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IMPACT OF MENTHOL ON GROWTH AND PHOTOSYNTHETIC FUNCTION OF *BREVIOLUM MINUTUM* (DINOFLAGELLATA, DINOPHYCEAE, SYMBIODINIACEAE) AND INTERACTIONS WITH ITS *AIPTASIA* HOST[1]

Sophie Clowez[2]

Department of Plant Biology, Carnegie Institution for Science, 260 Panama Street, Stanford, California 94305, USA

Christian Renicke, *John R. Pringle*

Department of Genetics, Stanford University, Stanford, California 94305, USA

and Arthur R. Grossman

Department of Plant Biology, Carnegie Institution for Science, 260 Panama Street, Stanford, California 94305, USA

Environmental change, including global warming and chemical pollution, can compromise cnidarian- (e.g., coral-) dinoflagellate symbioses and cause coral bleaching. Understanding the mechanisms that regulate these symbioses will inform strategies for sustaining healthy coral–reef communities. A model system for corals is the symbiosis between the sea anemone *Exaiptasia pallida* (common name *Aiptasia*) and its dinoflagellate partners (family Symbiodiniaceae). To complement existing studies of the interactions between these organisms, we examined the impact of menthol, a reagent often used to render cnidarians aposymbiotic, on the dinoflagellate *Breviolum minutum*, both in culture and in hospite. In both environments, the growth and photosynthesis of this alga were compromised at either 100 or 300 μM menthol. We observed reduction in PSII and PSI functions, the abundances of reaction-center proteins, and, at 300 μM menthol, of total cellular proteins. Interestingly, for free-living algae exposed to 100 μM menthol, an initial decline in growth, photosynthetic activities, pigmentation, and protein abundances reversed after 5–15 d, eventually approaching control levels. This behavior was observed in cells maintained in continuous light, but not in cells experiencing a light–dark regimen, suggesting that *B. minutum* can detoxify menthol or acclimate and repair damaged photosynthetic complexes in a light- and/or energy-dependent manner. Extended exposures of cultured algae to 300 μM menthol ultimately resulted in algal death. Most symbiotic anemones were also unable to survive this menthol concentration for 30 d. Additionally, cells impaired for photosynthesis by pre-treatment with 300 μM menthol exhibited reduced efficiency in re-populating the anemone host.

Key index words: **bleaching; cnidaria; *Exaiptasia pallida*; holobiont; sea anemone; *Symbiodinium*; symbiosis**

Abbreviations**: ASW, artificial sea water; Cas, enzymatic digested casein hydrolysate; ddH$_2$O, double distilled water; DTT, 1, 4-dithio-DL-threitol; FWHM, full width at half-maximum; F$_0$, minimum fluorescence (Q$_A$ fully oxidized); F$_m$, maximum fluorescence (Q$_A$ fully reduced); F$_v$, maximum variable chlorophyll fluorescence yield; F$_v$/F$_m$, maximum photochemical quantum yield of photosystem II; IMK, liquid medium of Daigo's IMK dissolved in artificial sea water; PVDF, polyvinylidene difluoride; RGB, red-green-blue color space; ROS, reactive oxygen species; TBST, Tris-buffered saline, pH 7.6 containing 0.1% (v/v) Tween 20**

The endosymbiosis between various cnidarian hosts and dinoflagellates of the former genus *Symbiodinium* (Pochon and Gates 2010, Lesser et al. 2013), now divided into different genera within the family Symbiodiniaceae (LaJeunesse et al. 2018), represents an important biotic association and serves as the foundation of coral–reef communities (Hoegh-Guldberg 1999, Weis 2008). To understand the symbiotic interactions between Symbiodiniaceae algae and their cnidarian hosts including corals (Stanley 2003), sea anemones (Trench 1993, Weis 2008, Davy et al. 2012, Voolstra 2013), and jellyfish (Freudenthal 1962, Davy et al. 2012), it is critical to develop model systems that allow for analyses of uptake, maintenance, and breakdown of the interactions between the algae and their animal partners. One such model is the sea anemone *Exaiptasia pallida* and its close relatives, collectively known as *Aiptasia* (Weis 2008, Davy et al. 2012). The advantages of this system include rapid growth under laboratory

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[2]Author for correspondence: e-mail: sclowez@carnegiescience.edu
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conditions and the ability of the sea anemones to survive in the aposymbiotic state. These features enable studies of the loss of the algae from host tissue under stress conditions, the initial interactions of different algal strains with their hosts, and events associated with colonization of the animal by the alga and its maintenance in the host tissue. We and others have been developing diverse biochemical, genetic, and cell biological approaches to facilitate the use of the cnidarian system for detailed analyses of key aspects of this cnidarian symbiotic association (e.g., Fitt and Warner 1995, Brown 1997, Douglas 2003, Lesser 2011, Xiang et al. 2020).

Several stresses can trigger the loss of algae from the cells of their cnidarian hosts. This phenomenon (often called "bleaching," but here "holobiont bleaching" to distinguish it from "algal bleaching," which is the loss of pigmentation from the algal cells themselves) is occurring at a global scale and is seriously threatening the survival of coral ecosystems (Hoegh-Guldberg et al. 2007, Hughes et al. 2018). Among the environmental factors (natural or experimentally administered) that provoke holobiont bleaching are elevated water temperatures (Belda-Baillie et al. 2002, Coffroth et al. 2010, Tolossa et al. 2011, Tolleter et al. 2013), strong UV radiation (Treignier et al. 2008), extended dark exposure (Yonge and Nicholls 1931, Schoenberg and Trench 1980, Hoegh-Guldberg and Smith 1989), and cold treatment (Steen and Muscatine 1987, Gates et al. 1992, Wang and Douglas 1999, Bieri et al. 2016). Furthermore, some chemical compounds, many considered environmental pollutants, can elicit bleaching; these include heavy metals and various pesticides (Owen et al. 2002, Råberg et al. 2003, Cantin et al. 2007, Gallen et al. 2013, Kuzminov et al. 2013, Hédouin et al. 2016), such as the commonly used herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea or DCMU (Jones 2004). Despite their negative ecological impacts, reagents that induce cnidarian bleaching can also be used to generate aposymbiotic organisms under controlled conditions, and those aposymbionts can then help elucidate the mechanisms critical for initial interactions, establishment, and maintenance of the symbiotic partnership.

The breakdown of the association between the endosymbionts and their hosts has previously been linked to compromised algal photosynthesis, although no direct causal relationship has been established (Kleppel et al. 1989, Porter et al. 1989, Fitt and Warner 1995, Jones et al. 1998, Warner et al. 1999, Takahashi et al. 2004, Tchernov et al. 2004, Perez and Weis 2006, Venn et al. 2006). Indeed, it has been difficult to determine whether impaired photosynthetic activity directly elicits algal expulsion from host tissue. For example, DCMU, a specific inhibitor of photosystem II (PSII) that blocks light-dependent, linear electron transport and photophosphorylation via direct association with a quinone binding site in PSII reaction centers, has been employed to study the effects of the inhibition of photosynthesis on symbiosis (Vandermeulen et al. 1972, Shick and Dykens 1984). However, DCMU also more generally impacts intracellular redox conditions (Joliot and Joliot 2002, Joliot et al. 2004), making interpretation of the results difficult.

Another chemical that can be used to elicit holobiont bleaching is menthol (Wang et al. 2012, Matthews et al. 2016, Wang et al. 2017), a cyclic terpene alcohol produced by some plants that shares the first several reactions of its biosynthetic pathway with that of carotenoids (Kirby and Keasling 2009, Kamatou et al. 2013). Menthol is well-known to impact sensory pathways in vertebrates by acting as a local anesthetic through activation of thermal receptors localized to the tips of sensory neurons (Haeseler et al. 2002, McKemy et al. 2002, Peier et al. 2002). Specifically, it binds to the menthol-responsive transient receptor potential melastatin 8 (TRPM8), which confers the sensation of cold (McKemy et al. 2002, Peier et al. 2002), and can interfere with neuronal signaling by altering Ca^{2+} levels (Okazawa et al. 2000, Tsuzuki et al. 2004). Interestingly, menthol also has a long history of use as an anesthetic for coral or sea anemone polyps (Duerden 1904, Waugh 1936, McCloskey 1970, Steele and Goreau 1977, Janes 2008) and has been evaluated for its effects on other aquatic organisms such as fish and clams (Norton et al. 1996, Kasai et al. 2014, Sykes and Wilson 2015). Due to its widespread application in agri-food and pharmaceutical products, menthol might enter the marine ecosystem, especially after insufficient treatment of wastewater (Richardson and Bowron 1985, Levine et al. 2006, Aleksandra et al. 2012), although studies are needed to systematically test the impacts of ecologically relevant menthol concentrations on marine life.

The experimental applications of menthol for anesthesia and holobiont bleaching make it vital to understand its effects both on the individual partners in the cnidarian–dinoflagellate symbiosis and on their interactions. Initial studies on the effects of menthol on coral samples and cultured endosymbiotic algae indicated that it causes a loss of algal PSII activity in hospite and has various other strain-specific effects (Wang et al. 2012, 2017). These experiments have focused mainly either on host responses or on the cultured, isolated algae. Yet, there is still little known about the specific processes in the algae that are impacted during menthol-induced bleaching of the holobiont.

In this report, we examine the ways in which menthol impacts growth, photosynthesis, and survival of the Symbiodiniaceae *Breviolum minutum* strain SSB01 (designated in the text as SSB01; Xiang et al. 2013), a facultative endosymbiont that can grow in culture both photoautotrophically and heterotrophically (Xiang et al. 2017). Moreover, we examined how menthol impacts the interactions of SSB01 with a compatible *Aiptasia* host with respect to both colonization by, and maintenance of, the algal endosymbiont.

