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A Multibiomarker Approach To Environmental Assessment

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Incorporation of ecologically relevant biomarkers into routine environmental management programs has been advocated as a pragmatic means of linking environmental degradation with its causes. Here, suites of biomarkers, devised to measure molecular damage, developmental abnormality and physiological impairment, were combined with chemical analysis to determine exposure to and the effects of pollution at sites within Southampton Water (UK). Test species included a filter feeder, a grazer, and an omnivore to determine the sensitivity of organisms occupying different trophic levels. Linear regression confirmed a significant association between incidence of intersex in *Littorina littorea* and tributyltin (TBT) concentrations ($R^2 = 0.954$) and between PAH metabolites in *Carcinus maenas* urine and PAHs in sediments ($R^2 = 0.754$). Principal component analysis revealed a gradient of detrimental impact to biota from the head to the mouth of the estuary, coincident with high sediment concentrations of heavy metals, PAHs, and biocides. Multidimensional scaling identified *C. maenas* as the organism most sensitive to contamination. Carboxylesterase activity, metallothionein and total haemolymph protein were the most discriminating biomarkers among sites. This holistic approach to environmental assessment is encouraged as it helps to identify the integrated impact of chemical contamination on organisms and to provide a realistic measure of environmental quality.

Introduction

The primary aim of ecological risk assessment is to predict the likely adverse effects of anthropogenic pollutants or activities on ecosystems and their components using an evidence-based approach. In marine ecosystems there are numerous tools available to environmental managers to achieve this aim (*1*). Historically these have centered on the determination of physical and chemical variables, and there is general agreement that current procedures have limited ability to determine the biological effects of exposure (*2*, *3*). Consequently, the use of biomarkers (functional measures of exposure to stressors expressed at the suborganismal, physiological, or behavioral level) (*4*) has been advocated as a vital addition to the risk assessment procedure (*5*, *6*). Much evidence supports the validity of many biomarkers as indicators of exposure and of biological response in laboratory studies or in relation to single parameters (*7*−*10*). Despite recommendations for the implementation of pilot studies to use biomarkers in environmental monitoring (*2*) and the interest of regulatory agencies (*11*, *12*), there is little published work to document the systematic use of multiple biomarkers to assess the health of organisms in complex ecosystems. In part, this is due to a lack of certainty in the interpretation of such studies. Calow and Forbes (*13*) have drawn attention to the problems of extrapolating from observations on a few individuals in a limited number of species to groupings of many individuals and species and the need to consider how the relative sensitivity of individual responses reflect the response of populations, communities and ecosystems. When consideration is given to the difficulties of applying epidemiological criteria to field surveys (*14*), and in the statistical analysis of multiple comparisons, it is easy to see why such studies are rarely attempted.

It is impractical to attempt a comprehensive assessment of each individual species within a given habitat. A more realistic approach is to establish the risk to key components in the ecosystem and assume that monitoring the adverse consequences for species occupying critical trophic positions will provide insight into the integrity of the ecosystem as a whole (*15*). By selecting diverse phyla exhibiting different feeding strategies (filter feeding, grazing, omnivory, predation) and measuring suites of biomarkers at the suborganismal and physiological level, the ecological relevance of pollutant exposures may be more readily determined and integrated into environmental management strategies (*16*−*18*). This weight of evidence approach is vital if environmental management is to develop better methods for dealing holistically with impacted biota as required by recent legislative changes such as the EC Water Framework Directive (*19*).

The aim of this study was to determine the viability of combining chemical analysis with suites of biomarkers to characterize the relationship between anthropogenic contamination and biotic response in Southampton Water, Southern England (Figure 1). The estuary was selected as it is subject to a high level of military, commercial, and industrial activity and serves a large population as a recreational amenity. Of particular note are the major sources of effluent discharge associated with the Esso refinery on the western shore and the persistence of the antifoulant tributyltin (TBT) at sites in the upper estuary (*20*). Invertebrate species were chosen for study as they are abundant components of most aquatic ecosystems and representatives of different trophic levels can be readily identified. Animals selected for these sites were the common shore crab, *Carcinus maenas*, an omnivore, the edible cockle *Cerastoderma edule*, a sediment dwelling filter and detritus feeder, and the periwinkle *Littorina littorea*, a grazer of microorganisms, detritus and algae inhabiting the upper to sublittoral shore. The testing regime included biomarkers of exposure and of effect. These encompassed cellular (cell viability, lysosomal integrity) and physiological (heart rate, haemolymph protein) status as well as measures of immunotoxicity (phagocytosis), genotoxicity (micronucleus formation), and endocrine disruption (intersex). Metal exposure was assessed by metallothionein

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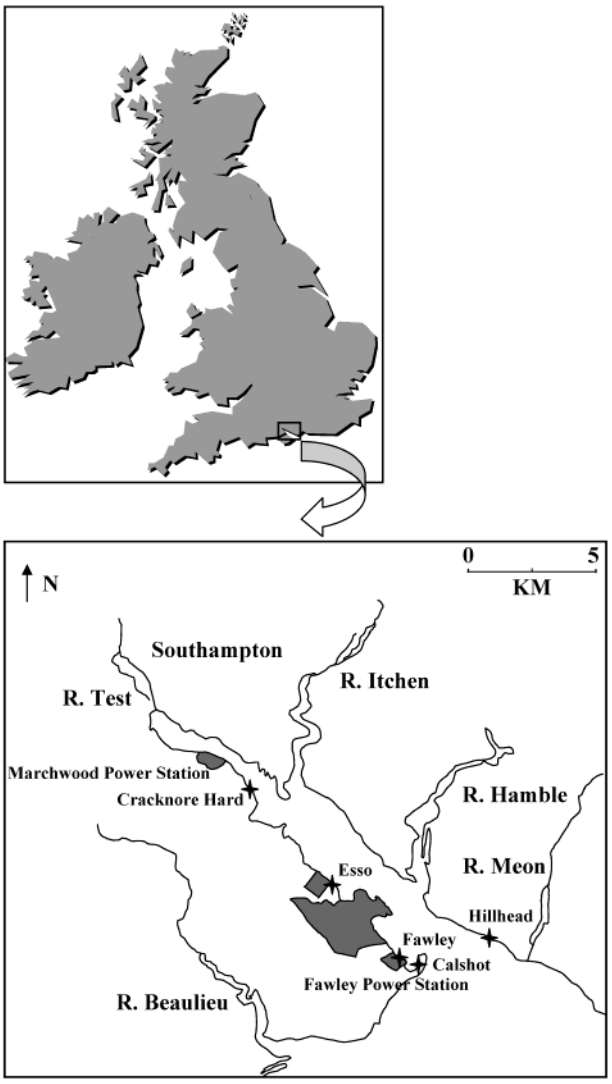

