed elevated NO production (A1, P = 0.021; B1, P < 0.001). Type C1 cells behaved differently, with exposure to 32°C for 48 h or 34°C for 24 h resulting in a significant down-regulation of NO synthesis relative to controls (32°C, P = 0.011; 34°C, P = 0.048).

**Effects of NO on Chl a fluorescence**

Treatment with the NO donors spermine NONOate (S-NONO) and S-nitrosoglutathione (GSNO) adversely affected PSII fluorescence yields and also revealed differences in the NO sensitivity of different Symbiodinium ITS2 types. The addition of NO donors at 100 μM caused Fmax/Fm to decline only in types B1 and C1; higher concentration (1 mM) was required to induce

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**Table 1** Statistical analysis of physiological parameters in three Symbiodinium ITS2 types exposed to temperature stress

<table>
<thead>
<tr>
<th>Parameter (treatment)</th>
<th>Test (interaction)</th>
<th>Statistic</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fmax/Fm (heat stress)</td>
<td>RM-ANOVA (time × temperature × type)</td>
<td>F5,17, 25,853 = 48.888</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Nitric oxide (no treatment)</td>
<td>UNIANOVA (type)</td>
<td>F2, 27 = 59.617</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Nitric oxide (heat stress)</td>
<td>RM-ANOVA (time × temperature × type)</td>
<td>F4, 42 = 12.022</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cell viability (heat stress)</td>
<td>RM-ANOVA (time × temperature × type)</td>
<td>F3, 36 = 5.073</td>
<td>&lt;0.001</td>
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significant declines in type A1 (Table 2, Fig. 3A). Furthermore, GSNO appeared to be a much more potent photosynthetic inhibitor than S-NONO. The specific NO scavenger cPTIO [2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imida-
zy1-1-oxy-3-oxide] was able to block the effects of 100 μM S-NONO and GSNO on Fv/Fm completely in B1 and C1 type cells, and of 1 mM S-NONO and GSNO in A1 cells (Fig. 3B), confirming the NO-dependent nature of the effects. To determine whether a cell’s own NO production might affect its PSII fluorescence, cultures were exposed to elevated temperature (36°C for A1—increased from 34°C to ensure photoinhibition of PSII—and 34°C for B1) with and without 2 mM cPTIO. Fv/Fm declined in both types at the higher temperature and this was prevented to an extent by the addition of cPTIO (Fig. 3C).

The application of NO donors also affected light-adapted PSII fluorescence, and significant declines in effective quantum yield (ΔF/Fm’) similar to those seen for Fv/Fm were observed (Table 2, Fig. 4A). Non-photochemical quenching (NPQ) was inhibited by S-NONO in all three Symbiodinium types, although declines in type C1 were typically less dramatic than for A1 or B1 (Table 2, Fig. 4B) and were absent in the GSNO treatment. Only in type A1 could the effects on NPQ of both NO donors be completely reversed by the addition of the NO scavenger cPTIO (Fig. 4B).

**Effects of temperature and NO on cell viability**

Cell mortality differed among the three types exposed to elevated temperatures (Fig. 5, Table 1). At 32°C only B1 displayed increases relative to controls (P < 0.001), and this was more dramatic at 34°C with up to 30% of cells being labeled as non-viable (P < 0.001). Increasing levels of cell death in other types were apparent only in type A1 exposed to 34°C for 48 h (P = 0.012), and even at this temperature mortality was relatively low (around 7% of treated cells). No significant changes were seen in the C1 population at either of the elevated temperatures, although a declining trend was evident (Fig. 5). Incubating cell suspensions at high temperature with the NO scavenger cPTIO confirmed NO as a causative agent of mortality in types A1 and B1 (Fig. 6, Table 2), wherein increases after 48 h at 32°C (B1) or 34°C (A1) were attenuated by cPTIO (Fig. 6).

**Discussion**

NO may be integral to the temperature-induced breakdown of the cnidarian–dinoflagellate symbiosis (Trapido-Rosenthal et al. 2005, Perez and Weis 2006, Weis 2008, Bouchard and Yamasaki 2009). This study confirms that symbiotic dinoflagellates produce NO and suggests that it could mediate their heat stress responses. It also highlights the physiological diversity of the genus Symbiodinium, even in the case of strongly conserved phenomena such as NO signaling.

