Biomarkers of Contaminant Exposure and Effect in Flatfish from Southern California

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ABSTRACT

Two biochemical indicators of contaminant exposure and effect (biomarkers) were measured in flatfish from areas of different contamination levels. Hornyhead turbot (Pleuronichthys verticalis) and English sole (Pleuronectes vetulus) were collected from stations near large municipal wastewater discharge zones off Palos Verdes and Orange County and from a reference site near Dana Point. Bile from each species was analyzed by high performance liquid chromatography (HPLC) to determine the concentration of fluorescent aromatic compounds (FACs), an indicator of recent polynuclear aromatic hydrocarbon exposure. The concentrations of stress proteins, indicators of contaminant effect, were measured in English sole livers using antibodies specific to these proteins. The concentrations of bile FACs were reflective of current sediment polynuclear aromatic hydrocarbon contamination levels. Significantly higher bile FAC concentrations were found in fish from contaminated sites near Palos Verdes than in fish from the Dana Point reference site. Similar FAC concentrations were found in fish from Dana Point and those collected near the Orange County wastewater discharge zones. Stress protein concentrations in English sole were not significantly different between sites.

INTRODUCTION

Fish are important indicators of marine environmental health. The soft-bottom habitat of the Southern California Bight supports a diverse assemblage of demersal fishes (Allen et al. 1998). These fish are key members of marine food webs, and are valued by the public as sources of food and recreation. Many species of demersal fish are chronically exposed to environmental contamination through activities such as feeding upon benthic infauna and contact with contaminated sediments or water.

Effects on demersal fish populations have been documented by previous studies of southern California waste discharge areas. These impacts, prevalent in the 1970s, have included alterations in species abundance (often related to changes in prey abundance), bioaccumulation of organic contaminants, and increased prevalence of diseases such as fin erosion (MBC Applied Environmental Sciences 1988, Stull 1995). Many of these impacts have been associated with municipal wastewater or sludge discharge areas in Santa Monica Bay or on the Palos Verdes Shelf.

Exposure of fish to contaminants has declined markedly since the 1970s. Improved waste treatment practices have produced dramatic declines in the inputs of contaminants from municipal wastewater discharge (Raco-Rands 1997), leading to reductions in sediment and fish tissue contaminant concentrations (Allen and Cross 1994, Stull 1995). Although sediment and fish tissue contamination is still widespread throughout the Southern California Bight (SCBPP Steering Committee 1998), adverse impacts on fish abundance and species composition are generally negligible and the incidence of external anomalies such as fin erosion has declined to background levels (Allen et al. 1998, Stull 1995).

Traditional monitoring methods for demersal fish may not have the sensitivity to assess present-day contamination impacts. While measurement of population and assemblage parameters have high ecological relevance, these measures are also strongly affected by environmental variables such as water temperature and prey abundance that can reduce the ability to identify impacts related to waste discharge.

An alternative approach for examining contaminant impacts on fish is to measure biomarkers that indicate changes at the cellular or biochemical level. The development of numerous biomarker techniques in the last decade has resulted in methods with the potential to assess stress in field-collected fish, detect effects leading to growth/reproductive impairment, and indicate exposure to rapidly metabolized contaminants (Huggett et al. 1992). Evidence has shown that contaminant-related impacts on fish physiology or reproduction are occurring.
in some areas of southern California (Hose et al. 1989, Applied Marine Sciences and Industrial Economics 1994). In addition, biomarker measurements of polynuclear aromatic hydrocarbon (PAH) exposure have revealed elevated values in local fish from contaminated locations (Varanasi et al. 1989).

Exposure to PAHs is a potentially important factor in determining the response of fish to contamination. Many PAHs are toxic and exposure to this group of compounds has been identified as a risk factor influencing the development of liver lesions in fish (Myers et al. 1998). Exposure to PAHs cannot be assessed by conventional tissue analyses because these compounds are rapidly metabolized by the liver and secreted into the bile. One biomarker that has been used to quantify the exposure in fish to PAHs is bile FACs (fluorescent aromatic compounds). Bile FAC concentrations have shown a strong correlation with sediment PAH concentrations (Collier et al. 1993). Bile FAC concentrations have been measured as an indicator of PAH exposure in fish from southern California and other locations including Puget Sound, Washington; Galveston Bay, Texas; and Tampa Bay, Florida (Varanasi et al. 1988, Willett et al. 1997, McCain et al. 1996).

