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ARTICLE *in* ENVIRONMENTAL SCIENCE AND TECHNOLOGY · AUGUST 2002

Impact Factor: 5.33 · DOI: 10.1021/es011433k · Source: PubMed

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Environ. Sci. Technol. **2002**, *36,* 2987−2993

Cellular Physiological Assessment of Bivalves after Chronic Exposure to Spilled *Exxon Valdez* Crude Oil Using a Novel Molecular Diagnostic Biotechnology

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The objective of this study was to determine the cellular physiological status of the bivalves *Mya arenaria* and *Mytilus trossulus* in an area experiencing a 10-yr chronic exposure of spilled *Exxon Valdez* crude oil in Prince William Sound. Bivalves were collected from well-characterized oiled and unoiled sites. We used a novel biotechnology (Environmental Cellular Diagnostic System) to determine (i) if bivalves were physiologically stressed, (ii) the nature of the altered physiological state, and (iii) whether the bivalves were responding to an exposure of polyaromatic hydrocarbons (PAH). Molecular diagnostic analysis indicated that bivalves at the oiled site were experiencing both oxidative and xenobiotic stress, resulting in increased protein turnover and chaperone activity. Bivalves from the impacted area were responding specifically to a PAH-xenobiotic exposure and accumulating protein−PAH adducts. Finally, species-specific responses were observed that could be related to the habitat preferences of each species. We conclude that bivalves inhabiting a site impacted by crude oil from the 1989 *Exxon Valdez* spill showed clear indications of cellular physiological stress.

Introduction

Short-term effects of crude oil spills are obvious and readily documented (*1, 2*). Long-term effects and recovery processes of impacted species and ecosystems are much more difficult to recognize and demonstrate (*3*−*7*). One general issue concerning both short- and long-term effects of oil exposure is the recovery of a population or an ecosystem after a spill event. In this context, we define "recovery" as a period of time when impacted populations stabilize and begin to track natural fluctuations observed at nonimpacted habitats (*8*−*13*). Measurements of recovery include, but are not limited to, convergence in community composition at impacted and nonimpacted sites, tracking of population dynamics, and assessment of physiological-level parameters (e.g., refs *5*, *7*, and *14*−*16*). Because of differences in the sensitivity and relevance of these diverse methods in assessing recovery, the results derived from these studies oftentimes can produce conflicting conclusions (e.g., refs *2*, *5*, *6*, and *13*).

In 1989, the T/V *Exxon Valdez* ran aground on Bligh Reef in Prince William Sound (PWS), AK, spilling nearly 42 million L of crude oil (*17*). By 1993, less than 2% of the spilled oil remained on PWS beaches, although residual oil still persisted on many of the impacted shorelines (*18*−*20*). The spill event resulted in the establishment of several long-term monitoring programs, which generally encompassed both oil-impacted and nonimpacted locations (*13*). Studies using various methodologies have grappled with the issue of physiological, population, community composition, and habitat level recovery from the *Exxon Valdez* spill event (e.g., refs *5*−*7* and *19*−*22*). Results from these studies reflected fundamental differences in the ability of different methods to assess recovery and, more importantly, indicated that different aspects of recovery progress at different rates (*5*, *6*, *22*−*24*). Such complexity in recovery processes makes assessing the long-term effects of environmental insults extremely difficult (*25*−*31*).

We examined cellular-level stress in two PWS bivalve species, *Mya arenaria* and *Mytilus trossulus*, using a novel cellular diagnostic technology. This technology, termed the Environmental Molecular Diagnostic System (ECDS), was developed to diagnose cellular physiological condition, monitor stress responses, identify putative stressors, and forecast outcomes of environmental problems (*32*−*35*). The ECDS assays specific cellular and molecular parameters indicative of physiological condition, including (but not limited to) membrane integrity and composition (e.g., lipid peroxidation products), anti-oxidant redox potential (e.g., glutathione redox status), chaperone activity (e.g., heat-shock proteins 60 and 70), enzymatic anti-oxidants (e.g., catalase, superoxide dismutases, glutathione peroxidases), xenobiotic detoxification pathways (e.g., cytochrome P450 family, P-glycoprotein 160), metal-regulatory proteins (e.g., metal-lothionein, ferritin, porphoryn), protein status and turnover (e.g., ubiquitin, protein carbonyl formation), and genomic and translational integrity (e.g., DNA abasic phosphate site formations). These parameters quantify specific cellular physiological functions, including (i) whether the structural integrity of the cell is compromised, (ii) the type or nature of the stress (e.g., oxidative stress), and (iii) whether defenses have been mounted against a particular stress (i.e., pesticide, acidity, heavy metal, PAH).