MATERIALS AND METHODS

Symbiodiniaceae strain and growth conditions. We chose the clonal, axenic *Breviolum minutum* strain SSB01 (Xiang et al. 2013, LaJeunesse et al. 2018) for this study to specifically investigate the direct responses of *B. minutum* to menthol and to eliminate potential effects (e.g., detoxification, metabolic conversions) caused by microbes present in xenic cultures. Stock axenic SSB01 cultures were grown in artificial sea water (ASW), made by mixing Coral Pro Salt (Red Sea) with deionized water to a concentration of 33.5, supplemented with Daigo's IMK and 0.4% (w/v) casein hydrolysate at 27°C (Xiang et al. 2013) without agitation and under 12:12 h light:dark diel illumination (10 μmol photons · m^{-2} · s^{-1} from Philips ALTO II 25 W bulbs, hereafter 12:12 h L:D). All manipulations of the cultures were performed following rigorous microbiological practices in a laminar flow cell-culture hood in a positively pressurized "algal growth" room. Both stock and experimental cultures were frequently checked microscopically for health and/or any microbial contamination and regularly tested for the cp23S genotype of the Symbiodiniaceae by Sanger sequencing (Xiang et al. 2013). Contamination could be detected visually as the microbes would overgrow the algae in the nutrient-rich, casein hydrolysate-supplemented IMK medium. They also would become apparent during microscopic examination, which was routinely performed, as well as by plating the cells on solid rich medium (Difco Marine Broth). The cultures were checked less frequently for microbial contamination by 16S-specific PCR (Xiang et al. 2013). Cell numbers in the cultures were quantified using a hemocytometer (Countess II FL Hemocytometer, Life Technologies).

Colonization of Aiptasia *and assessment of algal cell numbers in hospite.* Clonal aposymbiotic *Aiptasia* strain H2 sea anemones (Xiang et al. 2013) were used for colonization studies. Anemones were maintained in ASW at 25–27°C under 12:12 h L:D diel illumination (20–30 μmol photons · m^{-2} · s^{-1}), as described previously (Xiang et al. 2013). After a 10-d exposure of SSB01 cultures to various menthol concentrations (see below) in continuous light, 8 × 10^6 cells in 1 mL were added to 200 mL (4 × 10^4 cells · mL^{-1} final concentration) of menthol-free ASW harboring 10 aposymbiotic sea anemones. After incubation for 24 h, the medium was changed to algal-free ASW without menthol. Anemones were then maintained as above with a change in the ASW medium once per week and no feeding over the course of the experiment.

Algal populations in the hosts were monitored using a Leica MZ16 FA fluorescence stereomicroscope with images captured by a Leica DFC7000T RGB-CCD camera; individual symbiotic algae within the anemones were detected based on their red chlorophyll fluorescence upon blue-light excitation (Leica GFP Plus filter set; excitation at 480/40 nm), with the detected emission selected by a long-pass filter that blocks wavelengths < 510 nm. To quantify algal cell numbers in hospite, anemones that had been stored at –20°C in 500 μL of 0.01% (w/v) SDS in ddH_2O/Milli-Q water were homogenized, and the numbers of algae were determined using a Guava easyCyte HT 2 flow cytometer and InCyte v2.7 software (Millipore) as described previously (Krediet et al. 2015); algal numbers were then normalized to total soluble host protein content as determined using a Pierce BCA assay (Thermo Scientific) as described previously (Krediet et al. 2015).

Menthol treatments. For menthol treatment of free-living algae in culture, stock cultures were diluted and grown for 20 d to a density of ~ 10^6 cells · mL^{-1}, collected by centrifugation at 400*g* for 5 min at 27°C, and resuspended to a density of 10^5 cells · mL^{-1} in 50 mL of IMK medium supplemented with menthol (M2772, Sigma-Aldrich) from a 20% (w/v) stock that was dissolved in ethanol (Wang et al. 2012, Matthews et al. 2016) to the final menthol concentrations indicated in the text (30, 100, or 300 μM). The cultures were then grown either in continuous light (as above) or on a 12:12 h L:D regimen at 27°C. Control cultures were treated identically, except that ethanol without the menthol was added to the culture. The ethanol concentrations used (always less than 0.2%) had no detectable effect on algal growth or photosynthesis.

For menthol treatment of holobionts, SSB01-populated *Aiptasia*, strain H2 anemones were grown in 250 mL ASW either without (control) or with various concentrations of menthol (as above) added from the 20% stock solution. The ASW medium and menthol were changed every 2 d, and the sea anemones were fed once per week with freshly hatched *Artemia* nauplii in plain ASW for 4 h and then placed back in ASW containing menthol.

Assessment of cell viability. Aliquots of the cultured algae that had been grown for 15 d in the absence or presence of menthol were spread onto Difco Marine Broth agar (37.4 g · L^{-1} in water) supplemented with 28 mM glucose, maintained under a 12:12 h L:D regimen, and imaged after 2 weeks to determine the numbers of colonies formed. Viabilities are presented as the number of colonies on a plate divided by the total number of cells plated and then normalized to the number of colonies that grew on control plates (no menthol treatment and the same number of cells spread on the plate).

Assessment of photosynthetic function. Cultured cells grown at various menthol concentrations were collected by centrifugation at 400*g* for 5 min; replicate 50 mL cultures were used for each time point. To measure photosynthetic activity, algae were resuspended to ~ 10^7 cells · mL^{-1} in ASW containing 20% (w/v) Ficoll 400 (to slow cell settling). Spectroscopic measurements of PSII maximum quantum yield (Baker 2008) and PSI (P_{700}) activity (Joliot and Joliot 2005) were performed on whole cells using a JTS-10 spectrophotometer equipped with LED light sources (Bio-Logic). The maximal PSII quantum yield was measured after 20 min of dark acclimation according to the formula $F_v/F_m = (F_m–F_0)/F_m$, where F_m values were determined using a 250 ms saturating light flash of 2250 μmol photons · m^{-2} · s^{-1}. Prior to measuring PSI (P_{700}) activity, cells were incubated for 15 min in the dark with 20 μM DCMU to inhibit PSII. Samples were subjected to 10 s of excitation with 156 μmol photons · m^{-2} · s^{-1} illumination provided by orange LEDs (630 nm) followed by a saturating pulse (200 ms at 705 nm) to fully oxidize P_{700} and then a period of dark relaxation. Over the time course, absorbance changes were measured at 705 nm and corrected for nonspecific absorbance at 735 nm (i.e., P700 oxidation/reduction = $\Delta I/I$ 705 nm – $\Delta I/I$ 735 nm; Takahashi et al. 2013).

For photosynthetic measurements from algae in hospite, similar procedures were used with six replicate samples, each comprised of a single symbiotic anemone. The anemones were placed in the cuvettes and allowed to relax for 15 min in the dark before measurements were begun.

Immunodetection of proteins involved in photosynthetic functions. Samples of cultured algae were normalized for cell number and then centrifuged at 400*g* for 5 min, the supernatants were removed, and the cells were resuspended and boiled (1 min) in an SDS-PAGE extraction buffer (3 mM HEPES-KOH, pH 7.5, 60 mM DTT, 60 mM Na_2CO_3, 12% [w/v] sucrose, 2% [w/v] SDS). Solubilized polypeptides were resolved by SDS-PAGE in 10% polyacrylamide gels and then transferred from the gels to PVDF membranes (GE Healthcare) using a semidry blotting apparatus (Bio-Rad) at 0.8 mA $\cdot$ cm^{-2} for 45 min. Blotted membranes were blocked for 1 h in Tris-buffered saline, pH 7.6, containing 0.1% (v/v) Tween 20 (TBST) and 5% (w/v) powdered non-fat dry milk at room temperature, and then incubated overnight at 4°C with rabbit anti-α-tubulin (polyclonal; Agrisera AS10 680), rabbit anti-PsbA (polyclonal; Agrisera AS05 084), or rabbit anti-PsaB (polyclonal; Agrisera AS10 695) primary antibodies. Immunoreactive proteins on the membranes were detected by a chemiluminescence assay catalyzed by a horseradish peroxidase–linked goat anti-rabbit IgG secondary antibody (Agrisera AS09 602) after a 1 h incubation at room temperature with the secondary antibody in TBST containing 5% (w/v) powdered non-fat dry milk. After immunodetection, the membranes were incubated for 1 min with 0.1% (w/v) Coomassie Brilliant Blue G-250 in 50% (v/v) methanol and 10% (v/v) acetic acid; after removal of excess liquid, the gels were air-dried to visualize the protein profiles.

RESULTS

Impact of menthol on growth and pigmentation of cultured strain SSB01. To explore the impact of various menthol concentrations on the growth and pigmentation of SSB01 cells, we exposed algal cultures to 30, 100, or 300 μM menthol in continuous light and monitored cell densities and chlorophyll levels; menthol concentrations above 300 μM (e.g., 600 μM) were lethal within a few days of exposure (data not shown). Cells exposed to 30 μM menthol grew nearly as fast as the control cells and showed little or no reduction in pigmentation (Fig. 1, A and B), whereas cells exposed to 100 or 300 μM menthol exhibited significant growth reductions and loss of pigmentation starting by day 5 (Fig. 1, A and B). Interestingly, the cultures exposed to 100 μM menthol appeared to recover partially after longer periods of treatment (30 d), whereas the cultures exposed to 300 μM menthol showed no such recovery. Growth inhibition and loss of pigmentation in algal cells maintained in 300 μM menthol under continuous light were accompanied by a significant decline in the pH of the culture medium from 8.9 to 7.6 after 2 d and to 6.2 after 15 d (Fig. S1 in the Supporting Information). For 100 μM menthol, an intermediate pH change was observed that appeared to stabilize between 7.4 and 7.9. These differences in pH may reflect changes in the health and/or metabolism of the cultures, as addition of menthol in the absence of algae had no effect on the pH of the medium. We also assayed the viability of SSB01 cells following exposure to menthol for 15 d under continuous light. There was no detectable viability loss in 30 μM menthol, but 100 and 300 μM menthol caused decreases in viability to 84% and 78%, respectively (Fig. S2 in the Supporting Information).

The impact of menthol on SSB01 cells maintained on a diel regimen of 12:12 h L:D was also examined. In either the absence or presence of 30 μM menthol, the growth rate appeared slightly reduced relative to that in continuous light, but only the differences in cell densities between the two light regimens in the control at 15 d and in 30 μM menthol for 10, 15, and 30 d were statistically significant (Fig. 1, B and C and Table S1 in the Supporting Information). In 100 or 300 μM menthol, growth stopped after 10 d, and no recovery was observed in these cultures at later time points (Fig. 1C), unlike what was seen in 100 μM menthol under continuous light (Fig. 1, B and C).

