BIOMARKERS, 1999, VOL. 4, NO. 6, 400–414

Biomarker studies with juvenile oysters (*Crassostrea virginica*) deployed *in-situ*

AMY H. RINGWOOD[1]*, DEANNA E. CONNERS[2], CHARLES J. KEPPLER[1] and AUGUSTINE A. DINOVO[2]

[1] Marine Resources Research Institute, 217 Fort Johnson Road, Charleston, SC 29412, USA. e-mail: Ringwood A@MRD.DMR.State.SC.US

[2] Medical University of South Carolina, 171 Ashley Avenue, Charleston, SC 29452, USA

Hatchery-reared juvenile oysters (*Crassostrea virginica*) were deployed *in situ* for approximately 1 month from mid-May to mid-June of 1996 at sites that were classified as reference, agricultural, suburban, or urban/industrial. Cellular responses (lysosomal destabilization, glutathione concentrations, lipid peroxidation, heat shock proteins, metallothioneins, and multi-xenobiotic resistance proteins) were analysed, and their efficacy as biomarkers of stress was evaluated. Increased lysosomal destabilization, glutathione depletion, increased lipid peroxidation, and induction of heat shock proteins and metallothioneins were observed at many of the polluted sites, but increases in multi-xenobiotic resistance proteins were not. Significant correlations between sediment contaminants and lysosomal destabilization or glutathione concentrations were observed. Similarly, there were significant correlations between sediment cadmium and copper levels and metallothioneins. Although elevated lipid peroxidation products and heat shock proteins were observed at some of the contaminated sites, there were no significant correlations with contaminants. These studies suggest that lysosomal destabilization and glutathione depletion are sensitive, robust indicators of contaminant stress. Although lipid peroxidation and heat shock protein responses were not correlated with contaminants, they are still regarded as valuable indicators of stress. These studies demonstrate the value of using a suite of cellular biomarkers to identify and characterize stress responses related to anthropogenic perturbations.

Keywords: oysters, lysosomes, glutathione, lipid peroxidation, heat shock proteins, metallothioneins, multi-xenobiotic resistance proteins.

Introduction

The potential for increased stress in marine organisms due to anthropogenic pressures associated with increasing development (contaminant inputs, habitat alterations, etc.) demands careful monitoring of biological resources and development of strategies to minimize the impacts. Within the past decade, there has been an increasing emphasis on the potential use of biochemical, physiological, and histological indicators as biomarkers of exposure to or effects of anthropogenic impacts (Huggett *et al.* 1992, Decaprio 1997). The underlying premise of biomarker tools is that effects at higher levels of organization (populations and communities) represent the sum of effects on individuals that resulted from alterations in cellular and molecular responses (figure 1). Therefore, cellular responses should function as indicators for identifying individuals and populations for which conditions have exceeded compensatory mechanisms and are experiencing chronic stress, which, if unmitigated may progress to severe effects at higher levels of organization.

* Corresponding author: Amy H. Ringwood, Marine Resources Research Institute, 217 Fort Johnson Road, Charleston, SC, USA 29412.

Biomarkers ISSN 1354-750X print/ISSN 1366-5804 online © 1999 Taylor & Francis Ltd
http://www.tandf.co.uk/JNLS/bmk.htm
http://www.taylorandfrancis.com/JNLS/bmk.htm

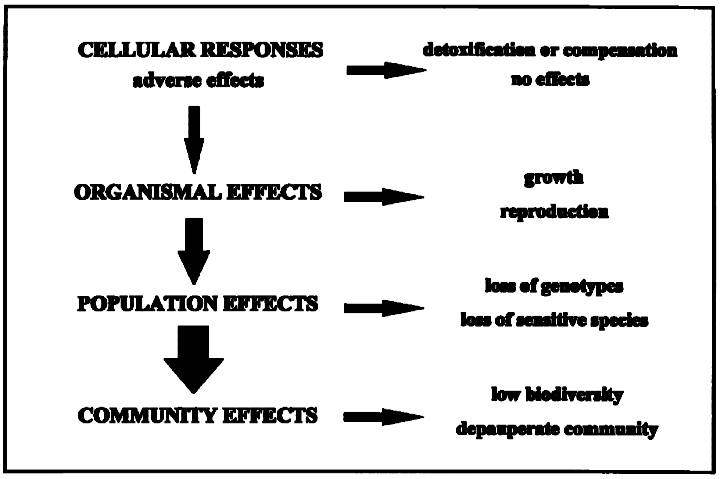

Figure 1. Generalized scheme depicting relationships between cellular responses and higher level effects.

The studies presented in this paper were conducted as part of a more extensive multi-disciplinary programme designed to determine the impacts of chemical contaminants on oysters from estuaries adjacent to different land-use activities. Juvenile hatchery-reared oysters (*Crassostrea virginica*) were deployed *in situ* at 15 sites that were classified *a priori* as either reference or potentially degraded based on existing data and/or perceived inputs. At the end of the 1 month deployment period, the effects on a variety of cellular responses (lysosomal destabilization, lipid peroxidation, glutathione concentrations, heat shock proteins, metallothioneins, and multi-xenobiotic resistance proteins) were determined. These results are presented, and their efficacy as indicators of stress is discussed. Therefore, the purposes of these studies were to identify cellular responses or suites of responses that were related to contaminant concentrations and evaluate their potential as valuable biomarkers of anthropogenic stress.

The cellular biomarkers evaluated in this study represent a range of damage and detoxification responses. Lysosomes are regarded as valuable indicators of pollutant-induced injury (Moore 1994). In tissues as well as in circulating haemocytes, lysosomes are involved in numerous functions, including cellular defence, tissue repair and turnover, and nutrition (Auffret 1988, Adema *et al.* 1991). There is a substantial body of literature validating that environmental pollutants (metals and organics) cause destabilization of lysosomes (Lowe *et al.* 1981, 1995, Moore 1985, 1990, Ringwood *et al.* 1998a). Lipid peroxidation reflects damage to cell membranes from free radicals. The peroxidation process is also a source of other cytotoxic products that may damage DNA and enzymes (Kehrer 1993, Yu 1994). Increased lipid peroxidation has been demonstrated in response to ischaemia-reperfusion events in mammalian tissues, paraquat and metal exposures in mussels, cadmium and PCB exposures in mullet, and exposures of catfish to PAH-contaminated sediments (Wenning *et al.* 1988, Wofford and Thomas 1988, Regoli 1992, Di Giulio *et al.* 1995).

