Diverse responses of *Symbiodinium* types to menthol and DCMU treatment

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To understand the mechanism of photosynthetic inhibition and generation of reactive oxygen species (ROS) in *Symbiodinium* types under stress, chemicals such as dichlorophenyl dimethylurea (DCMU) are widely used. Moreover, DCMU and recently menthol were used to generate aposymbiotic cnidarian hosts. While the effects of DCMU on *Symbiodinium* cells have been extensively studied, no studies have shown the mechanism behind menthol-induced coral bleaching. Moreover, no study has compared the effects of DCMU and menthol treatments on photosystem II (PSII) activity and generation of ROS in different *Symbiodinium* types. In this study, we utilized five freshly isolated *Symbiodinium* types (*S. minutum* (B1), *S. goreaui* (C1), C3, C15, and *S. trenchii* (D1a)) to compare the effects of DCMU and menthol treatments. *Symbiodinium* cells were exposed to DCMU and menthol at different concentrations for 4 h. Results showed that values of the 50% inhibitory concentration (IC_{50}) for PSII inhibition were 0.72~1.96 mM for menthol-treated cells compared to 29~74 pM for DCMU-treated cells. Diverse responses of *Symbiodinium* types were displayed in terms of PSII tolerance to menthol (*S. minutum*>*S. trenchii*=C15>C3=*S. goreaui*), and also in the response curves. In contrast, responses were not so diverse when the different types were treated with DCMU. Three of five menthol-treated *Symbiodinium* types showed instant and significant ROS generation when PSII activity was inhibited, compared to no ROS being generated in DCMU-treated *Symbiodinium* types. Both results indicated that menthol inhibited *Symbiodinium* PSII activity through *Symbiodinium* type-dependent mechanisms, which were also distinct from those with DCMU treatment. This study further confirmed that photosynthetic functions *Symbiodinium* have diverse responses to stress even within the same clade.

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Abstract

To understand the mechanism of photosynthetic inhibition and generation of reactive oxygen species (ROS) in *Symbiodinium* types under stress, chemicals such as dichlorophenyl dimethylurea (DCMU) are widely used. Moreover, DCMU and recently menthol were used to generate aposymbiotic cnidarian hosts. While the effects of DCMU on *Symbiodinium* cells have been extensively studied, no studies have shown the mechanism behind menthol-induced coral bleaching. Moreover, no study has compared the effects of DCMU and menthol treatments on photosystem II (PSII) activity and generation of ROS in different *Symbiodinium* types. In this study, we utilized five freshly isolated *Symbiodinium* types (*S. minutum* (B1), *S. goreaui* (C1), C3, C15, and *S. trenchii* (D1a)) to compare the effects of DCMU and menthol treatments. *Symbiodinium* cells were exposed to DCMU and menthol at different concentrations for 4 h. Results showed that values of the 50% inhibitory concentration (IC_{50}) for PSII inhibition were 0.72~1.96 mM for menthol-treated cells compared to 29~74 pM for DCMU-treated cells. Diverse responses of *Symbiodinium* types were displayed in terms of PSII tolerance to menthol (*S. minutum*>*S. trenchii*=C15>C3=*S. goreaui*), and also in the response curves. In contrast, responses were not so diverse when the different types were treated with DCMU. Three of five menthol-treated *Symbiodinium* types showed instant and significant ROS generation when PSII activity was inhibited, compared to no ROS being generated in DCMU-treated *Symbiodinium* types. Both results indicated that menthol inhibited *Symbiodinium* PSII activity through *Symbiodinium* type-dependent mechanisms, which were also distinct from those with DCMU treatment. This study further confirmed that *Symbiodinium* photochemical responses to stress are diverse even within the same clade.

INTRODUCTION

42 *Symbiodinium* spp. are associated with marine invertebrate hosts, including Protista, Porifera,
43 Cnidaria, and Mollusca (Coffroth & Santos 2005), and play important functional roles in
44 providing photosynthesis-derived carbon and conserving or recycling host nitrogen metabolites
45 (Davy et al. 2012). To date, nine (A~I) *Symbiodinium* clades and numerous subcladal types have
46 been identified with distinguishable genetic identities and physiological characteristics (reviewed
47 in Stat and Gates 2011). Among these, only clades A, B, C, and D are widely associated with
48 scleractinian corals. Different *Symbiodinium* types are known to show variations in their
49 photosynthesis functions (Rowan 2004; Tchernov *et al.* 2004; Robinson & Warner 2006;
50 Sampayo *et al.* 2008; Wang *et al*. 2012b; Suggett *et al.* 2015), resulting in different contributions
51 to their symbiotic associations (Sat *et al.* 2008; Yuyama & Higuchi 2014; Pernice *et al.* 2015).
52 Various stress tolerabilities among different *Symbiodinium* types were also revealed by high
53 antioxidant plasticity (Krueger *et al.* 2014).
54 The association between corals and *Symbiodinium* is highly vulnerable to physical and
55 chemical disturbances. For example, only 1～2°C above the summer average under moderate to
56 high irradiance is enough to disrupt the symbiotic relationship and end up with loss of symbionts
57 from coral hosts, resulting in 'coral bleaching' (Fitt *et al.* 2001; Lesser & Farrell 2004). The
58 mechanism of *Symbiodinium* depletion (reviewed in Weis 2008), from cnidarian host cells, is
59 generally attributed to damage by reactive oxygen species (ROS) generated from photoinhibition
60 of *Symbiodinium* during stress (e.g., high temperature, irradiance, or herbicides, e.g.,
61 dichlorophenyl dimethylurea (DCMU) treatment) (Jones *et al.* 1998; Jones 2004, 2005) or stress
62 on the coral host's metabolism (Jones 2004). Disruption of *Symbiodinium*-coral symbiosis can
63 also be achieved by some chemicals such as heavy metals (Jones 1997) and sunscreen
64 contamination (Danovaro *et al.* 2008). However, there is no direct evidence to indicate coral

bleaching caused by ROS generated by DCMU-treated *Symbiodinium* (Jones 2004).