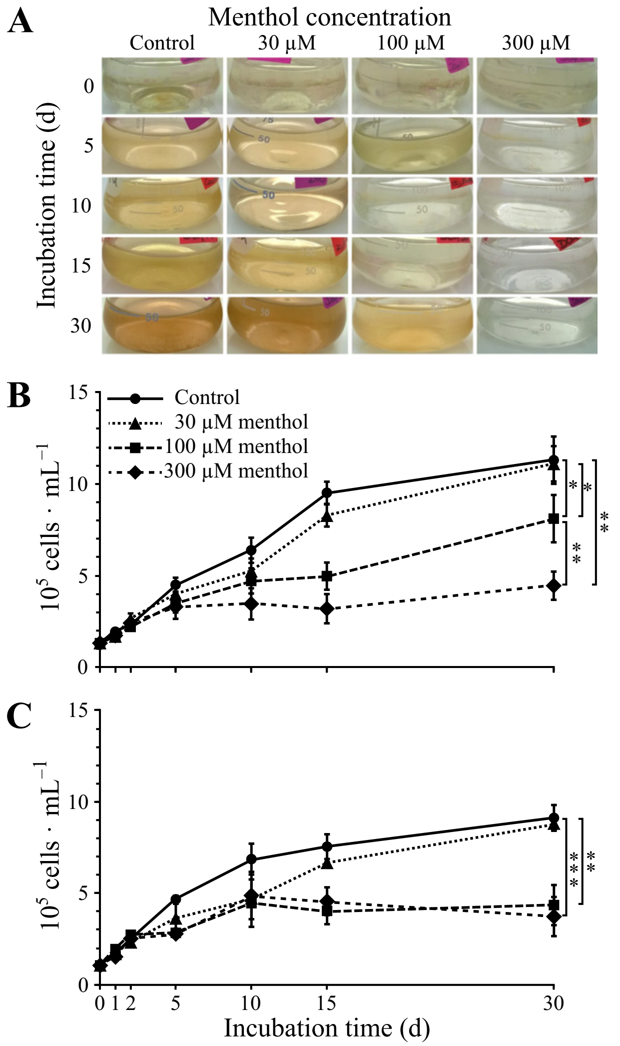

Fig. 1. Impact of menthol on growth and pigmentation of strain SSB01 in culture. Menthol was added to the medium to the indicated concentrations, and cultures were examined over 30 d of growth. (A) Images showing pigmentation of cell cultures and (B) changes in the cell densities of those cultures during growth under continuous light. (C) Changes in cell numbers during growth under a 12:12 h L:D regimen. In (B) and (C), means ± SEMs (n = 6) are shown, and statistical significances of the indicated differences in unpaired, two-tailed Student's t tests are shown for 30 d (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$); see Table S1 for detailed analysis.

The impact of menthol on chlorophyll levels in SSB01 cells cultured in continuous light was assessed by flow cytometry (Fig. 2). Within 5 d of exposure to menthol, a large proportion of SSB01 cells dropped below the normal lower limit of red chlorophyll fluorescence (broken line) at all concentrations of menthol tested. In 30 or 100 μM menthol, considerable recovery was observed by 15 or 30 d, respectively, but in 300 μM menthol, no recovery was observed and nearly all cells exhibited chlorophyll fluorescence that was well below the normal lower threshold (Fig. 2). These observations are consistent with the changes in pigmentation observed visually in the cultures (Fig. 1A).

Effect of menthol on PSI and PSII activities. Because bleaching of holobiont corals exposed to menthol has been associated with altered photosynthetic function (see Introduction), we examined the impact of menthol on photosynthetic activity in cultured SSB01 cells. In continuous light, 30 μM menthol had little effect on PSII maximal efficiency (F_v/F_m; Fig. 3A) or PSI activity (oxidation and reduction of P_{700}; Fig. 3, B–E), but 100 and 300 μM menthol elicited strong decreases in both parameters during the first 5 d of exposure (Fig. 3, A and B and Fig. S3 in the Supporting Information). In 100 μM menthol, both PSI and PSII activities began to recover by 10 d of menthol exposure, reaching control levels after 30 d of treatment; no such recovery was observed for cells in 300 μM menthol (Fig. 3, A–E).

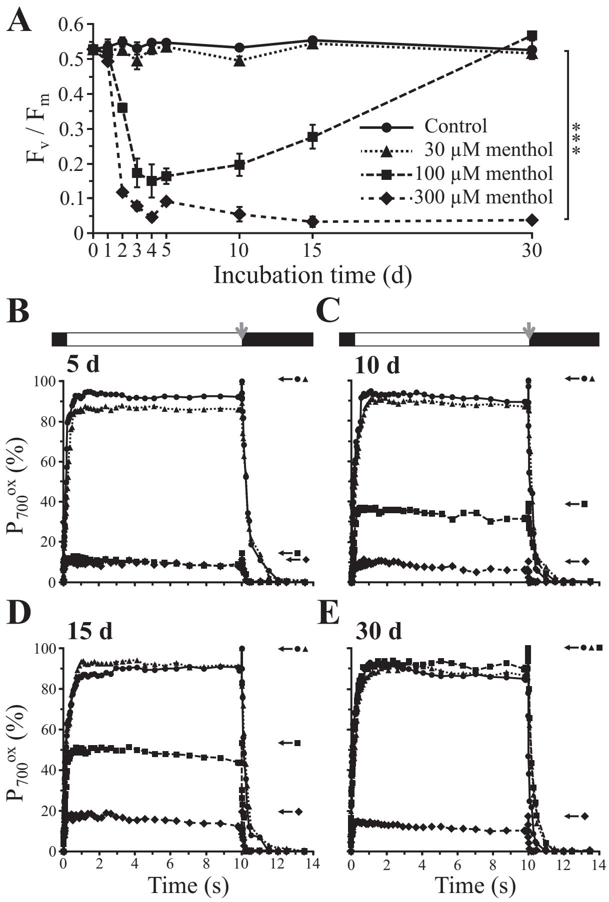

Fig. 3. Changes in PSII and PSI activities during growth of SSB01 in the presence of menthol in continuous light. Algal cultures from the experiment shown in Fig. 1, A and B were analyzed for (A) changes in PSII maximum efficiency based on F_v/F_m measurements and (B–E) changes in PSI activities in the light (white bar on top) based on oxidation–reduction kinetics of P_{700} (see Materials and Methods). In (A) means ± SEMs (n = 6) are shown, and the statistical significance of the difference between the control and 300 μM menthol in an unpaired, two-tailed Student's t test is shown for 30 d (***$P \leq 0.001$); see Table S1 for detailed analysis. In (B–E), the kinetics shown are averages of six independent biological replicates. Menthol concentrations are indicated by the same symbols given in (A). Bars above the plots showing PSI activity (B–E) depict the end of the initial dark incubation (blackened bar), 10 s of continuous illumination with actinic light (open bar), followed by a saturating light pulse (downward arrow; to induce maximum P_{700} oxidation), and finally a 4 s dark recovery phase (blackened bar). Black arrows on the sides of the plots mark the total oxidizable P_{700} levels, with the menthol concentrations indicated by the same symbols as used in (A).

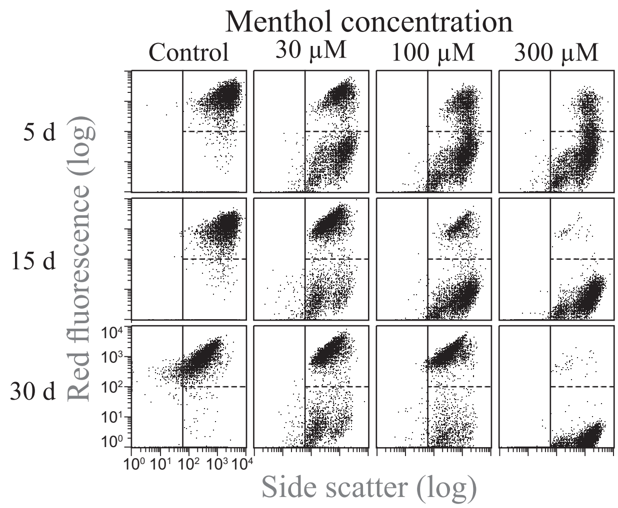

Fig. 2. Changes in chlorophyll fluorescence during growth of SSB01 in the presence of menthol in continuous light. Algal cultures from the experiment shown in Figure 1, A and B, were analyzed by flow cytometry for chlorophyll fluorescence (ordinate) and side scatter (abscissa; Krediet et al. 2015). Hatched lines indicate the lower limit of chlorophyll fluorescence for unbleached SSB01 cells, and the solid vertical line indicates the lower limit for the side scatter obtained from healthy cultures during exponential growth. The axis scales are identical for all plots (as labeled for the lower left plot).

When the menthol-treated SSB01 cells were grown on a 12:12 h L:D regimen, treatment with 30 μM menthol resulted in a reduction in F_v/F_m of ~ 0.1 compared to the control cells at 2 and 5 d, after which F_v/F_m recovered (exceeding control levels by 30 d; Fig. 4A), but had no prominent effect on PSI activity (Fig. 4B). In contrast, 100 or 300 μM menthol strongly inhibited both PSII and PSI activities, with no substantial recovery of either, even after 30 d (Fig. 4, A–E).

Losses of PSI and PSII core proteins correlated with the changes in photosynthetic activity. We examined the levels of PSI (represented by PsaB) and PSII (represented by PsbA) proteins in cells grown for various times in either the absence or presence of menthol under continuous light. We sought to use α-tubulin as a loading control but noted a strong reduction in the amount of α-tubulin in the cells maintained in 100 μM menthol at days 5 and 15 and in 300 μM menthol at days 5, 15, and 30 (Fig. 5). To assess whether this effect was specific or if general protein homeostasis was impacted by menthol, we used Coomassie Brilliant Blue to stain the total proteins transferred to the membrane. The decline in total protein levels was similar to that of α-tubulin (Fig. 5), even though cell viability was still ~ 80% after 15 d in either 100 or 300 μM menthol (Fig. S2), suggesting that the higher menthol concentrations had a global impact on general protein accumulation in the cells. For PsaB, under all conditions, there were changes in protein abundance that approximately resembled those of total proteins (Fig. 5). The losses in PsaB and total protein by 5 d in cells grown in 100 or 300 μM menthol paralleled the reduction of photosynthetic function (compare Fig. 5 with Fig. 3). Furthermore, the initial losses of total proteins and PsaB in 100 μM menthol were partially reversed by 15 d and fully so by 30 d (and maybe somewhat higher than at 0 d), again correlating with changes in photosynthetic activities (compare Fig. 5 with Fig. 3). In contrast, in 300 μM menthol, both PsaB and total protein levels continued to drop and were barely detectable by 30 d. Based on microscopy, by 30 d in 300 μM menthol, most of the cells were distorted and potentially lysed (as judged by their mostly transparent appearance), even though we were still able to count them (data not shown).