Glutathione, heat shock proteins, metallothioneins, and multi-xenobiotic resistance proteins function to ameliorate or facilitate detoxification of pollutants. Glutathione (GSH) is a ubiquitous tripeptide (L-γ-glutamyl-L-cysteinyl-glycine)

that is regarded as one of the most important non-protein thiols in biological systems (Kosower and Kosower 1978, Mason and Jenkins 1996). GSH functions as a very important overall modulator of cellular homeostasis, and serves numerous essential functions including detoxification of metal and organic pollutants, and free radicals (Meister and Anderson 1983, Christie and Costa 1984, Ketterer 1986). There is evidence that GSH depletion is associated with adverse effects in marine bivalves (Viarengo *et al.* 1990, Regoli and Principato 1995) as well as in mammalian models (Dudley and Klaasen 1984, Singhal *et al.* 1987, Prozialeck and Lamar 1995). Organisms may be more susceptible to additional stressors when GSH Is depleted, so GSH status may also function as a potential risk factor (Jones *et al.* 1995, Conners 1998). Heat shock proteins have been implicated as an important overall mechanism by which cells ameliorate damage due to chemical, temperature, and oxidative stress (Rothman 1989, Sanders, 1990, Welch 1992, Sanders and Martin 1993). Although multiple classes or isoforms have been identified, the Hsp 70s are regarded as the group that responds most commonly to environmental perturbations, and are believed to be potentially valuable general stress indicators (Stegeman *et al.* 1992). Metallothioneins (MTs) are ubiquitous metal-binding proteins (approximately 6500 Da) that play important roles in detoxification of toxic metals and are also believed to play important roles in normal metal metabolism (Hamer 1986, Engel and Brouwer 1989, Viarengo and Nott 1993). The fact that marine organisms, like virtually all phyla, respond to metal exposures, particularly Cd and Cu, by an increased expression of MTs has led to the perception that MTs may be used as potentially powerful biomarkers of metal toxicity (Benson *et al.* 1990, Roesijadi 1992, Stegeman *et al.* 1992, Viarengo *et al.* 1997). Multi-xenobiotic resistance (MXR) proteins, analogous to MDR or multi-drug resistance proteins described from mammalian systems, were recently discovered in marine organisms (Kurelec *et al.* 1992, Minier *et al.* 1993, Toomey and Epel 1993, Karnaky *et al.* 1993). MXR or MDR activity is conferred by the expression of a large cell surface glycoprotein, approximately 170 kDas. These proteins are believed to pump natural toxins as well as organic pollutants out of cells via an ATP-dependent mechanism. Under laboratory conditions, moderately hydrophobic compounds (including benzo(*a*)pyrene and naphthalene) have been identified as substrates for P-gp (Fairchild *et al.* 1987, Cornwall *et al.* 1995). It has been hypothesized that MXRs could be used as biomarkers of organic contaminant exposure.

Methods

Oyster deployments

Juvenile oysters, reared in the bivalve-culturing facilities at Marine Resources Research Institute (MRRI), were placed inside rigid Durothene® polyethylene spat bags, and deployed at 15 sites located in various estuaries along the South Carolina coast. Sites were classified *a priori* as either reference, or potentially degraded (characterized as impacted primarily by agricultural inputs, suburban development, or urban/industrial development) based on existing data and/or perceived inputs. The site categorizations and 3-letter designations are listed in table 1 (sites are described in more detail in Ringwood *et al.* 1998a). The bags (each containing approximately 100 hatchery-reared juvenile oysters) were deployed approximately 0.5 m off bottom in mid-May, 1996 and retrieved approximately 1 month later. At the time of deployment, oysters were 1.86 ± 0.20 cm in height (distance from hinge to shell margin) and 1.37 ± 0.19 cm in length (longest distance parallel to the hinge) (values are mean ± standard deviation; $n = 25$).

At the time of retrieval, oysters were returned to the laboratory and allowed to depurate overnight in water collected from each site. A subset of 20 live oysters was processed for the lysosomal destabilization

Table 1. List of deployed oyster sites. The name, 3-letter code, and category are shown.

Site name	Code	Category
Control Creek	CON	Reference
North Inlet	NIN	Reference
Fosters Creek	FOS	Reference
Kiawah Creek	KIA	Degraded—agricultural
Treatment	TRT	Degraded—agricultural
Trailer Park	TRP	Degraded—suburban
Flo's	FLO	Degraded—suburban
Alston Creek	ALS	Degraded—suburban
Shem Creek	SHM	Degraded—urban/industrial
New Market Creek	NMK	Degraded—urban/industrial
Noisette Creek	NOI	Degraded—urban/industrial
Plum Island	PLM	Degraded—urban/industrial
Koppers Creek	KOP	Degraded—urban/industrial
Diesel Creek	DIE	Degraded—urban/industrial
Shipyard Creek	SPY	Degraded—urban/industrial

assay. The remaining tissues from this group as well as tissues dissected from an additional 30 oysters were immediately frozen at –80 °C for later analyses of lipid peroxidation, GSH, Hsp70s, MTs, and MXRs.

Cellular responses

Lysosomal destabilization

A neutral red assay was used to evaluate lysosomal integrity (Lowe *et al.* 1992, Ringwood *et al.* 1998a). Briefly, cellular suspensions were prepared from pieces of digestive gland tissue (20–40 mg) dissected from individual oysters, and incubated in calcium/magnesium-free saline and trypsin to disaggregate the cells. An aliquot of the cell suspension was mixed with neutral red on a microscope slide, covered with a cover slip, and incubated in a humidity chamber at room temperature for 60 min. Digestive gland cells (6–12 μm in diameter) containing lysosomes were examined with a light microscope (100 X under oil immersion) to evaluate NR retention. Cells with NR retained in lysosomes were scored as stable and those with NR leaking into the cytoplasm were scored as destabilized. A minimum of 50 cells was counted for each preparation, and the data were expressed as destabilization indices (% destabilized lysosomes per individual oyster).

Lipid peroxidation

The thiobarbituric acid (TBA) test was used to measure lipid peroxidation (Gutteridge and Halliwell 1990). Digestive gland tissues were homogenized in 50 mM potassium phosphate buffer (pH 7.0) and centrifuged (14 000 rpm, 4 °C, 5 mins). A subsample of the supernatant was mixed with trichloroacetic acid containing TBA and butylated hydroxytoluene, heated at 100 °C for 15 min and centrifuged to remove the precipitate. The resulting malondialdehyde (MDA) was detected at 532 nm. Standards were prepared as described by Csallany *et al.* (1984), and the data were expressed as nM MDA per g wet weight.

Glutathione concentration (GSH)

Glutathione concentrations of individual oysters were determined by the DTNB-GSSG Reductase Recycling Assay (Anderson 1985). This assay is a sensitive and specific enzymatic procedure that follows the rate of 5-thio-nitrobenzoic acid (TNB) formation. Digestive gland tissues were homogenized in 10 volumes 5% sulphosalicyclic acid (SSA), and centrifuged (14 000 rpm, 5 min, 4 °C). Supernatants were diluted 1:1 with 5% SSA and mixed with the NADPH buffer containing DTNB. GSSG reductase was quickly added and the rate of TNB formation was monitored at 412 nm at 30 s intervals for 90 s. GSH concentrations were estimated from a standard curve and reported as nM GSH per g wet weight.

