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ALTERATION OF NORMAL CELLULAR PROFILES IN THE SCLERACTINIAN CORAL (*POCILLOPORA DAMICORNIS*) FOLLOWING LABORATORY EXPOSURE TO FUEL OIL

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Abstract—Petroleum contamination from oil spills is a continuing threat to our ocean's fragile ecosystems. Herein, we explored the effects of the water-soluble fraction of crude oil on a stony coral, *Pocillopora damicornis* (Linneaeus 1758). We developed methods for exposing corals to various concentrations of crude oil and for assessing the potential molecular responses of the corals. Corals were exposed to water-accommodated fraction solutions, and appropriate cellular biomarkers were quantified. When compared to the ''healthy'' control specimens, exposed corals exhibited shifts in biomarker concentrations that were indicative of a shift from homeostasis. Significant changes were seen in cytochrome P450 1-class, cytochrome P450 2-class, glutathione-*S*-transferase-pi, and cnidarian multixenobiotic resistance protein-1 biomarkers, which are involved the cellular response to, and manipulation and excretion of, toxic compounds, including polycyclic aromatic hydrocarbons. A shift in biomarkers necessary for porphyrin production (e.g., protoporphyrinogen oxidase IX and ferrochelatase) and porphyrin destruction (e.g., heme oxygenase-1 and invertebrate neuroglobin homologue) illustrates only one of the cellular protective mechanisms. The response to oxidative stress was evaluated through measurements of copper/zinc superoxide dismutase-1 and DNA glycosylase MutY homologue-1 concentrations. Likewise, changes in heat shock protein 70 and small heat shock proteins indicated an adjustment in the cellular production of proteins. Finally, the results of this laboratory study were nearly identical to what we observed previously among corals of a different species, *Porites lobata*, exposed to an oil spill in the field after the grounding of the Merchant Vessel *Kyowa Violet.*

Keywords—Coral Oil spill Biomarker Antibody Cellular diagnostics

INTRODUCTION

The expanding human interaction with the ocean environment has greatly affected the ocean's fragile ecosystems [1,2]. Specifically, petroleum contamination has been—and continues to be—a serious threat. Petroleum products, such as the petrochemicals used to make plastics and synthetic fibers and the oil and gas used for heat, energy, and fuel, are intimately involved with today's society. Despite the practical necessities, increased transport of petrochemicals over the world's oceans has the potential to affect our environment severely. In fact, oil spills are inevitable [3]. Although rapid action usually is taken either during or soon after spills to cleanse oil contamination on the water surface, water-soluble components of the oil remain fractionated within the water column or may be sequestered in sediments. This water-accommodated fraction (WAF) allows the contaminants to remain after the superficial cleaning activities and, thereby, causes a potential disruption in the homeostasis of any marine organisms it contacts.

It is well documented that petroleum products can affect aquatic organisms at different levels of organization. Studies of marine fishes, following environmental or laboratory exposure to petroleum products or by-products (e.g., polycyclic aromatic hydrocarbons [PAHs]), have provided strong evidence linking pollution to increases in the number of structural anomalies [4,5], developmental complications [6,7], behavioral changes [8,9], and diseases [10,11]. The time to appearance of these anomalies can range from several days to months after the initial exposure. However, cellular responses to pollutants are immediate and can indicate changes in the organism's cellular homeostasis.

The exposure of coral reef ecosystems to crude petroleum and, particularly, intermediate fuel oil (IFO) grade 180, a common grade of marine fuel used in tanker transport, is of interest following the grounding of the Merchant Vessel *Kyowa Violet* in December 2002, during which approximately 55,000 to 80,000 gallons spilled into the coral reef ecosystem off Colonia Harbor, Yap, Federated States of Micronesia. Our recent assessment of the corals affected by the spill suggested that the sublethal effects of this fuel-oil spill adversely affected coral reef health [12]. The present study was the logical extension of our previous in situ study and focused on whether the water-soluble fraction of marine fuel oil can cause a rapid shift in homeostasis of a widely distributed and well-studied coral following a controlled laboratory exposure. Specifically, we developed a methodology to expose corals to various concentrations of the water-soluble components of IFO 180 marine fuel oil, the same compounds that have been implicated in the *Kyowa Violet* spill. We utilized this approach to compare and contrast the response of exposed and reference specimens of the reef-building coral, *Pocillopora damicornis*.

