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P. K. Krishnakumar · E. Casillas · U. Varanasi

Effects of chemical contaminants on the health of *Mytilus edulis* from Puget Sound, Washington. II. Cytochemical detection of subcellular changes in digestive cells

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Abstract Activities of the enzymes NADPH-dependent ferrihemoprotein reductase (NFR), NADH-dependent DT-diaphorase (DTD), gamma-glutamyl transpeptidase (GGT), and catalase (CAT) and peroxisome proliferation (PP) in the digestive cells of *Mytilus edulis* from nine sites in Puget Sound, Washington (USA) sampled in September 1992 were measured cytochemically using image analysis. Mussels from these areas are known to be exposed to a wide range of chemical contaminants. At urban-associated sites, mussels generally showed increased activities of NFR, DTD, and CAT, suppressed GGT activity, and peroxisome proliferation, relative to mussels from the non-urban reference sites. Significant positive relationships were observed between tissue concentrations of polyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and DDTs and activities of NFR, DTD, CAT, and peroxisome proliferation. Structural changes in the digestive gland of mussels also appear to be more responsive to chemical contaminant exposure than changes in enzyme activity. These relationships suggest that NFR, CAT, and the induction of peroxisome proliferation represent complimentary indicators of biological effects from chemical-contaminant exposure in the marine bivalve *M. edulis*. The current findings support the use of selected cytochemically measured subcellular responses as biomarkers of contaminant exposure in environmental monitoring programs.

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P.K. Krishnakumar[1] · E. Casillas (✉) · U. Varanasi
Environmental Conservation Division,
Northwest Fisheries Science Center,
National Marine Fisheries Service,
National Oceanic and Atmospheric Administration,
2725 Montlake Boulevard East,
Seattle, Washington 98112–2097, USA

Present address:
[1] Central Marine Fisheries Research Institute,
P.O. Box 244, Bolar Mangalore 575–001, India

Introduction

The ability of bivalve molluscs to bioaccumulate high concentrations of polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) is well documented (see review by Livingstone 1991). The digestive cells in marine bivalves are a major site of accumulation, as well as detoxication and excretion of organic contaminants. Biochemical and cytochemical measures of various detoxicating and antioxidant enzyme activities in the digestive gland of field- and laboratory-exposed bivalve molluscs have shown potential for use as biomarkers of chemical-contaminant exposure (Bayne et al. 1988; Porte et al. 1991). The presence of cytochrome P450-mediated monooxygenase (MO) and flavin-containing monooxygenase (FMO) activities in bivalve molluscs have been established (Stegeman 1985; Livingstone 1988; Schlenk and Buhler 1989), and increased activities have been reported in the digestive gland of mussels exposed to chemical contaminants (Livingstone 1991). In addition, increased activity of NADPH-ferrihemoprotein reductase (NFR) has been reported in digestive gland of bivalves exposed to organic chemical contaminants (Moore 1988; Suteau and Narbonne 1988; Porte et al. 1991). NADPH-ferrihemoprotein reductase (cytochrome P450 reductase) is a flavoprotein (EC 1.6.2.4) required for electron transfer from NADPH to cytochrome P450 in the endoplasmic reticulum of animal tissues (Livingstone 1991). The cytochrome P450-dependent oxidative metabolism of organic xenobiotics could result in the formation of oxyradicals in the digestive cells of bivalves (Wenning and Di Giulio 1988; Livingstone et al. 1989; Winston et al. 1990). A number of biochemical perturbations such as lipid peroxidation, enzyme inactivation, and membrane and DNA

damage have been described as a consequence of oxyradical generation within animal cells.

There are several enzymes involved in the detoxification of oxyradicals that have potential to serve as biomarkers of anthropogenic contaminant exposure in bivalves. The flavoprotein, DT-diaphorase NAD(P)H-quinone oxydoreductase (EC 1.6.99.2) catalyzes the reduction of several peroxides and quinones, with NADH or NADPH serving as the electron donor (Livingstone et al. 1989). DT-diaphorase (DTD) is believed to act to prevent quinone toxicity, catalyzing a two-electron reduction of quinone compounds to the more stable hydroquinones (Lind et al. 1982). Aquatic organisms are exposed to several quinonoid pollutants, and activity of DTD has been measured and characterized in *Mytilus* sp. (Livingstone et al. 1989; Porte et al. 1991). Some recent studies have evaluated DTD and catalase (CAT) as potential biomarkers for xenobiotic exposure (Livingstone et al. 1989; Viarengo et al. 1991; Hasspeiler and Di Giulio 1992).

The enzyme gamma glutamyl transpeptidase (GGT; EC 2.3.2.2) is widely used as a marker of pre-neoplastic lesions in the rodent liver during chemical carcinogenesis (Pretlow et al. 1987; Parker et al. 1993). Gamma glutamyl transpeptidase catalyzes the transfer of a gamma glutamyl group to numerous peptide and amino acid acceptors and also participates in mercapturic acid formation during Phase II metabolism of xenobiotics (Hanigan and Pitot 1985). Very few studies have examined whether GGT activity is altered in bivalves exposed to chemical contaminants (Cajaraville et al. 1992).

Catalases (EC 1.11.1.6) are hematin-containing enzymes that facilitate the removal of hydrogen peroxide from the cell. In the cell, CAT activity is mainly associated with peroxisomes, which primarily function in fatty acid metabolism (Reddy and Lalwani 1983). The term peroxisome proliferator was introduced to designate a drug or xenobiotic that induces the proliferation of peroxisomes in liver cells. A number of structurally diverse chemicals such as hypolipidemic drugs, herbicides, leukotriene antagonists and plasticizers have been identified as peroxisome proliferators in rodents (Reddy and Azarnoff 1980; Reddy and Lalwani 1983). The induction of peroxisome proliferation has been reported in fishes (Di Giulio et al. 1989; Baldwin et al. 1990), but we are unaware of any studies evaluating peroxisome proliferation in marine bivalves.