Heat shock proteins (Hsp70)

Heat shock proteins were determined using a polyclonal antibody and dot-blot techniques. Gill tissues of individual oysters were homogenized in phosphate saline lysis buffer (40 nM Na_2HPO_4, 8 mM NaH_2PO_4, 150 mM NaCl, pH 7.4) with 1 mM PMSF and Triton X-100. After centrifugation (10 000 rpm, 15 min, 4 °C), the supernatants were adjusted to 1 mg protein ml^{-1} (protein concentrations were determined using the Bio-Rad protein assay), and 250 μg of protein was loaded onto a dot blot apparatus

(Bio-Rad Bio-Dot). The blots were incubated overnight in primary antibody (polyclonal Hsp70 rabbit anti-human, Accurate Chemical and Scientific Corporation), washed, and incubated in the secondary antibody (goat anti-mouse IgG) and detected using BCIP/NBT. Gel electrophoresis was used initially to verify the specificity of the Ab in the appropriate molecular weight position. The data-blots were photographed using a high resolution video camera (Canon L2 Hi-8 mm), digitized using Mocha (Jandel Scientific) and analysed using Sigma-Scan (Jandel Scientific). The results were expressed as relative pixel intensities mg^{-1} protein.

Multi-xenobiotic resistance proteins (MXR)

MXR proteins were determined using a monoclonal antibody and dot-blot techniques. Gill tissues of individual oysters were homogenized in phosphate saline lysis buffer and centrifuged. The supernatants were adjusted to 1 mg protein ml^{-1} (protein concentration were determined using the Bio-Rad protein assay), and samples were loaded onto the blotting apparatus (two replicates of 250 µl each). The blots were incubated overnight in the primary antibody (C219 monoclonal AB, Signet Laboratories Inc; 1:2000 in Tris-BLOTTO), rinsed and incubated in secondary antibody (goat α-mouse IgG, 1:500 in Tris-BLOTTO) for 2 h, and detected using BCIP/NBT. Gel electrophoresis was used initially to verify the specificity of the Ab in the appropriate molecular weight position. The blots were photographed, digitized, and analysed above, and results were expressed as relative pixel intensities mg^{-1} protein.

Metallothioneins (MT)

The metallothioneins of pooled oyster digestive gland tissues were isolated as described previously using FPLC (Ringwood and Brouwer 1993). Three to four replicate analyses (each composed of five oysters per pooled sample) were conducted for 11 of the 15 sites (not enough tissues were available for TRT, TRP, ALS, and NOI). Tissues were homogenized in 40 mM ammonium bicarbonate buffer (with PMSF and β-mercaptoethanol, bubbled with nitrogen. After centrifugation at 25 000 *g* for 1 h, the supernatants were filtered, loaded onto the size exclusion column (Superdex 10/50, Pharmacia), and eluted with the ammonium bicarbonate buffer. Prior to the analysis of the samples, purified rabbit MT (Sigma) was used to verify MT elution time. The metal concentrations (Cd and Cu) of the MT fractions were analysed by furnace AAS after acidification. Total protein concentrations were also determined (Bio-Rad assay). The results were expressed as Cu or Cd concentrations associated with the MT pool based on total proteins (i.e. nM Cu-MT or Cd-MT/mg^{-1} protein).

Sediment contaminant analyses

Sediment contaminant concentrations of surficial sediments collected from each site were analysed by NOAA/NOS, Charleston, SC. Sediment samples were collected at each site four times from May 1 to June 30. An extensive suite of PAH and metal contaminants as well as PCBs and a number of pesticide analytes were analysed using protocols and quality-assurance checks as defined by the NOAA National Status and Trends Program (Lauenstein and Cantillo 1993). Rigorous quality control measures were included to ensure the validity of the data, including analysis of standard reference materials (NIST 1941, Organics in Marine Sediments; NRB Canada MESS-2, Metals in Marine Sediments). Generally, there was little variation in contaminant levels over the different sampling periods, so averaged values were used for the regression analyses. Elevated pesticides and PCBs were very rare, so only the regressions with metal and PAH contaminants are presented in this paper.

Statistical analyses

Analysis of variance (ANOVA) and an *a posteriori* test (Student–Newman–Keuls) were used to identify treatments that were significantly different from CON and other reference sites for all data that passed normality and equal variance tests (true for all data except the MT results). A non-parametric Kruskal–Wallis test was used to analyse the MT data. Regression analyses were used to evaluate the relationships between cellular responses and sediment contaminants. The regression analyses passed normality and homoscedasticity tests. Studentized residuals were used to identify potential outliers, and those that were statistically significant (based on a table of critical values for tests of discordancy) were removed (Barnett and Lewis 1978). All analyses were conducted using Sigma Stat.

Results

Alterations in cellular responses were observed more often at the most contaminated sites. Lysosomal destabilization functioned as a very sensitive biomarker of contaminant stress. All but one of the potentially degraded sites had significantly higher lysosomal destabilization indices (figure 2). Glutathione (GSH) concentrations were also more frequently affected in oysters deployed at

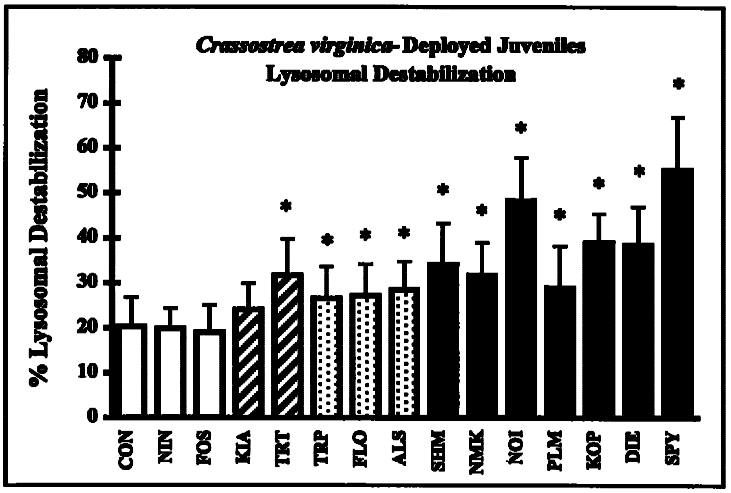

Figure 2. Lysosomal destabilization rates of hatchery-reared juvenile oysters (n = 20 individuals) deployed *in situ* for 1 month. See table 1 for site information. Data are means + standard deviations. The asterisks (*) indicate sites that were significantly different from CON. Site categories are identified as follows: M, reference; ▨, agricultural; ▩ suburban; m, urban/industrial.

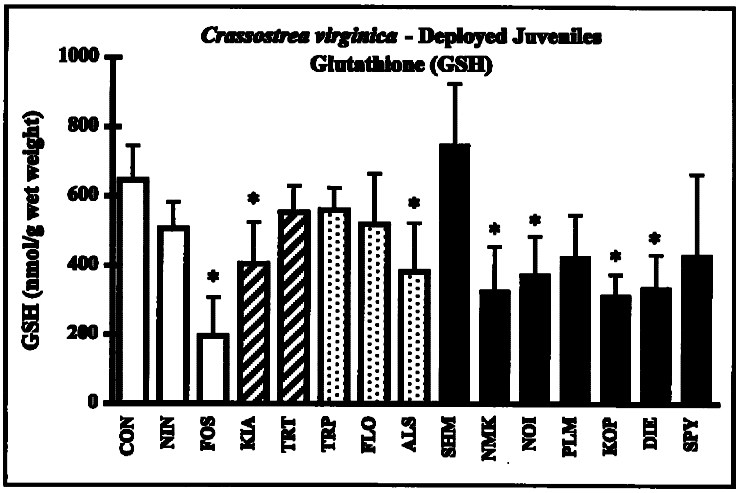

Figure 3. Glutathione (GSH) concentrations of hatchery-reared juvenile oysters (n = 5 individuals) deployed *in situ* for 1 month. Data are means + standard deviations. The asterisks (*) indicate sites that were significantly different from CON. Legend as described in figure 2.

