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Assessment of Seasonal Variability of Cytochemical Responses to Contaminant Exposure in the Blue Mussel *Mytilus edulis* (Complex)

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Abstract. A selected suite of cytochemical parameters in *Mytilus edulis* are altered in response to field and laboratory exposure to chemical contaminants. These biomarkers include lysosomal stability, nicotinamide adenine dinucleotide phosphate (NADPH)–ferrihemoprotein reductase activity, liposfuscin deposition, and accumulation of lysosomal and cytoplasmic unsaturated neutral lipid. Normal variations in physiological processes (influenced by exogenous seasonal changes in temperature, salinity, food availability, etc.) may alter the sensitivity of these biomarkers to contaminant exposure. To address this issue, *M. edulis* (complex) were sampled monthly from a reference nonurban site (Coupeville, Penn Cove) and a polluted urban site (Seacrest, Elliott Bay) in Puget Sound, WA, for a period of 15 months. Physiological measurements including total length, total weight, somatic and mantle weights (an indication of gonadal development and reproductive status), condition index, and the presence or absence of hemic neoplasia (HN, or leukemia) were recorded. Significant differences in lysosomal stability, lysosomal and cytoplasmic unsaturated neutral lipids, lipofuscin deposition, and NADPH–ferrihemoprotein reductase activity in cells of the digestive gland or digestive tubules were generally found in mussels taken throughout the year from Seacrest compared to mussels sampled from Coupeville, consistent with exposure to chemical contaminants. No seasonally influenced suppression of the entire suite of parameters as measures of contaminant exposure was evident. Therefore these biomarkers can be used to evaluate contaminant exposure in mussels throughout the entire year.

Marine bivalves, including the blue mussel (*Mytilus edulis* complex), are widely used in urban marine habitats to monitor levels of chemical contaminants (*e.g.*, O'Connor 1992; Goldberg *et al.* 1983; Farrington *et al.* 1983; Lauenstein 1988, 1995; Lauenstein and Dolvin 1992). Mussels are chosen as indicators because they are sessile filter feeders and can bioaccumulate high levels of contaminants (Livingston 1991; O'Connor and Lauenstein 1989; Pruell *et al.* 1987) in the digestive gland and surrounding tissues. The digestive gland (or digestive tubules) is the primary location of digestion and absorption of nutrients in mussels and is typically a site of high contaminant accumulation. Accumulation of contaminants can induce changes in a wide range of physiological and cytochemical parameters in the digestive gland (Bayne *et al.* 1988; Bayne 1989; Gray 1992; Krishnakumar *et al.* 1995). These laboratory and field studies show not only that exposure to chemical contaminants have biological consequences but moreover that these physiological responses may serve as indicators of contaminant exposure.

A study by Porte *et al.* (1991) showed that in mussels exposed to chemical contaminants in the field, biological and cytochemical measurements of the detoxifying enzymes present in the digestive cells can serve as indicators of chemical contaminant exposure. Laboratory studies confirm that lysosomal responses in the digestive cells show strong associations to contaminant exposure (Lowe *et al.* 1981; Moore and Clark 1982; Moore *et al.* 1985; Pipe and Moore 1986; Nott and Moore 1987; Axiak *et al.* 1988; Moore 1988, 1991; Cajaraville *et al.* 1989; Krishnakumar *et al.* 1990; Winston *et al.* 1991; Viarengo *et al.* 1992). Parameters that were consistently indicative of contaminant exposure included lysosomal stability, nicotinamide adenine dinucleotide phosphate (NADPH)–ferrihemoprotein reductase activity, lipofuscin deposition, and accumulation of lysosomal and cytoplasmic unsaturated neutral lipid.

These cytochemical responses were measured previously in mussels from various areas of Puget Sound, Washington (Krishnakumar *et al.* 1995). High levels of chemical pollutants, including polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), are found in sediments and biota at several of these locations (NOAA 1989; Stein *et al.* 1992). Krishnakumar *et al.* (1997) subsequently found in laboratory

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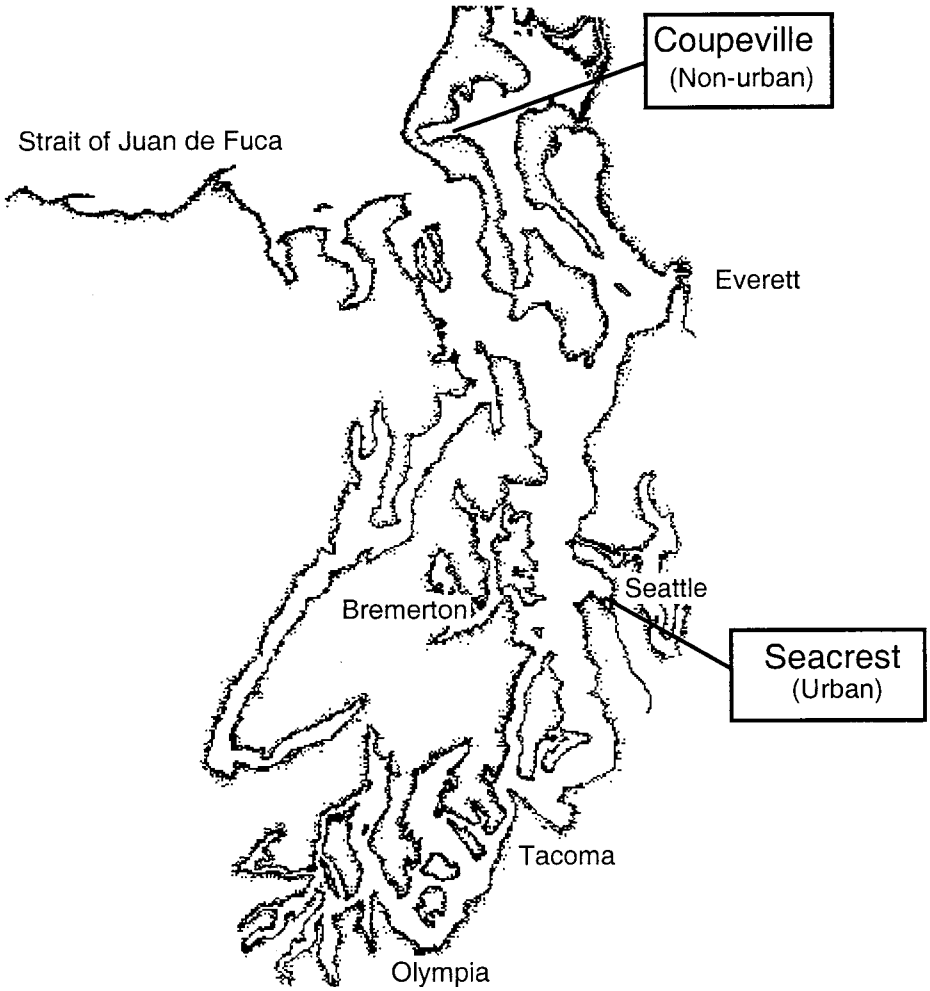