FIGURE 1. Location of study sites within Southampton Water, UK.

induction, pesticide exposure by the inhibition of esterase activities and PAH exposure by fluorescence of PAH metabolites. Concentrations of metals and PAHs in sediments were measured by ICP-MS and GC-MS, respectively. Relationships among the biological and chemical variables were determined using both univariate and multivariate statistical techniques.

Methods

Study Site. Southampton Water is a macrotidal coastal plain estuary fed by fresh water from the Rivers Test and Itchen. Its particular location, adjacent to the Isle of Wight, results in an unusual tidal regime with a double high water stand. The substratum is sand or muddy sand with a high percentage of organic matter; extensive salt marshes run along the west of the estuary. The influence of commercial shipping and maintenance facilities is evidenced by chronic TBT and antifoulant pollution. In sheltered sediment-based locations at the head of the estuary, concentrations of TBT close to the EQS limit of 2 ng L^{-1} occur (W. Langston, personal communication) (*21*). Major sources of effluent discharge into the estuary are shown in Figure 1 and include outfalls from the Esso oil refinery at Fawley and related industries. These have led to some of the highest recorded hydrocarbon levels in UK waters (48 μg L^{-1}, Cadland Bouy) (*3*). Copper is used as a catalyst in refinery processes, and spikes of copper contamination have been measured in adjacent sediments (*22*). Study sites (Figure 1) were chosen with the distribution of test organisms in mind and designed to encompass the urban and industrial outputs along the west side of the estuary, culminating at the Calshott Marshes, designated as an area of Special Scientific Interest.

Sample Collection and Preparation. Organisms were collected by hand from the sediments below the high water mark at each site between October 7 and 11th, 2002. Species selected were the shore crab, *Carcinus maenas*, (green intermoult males of carapace size 50−72 mm), the cockle *Cerastoderma edule* (size range 25−30 mm), and the periwinkle *Littorina littorea* (apex height approx 20 mm). Twenty-five individual cockles and crabs and 40 periwinkles were collected from each site and returned to the laboratory for analysis. Haemolymph was withdrawn from the posterior adductor muscle of *C. edule* and from the base arthrodial membrane of the third leg of *C. maenas* using a 21G syringe and stored on ice or snap frozen prior to analysis. Urine was collected from *C. maenas* as described by Bamber and Naylor (*23*). Sediments were sampled from the mudflats at each site using core sampling of the top 10 cm from bankside access points. Entry to Cadland Creek (Esso outfall site) was restricted, and sediment values for this site were based on those previously published by Rogers (*24*). Samples were placed in solvent rinsed 250 mL glass jars and stored at −80 °C prior to extraction and analysis.

Biomarkers. Esterase Activities. Enzyme activities were assayed at 25 °C in microtiterplate format using a method in which thiocholine or thioacetate derivatives are hydrolyzed by acetylcholinesterase or carboxylesterase, respectively (*25*). Sample or buffer blank (30−50 μL) was incubated for 5 min with 150 μL of 5-thio-2-nitrobenzoic acid, 270 μM in 50 mM sodium phosphate, pH 7.40. Enzyme activity was initiated by the addition of acetylthiocholine, 3 mM or phenylthioacetate, 800 μM and the absorbance recorded at 405 nm. Results were calculated as nmol substrate hydrolyzed min^{-1} mg^{-1} protein.

Lysosomal Stability. Stress-induced pathological change in the lysosomal compartment of blood cells was determined using the method of Lowe et al. (*26*) in which the reduced capacity of lysosomes to retain the dye Neutral Red, measured over time, was used as an indicator of membrane damage. Glass adhered cells were incubated for 15 min in Neutral Red, dissolved in physiological saline, and then viewed down the microscope at 15 min intervals to determine at what point in time there was evidence, in 50% of the cells, of dye loss from the lysosomes to the cytosol.

Metallothionein Analysis. Metallothionein concentration was determined by a minor modification to the spectroscopic method using 5,5-dithiobis-2-nitrobenzoic acid (DTNB) reported by Viarengo et al. (*27*). Whole tissue (*C. edule*) or gill (*C. maenas*) previously stored at −80 °C was ground to a fine powder in liquid nitrogen and 1 g (wet weight) of tissue diluted into a solution of 1 mM dithiothreitol containing 1 mM phenylmethylsulfonylfluoride. The solution was ultracentrifuged at 55 000 rpm at 4 °C for 70 min. Metallothionein was purified by extraction of the supernatant with ethanol and chloroform at 4 °C, and the resulting concentration was determined after addition of DTNB by recording the absorbance at 405 nm compared to a reduced glutathione standard.

Micronucleus Detection. The presence of micronucleated haemocytes was determined by adhering haemocytes to glass microscope slides with poly-L-lysine, fixing with methanol and staining with 5% Giemsa stain for 15−25 min, after which slides were rinsed and air-dried. The number of micronuclei per 1000 cells was determined at ×400 magnification using the following criteria: well-preserved cell cytoplasm, micronuclei displaced from the main nucleus and of smaller size (up to 1/3 diameter), and a similar or weaker staining as the main nucleus (*28*).

Heart Rate. Animals were returned to the laboratory and allowed to acclimate overnight. Heart rate was monitored for at least 24 h using a noninvasive computer-aided physiological monitoring system (CAPMON) (*29*). An infrared transducer was glued directly onto the shell or carapace of each animal. Animals were individually placed in 4 L of fully aerated seawater collected in situ for the duration of the recording.

