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Proteomic Assessment of Caffeine Effects on Coral Symbionts

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Caffeine is the most widely consumed and excreted psychoactive drug in the world. It is a ubiquitous tracer of urban wastewater, but its ecological effects are not well understood. We hypothesized that caffeine exposure is associated with coral bleaching. Here we report the effects of caffeine on four species of coral algae endosymbionts belonging to three widely distributed clades: Clade A *Symbiodinium microadriaticum* (A), Clade B *Symbiodinium* sp. from *Aiptasia pallida* (B6), Clade B *Symbiodinium* sp. from *Pseudoterogorgia bipinnata* (B7), and Clade C *Symbiodinium goreaui* (C). To assess the effect of caffeine on algal physiology we used two-dimensional polyacrylamide gel electrophoresis and peptide mass spectrometry to identify protein sensitive to caffeine exposure. The results show several upregulated and several downregulated polypeptides in all algae species tested. The heat-shock proteins are among the commonly affected proteins, suggesting that caffeine exposure associated with sewage discharge into natural waters may exacerbate the effects of stress from other environmental factors such as changes in ocean temperature and pH.

Introduction

Coral bleaching is a global environmental problem. The most current surveys have found 52%−90% bleaching in reefs worldwide (*1*). This is particularly alarming because these changes are not present in thousands of years of geologic records (*2*). Several hypotheses proposed to explain coral bleaching include UV-light exposure, anthropogenic global warming, overpopulation, and chronic pollution from sewage disposal (*2*−*4*). Although there are strong correlations between increased sea surface temperatures and bleaching, it is likely that a complex interaction of causes is responsible, and bleaching is still not completely understood (*2, 4*). In this article, we explore the potential ecological effects of caffeine, a ubiquitous component of domestic wastewater, for contribution to the destabilization of coral health through its effect on algal symbionts.

Caffeine (1,3,7-trimethyl-1*H*-purine-2,6(3*H*,7*H*)-dione or 1,3,7-trimethylxanthine) is a psychoactive stimulant consumed worldwide as part of the human diet or as part of specific pharmacological preparations. Caffeine is also the fourth most commonly found organic chemical and the most commonly found pharmaceutical product in surface waters (*5*). This is due to ubiquitous human excretion of caffeine and its recalcitrance in most wastewater treatment processes. The global average per capita consumption of caffeine is approximately 70 mg/person/day and up to 3% (w/w) is excreted intact (*6*). Caffeine is not sufficiently metabolized by wastewater microorganisms except by rare enzymes found in caffeine degrading bacteria such as *Pseudomonas putida* biotype A (ATCC 700097) (*7*). As such, caffeine has been successfully used as a reliable tracer of wastewater effluent discharge into natural water systems, including the oceans (*6, 7*). In many of these monitoring programs, caffeine concentrations are consistent but typically very low in the nanogram per liter range, and it is not clear if such low concentrations will have ecological effects alone or through synergistic interactions with other pollutants. We have estimated that up to 35 kg of caffeine are released daily into the Pacific Ocean, based on the discharge of approximately 946 million liters of treated wastewater from the Orange County Sanitation District in Southern California. In the Florida Keys, an urbanized region that is parallel to the third largest coral reef ecosystem in the world, Gardinali and colleagues have consistently detected caffeine at concentrations up to 200 ng per liter in coastal waters (*8*). The source of caffeine in this valuable reef environment is associated with the lack of adequate sewage treatment through the preponderance of private cesspits and underground injection wells that contribute to the degradation of coastal water quality.

Caffeine acts as a biochemical antagonist of adenosine receptors in the human brain, thereby increasing the synthesis of the neurotransmitter dopamine. Through its competitive inhibition of cyclic adenosine monophosphate (cAMP)-phosphodiesterase, caffeine causes the accumulation of cAMP in cells, which increases the activation of protein kinase A toward phosphorylation of enzymes involved in glucose metabolism (*9*). Caffeine is considerably more toxic to other organisms, including horses, dogs, parrots, and spiders, than to humans due to their underdeveloped capacity to metabolize the drug (*10*).