Fig. 1. Map of Puget Sound, WA, showing the two collection sites. Coupeville, on Whidbey Island, is the nonurban reference site and Seacrest, on Elliott Bay, is the urban polluted site

studies that mussels had similar cytochemical responses to both PAHs and PCBs. Collectively, these studies not only suggest that cytochemical responses have the potential to serve as biomarkers of stress but also serve specifically as biological indicators of exposure to organic (*e.g.*, PAHs and PCBs) and inorganic (*e.g.*, Cu) contaminants.

Other physiological changes in mussels exposed to chemical contaminants have been observed, including reductions in growth rate (scope-for-growth), altered condition indices (Cappuzzo and Leavitt 1988; Widdows and Johnson 1988; Widdows *et al.* 1990), and impaired growth and reproductive success proportional to the exposure level of chemical contaminants (Salazar and Salazar 1991).

The degree to which the biomarkers of exposure in the digestive gland are responsive to seasonal impacts and variations is currently unknown. It is likely that cytochemical changes and their responsiveness to contaminant exposure may vary seasonally. It has already been established that whole-organism responses to chemical contaminants may be strongly affected by seasonal conditions and seasonal variation (such as fluctuations in temperature, food availability, and reproductive status). Interpreting and attributing differences in organism responses to contaminants and to seasonal variation can be problematic. Previous field studies with mussels (Krishnakumar *et al.* 1994, 1995, 1997) could not address the potential interactive effect of these exogenous factors due to limited temporal sampling (mussels were only sampled at one time period).

The current study was completed to determine if seasonal variability affected the ability to use those cytochemcial parameters shown to be responsive to chemical contaminants throughout the year. Mussels were collected monthly from a reference nonurban site (Coupeville, Penn Cove) and a polluted urban site (Seacrest, Elliott Bay) in Puget Sound, WA. Physiological parameters known to vary with seasonal influences (Gosling 1992) were recorded. These included total length, total weight, somatic and mantle weights (an indication of gonadal development and reproductive status), and condition index. Hemic neoplasia (HN) was also sampled as a potential stressor that could influence biomarker response in mussels and could also be subject to seasonal variation. Lysosomal stability, NADPH–ferrihemoprotein reductase activity, lipofuscin deposition, and accumulation of lysosomal and cytoplasmic unsaturated neutral lipid were similarly measured monthly. In general, the differences in cytochemical responses between Coupeville and Seacrest mussels remained consistent over time despite seasonal fluctuations in the size, growth, condition index, and other related biological variables. These findings suggest that seasonal variability does not significantly influence biomarker results linked to contaminant exposure in mussels.

Table 1. Shell length (mm) and tissue wet weight (g) of mussels, *Mytilus edulis* complex (n = 25 per month, per site) sampled from a non-urban reference site (Coupeville) and an urban polluted site (Seacrest) (values are means ± SD)

	Coupeville			Seacrest		
Sampling Date	Length	Somatic Weight	Mantle (Gonad) Weight	Length	Somatic Weight	Mantle (Gonad) Weight
July 92	60 ± 4.9	4.9 ± 1.4	3.0 ± 1.0	46 ± 3.9	2.2 ± 0.5	1.2 ± 0.5
August 92	60 ± 4.3	5.4 ± 0.9	3.1 ± 0.9	43 ± 2.8	2.1 ± 0.4	1.0 ± 0.2
September 92	67 ± 6.5	6.1 ± 1.5	3.0 ± 0.8	47 ± 5.1	2.6 ± 1.0	1.2 ± 0.7
October 92	59 ± 3.2	4.7 ± 2.5	1.8 ± 0.5	39 ± 1.7	1.6 ± 0.3	0.6 ± 0.1
November 92	59 ± 3.8	4.0 ± 1.5	2.5 ± 1.1	38 ± 2.9	1.3 ± 0.3	0.5 ± 0.1
December 92	50 ± 4.8	2.6 ± 1.5	1.4 ± 0.2	37.9 ± 2.6	1.3 ± 0.3	0.4 ± 0.1
January 93	63 ± 6.0	4.5 ± 2.0	2.1 ± 0.4	38.6 ± 3.3	1.2 ± 0.3	0.4 ± 0.1
February 93	56 ± 4.7	3.7 ± 1.5	2.4 ± 0.3	37.8 ± 2.0	1.5 ± 0.5	0.6 ± 0.1
March 93	not sampled	not sampled	not sampled	not sampled	not sampled	not sampled
April 93	60 ± 4.5	7.8 ± 2.2	3.6 ± 1.2	42 ± 4.0	2.3 ± 1.0	1.3 ± 0.5
May 93	61 ± 3.9	7.2 ± 1.4	2.7 ± 0.7	38 ± 4.1	2.0 ± 0.9	0.8 ± 0.6
June 93	62 ± 5.7	4.7 ± 1.2	4.5 ± 1.5	38 ± 2.4	1.2 ± 0.3	1.4 ± 0.4
July 93	63 ± 6.2	4.4 ± 1.2	3.8 ± 0.8	37 ± 2.8	1.1 ± 0.3	1.1 ± 0.4
August 93	61 ± 4.1	5.0 ± 1.1	4.0 ± 1.1	39 ± 3.2	1.0 ± 0.5	1.5 ± 0.5
September 93	63 ± 5.1	5.5 ± 1.7	3.7 ± 1.4	41 ± 3.6	1.8 ± 0.6	1.1 ± 0.5