The purpose of the present study was to cytochemically measure induction of detoxicating and antioxidant enzymes and peroxisome proliferation in the digestive cells of mussels collected from various areas of Puget Sound, Washington. Surface sediments in some areas of Puget Sound are contaminated with high levels of chemical pollutants (NOAA 1989; Stein et al. 1992). Cytochemical techniques combined with automatic image-analysis were used to quantitatively measure the enzyme activities and proliferation of peroxisomes. Relationships among organic contaminant concentration in tissue (body burden) and the biological responses were then evaluated to demonstrate linkages between xenobiotic exposure of indigenous mussels in the marine environment and subcellular and cellular effects.

Materials and methods

Sampling

Tissue samples were taken from mussels (*Mytilus edulis*) used previously in a companion study of relationships between size and weight, cytochemical measures of lysosomal responses and chemical contaminant body burden of mussels from sites in Puget Sound, Washington (see Krishnakumar et al. 1994). As previously reported, *M. edulis* were collected from their natural beds from nine sites in Puget Sound (Fig. 1) during 21–24, September 1992 (Krishnakumar et al. 1994). Sites included the minimally contaminated areas of Oak Bay, Coupeville, and Double Bluff, in central and north Puget Sound, and Saltwater State Park in south Puget Sound. Mussels from these areas (hereafter called reference sites) were used to document the natural variability (range) for each parameter measured in this study. Chemical analyses of mussel tissues sampled from these reference sites showed that chemical contamination was low for these individuals and representative of concentrations for mussels from minimally-contaminated environments (O'Conner 1992; Krishnakumar et al. 1994). Contaminated sites that were sampled included Eagle Harbor, Seacrest and Four Mile Rock in Elliott Bay; City Waterway in Commencement Bay; and Sinclair Inlet near Bremerton, Washington. Mussels from these areas are considered to be chronically exposed to a variety of anthropogenic chemical contaminants. Areas within Eagle Harbor have been contaminated with

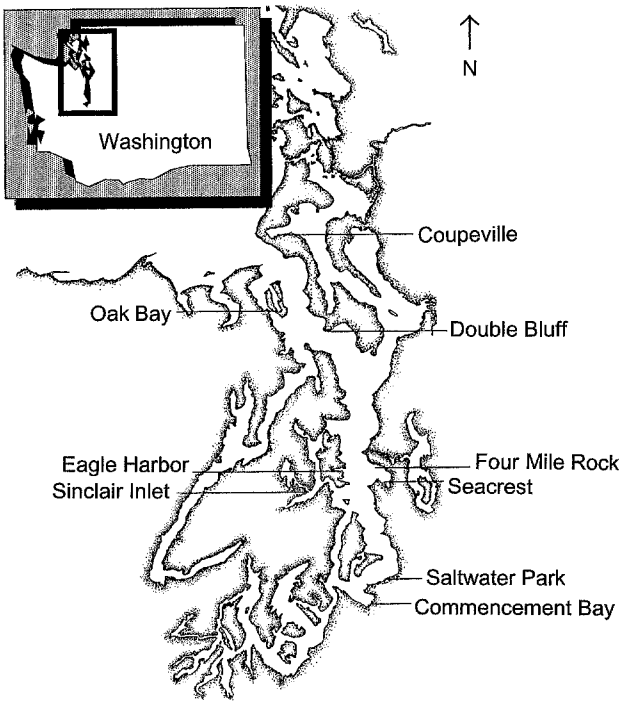

Fig. 1 Map of Puget Sound, Washington, USA, showing collection sites

high levels of creosote-associated polycylic aromatic hydrocarbons (PAHs) and both Commencement and Elliott Bays are high-density urban environments (Stein et al. 1992). The site at Four Mile Rock was one of the sampling areas in the National Status and Trends (NS&T) Mussel Watch Program (NOAA 1989), and was reported to contain mussels with some of the most contaminated tissues, particularly with regard to organic chemical contaminants.

Mussels ($n = 100$, shell length ≥ 40 mm) were haphazardly collected from each site during low tide and transported alive to the laboratory. Mussels < 40 mm in shell length were excluded because the amount of tissue was insufficient for all analyses. Water temperature, salinity, and particulate organic matter content at each site were measured, and reported previously (Krishnakumar et al. 1994) not to be substantially different. Mussels were acclimated in running seawater for 24 h before randomly subsampling (described in the next two subsections) to allow the gut contents to be cleared.

Tissue chemistry

The analyses of tissue contaminants of these mussels has been previously described (Krishnakumar et al. 1994). Thirty to 40 mussels were randomly selected from each site. Whole-mussel tissue samples were dissected, pooled, and kept at $-20\,^{\circ}C$ until chemical analysis. Samples were analyzed for organic chemical contaminants as described by Krahn et al. (1988) and Sloan et al. (1993). The results were expressed in ng g^{-1} dry tissue weight.

Cytochemistry

A small section of digestive gland was rapidly removed from each of ten randomly selected mussels, and the samples were placed in straight rows across the center of cryomolds (Tissue-Tek, Miles Inc, Elkhart, Indiana) (5 tissue samples/mold). Tissue samples were quickly embedded in O.C.T. compound (Tissue-Tek), supercooled in hexane precooled to $-70\,^{\circ}C$ in liquid nitrogen, and maintained at $-80\,^{\circ}C$ until analysis. The frozen tissues were sectioned serially at 10 μm in a cryostat at $-25\,^{\circ}C$. To insure that samples were read blindly, all samples were coded prior to freezing and decoded only after all measurements had been made. The source of all chemicals, unless otherwise indicated, was Sigma Chemicals (St Louis, Missouri).