There are few studies on the effect of caffeine on aquatic organisms spurned in part by the discovery of caffeine in most water systems. As far back as 1930, Brinley reported on the effect of caffeine on oxygen consumption in freshwater fish *Erimyson sucetta oblongus*, Mitchell and bullfrog tadpoles,

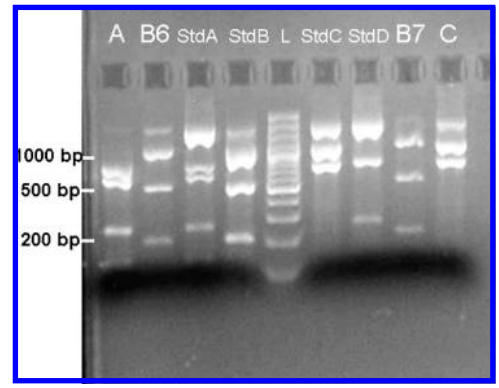

FIGURE 1. Results of PCR gel used to confirm the identification of the clades post experimentation. Confirms that the 4 strains tested, labeled above as A, B6, B7, and C, match their respective standard. Standards labeled above as StdA, StdB, StdC, and StdD. L is the molecular size control ladder.

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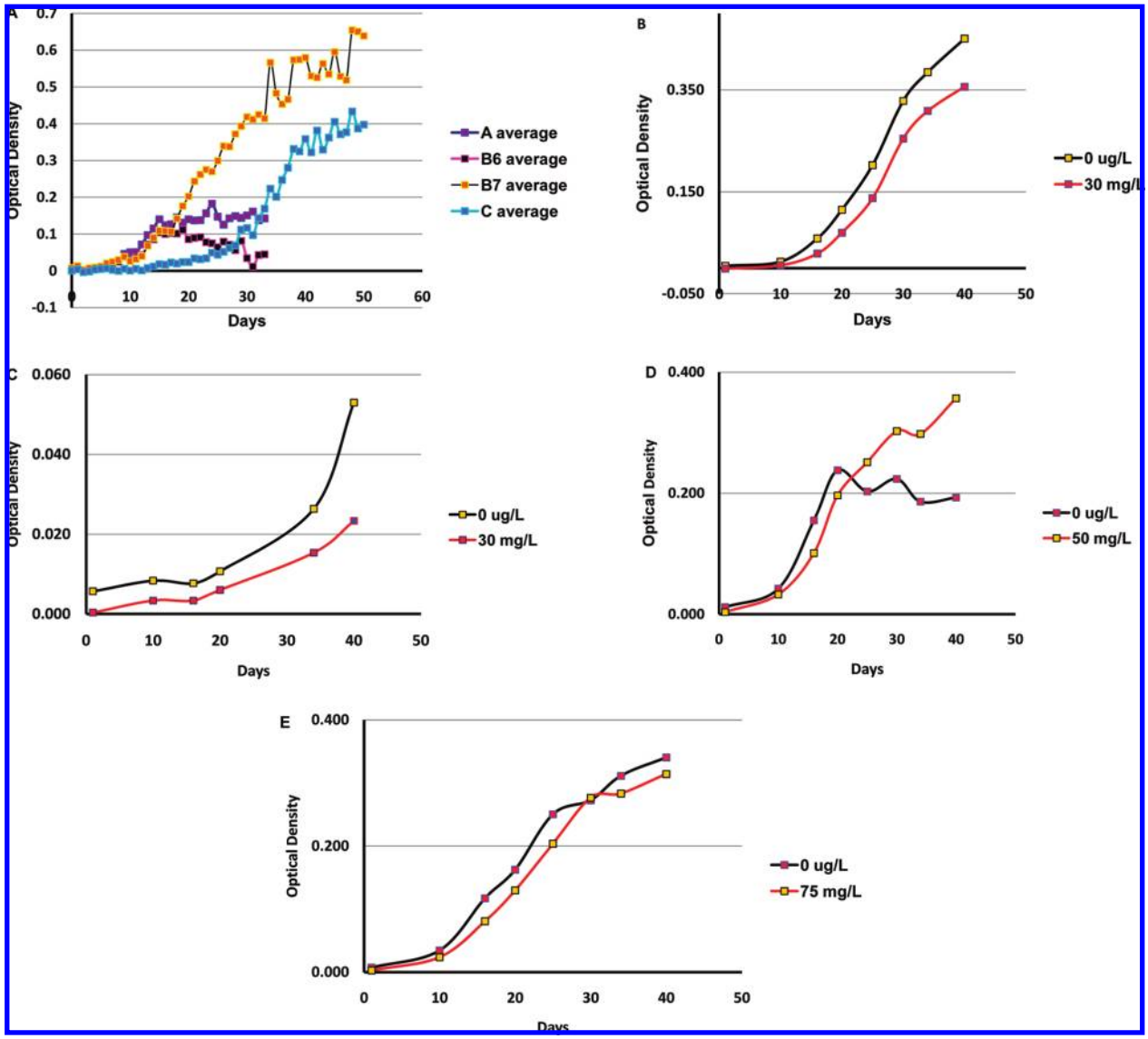