Materials and Methods

Collection

Mussels (*M. edulis* complex) (McDonald *et al.* 1991) were collected monthly from their natural beds at two sites in Puget Sound (Figure 1) from July 1992–September 1993. The first site was Seacrest, on Elliott Bay, which is an urban area of high boat traffic and industrial activity. Sediment from Seacrest contains elevated concentrations of PAHs and PCBs (Stein *et al.* 1992). High levels of these contaminants are known to bioaccumulate in the tissues of mussels at this site (> 500 ng/g dry weight of PAHs and PCBs) (Krishnakumar *et al.* 1994). The second site was a nonurban reference site located at Coupeville, on Whidbey Island, WA. Mussels from Coupeville have relatively low tissue concentrations of PAHs and PCBs (< 100 ng/g dry weight) and served as controls in studies examining the extent of biological impairment in mussels from urban estuaries (Krishnakumar *et al.* 1994). Following collection, mussels were depurated for 24 h in a flow-through seawater system at the Mukilteo Field Station, a National Marine Fisheries Service Facility located in Mukilteo, WA. Depuration facilitates the removal of any residual sediments in the soft tissues or body cavity. Following depuration, 35 mussels per site per month were analyzed (10 for cytochemical responses and 25 for biological measurements).

Biology

Barnacles and byssal threads, which can interfere with accurate measurements, were removed from the surface of mussels taken for biological analyses (n = 25). Length (umbo to farthest posterior margin), total weight, wet and dry weights for somatic and gonadal tissue, and sex were recorded for each mussel. This information was used to determine the condition indices. Condition indices normalize biological changes over time and can serve as an indication of the influence of seasonal fluctuations of reproductive status on biological measurements. They can also help assess the role of environmental factors, such as temperature and food availability, on the physiological status of bivalves. Length condition index (BCI [ln]) was calculated as follows: BCI (ln) = tissue wt (g)/shell length (mm) × 100.

The disease HN (or leukemia) is a biological parameter that is believed to influence the cytochemical parameters in mussels. To determine the prevalence of HN, 0.2–0.5 ml of hemolymph was drawn from the posterior adductor muscle of 20 mussels per month per site. Hemolymph was collected with a 23-gauge needle into a 3-cc syringe containing 0.5 ml Tris-EDTA hemocyte buffer (Krishnakumar *et al.* 1999). The contents of the syringe were then placed on a poly-L-lysine slide in a moist chamber and incubated at room temperature for 30 min. After incubation the slides were fixed in methanol and stained using the Schiff-Feulgen picromethyl blue procedure (Farley 1969) and then microscopically examined for the presence or absence of HN, as described by Elston *et al.* (1988).

Cytochemical Parameters

A section of the digestive gland was removed from 10 randomly selected mussels per site per month (Krishnakumar *et al.* 1994, 1995). Five tissue sections were placed in straight rows across the center of each cryomold (Tissue-Tek, Miles, Elkhart, IN). The molds were placed in a slurry of liquid nitrogen cooled (−70°C) hexane and embedded in OCT compound (Optimum Cutting Temperature, Tissue-Tek). The resulting frozen blocks were stored at −80°C until stained. For staining, blocks were placed in a cryostat at −20°C and 10-μm serial sections were cut. These sections were affixed to room-temperature slides and stained for each of the biomarker assays as described later. To ensure impartial results, all samples were randomly coded prior to analyses and decoded only after all measurements were made.

Duplicate neutral lipid sections from each cryomold, totaling 10 individual mussel samples per site per month, were prepared according to methods described in Bancroft (1967). Briefly, the samples were rinsed in distilled water and placed in 60% triethyl phosphate (v/v with distilled water) for 3 min and stained in a 1% solution of Oil Red O (Sigma, St Louis, MO) for 15 min. Sections were then washed and rinsed and mounted with glycerol gelatin (Sigma) under a coverslip for analyses. The slides were then examined using automated image analysis.

Samples for lipofuscin (degraded lipoprotein) analysis were also prepared in duplicate. The slides were fixed for 10 min in calcium-formal at 4°C, rinsed, and stained using the Schmorl reaction (Pearse 1972). Following staining in a solution of ferric chloride and potassium ferricyanide the slides were rinsed and mounted under coverslips