MATERIALS AND METHODS

Maintenance of corals

Pocillopora damicornis (Linnaeus 1758) colonies were obtained from Aquatic Aquaculture (Mardela Springs, MD,

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USA). Corals were maintained in 209-L aquaria at 26°C and a salinity of 38 ppt created with Instant Ocean™ Sea Salt mix (Aquarium Systems, Mentor, OH, USA) and deionized water. The closed aquarium system was illuminated by a Coralife™ metal 175-W halide bulb (Energy Savers Unlimited, Lafayette, CA, USA) on a 10:14-h light:dark photoperiod. Water changes of 10 L were made twice weekly with newly mixed seawater. In addition, 700 μl of Reef Success Vita Vitamins (Red Sea, Tel Aviv, Israel), 700 μl of Reef Success Iodine (Red Sea), and 700 μl of Reef Success Coral Trace (Red Sea) were added to the tank's protein skimmer with each water change. The corals were fed 5 ml of PhytoPlex (Kent Marine, GA, USA) once every other day.

Once the coral colonies were acclimatized to the laboratory setting for two weeks, 1- to 2-cm pieces were clipped from a single coral colony and glued individually to glass microscope slides using Instant Krazy Glue (Elmers, Columbus, OH, USA), which caused no deleterious effect to the coral (results not shown). The slides were returned to the aquaria, and the corals left to stabilize for two weeks before exposure. The experiment used a total of 16 coral fragments (i.e., four slides per exposure concentration).

Water-accommodated fraction

The WAF was generated using IFO 180 marine fuel oil samples, which were a generous donation from ExxonMobil Marine Fuels (New York, NY, USA) and Oil Testing Services (Lafayette, NJ, USA). Instant Ocean Sea Salt mix was combined with distilled water to create the 38-ppm salt water. Various concentrations of WAF were made separately using the variable-loading method instead of diluting a higher concentration solution [13,14]. One liter of salt water was added to 1-L, Teflon®-coated containers (Savillex, Minnetonka, MN, USA). Appropriate quantities of crude oil (0.25, 1, and 4 g) were weighed and added to each container along with the salt water. The solutions were mixed with a 4-cm, Teflon-coated stirbar on a magnetic stirrer for 24 h at ambient temperature. Additional solutions were prepared for each WAF concentration to allow water changes during the exposure period. Unaltered salt water was used as a control.

Exposure

One-liter glass beakers served as dosing chambers for the corals. The beakers were placed in heated water baths to maintain water temperature at 26°C, and glass Pasteur capillary pipettes were connected to individual aquarium air pumps and placed in each dosing chamber for aeration and water circulation. Four slides, each containing an individual coral piece, were placed at the bottom of each of the beakers. The slides were arranged in a square formation, as shown in Figure 1. Before addition of each WAF solution to the beaker, the solutions were poured into a separatory funnel to isolate the fraction of crude oil that did not dissolve in the seawater [14]. The coral samples were exposed to the various WAF concentrations for a 24-h period, with water changes performed every 8 h to avoid ammonia and nitrate accumulation for all exposed and reference groups [15].

Sample preparation

Samples were assayed as described previously [12,16]. After the 24-h exposure, each of the individual coral pieces was frozen and then ground to a fine powder in liquid nitrogen using a mortar and pestle. Approximately 0.5 g of the ground

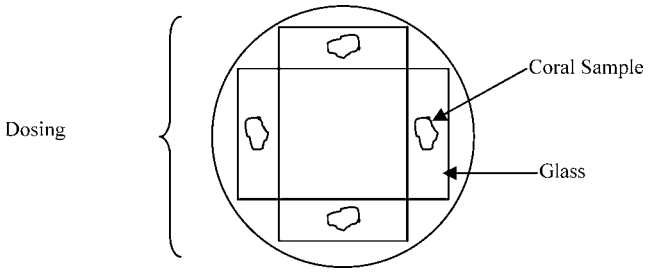

Fig. 1. Exposure of corals. Four slides, each containing an individual coral piece, were placed at the bottom of each of the exposure chamber. The slides were arranged in a square pattern as depicted. Corals were incubated with exposure solutions at 26°C with constant aeration.

sample powder was placed in a locking, 1.8-ml microcentrifuge tube along with 1,400 μl of a denaturing buffer consisting of 2% sodium dodecyl sulfate (SDS), 50 mM Tris(hydroxymethyl) aminomethane (pH 7.8), 15 mM dithiothreitol, 10 mM ethylenediaminetetra-acetate, 3% (w/v) polyvinylpolypyrrolidone, 0.005 mM salicylic acid, 0.001% dimethyl sulfoxide, 0.01 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 0.04 mM bestatin, 0.001 mM E-64, 2 mM phenylmethylsulfonyl fluoride, 2 mM benzamide, 5 μM amino-caproic acid, and 1 μg/100 μl of pepstatin A. Samples were then vortexed for 15 s, heated at 93°C for 6 min with occasional additional vortexing, and incubated at 25°C for 10 min. Samples were centrifuged (13,500 *g* for 8–10 min), and the middle-phase supernatant was aspirated and placed in a new Eppendorf tube [16] and subjected to a protein concentration assay according to the method described by Ghosh et al. [17].