Phagocytosis. The phagocytosis activity of haemocytes was determined by measuring the uptake of zymosan particles (from *Saccharomyces cerevisiae*) dyed with Neutral Red dye (*30*). Particle uptake by cell monolayers was estimated in microtiterplates by absorbance at 540 nm against a standard curve prepared using zymosan particles in the range $1.56-100 \times 10^7$ mL^{-1}. The protein concentration of each haemolymph sample from the phagocytosis assay was obtained using a commercial BCA protein assay.

Cellular Viability. The viability of haemocytes was determined by measuring their ability to retain Neutral Red dye (*31*). Samples of haemolymph (50 μL) were incubated in triplicate in flat-bottomed microtiterplates to allow a monolayer of cells to adhere to the wells. After 45 min, nonadhered cells were discarded, and the plates were washed with physiological saline. A solution of 0.4% Neutral Red dye in physiological saline (200 μL) was added, and after 3 h the wells were washed and an acidified solution of 1% acetic acid, 20% ethanol added to resolubilize the dye. The plate was gently shaken for 10 min before reading the absorbance at 540 nm.

Intersex. Snails were relaxed using 7% $MgCl_2$ in distilled water, and shell and aperture height were measured to the nearest 0.1 mm. Snails were removed from their shells and sexed and organisms exhibiting parasitism excluded. An intersex index was calculated as described by Bauer (*32*) as the average intersex stage of a population ($n = 50$ for each site) using Bauer's staging guidelines.

PAH Analysis of Urine. Fluorescence analyses were performed using a Hitachi F-4500 fluorescence spectrophotometer as described previously (*33*). Standards (pyrene and 1-OH pyrene), blanks and urine samples were diluted with 50% v/v ethanol/milliQ water. Fixed excitation wavelength fluorescence (FF) and synchronous excitation/emission fluorescence spectrometry (SFS) measurements were carried out with excitation and emission slit widths of 2.5 nm. The assigned wavelength pairs chosen and attributed to different PAH groups for FF were $\lambda_{ex/em} = 290/335$ for naphthalenes, $\lambda_{ex/em} = 345/382$ for pyrenes, and $\lambda_{ex/em}$ 380/430 for benzo[*a*]pyrene. These were selected after first analyzing a subset of samples and standards using a range of different excitation and emission wavelengths and calculating the mean emission wavelength of the dominant peak for both standard and sample spectra. A constant $\Delta\lambda$ of 37 nm was selected for the SFS analyses. Samples were quantified against 1-OH pyrene standards in the range $5-200$ $\mu g\ L^{-1}$ and are reported in terms of 1-OH pyrene equivalents for each of the wavelength pairs (naphthalene, pyrene, and benzo[*a*]pyrene groups). Values for each group were summed to provide an estimate of the total PAH metabolites present.

PAH Analysis of Sediments. Sediments were dried at 40 °C for 24 h, and water content was determined by loss in weight. Extraction, cleanup, and chromatographic procedures followed the method of Rowland et al. (*34*). In summary, wet sediments (approximately 30 g) were ground with anhydrous sodium sulfate and subjected for 24 h to Soxhlet extraction with dichloromethane (DCM). To measure hydrocarbon recoveries of authentic compounds from sediments using the extraction method, reference compounds (phenanthrene-d10, pyrene-d10, and chrysene-d12) dissolved in acetone were spiked into sediments. The purity of the compounds was >98%. The total hydrocarbon extracts were concentrated by rotary evaporation and fractionated into aliphatic, aromatic, and polar fractions by passage through a glass column packed with a hexane slurry of silica (60−100 mesh, 5% deactivated) under aluminum oxide (grade 1, neutral, 2% deactivated). The sample-to-adsorbent ratio was 1:200 (w/w). Sequential elution of the column with solvents of increasing polarity yielded F1 (aliphatic, 1.5 volumes hexane), F2 (aromatic, 2 volumes hexane:DCM) and F4 (polar, methanol)fractions. Fractions F1 and F2 were concentrated further by controlled evaporation before analysis by GC-MS in selective ion monitoring mode. GC-MS was carried out with a Hewlett-Packard MSD GC-MS fitted with a HP-1 Ultra, fused silica column 12 m × 0.2 mm i.d.; auto splitless injection (250 °C) was used and helium was the carrier gas (40 kPa head pressure). Oven temperature was programmed from 40 to 300 °C at 5 C min^{-1} and held at 300 °C for 10 min. Mass spectrometry operating conditions were: ion source temperature 250 °C, ionization energy 70 eV, SIM conditions were as follows: ions monitored *m*/*z*: 127, 128, 151, 152, 153, 154, 165, 166, 187, 188, 176, 179, 200, 211, 212, 226, 228, 236, 240, 252, 138, 276, 287, 279.

Metals Analysis. Dried sediments were sieved and the >1 mm fraction retained. A 1 g portion of each sediment sample was weighed and washed twice with 10 mL of 0.25% w/v EDTA, rinsed, and resuspended in 25 mL of distilled water. The fully quantitative analysis of the elements Fe, Zn, Cu, Cd, and As was obtained using Flame Atomic Absorption Spectrophotometry (Varian Spectra AA600) with instrument settings as previously described (*35*).

Statistical Analysis. Results were analyzed using the statistical packages Minitab and PRIMER (Plymouth Marine Laboratories). Data for each biomarker and each chemical were analyzed against each collection site using one way ANOVA where variances were homogeneous or by Kruskal Wallis for heterogeneous variances. Linear regression was used to analyze the association and potential causation between individual variables with stepwise linear regression applied to model the relationship between the variables. Multivariate statistical analysis was performed to summarize the patterns of variation and inter-relationships between biological and chemical variables and to detect and describe differences between sites. Differences at the <5% level were considered significant.