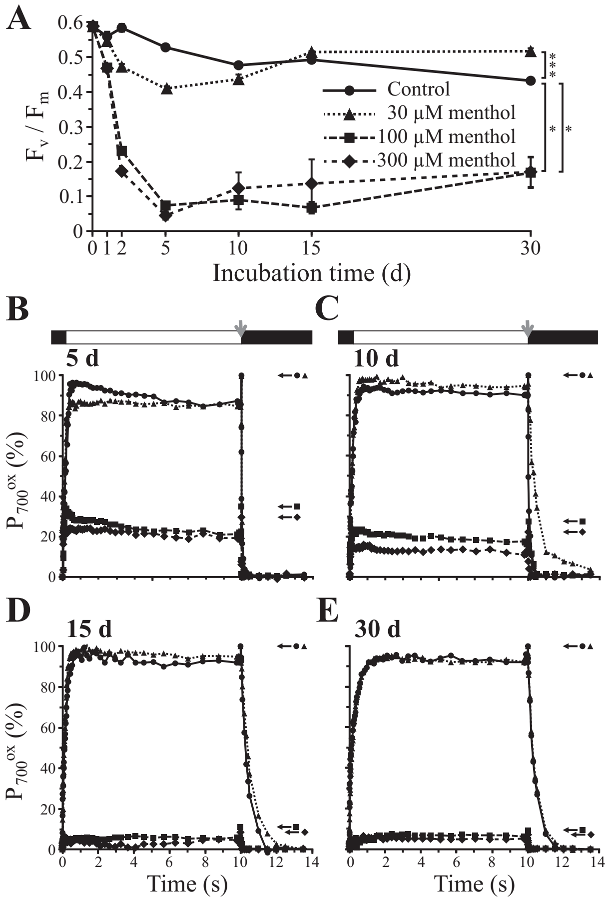

FIG. 4. Changes in PSII and PSI activities during growth of SSB01 cells in the presence of menthol under a 12:12 h L:D regimen. Algal cultures from the experiment shown in Figure 1C were analyzed as described in the legend for Figure 3. In (A) means ± SEMs of six replicates are shown except for 30 d at 100 μM and 15 and 30 d at 300 μM, where n = 4 (of n = 6, the cells in two of the cultures appeared dead). Statistical significances of the differences between measurements for the indicated samples in unpaired, two-tailed Student's t tests are shown for 30 d (*$P \leq 0.05$, ***$P \leq 0.001$); see Table S1 for detailed analysis. In (B–E), the kinetics shown are averages of six independent biological replicates, except for 30 d at 100 μM and 15 and 30 d at 300 μM, where n = 4.

The behavior of PsbA differed from that of PsaB. PsbA levels generally declined over the entire time course even when the cells were cultured in 0 or 30 μM menthol (Fig. 5). At 100 μM menthol, the abundance of PsbA diminished at 5 d, but then increased by 15 d and eventually decreased again by 30 d to a level slightly higher than that observed for untreated cells at the same time point. At 300 μM menthol, a decline and then a transient increase were observed, similar to those in 100 μM menthol, with the notable exception that after 30 d, we were unable to detect any PsbA protein, similar to the results with other individual proteins and with total protein (Fig. 5); as mentioned above, the cells were distorted and may have lysed by this time.

Loss of algae and photosynthetic function in menthol-treated, SSB01-containing sea anemones. To explore the effects of menthol on SSB01 cells living as

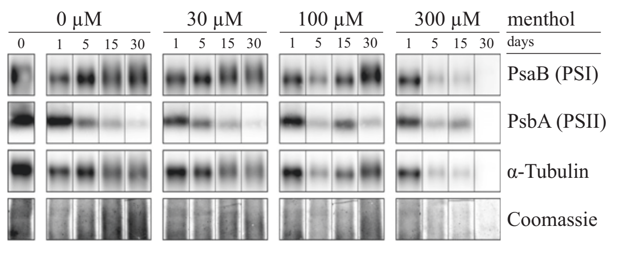

FIG. 5. Effect of menthol on abundances of PSI and PSII core subunits PsaB and PsbA, α-tubulin, and total cellular proteins in cultured SSB01 under continuous light. Immunodetection of PsaB, PsbA, and α-tubulin over a 30 d growth period in medium supplemented with menthol to the indicated concentrations. The same numbers of cells were sampled for all time points and conditions. Coomassie Brilliant Blue staining of the membranes was used to assess global changes in total protein levels. Note that the single bands/lanes were cropped from images of the full membranes and re-ordered for presentation.

endosymbionts, we subjected strain H2 polyps that were stably populated by SSB01 to various menthol concentrations. In one experiment, the sea anemones were examined at intervals using both fluorescence (Fig. 6A) and dark-field (Fig. S4 in the Supporting Information) microscopy. In a parallel experiment under identical conditions, anemones were sacrificed at intervals, and the numbers of endosymbiotic algae were determined by flow cytometry (Fig. 6B). Both approaches showed that 30 μM menthol had no significant effect on the algal numbers in hospite relative to the controls without menthol, and both fluorescence microscopy (Fig. 6A) and flow cytometry (data not shown) indicated that their chlorophyll fluorescence was not impacted. Moreover, some algal cell proliferation occurred in both these and control sea anemones over the first 6 d of the experiment. In contrast, exposure to either 100 or 300 μM menthol led to a rapid loss of algae from the hosts during the first 6 d, and the algal populations did not recover (Figs. 6B, S4). After 15 d at 300 μM menthol, the animals displayed severe tentacle retraction and shrinkage (Fig. S4), and by day 30, most were dead.

We also examined PSII function of SSB01 cells in hospite during exposure of the anemones to menthol and compared the results to those obtained in parallel for SSB01 cultured cells under identical light conditions (Fig. 7). The maximum PSII efficiency was about 8% higher in the untreated cultured cells compared to the cells in hospite (0.61 vs. 0.53), and both remained high over the 30 d incubation period in the absence of menthol or in the presence of 30 μM menthol (Fig. 7 and Fig. S5 in the Supporting Information). In contrast, after 5 d in 100 μM menthol or 3 d in 300 μM menthol, F_v/F_m for the algae in hospite dropped markedly. The algae in culture showed a strong decrease in F_v/F_m after 3 d in either 100 or 300 μM menthol, although the decline in 300 μM menthol was less pronounced than that of the algae in hospite. By 5 d, F_v/F_m was drastically reduced under either of these concentrations, with no significant differences in PSII efficiencies between cultured and in hospite algae.

Colonization of aposymbiotic sea anemones by SSB01 cells pre-treated with menthol. Because treatment of cultured SSB01 cells with 100 or 300 μM menthol led to a pronounced loss of chlorophyll and photosynthetic function, we assessed whether these changes altered the ability of the algae to colonize a host. We incubated aposymbiotic anemones for 24 h with either untreated algae or algae that had been pre-treated with menthol for 10 d (see Fig. 3 for changes in PSI and PSII activities associated with these treatments of the algae) and monitored colonization by fluorescence microscopy. Pre-treatment with 30 μM menthol had no detectable effect, but pre-treatment with 100 μM menthol markedly slowed colonization (Fig. 8; 15 d), with full colonization only apparent later. Moreover, pre-treatment with 300 μM menthol severely inhibited colonization during the first 15 d, and even at 30 d, only four of the 10 anemones were detectably colonized, and the algal density in these four was very low (Fig. 8).

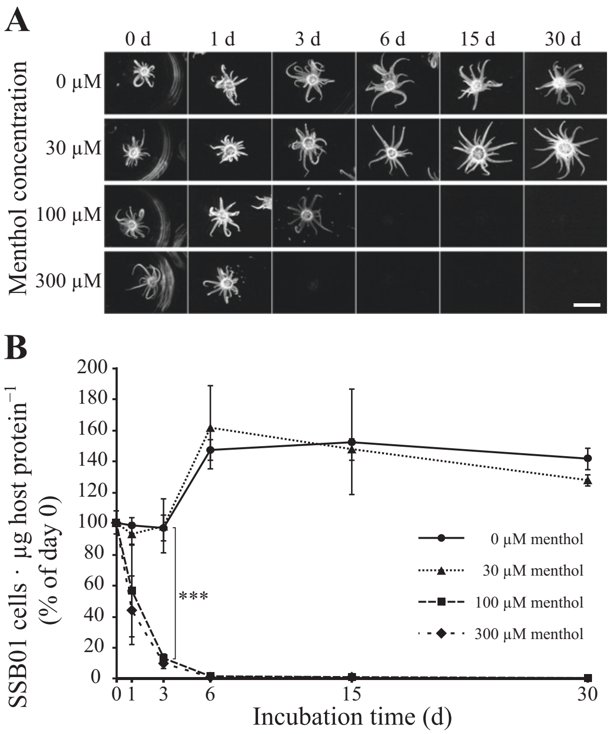

FIG. 6. Loss of algae from sea anemone hosts during exposure to menthol. Anemones of strain H2 containing SSB01 algae were exposed to the indicated concentrations of menthol in ASW, and changes in the numbers of algae in the host tissue were monitored by fluorescence stereomicroscopy (A) or (in a separate experiment) determined by flow cytometry (B; see Materials and Methods). (A) Images show only fluorescence recorded by the red camera channel (chlorophyll fluorescence) and were recorded and adjusted identically. Scale bar, 5 mm. (B) Shown are means ± SEMs for algal counts relative to total host protein on six (0 and 3 d) or three (all other time points) animals, normalized to the 0 d value. The statistical significance of the difference between the control and the sample treated with 100 μM menthol is shown for the 3 d samples (unpaired, two-tailed Student's t test; ***$P \leq 0.001$; see Table S1 for detailed analysis). No data are shown for 300 μM menthol at 30 d because all of the animals had died.