Electrophoresis

One-dimensional SDS–polyacrylamide gel electrophoresis (PAGE) and Western blot analysis were used to optimize the separation of target proteins and to validate the use of specific antibodies with *P. damicornis* protein extracts [18]. Total soluble protein (15–40 μg) from three randomly prepared samples from the same coral colony was loaded onto a 12.5% SDS-PAGE preparative gel. A Tris(2-carboxyethyl) phosphine (neutral pH) concentration of 0.001 M was added to the gels [19]. All gels were blotted onto polyvinylidene fluoride membranes using a wet-transfer system [20,21]. The membranes were blocked in 5% nonfat dry milk and assayed with a primary antibody for 1 h. The blots were then washed in Tris-buffered saline four times and incubated in a horseradish peroxidase–conjugated secondary antibody solution for 1 h at a 1:30,000 titer (Jackson ImmunoResearch Laboratories, Westport, PA, USA). Blots were washed again four times in Tris-buffered saline and developed using a Western Lightning Plus (New England Nuclear, Shelton, CT, USA) luminol/hydrogen peroxide–based chemiluminescent solution and documented using a Genegnome luminescent documentation system (Syngene, Frederick, MD, USA). To ensure a minimum of nonspecific cross-reactivity, blots were developed for at least 3 min. Calibration of a quantitative standard showed that 0.05 attomole of target protein could be detected at this level of sensitivity (results not shown).

Enzyme-linked immunosorbent assay experiments

Once validated, antibodies and samples were optimized for the enzyme-linked immunosorbent assay using 384-well microplates in an $8 \times 6 \times 4$ factorial design [17,22] and assayed using a Beckman Coulter Biomek 2000 Laboratory Automa-

Table 1. Biomarker expression[a]

Cellular parameter	Control	0.25 g/L	1 g/L	4 g/L
Protein Metabolic Condition				
Hsp70 (cnidarian)	0.077 ± 0.019A	0.201 ± 0.039B	0.253 ± 0.055BC	0.286 ± 0.054C
sHsp (cnidarian)	BDL	0.048 ± 0.015B	0.117 ± 0.046B	0.113 ± 0.034B
Oxidative Damage and Response				
Cu/ZnSOD (cnidarian)	0.003 ± 0.001A	0.007 ± 0.001A	0.022 ± 0.011B	0.007 ± 0.001A
MutY	0.004 ± 0.001A	0.009 ± 0.003B	0.013 ± 0.003B	0.013 ± 0.003B
Porphyrin Metabolism				
Protopophyrinogen oxidase IX	0.029 ± 0.004A	0.064 ± 0.010B	0.044 ± 0.008A	0.043 ± 0.004A
Ferrochelatase (cnidarian)	0.030 ± 0.004A	0.060 ± 0.004AB	0.091 ± 0.031B	0.079 ± 0.021B
Neuroglobin (invertebrate)	2.348 ± 0.342A	3.688 ± 0.723AB	4.236 ± 0.830B	4.742 ± 0.725B
Heme oxygenase-1	0.036 ± 0.016A	0.174 ± 0.150AB	0.265 ± 0.130B	0.484 ± 0.035C
Xenobiotic Response				
CYP P450 1-class	0.002 ± 0.001A	0.067 ± 0.041AB	0.116 ± 0.053B	0.089 ± 0.018B
CYP P450 2-class	0.074 ± 0.010A	0.037 ± 0.015B	0.052 ± 0.015AB	0.037 ± 0.012B
CYP P450 6-class	0.056 ± 0.011A	0.198 ± 0.267A	0.038 ± 0.007A	0.033 ± 0.008A
GST (cnidarian)	0.0015 ± 0.0006A	0.031 ± 0.012B	0.035 ± 0.010B	0.032 ± 0.012B
MXR-1 (cnidarian)	0.006 ± 0.003A	0.028 ± 0.013B	0.054 ± 0.011C	0.055 ± 0.007C

[a] Treatment means with different uppercase letters differed significantly at $\alpha = 0.05$ using the three different posthoc tests described in *Materials and Methods*. All units, except where noted, are expressed as femol target analyte/ng total soluble protein. Values are presented as the mean ± standard error ($n = 4$). BDL = below detection limit; Cu/ZnSOD = copper/zinc superoxide dismutase-1; CYP = cytochrome; GST = glutathione-*S*-transferese; Hsp70 = heat shock protein 70; MutY = DNA glycosylase MutY homologue; MXR-1 = multixenobiotic resistance protein-1; sHsp = small heat shock proteins.

