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1 **Nitric oxide and coral bleaching: is peroxynitrite generation required for**
2 **symbiosis collapse?**
3
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11 **Summary**
12
13 The temperature-induced collapse ("bleaching") of the coral-dinoflagellate symbiosis
14 is hypothesised to result from symbiont oxidative stress and a subsequent host innate
15 immune-like response. This includes the production of nitric oxide (NO), which is
16 involved in numerous microbial symbioses. Much of NO's cytotoxicity has been
17 attributed to its conversion, in the presence of superoxide (O_2^-), to highly reactive
18 peroxynitrite ($ONOO^-$). However, $ONOO^-$ generation has yet to be observed in either
19 a lower invertebrate or intracellular mutualism. Using confocal laser scanning
20 microscopy with the fluorescent $ONOO^-$ indicator aminophenyl fluorescein (APF), we
21 observed strong evidence that $ONOO^-$ is generated in symbiotic *Aiptasia pulchella*
22 under conditions known to induce thermal bleaching. However, a role for $ONOO^-$ in
23 bleaching remains unclear as treatment with a peroxynitrite scavenger had no
24 significant effect on thermal bleaching. Therefore, while $ONOO^-$ may have a potential
25 for cytotoxicity, *in vivo* levels of the compound may be insufficient to affect
26 bleaching.
27
28 **Keywords:** Reactive nitrogen species, endosymbiosis, oxidative stress, *Symbiodinium*,
29 cnidarian-dinoflagellate symbiosis, innate immunity.
30
31

32 **Introduction**

33

34 Microbial symbioses are ubiquitous in the natural world and sustain some of the most
35 diverse ecosystems on Earth. Among the most ecologically important associations are
36 those between reef corals (Cnidaria) and photosynthetic dinoflagellates. These algal
37 symbionts (genus *Symbiodinium*) provide the host with energy in exchange for
38 nutrients that are typically at low concentrations in the surrounding seawater (Davy et
39 al., 2012). The association underpins the existence of coral reefs but is being placed
40 under increasing strain by recent climatic changes, particularly rising sea surface
41 temperatures (Weis, 2008). It has long been known that excessive heating of corals
42 can result in the loss of their symbiotic algae (a process known as "bleaching"), yet
43 we still know little about the physiological events underpinning this symbiotic
44 collapse (Weis, 2008; Davy et al., 2012).

45

46 Coral bleaching has been linked to the overproduction of reactive oxygen species
47 (ROS; Lesser, 2006). Often associated with photosynthetic dysfunction, ROS have a
48 well-known capacity for cellular damage (Lesser, 2006), but at low concentrations
49 they also act as signalling compounds (Winterbourn, 2008). It has been hypothesised
50 that ROS leakage from photosynthetically compromised symbionts stimulates an
51 innate immune-like pathway resulting in host nitric oxide (NO) synthesis (Perez and
52 Weis, 2006; Weis, 2008), but very few data exist regarding ROS and cnidarian innate
53 immunity. NO is a ubiquitous signalling compound implicated in the regulation of
54 numerous microbial endosymbioses (Wang and Ruby, 2011). When produced
55 alongside ROS (specifically superoxide, O_2^-), however, NO can convert to highly
56 reactive peroxynitrite ($ONOO^-$), which is much more toxic and has a capacity to
57 irreversibly damage mitochondria, antioxidant enzymes, DNA, and lipid membranes
58 (Pacher et al., 2007). In fact, the reaction between NO and O_2^- occurs faster than that
59 of O_2^- with superoxide dismutase, thus the formation of $ONOO^-$ is regarded as
60 inevitable when NO and O_2^- are generated simultaneously (Pacher et al., 2007). The
61 likelihood of its generation under thermal stress has therefore led to $ONOO^-$ being
62 proposed as the effector of NO-mediated cnidarian bleaching (Perez and Weis, 2006;
63 Weis, 2008).