Determining the nominal (nonstressed) level of a particular biomarker within a species requires reference values from healthy individuals sampled from both laboratory and field studies for that species (*25*, *32*−*34*, *36*). Because of a current absence of information regarding nominal ranges for the diagnostic markers utilized in this study for these two species, we assumed that diagnostic marker ranges in a population from an unoiled area were representative of a healthy (i.e., unaffected by oil exposure) population, based on inferences from physical and biological studies done at these sites for the past 10 yr (*13*). To address the question of whether bivalve populations continue to be affected by oil from the *Exxon Valdez* spill, we examined differences in molecular marker profiles in populations of *M. arenaria* and *Mt. trossulus* inhabiting an *Exxon Valdez* oil-impacted spill

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10.1021/es011433k CCC: $22.00
Published on Web 05/25/2002

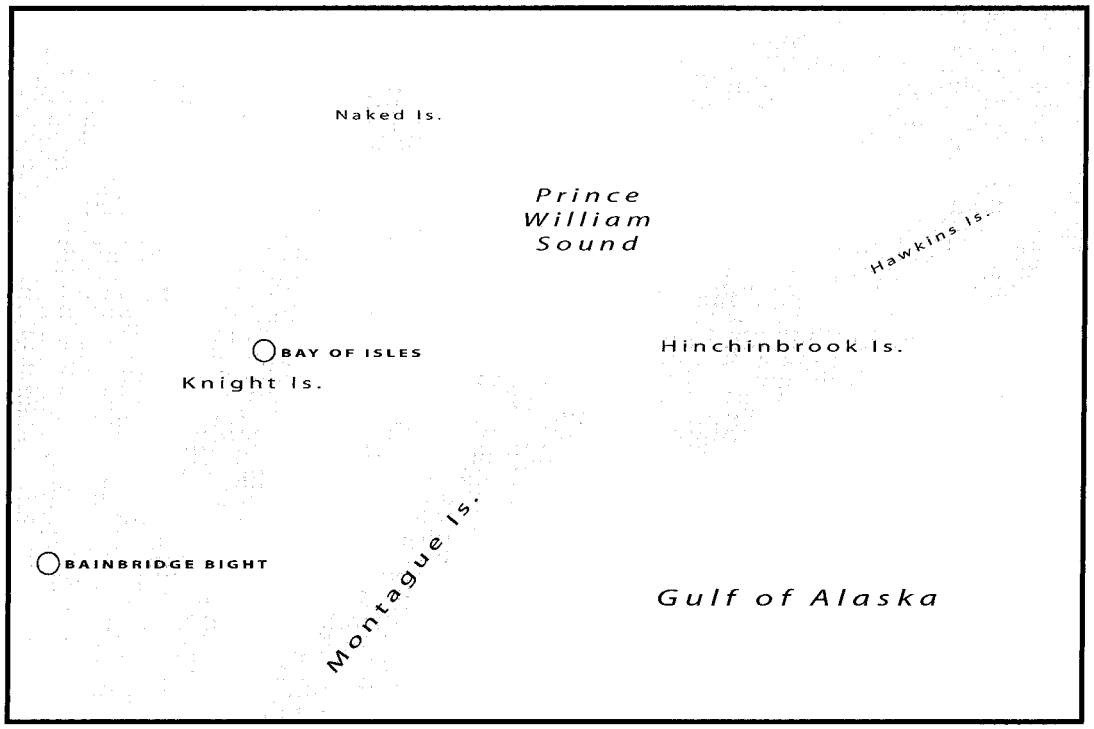

FIGURE 1. Map of Prince William Sound showing areas of bivalve collection.

site in Bay of Isles, Knight Island, and an unimpacted site along the northern coast of the Bainbridge Passage (Figure 1).

Experimental Section

All chemicals for buffered solutions were obtained from Sigma Chemicals Co. (St. Louis, MO). PVDF membranes and IP 96-well multiscreen plates were obtained from Millipore Corp. (Bedford, MA). Dot blot equipment was obtained from Bio-Rad Corp. (San Diego, CA). Antibodies against Cu/Zn superoxide dismutase (Cu/Zn SOD; catalog no. AB-S101-IU), cytochrome P450-2 class (CYP P450; catalog no. AB-P450-2A), heat-shock protein 60 (Hsp60; catalog no. AB-H100-N), heat-shock protein 70 (Hsp70; catalog no. AB-H101-MU1), heat-shock protein 90α (Hsp90; catalog no. AB-H102-MUA), glutathione peroxidase (GPx; catalog no. AB-G101-MU), glutathione *S*-transferase (GST; catalog no. AB-G100-MU), 4-hydroxy-2*E*-nonenal-adducted protein (HNE; catalog no. AB-HNE), malondialdahyde-adducted protein (MDA; catalog no. AB-MDA), manganese superoxide dismutase (Mn SOD; catalog no. AB-S100-MM), small heat-shock protein domain of all four classes of molluscan small heat-shock proteins (sHsp; catalog no. AB-H103), and ubiquitin (catalog no. AB-U100) were obtained from Envirtue Biotechnologies, Inc. (Walnut Creek, CA). Anti-rabbit conjugated horseradish peroxidase antibodies were obtained from Promega (Madison, WI). Quantitative standards for each of the ELISAs were obtained from Envirtue Biotechnologies, Inc.

