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Project Summary

Polynuclear Aromatic Hydrocarbons and Cellular Proliferative Disorders in Bivalve Molluscs from Oregon Estuaries

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This research project utilized indigenous populations of economically-important bivalve molluscs as monitors for detecting and quantifying 15 environmental polynuclear aromatic hydrocarbons (PNAH), including 11 compounds classified as carcinogens, 11 EPA Priority Pollutants, and 11 Toxic Pollutants. Cellular proliferative disorders resembling neoplasia were also studied in shellfish populations.

Baseline levels of PNAH were determined during a two-year period for mussels (*M. edulis*), clams (*M. arenaria* and *T. capax*) and oysters (*C. gigas*) from different sites in Yaquina, Coos, and Tillamook Bays, Oregon. Total concentrations of 15 unsubstituted PNAH were 30 to 60 μg/kg in shellfish from uncontaminated waters to greater than 1000 μg/kg in those from sites classified as contaminated.

A major effort was made to determine and evaluate relationships between PNAH and their concentrations in shellfish. Multiple regression and multiple correlation techniques were used to identify and evaluate interrelationships between PNAH. Certain relationships may be useful for predictive purposes in evaluating environmental PNAH and their concentrations in seafood products. Combined with other approaches, this may result in considerable cost reductions for long-term biological monitoring programs.

Cellular proliferative disorders resembling neoplastic conditions in vertebrates were found in mussels with the greatest PNAH concentrations. Further studies will be necessary to determine the significance of this correlation.

This Project Summary was developed by EPA's Environmental Research Laboratory, Gulf Breeze, FL, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

Considerable interest has been expressed recently about the presence of organic chemical carcinogens and mutagens in coastal estuaries and the potential direct effects on indigenous organisms that inhabit these productive environments, as well as the indirect effects on man. Foremost among these chemicals, PNAH are ubiquitous in the marine environment and may present a significant carcinogenic hazard. Scientists from several diverse disciplines have advocated using bivalve molluscs

as biomonitors for the detection and quantification of environmental contaminants, including carcinogens. The use of bivalve shellfish has received the widest support because these molluscs are permanent inhabitants of a specific environment and tend to concentrate toxic substances in their tissues. In addition, bivalve molluscs have been recommended for use in studies of environmental contaminants because many species from several different geographic locations have been reported to have cellular, perhaps neoplastic, proliferative disorders. Environmental pollutants were implicated as potential causative agents in several of those reports, although no cause-effect relationships have yet been established.

Quantitative field studies of PNAH concentrations in marine organisms have only recently been initiated despite earlier suggestions that extensive investigations of the marine environment, including chemical identification, monitoring and surveillance, and identification of fish and shellfish tumors may play an important role in the epidemiology of cancer. Of the PNAH, benzo(a) pyrene (BAP) has been the most extensively studied carcinogen in the marine environment. Excepting BAP, which has often been used as an index to indicate the presence of other PNAH, relatively little information is available on the presence or quantities of other unsubstituted PNAH in tissues of aquatic organisms. Considerable information, based on the use of advanced analytical methods capable of measuring nanogram quantities, is needed.

Associations between high tissue concentrations of PNAH, and other carcinogens, and the appearance of cellular proliferative disorders in shellfish populations should also be identified and carefully investigated. The existence of such associations would suggest the necessity of additional studies to fully evaluate potential cause and effect relationships.

Experimental Procedure

Clams, mussels, and oysters were sampled periodically from Coos, Yaquina, and/or Tillamook Bays in Oregon. Clams from the three bays were dug during low, approximately zero, tides, whereas mussels were collected during the entire ebb tide period, depending on location. Oysters were obtained from commercial growers and simply removed from the shucking tables. Immediately after collection, samples from each site were placed in labeled plastic bags, put on ice, and transported back to Corvallis. Animals were then removed from their shell and the pooled sample from each site was weighed. Each pooled sample was stored at -20°C until it was processed for PNAH analysis. Mussels and clams to be prepared for histological examination were placed in Davidson's fixative, processed in the usual way, sectioned at 6 μm, and stained with hematoxylin and eosin.