tion Workstation (Promega, Madison, WI, USA). Algal (i.e., dinoflagellate) and host extracts (cnidarian) were assayed using the following antibodies (with accompanying catalog numbers) from Envirtue Biotechnologies (Winchester, VA, USA): Algal anti–glutathione peroxidase (AB-1484), algal anti–manganese superoxide dismutase (AB-1501), algal anti–copper/zinc superoxide dismutase (AB-CZ1546), algal anti–glutathione-*S*-transferase (AB-1491), algal anti–heat shock protein 60 (AB-1506), anti–chloroplast small heat shock protein (AB-1), cnidarian anti–heat shock protein 60 (AB-1508), cnidarian anti–heat shock protein 70 (AB-Hsp70-1517), cnidarian anti–heat shock protein 90 (AB-Hsp90-1685), cnidarian anti–manganese superoxide dismutase (AB-1976), cnidarian anti–copper/zinc superoxide dismutase (AB-SOD-1516), cnidarian anti–glutathione peroxidase (AB-GPX-1433), cnidarian anti–small heat shock protein (AB-H105), cnidarian anti–ferrochelatase (AB-FC-1939), cnidarian anti–cytochrome P450 6-class homologue (AB-C6-2), cnidarian anti-metallothionein (AB-MM-10843), cnidarian anti–heme oxygenase-1 (AB-HO-1944), anti-ubiquitin (AB-U100), and anti–multixenobiotic resistance protein (ABC family of proteins). All samples were assayed in triplicate with intraindividual variation of less than 8% for the entire 384-well microplate (Table 1), and in addition, an eight-point calibration curve using a calibrant relevant to each antibody was plated in sextupilicate for each plate (results not shown). Based on data from our field study [12] and what we were able to detect with the antibodies listed above, we elected to focus our efforts on those biomarkers most likely to exhibit a response in this laboratory study.

Biomarkers

Cellular biomarkers serve as diagnostic tools to indicate variations in the physiological condition of an organism in response to environmental change. This is achieved by quantifying changes in the cellular and molecular processes of the cells. In the present study, certain biomarkers were grouped together for analysis. Each category encompasses biomarkers involved in a cellular process that would be expected to vary in response to specific types of stressors. The four diagnostic groups and incorporated biomarkers included Xenobiotic Response (cnidarian cytochrome P450 1-class [CYP P450 1-class], cnidarian cytochrome P450 2-class [CYP P450 2-class], cnidarian cytochrome P450 6-class [CYP P450 6-class], glutathione-*S*-transferase [GST-pi], and cnidarian multixenobiotic resistance protein-1 [MXR-1]), Porphyrin Metabolism (protoporphyrinogen oxidase IX [PPO], cnidarian ferrochelatase [FC], invertebrate neuroglobin homologue [globin], and heme oxygenase-1 [HO-1]), Oxidative Damage and Response (copper/zinc superoxide dismutase-1 [Cu/ZnSOD] and DNA glycosylase MutY homologue [MutY]), and Protein Metabolic Condition (heat shock protein 70 [Hsp70] and cnidarian small heat shock proteins [sHsp]).

Statistical analysis

Normality of the data was tested using the Kolmogorov–Smirnov test with Lilliefors' correction, and equality of variance was verified using the Levene median test. A one-way analysis of variance (ANOVA) was used if the data were found to be normally distributed and homogeneous. However, if the data did not meet the requirements for homogeneity of variances for the one-way ANOVA, the Kruskal–Wallis one-way ANOVA on ranks was used to compensate. The Tukey–Kramer honestly significant difference method or the Dunn's post hoc test was used when significant differences between the treatment means were found, depending on the variances [23,24]. Statistical significance was defined as $p < 0.05$.

We used canonical correlation analysis (CCA) as a heuristic tool to illustrate how biomarkers could be used to discriminate among populations. The CCA is a method of eigen analysis that reveals the basic relationship between two matrices—in our case, those of four exposures and the biomarker data. The CCA provided an objective statistical tool for determining if exposures were different from one another using sets of cellular biomarkers indicative of a cellular process and, if so, which biomarkers contributed to those differences. This analysis required combining data from all four exposures into one matrix, which we did by expressing biomarker response in a given population as a proportion of their mean levels.