Study Locations and Collection of Bivalves. Study sites were selected on the basis of known oiling history and presence of targeted bivalve species (*13*). To evaluate ECDS in the context of spill response and assessment, a heavily impacted site (defined by chemical profiling over time) was targeted for sampling. The Bay of Isles sampling location, on the east side of Knight Island in PWS, was particularly heavily oiled in 1989 (*13*). The tidal marsh presented several cleanup challenges because of extensive oiling, because of its restricted exposure to physical weathering, and because it retained a large amount of spilled oil in its peat-enriched substrate. By the year 2000, the remaining oil had decreased significantly in concentration, but pockets of oil could still be found and liberated to the water's surface in the form of sheen or discrete globules when sediments were disturbed (*13*). Despite the obvious residual oiling at this site, two species of bivalves were readily found: the mussel *Mt. trossulus* and the clam *M. arenaria*. Mussels were common on the surface of the marsh sediment and attached to the common intertidal brown alga *Fucus gardneri*. Clams were found in the upper 20 cm of the marsh sediment.

Physical and biological conditions at the Bay of Isles sampling location were compared to those at a site situated at the southwestern end of Bainbridge Passage, near Point Waters (*13*). The Bainbridge location was not oiled (confirmed by review of initial shoreline survey records and a decade of chemical monitoring under the NOAA long-term monitoring program; *13*), was relatively sheltered from wave exposure, and supported populations of the two bivalves (*Mytilus* and *Mya*) targeted for study.

At each of the two sampling locations, 10 bivalves of both species were collected, and the samples were split for ECDS analysis. Samples were collected in late June 2000.

Individuals were shucked, and whole soft tissues were sealed in individually labeled 1.8-mL cryogenic vials, which were placed in liquid nitrogen shippers for freezing, storage, and shipment. Samples were sent to the analytical facility under "blind" conditions and were stored in −75 °C freezers until analysis.

Sample Preparation, ELISA, Densitometric Analysis, and SDS−PAGE/Western Blotting. Bivalves (*M. arenaria* and *Mt. trossulus*) were individually ground in liquid nitrogen to a fine powder using a mortar and pestle and suspended in Envirtue's denaturing SDS buffer (catalog no. DB100). Individual samples were vortexed for 30 s, incubated at 85 °C for 5 min, revortexed, and then centrifuged at 10000*g* for 5 min. Total soluble protein concentration of samples was assayed by the method of Ghosh et al. (*38*).

Samples were assayed for BPDE, Cu/Zn SOD, CYP P450, GPx, GST, HNE, Hsp60, Hsp70, Hsp90, MDA, Mn SOD, sHsp, and ubiquitin using Envirtue Biotechnologies, Inc. ELISA-based analysis service with a chemiluminescent reporter

TABLE 1. Summary of Biomarker Analyses of *Mya arenaria* from Bainbridge and Bay of Isles Sites[a]

marker	nonimpacted area Bainbridge mean	nonimpacted area Bainbridge SE	impacted area Bay of Isles mean	impacted area Bay of Isles SE	significant difference?
BPDE (relative units/mg of TSP)	nd	nd	35.133	17.486	yes + ($p < 0.0001$)[w]
Cu/Zn SOD (ng of SOD/mg of TSP)	1.379	0.388	4.957	0.822	yes + ($p < 0.0001$)
Cyt P450 (ng of CYP/mg of TSP)	14.473	4.324	71.243	4.695	yes + ($p < 0.0001$)
GPX (ng of GPX/mg of TSP)	37.671	11.005	45.869	3.728	yes + ($p < 0.05$)
GST (ng of GST/μg of TSP)	113.381	55.744	134.568	35.740	ns
HNE (ng of HNE/mg of TSP)	16.028	1.466	39.660	13.065	yes + ($p < 0.002$)
Hsp60 (ng of Hsp60/μg of TSP)	6.024	3.313	10.004	1.180	yes + ($p < 0.005$)
Hsp70 (ng of Hsp70/μg of TSP)	36.688	30.259	318.557	73.893	yes + ($p < 0.0001$)
Hsp90 (ng of Hsp90/μg of TSP)	27.587	14.788	41.127	17.351	ns
MDA (ng of MDA/μg of TSP)	2.877	6.072	8.829	10.301	yes + ($p < 0.005$)[w]
MnSOD (ng of SOD/μg of TSP)	76.218	54.893	94.503	61.074	ns
sHsp (ng of sHsp/μg of TSP)	nd	nd	43.439	18.278	yes + ($p < 0.0001$)[w]
ubiquitin (ng of ubiquitin/mg of TSP)	6.852	1.381	49.574	9.753	yes + ($p < 0.0001$)

[a] For each biomarker, the comparison between nonimpacted area and impacted area was made using either a one-way ANOVA (for data that is parametric) or the Wilcoxon signed ranks test (for data that is nonparametric). Nonparametric analyses are denoted by the superscript w. Plus and minus signs denote an increase and decrease in biomarker levels, respectively. TSP denotes total soluble protein. ns refers to nonsignificant. nd stands for below the level of detectability.