While use of DCMU to generate aposymbiotic cnidarian hosts and the effects of DCMU on *Symbiodinium* cells have extensively been studied (Murata et al. 2007, Takahashi et al. 2013, Fransolet et al. 2014, Aihara et al. 2016, Parrin and Blackstone 2017), use of menthol as an efficient bleaching agent has only recently been established. In recent years, menthol was found to successfully induce bleaching in coral and sea anemone hosts (Wang *et al.* 2012a; Dani *et al.* 2016; Matthews *et al*. 2016).

Menthol is a cyclic terpene alcohol which can cause local anesthetic effects in neuronal and skeletal muscles by blocking voltage-operated sodium channels (Haeseler *et al.* 2002). This effect has led to its use as a marine anesthetic (Moore 1989; Lauretta *et al.* 2014). A variety of different membrane receptors are known to respond to menthol stimulation, including transient receptor potential (TRP)M8 that results in an increase in intracellular Ca^{2+} concentrations and causes a cold sensation in vertebrates (McKemy et al. 2002; Okazawa et al. 2000; Peier et al. 2002, Hans et al. 2012). Menthol is also a cytotoxic compound to plant tissues (Muller & Hauge 1967; Brown *et al.* 1987), causing a drastic reduction in the number of intact mitochondria and Golgi bodies in seedling roots (Lorber & Muller 1976), inhibiting respiration and photosynthesis (Pauly *et al.* 1981), and decreasing cell membrane permeability (Muller *et al.* 1969).

Recently, Wang et al. (2012a) demonstrated that menthol can be used to effectively to induce bleaching in the corals *Isopora palifera* and *Stylophora pistillata*. Dani et al (2016) further hypothesized that menthol-induced activation of a host TRP receptor might increase the intracellular calcium concentration. Modulation of calcium homeostasis, which is known to upregulate the autophagic pathway (Smaili et al. 2013), could therefore trigger *Symbiodinium* depletion through a phagolysosomal process. Whether the PSII breakdown in the endosymbiotic

Symbiodinium in corals is directly or indirectly caused by menthol needs to be clarified. Moreover, no studies have compared responses of different *Symbiodinium* types to both menthol and DCMU, which would investigate the important but under-explored topic of functional diversity among *Symbiodinium* species.

In this study, we tested the responses of different *Symbiodinium* types to DCMU and menthol exposure by evaluating the concentration for 50% inhibition of PSII activity and the generation of ROS. Results of this study will provide information on diverse responses among different *Symbiodinium* types to menthol treatment and is a first step towards understanding mechanisms of *Symbiodinium* cell depletion caused by menthol treatment.

MATERIALS AND METHODS

Symbiodinium were isolated from five coral species. Parts of colonies of *Acropora humilis*, *Galaxea fasicularis*, *Isopora palifera,* and *Porities lutea* corals were collected from reefs within Kenting National Park, Taiwan (21°55’54’’N, 120°44’45’’E). A sample collection permit (no. 488-100-01) was obtained from the Kenting National Park Authority as part of the long-term environmental monitoring project. Coral colonies were transferred to the laboratory within 3 h in an aerated plastic box, and maintained in an aquarium tank under conditions described in Wang *et al.* (2011). Corals were acclimatized to laboratory aquarium (Wang et al. 2012) settings (25 ºC and 70 µmol photons $m^{-2}s^{-1}$ light) for 1 week before initiating experiments. The glass sea anemone, *Exaiptasia pulchella* (Grajales and Rodrigues 2014), originally collected from a discharge trench of the Tongkun Fishery Research Institute, Taiwan, had been maintained in a

tank (45×30×30 cm) equipped with illumination and temperature control for more than 1 year.

To prepare freshly isolated *Symbiodinium* (FIS), coral fragments with 5~10 cm^2 of live tissue and excised tentacles from sea anemones were homogenized and washed with artificial seawater (ASW) prepared from Instant Ocean (Aquarium Systems, France) as described by Wang et al. (2011). The dominant FIS types used in this study were identified as *Symbiodinium* ITS2 types C1 (*S. goreaui*), C3, C15, D1a (*S. trenchii*), and B1 (*S. minutum*) from *S. pistillata*, *A. humilis*, *P. lutea*, *G. fasicularis* and *E. pulchella*, respectively, based on denaturation gradient gel electrophoresis (DGGE) according to Wang et al. (2011).

PSII activities of FIS were determined by the maximum quantum yield (F_v/F_m), and the minimum (F_0) and maximum (F_m) fluorescence levels were measured to calculate the variable fluorescence [F_v, ($F_v = F_m - F_0$)] using a DIVING-PAM fluorometer (Walz, Germany) at a setting of 8 for the measuring light and saturating flash of actinic light.

When determining values of the half-maximal inhibitory concentration (IC_{50}), triplicate samples with 5 ml of FIS (around 10^6 cells ml^{-1}) of each type showing F_v/F_m values of >0.6 (a value generally considered normal/healthy, Fitt et al. 2001) were transferred to a 50-ml Falcon tube and centrifuged at 417 ×*g* for 3 min to collect *Symbiodinium*. The algal pellet was then re-suspended in 5 ml of different concentrations of menthol- or DCMU-ASW and maintained at 25 °C under illumination of around 70 µmol photons $m^{-2}s^{-1}$ of photosynthetically active radiation (PAR). Wang et al. (2011) suggested that the PSII activity of FIS begins to fluctuate after incubation for 4 h, so the effects of menthol and DCMU were determined as the 4-h IC_{50}. F_v/F_m values of FIS in menthol or DCMU were directly determined with the DIVING-PAM fluorometer without dark adaptation at 4 h of incubation, and were converted to PSII inhibition by comparing to values in fresh isolates as below:

PSII inhibition (%) = $(1 - ((F_v/F_m)_{4h} / (F_v/F_m))_{0h}) \times 100\%$;

where $(F_v/F_m)_{0h}$ and $(F_v/F_m)_{4h}$ values were respectively measured at 0 and after 4 h of incubation. After plotting PSII inhibition against the logarithm-transformed concentration of menthol (or DCMU), the equation for calculating the IC_{50} was obtained from the best curve-fit-model provided in SigmaPlot 10.0 software.