Results

Biomarker Responses. Initially, the biomarkers measured for each organism were analyzed individually and statistically significant differences determined among sites (Table 1). Biomarkers of effect at the cellular level (lysosomal stability, cell viability, micronucleus formation, ANOVA, $p < 0.02$, 0.02, 0.02 respectively) were significantly different between Cracknore Hard and the remaining sites when measured in *C. edule* but showed no significant differences between sites for *C. maenas*. Conversely, biomarkers of exposure and of effect at the biochemical level (total haemolymph protein, metallothionein, PAH metabolites in urine, Kruskal −Wallis $p < 0.05$, ANOVA, $p < 0.002$, $p < 0.006$ respectively) showed significant differences between the Esso Outfall and the remaining sites for *C. maenas* but not for *C. edule*. Intersex index was the only variable measured in *L. littorea*, and this was significantly different between Cracknore Hard and the remaining sites ($p < 0.0001$). Given the number of measurements made, not all of them are illustrated graphically, and instead Figure 2 contains a representative example of the results obtained for the general health effects biomarkers in *C. maenas* and *C. edule*. Overall, a detrimental impact to biota was apparent at sites toward the head of the estuary (Cracknore Hard, Esso), whereas sites toward the mouth of the Estuary (Fawley, Calshot, Hillhead) appeared unaffected.

TABLE 1. Biomarker Responses Measured for Each Organism[d]

		organism		
	biomarker	*C. edule* filter-feeder	*L. littorea* grazer	*C. maenas* omnivore
molecular	esterase activity	$p > 0.05$	ND	$p > 0.05$
	metallothionein	$p > 0.05$	ND	**$p < 0.002$[b]**
	total haemolymph protein	$p > 0.05$	ND	**$p < 0.05$[c]**
	PAH metabolites in urine	ND	ND	**$p < 0.006$[b]**
cellular	micronucleus formation	**$p < 0.02$[a]**	ND	$p > 0.05$
	cell viability	**$p < 0.02$[a]**	ND	$p > 0.05$
	lysosomal stability	**$p < 0.2$[a]**	ND	$p > 0.05$
	phagocytosis	$p > 0.05$	ND	$p > 0.05$
physiological	heart rate	$p > 0.05$	ND	$p > 0.05$
	intersex index	ND	**$p < 0.0001$[a]**	ND

[a] Anova, between Cracknore Hard and remaining sites. [b] Anova, between Esso outfall and remaining sites. [c] Kruskal Wallis, between Esso Outfall and remaining sites. [d] $n = 8$ for each site, except for intersex index, for which $n = 50$. Significant differences between sites are shown in bold. ND = not determined.

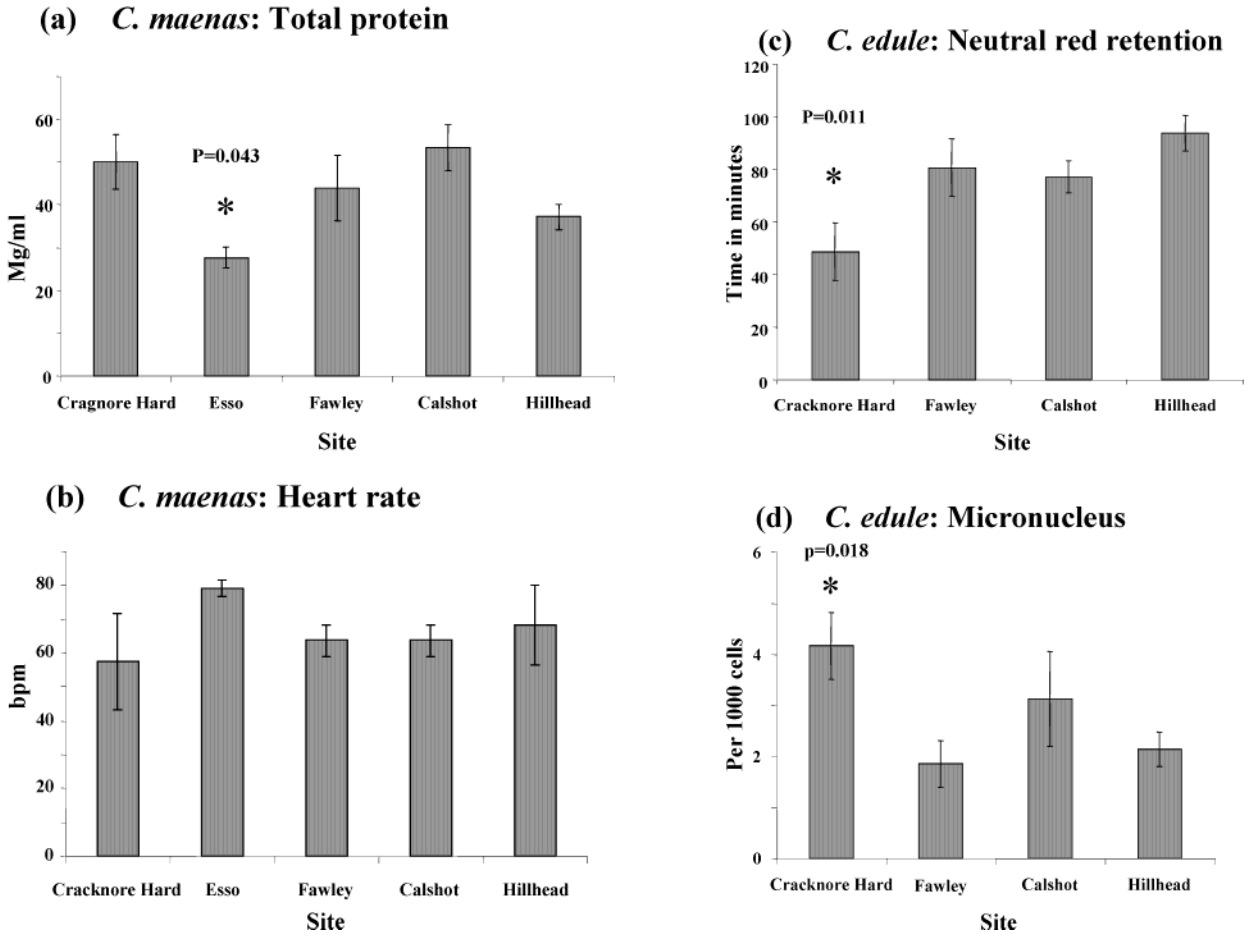

FIGURE 2. General health biomarkers presented according to site ($n = 8$). Statistical differences shown in the diagram are (a) Kruskal Wallis, between Esso and remaining sites (c) Anova, between Cracknore Hard and remaining sites (d) Anova, between Cracknore Hard and remaining sites.