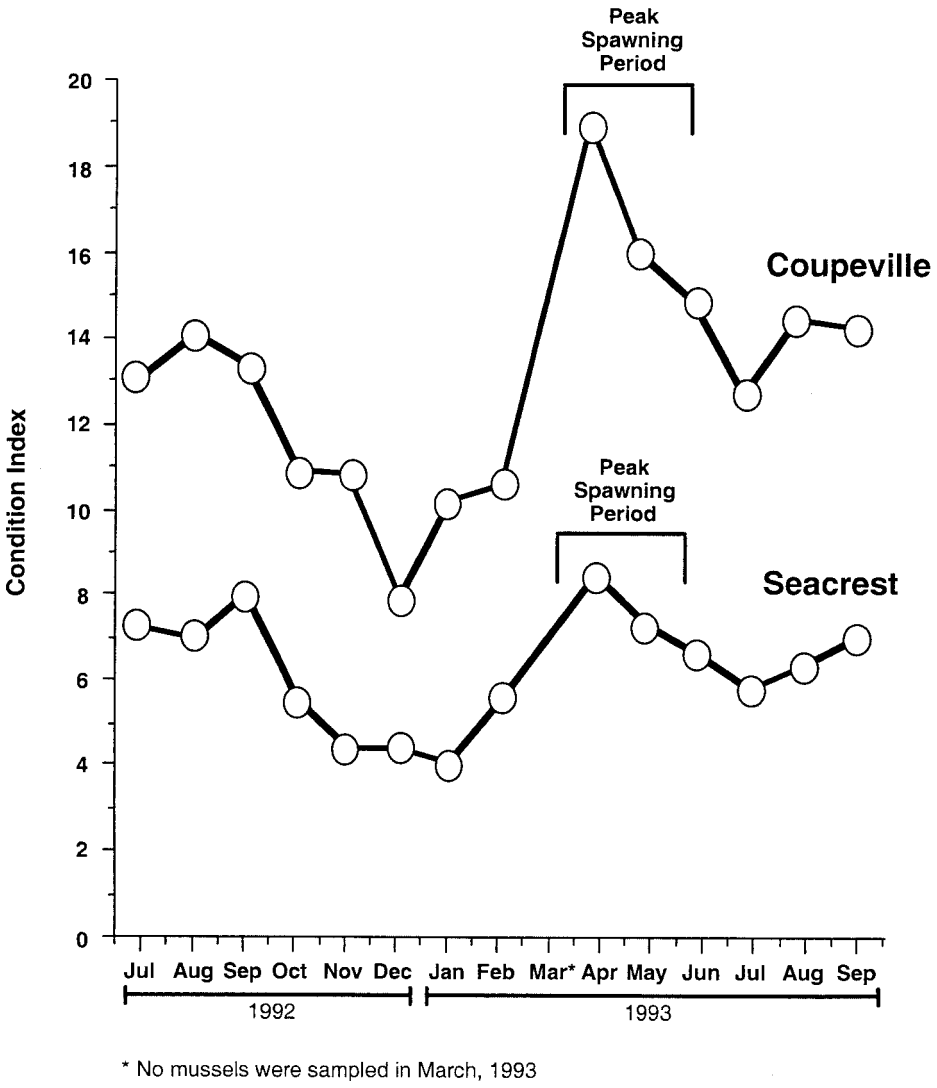

Fig. 2. Changes in the condition index of mussels (*Mytilus edulis* complex) from Coupeville and Seacrest (n = 25 per site, per month) from July 1992 to September 1993. The condition index is the somatic tissue wet weight (g)/(shell length [mm]) * 100

Table 2. The prevalence of hemic neoplasia in mussels (*Mytilus edulis* complex) from a nonurban reference site (Coupeville) and an urban polluted site (Seacrest) during monthly sampling April–September, 1993 (20 mussels were sampled during each month from each site)

Sampling Date	Coupeville (%)	Seacrest (%)
April 93	15	12
May 93	25	30
June 93	30	39
July 93	35	11
August 93	15	11
September 93	30	25

using glycerol gelatin. The slides were then analyzed using image analyses.

Lysosomal stability, or labilization period (the time necessary to labilize the responsive fraction of lysosomal hydrolase in an acid buffer), was determined using the marker enzyme N-acetyl B-hexosaminidase (Bitensky *et al.* 1973; Moore 1988). Multiple sections for each tissue sample were incubated at 37°C in a citrate buffer containing NaCl to labilize the lysosomal membrane. Serial sections were then allowed to labilize for exposure time periods of 0, 2, 5, 10, 15, 20, 25, and 30 min. After each time point sections were incubated for 20 min at 37°C in a reaction medium consisting of napthol AS-BI N-acetyl b-D-glucosamide (Sigma) dissolved in 2-methoxy ethanol and citrate buffer with NaCl and low-viscosity polypeptides (Sigma). Sections were then rinsed, placed in phosphate buffer, and stained with fast violet B (Sigma) at room temperature. After being stained for 10 min, the samples were fixed for 15 min in chilled calcium-formal, rinsed, and mounted with glycerol gelatin and a coverslip. The maximum reaction product for N-acetyl B-hexosaminidase (NAH) enzyme activity, corresponding to lysosomal activity, was then determined. This was accomplished by using automatic image analysis to examine the different levels of reaction product in the slides, recorded as pixel density, from the various incubation times.

NADPH–ferrihemoprotein reductase activity (NFR) is the detoxifying enzyme in bivalves similar in function to cytochrome P-450 in fish species. This enzyme reaction product was stained using the methods of Van Noorden and Butcher (1986) and Moore (1988). Duplicate cryomold tissue sections were incubated in total darkness for 30 min at 37°C in a sealed, oxygen-free atmospheric nitrogen chamber. The incubation medium consisted of 0.1 M HEPES buffer, 20 mM $MgCl_2$ (hexahydrate), 18% polyvinyl alcohol, 5 mM NADPH, 1% dimethyl