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65 Using the model cnidarian *Aiptasia pulchella*, this investigation sought to test the
66 hypothesis that cnidarian bleaching is dependent on NO's conversion to $ONOO^-$
67 during thermal stress. Filling this gap in our knowledge is important if we are to better
68 understand the cellular basis of coral bleaching and the breakdown of intracellular
69 mutualisms in general.
70
71 **Materials and Methods**
72
73 Symbiotic specimens of *Aiptasia pulchella* were maintained in glass bowls under a
74 12-h light:12-h dark cycle (80-100 μmol photons m^{-2} s^{-1} provided by cool white
75 fluorescent tubing; OSRAM DULUX L 36W 4000 K) at a temperature of 26°C, and
76 fed twice weekly with freshly hatched *Artemia* sp. nauplii. Prior to treatment,
77 anemones were starved for 48 h.
78
79 *Experimental induction of* $ONOO^-$ *in* Aiptasia pulchella *and its fluorometric*
80 *assessment.*
81
82 Peroxynitrite was detected using the fluorescent indicator aminophenyl fluorescein
83 (APF; Molecular Probes, Eugene, OR, USA). APF is specific for highly reactive
84 species and detects, alongside peroxynitrite, hydroxyl ($OH^·$) and hypochlorite (OCl^-)
85 radicals. By using specific scavengers of $ONOO^-$ and NO, however, one can
86 determine the extent to which APF fluorescence is $ONOO^-$-dependent. The suitability
87 of APF was determined by preparing 100 μl solutions of 10 μM APF in 'anemone
88 relaxing solution' (50% 0.22-μm filtered seawater (FSW), 50% 0.37 M $MgCl_2$) with
89 and without 2 mM urate (Sigma-Aldrich, Auckland, New Zealand), a peroxynitrite
90 scavenger that has been employed successfully in fluorescence-based assays (Tewari
91 et al., 2013). The peroxynitrite donor 3-morpholinosydnonimine (SIN-1; Invitrogen,
92 Auckland, New Zealand), which generates $ONOO^-$ through the simultaneous release
93 of NO and O_2^-, was then added (0 - 1 mM final SIN-1 concentration). APF
94 fluorescence (ex: 490 nm, em: 515 nm) was monitored over 2 h using a fluorescent
95 microplate reader (Enspire® 2300, Perkin-Elmer, Waltham, MA, USA). To determine
96 whether the effects of urate on APF signal were truly due to scavenging of $ONOO^-$
97 (rather than quenching of APF fluorescence), the SIN-1/APF incubation was repeated
98 with 2 mM urate added 30 min after SIN-1.

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The ability of APF to detect peroxynitrite *in vivo* was assessed by incubating individual anemones (n = 6) for 60 min with 10 µM APF in a) relaxing solution only, b) 1 mM SIN-1, c) 1 mM SIN-1 + 2 mM urate, d) 1 mM SIN-1 + 1 mM of the NO scavenger 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide (cPTIO; Life Technologies, Auckland, New Zealand).

Endogenous production of $ONOO^-$ by *A. pulchella* was investigated using the high temperature shock (HTS) method. Briefly, anemones (2-3 mm oral disc diameter) in FSW (n = 6 per treatment in individual glass beakers) were transferred to a 26°C water bath under 12-h light: 12-h dark cycle, (light: 70-90 µmol photons m^{-2} s^{-1} provided by a light-emitting diode (LED) light bankl 20 RoHS 5 W 6400 K) and allowed to acclimate for 48 h (fluorometric assessments of symbiont photosynthesis [see below] were conducted to ensure stability prior to treatment). Temperature was then increased (over < 1 h) to 33°C or kept constant at 26°C (control) and anemones were exposed to these conditions for 24 h. Some additional anemones (n = 6 per treatment) were HTS-treated in the presence of 2 mM urate or 1 mM cPTIO.

To quantify $ONOO^-$ in live *A. pulchella*, the FSW of experimental anemones was replaced with 10 µM APF in relaxing solution and anemones were incubated in the dark for 60 min. Individual anemones were transferred to glass-bottom dishes (MatTek Corporation, Ashland, MA, USA) and immobilised with 1% (w/v) low-melting agarose in relaxing solution, boiled and cooled to ca. 28°C. They were then visualised using an Olympus Fluoview FV-1000 inverted confocal LSM (Olympus, Center Valley, PA, USA) and ×40 0.9 NA water immersion lens. A 473 nm laser was used to excite APF, the fluorescence of which was measured at 510-530 nm. Symbiont chlorophyll autofluorescence was detected using 635 nm excitation and a 655-755 nm emission filter. Fifteen images in the z-plane were acquired for three tentacles of each replicate anemone and $ONOO^-$-dependent fluorescence was quantified by measuring the mean 510-530 nm fluorescence intensity of tentacle gastrodermis using ImageJ software (National Institutes of Health, Bethesda, USA). Unstained anemones were processed to control for tissue autofluorescence and laser intensity / LSM image acquisition settings remained unchanged throughout. Any non-