FIGURE 2. Growth data shown at 675 nm. Error bars are plus and minus one standard deviation. (A) Average growth curves of algae species: "A" is *Symbiodinium microadriaticum* Clade A from *Zoanthus sociatus* (*sensu* Trench). "B60" is *Symbiodinium* sp. Clade B, ITS-type B1 from *Aiptasia pallida* (*sensu* LaJeunesse). "B70" is *Symbiodinium* sp. from Clade B, ITS-type B1 from *Pseudoterogorgia bipinnata* (*sensu* LaJeunesse). "C" is *Symbiodinium goreaui* Clade C, ITS-type C1 from *Discosoma sancti-thomae* (*sensu* LaJeunesse). (B) Minimum Inhibitory Concentration (MIC) of 30 mg/L of caffeine for "C" Clade C *Symbiodinium goreaui*. Algae growth in the presence and absence of caffeine at (C) MIC of 30 mg/L of caffeine for "B7" Clade B *Symbiodinium* sp. from *Pseudoterogorgia bipinnata*, (D) MIC of 50 mg/L of caffeine for "A" Clade A *Symbiodinium microadriaticum*, and (E) MIC of 75 mg/L of caffeine for "B6" Clade B *Symbiodinium* sp. from *Aiptasia pallida*.

Rana catesbiana (*11*). More recently, Smith and Burgett (*12*) reported that caffeine at up to 600 μg/L had no statistically significant effect on the survivorship of the American toad, *Bufo americanus* tadpoles. There are even fewer studies on the physiological effects of caffeine in marine organisms. It has been shown that 5 g/L (25 mM) caffeine causes bleaching through its effect on protein phosphorylation (*13*). In *Aiptasia pulchella* 2 g/L (10 mM) of caffeine evoked release of symbiotic algae at 25 °C, and the proteins of caffeine-treated cnidarians show reduced phosphorylation compared to those of the control (*13*).

Based on the unresolved debate regarding the actual causes of coral bleaching, the ubiquitous distribution of caffeine, and previous research linking caffeine to cnidarian bleaching, we hypothesized that caffeine contributes to physiological stress experienced by coral ecosystems impacted by urban effluents. Here we show the effect of caffeine on growth and expression of proteins in algae associated with coral.

Methods Summary

Algal Strains. Four zooxanthellae species from three phylogenetic cladal groups of hard coral symbionts were investigated. Species "A" refers to Clade A *Symbiodinium microadriaticum* from the Canadian Center for the Culture of Microorganisms (CCCM) culture #411 (*sensu* Trench) isolated from the anemone *Zoanthus sociatus* off the coast of Florida. Species "B6" refers to Clade B, ITS-type B1 *Symbiodinium* sp. from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP) culture #2460 (*sensu* LaJuenesse) isolated from the anemone *Aiptasia pallida* off the coast of Florida. Species "B7" is Clade B, ITS-type B1 *Symbiodinium* sp. CCMP culture #2470 (*sensu* LaJuenesse) isolated from the octocoral *Pseudoterogorgia bipinnata* off the coast of Jamaica. Species "C" is Clade C, ITS-type C1 *Symbiodinium goreaui* CCMP culture #2466 (*sensu* LaJuenesse) isolated from the anemone *Discosoma sancti-thomae* off the coast of Jamaica. Polymerase chain