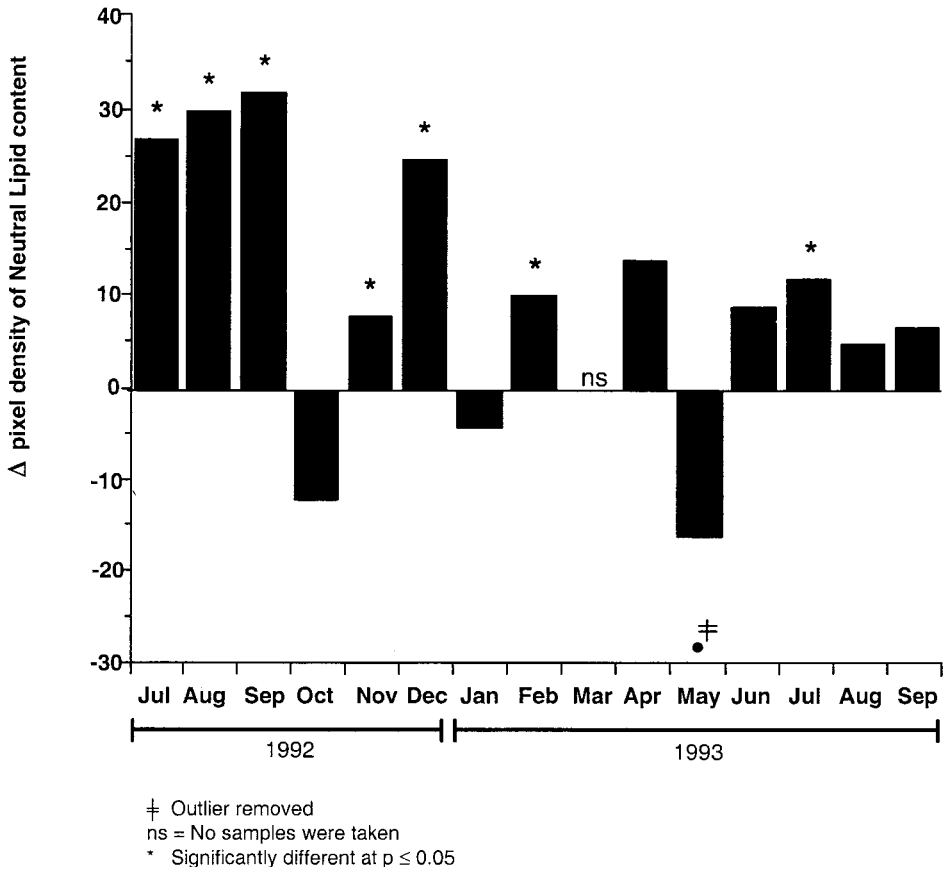

Fig. 3. Differences (Δ) in neutral lipid content (Seacrest mussel pixel density minus Coupeville mussel pixel density) from July 1992 to September 1993. Values for each month represent the average neutral lipid content of the digestive cells of *Mytilus edulis* complex (n = 10 per site, per month) from Coupeville and Seacrest measured as pixel density taken from image analysis

formamide, 1% ETOH, and 0.005 M neotetrazolium chloride. Following incubation, sections were rinsed twice in running and distilled water, respectively, and mounted using glycerol gelatin. Blank (background) values were determined by staining duplicate sections in a medium containing 1 mM NADP+. NFR enzyme activity is completely inhibited (55% to 69% inhibition) by 5 mM NADP+. The finished slides were then examined by automatic image analysis for overall pixel density.

Image Analysis

Tissue sections were quantitatively assessed for each of the cytochemical biomarkers (neutral lipid, lipofuscin, reductase, and lysosomal stability) using computer-enhanced automatic image analysis according to Krishnakumar *et al.* (1994). The system included a high-resolution charged couple device (CCD) color camera (COHU, mod. 8215.2000) mounted on a light microscope. Image analysis software (NIH v. 1.49) electronically captured the microscopic images displayed on a television screen and stored them on a Macintosh computer. Image intensity, or pixel density, was measured using a 255-unit gray scale (0 = absolute white and 255 = true black) with a 40× objective. Ten images of digestive tubules from the digestive gland were randomly taken from each duplicate section for each mussel (10 mussels per site per month). All images for each assay from both sites for each month were captured within a single day, during which all microscopic illumination and camera settings were kept constant. The digital image consisted of an eight-bit, 320 × 240 matrix of pixel bits. Average pixel densities were then calculated from the reaction products in the stored computer images. For each cytochemical reaction, a predetermined standard lower threshold level was used to separate reaction products from background values. The software program automatically converted the original image to a threshold picture, which is used to calculate the average gray value of the image. A mean pixel density was then determined for each individual mussel for every parameter measured.

Statistics

Differences in lysosomal responses, size, and condition indices of mussels between the two sites sampled monthly were tested using an unpaired single-tailed *t*-test (Zar 1984). The results shown in the figures are modified to represent the difference in the mean biomarker values of the reference site (Coupeville) subtracted from the contaminated site (Seacrest) at each monthly sampling point. The significance level for all statistical tests was set at $p \leq 0.05$.

Results

Biology

Mussel length remained consistent throughout the sampling period, mean lengths for mussels from Coupeville (the nonurban reference site) ranged from 50–67 mm and 37–47 mm in mussels from Seacrest (the urban polluted site) (Table 1). Although the length of collected mussels did not vary markedly over the sampling period at each site, there was an increase in somatic and mantle weight during the peak spawning period