contaminated sites (figure 3). All urban/industrial sites had lower GSH levels, and significant depletion was observed at three of the industrial sites, one suburban site, one agricultural site, and also at one of the reference sites, Fosters Creek (FOS). Increased lipid peroxidation was observed with oysters from some contaminated sites, but not at the most polluted industrial sites; and increased lipid peroxidation was also observed at FOS (figure 4). Induction of heat shock proteins (Hsp 70) was also observed at many of the polluted sites. Hsp 70 concentrations were significantly elevated at three industrial sites (NMK, PLM, KOP), one agricultural

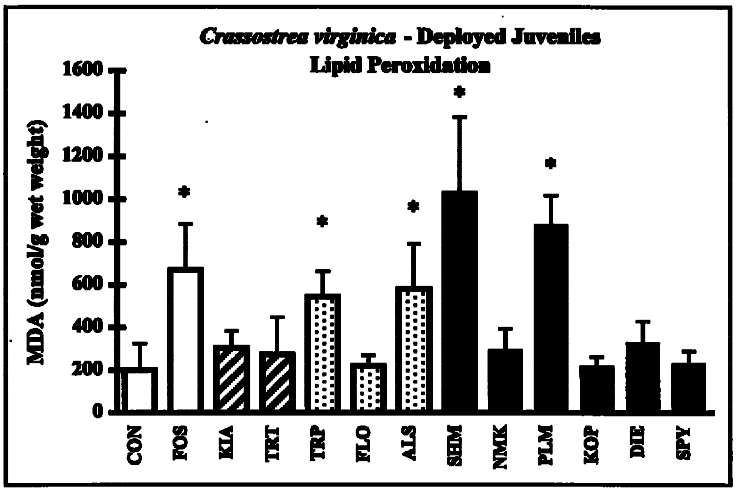

Figure 4. Lipid peroxidation (based on MDA concentrations) of hatchery-reared juvenile oysters (n = 5 individuals) deployed *in situ* for 1 month. Data are means + standard deviations. The asterisks (*) indicate sites that were significantly different from CON. The assay was not conducted for NIN because of insufficient tissues. Legend as described in figure 2.

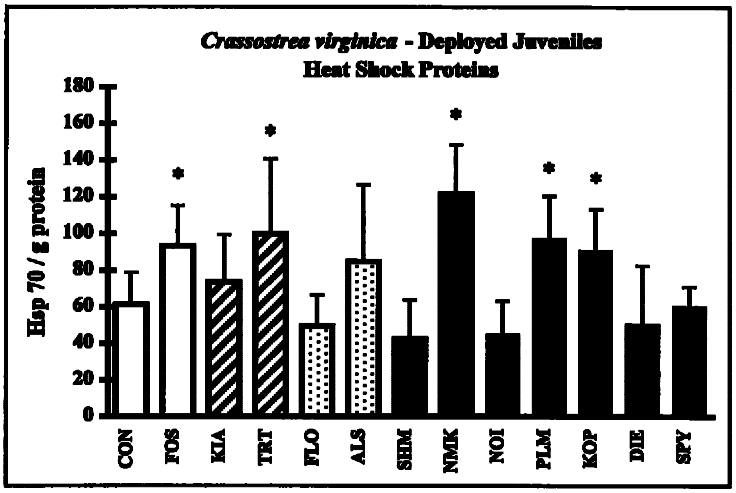

Figure 5. Heat shock proteins (relative pixel units g^{-1} protein) of hatchery-reared juvenile oysters (n = 5–10 individuals) deployed *in situ* for 1 month. Data are means + standard deviations. The asterisks (*) indicate sites that were significantly different from CON. The analyses were not conducted for NIN or TRP because of insufficient tissues. Legend as described in figure 2.

site, and one reference site (FOS) (figure 5). Significant inductions of MTs were detected at four of the six urban/industrial sites (figure 6). Evidence of significant MXR induction was detected at only one site (figure 7).

Examples of relationships between sediment contaminants and the various responses are illustrated in figures 8 and 9. These figures illustrate overall trends in which lysosomal destabilization and GSH concentrations were significantly related to both metals and PAHs, but lipid peroxidation and heat shock proteins were not. The correlation coefficients for a variety of other contaminants indicate significant associations between sediment contaminants and lysosomal destabilization or GSH

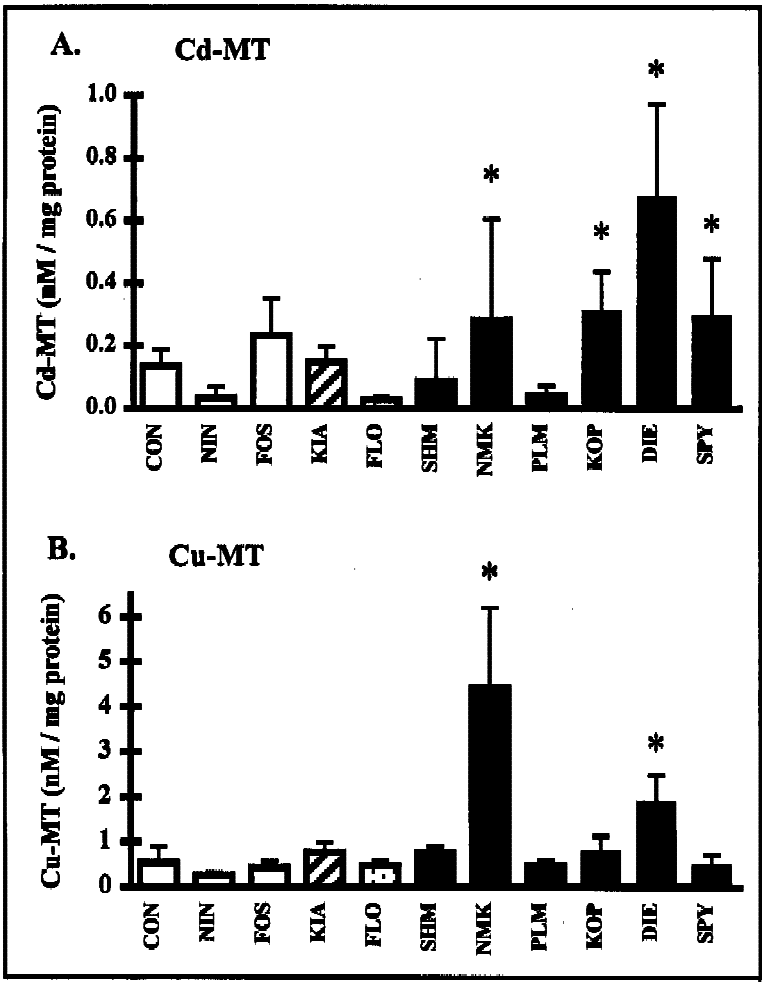

Figure 6. Metal concentrations associated with metallothioneins of hatchery-reared juvenile oysters deployed *in situ* for 1 month. Data are means + standard deviations (n = 3–4 composite replicates, composed of digestive gland tissues from 3–5 individuals). The asterisks (*) indicate sites that were significantly different from CON. (A) Cd MTs; (B) Cu MTs.

concentrations, but no significant correlations were observed with lipid peroxidation or heat shock proteins (table 2). Significant relationships between sediment Cd and Cu concentrations and MT associated metal concentrations were also observed (i.e. r^2 = 0.46 and 0.43 for sediment Cd and Cu relationships, respectively).