**Differential NO production in Symbiodinium ITS2 types**

Little is known regarding the roles of NO in microalgae, although its existence in these organisms has been recognized for some time (Sakihama et al. 2002, Vardi et al. 2003).
2006, Thompson et al. 2008). Heightened NO production in types A1 and B1, as seen in this study, supports the conclusions of recent investigations into NO synthesis by clade A Symbiodinium cells (Bouchard and Yamasaki 2008, Bouchard and Yamasaki 2009). Interestingly, the clade A cells used in the current work behaved differently from those examined in the study of Bouchard and Yamasaki (2009), in which exposure to 32°C induced chronic photoinhibition and persistent rather than ephemeral increases in NO production. Bouchard and Yamasaki (2009) acknowledged that their cells were approaching stationary growth phase, and it is possible that the stresses associated with aging algal cultures could account for their reduced thermal tolerance. Murik and Kaplan (2009) reported that the ability of Peridinium sp. dinoflagellates to withstand oxidative insults varied significantly with the population growth phase, and preliminary work carried out for the current investigation suggests that Symbiodinium cells in stationary phase may produce higher levels of NO than do younger populations (T.D. Hawkins unpublished data). Of course, it is also possible that these

Fig. 2 Effect of temperature (under 100–120 μmol photons m⁻² s⁻¹ cool white LED light) on the regulation of NO production in Symbiodinium ITS2 types A1 (A), B1 (B) and C1 (C). Values are means ± SEM, and asterisks indicate significant differences between each treatment and the control 26°C group (RM-ANOVA with pair-wise post-hoc, n = 3 per time point except for A1 at 26°C where n = 6, *P < 0.05; **P < 0.01; ***P < 0.001). Inset: phase contrast/TDI and confocal lase scanning microscopy micrographs of Symbiodinium cells exposed to 34°C for 48 h and incubated with either filtered seawater or 15 μM of the NO-sensitive probe DAF-FM-DA. NO-dependent fluorescence (510–530 nm) is labeled in green and the cells' Chl autofluorescence (>700 nm) is labeled in red. Scale bar = 10 μm.

Table 2 Statistical analysis (ANOVA, effect of treatment) of physiological parameters in three Symbiodinium ITS2 types exposed to combinations of NO donors, scavengers and heat stress

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Parameter</th>
<th>Statistic</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>NO donors ± cPTIO</td>
<td>A1, B1, C1</td>
<td>F₄/F₄₉m</td>
<td>F₄₆₉ = 245.983</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ΔF/F₄₉m</td>
<td>F₄₆₉ = 285.909</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NPQ</td>
<td>F₄₆₉ = 25.832</td>
</tr>
<tr>
<td>NO donor concentrations</td>
<td>A1, B1, C1</td>
<td>F₄/F₄₉m</td>
<td>F₄₆₉ = 193.048</td>
</tr>
<tr>
<td>High temperature ± cPTIO</td>
<td>A1, B1</td>
<td>F₄/F₄₉m</td>
<td>F₂₁₂ = 173.741</td>
</tr>
<tr>
<td>High temperature ± cPTIO</td>
<td>A1, B1</td>
<td>Cell viability</td>
<td>F₂₁₂ = 112.885</td>
</tr>
</tbody>
</table>
discrepancies simply reflect differences in thermal tolerance at the subcladal level (Robison and Warner 2006).

In spite of this, it seems likely that with both relatively ancestral (A) and derived (B and C) clades (Stat et al. 2006) producing NO, the ability to generate the compound is widespread in symbiotic dinoflagellates. Inabilities to detect NO in Symbiodinium in other studies (e.g. Perez and Weis 2006) might therefore reflect methodological differences. In any case, additional work on a wider range of Symbiodinium types would enable any phylogenetic patterns of NO synthesis in these algae to be determined.