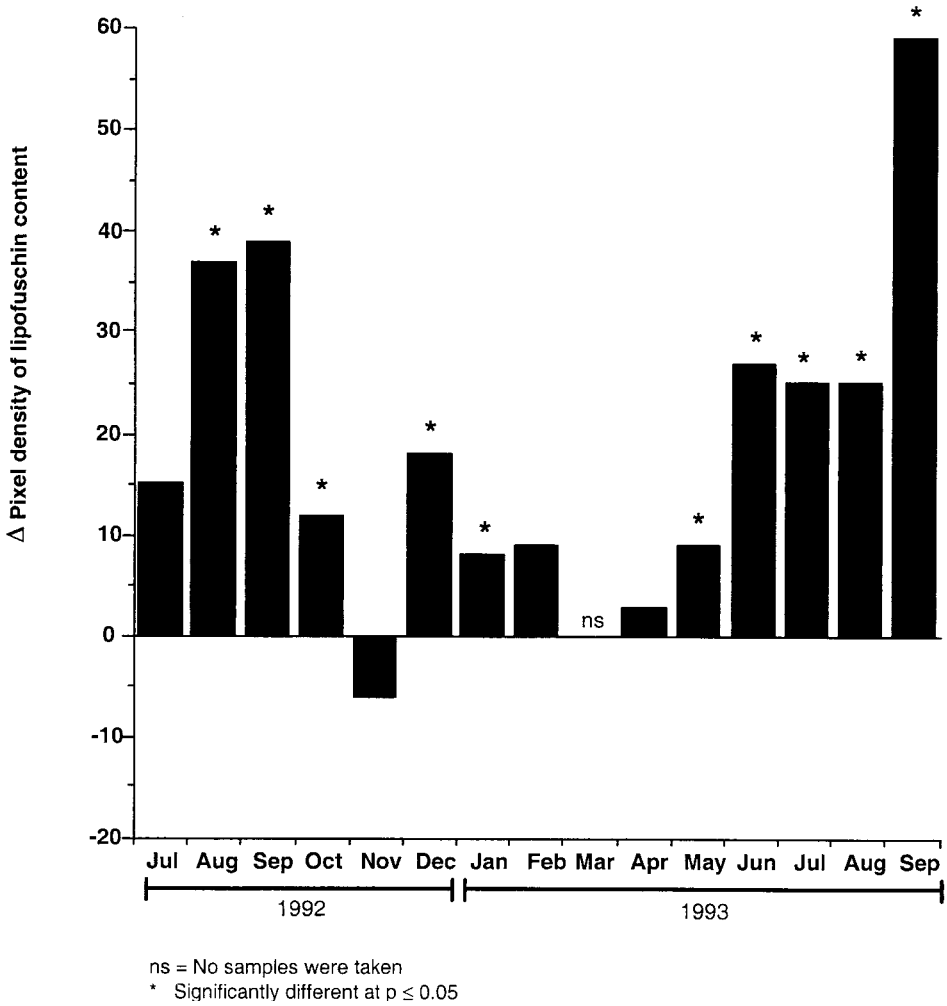

Fig. 4. Differences (Δ) in lipofuscin content (Seacrest mussel pixel density minus Coupeville mussel pixel density) from July 1992 to September 1993. Values for each month represent the average lipofuscin content of the digestive cells of *Mytilus edulis* complex (n = 10 per site, per month) from Coupeville and Seacrest measured as pixel density taken from image analysis

(April–May) compared to the other months. The mean somatic weight of the mussels from Coupeville ranged from 7.2–7.8 g during the peak spawning period (April–May) and was only 2.6–6.1 g for all the other months sampled. The mantle weight of the mussels also displayed a similar pattern. The mean somatic weight of mussels was 2.0–2.3 g from Seacrest for the spawning period and 1.0–2.2 g for the remaining months (excluding an unusually high value of 2.6 g for September 1992). The condition index mirrored these changes. There was an increase in the condition index of mussels from both sites during the peak spawning period, with the highest increase in mussels from Coupeville (Figure 2).

Over a 6-month period from April 1993 to September 1993, we examined 20 mussels each month from each site for HN (or leukemia). HN was found in mussels collected from both Coupeville and Seacrest (Table 2). Prevalence ranged from 15–30% in mussels from Coupeville and 11–39% in mussels from Seacrest. Although mussels from Seacrest did display the highest prevalence of 39% in June 1993, mussels from Coupeville showed a generally higher prevalence when comparing mussels between the sites during each monthly sampling. In fact, the prevalence of neoplasia in mussels was higher at Coupeville in 4 out of the 6 months sampled. The relationship between the prevalence of hemic neoplasia and site was not found to be significantly different using chi-square analysis at $p \leq 0.05$.

Cytochemical Parameters

Generally, either there were no significant differences in the various parameters tested between mussels from Coupeville or these mussels were more similar to each other than to mussels from Seacrest over time. Similarly, there were no significant temporal differences in the various parameters tested among mussels from Seacrest.

Monthly sampling of mussels from Seacrest from July 1992 through September 1993 generally (but not always) showed an increase in neutral lipid and lipofuscin content, increased NFR activity, and decreased lysosomal stability in the digestive cells when compared to levels found in mussels from Coupeville (Figures 3–6).

The mean (n = 10 per month per site) neutral lipid content of all the mussels from Coupeville was 65 ± 26 compared to 73 ± 18 (measured as pixel density taken from image analysis) for mussels from Seacrest. Neutral lipid levels in digestive cells of mussels from Seacrest were significantly higher than those