Discussion

Lysosomal integrity of oyster digestive gland (i.e. hepatopancreas) cells was adversely affected by elevated pollutants associated with anthropogenic activities. Hepatic tissues, which tend to be rich in lysosomes, are common targets for a variety of pollutants. These studies are consistent with other laboratory and field studies which have demonstrated that lysosomal membrane stability is very sensitive to contaminant exposures (Krishnakumar *et al.* 1994, Lowe *et al.* 1995,

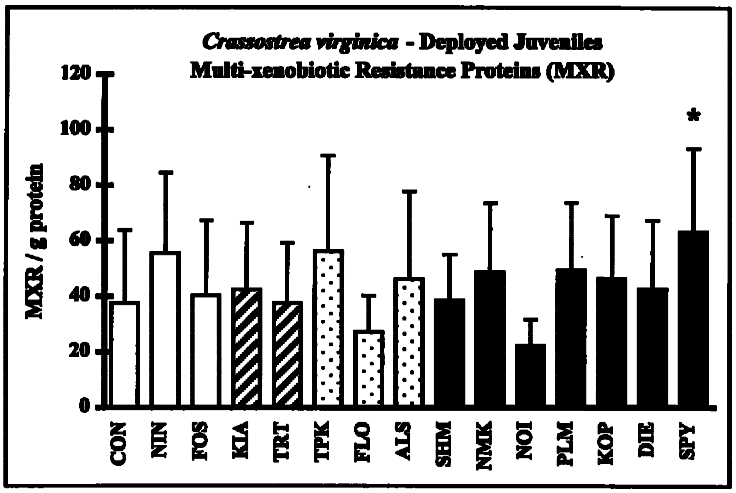

Figure 7. MXR protein concentrations measured in gills of hatchery-reared juvenile oysters (n = 9–29 individuals) deployed *in situ* for 1 month. Data are means + standard deviations. The asterisk (*) indicates the site that was significantly different from CON.

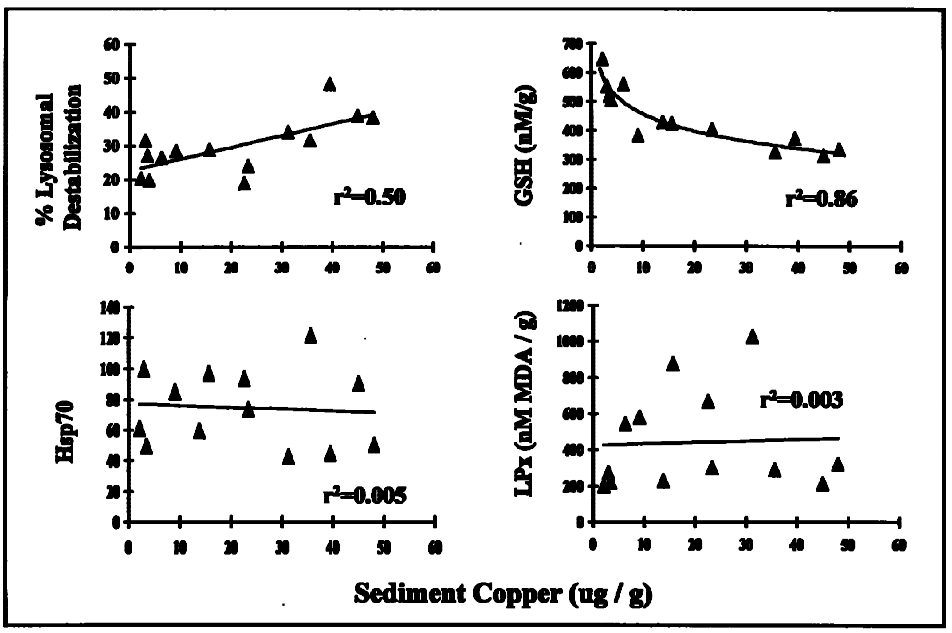

Figure 8. The relationships between sediment Cu concentrations and the various cellular responses. The correlation coefficients (r^2) are shown.

Regoli *et al.* 1998, Ringwood *et al.* 1998a). Pollutants may interact with phospholipids or other membrane components and affect permeability; or oxidation of metals or PAHs accumulated in lysosomes may generate oxyradicals that can damage lysosomes (Winston *et al.* 1991, 1996). Good correlations between lysosomal destabilization and contaminant concentrations continue to support the value of this response as a biomarker of contaminant exposure.

Good correlations were also observed between glutathione (GSH)

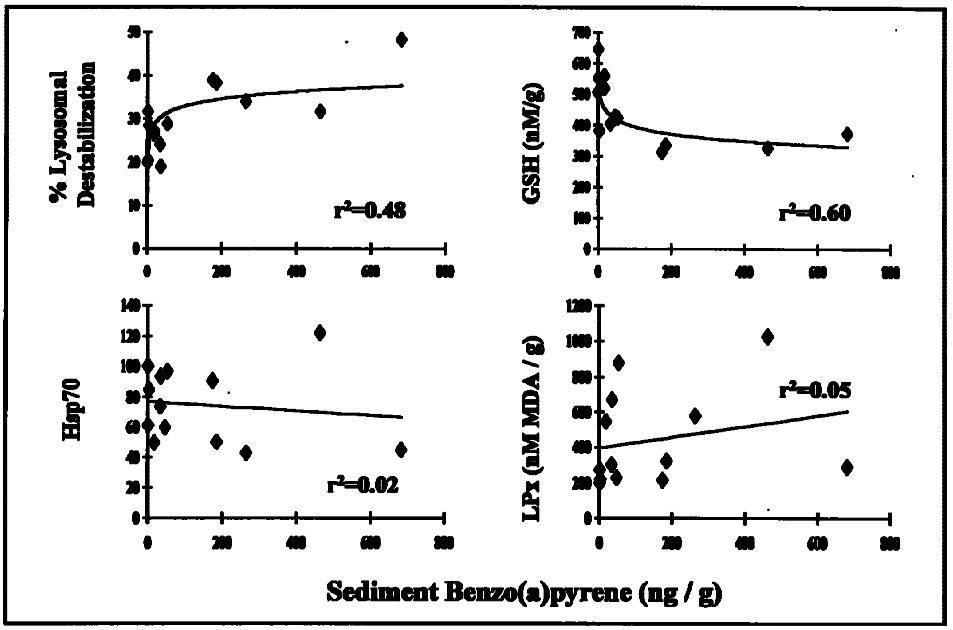

Figure 9. The relationships between sediment benzo(*a*)pyrene concentration and the various cellular responses. The correlation coefficients (r^2) are shown.

concentrations and pollutant levels. Glutathione performs important roles as an antioxidant and mediator of cellular homeostasis. Adverse effects on antioxidant systems may lead to antioxidant-mediated toxicities, including damage to membranes, proteins, and DNA (Winston and Di Giulio 1991). Therefore, depleted GSH may increase susceptibility to other contaminants or oxyradical-generating processes (Jones *et al.* 1995, Conners 1998).