NADPH-ferrihemoprotein reductase (NFR)

NADPH-ferrihemoprotein reductase was localized in cryostat sections as described by Van Noorden and Butcher (1986) and Moore (1988). The incubation medium contained 0.1 *M* HEPES buffer (pH 8.0), 20 m*M* $MgCl_2$, 20% polyvinyl alcohol, 5 m*M* neotetrazolium chloride and 6 m*M* NADPH. The control medium lacked NADPH. The medium was purged with nitrogen for 10 min. The incubation medium was placed on sections surrounded by plastic formers, and the sections were incubated in darkness for 30 min at 37 °C in a nitrogen atmosphere in an enclosed box kept moist by a bottom lining of damp tissue paper. After incubation, sections were rinsed in running tap water, rinsed in distilled water, and mounted in glycerol gelatin. NFR was deposited on the sections as a red and blue formazan product. The specificity of the cytochemical assays as representative of NADPH-ferrihaemoprotein reductase was confirmed using known inhibitors and stimultors (Table1). The presence of 5 m*M* $NADP^+$, a known inhibitor of NFR activity, in the incubation medium inhibited NFR activity by 61%, while 1 m*M* menadione, a known stimulator of NFR activity, enhanced the activity by 211%. NADPH-ferrihaemoprotein reductase activity in the mussel digestive cells was very low in the absence of substrate (negative control) in the reaction medium (Table 1).

Table 1 *Mytilus edulis.* Characterization of NADPH-ferrihemoprotein reductase activity in digestive cells of samples from Eagle Harbor, Washington. Values are means ± SD, $n = 5$ [See "Materials and methods – Cytochemistry – NADPH-ferrihemoprotein reductase (NFR)" for details]

Reaction conditions	Enzyme activity (pixel density)	% of positive control
Positive control (+ NADPH)	80 ± 23	—
Negative control (− NADPH)	12 ± 1	15
5 m*M* $NADP^+$ (inhibitor)	31 ± 8	39
1 m*M* dicumoral	78 ± 20	97
1 m*M* menadione (activator)	250 ± 52	311

Table 2 *Mytilus edulis.* Characterization of NADH-DT diaphorase activity in digestive cells of samples from City Waterway in Commencement Bay, Washington. Values are means ± SD, $n = 5$ [See "Materials and methods – Cytochemistry – NADH-DT diaphorase (DTD)" for details]

Reaction conditions	Enzyme activity (pixel density)	% of positive control
Positive control (+ NADH)	65 ± 11	—
Negative control (− NADH)	14 ± 1	23
1 m*M* dicumoral (inhibitor)	26 ± 6	40
1 m*M* menadione (activator)	210 ± 38	323

NADH-DT diaphorase (DTD)

The cytochemical detection of DTD was carried out as described by Straatsburg et al. (1989). Incubation medium and procedures were identical to those for NFR activity, save only that NADH was substituted for NADPH. DTD was deposited as a red and blue formazan product. The specificity of the cytochemical assays as representative of NADH-DT diaphorase was confirmed using known inhibitors and stimultors (Table 2). The presence of 1 m*M* dicumoral, a known inhibitor of DTD activity, in the incubation medium inhibited DTD activity by 60% while 1 m*M* menadione, a known stimulator enhanced the activity by 223%. NADH-DT diaphorase activity in the mussel digestive cells was reduced by 77% in the absence of substrate (negative control) in the reaction medium (Table 2).

γ-glutamyl transpeptidase (GGT)

Histochemical localization of GGT was carried out as described by Rutenburg et al (1969). Cryostat sections were fixed at 4 °C for 10 min in Baker's formal calcium containing 2.5% NaCl. Sections were incubated at 37 °C for 3 h in freshly prepared medium containing 2 ml (2.5 mg ml^{-1}) of L-glutamyl-a-4-metoxi-b-napthylamide (GMNA) in 10 ml of 0.1 *M* Tris-HCl buffer at pH 7.4 and 28 ml NaCl (2.5%) with 20 mg glycylglycine and 20 mg fast blue BB salt.

The GMNA was prepared as follows: GMNA (5 mg) is dissolved in 0.1 ml dimethyl sulphoxide, 0.1 ml 1*N* NaOH and 1.8 ml distilled water, to give a 2.5 mg ml^{-1} solution. Following incubation, sections were rinsed in 2.5% NaCl for 2 min and transferred to 0.1 *M* $CuSO_4$ solution for 2 min. Sections were again rinsed in 2.5% NaCl and distilled water and mounted in glycerol gelatin. GGT activity was shown by a red deposit on the sections.

Catalase (CAT)

The cytochemical detection of catalase was carried out as described by Angermuller and Fahimi (1981). Cryostat sections were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.2) containing 2.5% NaCl and 2 m*M* $CaCl_2$ for 3 h at 4 °C. Sections were washed in 0.1 *M* glycine-NaOH buffer (pH 10.5), and incubated in a medium containing 5 m*M* 3,3′-diaminobenzedine tetrachloride and 0.15% H_2O_2 in 0.1 *M* glycine-NaOH buffer at pH 10.5. Sections were incubated for 30 min at 37 °C in darkness. After incubation, sections were rinsed in a 0.1 *M* glycine-NaOH buffer for 5 min, rinsed in distilled water, and mounted in glycerol gelatin. Catalase activity was shown by a dark brown deposit on the sections.

Peroxisome proliferation

Peroxisome proliferation was assessed by microscopical examination, using catalase activity as the marker enzyme for peroxisomes in the digestive tubule in cryostat tissue sections (Beier and Fahimi 1987). Proliferation of peroxisomes was considered evident if $> 50\%$ of the digestive tubules in five fields randomly selected showed evidence of CAT activity. The incidence of peroxisome proliferation in each sample was reported as the percentage of mussels that exhibited catalase activity in $> 50\%$ of their digestive tubules. It should be noted that whereas CAT represents total activity in the digestive gland, proliferation of peroxisomes was considered evident in a mussel if $> 50\%$ of the digestive tubules showed evidence of CAT activity, irrespective of the intensity or total activity of CAT.