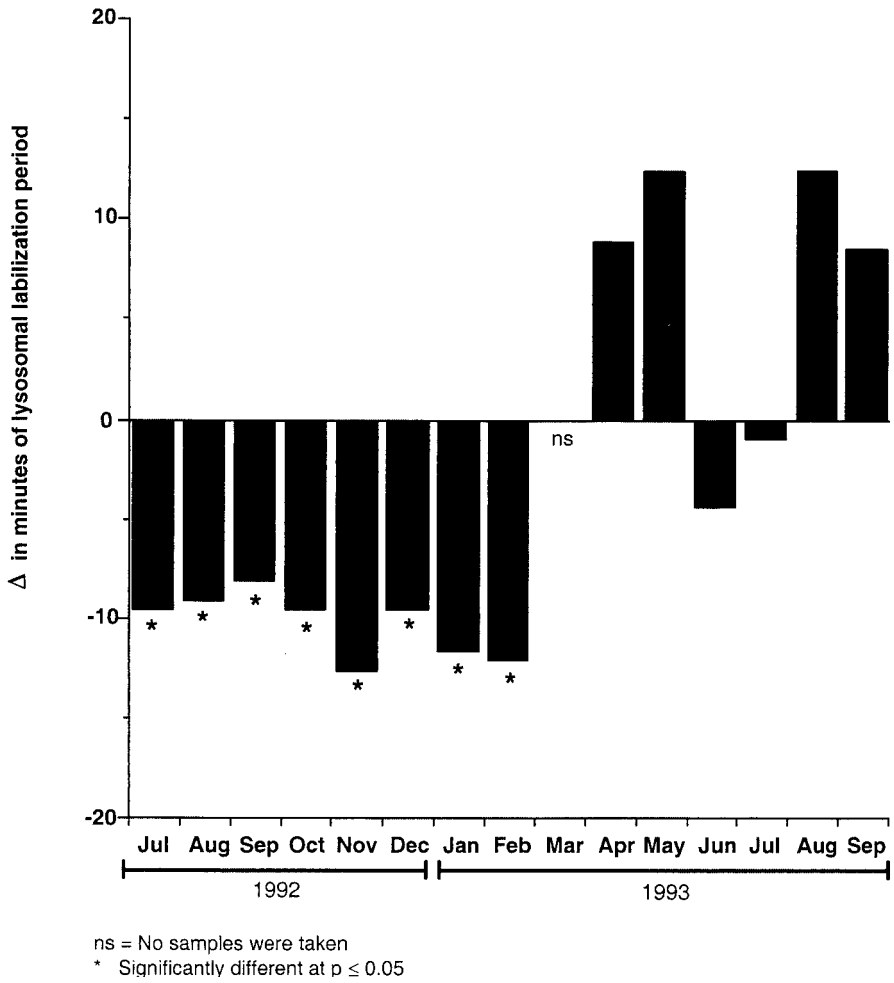

Fig. 5. Differences (Δ) in lysosomal stability (the labilization time in min of Seacrest mussels minus the labilization time in min of Coupeville mussels) from July 1992 to September 1993. Values represent the average lysosomal labilization period of five mussels (*Mytilus edulis* complex) per month per site measured in minutes taken from image analysis

for mussels from Coupeville for 7 months and higher (but not significantly) for an additional 4 months over the 14 months sampled. This relationship was reversed in October and January 1992 and again in May 1993, a peak spawning month for mussels in Puget Sound, WA (Figure 3).

The average lipofuscin content of the mussels from Coupeville and Seacrest was 49 ± 19 and 69 ± 24, respectively. Lipofuscin pixel density in mussels from Seacrest was significantly higher than in mussels from Coupeville for 10 of the 14 sampling months (Figure 4). Lipofuscin levels for mussels from Seacrest were lower than those for mussels from Coupeville for only one out of the 14 months sampled.

The mean lysosomal labilization period for mussels from Coupeville was 17 ± 5.0 min, whereas for Seacrest mussels the labilization period was 13 ± 6.0 min. The lysosomal stability was significantly shorter for Seacrest mussels than for corresponding mussels from Coupeville in over half (57%) of the months sampled and a shorter period overall in 10 of the 14 months (Figure 5). In April and May (periods of high spawning activity) and the last two months of 1993, the lysosomal labilization period was longer for mussels from Seacrest.

Similarly, NFR activity was significantly higher in mussels from Seacrest when compared to mussels from Coupeville for 4 of the 6 months in which data were available (Figure 6). Reductase levels in April, though not significant, also followed this trend. The only time this relationship was not evident was in June. In fact, the average reductase content in the mussel digestive cells from Coupeville was 60 ± 20 pixels, whereas NADPH activity was on average measured as 76 ± 20 pixels for mussels from the contaminated Seacrest site.

The trend indicated in each of the parameters above was even more evident when viewed together as a suite (Table 3). For example, when three biomarkers were considered together for the first eight months of the study, two or more exhibited a similar trend between the two sites to results documented previously in Krishnakumar *et al.* (1994, 1995). When all four biomarkers were available for the final 6 months, the same trend was exhibited in these parameters 75% of the time.

Discussion

Seasonal variations in physiological systems and digestive gland enzymes used as indicators of cellular alteration in mussels has been observed (Viarengo *et al.* 1991). The purpose of this study was to determine if seasonal variability would confound interpretation of the responses of cytochemical parameters in mussels that can be used to assess exposure to contaminants. This study revealed that the differences in cytochemical

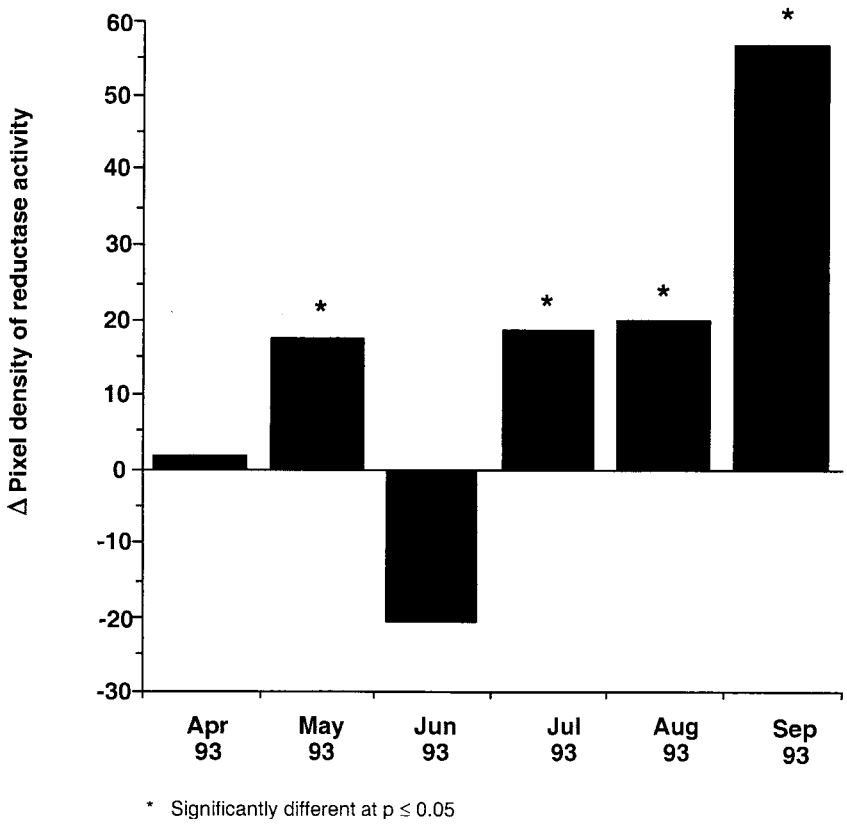

Fig. 6. Differences (Δ) in NADPH–ferrihemoprotein reductase activity (Seacrest mussel pixel density minus Coupeville mussel pixel density) from April 1993 to September 1993. Values for each month represent the average reductase content of the digestive cells of *Mytilus edulis* complex (n = 10 per site, per month) from Coupeville and Seacrest measured as pixel density taken from image analysis and corrected for background