Although increases in lipid peroxidation and heat shock proteins have been reported as a result of contaminant exposure, these responses did not correlate with metal or PAH levels. Increases in lipid peroxidation were not observed at some very polluted sites, although this may be explained by the significant induction of metallothioneins that was observed at these sites. Metallothioneins may also serve as free radical scavengers, so when significant metallothionein induction occurs, lipid peroxidation levels tend to diminish (Viarengo 1989, Ringwood *et al.* 1998b). No increases in lipid peroxidation were observed in oysters from the four urban/industrial sites that did have elevated MTs; likewise, MT induction was not observed in oysters from the two urban/industrial sites that had high peroxidation levels. Elevated heat shock proteins, like metallothioneins, may also ameliorate the damaging effects of free radicals.

South-eastern estuaries experience daily fluctuations in dissolved oxygen (DO) concentrations, which can be a source of natural DO stress, or may be exacerbated by contaminant or nutrient inputs. DO range deviations (the difference between the late night/early morning minimum and maximum concentrations measured during continuous deployments of > 24 h) may reflect the potential for reperfusion damage associated with increased generation of free radicals (Ringwood *et al.* 1996). DO profiles from contaminated tidal creek sites tended to have more severe DO swings. Sites located near sewage treatment plants (such as PLM) frequently

Table 2. Correlation coefficients (r^2) of regression analyses for metal and PAH contaminants and the various cellular responses: lysosomal destabilization (lyso), total glutathione concentrations (GSH), lipid peroxidation (LPx), and heat shock proteins (Hsp). An † indicates that SPY was identified as an outlier for the lysosomal results and was not included in the analyses. Both FOS and SHM were identified as outliers for the GSH results and were not included in the GSH analyses. An asterisk (*) indicates statistically significant r^2 values (i.e. $p \leq 0.05$).

	Lyso	GSH	LPx	Hsp
Metal				
Ag	0.36†*	0.72*	0.038	0.001
As	0.40†*	0.75*	0.156	0.002
Cu	0.50†*	0.86*	0.003	0.005
Cd	0.61*	0.72*	0.049	< 0.000
Cr	0.49*	0.77*	0.039	0.007
Hg	0.27†*	0.65*	0.015	0.096
Mn	0.31†	0.52*	0.107	0.017
Ni	0.36*	0.69*	0.037	< 0.000
Pb	0.51†*	0.74*	0.020	0.002
Sn	0.20†	0.50*	0.127	0.018
Zn	0.50†*	0.83*	0.007	< 0.000
PAH				
Acenapthene	0.45†*	0.45*	0.014	0.047
Anthracene	0.32†*	0.50*	0.125	0.031
Benzo(*a*)Pyrene	0.59†*	0.62*	0.008	0.016
Benzo(*a*)Anthracene	0.53†*	0.60*	0.000	0.001
Chrysene	0.62†*	0.62*	0.009	0.026
Fluorathene	0.59†*	0.47*	0.002	0.020
Fluorene	0.02	0.12	0.070	0.038
Naphthalene	0.31†*	0.73*	0.031	0.027
2-Me-Naphthalene	0.40†*	0.69*	0.087	0.004
Phenanthrene	0.26†*	0.14	0.003	0.021
Pyrene	0.60†*	0.49*	0.001	0.011

have DO concentrations that range from anoxia during late night/early morning low tides to super-saturation during the daytime. On the basis of previous DO data collected from a subset of these sites, there is some evidence that lipid peroxidation and heat shock proteins may be particularly valuable indicators of DO stress, but no significant relationships were observed between potential DO stress and lysosomal destabilization or GSH levels. Since GSH is involved in ameliorating free radical associated damage, the lack of a relationship between GSH and DO stress is somewhat surprising. While we do not mean to imply that GSH levels cannot be affected by increases in free radicals, or that Hsp 70s and lipid peroxidation do not increase due to contaminant exposures, these responses did tend to correlate with either contaminant or DO stress. These preliminary findings suggest the intriguing possibility that lysosomal destabilization and GSH may be particularly sensitive indicators of contaminant stress, while heat shock proteins and lipid peroxidation may be valuable indicators of DO stress. The potential associations between DO conditions and lipid peroxidation or heat shock proteins are currently being investigated to determine if different suites of biomarkers may be used to distinguish contaminant and DO stress.

The lack of MXR induction was somewhat surprising since many of the urban/industrial sites such as KOP and DIE have high concentrations of PAHs that have been identified as substrates for P-gp. However, subsequent seasonal studies conducted with native oysters from polluted and unpolluted sites indicated

that site-specific differences were more likely to be detected during the summer or autumn months, but spring levels were very similar from all sites (Keppler 1997). Since the deployed oyster studies presented here were conducted during the spring, studies should be conducted during the summer or autumn before the potential value of MXRs as a biomarker of organic pollutants can be determined.

Environmental monitoring programmes have typically used the strategy of comparing animals from reference sites to polluted sites. However, identification of suitable reference sites can be problematic, particularly if there are differences in 'natural stressors', i.e. salinity variations, desiccation stress, food availability, etc. It is therefore advantageous to have an adequate number of reference sites so that site-specific differences can be based on combined reference estimates, lending confidence to the identification of xenobiotic effects (Krishnakumar *et al*,. 1994). Evoking a biomedical model, the 'normal ranges' of cellular responses provide an important means of diagnosing stress or disease condition. Marine environmental toxicology would benefit from a similar approach. For example, based on a fairly extensive set of data (field and laboratory) for the lysosomal assay, the percent destabilized lysosomes typically ranges between 15 and 25%, and levels above 30% were consistently associated with elevated contaminants. Likewise, GSH levels of normal oysters ranged from 600 to 1200 nM g^{-1} and levels below 400 nM g^{-1} were consistently associated with contaminants. If normal ranges of cellular responses of marine organisms can be defined, the application of biomarkers as monitoring tools will be further advanced.

In conclusion, these studies demonstrate the potential value of suites of cellular responses as indicators of stress. A variety of perturbed responses was observed at many of the most polluted sites, whereas fewer perturbed responses were observed at less polluted sites. Multiple indices improve the ability to conclude with a relatively high degree of certainty that organisms from a site are stressed by anthropogenic activities. When multiple responses that reflect both oxidative stress and chemical stress are observed, this may suggest that a site is experiencing both pollutant and dissolved oxygen stress, or may indicate extensive degradation. The interpretation of individual responses is improved when evaluated in the context of a suite of cellular biomarkers.

Acknowledgements

We gratefully acknowledge J. Hameedi and NOAA (National Oceanic and Atmospheric Administration, Office of Ocean Resources Conservation and Assessment) who provided funding for these studies (Cooperative Agreement No. NA57OA0486); Geoff Scott and Dan Bearden of NOAA/NOS, Charleston, SC for the sediment contaminant analyses and other general assistance with these studies.