ROS generated in menthol- or DCMU-treated FIS were determined with a 2′,7′-dichlorofluorescin diacetate (H_2DCFDA) probe. FIS (around 10^6 cells ml^{-1} , cells counted as in Wang et al. 2012b) of each *Symbiodinium* type was incubated in ASW containing 1.73 mM menthol (for *S. minutum*, this was 2.43 mM) or 130 pM DCMU as the positive control, for 4 h in which the PSII activity of FIS would be completely inhibited. The mentioned concentration used to examine ROS generation refers to the highest concentration causing maximum inhibition of PSII activity. Also, during the trials of menthol-induced bleaching of the coral hosts, the effective concentration varied between different hosts associated with different *Symbiodinium* types, but all displayed significant photosystem II (PSII) breakdown in the endosymbiotic *Symbiodinium* (Fig. S1). ROS in *Symbiodinium* were detected by incubating 1 ml of an algal suspension with 5 µl H_2DCFDA (10 mM in dimethyl sulfoxide) for 30 min in the dark, followed by fluorescence microscopic examination (Mydlarz & Jacobs 2004) and fluorescence determination (Wang et al. 2011). The fluorescence data of ROS signals are expressed in arbitrary fluorescence units per cell.

Dose effects (4-h IC_{50}) of menthol and DCMU on PSII inhibition of FIS were calculated from samples of each treatment. The curve equations for IC_{50} calculation were determined by the best-fit regression curve of PSII inhibition (%) against the logarithm-transformed reagent concentration, which are described in equation (1) for menthol and (2) for DCMU treatment:

157 $$y = y_0 + \frac{a}{1 + e^{\left(\frac{-(x - x_0)}{b}\right)}}$$ (1) and

158 $$y = \frac{a}{1 + e^{\left(\frac{-(x - x_0)}{b}\right)}}$$ (2)

159 Parameters and regression coefficients of the equations are listed in Table 1.

160 Comparisons of 4-h IC_{50} values between *Symbiodinium* types were made using a one-way
161 analysis of variance (ANOVA) followed by Fisher's least significance difference (LSD) test,
162 with a significance level of 0.05. The coefficient of variance (CV) was used to evaluate the
163 variation in IC_{50} measurements between replicates.

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165

166 **RESULTS**

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168 When *Symbiodinium* algae were incubated in menthol-supplemented ASW for 4 h, all five
169 *Symbiodinium* types (*S. minutum*, *S. goreaui*, C3, C15, and *S. trenchii*) displayed typical dose-
170 response curves under menthol concentrations of 0.19~2.43 mM (Fig. 1A). In contrast to
171 menthol inhibition at millimolar levels, DCMU-treated samples displayed PSII inhibition of FIS
172 at picomolar levels (4~129 pM) (Fig. 1B).

173 Regression coefficients for curve fitting ranged 0.989~0.999 for menthol treatments and
174 0.995~0.999 for DCMU treatments. When converting parameter "b" in Table 1 to a slope factor
175 (curve steepness) as described by Motulsky and Christopoulos (2003), values for menthol
176 treatments were divided into two groups which included high (20.0~30.3 for *S. minutum,* types
177 C3, and C15) and low slope factors (8.7~9.5 for *S. goreaui* and *S. trenchii*). But those for
178 DCMU-treated FIS displayed only one group of slope factors that ranged 4.3~6.3. The 4-h IC_{50}
179 values for menthol and DCMU treatments on five *Symbiodinium* types were calculated, and
180 results are listed in Table 2. Mean 4-h IC_{50} values for menthol ranged 0.72~1.96 mM with CV
181 values ranging 1.1%~12.3%, and those for DCMU ranged 29~74 pM with CV values of
182 2.3%~14.1% (Table 2). Two sets of data significantly varied among *Symbiodinium* types
183 (menthol, $F_{4,11} = 17.54$, $p<0.001$; DCMU, $F_{4,10} = 12.00$, $p<0.01$), and *S. minutum* was found to
184 be the most tolerant type to both menthol and DCMU irritation ($p<0.05$). In contrast to the
185 comparable tolerability to DCMU among different *Symbiodinium* types, *S. trenchii* and type C15
186 were significantly more tolerant to menthol than were *S. goreaui* and C3 ($p<0.05$).

187 In order to explore if ROS are the cause of menthol inhibition of PSII activity of FIS, the
188 generation of algal ROS under a condition of complete PSII activity inhibition by menthol or

DCMU treatment was further examined. According to a fluorescence microscopic examination, no DCMU-treated type displayed green fluorescence, indicating no ROS signal, as found in the ASW control (Fig. S2). However, ROS generation in menthol-treated *Symbiodinium* varied among types. As shown in Fig. 2B, *S. minutum*, consistent with the ASW control in Fig. 2A, showed almost no ROS signal. For *S. trenchii*, there were only mild ROS signals detected (Fig. 2C). In contrast to *S, minutum* and *S. trenchii*, as shown in Fig. 2D-F, types C15, C3, and *S. goreaui* displayed intense green fluorescence, representing considerable ROS generation. Direct measurements of the fluorescence intensity derived from an ROS probe also indicated that the five *Symbiodinium* types had varied responses to menthol irritation (Table 3). Menthol-treated *S. goreaui*, C3, and C15 types displayed almost double the extent of ROS fluorescence (with mean values of 58~68 fluorescence units cell^{-1}) of that of *S. trenchii* (35±3 fluorescence units cell^{-1}), and more than 3-times that of *S. minutum* (18±2 fluorescence units cell^{-1}) (Table 3). ROS fluorescence intensities obtained from menthol-treated *S. minutum* and all DCMU-treated types were comparable to background levels (with mean values of 18~28 fluorescence units cell^{-1}), even though F_v/F_m values were reduced to < 0.1. In order to monitor the times needed for *Symbiodinium* types to respond to menthol irritation, F_v/F_m values and ROS levels of the C3 type were examined every 5 or 10 min after suspending the alga in menthol-supplemented ASW. As shown in Fig. 3A, the F_v/F_m value was significantly reduced from 0.685 to < 0.2 within 5 min of incubation, and to nearly 0 after 10 min of incubation. As with the decrease in F_v/F_m value, ROS generation in the menthol treated *Symbiodinium* reached a maximum within 5 min of incubation (Fig. 3B).