The stress protein response is a biomarker that has shown potential for use as an effects indicator (Sanders et al. 1991). Stress proteins are believed to be part of the cellular protection and repair mechanisms that are induced in response to various environmental contaminants (Stegeman et al. 1992). These proteins are rapidly produced during stress to restore and protect normal cell function by refolding damaged proteins, resolubilizing protein aggregates, and helping to protect protein synthesis (Sanders et al. 1994). Because stress proteins are an integral part of cellular repair and protection, they are believed to be relevant to overall organismal health, and as such are good candidate biomarkers of contaminant effect (Sanders et al. 1991). The two stress proteins studied most often are hsp60 and hsp70. Most previous work with stress proteins in fish have used laboratory exposures (Dyer et al. 1993, Theodorakis et al. 1992, Sanders et al. 1995). One study by Sanders and Martin (1993) did find elevated stress protein levels in field-collected fish from southern California. However, the sampling design of that study precluded interpretation of the relationship between stress protein concentrations and sediment contamination gradients in southern California.

In this article, we report on the application of two biomarkers to examine contaminant exposure and effects in two species of flatfish. The objective of this research was to determine whether PAH exposure and cellular stress were elevated in fish living near municipal wastewater discharge zones.

**METHODS**

**Sampling Design**

Biomarkers were measured in flatfish from four sites with varying sediment contamination levels (Figure 1, Table 1). Dana Point Station R52 was selected as a reference site because fish tissue and sediment contaminant concentrations at this location are historically low (Allen and Cross 1994, Bay et al. 1994). Station R52 is approximately 6 km from the South East Regional Reclamation Authority municipal wastewater discharge site and approximately 8 km from the Dana Point Harbor, the two nearest discrete contamination sources. Two Palos Verdes stations (7C and 9C), located near the County Sanitation Districts of Los Angeles County municipal wastewater discharge site, were selected because their sediments contain high concentrations of many contaminants, including PAHs, PCBs, DDTs, and metals. The Orange County station (T1), located near the Orange County Sanitation District municipal wastewater discharge site, was selected as an area of intermediate contaminant exposure. Elevated sediment concentrations of PAHs and metals are present near the Orange County discharge areas, but the concentrations and spatial extent are much less than at Palos Verdes (CSDOC 1997).

Samples were collected from two species of flatfish: English sole (Pleuronectes vetulus) and hornyhead turbot (Pleuronichthys verticalis). These species were selected because they have a close association with the sediment and prior data on bile FACs are available for comparison (Krahn et al. 1984, 1986; Varanasi et al. 1989). Data for fish from Stations 7C and 9C were combined for statistical analysis because these stations represent the same contamination source.

**Field Sampling**

English sole and hornyhead turbot were collected from Stations 7C and 9C on April 22, 1996, and from Station R52 on May 16, 1996 (Figure 1). Hornyhead turbot were also collected from Station T1 on May 7, 1996. All fish were captured using an otter trawl in a cooperative effort with a concurrent Southern California Coastal Water Resource Project (SCCWRP) study of PCBs and DDTs in sediments and fish livers (see Distribution and bioaccumulation of persistent chlorinated hydrocarbons in the food chain of hornhead turbot). Four to seven individuals of each species were collected at each station.
Livers (for stress proteins) and gall bladders (containing the bile for FACs) were dissected from English sole at sea and stored on dry ice. Although we planned to analyze stress protein concentrations in both English sole and hornyhead turbot, the sampling constraints of the concurrent bioaccumulation study prevented us from dissecting hornyhead turbot at sea. Stress proteins rapidly degrade unless tissues containing these proteins are stored at very low temperatures. Therefore, hornyhead turbot specimens, which were stored on wet ice and dissected the same day after transport to the lab, were analyzed only for bile FACs. The PAH metabolites are not degraded from the temporary storage of fish on wet ice, unlike the stress proteins. All tissue samples were stored in the laboratory at -80°C until they were analyzed.