References

ADEMA, C. M., VAN DER KNAPP, W. P. W. and SMINIA, T. 1991, Molluscan hemocyte-mediated cytotoxicity: the role of reactive oxygen intermediates. *Reviews in Aquatic Science* , **4**, 201–223.

ANDERSON, M. E. 1985, Determination of glutathione and glutathione disulfide in biological samples. *Methods in Enzymology*, **113**, 548–555.

AUFFRET, M. 1998. Bivalve hemocyte morphology. *American Fisheries Society Special Publication*, **18**, 169–177.

BARNETT, V. and LEWIS, T. 1978, *Outliers in Statistical Data* (Chichester: John Wiley & Sons).

BENSON, W. H., BAER, K. N. and WATSON, C. F. 1990, Metallothionein as a biomarker of environmental

metal contamination. species-dependant effects. In *Biomarkers of Environmental Contamination*, J. F. McCarthy and L. R. Shugart, eds (Chelsea, MI: Lewis Publishers).

CHRISTIE, N. T. and COSTA, M. 1984, *In vitro* assessment of the toxicity of metal compounds. IV. Disposition of metals in cells: interactions with membranes, glutathione, metallothionein, and DNA. *Biology of Trace Elements Research*, **6**, 139–158.

CONNERS, D. E. 1998, The toxicological significance of glutathione depletion in oysters *Crassostrea virginica*, exposed to copper under laboratory and field conditions. Masters thesis, Medical University of South Carolina, Charleston, SC, 69 pp.

CORNWALL, R., TOOMEY, B. H., BARD, S., BACON, C., JARMAN, W. M. and EPEL, D. 1995, Characterization of multixenobiotic/multidrug transport in the gills of the mussel *Mytilus californianus* and identification of environmental substrates. *Aquatic Toxicology*, **31**, 277–296.

CSALLANY, A. S., GUAN, M. D., MANWARING, J. D. and ADDIS, P. B. 1984, Free malonaldehyde determination in tissues by high-performance liquid chromatography. *Analytical Biochemistry*, **142**, 277–283.

DECAPRIO, A. P. 1997, Biomarkers: Coming of age for environmental health and risk assessment. *Environmental Science & Technology*, **31**, 1837–1848.

DI GUILIO, R. T., BEHAR, J. V., CARLSON, D. B., HASSPIELER, B. M. and WATSON, D. E. 1995, Determinants of species susceptibility to oxidative stress: a comparison of channel catfish and brown bullhead. *Marine Environmental Research*, **39**, 175–180.

DUDLEY, R. E. and KLAASSEN, C. D. 1984,. Changes in hepatic glutathione concentration modify cadmium-induced hepatotoxicity. *Toxicology and Applied Pharmacology*, **72**, 530–538.

ENGEL, D. W. and BROUWER, M. 1989, Metallothionein and metallothionein-like proteins: physiological importance. *Advances in Comparative Environmental Physiology*, **5**, 53–75.

FAIRCHILD, C. R., IVY, S. P., RUSHMORE, T., LEE, G., KOO, P., GOLDSMITH, M. E., MYERS, C. E., FARBER, E. and COWAN, K. H. 1987. Carcinogen-induced mdr overexpression is associated with xenobiotic resistance in rat preneoplastic liver nodules and hepatocellular carcinoms. *Proceedings of the National Academy of Sciences USA*, **84**, 7701–7705.

GUTTERIDGE, J. M. C. and HALLIWELL, B. 1990, The measurement and mechanism of lipid peroxidation in biological systems. *Trends in Biological Science*, **15**, 129–135.

HAMER, D. H. 1986. Metallothionein. *Annual Reviews in Biochemistry*, **55**, 913–51.

HUGGETT, R. J., KIMERLE, R. A., MEHRIE, P. M. and BERGMAN, H. L. 1992, *Biomarkers: Biochemical, Physiological, and Histological Markers of Anthropogenic Stress* (Boca Raton: Lewis Publ.), 347.

JONES, D. P., BROWN, L. S. and STERNBERG, P. 1995, Variability in glutathione-dependent detoxification *in vivo* and its relevance to detoxification of chemical mixtures. *Toxicology*, **105**, 267–274.

KARNAKY, K., SUGGS, K., FRENCH, S. and WILLINGHAM, M. C. 1993, Evidence for the multidrug transporter, P-glycoprotein, in the killifish, *Fundulus heteroclitus*. *Bulletin Mt. Desert Island Biological Laboratory*, **32**, 61–62.

KEHRER, J. P. 1993, Free radicals as mediators of tissue injury and disease. *Critical Reviews in Toxicology*, **23**, 21–48.

KEPPLER, C. 1997, Expression of multixenobiotic resistance proteins and total proteins in the gills of the oyster, *Crassostrea virginica*. Ms thesis, University of Charleston-South Carolina, 96 pp.

KETTERER, B. 1986, Detoxification reactions of glutathione and glutathione transferases. *Xenobiotica*, **16**, 957–973.

KOSOWER, N. S. and KOSOWER, E. M. 1978, The glutathione status of cells. *International Reviews in Cytology*, **54**, 109–160.

KRISHNAKUMAR, P. K., CASILLAS, E. and VARANASI, U. 1994, Effects of environmental contaminants on the health of *Mytilus edulis* from Puget Sound, Washington, USA. I. Cytochemical measures of lysosomal responses in the digestive cells using automatic image analysis. *Marine Ecology Progress Series*, **106**, 249–261.

KURELEC, B., KRČA, S., B., PIVCEVIC, B., UGARKOVIC, D., BACHMANN, M., IMSIECKE, G. and MULLER, W. E. G. 1992, Expression of P-glycoprotein gene in marine sponges. Identification and characterization of the 125 kDa drug-binding glycoprotein. *Carcinogenesis*, **13**, 69–76.

LAUENSTEIN, G. G. and CANTILLO, A. Y. 1993, Sampling and analytical methods of the National Status and Trends Program, National Benthic Surveillance and Mussel Watch projects. NOAA Technical Memorandum NOS ORCA 71.

LOWE, D. M., MOORE, M. N. and CLARK, K. R. 1981, Effects of oil on digestive cells in mussels: quantitative alterations in cellular and lysosomal structure. *Aquatic Toxicology*, **1**, 213–226.

LOWE, D. M., MOORE, M. N. and EVANS, B. M. 1992, Contaminant impact on interactions of molecular probes with lysosomes in living hepatocytes from dab *Limanda limanda*. *Marine Ecology Progress Series*, **91**, 135–140.

LOWE, D. M., FOSSATO, V. U. and DEPLEDGE, M. H. 1995, Contaminant-induced lysosomal membrane damage in blood cells of mussels *Mytilus galloprovincialis* from the Venice Lagoon: an *in vitro* study. *Marine Ecology Progress Series*, **129**, 189–196.

MASON, A. Z. and JENKINS, K. D. 1996, Metal detoxification in aquatic organisms. In *Metal Speciation*

and Bioavailability in Aquatic Systems, A. Tessier and D. R. Turner, eds (Chichester: John Wiley & Sons), pp. 479–608.