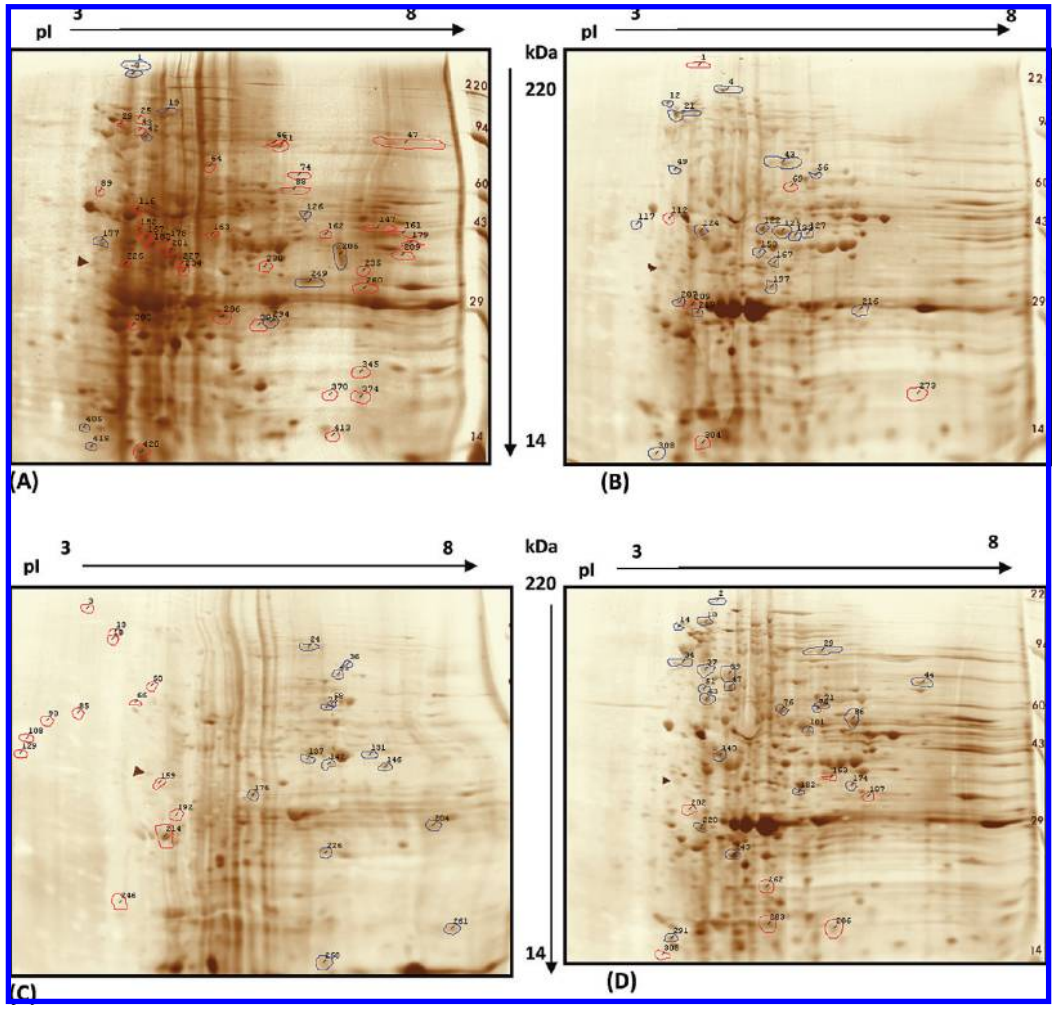

FIGURE 3. Differential display of 2-D gel images showing the influence of caffeine at 60 mg/L. 200 μg aliquots of polypeptide extracts were loaded onto the gels. Polypeptide spots that increased in size with respect to caffeine exposure by more than 1.7-fold or difference and/or *p* values < 0.05 are outlined in blue, whereas polypeptide spots that decreased in response to caffeine exposure according to the same criteria are outlined in red. (A) Differential display of polypeptides for Clade C *Symbiodinium goreaui*. (B) Differential display of polypeptides for Clade B *Symbiodinium* sp. from *Pseudoterogorgia bipinnata*. (C) Differential display of polypeptides for Clade A *Symbiodinium microadriaticum*. (D) Differential display of polypeptides for Clade B *Symbiodinium* sp. from *Aiptasia pallida*.

reaction (PCR) based on the following primer pair was used to confirm the identification of the different clades: (5′-TCAGTACAAATAATATGCTG-3′) and (5′-GGATAACAATTTCACACAGGTTATCGCCCCAATTAAACAGT-3′).