**Bile FACs Analysis**

Concentrations of bile FACs were determined following the methods of Krahn et al. (1986), using reverse-phase high performance liquid chromatography (HPLC) and fluorescence detection. Five µl of untreated bile from each English sole and hornyhead turbot were injected onto the HPLC. Bile proteins and other bile components that could potentially interfere with PAH quantification are water soluble, and were passed through the nonpolar reverse phase column, while the hydrophobic PAHs and metabolites were adsorbed onto the column. The PAHs and metabolites were then eluted from the column with methanol, and measured using fluorescence detection. Fluorescence of the PAHs was measured at the excitation/emission wavelength pair (380/430 nm) appropriate for PAHs with similar structure as benzo[a]pyrene (BaP) and its metabolites. The higher molecular weight PAHs (products of combustion) and their metabolites tend to fluoresce at the same wavelength, and are semi-quantitated collectively as BaP equivalents. Fluorescence values were converted to ng BaP equivalents using a BaP standard curve. Quality assurance procedures included measuring at least one BaP external standard, a method blank and an English sole bile composite, before each set of samples.

**Stress Protein Analysis**

Samples were processed according to Sanders et al. (1994). Livers were first homogenized in a hypotonic solution that lysed both the cell nucleus and mitochondria. The resulting cell fraction was centrifuged at high speed, and the supernatant containing the cytoplasmic proteins was collected. The proteins were then precipitated with trichloroacetic acid and centrifuged again. The supernatant containing the precipitated proteins was then dialyzed against a buffer containing sodium chloride and sodium phosphate. The proteins were then subjected to two-dimensional gel electrophoresis, and the gel was stained with Coomassie blue to visualize the proteins. The gel was then scanned to quantify the relative abundance of each protein band. The data was analyzed using statistical software to identify proteins that were differentially expressed between the two species.

**TABLE 1. Contaminant concentrations in sediment and hornyhead turbot liver from the study sites.**

<table>
<thead>
<tr>
<th>Station</th>
<th>Metals (mg/kg dry wt)b</th>
<th>Organics (ng/g dry wt)</th>
<th>Organics (ng/g wet wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cd</td>
<td>Cu</td>
<td>Zn</td>
</tr>
<tr>
<td>R52</td>
<td>0.17</td>
<td>14.4</td>
<td>85.9</td>
</tr>
<tr>
<td>T1</td>
<td>0.42</td>
<td>16</td>
<td>45.4</td>
</tr>
<tr>
<td>7C</td>
<td>10</td>
<td>140</td>
<td>380</td>
</tr>
<tr>
<td>9C</td>
<td>2.98</td>
<td>59.6</td>
<td>160</td>
</tr>
</tbody>
</table>

³Data for hornyhead turbot livers are median values.

bMetals and PAH data are for samples collected on different dates as part of monitoring or other research programs (Bay et al. 1994, CSDOC 1996, CSDLAC 1996).

cThe PCB congener and DDT concentrations for sediments and hornyhead turbot livers were measured on samples collected in this study (see Distribution and bioaccumulation of persistent chlorinated hydrocarbons in the food chain of hornyhead turbot).

NA = Not available.
ND = Not detected.
dria, organelles where induced stress proteins are localized. The homogenates were then centrifuged to isolate the stress proteins from larger cell components. Supernatants, containing the stress proteins, were collected and total protein concentrations were determined with the BioRad DC protein assay.

Using a technique called slot blotting, 15 µg total sample protein from each fish was directly applied and immobilized onto nitrocellulose paper. Blots were prepared in triplicate for each stress protein of interest (hsp60, hsp70). Stress proteins were detected using an antibody technique that indirectly labels the proteins with an insoluble purple stain. The intensity of the stain is related to the amount of stress protein present in each sample. Relative stress protein concentrations were estimated by comparing intensity values obtained from digital image analysis for each fish sample to a standard curve constructed with an English sole dilution series, included on each blot. Data are expressed as the relative stress protein level/µg total protein.