MEISTER, A. and ANDERSON, M. E. 1983, Glutathione. *Annual Reviews in Biochemistry.*, **52**, 711–760.

MINIER, C., AKCHA, F. and GALGANI, F. 1993, P-glycoprotein expression in *Crassostrea gigas* and *Mytilus edulis* in pollution seawater. *Comparative Biochemistry and Physiology*, **106B**, 1029–1036.

MOORE, M. N. 1985, Cellular responses to pollutants. *Marine Pollution Bulletin*, **16**, 134–139.

MOORE, M. N. 1990, Lysosomal cytochemistry in marine environmental monitoring. *Histochemistry Journal*, **22**, 187–191.

MOORE, M. N. 1994, Reactions of molluscan lysosomes as biomarkers of pollutant-induced cell injury. In *Contaminants in the Environment. A Multidisciplinary Assessment of Risks to Man and Other Organisms*, A. Renzoni, N. Mattei, L. Lorena and M. C. Fossi, eds (Boca Raton: CRC Press, Lewis Publishers), pp. 111–123.

PROZIALECK, W. C. and LAMAR, P. C. 1995, Effects of glutathione depletion on the cytotoxic actions of cadmium in LLC-PK1 cells. *Toxicology and Applied Pharmacology*, **134**, 285–295.

REGOLI, F. 1992, Lysosomal responses as a sensitive stress index in biomonitoring heavy metal pollution. *Marine Ecology Progress Series*, **84**, 63–69.

REGOLI, F. and PRINCIPATO, G. 1995, Glutathione, glutathione-dependent and antioxidant enzymes in mussel, *Mytilus galloprovincialis*, exposed to metals under field and laboratory conditions: implications for the use of biochemical biomarkers. *Aquatic Toxicology*, **31**, 143–164.

REGOLI, F., NIGRO, M. and ORLANDO, E. 1998, Lysosomal and antioxidant responses to metals in the Antarctic scallop *Adamussium colbecki*. *Aquatic Toxicology*, **40**, 375–392.

RINGWOOD, A. H. and BROUWER, M. 1993, Expression of constitutive and metal-inducible metallothioneins in oyster embryos *Crassostrea virginica*. *Comparative Biochemistry and Physiology,* **106B**, 523–529.

RINGWOOD, A. H., HOLLAND, A. F., KNEIB, R. and ROSS, P. 1996, EMAP/NS & Pilot Studies in the Carolinian Province: Indicator Testing and Evaluation in Southeastern Estuaries. NOAA Technical Memorandum NOS ORCA 102. 115 p.

RINGWOOD, A. H., CONNERS, D. and HOGUET, J. 1998a, The effects of natural and anthropogenic stressors on lysosomal destabilization in oysters, *Crassostrea virginica*. *Marine Ecology Progress Series*, **166**, 163–171.

RINGWOOD, A. H., CONNERS, D. E. and DINOVO, A. 1998b, The effects of copper exposures on cellular responses in oysters. *Marine Environmental Research*, **46**, 591–595.

ROESIJADI, G. 1992, Metallothioneins in metal regulation and toxicity in aquatic animals. *Aquatic Toxicology*, **22**, 81–114.

ROTHMAN, J. E. 1989, Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells. *Cell*, **59**, 591–601.

SANDERS, B. M. 1990. Stress proteins: Potential as multitiered biomarkers. In *Biomarkers of Environmental Contamination*, L. Shugart and J. McCarthy, eds (Boca Raton, FL: Lewis Publishers), pp. 165–191.

SANDERS, B. M. and MARTIN, L. S. 1993, Stress proteins as biomarkers of contaminant exposure in archived environmental samples. *Science of the Total Environment*, **139/140**, 459–470.

SINGHAL, R. K., ANDERSON, M. E. and MEISTER, A. 1987, Glutathione, a first line of defense against cadmium toxicity. *FASEB Journal*, **1**, 220–223.

STEGEMAN, J. J., BROUWER, M., DI GIULIO, R. T., FORLIN, L., FOWLER, B. A., SANDERS, B. M. and VAN VELD, P. A. 1992, Molecular responses to environmental contamination: Enzyme and protein systems as indicators of chemical exposure and effect. In *Biomarkers: Biochemical, Physiological, and Histologic Markers of Anthropogenic Stress*, R. Huggett, R. A. Kimerle, P. H. Mehne and H. L. Bergman, eds (Boca Raton: Lewis Publishers), pp. 235–335.

TOOMEY, B. H. and EPEL, D. 1993, Multixenobiotic resistance in *Urechis caupo* embryos: protection from environmental toxins. *Biological Bulletin*, **185**, 355–364.

VIARENGO, A. 1989, Heavy metals in marine invertebrates: mechanisms of regulation and toxicity at the cellular level. *Reviews in Aquatic Sciences*, **1**, 295–317.

VIARENGO, A. and NOTT, J. A. 1993, Mechanisms of heavy metal cation homeostasis in marine invertebrates. *Comparative Biochemistry and Physiology*, **104C**, 355–372.

VIARENGO, A., CANESI, L., PETRICA, M., POLI, G., MOORE, M. N. and ORUNESU, M. 1990, Heavy metal effects on lipid peroxidation in the tissues of *Mytilus galloprovincialis* Lam. *Comparative Biochemistry and Physiology*, **9C**, 37–42.

VIARENGO, A., PONZANO, E., DONDERO, F. and FABBRI, R. 1997, A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic molluscs. *Marine Environmental Research*, **44**, 69–84.

WELCH, W. J. 1992, Mammalian stress response: cell physiology, structure/function of stress proteins, and implications for medicine and disease. *Physiological Reviews*, **72**, 1063–1081.

WENNING, R. J., DI GIULIO, R. T. and PAGE, D. S. 1998, Oxidant-mediated biochemical effects of paraquat in the ribbed mussel, *Geukensia demissa*. *Aquatic Toxicology*, **12**, 157–170.

WINSTON, G. W. and DI GIULIO, R. T. 1991, Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquatic Toxicology*, **19**, 137–161.

WINSTON, G. W., MOORE, M. N., STRAATSBURG, I. and KIRCHIN, M. A. 1991, Decreased stability of digestive gland lysosomes from the common mussel *Mytilus edulis* L. by in vitro generation of oxygen-free radicals. *Archives of Environmental Contamination and Toxicology*, **21**, 401–408.

WINSTON, G. W., MOORE, M. N., KIRCHIN, M. A. and SOVERCHIA, C. 1996, Production of reactive oxygen species by hemocytes from the marine mussel, *Mytilus edulis*: lysosomal localization and effect of xenobiotics. *Comparative Biochemistry and Physiology*, **113C**, 221–229.

WOFFORD, H. W. and THOMAS, P. 1988, Peroxidation of mullet and rat liver lipids in vitro: Effects of pyridine nucleotides, iron, incubation buffer, and xenobiotics. *Comparative Biochemistry and Physiology*, **89C**, 201–206.

YU, B. P. 1994, Cellular defenses against damage from reactive oxygen species. *Physiological Reviews*, **74**, 139–162.