TABLE 2. Summary of Biomarker Analyses of *Mytilus trossulus* from Bainbridge and Bay of Isles Sites[a]

marker	nonimpacted area Bainbridge mean	nonimpacted area Bainbridge SE	impacted area Bay of Isles mean	impacted area Bay of Isles SE	significant difference?
BPDE (relative units/mg of TSP)	nd	nd	nd	nd	
Cu/Zn SOD (ng of SOD/mg of TSP)	1.762	0.382	0.764	1.421	yes − ($p < 0.05$)
CytP450 (ng of CYP/mg of TSP)	11.465	10.533	57.605	10.103	yes + ($p < 0.0001$)
GPX (ng of GPX/mg of TSP)	127.200	46.206	41.245	7.662	yes − ($p < 0.0003$)
GST (ng of GST/μg of TSP)	47.408	28.688	127.189	78.195	yes + ($p < 0.008$)
HNE (mg of HNE/mg of TSP)	7.960	4.090	39.029	12.578	yes + ($p < 0.0001$)
Hsp60 (ng of Hsp60/μg of TSP)	6.359	0.112	7.816	3.218	ns
Hsp70 (ng of Hsp70/μg of TSP)	112.590	23.588	228.718	59.775	yes + ($p < 0.0001$)
Hsp90 (ng of Hsp90/μg of TSP)	14.672	4.856	39.477	48.001	ns
MDA (ng of MDA/μg of TSP)	nd	nd	17.026	14.873	yes + ($p < 0.0001$)[w]
Mn SOD (ng of SOD/μg of TSP)	11.825	13.770	222.405	267.416	yes + ($p < 0.03$)
sHsp (ng of sHsp/μg of TSP)	4.157	1.942	29.527	12.586	yes + ($p < 0.0001$)
ubiquitin (ng of ubiquitin/mg of TSP)	4.606	1.480	13.298	6.134	yes + ($p < 0.0009$)

[a] For each biomarker, the comparison between nonimpacted area and impacted area was made using either a one-way ANOVA (for data that is parametric) or the Wilcoxon signed ranks test (for data that is nonparametric). Nonparametric analyses are denoted by the superscript w. Plus and minus signs denote an increase and decrease in biomarker levels, respectively. TSP denotes total soluble protein. ns refers to nonsignificant. nd stands for below the level of detectability.

system. Five hundred nanograms of total soluble protein (TSP) were loaded per well per sample. All samples for each assay were done in triplicate. Quantitative standard series also were done in triplicate for each assay. ELISA procedures were comparable to those reported in refs *32*−*35*.

Once developed, blots or plates were scanned into a computer and analyzed using NIH image software (http://rsb.info.nih.gov/nih-image). A serial dilution of purified protein for each cellular parameter was included in each assay to allow sample quantification and quality control. Concentration standards for each assay were determined, and a quadratic or polynomial equation was used to determine the concentration of each sample.

Three samples from each population were subjected to SDS−PAGE/immunoanalysis for each of the antibodies to ensure that antibodies cross-reacted with the target protein and that nonspecific cross-reactivity was absent as described in refs *33*−*35* (data not shown; for a copy of the report that includes these data, please contact Gary Shigenaka, U.S. NOAA, at gary.shigenaka@noaa.gov).

Statistical Analysis. This study used 10 animals per site with only one set of sampling during late June. Power calculations were made using preliminary studies of oil exposure in bivalves in laboratory settings and determined that 6−7 samples per condition would provide the necessary statistical power for identifying statistical differences. All biomarker measurements were made in triplicate on each individual sample. Means and standard deviations were calculated for each sample for each biomarker. Means and standard error of means of each site for each biomarker were calculated using the means from each sample for each biomarker and are presented in Tables 1 and 2.

Statistical analyses were performed to determine whether individual cellular biomarkers in clams (*M. arenaria*) or in mussels (*Mt. trossulus*) differed between a nonimpacted site (Bainbridge) and an impacted site (Bay of Isles). Two statistical methods were used to test this question. If the data were normally distributed and homogeneous, a one-way analysis of variance (ANOVA) was employed. Homogeneity of variances was tested by Hartley's F_{max} test (*39*), and normality was tested by graphical assessment of goodness of fit using JMP software (version 4.0.4). If the data were not parametric, as for cases in which undetectable levels of biomarkers resulted in means and variances of zero, the nonparametric Wilcoxon signed ranks test was used (*39*). To maintain the experiment-wise error rate at the desired level ($\alpha = 0.05$), a Dunn−ŠidáK method was used to calculate a new $\alpha' = 0.0036571$ for each hypothesis test (*39*). Combined,

these methods formed a conservative analysis of the data. No additional tests for correlations between the various biomarkers were performed, although results from this study may provide the basis for future explorations, using additional specific biomarkers, into correlations between those biomarkers that are connected in cellular metabolic pathways.