DISCUSSION

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213 This study indicated that millimolar levels of menthol significantly inhibited PSII activities of
214 five freshly isolated *Symbiodinium* types. Loss of photosynthetic activity in *Symbiodinium* might
215 explain the coral bleaching phenomenon of menthol-treated corals and sea anemones (Wang *et al*.
216 2012a; Dani *et al.* 2016; Matthews *et al*. 2016). However, various sensitivities and response
217 modes to menthol irritation indicate that different *Symbiodinium* types did not react uniformly to
218 the chemicals. Diverse physiological performances among different *Symbiodinium* types were
219 observed with respect to thermal tolerance (Rowan 2004; Tchernov *et al.* 2004; Robinson &
220 Warner 2006; Sampayo *et al.* 2008; Wang *et al*. 2012b; Suggett *et al.* 2015), thermal stress-
221 induced reactive oxygen release and antioxidant plasticity (Krueger *et al.* 2014), and expression
222 of photosynthesis-related genes (Parkinson *et al.* 2016). Therefore, it is reasonable to expect that
223 different *Symbiodinium* types would exhibit diverse sensitivities to menthol (Fig. 1, Table 1).
224 However, when PSII activities were completely shut down by menthol, responses of the
225 *Symbiodinium* types could be divided into two groups, ROS generating (*S. goreaui*, C3, and C15)
226 and non-ROS generating (*S. minutum* and *S. trenchii*). The reason for such a distinction needs to
227 be addressed in future studies.

228 Menthol and other monoterpenes, because of their lipophilic nature, are known to inhibit
229 growth of plant tissues by inducing lipid oxidation, which affects the membrane structure and
230 function (Zunino & Zygadlo 2004; Singh *et al*. 2006; Kaur *et al*. 2011). As proposed by Wang et
231 al. (2012a), the mechanism of menthol-induced bleaching might be attributable to Ca^{2+}
232 stimulated exocytosis. This has been demonstrated in previous results from transcriptomic
233 studies suggesting the disruption of calcium homeostasis in both corals and sea anemones during
234 stress-induced bleaching (De Salvo et al. 2008, Moya et al. 2012). Also, Dani et al (2016) have

proposed that menthol treatment could be responsible in inducing symbiophagy through Ca^{2+}-triggered mechanisms. Preliminary experiments conducted by Wang et al. (2012a) also suggest that menthol inhibition of *Symbiodinium* PSII activity may play a role in the expulsion of the algal cells or the digestion of the *Symbiodinium* cells by the host.

Generation of ROS in the menthol-treated clade C *Symbiodinium* might be derived from a lipid-oxidation process or a reaction of a TRPM8-like channel receptor. In *Symbiodinium* C3, ROS generation was initiated and reached a maximum within 5 min of incubation (Fig. 3B), indicating a typical ROS burst reaction as found in infected plants (Wojtaszek 1997). The production of large amounts of ROS indicates a quick defensive response to stress. As to *S. minutum*, the lack of ROS detected in the menthol-induced PSII shutdown might be attributed to cells containing higher antioxidant activities and/or a lack of a TRPM8-like channel receptor. Comparisons of antioxidant networks revealed that *S. minutum* produced more superoxide dismutase and ascorbate peroxidase than *S. goreaui* when confronting heat stress (Krueger *et al*. 2014). No ROS generation in menthol-treated *S. trenchii* might be attributed to the "stress-tolerant" nature of members of *Symbiodinium* clade D (Toller *et al*. 2001; LaJeunesse *et al*. 2008), in which ROS levels were found to remain unaffected by heat stress (McGinty *et al*. 2012).

In conclusion, activity of (TRP)M8 might not be a general phenomenon. We observed diverse responses based on *Symbiodinium* types to menthol treatment. In addition to (TRP)M8, menthol might also be inducing symbiophagy (Dani et al. 2016) in the hosts of certain *Symbiodinium* types. Although, studies (including this one) have tried to understand the mechanisms involved in loss of *Symbiodinium* cells when treated with menthol, the clarity as to what really happens is still lacking and needs to be explored in more detail in future studies.

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266 **References**

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268 Coffroth MA, Santos SR. 2005. Genetic diversity of symbiotic dinoflagellates in the genus
269 *Symbiodinium*. *Protist* 156: 19-34.

270 Dani V, Priouzeau F, Pagnotta S, Carette D, Laugier J-P, Sabourault C. 2016. Thermal and
271 menthol stress induce different cellular events during sea anemone bleaching. *Symbiosis* 69:
272 175-192.

273 Davy SK, Allemand D, Weis VM. 2012. Cell biology of cnidarian-dinoflagellate
274 symbiosis. *Microbiology and Molecular Biolology Reviews* 76: 229–261.

275 Desalvo MK, Voolstra CR, Sunagawa S, Schwarz JA, Stillman JH, Coffroth MA, Szmant AM,
276 Medina M. 2008. Differential gene expression during thermal stress and bleaching in the
277 Caribbean coral *Montastrea faveolata*. *Molecular Ecology* 17: 3952-3971.

278 Douglas AE. 2010. *The symbiotic habit*. New Jersey, Princeton University Press.

279 Grajales A, Rodríguez E. 2014. Morphological revision of the genus Aiptasia and the family
280 Aiptasiidae (Cnidaria, Actiniaria, Metridioidea). *Zootaxa* 3826: 055–100.

281 Hans M, Wilhelm M, Swandulla D. 2012. Menthol suppresses nicotinic acetylcholine receptor
282 functioning in sensory neurons via allosteric modulation. *Chemical Senses* 37: 463-469.

283 Jones RJ. 2004. Testing the 'photoinhibition' model of coral bleaching using chemical inhibitors.
284 *Marine Ecology Progress Series* 284: 133-45.

285 Jones R. 2005. The ecotoxicological effects of Photosystem II herbicides on corals. *Marine*
286 *Pollution Bulletein* 51: 495-506.

287 Jones RJ, Hoegh-Guldberg O, Larkum AWD, Schreiber U. 1998. Temperature-induced
288 bleaching of corals begins with impairment of the CO_2 fixation mechanism in
289 zooxanthellae. *Plant Cell & Environment* 21: 1219-30.