Image analysis

Activities of NFR, DTD, GGT and CAT in the tissue sections were quantified by image analysis. The image system consisted of a high-resolution CCD (charge coupled device) color camera (Model 8215.2000, COHU, Inc., San Diego) mounted on a light microscope with a 40× objective. The image was displayed on a television screen and captured with an image-analysis program (NIH image 1.44) with Macintosh software on a Macintosh IICX computer. Five images of digestive tubules for each cytological analysis were randomly taken from each duplicate section of each mussel sample. Images for each cytochemical test were captured in one session during which the microscope illumination and camera setting were constant. The digital image consisted of a 8 bit, 320 × 240 matrix of picture elements (pixels), where each pixel consisted of a number between 0 and 255 representing the intensity of transmitted light (or grey level) at a point. Stored images were later analyzed to determine the average pixel density of the reaction products in each image, as described by Krishnakumar et al. (1994).

Statistical analyses

Differences in NFR, DTD, GGT and CAT activities in the digestive cells of mussels among sites were tested by analysis of variance

Table 3 *Mytilus edulis*. Concentration of organic contaminants in whole tissue (ng g^{-1} dry tissue wt). Samples were analyzed by gas chromatography/mass spectroscopy for polycyclic aromatic hydrocarbons (*PAHs*) and gas chromatography/electron-capture detection for chlorinated hydrocarbons. See Krishnakumar et al. (1994) for more details on chemical contaminant burden of these mussels [*low-molecular weight PAHs* include summed concentrations for napthalene, acenapthylene, acenapthene, fluorene, phenanthrene, anthracene, and 2-methylnapthalene; *high-molecular weight PAHs* include summed concentrations for fluoranthene, pyrene, benz(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benz(e)pyrene, benzo(a)pyrene, indeno (1,2,3,-c,d)pyrene, dibenzo (a,h)anthracene, and benzo(g, h, i)perylene; *total DDTs* include summed concentration of DDT, DDE, and DDD; *other pesticides* include summed concentration of aldrin, chlordane, dieldrin, heptachlor, and lindane]

Organic contaminants	Eagle Harbor	City Waterway	Seacrest	Four mile Rock	Sinclair Inlet	Oak Bay	Saltwater Park	Coupeville	Double Bluff
Polycycic aromatic hydrocarbons									
Σ low-molecular weight PAHs	26 000	750	400	320	100	70	80	80	70
Σ high-molecular weight PAHs	37 500	5010	1230	820	120	40	90	50	50
Chlorinated hydrocarbons									
polychlorinated biphenyls	400	490	500	290	180	60	140	50	110
total DDTs	21	29	28	19	15	11	9	11	11
other pesticides	16	33	22	17	11	10	17	12	11

(ANOVA). Dunnett's multiple-comparison test was used to determine differences in enzymatic activity and percent peroxisome proliferation of mussels from all reference sites combined ("combined reference") in comparison to mussels from each of the urban-associated sites. Linear regression analysis (Zar 1974) was used to initially evaluate relationships between the mean NFR, DTD, GGT and CAT activity and peroxisome proliferation in the digestive cells of mussels and the tissue burden of chemical contaminants. Biological effects and toxic chemicals typically show a sigmoidal dose-response relationship (Klaassen 1986). For those relationships that appeared to be the strongest, non-linear regression analysis was used to further evaluate the relationship. A sigmoid exponential saturation curve ($y = A/(1 + Be^{nx})$) was used to model the relationships between biological effects and tissue-contaminant burden. The non-linear regression algorithm, which obtains a least-squares estimate of the equation parameters (the Levenberg–Marquardt method), was applied using DeltaGraph (DeltaPoint, Inc., Monterey, California). Findings were considered significant at $\alpha \leq 0.05$.

Results

Tissue chemical-contaminant burden

Concentrations (ng g^{-1} dry tissue wt) of organic contaminants in *Mytilus edulis* from the nine sites are shown in Table 3 as summed (Σ) concentrations of individual PAH and CH (chlorinated hydrocarbon) analytes. A more detailed description of the concentrations of organic and metal contaminants in mussel tissues from these sites has been given by Krishnakumar et al. (1994).

Tissue concentrations of ΣPAHs, ΣPCBs, total DDTs, and Σ pesticides were higher in mussels from the urban-associated sites of Eagle Harbor, City Waterway, Seacrest, Four Mile Rock and Sinclair Inlet than in mussels from non-urban associated sites (Oak Bay, Saltwater Park, Coupeville and Double Bluff. Concentrations of ΣPAHs were greatest in tissue of mussels from Eagle Habor, nearly 500 times higher than in mussels from the reference sites. Concentrations of ΣPAHs in tissues of mussels from reference sites were comparable to levels observed in mussels from minimally contaminated areas in the near coastal environment of the USA (O'Conner 1992). Chlorinated hydrocarbon concentrations were highest in tissues of mussels from City Waterway and Seacrest, nearly five times greater than observed in mussels from the reference sites. There was a significant amount of covariance among the chemical contaminants in mussels from all nine sites. Simple correlation coefficients were significant for all possible comparisons of the organic contaminants except for the relationship between the summed concentration of pesticides (excluding DDTs) and the summed concentration of PAHs, which was not significant. Correlation coefficients for all other comparisons ranged from 0.72 to 0.97.

Cytochemical measurements

Activities of NFR, DTD, GGT and CAT and the incidence of peroxisome proliferation in the digestive cells of *Mytilus edulis* from the nine sites are summarized in Table 4. In general, activities of NFR, DTD, GGT, CAT and peroxisome proliferation were similar among mussels from the minimally-contaminated reference sites and significantly different from mussels from the urban-associated sites (ANOVA; Fisher's protected least-significant-difference test). The NFR and CAT activities were significantly higher in mussels from the contaminated sites of Eagle Harbor, City Waterway, Seacrest and Four Mile Rock compared to mussels from all the reference sites combined (combined reference ANOVA; Dunnett's multiple-comparison test). Similarly, DTD activity was significantly higher in mussels from the two most contaminated sites (Eagle