Despite similarities in photomicrographs of the algae, their genotypic fingerprints are quite distinct and recognizable and are used to confirm the integrity and purity of the cultures (*14, 15*) (Figure 1).

The four species were cultivated in a natural seawater medium f/2-Si obtained from CCMP, West Boothbay Harbor, Maine. Cultures (100 mL) were incubated at 24 °C on a 12:12 light:dark cycle using an exclusive light intensity of 50 μmol photons m^{-2} s^{-1}. All glassware used for culturing and testing were washed with a 10% HCl solution, repeatedly rinsed with deionized water, and sterilized.

Effect of Caffeine on Algal Growth. To measure the effect of caffeine on algal growth, triplicate cultures were incubated with caffeine at 0, 50 μg/L, 500 μg/L, 5 mg/L, 30 mg/L, 50 mg/L, 75 mg/L, and 100 mg/L. Culture growth was monitored spectrophotometrically at 675 nm (*16, 17*), and caffeine was monitored at 275 nm (7) with Spectramax 250 (Molecular Dynamics, Palo Alto, CA).

Effect of Caffeine on Differential Polypeptide Accumulation. Algae cultures in the presence or absence of 60-mg/L caffeine (four replicates each) were prepared for protein extraction at the peak of the exponential growth phase, after 35 days incubation for C, 28 days for B7, 24 days for A, and 22 days for B6. Proteins were extracted according to Weis et al., 2002 (*18*). Protein concentration in the extracts was determined using Bradford assay (USB Corporation, Cleveland, Ohio). Two-dimensional electrophoresis and silver nitrate staining were performed according to standard methods (Kendrick Laboratories, Inc. Madison, WI). Polypeptide concentrations within the gels were determined through densitometry (Model PDSI, Molecular Dynamics Inc., Sunnyvale, CA), and differential display images were analyzed using Progensis PG240 software including image warping with TT900 software (version 2006, Nonlinear Dynamics).

Next, three polypeptide spots per species were chosen for LC-MS/MS analysis on the basis of the most statistically significant (and a good visual difference) for the following: The polypeptide spot with the largest increase in spot density in cultures with caffeine, the polypeptide spot with the largest decrease in spot density in cultures with caffeine, and the polypeptide spot with the largest change of spot density within the molecular weight range of cytochrome P450 enzymes which are known to metabolize caffeine in many organisms including some algae. Algae typically have P450 in the molecular mass range of 46−67 kDa (*19*). The three polypeptides from each culture were digested with trypsin

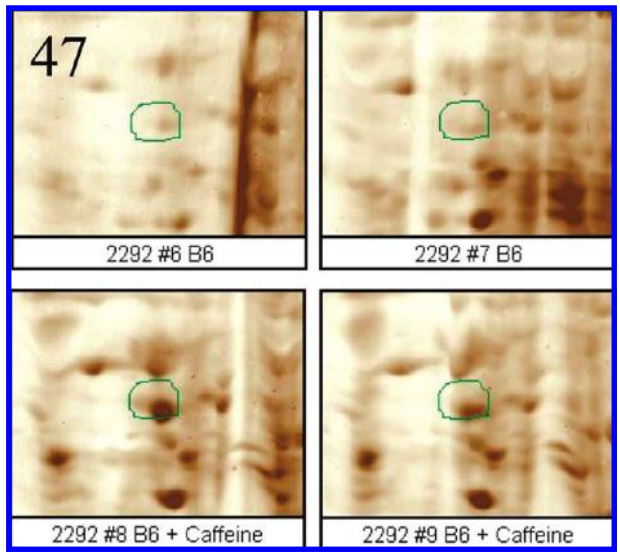

FIGURE 4. Example of 2-D PAGE display panels showing the effect of caffeine on heat-shock proteins for Clade B *Symbiodinium* sp. from *Aiptasia pallida*. The two panels are duplicates. The prefix 2292 represents the gel identification number.

and analyzed by LC-MS/MS at Columbia University's Protein Core Facility. The sequences were searched against the NCBI database to generate mascot reports and partial peptide sequence matches.