RESULTS

Significant changes were seen in CYP P450 1-class, CYP P450 2-class, GST-pi, and MXR-1, all of which are involved in the cellular response, manipulation, and excretion of toxicants. Likewise, we observed changes in the expression of biomarkers involved in porphyrin production (e.g., PPO and FC) and porphyrin destruction (e.g., HO-1 and globin), which are necessary for cell protection. The corals' responses to oxidative stress and damage were evaluated through alterations in Cu/ZnSOD and MutY concentrations, and changes in Hsp70 and sHsp indicated an adjustment in the cells' production of proteins. Finally, the present results are consistent with preliminary results from our earlier trials at these exposure levels.

Xenobiotic response

Although CYP P450 1-class expression was elevated for all the exposure levels (Table 1), significant differences between the control and the WAF concentrations were seen only in the 1 and 4 g/L treatments. Levels in the 0.25 g/L exposure were not significantly increased. In contrast, the dose responses for CYP P450 2-class showed a significant decrease in expression from the control at exposures of 0.25 and 4 g/L, but not for the 1 g/L (0.052 ± 0.015 fmol/ng total soluble protein) exposure (Table 1). No difference from the control was observed among any of the CYP P450 6-class exposures (Table 1).

The expression of GST-pi increased significantly for all exposures in a nearly identical manner. The MXR-1 expression was significantly higher than that in the control for all the exposures. Among the exposures, a twofold increase was observed between 0.25 g/L and the two higher exposures of 1 and 4 g/L.

Porphyrin metabolism

Protoporphyrinogen oxidase IX expression among control specimens was not significantly different from that in corals in the 1 and 4 g/L exposures. However, expression in the 0.25 g/L exposure was significantly higher than that observed in all other exposures (Table 1). Conversely, FC expression was significantly elevated in the 1 and 4 g/L exposures, but not in the 0.25 g/L treatment. Likewise, globin was significantly increased in the 1 and 4 g/L exposures, but not in the 0.25 g/L exposure (Table 1). Heme oxygenase-1 expression, despite the apparent increase between the control and 0.25 g/L exposure, was not significantly different among treatments. However, levels in corals from the 1 and 4 g/L exposures were significantly increased compared to those of the control corals and to each other (Table 1).

Oxidative damage and response

Copper/zinc superoxide dismutase-1 control expression was not significantly different from corals in the 0.25 and 4 g/L exposures. However, the 1 g/L treatment samples showed a significant elevation from the control and the other exposures. The MutY expression was significantly increased from the control in all exposures (Table 1).

Protein metabolic condition

The Hsp70 expression was significantly elevated for all exposures compared to the control coral specimens. Moreover, a statistically significant increase was further noted at concentrations higher than 0.25 g/L. Despite a significant difference from the control, the 1 g/L exposure did not differ from the 0.25 and 4 g/L exposures. The sHsp expression revealed a significant increase in all exposures (Table 1).

DISCUSSION

Exposure to petroleum mixtures may result in a number of pathologies, ranging from anemia (interference in heme metabolism and function) to degeneration of cilia in gastrodermal cells in both mammals and cnidarians [25]. These pathologies arise as a result of mechanistic toxicities occurring on the biochemical and subcellular levels. Examining biomarkers that reflect the performance of these biochemical and subcellular processes can generate a dose-dependent profile of the types of stressors affecting the organism and facilitate a deeper comprehension of the nature of the toxicological mechanisms associated with a petroleum exposure. A serial concentration-exposure experimental design, as reported herein, allowed us to test for the occurrence of a dose–response behavior of the organism to the toxicant and demonstrated the occurrence of an emergent response to increasing concentrations (e.g., accumulation of protoporphyrin at low-concentration exposure and hemolytic anemia at higher-concentration exposure) [26]. Furthermore, understanding how key metabolic and cellular responses are affected by a serial exposure to IFO 180 marine fuel also may support a more accurate prediction of how higher-order physiological processes may behave.

Xenobiotic response

When any noxious pollutants or xenobiotics enter a cell and its compartments, the cell initiates the process of biotransformation to avoid injury. This biological process is composed of three sequential steps or phases, involving specific proteins.

Phase-1 responses include enzymatic reactions that alter the xenobiotics through addition or exposure of polar groups, such as hydroxyl, carboxyl, thiol, and amino, on the toxicant. This is a necessary step for the phase-2 proteins to interact with the altered xenobiotics. Reactions such as oxidation, reduction, and hydrolysis are used for the polar group tags [27–29]. Phase-3 enzymes allow the xenobiotics to be exported out of the cell. However, it is possible, depending on the reaction, that an even more toxic metabolite is formed [29]. Our study focused predominantly on the superfamily of cytochrome P450s, which are mainly associated with oxidative and hydrolytic processes during phase 1 [28].

