Effect of Anthropogenic Caffeine on Cnidarian Bleaching

Introduction:

Recently, coral biologists have observed an increase in the frequency of coral bleaching (Berkelmans 1998). Coral reefs are hotspots of biodiversity, containing organisms which can be found no where else on the planet. With this great biodiversity, comes the potential for new medicines. Coral reefs are home to many species of fish which provide food for people in developing countries such as Indonesia (Jones et al. 1999). In addition to supporting economies based on tourism and providing sources for medicine, coral reefs serve as a barrier between deadly storms and waves. The destruction of coral reefs presents a great challenge to human health, injury prevention, and pharmaceutical research; therefore, it must be stopped. One solution to this problem is to employ techniques of multidisciplinary and interdisciplinary research to discover the causes of this bleaching and employ principles of social ecology and public policy-making to lessen its possible anthropogenic causes (Douglas 2003).

The phenomenon known as bleaching occurs when the cells of a cnidarian release their symbiotic algae, or when the photosynthetic pigments of these brown *Sybiodinium* algae, otherwise known as zooxanthellae, are degraded (Douglas 2003). Cnidarians depend on their symbionts for about sixty-percent of their nutrients, and although they can recover from bleaching, it often leads to the death of the cnidarian. Bleaching has many known causes, including increases and decreases in temperature, high irradiance, darkness, heavy metals, and pathogenic microorganisms (Douglas 2003). Chronic pollution is another possible cause, but many ocean pollutants remain to be tested for their possible contributions to cnidarian bleaching (Douglas 2003). It is the challenge of today's interdisciplinary scientists to employ *Symbiodinium* symbiosis, coral ecology, and techniques of reef management to determine possible anthropogenic causes of bleaching and work, through the implementation of policy, to reduce bleaching (Douglas 2003).

Caffeine, perhaps the most widely consumed drug because of it's extensive use in beverages, food, and pharmaceuticals, has been proven to be an effective marker for tracing surface water pollutants which come from human wastewater (Buerge et al. 2003). It does not appear to be degraded in ocean water and its use is not predicted to decline in the near future (Buerge et al. 2003). Consumption of caffeine in the United States alone is averaged at 210 mg of caffeine per person per day (Ogunseitan 1996). Current waste treatment methods do not remove a significant amount of caffeine and so much of it enters the ocean (Ogunseitan 1996). Enough evidence exists to show that caffeine is spatially and temporally distributed in high enough concentrations to pollute coral reefs.

In their 2001 study on how differences in temperature can cause bleaching in cnidarians, Sawyer and Muscatine also tested a variety of pharmaceutical products, one of which being caffeine, for their effects on bleaching in the Hawaiian reef coral *Pocillopora damicornis* and the sea anemone *Aiptasia pulchella*. They proved that, at concentrations of 25mM, bleaching does occur in living coral. This occurs because caffeine degrades proteins in the host cells of the cnidarians, and thus affects their adhesion to their algal symbionts. The corals realeased a significant amount of the algae after just 1.5 hours. Caffeine also retards the efficiency of photosynthesis in the zooxanthellae by increasing the amounts of cyclic AMP, which then subsequently decreases the productivity of photosystem II.

Work to Date:

Based on previous research which has shown that 25mM of caffeine causes bleaching, Dr. Ogunseitan's graduate student, Kelly Pollack, has studied the effect of smaller (more environmentally realistic) caffeine concentrations with respect to biomass production and photosynthetic activity of the coral symbiont zooxanthellae, *Symbiodinium microadriaticum*. The results of her studies to date show that peak algal growth is retarded by 2.5% at 2.5x10^(-7) mM caffeine to 81% at 3.0 mM caffeine. Caffeine influenced algal growth for concentrations between 0 and 0.5 mM, R=.901 ($p<.001$). There was a negative correlation between chlorophyll synthesis and caffeine concentration between 0.5 and 3mM. For chlorophyll-*a*, R=0.719 (p=0.008); and for chlorophyll-*c*, R=.652 (p=0.022). There was also significant negative correlation between chlorophyll-*c* synthesis and the amount of caffeine from 2.5x10^(-7) to 0.5mM, R=.533 (p=.017). The caffeine Kelly Pollack measured in treated sewage effluent from the Sanitation District of Orange County (O.C.S.D.), California, was at a concentration of 1.94x10^(-4) plus or minus 4.9x10^(-5) mM (37.5 plus or minus 9.5 micro-g per liter). Approximately 946 million liters of treated effluent is released into the Pacific Ocean by this facility per day. The caffeine entering the ocean from O.C.S.D. alone is estimated at 35.5 plus or minus 9.5 kg/day. These results provide a framework for the hypothesis that caffeine from domestic sources contributes to coral bleaching.