Table 4 *Mytilus edulis*. Cytochemical responses in digestive cells (means ± SD; $n = 10$) assessed by automatic image-analysis. Peroxisome proliferation expressed as percent of mussels ($n = 10$) showing increased proliferation from each site, all other data as pixel density [* significantly ($p < 0.05$) different than for mussels from all of reference sites combined: ANOVA-Dunnetts' multiple comparison one-tailed test or chi-square analyses (peroxisome proliferation only)]

Site	NADPH-ferrihaemoprotein reductase	NADH-DT-diaphorase	Gamma-glutamyl transpeptidase	Catalase	Peroxisome proliferation (%)
Urban					
Eagle Harbor	71 ± 8*	66 ± 11*	25 ± 10*	20 ± 4*	90*
City Waterway	70 ± 16*	72 ± 15*	69 ± 13	15 ± 3*	60*
Seacrest	67 ± 11*	56 ± 19	26 ± 7*	16 ± 3*	70*
Four Mile Rock	66 ± 9*	46 ± 12	26 ± 9*	15 ± 3*	60*
Sinclair Inlet	40 ± 20	49 ± 18	55 ± 20	11 ± 4	60*
Reference					
Oak Bay	40 ± 6	47 ± 9	61 ± 20	10 ± 2	10
Saltwater Park	47 ± 14	50 ± 17	91 ± 17	13 ± 2	30
Coupeville	45 ± 18	39 ± 15	41 ± 12	11 ± 3	20
Double Bluff	34 ± 5	39 ± 16	90 ± 24	11 ± 3	30
Combined reference	41 ± 12	44 ± 15	71 ± 28	11 ± 3	22.5

Table 5 *Myilus edulis.* Correlation coefficients between tissue concentrations of polycyclic aromatic hydrocarbons (*PAHs*) and polychlorinated hydrocarbons (*PCBs*) and cytochemical changes in digestive gland. Concentration of PAHs were initially log-transformed before linear relationships were evaluated [*Other pesticides* summed concentration of pesticides described in Table 1; * significant relationship ($p \leq 0.05$) between bioindicator response and selected organic contaminants measured in whole tissue of mussels sampled from Puget Sound, Washington]

Bioindicator	Contaminant			
	ΣPAHs	PCBs	Total DDTs	Other pesticides
NADPH-ferrihemoprotein reductase	0.89*	0.90*	0.85*	0.74*
DT-diaphorase	0.84*	0.83*	0.78*	0.78*
γ-glutamyl transpeptidase	−0.53	−0.45	−0.49	−0.53
Catalase	0.94*	0.81*	0.67*	0.52
Peroxisome proliferation	0.87*	0.84*	0.75*	0.46

Harbor and City Waterway) than in mussels from all the reference sites combined. In contrast, GGT activity was significantly lower in mussels from the contaminated sites of Eagle Harbor, Seacrest and Four Mile Rock than in mussels from all the reference sites combined. The prevalence of peroxisome proliferation was 60 to 90% in the digestive cells of mussels from contaminated sites, while it was only 10 to 30% among mussels from the reference sites.

Relationship between tissue chemical-contaminant burden and cytochemical measures

Significant relationships were obtained between measures of anthropogenic contaminant exposure (e.g. body burden of PAHs and PCBs) and activities of enzymes typically involved in the biotransformation of organic xenobiotics and peroxisome proliferation in the digestive gland in mussels from Puget Sound (Table 5). For example, as the level of ΣPAHs (log-transformed), PCBs, or total DDTs in mussel tissue increased, the mean NFR, DTD, and CAT activities and proliferation of peroxisomes in their digestive gland increased significantly ($p \leq 0.05$). Additionally, the summed concentration of other pesticides, which include aldrin, chlordane, dieldrin, heptachlor, and lindane, were significantly related to increased NFR and DTD activities in the digestive gland of mussels; however, these pesticides were not associated with increased CAT activity or proliferation of peroxisomes as was observed for the other contaminant classes. Even though GGT activity was significantly depressed in mussels from three of the five urban-associated areas, no significant inverse relationship could be identified between any of the classes of contaminants in the tissues of mussels and GGT activity in their digestive gland.

The tissue concentrations of ΣPAHs and ΣPCBs in tissues of mussels from several of the urban-associated areas of Puget Sound were similar to high levels found in mussels from the NOAA's Mussel Watch Program (O'Conner 1992), and the correlations observed

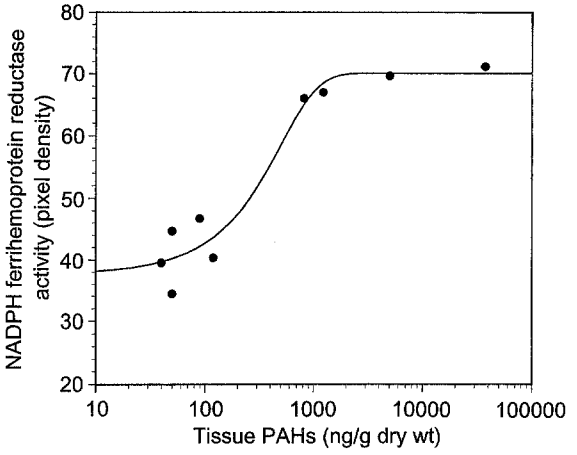

Fig. 2 *Mytilus edulis.* Relationship between Σ high-molecular weight PAHs and NADPH-ferrihemoprotein reductase activity ($R^2 = 0.95$) in digestive cells of mussels from variety of sites in Puget Sound, Washington