Results and Discussion

In clinical diagnostics, deviation of levels for a cellular parameter from the "nominal" range indicates an altered state. This altered state is defined as a "diseased" state for the individual or population only if that phenotype is associated with a deteriorating condition that ultimately affects the "performance" of the individual or population. Performance is defined by individual fitness, reproductive fitness, immunological fitness, metabolic integrity, and other similar parameters (*40*). Hence, a diseased state for an individual or population may be defined as a condition of *abnormal vital function involving any structure, part, or system of that individual or population* (*41*). On the basis of a number of monitoring and experimental studies, we assumed that individuals from the Bainbridge reference site would possess nominal nonoil-impacted levels for each parameter.

Benzo[*a*]pyrene can be enzymatically converted into an activated diol epoxide that can adduct with DNA, RNA, and protein and is a reliable biomarker of biologically relevant PAH exposure (*42*−*45*). BPDE can form adducts with specific amino acid residues of proteins, causing inactivation of enzymatic or functional activity (*42*). Detectable levels of benzo[*a*]pyrene diol epoxide-adducted protein (BPAP) in *M. arenaria* in the Bay of Isle site indicate a high exposure to PAHs, especially when contrasted with BPAP being undetectable in Bainbridge population (Table 1; *43*). The presence of BPAP indicates metabolic activation of benzo[*a*]pyrene, suggesting an increase in reactive oxygen species production, which may lead to increased oxidative damage (*42*). The presence of BPAP also suggests the presence and accumulation of benzo[*a*]pyrene dio epoxide-adducted DNA (BPA−DNA), a known mutagen and possible carcinogen (*46*). This in turn raises the possibility that the Bay of Isles population may be experiencing an increased mutation rate and perhaps a faster rate of senescence due to DNA damage and an increased deleterious mutation rate (*47*, *48*). Undetectable BPAP levels in *Mt. trossulus* at both sites does not exclude the possibility that this species is exposed to a PAH source, especially since the Bay of Isles site is documented to contain significant levels of residual oiling (*13*, *14*) and that lower limit detection levels for this assay are 80 nmol of BPAP/μg TSP (Downs and Robinson, unpublished results).

Besides possible differences in metabolic activation/clearance of benzene, benzo[*a*]pyrene, and BPAP between the two species, there are differences in habitat preference that would affect oil exposure in the two species (*13*). *M. arenaria* inhabits the top 20 cm of sediment while *Mt. trossulus* has a wider distribution, ranging from subtidal areas (where they tend to be "grazed" by invertebrate predators) to substrate several meters above the high tide line. Proximity of an individual to oiled sediment has a direct relation to the concentration of exposure (*49*). Hence, there may be differences in concentration exposure for the two species—*M. arenaria* having the closest association with oiled sediment and therefore the highest level of exposure, producing a higher level of BPAP accumulation than *Mt. trossulus*.

During formation of BPAP, reactive oxygen species are produced as a byproduct of monooxygenase activity (*42*). Since BPAP and BPA−DNA can inhibit activity and accumulation of anti-oxidant enzymes and their consequent metabolic pathways, chronic exposure of PAHs may produce a level of oxidative stress (e.g., Asada−Halliwell pathway; *50*). Oxidative stress by itself can cause severe denaturation and inhibition of enzymatic activity as well as being an origin for mutagenesis. Several well-established theories concerning oxidative stress posit that chronic exposure to oxidative stress can result in decreased reproductive fitness, accelerated senescence, decreased resistance to pathogens, and decreased tolerance to environmental stressors (for review, see ref *47*).

Two end products of lipid peroxidation (i.e., oxidative stress) are hydroxynonenal (HNE) and malondialdehyde (MDA) (*51*, *52*). Both HNE and MDA are reactive molecules that will form adducts with DNA and proteins (*51*−*55*). To determine if populations in the Bay of Isles site were experiencing higher levels of oxidative damage, we assayed HNE and MDA adducted with protein. Levels of HNE in both species were significantly higher at the Bay of Isles site as compared to levels found at the Bainbridge site (Tables 1 and 2). MDA levels also were significantly higher in the Bay of Isles populations for both species compared to the levels seen at the Bainbridge site (Tables 1 and 2). These two assays corroborate that both species at the impacted site are experiencing an increased rate of lipid peroxidation as well as suggesting that anti-oxidant defenses are being overwhelmed.