DISCUSSION

Our understanding of coral biology should benefit greatly from the availability of a versatile and accessible model system for cnidarian symbiosis, such as that provided by *Aiptasia* in combination with different strains of Symbiodiniaceae. This system facilitates rigorous examination of the cnidarian and its dinoflagellate symbionts, and, importantly, also allows studies of the partner organisms independently (Weis 2008, Davy et al. 2012).

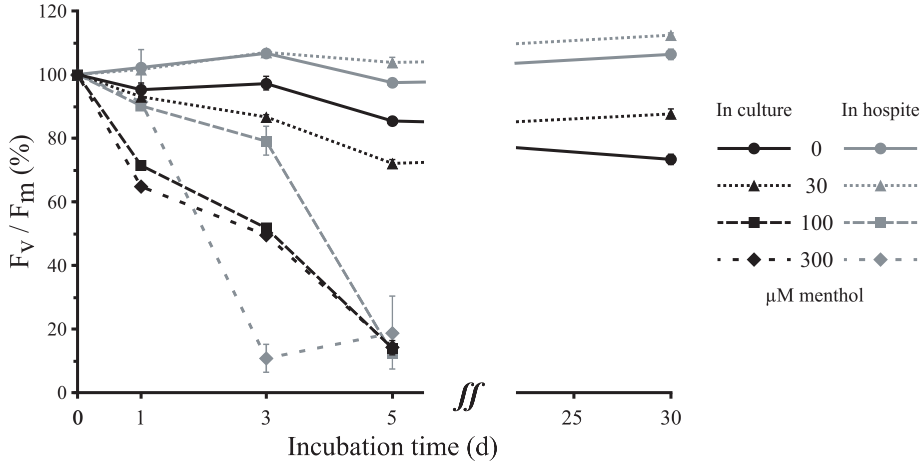

FIG. 7. Changes in PSII activity during exposure of symbiotic sea anemones to menthol. Strain H2 anemones containing SSB01 algae were exposed to the indicated concentrations of menthol on a 12:12 h L:D regimen. In a parallel experiment, SSB01 cells were cultured in IMK medium under the same conditions. The changes in maximal PSII efficiencies (based on F_v/F_m measurements) were determined at the indicated times and compared between the in hospite and cultured algae. The data are given as percentages of the day 0 control values (0.53 in hospite, 0.63 in culture). A break in the graph is indicated between 5 d and 25 d. Means ± SEMs (n = 6) are shown for both anemones and cultures. Absolute values are shown in Figure S5.

Furthermore, the ability to generate and maintain aposymbiotic *Aiptasia* facilitates the exploration of host–symbiont interactions through re-population of the sea anemone with both native and non-native algal strains (Hambleton et al. 2014, Matthews et al. 2018, Rädecker et al. 2018, Gabay et al. 2019, Tivey et al. 2020, Xiang et al. 2020). One effective method to elicit algal loss from both coral and sea anemone hosts, and thus establish aposymbiotic animals, is through the administration of menthol (Wang et al. 2012, 2017, Matthews et al. 2016).

In this study, we focused on the impact of menthol on *Breviolum minutum* strain SSB01, either grown as a free-living organism in culture or maintained in hospite within *Aiptasia*. At a concentration of 30 µM menthol, the lowest concentration used, in continuous light, there was little effect on the cultured algae except for a slight reduction in pigment/chlorophyll content (Figs. 1, A and B, and 2). In contrast, in the cultures exposed to 300 µM menthol, the highest concentration used, there was a dramatic impact over a 30 d period that resulted in an almost complete loss of pigmentation and photosynthetic functions and a marked decline in the levels of total proteins, including PsaB and PsbA; this concentration of menthol was toxic to the algae over the 30 d growth period (Figs. 1, A and B, 2, 3, 5, S2; and data not shown). Exposure of the cultures to 100 µM menthol had a detectable but less pronounced impact: By 5 d, there was a loss of pigmentation, decreased PSI and PSII activities, and a reduction in both total protein and the individual proteins analyzed (Figs. 1, 2, 3 and 5); by 15 d, there was also a slight effect on cell viability (Fig. S2). Interestingly, the cells treated with 100 µM menthol showed recovery of protein levels at 15 d in continuous light, with the PsbA abundance even exceeding that of the control (Fig. 5). This recovery was sustained over the 30 d growth period and was congruent with the reversal of the negative effects of 100 µM menthol on photosynthetic activities and pigmentation (Figs. 1, A and B, 2 and 3).

Interpretation of the variations in the abundance of the PSII reaction-center protein PsbA at the different menthol concentrations is complicated because the variations may reflect multiple factors. First, there is a menthol-independent, steady decrease in PsbA levels as the cultures become denser over time (note the changes at 0 µM menthol). Such a shift in the stoichiometry of PSI and PSII and in PsbA abundance frequently occurs in photosynthetic organisms in response to changes in light intensity (e.g., as the density of algal cultures changes; Murakami et al. 1997, Tullberg et al. 2000, Trebitsh and Danon 2001, Mulo et al. 2012, Sharpe et al. 2012, Einbinder et al. 2016). This general acclimation response is also reflected by an increase in F_0/F_m in untreated cells, mostly due to a decrease in F_m, which is potentially a consequence of quenching of chlorophyll fluorescence (Fig. S6 in the Supporting Information). Second, the toxicity of 100 or 300 µM menthol accelerated the decline within the first 5 d. Third, the cells appeared to develop a tolerance to menthol, resulting in an increase in PsbA levels between 5 and 15 d, slightly surpassing the levels in the untreated cells (although the F_v/F_m was still significantly depressed in the presence of menthol; see Fig. 3). Finally, PsbA levels dropped again by 30 d at 100 µM menthol, which is probably a consequence of both the acclimation response and the establishment of

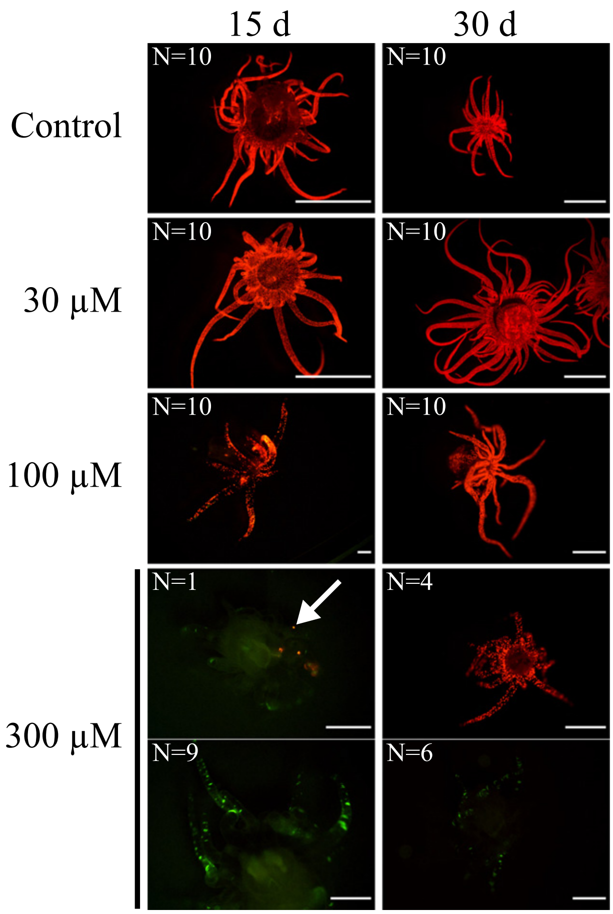

FIG. 8. Colonization of sea anemones by SSB01 cells pre-treated with menthol. SSB01 cells were grown for 10 d in continuous light in the indicated concentrations of menthol and then incubated with aposymbiotic anemones of strain H2 for 24 h. The anemones were then washed and grown for an additional 14 or 29 d, and algal chlorophyll was then visualized by fluorescence microscopy. Images are merges of the red (chlorophyll fluorescence) and green (unknown fluorescing species from the host) camera channels at varying acquisition settings. The animals shown are representative of 10 individuals examined per treatment. The white arrow marks the presence of a few algae in the tissue of one anemone among the 10 in this group.

menthol tolerance; at this point, F_v/F_m was similar to that of the control cells and those treated with 30 μM menthol (Fig. 3). In contrast, 300 μM menthol exceeded the concentration that the algal cells could tolerate. Although the cells maintained a visible outline for 30 d, they appeared potentially lysed, in agreement with the marked loss of detectable protein and pigmentation (see Figs. 1A and 2).

Our initial experiments on cultured cells were performed in continuous light, but we also examined the impact of menthol on cells maintained under a 12:12 h L:D regimen to better compare the results in culture with those observed in hospite (the symbiotic sea anemones were maintained on a 12:12 h L:D regimen). Unlike cultures in continuous light, those maintained under the light–dark regimen were unable to recover photosynthetic activity in medium containing 100 μM menthol (Fig. 4). Even 30 μM menthol caused a decrease in PSII efficiency in these cultures during the first 10 d, although we observed no impact on PSI activity. These results suggest that sustaining a continuous light input throughout the menthol treatment may facilitate detoxification/degradation of the menthol by the algae or allow the cells to acclimate and attain a state of high menthol tolerance.