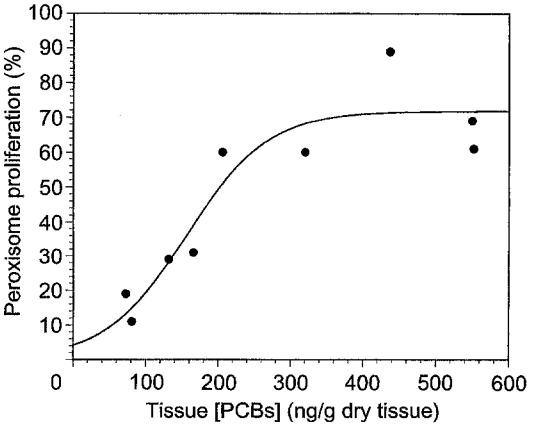

Fig. 3 *Mytilus edulis.* Relationship between total PCBs and percent peroxisome proliferation ($R^2 = 0.86$) in digestive cells of mussels from variety of sites in Puget Sound, Washington

between the tissue burdens of ΣPAHs or ΣPCBs and biological effects were highly significant; examples of these relationships are presented. In one example (Fig. 2), the level of ΣPAHs in mussel tissues is significantly correlated with the mean NFR activity in the digestive gland ($R^2 = 0.95$, $p \leq 0.05$, ANOVA). In another example (Fig. 3) representing a strong relationship, tissue levels of PCBs are significantly correlated with the percent increase in proliferation of peroxisomes ($R^2 = 0.86$, $p \leq 0.05$, ANOVA).

Discussion

The results of our investigation demonstrate that the enzymes, NFR, DTD and CAT, which are involved in the biotransformation of organic xenobiotics (Livingstone 1991), and the incidence of peroxisome proliferation were increased in the digestive gland of the mussel *Mytilus edulis* sampled from urban areas of Puget Sound, Washington. The strong association between chemical-contaminant body burden and these subcellular changes suggest that increased exposure to organic chemicals was a causative factor. Although GGT activity was significantly decreased in mussels from some of the urban-associated sites, no association between chemical-contaminant body burden and GGT activity was observed. The current findings support the use of selected cytochemically-measured subcellular responses as biomarkers of contaminant exposure in environmental monitoring programs.

The correlation between organic chemical contaminants such as PAHs and PCBs in tissues and increased NFR activity and proliferation of peroxisomes suggests that xenobiotics may be the causative agents. Because of co-occurrence of major classes of contaminants, it is difficult to attribute cause-and-effect relationships to any single class of chemical contaminants. However, ultrastructural, cytochemical and morphometric results of experimental and field studies have shown that organic contaminants such as PAHs and PCBs induce substantial alterations in both structure and function of the digestive cells of bivalve molluscs (Bayne et al. 1988; Moore 1991). Other investigators have reported consistent, positive relationships between prevalences of certain hepatic lesions in fish and PAH exposure (Myers et al. 1994), suggesting potential causal links between exposure to these contaminants and biological effects. Moreover, the concentrations of ΣPAHs and ΣPCBs in tissue of mussels from several of the urban-associated areas of Puget Sound were comparable to the highest concentrations found in mussels from the NOAA's Mussel Watch Program (O'Conner 1992), whereas the tissue burden of total DDTs and the summed concentration of other pesticides were relatively low and not comparable to high concentrations found in mussels from the NOAA's Mussel Watch Program (O'Conner 1992). Thus, the consistent enzymatic and cytological changes observed in the present study in the digestive cells of *Mytilus edulis* collected from urban-associated sites in Puget Sound, Washington, may be attributable to the elevated tissue concentrations of PAHs and PCBs.

The enzyme activities that exhibited a substantial range of response to contaminant exposure were NFR and CAT. For example, NFR activity in the digestive cells of mussels from four of the urban-associated sites were 67% greater than activities in mussels from the reference sites, while the activities of the oxidase enzyme, CAT, were 50% greater than mussels from the reference sites. Increases in NFR enzyme activity in digestive-gland microsomes have been reported in several bivalve species exposed to pollutants (Moore 1988; Schlenk and Buhler 1989; Porte et al. 1991). Elevation of oxyradical production following exposure to organic chemical contaminants and increased activities of DTD and CAT have been detected in homogenates of digestive gland of several molluscan species (Porte et al. 1991; Cajaraville et al. 1992).

Peroxisome proliferation also was significantly elevated in *Mytilus edulis* from the urban sites. Coincident with peroxisome proliferation may be alterations in the peroxisomal membrane permeability to hydrogen peroxide. The diffusion of hydrogen peroxide from peroxisomes may lead to the production of the biologically damaging free radical (•OH) in the cytosol (Reddy and Lalwani 1983). Excessive accumulation of lipofuscin in the liver of rats exposed to peroxisome proliferators was reported as evidence for the increased production of free radicals as a result of hydrogen peroxide generated by sustained proliferation of peroxisomes (Reddy et al. 1982). Similarly, we have reported an excessive accumulation of lipofuscin in the digestive cells of mussels collected from the same contaminated sites (Krishnakumar et al. 1994). These findings suggest that peroxisome proliferation in *M. edulis* may lead to increased levels of cellular hydrogen peroxide which can alter cellular macromolecules.

Although a significant relationship between tissue burden of PAHs and PCBs and DTD activity was identified, significant increases in the activity of DTD was found only in mussels collected from Eagle Harbor and City Waterway, the sites where mussels exhibited the highest tissue concentrations of PAHs. Organic contaminants such as PAHs have been shown to induce the activities of DTD in mussels (Livingstone et al. 1989; Porte et al. 1991). Benzo(a)pyrene, a model PAH, is metabolized predominantly to quinones by digestive-gland microsomes of *Mytilus edulis* (Stegeman 1985; Livingstone 1988). The DTD-mediated reduction of quinones to hydroquinones and subsequent conjugation and excretion reduces the concentrations of quinones available for redox cycling (Livingstone et al. 1989). Thus, the present results appear to suggest that the tissue burdens of PAHs in mussels from Eagle

Harbor and City Waterway were sufficient to induce increased DTD activity. In contrast to observations of increased activities of NFR, DTD, and CAT in the digestive cells of mussels from urban-associated sites, a significant decrease in the activities of GGT was observed in mussels from the contaminated sites of Eagle Harbor, Seacrest and Four Mile Rock compared to the reference sites. Despite these observations, no relationship between chemical-contaminant body burden and GGT activity was identified.