Besides supporting the argument for increased oxidative stress levels at the impacted site, higher levels of lipid peroxidation products can adversely affect other cellular functions. HNE and MDA can adduct with proteins and DNA. In addition to altering the pK_a of a protein, HNE will cross-link two or more proteins (*56*). Cross-linking not only causes denaturation and inactivation of the protein, but it may also prevent its degradation by the ubiquitin/proteosome pathways (e.g., prevention of ubiquination of the protein, degradation by proteosome complexes; *56*). This nondegradative quality can result in the accumulation of HNE-adducted denatured proteins in the cell, resulting in inclusion body formations, inhibition of mitochondrial function, and ultimately cell death (*53*, *57*, *58*). MDA can also act as a mutagen and a carcinogen (*50*).

Examination of HNE and MDA levels does not conclusively answer the question of oxidative stress, although it does strongly argue that bivalves at the oiled site are experiencing an oxidative stress, at least more so than bivalves at the unoiled site. There are several ways to better resolve this issue. One method would be to increase the sensitivity of the HNE and MDA assays to detect sub-nanogram concentrations for these two parameters, thereby increasing the power and rigor of the statistical analysis. An additional strategy would be to examine a number of other parameters of oxidative stress: DNA oxidation products, amino acid oxidation products, anti-oxidant capacity (e.g., total GSH:GSSG ratio), and other byproducts of lipid peroxidation in order to corroborate an oxidative damage assessment.

In response to increased levels of reactive oxygen species and oxidative damage, cells will usually upregulate the accumulation of a number of enzymatic anti-oxidants (*34*, *35*). We used Cu/Zn SOD, Mn SOD, and GPX as markers for determining cellular responses to increased reactive oxygen species (*58*−*62*). Superoxide disumtases catalyze the reaction of superoxide ions and two protons to form hydrogen peroxide (*58*). Both species of SOD accumulate in response to oxidative stress and are one of the primary anti-oxidant defense pathways (*59*). Glutathione peroxidase detoxifies hydroperoxides and organic peroxides, and changes in GPX levels are also used as a measure of an oxidative stress response (*59*−*62*). In *M. arenaria*, Cu/Zn SOD levels were significantly higher at the impacted site than the nonimpacted site (Table 1). Cu/Zn SOD in *Mt. trossulus* exhibited an opposing pattern; levels were significantly lower in the impacted site as compared to the nonimpacted site (Table

2). The pattern of GPX accumulation was similar for the two species (Tables 1 and 2). In the impacted site, *M. arenaria* had higher GPX levels as compared to the nonimpacted site (Table 1). As with Cu/Zn SOD, *Mt. trossulus* had lower levels of GPX at the impacted site than at the nonimpacted site (Table 2). The pattern of GPX and Cu/Zn SOD supports the argument that *M. arenaria* is responding to an oxidative stress, though it is difficult to readily explain the pattern of GPX and Cu/ZnSOD for *Mt. trossulus*.

Examination of MnSOD levels in *Mt. trossulus* supports the argument that the Bay of Isles population was responding to an oxidative stress, at least on the level of the mitochondria (Table 2). Levels of MnSOD between the impacted and nonimpacted site for *M. arenaria* were not significantly different (Table 1). This suggests that the location of benzo-[*a*]pyrene (BP) activation to an electrophilic metabolite may be different for the two species. Benzopyrene activation can occur by a number of different monooxygenases (e.g., in humans, the subfamily CYP 1A1, CYP2C9, and CYP3A4) and by prostaglandin H synthases (*37*, *38*, *63*). Some isoforms of the monooxygenase family occur within the mitochondria as well as in microsomes (*64*). One possibility is that adaptive monooxygenase for *Mt. trossulus* occurs predominantly in the mitochondria, while in *M. arenaria* it occurs predominantly in microsomes that are dispersed throughout the cytoplasm (*64*).

To determine if bivalves were responding to a xenobiotic stressor in the Bay of Isles site, we examined two separate components of a cell's detoxification system: CYP P450 2E immunohomologue and glutathione *S*-transferase (GST). Cytochrome P450 2E has both physiologically relevant oxidative and reductive reactions and is known to associate and catalyze as many as 60 xenobiotic-based substrates (*64*, *65*). A primary reason for using the 2E class of cytochrome P450s is that it is not induced by heat stress (*34*, *35*, *66*). Cytochrome P450 levels were significantly higher for both species at the impacted site as compared to the nonimpacted site (Tables 1 and 2). Cytochrome P450 2E can be found in both microsomal and mitochondrial fractions (*60*, *61*). Differential induction of organellar isoforms by organellar fractionation may resolve the issue concerning location of BP activation and site-specific oxidative stress, especially for these two species.

The role of glutathione transferases is associated with cellular detoxification of genotoxic and cytotoxic xenobiotic electrophiles (*67*). During a xenobiotic challenge, glutathione may be conjugated to a xenobiotic by GST (*68*). Additionally, GST may detoxify DNA hydroperoxides and thus play an important role in DNA repair as well as detoxifying HNE in the cell (*69*, *70*).