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132 fluorescing anemones were incubated for 30 min with 1 mM SIN-1 to confirm
133 successful loading of APF.
134
135 *Examining the role of $ONOO^-$ in the thermal bleaching of* Aiptasia pulchella.
136
137 The role of $ONOO^-$ in temperature-induced bleaching was investigated by incubating
138 *A. pulchella* (n = 5 in individual glass beakers) for 24 h at 26°C in FSW containing 1
139 mM SIN-1 with and without 2 mM urate. In addition, six replicate anemones were
140 HTS-treated with and without 2 mM urate. After 24 h exposure to high temperature or
141 SIN-1 / urate, anemones were returned to control conditions for a further 24 h (to
142 allow time for bleaching) and then processed for symbiont density assays as described
143 below.
144
145 *Assessments of symbiont photosystem II fluorescence and host bleaching.*
146
147 Fluorescence yields of photosystem II (PSII) were monitored regularly (midday and
148 30 min after lights-off) using pulse amplitude modulation fluorometry (Diving-PAM,
149 Walz, Effeltrich, Germany). Symbiont densities were determined as follows. Whole
150 anemones were homogenised using a tissue grinder in a small volume of buffer (50
151 mM potassium phosphate, pH 7.8, 1 mM EDTA). An aliquot was removed for
152 haemocytometer counts (at least 6 per sample; Improved Neubauer, Boeco, Germany)
153 and the remainder was centrifuged (16000 × g for 20 min) and analysed for soluble
154 protein content (Bradford assay). Changes in symbiont density relative to host soluble
155 protein ("% symbiont loss") after 48 h were calculated relative to mean pre-treatment
156 (t = 0) values.
157
158 *Statistical analysis.*
159
160 Data analysis was carried out using PASW Statistics 18.0 (IBM, Armonk, NY, USA).
161 Where appropriate, data were analysed using repeated measures analysis of variance
162 (RMANOVA). RMANOVA reports represent time × treatment interactions. All other
163 analyses were carried out using one-way ANOVA. In all cases data were examined
164 for normality and transformed where necessary.
165

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166 **Results and Discussion**

167

168 *Detection of ONOO⁻ in symbiotic* Aiptasia pulchella *using aminophenyl fluorescein*
169 *(APF).*

170

171 Aminophenyl fluorescein fluorescence (515 nm) successfully responded to SIN-1-
172 derived peroxynitrite in a dose-dependent manner (Fig. S1A). Addition of the $ONOO^-$
173 scavenger urate (2 mM) prevented this increase, and adding urate after 30 min
174 confirmed that this was due to $ONOO^-$-scavenging rather than quenching of
175 fluorescence (Fig S1B). The effects of SIN-1 and urate on APF fluorescence *in vitro*
176 are shown in electronic supplementary Figure S1.

177

178 Incubation of APF-loaded *A. pulchella* with the peroxynitrite donor SIN-1 resulted in
179 significant increases in tissue 510-530 nm fluorescence (one-way ANOVA, $F_{3, 20}$ =
180 59.753, $p < 0.001$, Fig. 1A) that were absent in the presence of 2 mM urate or 1 mM
181 cPTIO (Fig. 1A). HTS treatment of *A. pulchella* also induced increases (one-way
182 ANOVA, $F_{3, 19} = 14.679$, $p < 0.001$), which were absent in the presence of urate or
183 cPTIO (Fig. 1B). The declines in fluorescence intensity when anemones were treated
184 with scavengers either of $ONOO^-$ itself (urate) or its precursor NO (cPTIO) confirm
185 that APF signal in *A. pulchella* was an accurate reflection of $ONOO^-$ generation rather
186 than the dye's interactions with other highly reactive compounds.

187

188 The generation of peroxynitrite ($ONOO^-$) has been proposed as a significant step in
189 the cellular cascade underpinning coral bleaching (Perez and Weis, 2006; Weis, 2008)
190 and this study provides strong evidence that $ONOO^-$ generation occurs in thermally
191 stressed cnidarians. To the authors' knowledge, it also represents the first observation
192 of $ONOO^-$ in either a lower invertebrate (e.g. Porifera, Cnidaria, or Ctenophora) or an
193 intracellular mutualism.