290 Kaur S, Rana S, Singh HP, Batish DN, Kohli RK. 2011. Citronellol disrupts membrane integrity
291 by inducing free radical generation. *Zeitschrift für Naturforschung C* 66: 260–266.
292 Krueger T, Becker S, Pontasch S, Dove S, Hoegh-Guldberg O, Leggat W, Fisher PL, Davy SK.
293 2014. Antioxidant plasticity and thermal sensitivity in four types of *Symbiodinium* sp.
294 *Journal of Phycology* 50: 1035–1047.
295 LaJeunesse T, Reyes-Bonilla H, Warner M, Wills M, Schmidt GW, Fitt WK. 2008. Specificity
296 and stability in high latitude eastern Pacific coral–algal symbioses. *Limnology and*
297 *Oceanography* 53: 719–727.
298 Matthews JL, Sproles AE, Oakley CA, Grossman AR, Weis VM, Davy SD. 2016. Menthol-
299 induced bleaching rapidly and effectively provides experimental aposymbiotic sea
300 anemones (*Aiptasia* sp.) for symbiosis investigations. *Journal of Experimental Biology* 219:
301 306-310.
302 McGinty ES, Pieczonka J, Mydlarz LD. 2012. Variations in reactive oxygen release and
303 antioxidant activity in multiple *Symbiodinium* types in response to elevated temperature.
304 *Microbial Ecology* 64: 1000-1007.
305 McKemy DD, Neuhausser WM, Julius D. 2002. Identification of a cold receptor reveals a
306 general role for TRP channels in thermosensation. *Nature* 416: 52-58.
307 Motulsky HJ, Christopoulos A. 2003. *Fitting models to biological data using linear and*
308 *nonlinear regression*: *A practical guide to curve fitting.* GraphPad Software Inc., San
309 Diego CA, www.grappad.com.
310 Moya A, Ganot P, Furla P, Sabourault C. 2012. The transcriptomic response to thermal stress is
311 immediate, transient and potentiated by ultraviolet radiation in the sea anemone *Anemonia*
312 *viridis*. *Molecular Ecology* 21: 1158–1174.

313 Mydlarz LD, Jacobs RS. 2004. Comparison of an inducible oxidative burst in free-living and
314 symbiotic dinoflagellates reveals properties of the pseudopterosins. *Phytochemistry* 65:
315 3231-3241.
316 Okazawa M, Terauchi T, Shiraki T, Matsumura K, Kobayashi S. 2000. l-Menthol-induced
317 [Ca^{2+}]i increase and impulses in cultured sensory neurons. *Neuroreport* 11: 2151-2155.
318 Pang ZP, Südhof TC. 2010. Cell biology of Ca2+-triggered exocytosis. *Current Opinion in Cell*
319 *Biology* 22: 496-505.
320 Parkinson JE, Baumgarten S, Michell CT, Baums IB, LaJeunesse TC, Christian R, Voolstra CR.
321 2016. Gene expression variation resolves species and individual strains among coral-
322 associated dinoflagellates within the genus *Symbiodinium*. *Genome Biology & Evolution* 8:
323 665-680.
324 Peier AM, Moqrich A, Hergarden AC, Reeve AJ, Andersson DA, Story GM, Earley TJ, Dragoni
325 I, McIntyre P, Bevan S. 2002. A TRP channel that senses cold stimuli and menthol. *Cell*
326 108: 705-715.
327 Pernice M, Dunn SR, Tonk L, Dove S, Domart-Coulon I, Hoppe P, Schintlmeister A, Wagner M,
328 Meibom A. 2015. A nanoscale secondary ion mass spectrometry study of dinoflagellate
329 functional diversity in reef-building corals. *Environmental Microbiology.* 17: 3570-3580.
330 Pochon X, Putnam HM, Gates RD. 2014. Multi-gene analysis of *Symbiodinium* dinoflagellates: a
331 perspective on rarity, symbiosis, and evolution. *PeerJ* 2: e394.
332 Robinson JD, Warner ME. 2006. Differential impacts of photoacclimation and thermal stress on
333 the photobiology of four different phylotypes of *Symbiodinium* (Pyrrhophyta). *Journal of*
334 *Phycology* 42: 568-579.
335 Rowan R. 2004. Thermal adaptation in reef coral symbionts. *Nature* 430: 742.

336 Sampayo EM, Ridgway T, Bongaerts P, Hoegh-Guldberg O. 2008. Bleaching susceptibility and
337 mortality of corals are determined by fine-scale differences in symbiont type. *Proceedings*
338 *of National Academy of Sciences of the United States of America* 105: 1444-1449.
339 Stat M, Morris E, Gates RD. 2008. Functional diversity in coral– dinoflagellate symbiosis.
340 *Proceedings of National Academy of Sciences of the United States of America* 105: 9256-
341 9261.
342 Stat M, Gates RD. 2011. Clade D Symbiodinium in scleractinian corals: a "nugget" of hope, a
343 selfish opportunist, an ominous sign, or all of the above? *Journal of Marine Biology*
344 2011:730715. doi:10.1155/2011/730715
345 Singh HP, Batish DR, Kaur S, Arora K, Kohli RK. 2006. α-Pinene inhibits growth and induces
346 oxidative stress in roots. *Annals of Botany* 98: 1261 – 1269.
347 Smaili SS, Pereira GJS, Costa MM, Rocha KK, Rodrigues L, do Carmo LG, Hirata H, Hsu Y_T.
348 2013. The role of calcium stores in apoptosis and autophagy. *Current Molecular Medicine*
349 13: 252-265.
350 Suggett DJ, Goyen S, Evenhuis C, Szabó M, Pettay DT, Warner ME, Ralph PJ. 2015. Functional
351 diversity of photobiological traits within the genus *Symbiodinium* appears to be governed by
352 the interaction of cell size with cladal designation. *New Phytologist* 208: 370–381.
353 Tchernov D, Gorbunov MY, de Vargas C, Yadav SW, Milligan AJ, Häggblom M, Falkowski PG.
354 2004. Membrane lipids of symbiotic algae are diagnostic of sensitivity to thermal bleaching
355 in corals. *Proceedings of National Academy of Sciences of the United States of America*
356 101: 13531-13535.
357 Toller W, Rowan R, Knowlton N. 2001. Zooxanthellae of the *Montastraea annularis* species
358 complex: patterns of distribution of four taxa of *Symbiodinium* on different reefs and across