GST levels were not significantly different between nonimpacted and impacted sites for *M. arenaria* (Table 1). Several viable hypotheses can be proposed for this lack of response. The first is that PAH xenobiotics do not differentially upregulate GST accumulation. Another hypothesis is that GST isoforms detected by the antibody are not the dominant GST isoforms that are upregulated in response to electrophilic metabolites (*67*). Another possible explanation is that GST has a high basal expression level in this species; hence, exposure of electrophilic metabolites at the level this species is experiencing does not necessitate higher levels of this enzyme (*70*). GST levels in *Mt. trossulus* were significantly higher at the impacted site as compared to the nonimpacted site (Table 2). These data support a xenobiotic response, specifically one of an electrophilic nature, as would be expected for a PAH exposure. The data also support the hypothesis that bivalves in the Bay of Isles site were exposed and responding to a PAH source and were experiencing and responding to an oxidative stress.

The next pertinent diagnostic issue is whether bivalves in the impacted site were responding to increased protein denaturation conditions. Ubiquitin levels in *M. areneria* are almost seven times higher at the impacted site as compared to the nonimpacted site (Table 1). Ubiquitin levels in *Mt. trossulus* were three times higher (Table 2). Increased ubiquitin levels indicate increased protein turnover, likely resulting from an irreversible denaturing stress (*71*−*75*). This interpretation is supported by the pattern of chaperonins, Hsp60 and Hsp70, which are accumulated in response to both a protein denaturing stress and an increase in protein synthesis (*76*, *77*). In *M. arenaria*, Hsp60 levels were significantly higher at the impacted site (Table 1). Hsp70 levels were also significantly higher at the impacted site as compared to the nonimpacted site for *M. arenaria* (Table 1). In *Mt. trossulus*, Hsp60 levels were not significantly different between nonimpacted and impacted sites (Table 2). In contrast to Hsp60, Hsp70 levels in *Mt. trossulus* were significantly higher at the impacted site than at the nonimpacted site (Table 2). Increased expression of Hsp70 for both bivalves, in conjunction with significantly increased accumulation of ubiquitin, at the impacted site strongly argues that both species are experiencing an insult to the protein component of cellular integrity and that an increased energy expenditure is required to maintain cellular homeostasis (*40*, *78*). Increased levels of Hsp60 in *M. arenaria* suggest that there is either increased mitochondrial protein denaturation, increased protein synthesis specific to the mitochondria, or both (*77*).

The presence of BPDE−protein, high levels of CYP P450, oxidative damage products, and protein turnover components strongly supports a model proposed by Fucci and coworkers concerning a mixed-function oxidation centered around a P450 monooxygenase (especially a CYP P450 type 2 subclass or a CYP P450 type 1 subclass) resulting in enzyme inactivation and increased protein turnover (*78*). This model ascribes a mechanism for an increased rate of degeneration (senescence), in that many of the enzymes modified and inactivated by these oxidation reactions are the same enzymes that are inactivated during senescence (*78*, *79*). If this model is true in the case of these two bivalves at the Bay of Isles site, it suggests that both species at the impacted locality would have a reduced metabolic capacity, possibly resulting in a reduced reproductive fitness capacity, a reduced immunocompetence capacity, and a general reduction in individual fitness (e.g., competition). This oxidation model would also infer, with some supporting evidence, that the bivalves would have reduced genomic integrity, which in turn may directly affect fecundity and offspring viability (*45*, *48*).

Are the bivalves stressed and are they responding to a stress? Small heat-shock proteins are often used as a single proxy marker for organismal stress (*80*, *81*). They are a ubiquitous family of proteins, and their presence usually is only induced by a stress (*64*). Several studies have demonstrated that various sHsp isoforms are induced by incapacitating insults to key metabolic pathways, such as oxidative phosphorylation or photosynthesis—metabolic pathways central to cellular homeostasis (*80*, *81*). The antibody used in this study detected the four major isoforms of invertebrate sHsps. One sHsp isoform, Hsp22, localizes specifically to mitochondria, especially those in neural and reproductive tissue, and protects complex I of oxidative phosphorylation (*82*; C. Downs, Woodley, A. Downs, Towers, and Chandler, unpublished results). In *M. arenaria*, sHsps levels were undetectable at the unimpacted site but reached as much as 40 ng/μg of TSP at the impacted site (Table 1). Low levels of sHsp expression were detected in *Mt. trossulus* at the nonimpacted site, but sHsp levels were 5-fold higher at the impacted site (Table 2). SDS−PAGE/immunoanalysis comparing *Mt. trossulus* samples from Bainbridge and Bay of

Isles indicated that the sHsp22 homologue was induced in only Bay of Isles individuals (data not shown), supporting the interpretation that integrity of oxidative phosphorylation may be compromised (*83*). High expression of sHsps at the Bay of Isles indicates that both species were stressed and that the stress impacted mitochondrial function and perhaps cytoskeletal integrity (*83*, *84*).