To confirm the identity of heat-shock proteins, we performed Western immuno-blotting using PVDF membranes. After staining with Coomassie Brilliant Blue R-250, the blot was blocked for 2 h in 5% Carnation Nonfat Dry Milk (NFDM) in Tween-20 tris buffer saline (TTBS) and rinsed in TTBS. The membrane was incubated in primary antibody (anti-HSP 70 Product SPA-812 Lot # 03200632), diluted 1:25,000 in 2% NFDM TTBS overnight, and rinsed three times for 10 min each in TBBS. The blot was then incubated in secondary antibody (anti-Rabbit IgG HRP (Amersham, Batch 364579), diluted 1:2000 in TTBS for 2 h, rinsed in TTBS, and exposed to X-ray film.

Caffeine Stability in Seawater. Water samples were collected from San Diego Creek and adjacent Pacific Ocean at Corona Del Mar State Beach and transported to the laboratory within 2 h for processing. Caffeine was added to 250 mL of water samples for a final caffeine concentration of 20 mg/L in triplicate in Teflon flasks incubated either in darkness or light (50 μmol photons m^{-2} s^{-1}) on a 12:12 light: dark cycle. Residual caffeine concentration was monitored daily at 274 nm using a Spectramax 250 spectrometer for 30 days.

Results and Discussion

There are several reasons to explore the potential ecological effects of caffeine, including its prevalence as an environmental contaminant associated with urban wastewater effluent and its noted physiological effects. With respect to the ocean, caffeine is also known to affect calcium metabolism, which may further exacerbate the leaching of calcium associated with the ocean acidification resulting from the effects of the increasing atmospheric carbon dioxide concentration in the atmosphere and global warming on the quality of coral ecosystems (*1, 20*). With this research, we tested the hypothesis that caffeine exposure also affects growth and physiological processes in marine algae associated with corals and that the introduction of caffeine into the ocean through wastewater discharge may be contributing in various ways to coral bleaching.

Effect of Caffeine on Algal Growth. We discovered that the normal growth rate of algae vary considerably, regardless of the clade affiliation (Figure 2, panel a). Algae belonging to clades A and B6 grew the quickest, reaching stationary phase after 15 days, whereas clade C alga achieved stationary phase after 40 days and clade B7 after 50 days of incubation. Caffeine significantly suppressed algae growth during the logarithmic phases (Figure 2, panels b-e). The minimum inhibitory concentration (MIC) of caffeine for each alga was 30 mg/L for C and B7, 50 mg/L for A, and 75 mg/L for B6.

Apparently some algae are more resistant to the effects of caffeine than others, a variation in tolerance that has been shown for other organisms. For example, after 22 days of incubation with caffeine, the growth of alga A appears to increase, suggesting that this organism is able to utilize caffeine as a nutrient.

The variation in algae sensitivity to a potentially toxic chemical such as caffeine is interesting and might be informative in attempts to re-establish corals that have been adversely affected by pollutants. We focused on *Symbiodinium* because algae in this group are the most common endosymbionts among coral reef-building cnidarians, platyhelminths, mollusks, and protists (*21*), although more information will be needed before strategic coral-reseeding programs can be implemented. These symbiont populations may reach densities of several million or more organisms per cm^2 of host tissue (*22*). Several *Symbiodinium* species exhibit specific biogeographic distribution patterns and live within multiple scleractinian coral hosts. Specifically, members of the *Symbiodinium* clades A and B are typically found in higher latitudes, whereas clade C *Symbiodinium* is abundant in tropical latitudes (*23*). Due to a large amount of variability, subcladal species are not yet well-distinguished (*24*), and the biogeographical distribution pattern and host specific distribution of the *Symbiodinium* genus is not completely understood and is highly debated (*25*).

Effect of Caffeine on Differential Polypeptide Accumulation. Figures 3 and 4 show the differential display of algae polypeptide pools produced in the presence and absence of caffeine. In clade A, 279 polypeptide spots were detected in caffeine-exposed cells. Of these, 14 were significantly upregulated and 13 were significantly downregulated relative to the control ($p < 0.05$.) In clade B6, 319 polypeptide spots were detected, with 22 upregulated and 7 downregulated spots significantly affected relative to the control. In B7, 319 polypeptide spots were detected, and 19 upregulated and 6 downregulated proteins were significantly altered. In C, 431 polypeptide spots were detected, of which 12 were upregulated and 37 were downregulated. From these, we selected protein spots with quantities that were statistically significantly different between caffeine-exposed and unexposed cultures ($p \leq 0.043$) and that met the criteria described in the methods. The results show that these proteins ranged widely in size from 23 kDa to 186 kDa. The isolectric points (pI) ranged from 4.6 to 7.4. Quantitative analysis also shows that there was a -9.2 and $+3.3$ fold difference range in quantity of protein in these particular polypeptide spots (Table 1). These data show that caffeine influences a wide range of proteins both within the same strain and between different strains and clades.