**NO’s effects on Symbiodinium cells**

Research into NO synthesis in protists is in its infancy, but even less is known about NO’s physiological functions in these organisms. In plants, NO is often protective (Delledonne 2005) but, conversely, it can inhibit photosynthesis (Takahashi and Yamasaki 2002) in a manner similar to its inhibition of mitochondrial electron transport (Brown 1999). This investigation suggests an inhibitory capability of NO in the photosynthesis and photoprotection of some symbiotic dinoflagellates. Furthermore, the alleviation of temperature-induced declines in maximum PSII fluorescence yields in the presence of an NO scavenger suggests that some symbiont types’ own NO production at elevated temperatures may be playing a role in the photoinactivation of PSII. Further work is required, however, before any mechanistic conclusions can be drawn; in particular whether NO’s apparent inhibition of Symbiodinium PSII is due to its direct actions on photosynthetic machinery or to more generalized cellular damage. In addition, the situation

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**Fig. 3** Effects of NO on the relative maximum fluorescent yields of PSII ($F_v/F_m$) in Symbiodinium cells under 100–120 μmol photons m$^{-2}$ s$^{-1}$ cool white LED light and (A) treated with differing concentrations of S-NONO and GSNO for 3 h; (B) exposed to the NO donors S-NONO and GSNO (1 mM for ITS2 type A1, 100 μM for B1 and C1) for 3 h with and without the NO scavenger cPTIO (at concentrations equal to that of the donor); and (C) incubated at elevated temperature (36°C for A1, 34°C for B1) for 6 h with and without 2 mM cPTIO. Values are means ± SEM, and letters represent bars that are significantly different within each type (ANOVA with pair-wise post-hoc, $n = 3$, $P < 0.01$).

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**Fig. 5** Effect of temperature (under 100–120 μmol photons m⁻² s⁻¹ cool white LED light) on the viability of *Symbiodinium* ITS2 types A1 (A), B1 (B) and C1 (C). Values are means ± SEM, and asterisks indicate significant differences between each treatment and the control (26°C) group (RM-ANOVA with pair-wise post-hoc, n = 3, *P < 0.05; **P < 0.01; ***P < 0.001). Inset: phase contrast/TD1 and confocal laser scanning...
appears different for type C1, as this displayed progressive photoinhibition at 34°C (Fig. 1) alongside decreasing NO synthesis (Fig. 2). The observed inactivation of PSII in these cells may therefore have been purely temperature driven, while in types A1 and B1 it could have resulted from increasing NO synthesis (as a general temperature response) exacerbating thermal photoinhibition. This is supported by the fact that $F_v/F_m$ in these types could not be restored to control levels at high temperature (Fig. 3C) even following the addition of an NO scavenger.

Bouchard and Yamasaki (2009) proposed NO as a cytotoxic molecule mediating PCD in Symbiodinium cells, and our data confirm NO’s involvement in the temperature-induced mortality of some Symbiodinium types. Preliminary results obtained during this study suggested that a significant component of the observed mortality was indeed PCD-like (T.D. Hawkins unpublished data), though it is difficult to attribute cellular characteristics conclusively to a particular mode of cell death (Moharikar et al. 2006, Jimenez et al. 2009) and hence these data are not shown here. In the context of symbiont diversity, however, the fact that A1- and B1-type Symbiodinium cells produced similar levels of NO at the highest temperature but suffered dramatically different fates in terms of their photosynthetic competence and mortality lends further support to our finding that these types are differentially susceptible to NO and to the hypothesis that this could underpin their thermal sensitivities.

**Conclusions**

NO appears harmful to Symbiodinium cells’ photosynthetic function during heat stress and this may be important in the context of coral bleaching, as chronic photoinhibition can lead to oxidative stress (Liu et al. 2004, Suggett et al. 2008) that may, alongside NO, trigger host responses resulting in symbiont ejection (Perez and Weis 2006, Weis 2008). Together with the varying NO production of different symbiont types, their differential NO tolerance may affect the levels of ROS leakage in hospite, cell mortality and, potentially, the host’s bleaching response.