Data obtained from this research support the argument that bivalves exposed to 10-year-old residual oil from the *Exxon Valdez* continue to show signs of cellular physiological stress relative to bivalves from an unoiled site. Furthermore, our data indicate that bivalves from the Bay of Isles site were responding to both a xenobiotic and an oxidative stress. Results of the BPAP analysis suggest that at least one of the environmental stressors is a PAH.

We strongly caution that this data set and its interpretations are preliminary. A number of caveats for this study need to be addressed before conclusive insights can be made about impact and recovery. One major caveat is that there was only a single, sampling time point. This synoptic "snapshot" may not accurately assess the long-term recovery process in an inherently dynamic system. To monitor systems that change over time, several temporal samples are required to correctly gauge the changes. Timing of sampling also significantly affects the ability to gauge the recovery process. We analyzed samples collected in late June, a time when both species can be in reproductive conditions during which many organisms may accumulate novel Hsp60, Hsp70, and sHsp isoforms that are not stress induced (*85*). This in turn can result in a confounding effect for interpretation, because levels of these markers may be abnormally high during reproduction than other seasonal periods. One strategy of partially overcoming this problem is to create isoform-specific ELISAs that do not assay reproductive-associated isoforms. A more robust strategy would be to include sampling throughout the year, thus obtaining seasonal profiles for that population and rigorously tracking molecular marker fluctuations.

A second caveat is that this study used a limited number of markers. Incorporation of additional markers would allow more robust and subtle interpretations and distinctions. For example, direct measurement of benzo[*a*]pyrene diol epoxide-adducted DNA would allow for a more accurate interpretation and assessment of the effects of PAH exposure to the bivalves' genomes. This line of inquiry can be extended to markers that specifically reflect genomic integrity and mutation rate, such as micronuclei formation and DNA abasic site formation. This information, in turn, can better estimate or forecast higher-order phenomena, such as homeostatic capacity, reproductive fitness, and competitive ability.

A third caveat is that the populations collected at the Bainbridge site were assumed to represent a "healthy" population. One way to better approximate the nominal range for each of these cellular parameters would be to sample a number of different nonimpacted sites. This concept can be extended to the sampling collection scheme for a particular site. Collection transects that account for tidal depth and spatial distribution will provide more information about within-site heterogeneity.

Substrate and microhabitat differences between the two study sites constitute the basis for the fourth caveat. Bay of Isles represents a habitat type uncommon in PWS; a protected tidal marsh. Because there are very few of these areas in PWS, a completely comparable reference site was not available. While the Bainbridge study location was physically similar to the Bay of Isles site (affirmed biologically by the occurrence of the two targeted bivalve species at both places), subtle differences in the physical characteristics of the two areas may have influenced cellular marker results in the two sampled populations (*13*).

The data presented herein demonstrates the potential of the ECDS as an important means of assessing impact and recovery in habitats where anthropogenic stress is suspected. Used in an appropriate study design and in conjunction with other methods, the ECDS can be an important tool for approximating both environmental "health" status and progress toward a specified recovery or restoration endpoint. Furthermore, this technology can be expanded and augmented to address specific issues and concerns within a given environmental system. Finally, because this technology addresses cellular level processes, it has the potential to forecast higher levels of biological organization, such as consequences to populations, communities, and entire ecosystems (*26*, *35*, *86*, *87*).

Acknowledgments

This research was supported in part by the *Exxon Valdez* Restitution Fund. Prince William Sound field operations were conducted aboard the M/V *Auklet*, operated by David Janka. We sincerely thank Cheryl M. Woodley, Hal Helbock, Virgil D. Downs, Bruce N. Ames, Sylvia Galloway, R. Pavia, Carol Ann Manen, and the anonymous reviewers for critical discussion and comments on the manuscript. We would also like to thank the University of California−Berkeley Department of Statistics for critical discussion and advice. Partial support for statistical analysis was provided by EPA Grant R825795 and NSF Grant DEB9727939 to J.E.F. This is Envirtue Biotechnologies, Inc. Manuscript No. 008. The views expressed in this manuscript do not necessarily represent those of the U.S. Government or the National Oceanic and Atmospheric Administration. Mention of trade names or commercial products does not constitute endorsement or recommendation for their use. The U.S. National Oceanic and Atmospheric Administration's National Ocean Service (NOS) does not approve, recommend, or endorse any proprietary product or material mentioned in this publication. No reference shall be made to NOS, or to this publication furnished by NOS, in any advertising or sales promotion that would indicate or imply that NOS approves, recommends, or endorses any proprietary product or proprietary material mentioned herein or that has as its purpose an intent to cause directly or indirectly the advertised product to be used or purchased because of NOS publication.

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Received for review November 20, 2001. Revised manuscript received April 29, 2002. Accepted April 29, 2002.

ES011433K