Biomarkers of Exposure. The relationship between biomarkers of exposure and contaminant concentration at each site was investigated using regression analysis. Least squares linear regression analysis of the relationship between intersex index (the average intersex stage calculated for groups of 50 snails) in *L. littorea* and sediment TBT concentration gave an adjusted R^2 of 0.954. This indicates a very strong relationship between the two variables (intersex index = 0.239 + 3.02 TBT concentration $\mu g\ g^{-1}$ dry weight). A similar analysis applied to the measurement of PAH metabolites in the urine of *C. maenas* by fixed fluorescence and the total PAH residues in sediments by GC-MS at each site yielded an R^2 of 0.751 (urinary PAH metabolites log peak area = 1973 + 0.0950 PAH $\mu g\ g^{-1}$ dry weight). This indicated a moderately strong relationship between the two measurements. To examine the relationship between metallothionein induction and sediment metal concentration, stepwise linear regression was used based on the significance of individual coefficients (Table 2). Using this model to describe the relationship between metals and metallothionein measured in the gills of *C. maenas*, an adjusted R^2 of 0.927 was obtained indicating that in crabs, 92.7% of the variability in metallo-

TABLE 2. Stepwise Regression Analysis of Metallothionein Induction and Sediment Metal Concentrations[a]

sediment metal (μg g^{-1})	metallothionein (μg g^{-1}) *C. maenas* gill	*C. edule* whole body
Cu	0.787	
Fe	0.927	0.732
Cd	0.759	
Zn	0.679	

[a] Results are the R^2 value adjusted for degrees of freedom.

thionein could be explained by the sediment Fe concentration ($p < 0.006$). Best alternative subsets were, in descending order, Cu, Cd, and Zn. A similar analysis for metallothionein measured in whole body homogenates of *C. edule* revealed that the sediment Fe was again the most significant variable in determining metallothionein concentration, although the significance was reduced ($p < 0.05$). A draughtsman's plot revealed the only significant covariance to be between Fe and Cu (0.952). Thus each of the biomarkers of exposure showed a strong relationship with specific sediment contaminant residues.

Multivariate Analysis. Univariate analysis provides a means of describing associations and relationships between individual variables but, by analyzing all of the responses together using multivariate techniques, more powerful statistical tests can be generated for the combined effects of pollutants and other environmental variables. This enables the patterns of variation and the inter-relationships in the whole data set to be synthesized and summarized simultaneously.

To determine whether the study sites could be distinguished on the basis of their chemistry, a Principal Component Analysis (PCA) was performed. Ordination techniques such as PCA aim to replace large, complex data sets with a much smaller set of derived variables that still retain most of the relevant information. In PCA, the derived variables are presented as axes that represent a best line fit through the data. The process is continued through successive principal components until all of the variation has been explained, the rationale being that this will also encompass most of the important information in the data. A PCA plot was derived from the physicochemical variables measured at each site. Figure 3 shows how the PCA has separated the sites in a gradient from Hillhead at the mouth of the estuary to Cracknore Hard and Esso toward the head of the estuary. The highest Eigen values (coefficients in the linear combinations of variables making up the principal components) identified by the PCA were for Fe and total PAH, although the spread of the results was fairly even across each of the chemicals measured (Table 4). A graphical plot has then been superimposed onto this putative pollution gradient of each of the biological variables, with the diameter of the circular plot representing the mean value, allowing the results for each of the biomarkers at each site to be visualized. This can be done in turn for all of the organisms and biomarkers included in the study. In Figure 3 the responses for three of the biomarkers (total protein, metallothionein, and urinary PAH metabolites) measured in *C. maenas* are shown as an example.

We then sought to determine which of the biomarkers contributed most to site discrimination for each organism. A similarity matrix was constructed and BioEnv, a biota and environment matching program, was used to provide a rank correlation of the biological responses contributing most to the difference between sites. Table 5 provides a ranking of the four most discriminating biomarkers for *C. maenas* and *C. edule*. This was performed both with and without the biomarkers of exposure (PAH metabolites, metallothionein) to determine their contribution to the ranking. The results show that carboxylesterase activity, metallothionein, and haemolymph protein were the most discriminating biomarkers for *C. maenas*. For *C. edule* the ranking included heart rate, cell viability, and lysosomal stability.

TABLE 3. Average Metal and Total PAH Concentrations (in μg g^{-1} Dry Weight) at Sites within Southampton Water

metal	Cracknore Hard	Esso Outfall	Fawley Power Station	Calshott Marsh	Hillhead
		Metals			
Fe	395	785	314	395	185
Zn	75	87	8.4	27	3.6
Cu	32	81	3.5	10.5	0.7
Cd	0.16	0.34	0.05	0.16	0.11
As	0	0.75	0	0	0
Sn[a]	0.43	ND	0.08	0.04	0.06
		PAHs			
total PAHs	2646	24092[b]	130	329	16.8

[a] Langston et al., 1998, as sediment TBTO. [b] Rogers et al., 1998. Metals were measured by atomic absorption spectroscopy. PAHs were measured by GC-MS.