359 depths. *Biological Bulletin* 201: 348–359.
360 Wang J-T, Meng P-J, Sampayo E, Tang S-L, Chen CA. 2011. Photosystem II breakdown
361 induced by reactive oxygen species in freshly-isolated *Symbiodinium* from *Montipora*
362 (Scleractinia; Acroporidae). *Marine Ecology Progress Series* 422: 51-62.
363 Wang J-T, Chen Y-Y, Tew KS, Meng P-J, Chen CA. 2012a. Physiological and biochemical
364 performances of menthol-induced aposymbiotic corals. *PLoS ONE* 7(9): e46406.
365 Wang J-T, Meng P-J, Chen Y-Y, Chen CA. 2012b. Determination of the thermal tolerance of
366 *Symbiodinium* using the activation energy for inhibiting Photosystem II activity. *Zoological*
367 *Studies* 51: 137-142.
368 Wojtaszek P. 1997. Oxidative burst: an early plant response to pathogen infection. Biochemistry
369 Journal 322: 681-692.
370 Yuyama I, Higuchi T. 2014. Comparing the effects of symbiotic algae (*Symbiodinium*) clades C1
371 and D on early growth stages of *Acropora tenuis*. *PLoSONE* 9(6): e98999.
372 Zunino MP, Zygadlo JA. 2004. Effects of monoterpenes on lipid oxidation in maize. *Planta* 219:
373 303-309.
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375
376

Figure legend

Figure 1. Dose effects of menthol and dichlorophenyl dimethylurea (DCMU) on inactivation of photosystem II (PSII) function in freshly isolated *Symbiodinium*.

The means of PSII inhibition (n=3) after 4 h of incubation in menthol (A) or DCMU (B) are plotted in the presence of various reagent concentrations.

Figure 2. Diverse reactive oxygen species (ROS) generation levels among *Symbiodinium* types when treated with artificial seawater (ASW) containing menthol. Freshly isolated *Symbiodinium* (FIS) was incubated in menthol-supplemented ASW for 4 h, followed by 2′,7′-dichlorofluorescin diacetate labeling and microscopic examination of fluorescence. Menthol concentrations used in FIS incubation were 1.73 mM for types C1 (*S. goreaui*), C3, C15, and D1a (*S. trenchii*), and 2.43 mM for B1 (*S. minutum*), which would cause complete breakdown of PSII activity in the algae. ROS signals in type C3 treated with the ASW control (A) and menthol-treated *Symbiodinium* types B1, D1a, C15, C3, and C1 (B~F) are presented with a representative photo. n=3, Scale bar in the photo represents 50 μm.

Figure 3. The time course of photosystem II (PSII) activity decline and reactive oxygen species (ROS) generation when treating the *Symbiodinium* C3 with menthol and artificial seawater (ASW). Changes in PSII activity (A) and ROS levels (B) in type C3 treated with 1.73 mM menthol were determined and plotted with incubation time. ASW-incubated congeneric *Symbiodinium* was used as a control.

399 **Table 1**. Parameters and regression coefficients of equations for dose-response curves derived
400 from menthol and dichlorophenyl dimethylurea (DCMU) (equations (1) and (2) in Material and
401 Methods) treatments on photosystem II (PSII) inhibition of different types of freshly isolated
402 *Symbiodinium*. Data are presented as mean values (n=3).
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Phylotype	Menthol					DCMU			
	a	b	x_0	y_0	r^2	a	b	x_0	r^2
B1 (*S. minutum*)	53.71	0.045	0.23	6.33	0.992	86.57	0.18	2.06	0.997
C1 (*S. goreaui*)	98.35	0.115	-0.11	8.13	0.995	72.86	0.21	1.84	0.997
C3	94.45	0.050	-0.06	6.44	0.996	74.39	0.23	1.59	0.999
C15	96.63	0.033	0.01	2.66	0.999	75.73	0.16	1.86	0.995
D1a (*S. trenchii*)	111.22	0.105	0.07	10.15	0.989	73.53	0.22	1.57	0.998

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408 **Table 2**. Values of 4-h 50% inhibitory concentration (IC_{50}) of menthol and dichlorophenyl
409 dimethylurea (DCMU) to inactivate photosystem II (PSII) activity of freshly isolated
410 *Symbiodinium* types. Data are the mean ± SD (n=3), and those with the same superscripts do not
411 significantly differ at p=0.05 (by Fisher's least significance difference test). CV indicates the
412 coefficient of variation.

Phylotype	Cnidarian host	Menthol		DCMU	
		mM	CV (%)	pM	CV (%)
B1 (*S. minutum*)	*Exaiptasia pulchella*	1.96 ± 0.13[a]	6.4	74 ± 8[a]	10.9
C1 (*S. goreaui*)	*Stylophora pistillata*	0.72 ± 0.09[b]	12.3	29 ± 3 [b]	9.8
C3	*Acropora humilis*	0.86 ± 0.02[b]	2.7	37 ± 1[b]	2.3
C15	*Porities lutea*	1.01 ± 0.01[c]	1.1	33 ± 3[b]	10.1
D1a (*S. trenchii*)	*Galaxea fasicularis*	1.07 ± 0.07[c]	6.2	52 ± 7 [b]	14.1

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427 **Table 3**. Reactive oxygen species (ROS) signals detected in menthol- or dichlorophenyl
428 dimethylurea (DCMU)-treated freshly isolated *Symbiodinium* (FIS) labeled with 2′,7′-
429 dichlorofluorescin diacetate. Detailed conditions of menthol and DCMU treatment are described
430 in Fig. 2, except that the concentration used for DCMU was 0.13 mM. ROS data are expressed
431 as the fluorescence intensity of 2',7'-dichlorofluorescein, and are presented as the mean ± SD
432 (n=3).