This study is the first to examine NO production by different types of symbiotic dinoflagellates, and it is clear that the genetic diversity of these algae is reflected in their physiology even in the case of the strongly conserved phenomenon of NO signaling. Despite this, NO seems to be involved in the temperature-induced photoinhibition and mortality of some Symbiodinium types. As such, symbiont-derived NO may play a significant role in the coral bleaching phenomenon, whether directly—inducing mortality in the symbionts (Strychar et al. 2004, Dunn et al. 2004)—or indirectly—through leakage of itself or ROS into the host leading to an immune-like rejection response (Perez and Weis 2006, Weis 2008). The significance of NO in the longer term and typically less acute temperature stresses that characterize most natural coral bleaching events, however, remains unclear. Further investigation of intact symbioses would yield vital information regarding the involvement of NO signaling pathways in the cnidarian bleaching phenomenon.

**Materials and Methods**

**Culture of Symbiodinium dinoflagellates**

The three Symbiodinium types used in this experiment were originally isolated from taxonomically and geographically distinct host populations (Table 3), and had been maintained in culture for at least 2 years. Their ITS2 types were identified by the methods of Star et al. (2009). Prior to experimentation, Symbiodinium cell cultures were grown at 26°C under a 12 h:12 h light:dark cycle (100–120 μmol photons m⁻² s⁻¹ provided by cool white fluorescent tubing; OSRAM DULUX L 36 W 4000 K). Flasks containing Guillard’s f/2 medium (~Si) were inoculated 20 d prior to experimental treatment and cells were maintained in exponential growth phase (50,000–150,000 cells ml⁻¹) by regular dilution with fresh f/2 medium.

Cultures were diluted to a concentration of 30,000 cells ml⁻¹ 72 h prior to treatment and left undiluted throughout each experiment.

**Experiments**

To investigate the effects of temperature on PSII, NO production and cell viability, Symbiodinium cell suspensions (three
Replicate flasks per ITS2 type) were transferred to an illuminated water bath set at 26°C and an irradiance of 100–120 μmol photons m⁻² s⁻¹ [provided by a light-emitting diode (LED) light bank—20 RoHS 5 W 6400 K—which provided illumination for all subsequent experiments], and allowed to acclimate for 48 h. The temperature was then either maintained at 26°C (control) or increased over a short period (<2h) to 32 or 34°C (see below for more details). Cells were exposed to the new conditions for 48 h.

The effects of NO on photosynthetic performance were examined by exposing Symbiodinium cells to different NO donor and scavenger combinations, at either control (26°C) or elevated temperatures under an irradiance of 100–120 μmol photons m⁻² s⁻¹. To ensure that the more thermally tolerant A1 cells would be photoinhibited by the elevated temperature treatment, they were exposed to a higher temperature (36°C) than were B1 cells (34°C). The B1 and C1 cells’ sensitivity also precluded the use of donor concentrations >100 μM. S-NONO (Invitrogen) and GSNO (Sigma-Aldrich) provided NO, and c-PTIO (Invitrogen) was used as a specific NO scavenger in order to examine the NO dependency of a response.

To assess the effects of NO on cell mortality, type A1 and B1 cells were exposed to control (26°C) and elevated temperatures (A1, 34°C; B1, 32°C) with and without 2 mM cPTIO.

All NO donor and scavenger treatments took place in 0.22 μm filtered seawater (FSW) in 1.5 ml tubes with cell suspensions at a concentration of ~1 x 10⁶ cells ml⁻¹.

### Fluorometric assessment of photosynthetic competence

Fluorescence induction curves were conducted using a Water-PAM fluorometer (Walz). Cells were dark adapted for 30 min prior to measurement. Thus, values were obtained for maximum fluorescent yield of PSII (F₀/Fₘ). During induction curves, cells were exposed to 250 μmol photons m⁻² s⁻¹ (irradiance increased to stimulate the cells’ NPQ response), and the effective quantum yield of PSII (ΔF/Fₘ) was measured until steady state. Using these data, NPQ was calculated as (Fₘ – Fₗ)/Fₘ.

In order to ensure consistency between treatments, as well as to increase conservatism, comparisons of PSII fluorescence yields and NPQ refer to values relative to pre-treatment (t = 0 h) conditions. In all cases, only algal cultures with initial dark-adapted yields (F₀/Fₘ) > 0.5 were used.