Several of the proteins featured in Table 1 function in physiological stress response, photosynthesis, or glycolysis. Heat-shock proteins (Hsp60, Hsp70, and Hsp90) were the most common significantly affected proteins under caffeine exposure, upregulated 2−3-fold in clade B and downregulated up to 9-fold in clade C ($p < 0.01$). Heat-shock proteins are involved in the folding of newly synthesized proteins, transport of proteins across membranes, refolding misfolded or aggregated proteins, and control activity of regulatory proteins (*25*). Under stressful environmental conditions such

TABLE 1. Catalog of Polypeptides and Their Attributed Functions Identified in Caffeine-Exposed Algae[a]

species	MW (Daltons)	pI	significant change in protein concn upregulated (+) or downregulated (-) *$p < 0.05$; **$p < 0.01$	"Match Score" *=matched to a *Symbiodinium* sp. in the database	number of similar peptides included in matched score	putative protein identification	putative protein function
Clade A *Symbiodinium microadriaticum* (A)	160,420	4.6	−4.6 *	121*	3	peridinin chlorophyll-a binding protein	photosynthesis
	45,234	7.4	+2.9 *	272*	5	glyceraldehyde-3-phosphate dehydrogenase	glycolysis; programmed cell death
	26,276	7.2	+3.2 **	152*	3	glyceraldehyde-3-phosphate dehydrogenase	glycolysis; programmed cell death
Clade B *Symbiodinium* sp. from *Pseudoterogorgia bipinnata* (B7)	186,592	6.1	+3.3 *	ND[b]	ND[b]	ND[b]	ND[b]
				210	3	**Hsp70**	**heat-shock response**
				155*	2	ribulose bisphosphate carboxylase	photosynthesis
				75	1	cystathione gamma lyase	cysteine biosynthesis
				69	1	methionine adenosyltransferase	S-adenosyl methionine synthesis
	43,022	6.5	+1.9 **	63	1	acetyl-CoA acetyltransferase	lipid metabolism
				781*	17	peridinin chlorophyll-a binding protein	photosynthesis
				185	3	oxygen evolving enhancer 1	photosynthesis
				91*	3	glyceraldehyde-3-phosphate dehydrogenase	glycolysis; programmed cell death
	30,479	5.5	−2.0 *	75	1	fructose-1,6-bisphosphate aldolase	glycolysis
Clade B *Symbiodinium* sp. from *Aiptasia pallida* (B6)				215	4	**Hsp70**	**heat-shock response**
				116	2	**Hsp60**	**heat-shock response**
				90	2	**Hsp90**	**heat-shock response**
	75,950	5.9	+2.9 *	69	1	vacuolar ATP synthase catalytic subunit A	peripheral V1 complex of ATPase
				118	1	cytochrome b5	enhances cytochrome P450 isoforms
				116	1	methionine adenosyltransferase	S-adenosyl methionine synthesis
				108	2	S-adenosylmethionine synthetase	S-adenosyl methionine synthesis
				103	1	ATP synthase subunit alpha	ATP synthesis
	55,087	7.1	+2.2 *	98	1	fumarate reductase	succinate synthesis
	23,025	6.2	−2.2 *	121*	3	peridinin chlorophyll-a binding protein	photosynthesis
Clade C *Symbiodinium goreaui* (C)				202	1	**endoplasmin (Hsp90-like protein)**	**heat-shock response**
				165	3	**Hsp70**	**heat-shock response**
				144	3	carbon monoxide dehydrogenase	carbon metabolism
				118	3	aminopeptidase N	protein maturation and degradation
	128,105	5.7	−5.3 **	102	2	aldehyde oxidase and xanthine dehydrogenase, molybdopterin binding	caffeine metabolism
				456	7	cell division cycle protein 48	ubiquitinated proteins degradation
				337*	6	**Hsp 90**	**heat-shock response**
				230	4	**Hsp70**	**heat-shock response**
	12,134	5.7	−9.2 **	155	3	carbon monoxide dehydrogenase	carbon metabolism
				257*	5	glyceraldehyde-3-phosphate dehydrogenase	glycolysis; programmed cell death
	48,724	6.5	−2.6 **	78	1	chloroplast ferredoxin-NADP{+) reductase	ATP homeostasis