Western blotting was conducted according to Laemmli (1970) and Towbin et al. (1979) to confirm the specificity of the antibodies used in the above technique. Western blotting of English sole liver samples detected proteins that corresponded to purified stress proteins used as positive controls. Thus, the antibodies were able to identify the stress proteins among the other components in the tissue homogenates.

RESULTS

Bile FACs were detected in all fish examined. The concentrations of these PAH metabolites followed the trend of sediment-associated PAHs (Table 1), with the highest levels present in Palos Verdes fish (Figure 2). The FAC concentrations in hornyhead turbot were higher than in English sole from the same station.

The concentrations of bile FACs in fish from Palos Verdes were significantly higher than the concentrations in specimens from Dana Point for both English sole (p = 0.016, Mann-Whitney test) and hornyhead turbot (p ≤ 0.05, Kruskal-Wallis test / Dunn’s method). Bile FACs in hornyhead turbot from the Orange County discharge zones were not significantly different from Dana Point fish (Dunn’s method).

Both stress proteins (hsp60 and hsp70) were detected in all fish examined (Figure 3). The concentrations were similar for each protein and no significant differences were present between stations (p > 0.2, ANOVA). No significant relationships were found between bile FACs and hsp60 ($r^2 = 0.11$, Pearson Product Moment Correlation), or hsp70 ($r^2 = 0.11$).

DISCUSSION

The results of this study show that English sole and hornyhead turbot at Palos Verdes are exposed to elevated levels of PAHs. These data are consistent with DDT and PCB concentrations found in hornyhead turbot livers (Table 1), indicating that fish at Palos Verdes are exposed to high levels of other organic contaminants. While the concentrations of bile FACs in fish from Palos Verdes were elevated compared to surrounding areas in southern California, higher concentrations have been found for English sole from other locations. For example, the mean bile FAC concentration measured in English sole from Oakland Estuary, California, by the National Marine Fisheries Service in 1990 is approximately three times the concentration measured in English sole from Palos Verdes in this study (Tom Hom, personal communication).
In the present study, PAH exposure in fish was shown to be influenced by the PAH exposure gradients associated with sediment contamination levels around a large wastewater outfall. Similar results have been found in earlier studies in southern California. For example, the mean FAC concentration in hornhead turbot captured near the City of Los Angeles’ wastewater outfall system in Santa Monica Bay in 1986 was four times the mean concentration measured in hornhead turbot from Dana Point collected in 1984-1986 (Varanasi et al. 1989).

Despite evidence that fish at Palos Verdes are exposed to elevated levels of contaminants, there was no indication of cellular stress using these methods. Concentrations of the stress proteins hsp60 and hsp70 in English sole did not differ significantly between Dana Point and Palos Verdes. Sanders and Martin (1993) did find elevated stress protein levels in a hornhead turbot from Santa Monica Bay. However, because of the units that were used to express stress protein concentrations, we cannot compare the stress protein response in that study to our results.

While biomarkers such as stress proteins provide a sensitive measure of contaminant effects, their specificity can also result in a failure to detect some toxic effects. Just as the measurement of a single compound (e.g., Chlordane) does not provide a complete description of contamination patterns at a site, measurement of a single biomarker may not provide a complete assessment of effects. Biomarker responses may also be sensitive to the timing of exposure. For example, short-term laboratory experiments demonstrate a consistent induction of fish stress proteins in response to contaminants (Dyer et al. 1993, Sanders et al. 1995), but long-term exposures produce inconsistent results (Theodorakis et al. 1992).

The biomarker data produced by this study are consistent with the complement information from other chemical and biological measurements at Palos Verdes. Measurements of bile FACs indicate that the PAHs present in sediments near Los Angeles County’s municipal wastewater outfall system at Palos Verdes provide an increased exposure of potentially toxic compounds to resident fish. Stress protein measurements failed to detect an adverse biological response in these fish, however. The lack of effects is consistent with results of recent monitoring, which show little evidence of adverse contaminant effects on fish health or assemblages in the area (CSDLAC 1996).

LITERATURE CITED


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