TABLE 4. Statistical Parameters Generated by PCA To Describe the Chemical Variability between Sites in (a) the Eigenvectors, the Coefficients in the Linear Combination of Variables Making up the PC, Are Listed and in (b) the Principal Component Scores Are Shown

	Section a eigenvectors	
variable	PC1	PC2
Fe	−0.421	−0.139
Zn	−0.361	−0.321
Cu	−0.400	−0.222
Cd	−0.399	−0.021
PAH	−0.414	−0.139

	Section b	
sample	score 1	score 2
Cracknore Hard	−0.092	−1.228
Esso	−3.770	0.926
Fawley	1.081	0.782
Calshot	0.441	−1.925
Hillhead	2.340	1.445

To identify which of the organisms showed the greatest discrimination between sites, we used multidimensional scaling (MDS), an ordination technique that describes similarity (or dissimilarity) between pairs of units. Axes are derived by the ordination procedure to give the best agreement of the relative distances between pairs of units. This results in a 2-dimensional (2-D) plot representing the similarity between sites. A benefit of MDS is its flexibility in dealing with data sets that have many different kinds of variable, such as may occur in field surveys. The MDS plot presented in Figure 4a has been constructed by including four of the biomarkers (heart rate, total haemolymph protein, esterase activity, metallothionein) for both *C. maenas* and *C. edule*. The spread of the results (a measure of dissimilarity) can be interpreted as an indication of the sensitivity of each organism to the effect of pollutants at each of the four sites included in the analysis. The wider 2-D spread of results obtained using *C. maenas* indicates that, when analyzed together, the crab responses were more sensitive to the contaminants present at each site compared to cockles. By removing biomarker responses sequentially, a visual rep-

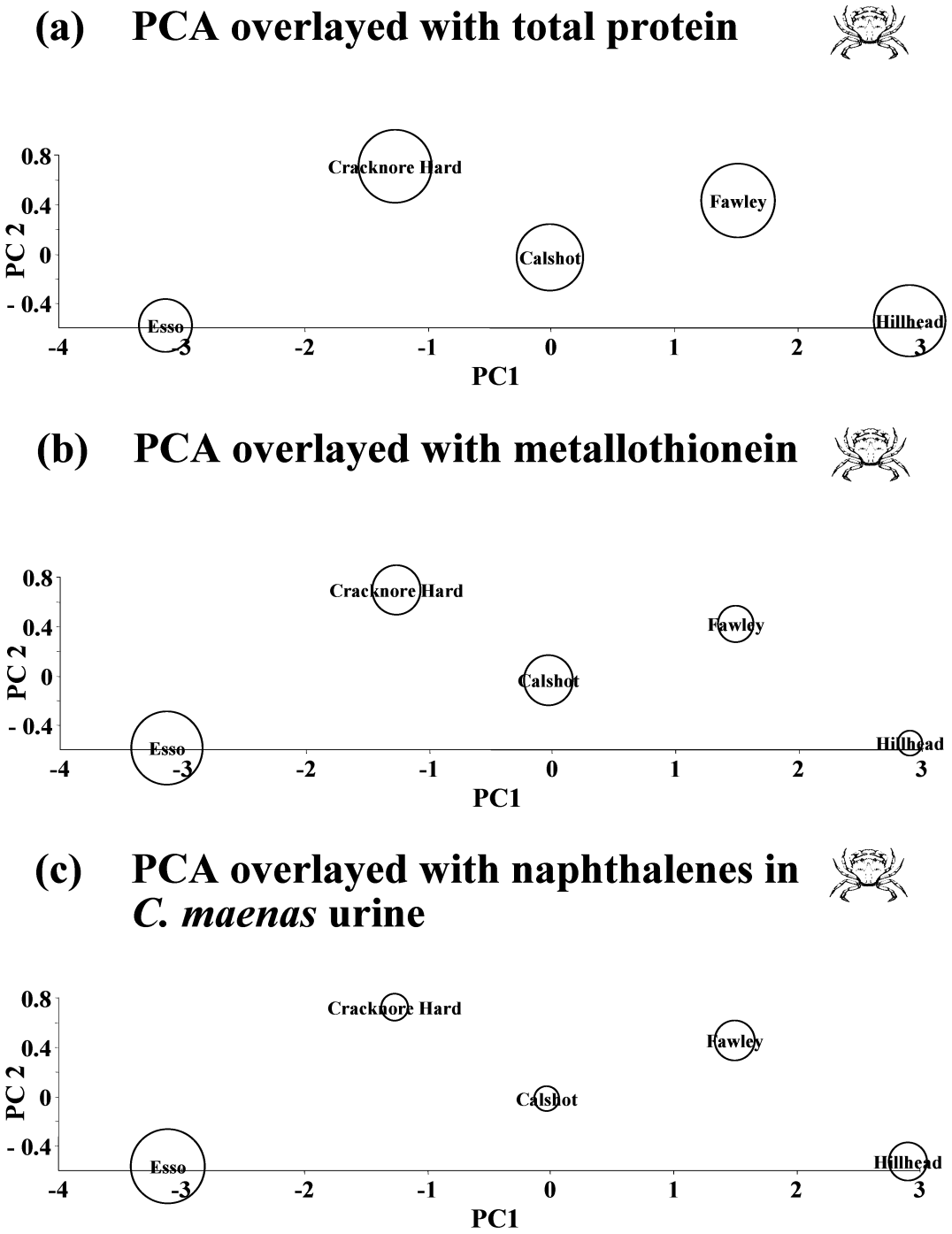

FIGURE 3. A PCA plot derived from the log transformed chemical variables for each site. Axes 1 and 2 accounted for 65.8% and 93.2% of the variability, respectively. The PRIMER software package has been used to superimpose biological variables for each site individually, with the diameter of the plot representing the mean value. Shown here are results for total protein, metallothionein, and urinary naphthalene equivalents measured in *C. maenas*.

resentation of the contribution of each biomarker to the overall dissimilarity can be obtained. For example, by removing metallothionein from the equation (Figure 4b), the differentiation between sites is increased for *C. edule*, whereas for *C. maenas*, the displacement between sites is reduced. Thus metallothionein was redundant from site differentiation for *C. edule* but was a discriminating factor in the differentiation between sites for *C. maenas*. This provides a graphical indication of the contribution to site discrimination provided by each biomarker.