Menthol is known for its antibiotic properties against a variety of bacteria and fungi (Pattnaik et al. 1997, Işcan et al. 2002, Trombetta et al. 2005, Soković et al. 2009), but it has also been shown to be toxic to some invertebrate animals (Erler and Tunç 2005, Samarasekera et al. 2008). Due to its hydrophobicity, menthol can insert into the lipid bilayers of biological membranes, change their physicochemical properties, and disrupt their integrity (Sikkema et al. 1994, Trombetta et al. 2005, Turina et al. 2006). Similarly, menthol might disrupt vital, membrane-dependent photosynthetic reactions by compromising thylakoid membrane structure. On the other hand, some microorganisms are not only resistant to high concentrations of menthol but are also able to use it as their sole carbon source during growth and metabolize it to carbon dioxide (Williams and Trudgill 1994, Harder and Probian 1995, Foss and Harder 1998). The menthol-dependent pH changes observed in the media in which the algae were grown might reflect menthol detoxification and/or its metabolic breakdown by the algae but could also be an indirect result of an effect on various metabolic processes in the cells including photosynthesis, fermentation, or even cell lysis. Attempts to assess the menthol concentrations in the growth medium after 30 d of algal growth using UV-Vis spectroscopy and a colorimetric assay (Safronova et al. 1982) were unsuccessful, probably because the concentrations of menthol used in this study were below the detection limits of the assays. In animal and human studies, menthol was shown to be eliminated from the system mainly by glucuronosylation and/or hydroxylation followed by excretion of the respective products (Madyastha and Srivatsan 1988, Yamaguchi et al. 1994, Gelal et al. 1999, Miyazawa et al. 1999, 2011). In plant cell cultures, biotransformation of menthol to products that have a higher solubility in water might reduce its toxicity (Suga et al. 1987, Furuya et al. 1989). It is possible that *Breviolum minutum* can use similar mechanisms for detoxification of menthol if provided with sufficient energy/ATP and reductant through photosynthesis. Another route for detoxification of menthol may directly involve photosynthetic electron transport and the generation of ROS, which could potentially react with menthol and result in less toxic products. These possibilities could account for the differential menthol sensitivity of the algae in continuous light compared to a light–dark regimen. However, the experiments conducted to date do not allow direct conclusions about the underlying mechanisms for menthol tolerance in SSB01. Importantly, by using axenic cultures we can conclude that the observed menthol tolerance was directly related to algal cellular functions and not caused by metabolic processes of any associated microbes, as would be possible if xenic algal cultures were used. Yet, the effects of menthol on Symbiodiniaceae in the context of their microbiomes (as found in nature) could be an interesting aspect for future studies.

The cnidarian–dinoflagellate mutualism is complex and relies on the controlled exchange of nutrients between the partner organisms (Muscatine 1990, Davy et al. 2012, Matthews et al. 2018), which probably demands marked physiologic accommodations. Changes in photosynthetic activity elicited in the endosymbiotic algae by menthol may result in suboptimal interactions, aberrant metabolite exchange, and/or potential accumulation of ROS (e.g., Lesser and Farrell 2004, Schieber and Chandel 2014, Matthews et al. 2016). The finding that exposure to 300 μM menthol for 30 d impacts total protein content, cell integrity, and algal as well as host viability suggests that high levels of menthol can directly or indirectly disrupt critical cellular processes such as protein synthesis, especially during an extended exposure to the compound, which could ultimately result in the death of both the algae and the animal.

The loss of Symbiodiniaceae algae from host tissue has been associated with impaired PSII function (Warner et al. 1999, Hill et al. 2004, Tolleter et al. 2013). However, in the presence of 100 or 300 μM menthol, we observed a marked decrease in the SSB01 population in hospite even after 1 d (Fig. 6B), although PSII activity remained relatively high at this time (Figs. 7 and Figure S5). Interestingly, residence in the host tissue may have provided some short-term protection against the effects of 100 or 300 μM menthol, as the decline in PSII function appeared to be somewhat delayed compared to that in the cultured algae. Moreover, colonization of aposymbiotic sea anemones by SSB01 cells pre-treated with 100 or 300 μM menthol showed that even when PSI and PSII functions of the cultured algae were impaired (Fig. 3C), the algae were still able to populate the host (Fig. 8), although less effectively. Taken together, our results suggest that full photosynthetic function may not be required for the maintenance of the association or initial colonization of the host, although it might favor more rapid colonization and higher final cell density within the host.

In this study, we have shown that menthol elicits a loss of pigmentation in cultured SSB01 cells, has inhibitory effects on both PSI and PSII activities, and can impact protein abundances and algal cell viability, especially at 300 μM. Surprisingly, the algae can abrogate the impact of an intermediate menthol concentration (100 μM) when maintained in continuous light. Furthermore, although menthol also causes a decrease in photosynthetic activity in the algae in hospite, this reduction does not correlate closely with the loss of algae from the host tissue. The results of this initial analysis of how menthol affects the complex interactions between a model cnidarian and its dinoflagellate symbiont suggest that menthol may impact a number of different biological and/or metabolic processes, which eventually leads to a decline in endosymbiont photosynthetic activity and to holobiont bleaching. Furthermore, the information provided here should allow researchers to make more informed decisions concerning the concentrations and duration of incubation with menthol that would be required to generate aposymbiotic organisms and to select the most suitable parameters to monitor when examining the impact of menthol on the physiology of the endosymbiotic alga.

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Aleksandra, J., Meritxell, G., Mira, P., Antoni, G. & Damia, B. 2012. Occurrence and elimination of pharmaceuticals during conventional wastewater treatment. *In* Guasch, H., Ginebreda, A. & Geiszinger, A. [Eds.] *The Handbook of Environmental Chemistry*. Springer, Berlin, Heidelberg, 19:1–23.

Baker, N. R. 2008. Chlorophyll fluorescence: A probe of photosynthesis in vivo. *Annu. Rev. Plant. Biol.* 59:89–113.

Belda-Baillie, C. A., Baillie, B. K. & Maruyama, T. 2002. Specificity of a model cnidarian-dinoflagellate symbiosis. *Biol. Bull.* 202:74–85.

Bieri, T., Onishi, M., Xiang, T., Grossman, A. R. & Pringle, J. R. 2016. Relative contributions of various cellular mechanisms to loss of algae during cnidarian bleaching. *PLoS ONE* 11: e0152693.

Brown, B. 1997. Coral bleaching: Causes and consequences. *Coral Reefs* 16:129–38.

Cantin, N. E., Negri, A. P. & Willis, B. L. 2007. Photoinhibition from chronic herbicide exposure reduces reproductive output of reef-building corals. *Mar. Ecol. Progr. Ser.* 344:81–93.

Coffroth, M. A., Poland, D. M., Petrou, E. L., Brazeau, D. A. & Holmberg, J. C. 2010. Environmental symbiont acquisition may not be the solution to warming seas for reef-building corals. *PLoS ONE* 5:e13258.

Davy, S. K., Allemand, D. & Weis, V. M. 2012. Cell biology of cnidarian-dinoflagellate symbiosis. *Microbiol. Mol. Biol. Rev.* 76:229–61.

Douglas, A. 2003. Coral bleaching-How and why? *Mar. Pollut. Bull.* 46:385–92.

Duerden, J. E. 1904. The coral *Siderastrea radians* and its postlarval development. Monograph Series: Carnegie Institution of Washington Publication No. 20, Washington, D.C., 130 pp. plus figures.

Einbinder, S., Gruber, D. F., Salomon, E., Liran, O., Keren, N. & Tchernov, D. 2016. Novel adaptative photosynthetic characteristics of mesophotic symbiotic microalgae within the reef-building coral, *Stylophora pistillata*. *Front. Mar. Sci.* 3:1–9.

Erler, F. & Tunç, I. 2005. Monoterpenoids as fumigants against greenhouse pests: Toxic, development and reproduction-inhibiting effects. *Z. Pflanzenkr. Pflanzenschutz.* 112:181–92.

Fitt, W. & Warner, M. 1995. Bleaching patterns of four species of Caribbean reefs corals. *Biol. Bull.* 189:298–307.

Foss, S. & Harder, J. 1998. *Thauera linaloolentis* sp. nov. and *Thauera terpenica* sp. nov., isolated on oxygen-containing monoterpenes (linalool, menthol, and eucalyptol) and nitrate. *Syst. Appl. Microbiol.* 21:365–73.

Freudenthal, H. D. 1962. *Symbiodinium* gen. nov. and *Symbiodinium microadriaticum* sp. nov., a zooxanthella: Taxonomy, life cycle, and morphology. *J. Protozool.* 9:45–52.

Furuya, T., Orihara, Y. & Miyatake, H. 1989. Biotransformation of (–)-menthol by *Eucalyptus perriniana* cultured cells. *J. Chem. Soc. Perkin Trans.* 53:1711–9.

Gabay, Y., Parkinson, J. E., Wilkinson, S. P., Weis, V. M. & Davy, S. K. 2019. Inter-partner specificity limits the acquisition of thermotolerant symbionts in a model cnidarian-dinoflagellate symbiosis. *ISME J.* 13:2489–99.

Gallen, C., Devlin, M., Paxman, C., Banks, A. & Mueller, J. 2013. Pesticide monitoring in inshore waters of the Great Barrier Reef using both time-integrated and event monitoring techniques (2012 - 2013). The University of Queensland, The National Research Centre for Environmental Toxicology (Entox), pp. 1-94.

Gates, R. D., Baghdasarian, G. & Muscatine, L. 1992. Temperature stress causes host cell detachment in symbiotic cnidarians: Implications for coral bleaching. *Biol. Bull.* 182:324–32.

Gelal, A., Jacob, P., Yu, L. & Benowitz, N. L. 1999. Disposition kinetics and effects of menthol. *Clin. Pharmacol. Ther.* 65:145.

Haeseler, G., Maue, D., Grosskreutz, J., Bufler, J., Nentwig, B., Piepenbrock, S., Dengler, R. & Leuwer, M. 2002. Voltage-dependent block of neuronal and skeletal muscle sodium channels by thymol and menthol. *Eur. J. Anaesth.* 19:571–79.

Hambleton, E. A., Guse, A. & Pringle, J. R. 2014. Similar specificities of symbiont uptake by adults and larvae in an anemone model system for coral biology. *J. Exp. Biol.* 217:1613–9.

Harder, J. & Probian, C. 1995. Microbial degradation of monoterpenes in the absence of molecular oxygen. *Appl. Environ. Microbiol.* 61:3804–8.

Hédouin, L. S., Wolf, R. E., Phillips, J. & Gates, R. D. 2016. Improving the ecological relevance of toxicity tests on scleractinian corals: Influence of season, life stage, and seawater temperature. *Environ. Pollut.* 213:240–53.