194

195 *Involvement of peroxynitrite in thermal photoinhibition and bleaching.*

196

197 The addition of urate (2 mM) significantly alleviated declines in PSII fluorescence
198 yield (RMANOVA, $F_{8, 60} = 12.491$, $p < 0.001$, Fig. 2A). Peroxynitrite strongly
199 inhibits mechanisms of electron transport (Pacher et al., 2007) and this presumably

200 includes the components of photosynthesis within symbionts' chloroplasts. As we
201 know very little about the role of reactive nitrogen species in microalgal
202 photoinhibition, this is an attractive area for future work.
203

204 Peroxynitrite can be cytotoxic to the host in numerous ways (see Pacher et al., 2007
205 for review) but, in the light of recent investigations (e.g. Dunn et al., 2012), its
206 interactions with cnidarian host mitochondria may be most important. Differences in
207 the reactivity of NO and O_2^- and their potential for diffusion mean that peroxynitrite
208 generation occurs primarily at the sites of O_2^- production (Pacher et al., 2007), the
209 most significant of which (in the host at least) is the inner membrane of mitochondria
210 (Lesser, 2006). Current research is linking mitochondrial dysfunction and the
211 associated apoptotic pathways to cnidarian bleaching (Dunn et al., 2012) and as noted
212 above, $ONOO^-$ is a potent inhibitor of mitochondrial electron transport. Whether this
213 occurs at *in vivo* $ONOO^-$ concentrations has been the subject of debate (Pacher et al.,
214 2007), however, and we still know little about $ONOO^-$ generation beyond mammals.
215 For example, $ONOO^-$'s extremely high reactivity and relatively short half-life in
216 biological systems (Lesser, 2006; Pacher et al., 2007) could very well limit its
217 capacity for diffusion and thus the number of potential target molecules. In this study
218 the peroxynitrite donor SIN-1 (1 mM in FSW) induced significant bleaching of *A.*
219 *pulchella* at control temperatures (one-way ANOVA, $F_{4, 27} = 20.94$, $p < 0.001$, Fig
220 2B; Tukey HSD *post-hoc vs.* "control" $p = 0.006$) and the $ONOO^-$ scavenger urate
221 restored levels of symbiont loss to those of the controls (Tukey HSD *post-hoc vs.*
222 "control" $p = 0.365$). It would appear, therefore, that addition of $ONOO^-$ to *A.*
223 *pulchella* can stimulate bleaching. This is not entirely surprising, as a bolus dose of an
224 NO and O_2^- donor would be expected to induce significant physiological stress
225 (Lesser, 2006; Weis, 2008). However, there was no significant effect of the $ONOO^-$
226 scavenger urate on thermal bleaching intensity (Tukey HSD *post-hoc vs.* "HTS", $p >$
227 0.999), and this was not due to heat-induced degradation of the scavenger; application
228 to APF/SIN-1 solutions of FSW/urate pre-heated to 33°C for 24 h strongly inhibited
229 APF fluorescence (Fig. S1C). Together, these findings suggest that while $ONOO^-$ has
230 a bleaching-inducing capacity, either the levels of $ONOO^-$ generated *in vivo* are not
231 sufficient to influence symbiont loss, or alternative pathways (perhaps involving ROS
232 or NO directly) are more critical.
233

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234 In conclusion, we propose that any mediation of temperature-induced cnidarian
235 bleaching by nitric oxide occurs independently of its conversion to peroxynitrite. NO
236 has the capacity to directly influence the cell death pathways implicated in bleaching
237 (Snyder et al., 2009) so $ONOO^-$ generation may be unnecessary in this regard. The
238 situation may of course be different in reef corals undergoing bleaching in the field,
239 where light intensities greater than those employed in the present study could
240 exaggerate NO / ROS synthesis in both host and symbiont. In any case, investigating
241 where in the symbiosis $ONOO^-$ is produced, and how such a potent radical can have
242 such modest effects during temperature stress are important subjects for future study.
243

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245

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250

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257

258 **References**

259

260 **Davy, S. K., Allemand, D. and Weis, V. M.** (2012). The cell biology of cnidarian-
261 dinoflagellate symbiosis. *Microbiology and Molecular Biology Reviews* **76**, 229-
262 261.
263 **Dunn, S. R., Pernice, M., Green, K., Hoegh-Guldberg, O. and Dove, S. G.** (2012).
264 Thermal stress promotes host mitochondrial degradation in symbiotic
265 cnidarians: Are the batteries of the reef going to run out? *PLoS ONE* **7**, e39024.
266 **Lesser, M. P.** (2006). Oxidative stress in marine environments. *Annual Reviews of*
267 *Physiology* **68**, 253-278.
268 **Pacher, P., Beckman, J. S. and Liaudet, L.** (2007). Nitric oxide and peroxynitrite
269 in health and disease. *Physiological Reviews* **87**, 315-424.