Cytochrome P450s are heme-containing monooxygenases. In many species, most members of this superfamily are involved in the metabolism of xenobiotics; however, a limited number of cytochrome P450s are associated with biosynthetic pathways of steroid and bile acid production [30,31]. In the present study, we focused on specific families of cytochrome P450s, namely the CYP P450 1-, 2-, and 6-classes. These particular classes were chosen because of their metabolic interaction with specific kinds of molecules. Cytochrome P450 1-class associates with PAHs [31], whereas CYP P450 2-class interacts with a wide range of steroids and xenobiotic substances [32] and CYP P450 6-class with pesticides containing chlorinated side chains [32,33].

Canonical correlation analysis indicated significant changes in the response to the WAF exposure (Fig. 2). For the cytochrome P450s, these changes were found in the responses of CYP P450 1- and 2-class comparisons (Fig. 2A). In the CYP P450 1-class, this response suggests that the corals were reacting to the PAH as well as to benzene, toluene, ethylbenzene, and xylene, all of which are components of the IFO 180. It

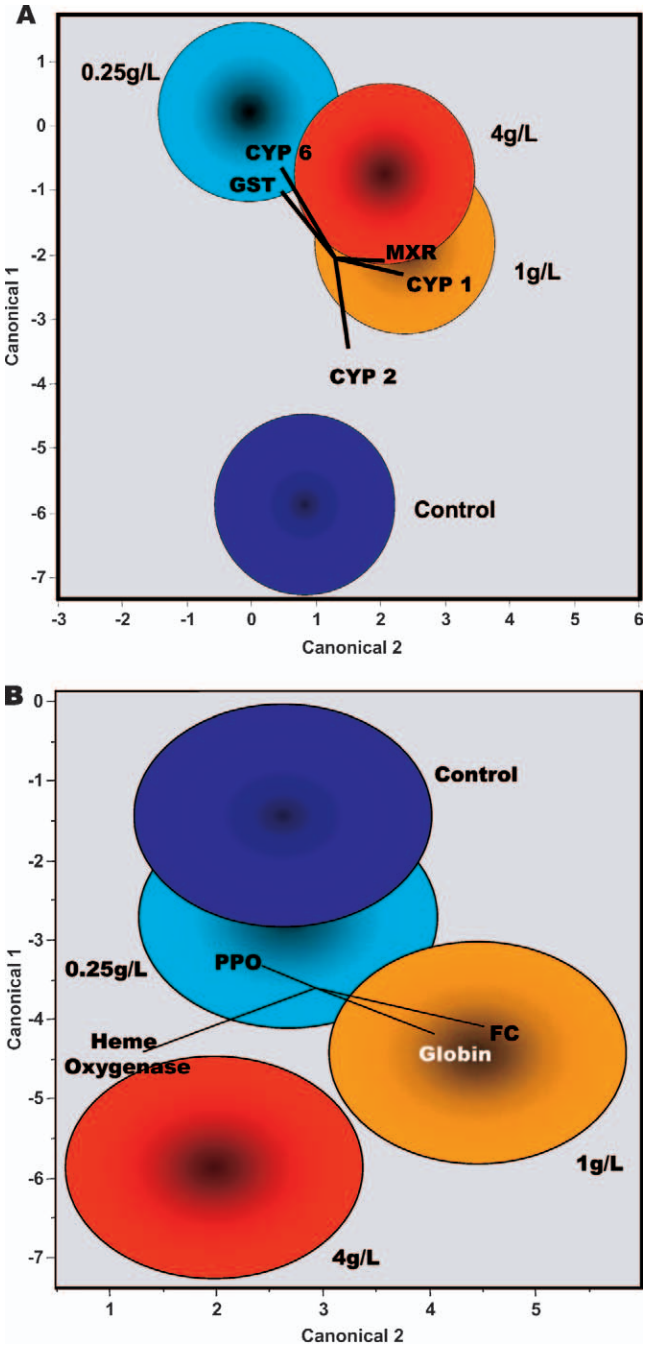

Fig. 2. Xenobiotic response and porphyrin metabolism biomarkers. Original variates were biomarker levels expressed as a percentage of the control value in each treatment. Circles show the 95% confidence intervals around the distribution centroid of each stressor. Biplot rays radiating from the grand mean show the directions of the original biomarker responses in canonical space. Overlapping centroids indicates that those populations are not significantly different from one another; nonoverlapping centroids indicate a statistically significant difference ($p < 0.05$). (**A**) Canonical centroid plot of Xenobiotic Response biomarkers (see *Materials and Methods*). (**B**) Canonical centroid plot of Porphyrin Metabolism biomarkers (see *Materials and Methods*). CYP = cytochrome; FC = cnidarian ferrochelatase; GST = glutathione-*S*-transferase; MXR = multixenobiotic resistance protein 1; PPO = protoporphyrinogen oxidase IX.