Chemical Analysis of Sediments. Contaminant residues in the sediments collected from each site are shown in Table 3. Metal concentrations were within the available OSPAR-recommended environmental assessment criteria (EAC) for all metals and sites except for the copper concentration at the Esso site (EAC 5−50 μg g^{-1} dry weight, measured value 81 μg g^{-1} dry weight). Aromatic hydrocarbons are presented in Table 3 as total PAH μg g^{-1} dry weight. Inspection of the individual results for each of the 16 PAH compounds listed by the US EPA as priority pollutants revealed that sediment concentrations at Fawley, Calshott, and Hillhead were within EAC limits. The PAH sediment burden at Cracknore Hard was elevated compared to these sites but was still within EAC limits for each compound. The highest PAH concentra-

TABLE 5. Contribution of Each Biomarker to Site Discrimination[a]

with exposure biomarkers		excluding exposure biomarkers	
response	correlation coefficient	response	correlation coefficient
		C. maenas	
carboxylesterase activity	0.943	esterase activity	0.943
haemolymph protein	0.771	metallothionein	0.943
acetylcholinesterase activity	0.714	PAH naphthalene metabolites in urine	0.829
heart rate	0.600	acetylcholinesterase activity	0.829
		C. edule	
heart rate	0.943	heart rate	0.943
cell viability	0.886	cell viability	0.943
lysosomal stability	0.886	lysosomal stability	0.943
acetylcholinesterase activity	0.771	acetylcholinesterase activity	0.943

[a] A similarity matrix was constructed based on normalized Euclidian distances and a rank correlation of biological responses produced to best describe the difference between sites. All nine biomarkers were included in the analysis. Biomarkers of exposure (PAH metabolites in urine, metallothionein) were excluded from the second column. Results are shown for the four most discriminating biomarkers for each category in descending order.

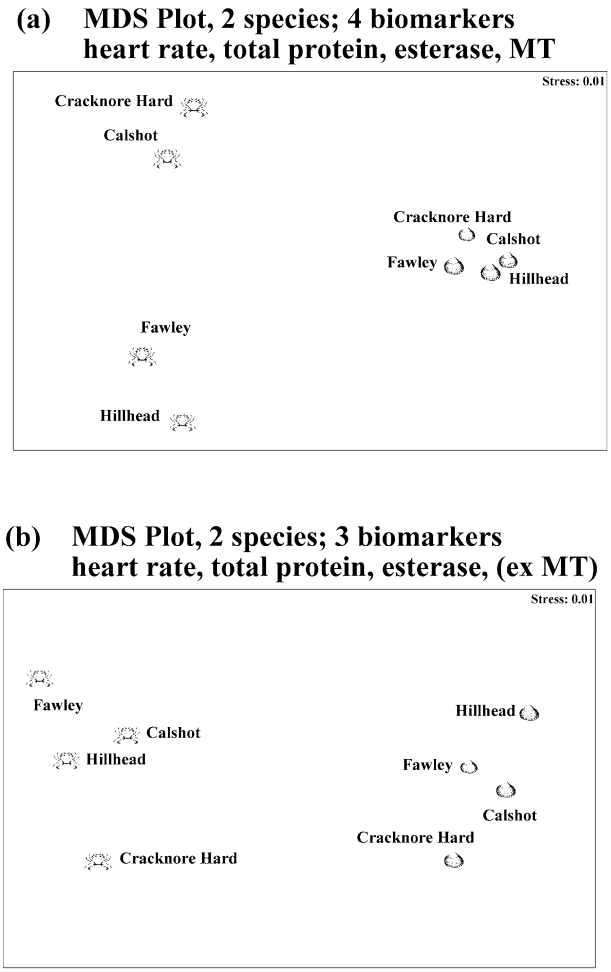

FIGURE 4. Multidimensional scaling representation of similarity between sites. The analysis is based on a similarity matrix constructed using normalized Euclidian Distances on log transformed data and includes chemical variables for each site and biological variables for *C. maenas* and *C. edule*.

tion was reported at the Esso outfall at Cadland Creek (*24*). Cracknore Hard and Esso sites were statistically different from each of the other sites and from each other (Kruskal-Wallis, $p < 0.01$).

Discussion

Estuarine waters such as Southampton Water that are subject to multiple chemical inputs may exhibit chronic biological effects that are not always demonstrated by conventional bioassay-based monitoring (*3*). Yet controversy continues over how best to integrate biomarkers into monitoring programs to determine the extent of suspected environmental degradation. Different schemes have been proposed, many based on the definition and classification system advocated by the National Research Council (*2*, *36*−*38*). In the approach used here, background information on contaminant discharges was used to identify sites of concern and to instigate a rapid assessment of the extent of contamination and biotic response. By using a suite of biomarkers to determine the integrated impact of multiple contaminants and relate exposure to ensuing physiological impairment in species occupying different trophic levels, sublethal effects associated with a gradient of contamination in the upper estuary of Southampton water were clearly identified.

There is no single biomarker that can unequivocally measure environmental degradation, and, if there were, it is unlikely that the differentiation among clean and polluted sites would be complete. Although not all the biomarkers employed in the present study revealed inter site differences, by combining biomarkers of exposure and of effect at the molecular, cellular, and whole organism level, a compelling weight of evidence has been revealed for the existence of environmental stress in the contaminated upper estuary. If an evidence-based approach is to be adopted in environmental assessment, then all types of evidence must be considered. The quality assurance, sensitivity, and specificity of a particular biomarker may be readily available from the literature or from laboratory studies. What is rarely available is evidence of outcome, i.e., does the use of a particular biomarker in a given species make any difference at all to the differentiation of impacted sites. Multiple biomarker field studies have tended to adopt a univariate approach to describe associations and relationships between individual variables (*39*, *40*). A series of pairwise comparisons can be a powerful means of describing associations and relationships between individual variables but gives no indication of how the results may be used in a quantitative manner to discriminate between sites. Multivariate procedures are used commonly in ecological studies of species composition (*41*) and for analyzing the responses of biological communities (*42*). They are often used in clinical diagnosis, where discriminant analysis (*43*), and other techniques such as artificial neural networks (*44*) or Bayesian belief networks (*45*), can be used to set objective decision criteria in order to include or exclude specific diagnoses.