Table 3. Seasonal differences in the responses of a suite of biomarkers in *Mytilus edulis* complex

Month	Neutral Lipid	Lipofuscin	Lysosomal Stability	NADPH Reductase	Total SD[a]	Total Trend[b]
July 92	+*	+	−*	NA	2	3
August 92	+*	+*	−*	NA	3	3
September 92	+*	+*	−*	NA	3	3
October 92	−	+*	−*	NA	2	2
November 92	+*	−	−*	NA	2	2
December 92	+*	+*	−*	NA	3	3
January 93	−	+*	−*	NA	2	2
February 93	+*	+	−*	NA	2	3
March 93	NA	NA	NA	NA	NA	NA
April 93	+	+	+	+	0	3
May 93	−	+*	+	+*	2	2
June 93	+	+*	−	−	1	3
July 93	+*	+*	−	+*	3	4
August 93	+	+*	+	+*	2	3
September 93	+	+*	+	+*	2	3
% trends SD[c]	50	71	57	66	93	

Values are expressed as positive (+) or negative (−) by comparing biomarker responses in mussels from Seacrest to biomarker responses in mussels from Coupeville (n = 10 per site, per month).

* Significantly different at p ≤ 0.05 (one-tailed).

[a] Total number of biomarker responses in mussels that were significantly different between the sites.

[b] Total number of biomarker responses that displayed a similar trend between the two sites to results documented previously in Krishnakumar *et al.* (1994, 1995).

[c] Percentage of individual biomarker responses that displayed a similar trend between the two sites to results documented previously in Krishnakumar *et al.* (1994, 1995) and were significantly different.

responses between mussels from the reference (Coupeville) and contaminated (Seacrest) sites generally remained consistent over time. Although each of the cytochemical parameters in mussels from Seacrest were not always significantly different from mussels from Coupeville, they were significant in over half the time points sampled for every parameter exam-

ined, which is consistent with previous research efforts (Krishnakumar *et al.* 1994, 1995, 1997). Statistically, some of the individual parameters (such as lysosomal stability) were weakest during April–June, a period of gonadal development and spawning activity. This may be related to energy spent on reproduction, which is normally dedicated to resisting the effects of chemical contaminants during other periods of the mussel life cycle. It is important to note, however, that although individual biomarker responses may not remain significant during peak spawning activity, the general trend between biomarker response from the two sites remains. Moreover, there has long been demand to consider combining cytochemical tests as a suite (Moore 1985, 1990) when they are applied in the field of environmental monitoring. When considered as a suite of parameters, at least one (and often two or more) of the parameters indicated exposure to chemical contaminants in mussels sampled from Seacrest when compared with mussels from Coupeville at all sampling times (Table 3). This indicates that seasonal variability does not appear to entirely suppress the ability of a suite of bioindicators to serve as indicators of contaminant exposure in mussels from urban sites, despite seasonal fluctuations influencing the condition index and other whole-organism indices in the mussels sampled.

The influence of seasonal variability on mussel physiology was most clearly observed as an increase in the condition index and to a lesser extent an increase in the somatic and mantle tissue weights from both sites during peak spawning periods (Figure 2). Coupeville mussels had the most dramatic increases in somatic weight and the largest peak of the condition index during spawning (Table 1). This suggests that mussels from nonreference sites are able to devote more energy to reproduction when environmental conditions are favorable.

In addition to gonadal development, the prevalence of HN may also confound the use of the cytochemical parameters as measures of chemical contaminant exposure. Previous research (Krishnamkumar *et al.* 1999) showed that HN did not vary in response to chemical contaminant exposure; however, it was not determined whether HN could alter the response of cytochemical parameters to contaminant exposure. HN can infect a high proportion of mussels in Puget Sound and could therefore be a confounding factor in interpreting the results. Although mussels from Seacrest did display the single highest prevalence of HN at an individual time point, mussels from Coupeville had a higher prevalence in 4 out of the 6 months sampled (Table 2). There was no apparent seasonal variation in the prevalence of HN within each of these populations. Because the values of HN observed from both sites did not change consistently with seasonal variations, HN is not likely to have variably affected the response of the parameters measured in mussels from this study.

As mentioned, differences in the four biomarkers of cytochemical responses between the reference and contaminated mussels generally remained consistent over time. When considered as a suite of biomarkers, rather than independently, this trend becomes clearer (Table 3). For example, significant differences between mussels from the two sites was only apparent 50%, 57%, 66%, and 71% of the time for the neutral lipid, lysosomal stability, NADPH reductase, and liposfuscin biomarkers, respectively. Yet when all four assays are considered together as a combined monitoring suite, the significant differences in at least one biomarker response between mussels from the two sites is over 92%. Similarly, when comparing mussels from the two sites for trends that are similar to those found previously (Krishnakumar *et al.* 1994, 1995), values ranged from 71–93%, depending on the individual parameter. When viewed as a suite, at least one of the three or four biomarkers assessed always (100%) revealed differences in cytochemical responses to contaminant exposure between mussels from the two sites during every month sampled, irrespective of season.

In summary, neutral lipid, liposfuscin, lysosomal stability, and NFR are cytochemically derived measures of subcellular and cellular events and, when applied as a suite of measures, can be used during any season to assess contaminant exposure in mussels despite fluctuations in physiological conditions. These findings support the continued use of this selected suite of parameters in field monitoring and laboratory research programs to determine the effects of contaminants in bivalves.

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