also was observed that a significant toxicity response was generated above the 1 g/L level. However, no dose–response effect was noticed as the WAF concentrations increased (Table 1). The response levels could be related to the amount of WAF of the fuel oil in the solution. In the 0.25 g/L solution, the concentration of aromatic hydrocarbons would be less than that found in preparations at the higher concentrations. It may be that CYP P450 1-class is a good indicator of the levels of aromatic hydrocarbon compounds in the environment. There also is a possibility that the cell becomes saturated in the process of producing CYP P450 1-class regardless of an increase in PAH exposure. The CYP P450 2-class levels were decreased for all doses tested (Table 1). This decrease in expression could correspond to a shift in subcellular response systems to compensate for the increase in the CYP P450 1-class elevation. Some fuel oil compounds in IFO 180 also could have adverse effects on the ability of cells to produce CYP P450 2-class enzymes, causing the observed decrease. No change in expression was seen for the CYP P450 6-class. This was expected, because the exposure to IFO 180 fuel oil lacks the chlorinated compounds that would elicit a response.

The new polar metabolites from phase 1 are a threat to the cell, because they can interact with or become embedded in the membrane. To prevent this, in phase 2, the metabolites are conjugated with endogenous substrates, such as glutathione, sulfates, acetates, and glucuronides [34]. The reactions of this phase cause the compounds to become more water soluble and, thus, capable of being excreted from the cell [35,36]. Of particular interest is the enzyme family of glutathione-*S*-transferases, which has been associated with the detoxification process in aerobic animals. The phase-2 enzyme, glutathione-*S*-transferase, primarily catalyzes the conjugation of electrophilic compounds, such as PAHs, with the thiol group of glutathione [35]. This reaction decreases the reactivity of the compounds with other molecules in the cell [37]. We focused on one of the four main classes of glutathione-*S*-transferase, GST-pi, which has been described in cnidarians [25]. Activity of GST-pi in our experiment was significantly elevated in response to exposure to the WAF, revealing activation of the detoxification response.

The third and final phase is the elimination of the altered metabolites. Depending on the end product, several different pathways exist. Compounds can be transported to the lysosome for degradation, sequestered in lysosome-like structures for containment, or ultimately, excreted from the cell [38,39]. Specific proteins, which help in the transport of xenobiotics out of the cell, are adenosine triphosphate–binding cassette transporters. One such phase-3 enzyme is MXR-1, which is used to export glutathione-conjugated compounds out of the cell [38]. An increase in the CYP P450 1-class (phase-1) as well as the GST-pi (phase-2) enzymes leads to an increase in the expression of MXR-1, as was observed. Although no dose–response effect was observed, the significant difference in the highest doses of the WAF (1 and 4 g/L) is in agreement with the responses seen in CYP P450 1-class.

Porphyrin metabolism

Is there a significant shift in porphyrin metabolism and catabolism as a result of increasing concentrations of IFO 180 WAF? Canonical correlation analysis indicated that the 0.25 g/L of IFO 180 WAF treatment did not cause a significant shift in porphyrin metabolism, but higher concentrations of IFO 180 WAF did (Fig. 2B). Porphyrins are heterocyclic molecules consisting of four pyrrole rings joined by methane bridges. Depending on the porphyrin, different substituents can be found on the ring. The biomarkers assessed included enzymes responsible for the synthesis of porphyrins or their deg-

radation. We focused on the final two steps of the porphyrin-synthesis pathway. Protoporphyrinogen oxidase IX is involved with the penultimate step of porphyrin production, and FC catalyzes the final step. In the penultimate step of porphyrin synthesis, coproporphyrinogen III is oxidized into protoporphyrin IX [40]. The final step is the insertion of iron into the porphyrin ring by FC to form a hemin [41]. An increase in protein levels of PPO and FC would be indicative of an increased demand in porphyrin anabolism, or it could result as a compensatory reaction of inhibited activities of PPO and FC enzymes. An increase in globin concentrations indicates either an increased demand for cellular oxygen, or it reflects increasing inhibitory activity of globin by petroleum components. The behavior of the protein levels of HO-1 in response to IFO 180 WAF exposure is similar to the behavior seen in other studies of HO-1 and petroleum exposure, suggesting an increased demand in heme degradation, most likely as a result of iron/porphyrin dissociation via PAH interactions [42].