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

270 **Perez, S. and Weis, V. M.** (2006). Nitric oxide and cnidarian bleaching: An
271 eviction notice mediates breakdown of a symbiosis. *Journal of Experimental*
272 *Biology* **209**, 2804-2810.
273 **Snyder, C. M., Shroff, E. H., Liu, J. and Chandel, N. S.** (2009). Nitric oxide
274 induces cell death by regulating anti-apoptotic Bcl-2 family members. *PLoS ONE*
275 **4**, e7059.
276 **Tewari, R., Prommer, J. and Watanabe, M.** (2013). Endogenous nitric oxide
277 generation in protoplast chloroplasts. *Plant Cell Rep* **32**, 31-44.
278 **Wang, Y. L. and Ruby, E. G.** (2011). The roles of NO in microbial symbioses.
279 *Cellular Microbiology* **13**, 518-526.
280 **Weis, V. M.** (2008). Cellular mechanisms of cnidarian bleaching: Stress causes
281 the collapse of symbiosis. *Journal of Experimental Biology* **211**, 3059-3066.
282 **Winterbourn, C. C.** (2008). Reconciling the chemistry and biology of reactive
283 oxygen species. *Nature Chemical Biology* **4**, 278-286.
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287 **Figure Legends**

288 **Figure 1. Detection of peroxynitrite ($ONOO^-$) in live *Aiptasia pulchella*.**
289 Fluorescence intensities of anemone gastrodermis loaded with 10 μM aminophenyl
290 fluorescein (APF) and (**A**) incubated with and without 1 mM of the $ONOO^-$ donor
291 SIN-1, 2 mM of the $ONOO^-$ scavenger urate, or 1 mM of the NO scavenger cPTIO, or
292 (**B**) exposed to high temperature stress (HTS) with and without 2 mM urate or 1 mM
293 cPTIO. Values in both panels are means ± SE (n = 6) and asterisks denote significant
294 differences relative to the control group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). **C**)
295 Confocal LSM micrographs of anemone tentacles exposed to control conditions, HTS,
296 HTS + 2 mM urate, and HTS + 1 mM cPTIO. Green indicates APF fluorescence,
297 symbiont chlorophyll autofluorescence is labelled red. Scale bar: 100 μm.
298

299 **Figure 2. Involvement of peroxynitrite ($ONOO^-$) in temperature-induced**
300 **photoinhibition and bleaching in *Aiptasia pulchella*. A**) Quantum yields of PSII
301 over 48 h. Shaded areas represent periods of darkness and the dotted line indicates the
302 time at which anemones were returned to control (26°C) conditions (from 33°C). **B**)
303 Percentage bleaching of anemones treated for 24 h either with 1 mM of the $ONOO^-$
304 donor SIN-1 or exposed to high temperature stress (HTS - 33°C), both treatments
305 with and without 2 mM urate. Treatments lasted for 24 h before anemones were
306 returned to control conditions. Values in both panels are means ± SE (n = 6 for
307 controls and HTS ± urate, n = 5 for SIN-1 ± urate) and asterisks denote significant
308 differences relative to the control group (** $p < 0.01$, *** $p < 0.001$).

309

310 **Figure S1. The suitability of aminophenyl fluorescein (APF) for detection of**
311 **peroxynitrite ($ONOO^-$). A**) Fluorescence kinetics after addition of various
312 concentrations of the $ONOO^-$ donor SIN-1 to APF (10 μM in relaxing solution - see
313 text). **B**) Fluorescence kinetics of APF after addition of 1 mM SIN-1 in the presence
314 or absence of 2 mM urate (a peroxynitrite scavenger). The urate was added 30 min
315 after SIN-1, such that previously activated APF remained fluorescent. **C**)
316 Fluorescence kinetics of APF after addition of 1 mM SIN-1 in the presence or absence
317 of 2 mM freshly prepared or heat-treated urate. Values are means ± SE (n = 4
318 independent experiments).