Hill, R., Larkum, A. W. D., Frankart, C., Kuhl, M. & Ralph, P. J. 2004. Loss of functional photosystem II reaction centers in zooxanthellae of corals using fluorescence rise kinetics. *Photosynth. Res.* 82:59–72.

Hoegh-Guldberg, O. 1999. Climate change, coral bleaching and the future of the world's coral reefs. *Mar. Freshwater. Res.* 50:839–66.

Hoegh-Guldberg, O., Mumby, P. J., Hooten, A. J., Steneck, R. S., Greenfield, P., Harvell, C. D., Sale, P. F. et al. 2007. Coral reefs under rapid climate change and ocean acidification. *Science* 318:1737–42.

Hoegh-Guldberg, O. & Smith, G. J. 1989. The effect of sudden changes in temperature, light and salinity on the population density and export of zooxanthellae from the reef corals *Stylophora pistillata* Esper and *Seriatopora hystrix* Dana. *J. Exp. Mar. Biol. Ecol.* 129:279–303.

Hughes, T. P., Anderson, K. D., Conolly, S. R., Heron, S. F., Kerry, J. T., Lough, J. M., Baird, A. H. et al. 2018. Spatial and temporal patterns of mass bleaching of corals in the Anthropocene. *Science* 359:80–83.

Işcan, G., Kirimer, N., Kürkcüoğlu, M., Başer, K. H. C. & Demirci, F. 2002. Antimicrobial screening of *Mentha piperita* essential oils. *J. Agric. Food Chem.* 50:3943–6.

Janes, M. P. 2008. Laboratory methods for the identification of soft corals (Octocorallia: Alcyonacea). *Adv. Coral Husband. Public Aqua.* 2:413–26.

Joliot, P., Beal, D. & Joliot, A. 2004. Cyclic electron flow under saturating excitation of dark-adapted *Arabidopsis* leaves. *Biochim. Biophys. Acta.* 1656:166–76.

Joliot, P. & Joliot, A. 2002. Cyclic electron transfer in plant leaf. *Proc. Natl. Acad. Sci. USA* 99:10209–14.

Joliot, P. & Joliot, A. 2005. Quantification of cyclic linear flows in plants. *Proc. Natl. Acad. Sci. USA* 102:4913–18.

Jones, R. J. 2004. Testing the 'photoinhibition' model of coral bleaching using chemical inhibitors. *Mar. Ecol. Progr. Ser.* 284:133–45.

Jones, R., Hoegh-Guidberg, O., Larkum, A. & Schreiber, U. 1998. Temperature-induced bleaching of corals begins with impairment of the CO_2 fixation mechanism in zooxanthellae. *Plant Cell Environ.* 21:1219–30.

Kamatou, G. P. P., Vermaak, I., Viljoen, A. M. & Lawrence, B. M. 2013. Menthol: A simple monoterpene with remarkable biological properties. *Phytochemistry* 96:15–25.

Kasai, M., Hososhima, S. & Yun-Fei, L. 2014. Menthol induces surgical anesthesia and rapid movement in fishes. *Open Neurosci. J.* 8:1–8.

Kirby, J. & Keasling, J. D. 2009. Biosynthesis of plant isoprenoids: Perspectives for microbial engineering. *Annu. Rev. Plant. Biol.* 60:335–55.

Kleppel, G. S., Dodge, R. E. & Reese, C. J. 1989. Changes in pigmentation with the bleaching of stony corals. *Limnol. Oceanogr.* 34:1331–35.

Krediet, C. J., DeNofrio, J. C., Caruso, C., Burriesci, M. S., Cella, K. & Pringle, J. R. 2015. Rapid, precise, and accurate counts of *Symbiodinium* cells using the Guava Flow Cytometer, and a comparison to other methods. *PLoS ONE* 10:e0135725.

Kuzminov, F. I., Brown, C. M., Fadeev, V. V. & Gorbunov, M. Y. 2013. Effects of metal toxicity on photosynthetic processes in coral symbionts, *Symbiodinium* spp. *J. Exp. Mar. Biol. Ecol.* 446:216–27.

LaJeunesse, T. C., Parkinson, J. E., Gabrielson, P. W., Jeong, H. J., Reimer, J. D., Voolstra, C. R. & Santos, S. R. 2018. Systematic revision of Symbiodiniaceae highlights the antiquity and diversity of coral endosymbionts. *Curr. Biol.* 16:2570–80.

Lesser, M. P. 2011. Coral bleaching: Causes and mechanisms. *In* Dubinsky, Z. & Stambler, N. [Eds.] *Coral Reefs: An Ecosystem in Transition.* Springer, Dordrecht, pp 405–19.

Lesser, M. P. & Farrell, J. H. 2004. Exposure to solar radiation increases damage to both host tissues and algal symbionts of corals during thermal stress. *Coral Reefs* 23:367–77.

Lesser, M., Stat, M. & Gates, R. D. 2013. The endosymbiotic dinoflagellates (*Symbiodinium* sp.) of corals are parasites and mutualists. *Coral Reefs* 32:603–11.

Levine, A. D., Meyer, M. T. & Kish, G. 2006. Evaluation of the persistence of micropollutants through pure-oxygen activated sludge nitrification and denitrification. *Water Environ. Res.* 78:2276–85.

Madyastha, K. M. & Srivatsan, V. 1988. Studies on the metabolism of l-menthol in rats. *Drug Metab. Dispos.* 16:765–72.

Matthews, J. L., Oakley, C. A., Lutz, A., Hillyer, K. E., Roessner, U., Grossman, A. R., Weis, V. M. & Davy, S. K. 2018. Partner switching and metabolic flux in a model cnidarian–dinoflagellate symbiosis. *Proc. Roy. Soc. B-Biol. Sci.* 285:20182336.

Matthews, J. L., Sproles, A. E., Oakley, C. A., Grossman, A. R., Weis, V. M. & Davy, S. K. 2016. Menthol-induced bleaching rapidly and effectively provides experimental aposymbiotic sea anemones (*Aiptasia* sp.) for symbiosis investigations. *J. Exp. Biol.* 219:306–10.

McCloskey, L. R. 1970. The dynamics of the community associated with a marine scleractinian coral. *Int. Rev. Ges. Hydrobio.* 55:13–81.

McKemy, D. D., Neuhausser, W. M. & Julius, D. 2002. Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature* 416:52–58.

Miyazawa, M., Kumagae, S. & Kameoka, H. 1999. Biotransformation of (+)- and (–)-menthol by the larvae of common cutworm (*Spodoptera litura*). *J. Agric. Food Chem.* 47:3938–40.

Miyazawa, M., Marumoto, S., Takahashi, T., Nakahashi, H., Haigou, R. & Nakanishi, K. 2011. Metabolism of (+)- and (–)-menthols by CYP2A6 in human liver microsomes. *J. Oleo Sci.* 60:127–32.

Mulo, P., Sakurai, I. & Aro, E. M. 2012. Strategies for *psbA* gene expression in cyanobacteria, green algae and higher plants: From transcription to PSII repair. *Biochim. Biophys. Acta.* 1817:247–57.

Murakami, A., Fujita, Y., Nemson, J. A. & Melis, A. 1997. Chromatic regulation in *Chlamydomonas reinhardtii*: Time course of photosystem stoichiometry adjustment following a shift in growth light quality. *Plant Cell Physiol.* 38:188–93.

Muscatine, L. 1990. The role of symbiotic algae in carbon and energy flux in reef corals. *Coral Reefs* 25:75–87.

Norton, J. H., Dashorst, M., Lansky, T. M. & Mayer, R. J. 1996. An evaluation of some relaxants for use with pearl oysters. *Aquaculture* 144:39–52.

Okazawa, M., Terauchi, T., Shiraki, T., Matsumura, K. & Kobayashi, S. 2000. *l*-Menthol-induced $[Ca^{2+}]_i$ increase and impulses in cultures sensory neurons. *NeuroReport* 11:2151–55.

Owen, R., Knapa, A., Toaspern, M. & Carbery, K. 2002. Inhibition of coral photosynthesis by the antifouling herbicide irgarol 1051. *Mar. Pollut. Bull.* 44:32.

Pattnaik, S., Subramanyam, V. R., Bapaji, M. & Kole, C. R. 1997. Antibacterial and antifungal activity of aromatic constituents of essential oils. *Microbios* 89:39–46.

Peier, A. M., Moqrich, A., Hergarden, A. C., Reeve, A. J., Anderson, D. A., Story, G. M., Earley, T. J., Dragoni, I., McIntyre, P., Bevan, S. & Patapoutain, A. 2002. A TRP channel that senses cold stimuli and menthol. *Cell* 108:705–15.

Perez, S. & Weis, V. M. 2006. Nitric oxide and cnidarian bleaching: an eviction notice mediates the breakdown of symbiosis. *J. Exp. Biol.* 209:2804–10.

Pochon, X. & Gates, R. D. 2010. A new *Symbiodinium* clade (Dinophyceae) from sotitid foraminifera in Hawaii. *Mol. Phylogenet. Evol.* 56:492–7.

Porter, J. W., Fitt, W. K., Spero, H. J., Rogers, C. S. & White, M. W. 1989. Bleaching in reef corals: Physiological and stable isotopic responses. *Proc. Natl. Acad. Sci. USA* 86:9342–6.

Råberg, S., Nyström, M., Erös, M. & Plantman, P. 2003. Impact of the herbicides 2, 4-D and diuron on the metabolism of the coral *Porites cylindrica*. *Mar. Environ. Res.* 56:503–14.

Rädecker, N., Raina, J. B., Pernice, M., Perna, G., Guagliardo, P., Kilburn, M. R., Aranda, M. & Voolstra, C. 2018. Using *Aiptasia* as a model to study metabolic interactions in Cnidarian-Symbiodinium symbioses. *Front Physiol.* 9:214.

Richardson, M. L. & Bowron, J. M. 1985. The fate of pharmaceutical chemicals in the aquatic environment. *J. Pharm. Pharmacol.* 37:1–12.

Safronova, T. O., Popov, D. M. & Shevchenko, T. G. 1982. Photocolorimetric determination of menthol in oil and tincture of peppermint. *Pharm. Chem. J.* 16:68–70.