Oxidative damage and response

Reactive oxygen species, such as superoxide radical anions and hydroxyl radicals, can stress an organism and cause cellular damage. Cells respond to this stress by releasing antioxidants to suppress these oxygen species [43]. Copper/zinc superoxide dismutase-1 is an antioxidant enzyme that protects the cell by catalyzing superoxides into hydrogen peroxide, which is then scavenged by further antioxidant pathways for further disposal [44]. In the present study, we observed a significant elevation of Cu/ZnSOD in the 1 g/L exposure concentration, whereas corals in the other exposures only exhibited a slight increase that was not statistically significant. The most reasonable explanation is that the cells became saturated at higher doses, which in turn inhibited the Cu/ZnSOD mechanism.

Reactive oxygen species also can cause damage at the subcellular level, producing lesions on the DNA. For example, MutY is involved in the base excision repair pathway of cells, and it is responsible for repairing oxidative damage to DNA [45]. The significant elevation that we observed among all the exposures could be indicative that DNA repair is occurring.

Protein metabolic condition

Protein production and turnover is a vital part of cellular homeostasis. Under normal conditions, when proteins leave the ribosome, they are bound by chaperones that help to ensure that the protein correctly folds into the native form. The chaperones also bind to misfolded or denatured proteins and prevent them from aggregating and causing cell damage. However, under stress conditions, the number of denatured proteins increases. In response, the cell up-regulates the genes that express the chaperones and are indicative of protein metabolic stress. Heat shock protein 70 is a cytosolic chaperone that binds to denatured proteins [46]. Small heat shock proteins are intrinsic chaperones, which are vital to protein conformation. The Hsp70 exhibited a significant dose–response effect as the IFO 180 WAF concentrations increased. Likewise, sHsp expression was significantly elevated in corals at all doses tested. However, no differences in expression among the increasing WAF concentrations were observed. Although this can signify that the coral samples were, indeed, stressed in their exposure to the WAF solutions and that a shift from metabolic homeostasis had occurred, both heat shock protein biomarkers are indicative of a general, nonspecific stress response that may be caused by other environmental factors.

Although not unexpected, the present results bear a remarkable similarity to those of our previous study of an oil spill on a coral reef in Yap [12]. In that study, we collected coral samples (*Porites lobata*) approximately 75 d after the spill, and we assayed a suite of biomarkers, including many of those investigated during the present study. Among biomarkers of xenobiotics response, we noted that all the biomarkers that were elevated in the present study (e.g., CYP 450 1-class, CYP 450 2-class, MXR-1, and GST-pi) also were elevated in corals collected at Yap approximately 75 d after the oil spill. We also noted that CYP 450 6-class was not elevated in our present study, and the results were equivocal in the corals collected from the impacted site at Yap. The CYP 450 6-class levels were not significantly different from those expressed by corals at one of the reference sites. Again, this is not surprising, in that CYP 450 6-class interacts with the chlorinated side chains of pesticides [32,33]. It was expected that the level of expression of the various biomarkers (e.g., CYP 450 1-class) would vary between our field [12] and laboratory studies. Such differences likely result from interspecific variation, duration of exposure, laboratory versus environmental conditions, and so on. Finally, the narrow range of biomarkers examined in the present study was informed by the results from the field study and was adequate to demonstrate the impact of oil on corals under laboratory conditions.

Likewise, PPO levels did not differ significantly from control samples in our laboratory study, and the levels were not elevated among impacted corals in Yap when compared to corals collected from two reference sites. However, levels of FC and HO-1 were significantly elevated in exposed corals in both studies. Thus, porphyrin metabolism appears to be affected in a similar manner in both situations.

Our comparative evaluation of oxidative damage and response exhibited a remarkable similarity to our observation of xenobiotic response. In fact, MutY was elevated in both laboratory-exposed corals and in situ–exposed corals. However, levels of Cu/ZnSOD produced somewhat ambiguous results in the laboratory, because control samples did not differ significantly in corals from high and low exposure concentrations but did differ significantly from the intermediate exposure level. In the field study, we observed similar ambiguity, because the elevations observed in the exposed corals only differed significantly from one of the two reference sites utilized. The low number of samples and inherent variability of the data likely limited our ability to detect significant differences.

Finally, levels of two key protein chaperones, Hsp70 and sHsp, were elevated in both exposure situations compared to levels in the control/reference corals. Again, these results also demonstrate a remarkable similarity between independent laboratory and field studies.

These results clearly demonstrate the utility of this approach across the two coral species. Specifically, it is of considerable significance that almost without exception, we observed a parallel response in two different species of corals to laboratory and in situ oil exposures for a variety of biomarkers, the obvious implication being that biomarkers and, in particular, suites of biomarkers have the potential to accurately predict toxic insults in coral reef ecosystems.

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