### Flow cytometric (FCM) assessment of nitric oxide production and mortality in Symbiodinium cells

NO production was measured with the fluorescent nitric oxide indicator 4-amino-5-methylamino-2,7’-difluorofluorescein diacetate (DAF-FM-DA; Molecular Probes) at a final concentration of 15 μM in FSW. DAF-FM-DA is more sensitive (~3 nM detection limit), photostable and cell permeable than previously available fluorescent dyes and has been successfully used to measure the low levels of NO present in microalgae (e.g. Thompson et al. 2008). After 90 min incubation in the dark, cells were washed twice by repeated centrifugation (800 x g for 5 min) and resuspension in FSW in order to remove excess probe. They were then kept in the dark for a further 30 min to allow cleavage of the DAF-FM-DA dye to its active DAF-FM form (Nagano and Yoshimura 2002). The fluorescent product of DAF-FM’s reaction with NO has an emission maximum at 515 nm when excited with blue light (Nagano and Yoshimura 2002), and this was measured using the FL1 (515–545 nm) channel on a FACScan three-channel flow cytometer (Becton-Dickinson). Excitation light was provided by a 488 nm argon laser and cells were gated on the basis of their Chl fluorescence (measured with a >650 nm detector). Blank, unlabeled samples were processed alongside probed cells to control for autofluorescence, and the NO-dependent fluorescence was calculated by subtracting the mean signal of approximately 50,000 blank cells from its equivalent DAF-FM value. Positive controls incubated for 30 min with 1 mM of the NO donor sodium nitroprusside (SNP; Sigma-Aldrich) were prepared to ensure successful loading of the DAF-FM dye. To standardize fluorescence across treatments and types, volumetric measurements of individual cells (modeled as ellipsoids) were taken using an eyepiece graticule and a ×100, 1.25 numerical aperture (NA) oil immersion objective. At least 50 cells were measured per sample.

Propidium iodide (PI; Sigma Aldrich) was used to selectively label non-viable Symbiodinium cells (Strychar et al. 2004). PI cannot permeate the plasma membranes of live cells, but can diffuse through the dysfunctional membranes of dead and dying cells, wherein it binds strongly to nucleic acids and displays increased fluorescence. Upon excitation with a 488 nm argon laser and gating by Chl fluorescence (see above), PI emission was detected at 560–605 nm. Percentages of PI-positive (non-viable) cells were calculated using graphical analysis of probed and blank samples (Weasel FCM analysis software, Walter & Eliza Hall Institute, Melbourne, Australia). All gain, threshold and compensation settings were kept constant throughout both NO and cell viability assays.

### Confocal microscopy of Symbiodinium cells

To confirm the cellular localization of fluorescence, the dyes were visualized using an Olympus Fluoview FV-1000 inverted confocal laser scanning microscope and a ×100, 1.45 NA oil immersion lens. Cells were aliquoted onto poly-l-lysine-coated glass bottom dishes (MatTek Corporation) and left to settle for 15 min. The medium was then replaced with 1% (w/v) carboxymethylcellulose in FSW in order to immobilize the cells for imaging. DAF-FM was excited with a 473 nm diode laser and its fluorescence was detected at 485–545 nm. PI was excited by a 559 nm diode laser and fluorescence was detected at 595–645 nm. A 635 nm diode laser was used to visualize Chl autofluorescence, which was detected at 655–755 nm. Laser intensity, pinhole aperture (1 Airy unit), detector
gain and offset were kept constant between blank and probed samples.

**Statistical analyses**

Data analyses were carried out using a PASW Statistics 18.0 package (IBM). Repeated measures analysis of variance (RM-ANOVA) was used to test null hypotheses regarding photosynthetic yields, NO production and cell death for the three ITS2 types and treatments. Two-way univariate ANOVA was used to examine the effects of NO on cell physiology. Post-hoc analyses represent pair-wise comparisons of estimated marginal means between treatments and controls at a particular time point, unless otherwise indicated. Data were examined for normality and equal variance prior to any parametric analysis, and were transformed where necessary to fulfill these assumptions. In the case of sphericity (the variance parameter for RM-ANOVA), the Greenhouse–Geisser correction was used whenever Mauchly’s test returned a significant result.

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**References**