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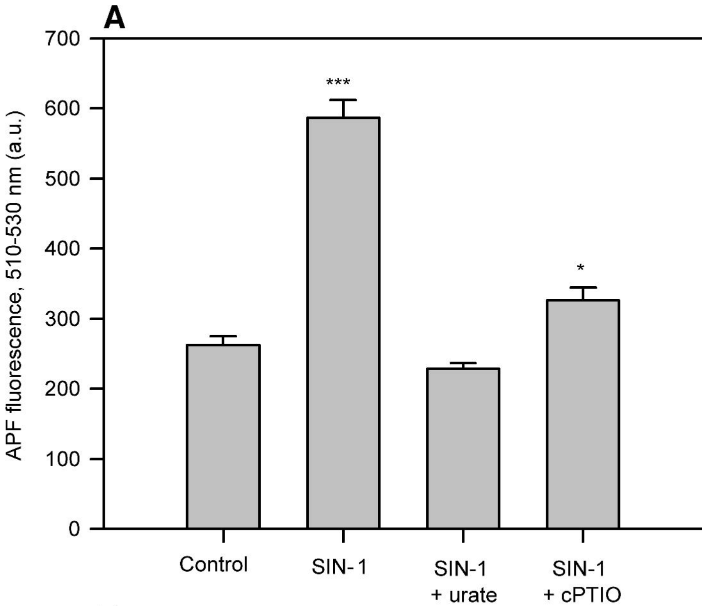
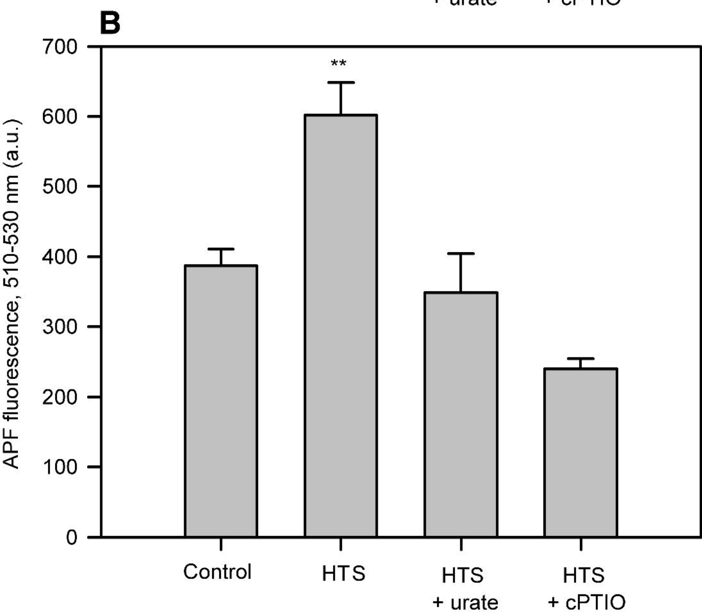
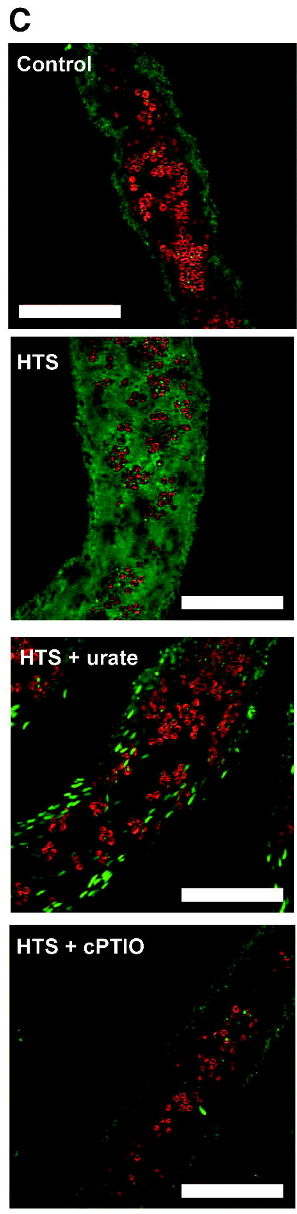

A
APF fluorescence, 510-530 nm (a.u.)
0
100
200
300
400
500
600
700
***
*
Control
SIN-1
SIN-1
+ urate
SIN-1
+ cPTIO


B
APF fluorescence, 510-530 nm (a.u.)
0
100
200
300
400
500
600
700
**
Control
HTS
HTS
+ urate
HTS
+ cPTIO


C
Control
HTS
HTS + urate
HTS + cPTIO

A

Quantum Yield of PSII

26°C
33°C
33°C + urate

0.7
0.6
0.5
0.0

***
***
***
***
***
**

0
12
24
36
48

Time (h)

B

% Symbiont loss

60
40
20
0
-20

**
***
***

26°C
- urate
+ urate
- urate
+ urate
26°C + SIN-1
33°C