The response of peroxisomes in digestive cells of mussels exposed to contaminants is generally similar to the effects observed in the hepatocytes of fish and rodents. Chemicals identified as peroxisome proliferators are reported to suppress the activities of GGT in the hepatocytes of rodents (Pretlow et al. 1987). Proliferation of peroxisomes in hepatocytes of fishes and rodents exposed to xenobiotics was accompanied by increased CAT activity and decreased GGT activity (Yang et al. 1990; Garberg et al. 1992). In the present study, we also observed a significant increase in CAT, a marker of peroxisome proliferation, and a suppression of GGT activity in mussels from the urban-associated sites compared to those from the reference sites. Although the observed proliferation of peroxisomes could be a secondary response to nutritional differences amongst the sites, we reported in our previous study that particulate organic matter at all the sites were sufficient for normal growth (Krishnakumar et al. 1994). Furthermore, in a preliminary laboratory study, we observed increased proliferation of peroxisomes in *Mytilus edulis* exposed via diet to a mixture of PAHs or PCBs (Krishnakumar unpublished data), supporting our field study results.

Structural changes in the digestive gland of mussels appear to be more responsive to chemical contaminant exposure than changes in enzyme activity. In the present study, we observed increased proliferation of peroxisomes but no changes in enzyme activity in the digestive cells of mussels from Sinclair Inlet. Previously, we reported evidence for impaired lysosomal stability in mussels collected from Sinclair Inlet (Krishnakumar et al. 1994). Lysosomal changes were also found to be related to the tissue burden of PAHs and PCBs in mussels (Krishnakumar et al. 1994). Although tissue PAH- and PCB-contaminant burden was higher in mussels from Sinclair Inlet than in mussels from the non-urban reference sites, the tissue chemical-contaminant burden was much less than that of mussels from the other urban sites. Based on this evidence, cytological changes appear to be more responsive to contaminant exposure than are activities of selected enzymes involved in the biotransformation of xenobiotics; however, the dose-response of cytological and enzymatic changes in the digestive cells of mussels exposed to chemical contaminants is necessary to evaluate differential sensitivity of cytological and enzymatic changes.

In summary, the present study demonstrated that mussels from urban-associated areas of Puget Sound, Washington, exhibited significant alterations in cytochemically measured peroxisome proliferation and activities of selected enzymes involved in the biotransformation of xenobiotics compared to mussels from minimally-contaminated reference areas, and that these changes were positively and significantly related to levels of chemical contaminants in tissues. This suggests that these parameters may be useful indicators of contaminant-induced effects in natural populations of mussels. Although further studies are needed to delineate the specificity to chemical contaminant exposure and to assess the influence of environmental factors (seasonal effects) and physiological changes (e.g. reproduction), the results of the present study suggests that NADPH-dependent ferrihemoprotein reductase activity, catalase activity, and the induction of peroxisome proliferation represent promising and complimentary biomarkers of contaminant exposure in the marine bivalve *Mytilus edulis*.

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References

Angermuller S, Fahimi HD (1981) Selective cytochemical localization of peroxidase, cytochemical localization of peroxidase, cytochrome oxidase and catalase in rat liver with 3,3' diaminobenzidine. Histochemistry 71: 33–44

Baldwin LA, Calabrese EJ, Kostecki PT, Yang JH (1990) Isolation of peroxisomal enoyl-CoA hydratase in rainbow trout and immunochemical identification with the bifunctional enzyme. Fish Physiol Biochem 8: 347–351

Bayne BL, Clarke KR, Gray GS (1988) Biological effects of pollutants: the results of a practical workshop. Mar Ecol Prog Ser 46: 1–278

Beier K, Fahimi HD (1987) Application of automatic image analysis for morphometric studies of peroxisomes stained cytochemically for catalase. II. Light-microscopic application. Cell Tissue Res 247: 179–185

Cajaraville MP, Uranga JA, Angolu E (1992) Comparative effects of the water accommodated fraction of three oils on mussels. Quantitative histochemistry of enzymes related to the detoxication metabolism. Comp Biochem Physiol 103C: 369–377

Di Giulio RT, Halaig C, Wolf T (1989) Phase I and phase II biotransformation enzyme activities in channel catfish exposed to contaminated sediment. Toxicologist 9: p 43

Garberg P, Stenius U, Nilsson K, Hogberg J (1992) Peroxisome proliferation and resistance to hydrogen peroxide in rat hepatocytes: is development of resistance an adaptation to cytotoxicity? Carcinogenesis 13: 1751–1758

Hanigan MH, Pitot HC (1985) Gamma-glutamyl transpeptidase – its role in hepatocarcinogenesis. Carcinogenesis 6: 165–172

Hasspeiler BM, Di Giulio RT (1992) DT diaphorase [NAD(P)H]: (quinone acceptor oxidoreductase): kinetics and distribution. Aquat Toxic 24: 143–152

Klaassen CD (1986). Principles of toxicology. In: Klaassen CD, Amdur MO, Doull J (eds) Casarett and Doull's toxicology; the basic science of poisons. Macmillan Publishing Co, New York, pp 11–32

Krahn MM, Moore LK, Bogar RG, Wigrens CA, Chan SL, Brown DW (1988) High performance liquid chromatographic methods for isolating organic contaminants from tissue and sediment extracts. J Chromat 437: 161–175

Krishnakumar PK, Casillas E, Varanasi U (1994) Effect of environmental contaminants on the health of *Mytilus edulis* from Puget Sound, Washington: cytochemical measures of lysosomal responses in the digestive cells using automatic image analysis. Mar Ecol Prog Ser 106: 249–261

Lind C, Hochstein P, Ernster L (1982) DT-diaphorase as a quinone reductase: a cellular control device against semiquinone and superoxide radical formation. Archs Biochem Biophys 216: 175–185

Livingstone DR (1988) Responses of microsomal NADPH-cytochrome reductase activity and cytochrome P-450 in digestive glands of *Mytilus edulis* and *Littorina littorea* to environmental and experimental exposure to pollutants. Mar Ecol Prog Ser 46: 37–43