Samarasekera, R., Weerasinghe, I. S. & Hemalal, K. P. 2008. Insecticidal activity of menthol derivatives against mosquitoes. *Pest Manag. Sci.* 64:290–5.

Schieber, M. & Chandel, N. S. 2014. ROS function in redox signaling and oxidative stress. *Curr. Biol.* 24:R453–62.

Schoenberg, D. A. & Trench, R. K. 1980. Genetic variation in *Symbiodinium* (=*Gymnodinium*) *microadriaticum* Freudenthal, and specificity in its symbiosis with marine invertebrates. III. Specificity and infectivity of *Symbiodinium microadriaticum*. *Proc. Roy. Soc. B Biol. Sci.* 207:445–60.

Sharpe, S. C., Koester, J. A., Loebl, M., Cockshutt, A. M., Campbell, D. A., Irwin, A. J. & Finkel, Z. V. 2012. Influence of cell size and DNA content on growth rate and photosystem II function in cryptic species of *Ditylum brightwellii*. *PLoS ONE* 7: e52916.

Shick, J. M. & Dykens, J. A. 1984. Photobiology of the symbiotic sea anemone *Anthopleura Elegantissima*: Photosynthesis, respiration, and behavior under intertidal conditions. *Biol. Bull.* 166:608–19.

Sikkema, J., De Bont, J. A. M. & Poolman, B. 1994. Interactions of cyclic hydrocarbons with biological membranes. *J. Biol. Chem.* 269:8022–8.

Soković, M. D., Vukojević, J., Marin, P. D., Brkić, D. D., Vajs, V. & Van Griensven, L. J. L. D. 2009. Chemical composition of essential oils of thymus and mentha species and their antifungal activities. *Molecules* 14:238–49.

Stanley, G. D. Jr. 2003. The evolution of modern corals and their early history. *Earth Sci. Rev.* 60:195–225.

Steele, R. D. & Goreau, N. I. 1977. The breakdown of symbiotic zooxanthellae in the sea anemone Phyllactis (=*Oulactis*) *flosculifera* (Actiniaria). *J. Zool.* 181:421–37.

Steen, R. G. & Muscatine, L. 1987. Low temperature evokes rapid exocytosis of symbiotic algae by a sea anemone. *Biol. Bull.* 172:246–63.

Suga, T., Hamada, H., Hirata, T. & Izumi, S. 1987. Stereoselectivity in oxidative and reductive transformations of p -menthane derivatives with the cultured cells of *Nicotiana tabacum*. *Chem. Lett.* 16:903–6.

Sykes, C. L. & Wilson, W. D. 2015. Challenges of developing a molluscicide for use on *Dreissena rostriformis bugensis* veligirs in fish transport tanks. *In* Gerstenberger, S. L. & Wong, W. H. [Eds.] *Biology and Management of Invasive Quagga and Zebra Mussels in the Western United States*. CRC Press, Boca Raton, pp. 487–504.

Takahashi, H., Clowez, S., Wollman, F. A., Vallon, O. & Rappaport, F. 2013. Cyclic electron flow is redox-controlled but independent of state transition. *Nat Commun.* 4:1–8.

Takahashi, S., Nakamura, T., Skamizu, M., Van Woesik, R. & Yamasaki, H. 2004. Repair machinery of symbiotic photosynthesis as the primary target of heat stress for reef-building corals. *Plant Cell Physiol.* 45:251–5.

Tchernov, D., Gorbunov, M. Y., De Vargas, C., Narayan Yadav, S., Milligan, A. J., Haggblom, M. & Falkowski, P. G. 2004. Membrane lipids of symbiotic algae are diagnostic of sensitivity to thermal bleaching in corals. *Proc. Natl. Acad. Sci. USA* 101:13531–5.

Tivey, T. R., Parkinson, J. E. & Weis, V. M. 2020. Host and symbiont cell cycle coordination is mediated by symbiotic state, nutrition, and partner identity in a model cnidarian-dinoflagellate symbiosis. *MBio* 11:1–17.

Tolleter, D., Seneca, F. O., DeNofrio, J. C., Krediet, C. J., Palumbi, S. R., Pringle, J. R. & Grossman, A. R. 2013. Coral bleaching independent of photosynthetic activity. *Curr. Biol.* 23:1782–6.

Tolossa, I., Treignier, C., Grover, R. & Ferrier-Pagès, C. 2011. Impact of feeding and short-term temperature stress on the content and isotopic signature of fatty acids, sterols, and alcohols in the scleractinian coral *Turbinaria reniformis*. *Coral Reefs* 30:763–74.

Trebitsh, T. & Danon, A. 2001. Translation of chloroplast *psbA* mRNA is regulated by signals initiated by both photosystems II and I. *Proc. Natl. Acad. Sci. USA* 98:12289–94.

Treignier, C., Grove, R., Ferrier-Pagès, C. & Tolosa, I. 2008. Effect of light and feeding on the fatty acid and sterol composition of zooxanthellae and host tissue isolated from the scleractinian coral *Turbinaria reniformis*. *Limnol. Oceanogr.* 56:2702–10.

Trench, R. K. 1993. Microalgal-invertebrate symbiosis: A review. *Endocytobiosis Cell. Res.* 9:135–75.

Trombetta, D., Castelli, F., Sarpietro, M. G., Venuti, V., Cristani, M., Daniele, C., Saija, A., Mazzanti, G. & Bisignano, G. 2005. Mechanisms of antibacterial action of three monoterpenes. *Antimicrob. Agents Chemother.* 49:2474–8.

Tsuzuki, K., Xing, H., Ling, J. & Gu, J. G. 2004. Menthol-induced Ca^{2+} release from presynaptic Ca^{2+} stores potentiates sensory synaptic transmission. *J. Neurosci.* 24:762–71.

Tullberg, A., Alexciev, K., Pfannschmidt, T. & Allen, J. F. 2000. Photosynthetic electron flow regulates transcription of the *psaB* gene in pea (*Pisum sativum* L.) chloroplasts through the redox state of the plastoquinone pool. *Plant Cell Physiol.* 9:1045–54.

del Turina, A. V., Nolan, M. V., Zygadlo, J. A. & Perillo, M. A.2006. Natural terpenes: Self-assembly and membrane partitioning. *Biophys. Chem.* 122:101–13.

Vandermeulen, J. H., Davis, N. D. & Muscatine, L. 1972. The effect of inhibitors of photosynthesis on zooxanthellae in corals and other marine invertebrates. *Mar. Biol.* 16:185–91.

Venn, A. A., Wilson, M. A., Trapido-Rosenthal, H. G., Keely, B. J. & Douglas, A. E. 2006. The impact of coral bleaching on the pigment profile of the symbiotic alga. *Symbiodinium. Plant Cell Environ.* 29:2133–42.

Voolstra, C. R. 2013. A journey into the wild of the cnidarian model system *Aiptasia* and its symbionts. *Mol. Ecol.* 22:4366–8.

Wang, J. T., Chan, Y. Y., Tew, K. S., Meng, P. J. & Chan, C. A. 2012. Physiological and biochemical performances of menthol-induced aposymbiotic corals. *PLoS ONE* 7:e46406.

Wang, J. T. & Douglas, A. E. 1999. Essential amino acid synthesis and nitrogen recycling in an alga-invertebrate symbiosis. *Mar. Biol.* 135:219–22.

Wang, J. T., Keshavmurthy, S., Chu, T. Y. & Chen, C. A. 2017. Diverse responses of *Symbiodinium* types to menthol and DCMU treatment. *PeerJ* 3843:1–15.

Warner, M. E., Fitt, W. K. & Schmidt, G. B. 1999. Damage to photosystem II in symbiotic dinoflagellates: a determinant of coral bleaching. *Proc. Natl. Acad. Sci. USA* 96:8007–12.

Waugh, P. 1936. The variation and species of Red Sea *Turbinaria* and *Astraeopora* with a discussion of the genera. *J. Zool.* 913:29.

Weis, V. M. 2008. Cellular mechanisms of cnidarian bleaching: stress causes the collapses of symbiosis. *J. Exp. Biol.* 211:3059–66.

Williams, D. R. & Trudgill, P. 1994. Ring cleavage reactions in the metabolism of (–)-menthol and (–)-menthone by a *Corynebacterium* sp. *Microbiology* 140:611–6.

Xiang, T., Hambleton, E. A., DeNofrio, J. C., Pringle, J. R. & Grossman, A. R. 2013. Isolation of clonal axenic strains of the symbiotic dinoflagellate *Symbiodinium* and their growth and host specificity. *J. Phycol.* 49:447–58.

Xiang, T., Jinkerson, R. E., Clowez, S., Tran, C., Krediet, C. J., Onishi, M., Cleves, P. A., Pringle, J. R. & Grossman, A. R. 2017. Glucose-induced trophic shift in an endosymbiont dinoflagellate with physiological and molecular consequences. *Plant Physiol.* 176:1793–807.

Xiang, T., Lehnert, E., Jinkerson, R. E., Clowez, S., Kim, R. G., DeNofrio, J. C., Pringle, J. R. & Grossman, A. R. 2020. Symbiont population control by host-symbiont metabolic interaction in Symbiodiniaceae-cnidarian associations. *Nature Comm.* 11:108–16.

Yamaguchi, T., Caldwell, J. & Farmer, P. B. 1994. Metabolic fate of [^{3}H]-*l*-menthol in the rat. *Drug Metab. Dispos.* 22:616–24.

Yonge, C. M. & Nicholls, A. G. 1931. Studies on the physiology of corals: V. The effect of starvation in light and in darkness on the relationship between corals and zooxanthellae. *Sci. Rep. Great. Barrier Reef Exped.* 1:177–211.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Effect of menthol addition on pH of SSB01 cultures.

Figure S2. Survival of cultured SSB01 cells during menthol treatment.

Figure S3. Impact of menthol treatment on PSI activity in cultured SSB01.

Figure S4. Changes in size and morphology of symbiotic sea anemones during menthol treatment.

Figure S5. Changes in the maximum PSII quantum yields (Fv/Fm) during exposure of symbiotic sea anemones and cultured algae to menthol.

Figure S6. Changes of PSII antenna size or connectivity in aging cultures.

Table S1. Detailed results of statistical testing of quantitative data.