Phylotypes	ROS signal	
	Menthol	DCMU
	(fluorescence units cell^{-1})	
B1 (*S. minutum*)	18 ± 2	15 ± 2
C1 (*S. goreaui*)	64 ± 6	12 ± 1
C3	68 ± 9	18 ± 2
C15	58 ± 8	18 ± 1
D1a (*S. trenchii*)	35 ± 3	17 ± 2

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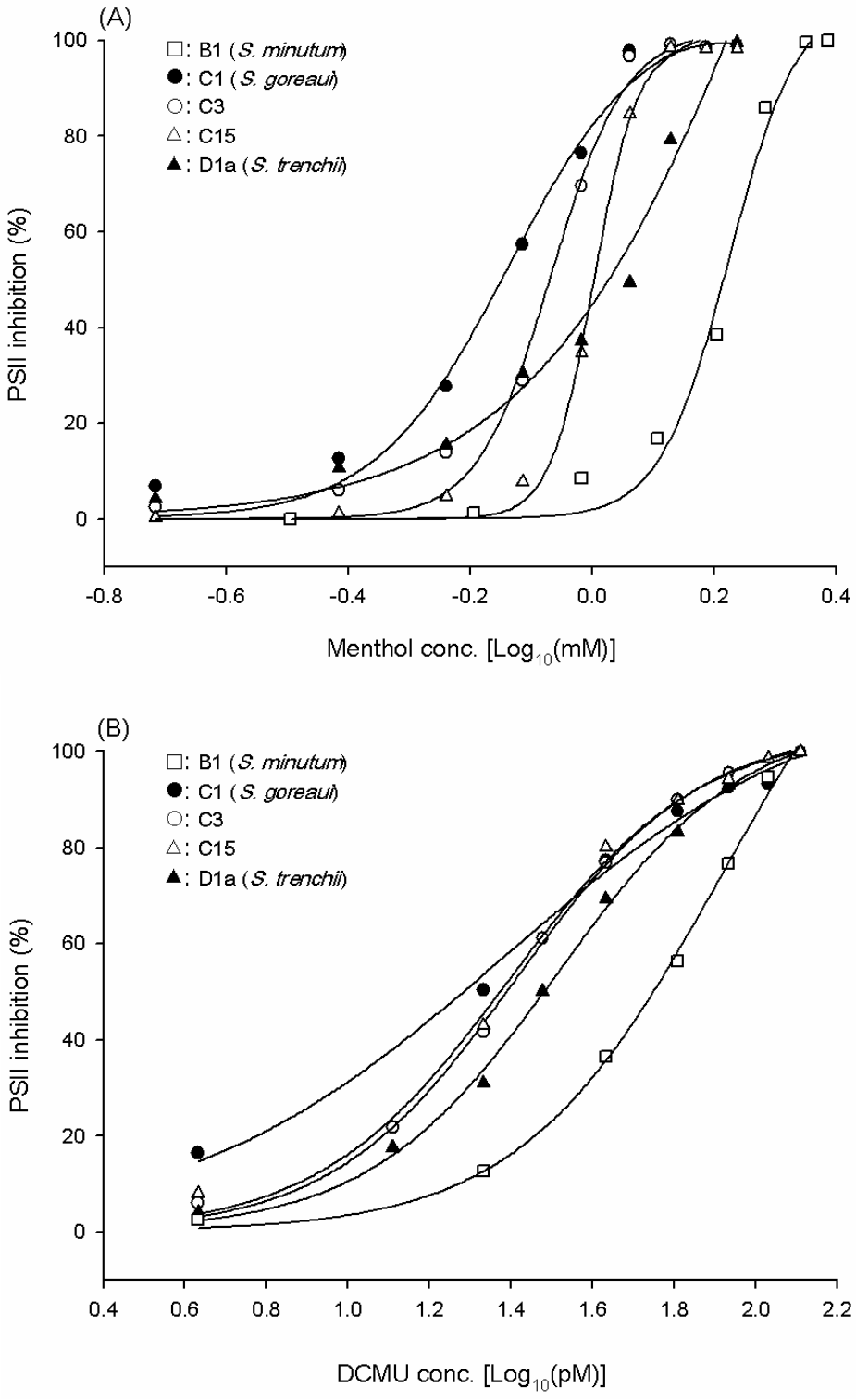