Livingstone DR (1991) Organic xenobiotics metabolism in marine invertebrates. In: Gilles R (ed) Advances in comparative and environmental physiology. Springer-Verlag, Berlin, pp 45–185

Livingstone DR, Martinez PG, Winston GW (1989) Menadione-stimulated oxyradical formation in digestive gland microsomes of the common mussel, *Mytilus edulis* L. Aquat Toxic 15: 213–236

Moore MN (1988) Cytochemical responses of the lysosomal system and NADPH ferrihemoprotein reductase in molluscan digestive cells to environmental and experimental exposure to xenobiotics. Mar Ecol Prog Ser 46: 81–89

Moore MN (1991) Environmental distress signals: cellular reactions to marine pollution. In: Graumann W, Drukker J (eds) Histo- and cytochemistry as a tool in environmental toxicology. Prog Histochem Cytochem 23: 2–19

Myers MS, Stehr CM, Olson OP, Johnson LL, McCain BB, Chan S-L, Varanasi U (1994) Relationships between toxicopathic hepatic lesions and exposure to chemical contaminants in English sole (*Pleuronectes vetulus*), starry flounder (*Platichthys stellatus*), and white croaker (*Genyonemus lineatus*) from selected marine sites on the Pacific coast, USA. Envir Hlth Perspectives 102: 200–215

NOAAC (National Oceanic and Atmospheric Administration) (1989) National Status and Trends Program for Marine Enviromental Quality: a summary of data on tissue concentration from the first three years (1986–1988) of the Mussel Watch project. NOAA natn mar Fish Serv tech Memo US Dep Commerce NOS/OMA-49: 1–22

O'Conner TP (1992) Mussel Watch: recent trends in coastal environmental quality. National Oceanic and Atmospheric Administration, Silver Spring, Maryland

Parker LM, Lauren DJ, Hammock BD, Winder B, Hinton DE (1993) Biochemical and histochemical properties of hepatic tumors of rainbow trout, *Oncorhynchus mykiss*. Carcinogenesis 14: 211–218

Porte C, Sole, Albaiges MJ, Livingstone DR (1991) Responses of mixed-function oxygenase and antioxidase enzyme system of *Mytilus* sp. to organic pollution. Comp Biochem Physiol 100C: 183–186

Pretlow TP, Grane RW, Goehring PH, Lapinsky AS, Pretlow II TP (1987) Examination of enzyme-altered foci with gamma-glutamyl transpeptidase, aldehyde dehydrogenase, glucose-6-phosphate dehydrogenase, and other markers in methacrylate-embedded liver. Lab Invest 56: 96–100

Reddy JK, Azarnoff DL (1980) Hypolipidaemic hepatic peroxisome proliferators form a novel class of chemical carcinogens. Nature, Lond 283: 397–398

Reddy JK, Lalwani ND (1983) Carcinogenesis by hepatic peroxisome proliferators: evaluation of the risk of hypolipidemic drugs and industrial plasticizers to humans. CRC critical Rev Toxic 12: 1–58

Reddy JK, Lalwani DD, Reddy MK, Quereshi SA (1982) Excessive accumulation of autofluorescent lipofuscin in the liver during hepatocarcinogenesis by methyl clofenapate and other hypolipidemic peroxisome proliferators. Cancer Res 42: 259–266

Rutenburg AM, Kim H, Fischbein JW, Hanker JS, Wasserkrug HL, Seligman AM (1969) Histochemical and ultrastructural demonstration of γ-glutamyl transpeptidase activity. J Histochem Cytochem 17: 517–526

Schlenk D, Buhler DR (1989) Xenobiotioc biotransformation in the Pacific oyster (*Crassostrea gigas*). Comp Biochem Physiol 94C: 469–475

Sloan CA, Adams NG, Pearce RW, Brown DW, Chan S-L (1993) Northwest Fisheries Science Center analytical procedures. NOAA natn mar Fish Serv tech Memo NOS/ORCA 71: 53–97

Stegeman JJ (1985) Benzo[a]pyrene oxidation and microsomal enzyme activity in the mussel (*Mytilus edulis*) and other bivalve mollusc species from the Western North Atlantic. Mar Biol 89: 21–30

Stein JE, Collier TK, Reichert WL, Casillas E, Hom T, Varanasi U (1992) Bioindicators of contaminant exposure and sublethal effects: studies with benthic fish in Puget Sound, WA. Envir Toxic Chem 11: 701–714

Straatsburg IH, DeGraff F, Van Nordeen CJF, Van Raamsdonk WM (1989) Enzyme reaction rate studies in electromotor neurons of the weakly electric fish *Apternotus leptorhynchus*. Histochem J 21: 609–617

Suteau PM, Narbonne JF (1988) Preliminary data on PAH metabolism in the marine mussel *Mytilus galloprovincialis* from Arachon Bay, France. Mar Biol 98: 421–425

Van Noorden CJF, Butcher RG (1986) A quantitative histochemical study of NADPH-ferrihemoprotein reductase activity. Histochem J 18: 364–370

Viarengo A, Canesi L, Pertica M, Livingstone DR (1991) Seasonal variations in the antioxidant defence systems and lipiod peroxidation of the digestive gland of mussels. Comp Biochem Physiol 100C: 187–190

Wenning RJ, Di Giulio RT (1988) Microsomal enzyme activities, superoxide production, and antioxidant defenses in ribbed mussels (*Geukensia demissa*) and wedge clams (*Rangia cuneata*). Comp Biochem Physiol 90C: 21–28

Winston GW, Livingstone DR, Lips F (1990) Oxygen reduction metabolism by the digestive gland of the common marine mussel, *Mytilus edulis* L. J exp Zool 255: 296–308

Yang JH, Kostecki PT, Calabrese EJ, Baldwin LA (1990) Induction of peroxisome proliferation in rainbow trout exposed to ciprofibrate. Toxic appl Pharmac 104: 476–482

Zar JH (1974) Biostatistical analysis. Prentice-Hall, Englewood Cliff, New Jersey