Specific Aims:

A. Cnidarian Testing at Low Levels of Caffeine Exposure: Kelly Pollack's initial studies showing gross growth changes in zooxanthellae subjected to low levels of caffeine, and previous research showing that caffeine initiated bleaching in coral at 25mM, lead to the goal to discover the minimum concentration of caffeine that can initiate a bleaching event.
B. Zooxanthellae Protein Analysis: Experiments done in the lab thus far have helped narrow down a range of concentrations in which caffeine significantly affects the growth of the zooxanthellae. However, important physiological changes may occur before gross alterations can be perceived. Analyzing the proteins in the algal samples is

necessary to fully understand the impact of caffeine on the cnidarian's symbiont, and to pinpoint the caffeine concentration at which *Symbiodinium microadriaticum* is affected.

C. Direct Reef Environmental Measurements: Although caffeine has been extracted from coral, and has been measured in open ocean waters, there is extremely limited data regarding how much caffeine can be found in reef waters. Expanding upon this knowledge is fundamental to understanding what role caffeine may have in coral bleaching. Solid-phase extraction techniques may be guides for water sampling of caffeine concentrations in mid-reef post-dispersion locations. These can be compared to caffeine concentrations in wastewater effluent for the area sampled and coral and algal cellular concentrations. Maps can then be generated showing areas of coral reef density in proximity to dense urban areas and locations of sewage effluent released into the ocean.

Materials and Methods:

Caffeine Stock Solutions

By adding different low concentrations of caffeine to each tank, we can create the variable condition for our experiments. Stock solutions of caffeine ranging from 0mM (for the control tank) to 0.5 mM will be prepared and will be introduced into the reef tanks.

Pocillopora Coral (*Pocillopora damicornis)*:

This is one of the corals we intend to use in the lab (it is important to note that the zooxanthellae we intend to use for protein analysis come living on this coral as they are its natural mutualistic symbionts). This is a coral which requires minimal maintenance and Kelly Pollack has been able to secure sufficient quantities of it from The Long Beach Aquarium of the Pacific. The tanks must be monitored frequently to make sure that optimal conditions for its health are being maintained. This coral must be kept under moderate to high lighting, medium water flow, 72-78 degrees F, 1.023-1.025 sg, a pH of 8.1-8.4, and a dKH of 8-12. It also must be supplemented with calcium, strontium, and trace elements (<www.etropicals.com>).

Reef Tanks:

Coral must be kept in well-maintained reef tanks for optimal chances of survival. The corals will spend the duration of the study in four or more reef tanks in the lab. The largest tank is one-hundred-twenty gallons and there are also three other smaller tanks which hold five gallons of salt water. The tanks are maintained at the optimal conditions for the coral through the use of halide lighting, appropriate water flow, and a constant temperature. In addition to maintaining the tanks for the health of the corals, consistent monitoring to ensure constant tank conditions will help eliminate any other outside causes of bleaching; e.g., the temperature will be closely monitored to ensure that

bleaching does not occur due to a change in temperature, but only occurs due to variable caffeine concentrations. To make sure that bleaching does not occur for any cause other than caffeine, we have corals living in the tanks and will monitor their health for several weeks before caffeine is added.

Two- Dimensional Gel Electrophoresis using a Pharmacia Biotech Multiphor II apparatus:
It is important to measure the changes in the algal proteins as varying concentrations of caffeine are added to the tank systems because changes in the proteins could reflect changes in the photosynthetic output of the zooxanthellae. Throughout the study, the proteins will be centrifuged out of the zooxanthellae and two-dimensional gel electrophoresis will be employed to look for these changes. The gel and phosphorus isotope will be used to the manufacturer's instructions. The proteins will be put in the reservoir on top of the apparatus and go down the gel towards the bottom and, due to their relative gravitational pull based on their mass, or simply their differing weights, will be separated by different kinds of proteins. The gel will be dried and the resulting protein bands will be analyzed for changes throughout experiments (Sawyer and Muscatine 2001).

Pulse Amplitude Modulation (PAM) Chlorophyll Fluorometry:
This is an advanced instrument which has been rented from the manufacturer Heinz Walz in Germany because it is necessary for the measure of the productivity of the *Symbiodinium in situ*. The PAM device can be immersed in the reef tanks to measure the degree of bleaching occurring in the living coral tissue. The fluorescence comes from the antennae pigments of photosystem II in the algae (Jones et al. 1999). The reaction centers of photosystem II will be opened in an algal sample which has been in a dark environment with a pulse of red light and the initial fluorescence of the sample will be measured. Next, a pulse of white light will be administered to the coral, closing the reaction centers, and the resulting maximum fluorescence will be measured. The rates of photosynthesis can be used to determine the percent bleaching of the coral samples.

Expected Results:
This research will have implications for wastewater treatment standards and will provide information as to what role anthropogenic caffeine may have on the bleaching of cnidarians. In addition, due to the great biodiversity of cnidarians and coral reefs and the worldwide use of caffeine, this research will have important implications for health and disease/injury prevention on both national and international levels. The knowledge gained will benefit conservation, reef management, and policy efforts worldwide in the areas of waste treatment and pharmaceuticals. This research will help the scientific community understand the effects humans have on marine ecosystems and, in turn, help create an environment conducive to human health. In addition, I hope to expand this project further in the future and study the effectiveness of public policies to reduce the pollution of coral reefs by humans and study the relationship between

the health of marine ecosystems and the promotion of humankind's physical, mental, and social well-being.