Fig. 1

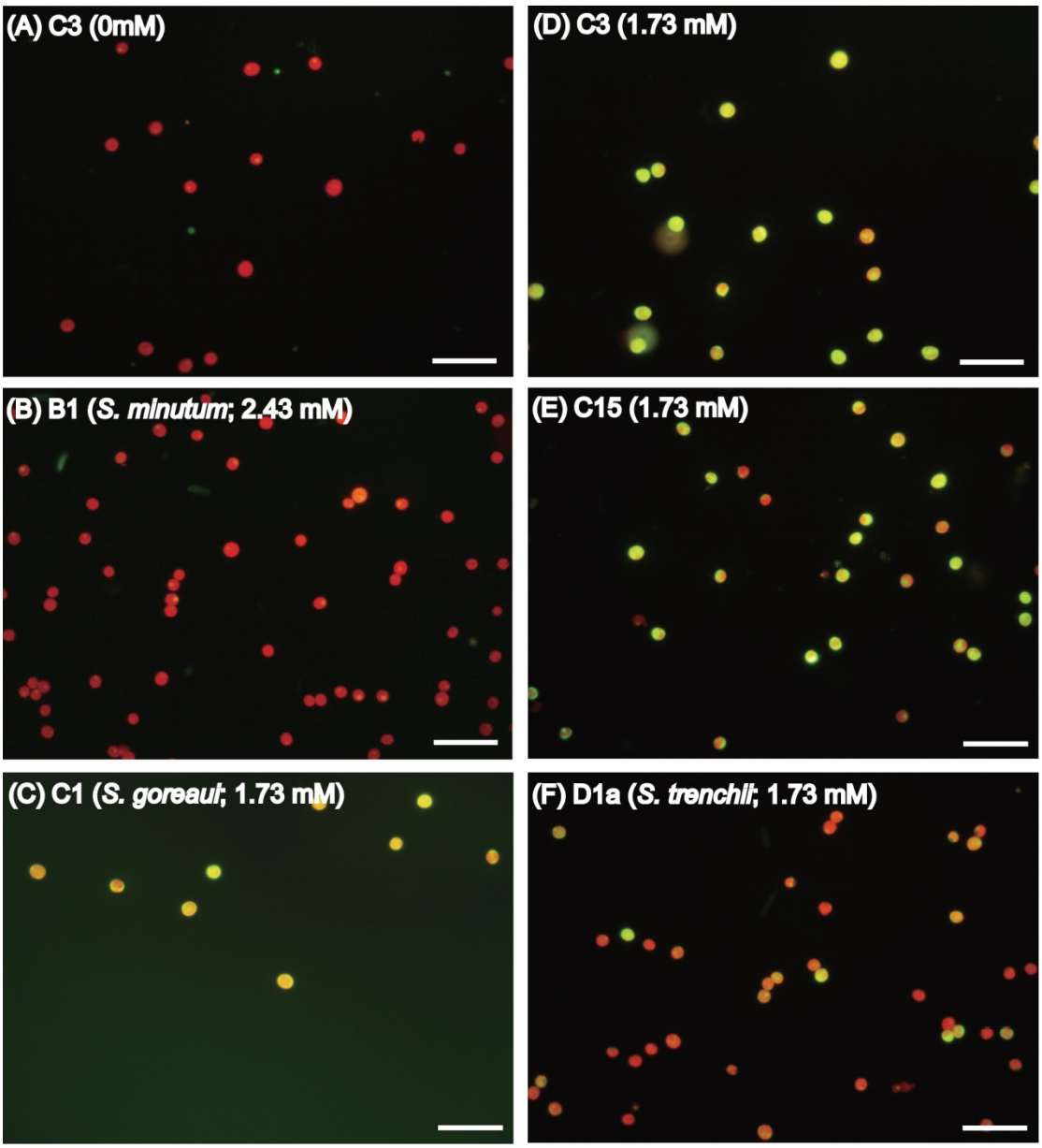
(A) C3 (0mM)
(D) C3 (1.73 mM)
(B) B1 (S. minutum; 2.43 mM)
(E) C15 (1.73 mM)
(C) C1 (S. goreaui; 1.73 mM)
(F) D1a (S. trenchii; 1.73 mM)

Fig. 2

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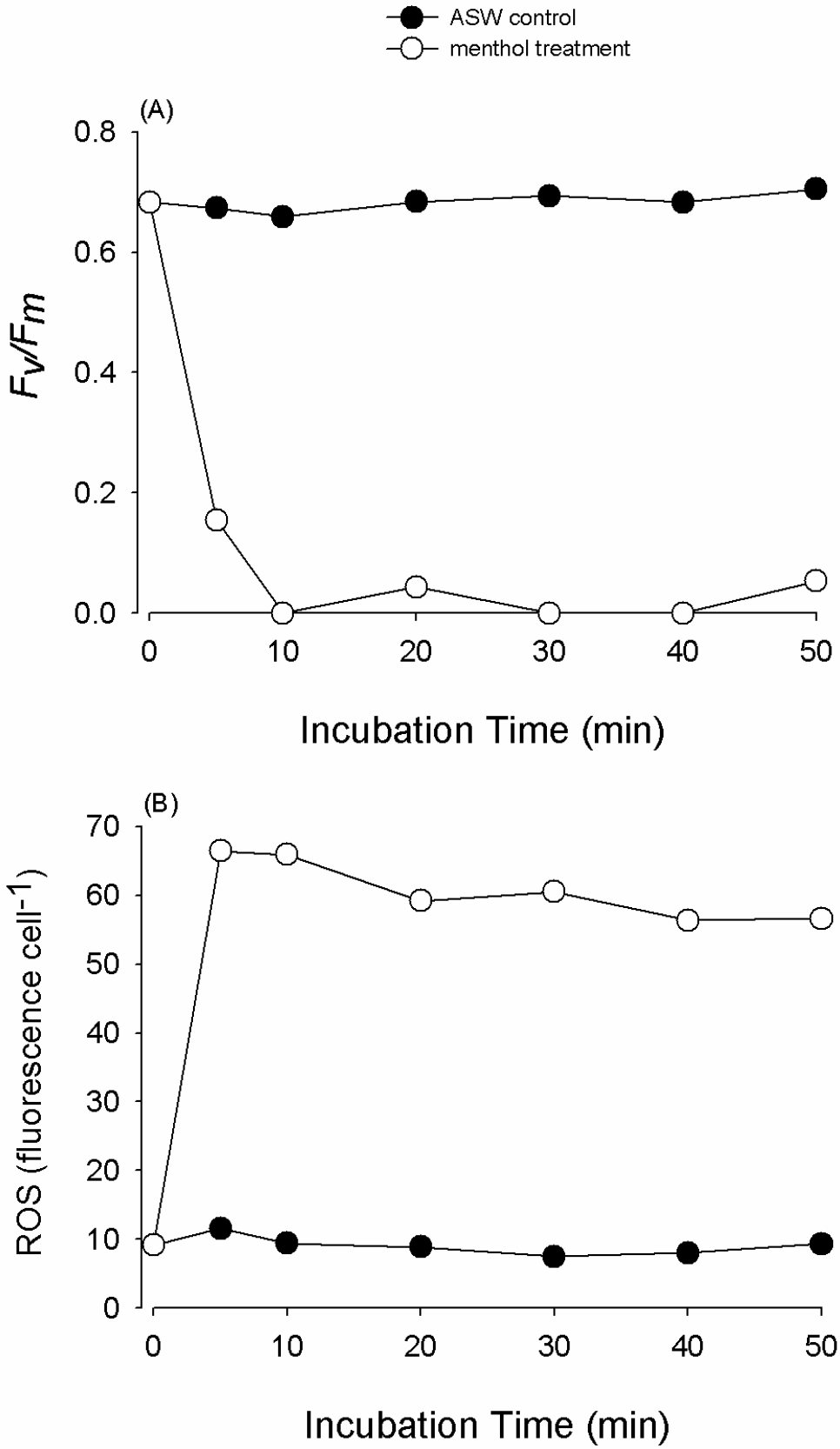

Fig. 3