The present study indicates how multivariate techniques, such as MDS, can be used in ecotoxicological studies to identify suites of biomarkers that most complement each

other, indicating the biomarkers that contribute most toward site discrimination and those that are redundant for any particular site or organism. Discriminating biomarkers in *C. maenas* included biomarkers of exposure and of effect, carboxylesterase activity, metallothionein, PAH metabolites in urine, and total haemolymph protein. For *C. edule*, biomarkers of effect, heart rate, and lysosomal stability, distinguished most between sites. These results can be compared with those of Astley et al. (*46*) who conducted a multibiomarker deployment study of the highly contaminated Tees estuary and analyzed the results using a similar multivariate procedure. Lysosomal stability contributed the most to the discrimination between sites and metallothionein contributed the least. These different intrinsic patterns of response at sites experiencing multiple contaminant impacts may offer a potential means of identifying the types of pollutant contributing most to the observed effect.

It was hypothesised that monitoring the responses of sensitive species might provide a valuable insight into the status of the entire impacted ecosystem. Here, the biomarker responses of the omnivorous predator *C. maenas* were the most sensitive to environmental variables. Although it is tempting to view this as an illustration of biomagnification, it is more likely a reflection of many interacting stress and species-specific factors. Biomagnification of PAHs through the food chain does not tend to occur due to the ability of most organisms to metabolize them. However, an invertebrate with relatively low metabolic capacity such as *C. maenas* ingesting contaminated molluscs as food will be exposed to an added burden of contamination. The acute toxicity of PAHs to invertebrates is due partly to interference in cellular membrane function and membrane-associated enzyme systems and a generalized narcosis is seen above concentrations of around 10 μg g^{-1} dry weight (*47*). Narcosis might be expected to compromise the physiological function of an active predator more acutely than a sessile filter feeder, contributing toward a decrease in predatory behavior, feeding rate, and viability. Rainbow (*48*) has suggested that the dynamic balance of uptake, detoxification, and excretion of metals by aquatic invertebrates is more important from the point of view of toxicity than the actual body burden. The uptake and distribution of copper and iron, both identified in the contaminated sediments at elevated levels, is strongly influenced in *C. maenas* by nutritional state (*49*), illustrating the many confounding and interacting factors that can impact upon the general health and toxicological responses of organisms. While causality cannot be unequivocally attributed in a complex field situation, the suites of biomarkers used in this study provide a valuable means of integrating the net effects of multiple environmental variables.

It was evident that each of the biomarkers of exposure represented a valid surrogate of contamination. Crabs are capable of metabolizing PAHs. For example, in studies of *C. maenas*, benzo[*a*] pyrene was metabolized rapidly to primary and conjugated metabolites (*50*). Therefore, monitoring the metabolites of PAHs in the body fluids of crabs represents a realistic means of monitoring their bioavailability (*33*). The use of UV/f has been viewed more as a preliminary screening method, due, in part, to interference from other compounds that may be present in extracted samples such as lipid or unsaturated hydrocarbons (*47*). Urine offers the distinct advantage of requiring no prior extraction due to the physiological filtration afforded by the kidney and comparison of UV/f with immunoassay has yielded good correlations (*51*). The method described here provides a novel, rapid, and nondestructive biomarker of PAH exposure in *C. maenas*.

The established European imposex species for TBT biomonitoring, the neogastropod mollusc *Nucella lapillus*, has disappeared from Southampton Water due to the persistence of contamination, hence the use of *Littorina littorea* as an alternative (*32*). In *L. littorea*, intersex is defined as any disturbance of the congruity between gonad and genital tract, while imposex in *N. lapillus* is a superimposition of male sex organs (penis and/or vas deferens) onto females (*52*). During the development of intersex, there is no direct superimposition of male characteristics but rather the organs of the pallial oviduct are modified gradually toward a male morphological structure. Besides TBT, copper and other heavy metals can induce alterations in gonadal maturation in molluscs (*53*) although regression analysis indicated no correlation here between the other heavy metals measured and intersex index. Our results support Bauer's estimates of the most probable TBT threshold concentration for intersex development of around 0.4 μg g^{-1} dry weight (*32*). Interestingly, the water TBT concentration measured at the site (3−7 ng L^{-1}) (*21*) has been suggested to pose no toxic threat to the embryonic/larval development of other species (*54*). Evidently, despite legislation to reduce TBT inputs from leisure craft, the Cracknore Hard boatyard remains impacted by high TBT concentrations most likely associated with organic matter and paint particles in the sediments (*20*). The half-life of TBT in undisturbed muddy sediments such as those in the present study may be very long, $t_{1/2} = 1-20$ years, (*55*), representing a persistent reservoir that poses a potential long-term risk to benthic organisms.

Chapman (*56*) reviewed data from a range of laboratory experiment and field or modeling studies involving different organisms to predict that 2−12 μg g^{-1} dry weight of a PAH in sediments would represent a threshold value between biological effects and no effects. The results presented here corroborate this suggestion. The total PAH concentrations (0.3−24 μg g^{-1} dry weight) are typical of those found in similar industrialized estuaries in the USA and Europe and confirm a fall in sediment PAH concentrations in Southampton Water from earlier reports (*57*). Sediment PAH assemblages tend to be persistent and have been used as indices of the rate of PAH input to aquatic environments. The Esso refinery operates with facilities which clean aqueous effluents before discharge into Southampton Water, and the authorized limits for mass releases have steadily reduced to reflect improved performance in effluent control. This also applies to the release of metals. For example, the oil refinery is the main contributor of copper in Southampton Water, and the results presented here for the Esso Outfall site (81 μg g^{-1} dry weight) compare with highly elevated concentrations of up to 1022 μg g^{-1} dry weight reported previously (*57*).

In conclusion, combining chemical analysis with suites of biomarkers addresses the need for more pragmatic environmental assessment techniques linking environmental degradation with its causes. Further work is needed, for example to define more fully the range of responses considered normal for different sites and to identify periods of sensitivity to seasonal influences. This holistic approach provides a means of identifying the integrated impact of chemical contamination on different levels of biological function and could make a viable addition to routine management protocols for protecting the environment.

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