Project Timeline:

Week 1: *Cnidarian Testing at Low Levels of Caffeine Exposure*: Perform water quality tests on tanks. Learn how to use diving-PAM for fluorescence analysis. Create stock solutions of caffeine. Help with tank measurements, e.g. water changes and lighting. Clean tanks. Monitor coral health.

Week 2: In addition to monitoring the tank conditions and coral health, I will be using the diving-PAM for fluorescence analysis and entering the data from this process. I will also be ordering additional equipment for our next experiments. I will be inoculating algae so that they can grow sufficiently for our protein testing in the next few weeks.

Week 3: I will continue all previously mentioned tasks as well as learn how to use the technique of gel electrophoresis for the upcoming weeks of protein analysis. I will review current research literature relevant to our topic and discuss it with Kelly Pollack.

Week 4: In addition to monitoring tanks and coral health, I will be helping Kelly Pollack analyze the data from our fluorometry tests. This will require complex mathematical procedures in concert with Microsoft Excel.

Week 5: *Zooxanthellae Protein Analysis*: Begin protein analysis of the zooxanthellae using gel electrophoresis. Clean reef tanks as part of regularly scheduled maintenance. Wash glassware. Thoroughly inspect coral to insure their optimum health.

Week 6: Continue protein analysis and data entry. Discuss relevant literature with Kelly Pollack. Meet with Dr. Ogunseitan to discuss our progress. Begin gathering images for my poster presentation at next year's UROP Symposium.

Week 7: Continue protein analysis and data entry. Begin analyzing results from the electrophoresis tests. Order more equipment for upcoming experiments as needed. Begin disassembly of reef tanks. Clean glassware and dispose of algae samples.

Week 8: *Direct Reef Environmental Measurements*: Help prepare ocean water samples for testing. Test ocean water samples for their caffeine content. Finish disassembling reef tanks and analysis of zooxanthellae protein data. Meet with Dr. Ogunseitan to discuss results from the first two experiments.

Week 9: Enter data from water quality samples, take inventory of lab supplies, and maintain the lab while Kelly Pollack is in Australia getting ocean water samples from near the Great Barrier Reef. Develop text for poster to present at the UROP Symposium. Read relevant literature.

Week 10: Help test the water samples from Australia and enter data. Help analyze results from all water quality testing. Clean glassware and help organize the lab. Finish poster for UROP Symposium. Meet with Dr. Ogunseitan to discuss further involvement with this project and future projects. After working in Kelly Pollack's lab this summer, I will have the opportunity to expand on this project or create my own related project.

Student's Level of Preparation/ Participation:
I have done extensive reading on corals, wastewater treatment, algae, and caffeine, as well as basic instructions for all necessary procedures. Throughout Spring Quarter, I will come to the lab for training on the procedures as the necessary equipment arrives. I will observe Kelly Pollack as she performs coral testing. I will start with tank maintenance and begin water quality testing. I will aid in furnishing supplies for this lab by submitting a proposal for UROP next month. In addition to this lab work, I am taking classes which will enrich my knowledge of research, including; Social Ecology of Health Promotion, Bio Sci Safety and Ethics, Viruses and Disease with Professor David Camerini, and Chemistry 1C Lab. I will finish my first year of core courses by this summer including biology classes (one based on molecular scale and the other based on ecological scale), General Chemistry, Writing, and Calculus. All of these courses are crucial to understanding laboratory procedures, writing successful proposals for funds and supplies, and data analysis. I am volunteering at the UROP Symposium this year and will use part of this summer to prepare for my presentation at this event next year. I am committed to this project and confident that I will be sufficiently prepared to enter this lab full-time by summer.

My responsibilities are divided by type (or goal) of task as it relates to the research. Some tasks, (like water quality testing) could be done without Kelly Pollack present.

Coral testing:
- Perform water quality tests on tanks (every other day to weekly)
- Learn how to use diving-PAM for fluorescence analysis and assist Kelly Pollack in measurements
- Create stock solutions of caffeine
- Help with tank maintenance, e.g. water changes, cleaning, lighting, etc.
- Monitor coral health

Ocean water samples:
- Help prepare samples for testing
- Test samples

Algae protein analysis:
- Inoculate algae into new test flasks to keep alive and for testing
- Create stock solutions of caffeine
- Assist with protein (gel electrophoresis) analysis as needed
- Wash glassware as needed

General/Miscellaneous:
- Appropriate reading and literature review
- Meet with Kelly Pollack to discuss current literature and research relevant to the study
- Equipment ordering

- Meet with Dr. Ogunseitan to discuss the progress of the three experiments

References:

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Thank you for your consideration. I am submitting this proposal for both ID SURE and SURP and have not personally received any other outside sources of funding.