[a] Selected polypeptides were characterized through mass spectrometry and amino acid sequence analysis. Sequence comparisons to depository data revealed matches to one or more polypeptides in *Symbiodinium* and/or other species. [b] ND = Not detected.

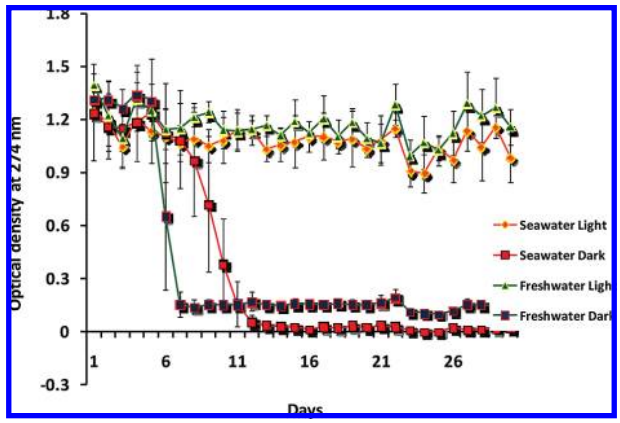

FIGURE 5. Fate of 0.10 mM caffeine in ocean water and freshwater under light and dark conditions.

as elevated ambient temperatures which have been associated with coral bleaching, heat-shock proteins are known to be induced in coral (*26*). Hsp 70 levels have been found to increase markedly in tropical reef coral *G. djiboutiensis* following 2 h heat shocks at 33, 36, 38, and 40 °C (*27*). Our results show now that the heat-shock response is also induced in coral endosymbionts exposed to caffeine. We confirmed the identity of putative heat-shock proteins using Western immunoblotting. This result suggests that caffeine may exacerbate the effects of other stressors that induce similar responses such as elevated temperatures and acidified oceans in a synergistic way to increase the pace and intensity of coral bleaching.

As noted by Imre and colleagues (*28*) up to 0.045% caffeine was measured in certain corals. However, caffeine and chemically related compounds are common plant metabolites and therefore may have been produced *in situ*. In order to explore the chemical fate of caffeine in ocean waters and whether caffeine is sufficiently stable to accumulate to levels that can induce stress response, we used microcosm experiments, and the results are shown in Figure 5. The results show that caffeine degrades completely in the dark after 6 days in freshwater and 11 days in ocean water. We did not detect significant caffeine degradation when microcosm flasks were incubated under lighted conditions. We are exploring this discrepancy further, as it may have to do with the formation of yet uncharacterized byproducts of caffeine metabolism. In all samples where caffeine degradation occurred, we were able to isolate *Pseudomonas putida*, which has been shown previously to utilize caffeine as the sole carbon source, but this organism is not especially adapted to marine ecosystems (*6, 7*).

This study contributes important information about the synthesis of Hsp in coral endosymbionts, as a potential addition to the repertoire of molecular tools for assessing ecosystem health and the effects of environmental stressors (*29, 30*). We acknowledge that the concentrations of caffeine determined to be influential in this study are orders of magnitude higher than ambient levels of caffeine routinely found in marine ecosystems inhabited by coral reefs (*8*). Nevertheless, the central importance and sensitivity of the stress response pathway provide a strong rationale for further studies on the potential impacts of chronic exposure to low doses of caffeine and the possibility of synergistic effects in the presence of other stressors and chemical pollutants. Based on the results presented here, we cannot rule out the possibility that caffeine associated with urban sewage effluent (*31*−*35*) can exacerbate the effects of stress from other environmental factors (e.g., global warming) and act as additive stressors in the coral reef environment. This research has important implications to wastewater processing and management and coral reef management globally. However, more work needs to be done to better understand the role of Hsp within coral and its algal symbionts.

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