For PNAH analysis, tissues were saponified in ethanol/potassium hydroxide for 1.5 hr and the supernate liquid-liquid extracted into 2,2,4-trimethylpentane. The organic phase was passed through a column of partially deactivated florisil (4% water added w/w). The PNAH were eluted from the florisil with benzene and the eluate liquid-liquid extracted with dimethylsulfoxide. The sample was then cleaned up by column chromatography on Sephadex LH-20, concentrated to 100 μl and analyzed by reverse phase high performance liquid chromatography (HPLC).

Only a brief description of the HPLC operating conditions are included below.

Liquid chromatography: Spectra-Physics Model 8000 with data system, Valco injector with 10 μl loop;

Column: Perkin Elmer HC-ODS for PNAH, 0.26x25 cm, 10 μm C_{18} packing with an Alltech C_{18} guard column, 0.46x10 cm;

Mobile phase: acetonitrile/water gradient constant flow mode, 0.8 ml/min. temp. 20°C (Time-%MeCN-%H_2O were as follows: 0 min-60-40, 4 min-60-40, 20 min-60-40, 45 min-100-0, 55 min-20-80, 65 min-20-80, 70 min-60-40, and 80 min-60-40);

Detector 1: Schoeffel Model 770 variable wavelength UV detector, 296 nm, range 0.02 and 254 nm, range 0.02;

Detector 2: Schoeffel Model 970 variable wavelength fluorescent detector, 326 nm excitation, greater than 412 emission (cutoff filter), range 0.1.

Known standard concentrations were made and the data stored by the HPLC. Subsequently, those data were used to calculate the concentrations of PNAH in the samples. Sample losses were accounted for by using an internal spike of ^{3}H-BAP. Total PNAH reported in the results represent the sum of the concentrations of phenanthrene, fluoranthene, pyrene, benzo(c)phenanthrene, triphenylene, benz(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k) fluoranthene, dibenz(a,c)anthracene, BAP, dibenz(a,h)anthracene, benzo(g,h,i) perylene, indeno(1,2,3-c,d)pyrene, and coronene.

Conclusions

Baseline levels of PNAH in indigenous bivalve molluscs used as biomonitors reflected the degree of human onshore activity at the various sample sites and, presumably, the level of water contamination. PNAH concentrations in shellfish from relatively pristine areas ranged from 30 to 60 μg/kg, whereas those from industrialized areas contained 500 to 1500 μg/kg. The data collected during the present study tend to confirm that bivalve molluscs make excellent biomonitors for detecting and measuring PNAH in estuaries.

Multiple regression and multiple correlation techniques were used to identify and evaluate relationships between PNAH. *Identification and evaluation* of quantitative and qualitative relationships between individual PNAH and between PNAH and their concentrations in bivalve molluscs indicated that a significant potential exists for developing predictive models for PNAH in aqueous environments and their concentrations in bivalve molluscs. Statistical analyses also indicated that concentrations of individual PNAH were greater for the isomer which had the greatest solubility in water.

Different populations of shellfish were examined histologically for the presence of cellular proliferative disorders. Clams from Coos Bay and mussels from Tillamook Bay were not found with the large, abnormal cells that characterize the conditions. The disorder was present in a significant number (mean prevalence = 10%) of Yaquina Bay mussels with the highest concentrations of PNAH measured in this study, whereas it rarely appeared in a second population at a "clean" site across the bay. The correlation between the degree of PNAH contamination and the prevalence of the cellular disorders may be significant, but no cause-effect relationship has been established. It remains to be determined if carcinogenic metabolites can be formed by this species.

Discussion

An analytical method, utilizing HPLC, was developed and used for qualitative

and quantitative determination of 15 unsubstituted PNAH isomers. The method resolved most members of the benzpyrene group. Perylene was not identified because it does not absorb UV light at the wavelength used and benzo(j) fluoranthene and benzo(e)pyrene could not be separated.

The identification of multiple PNAH in indigenous organisms represents a significant advance in studying environmental levels of PNAH in marine organisms. The presence of PNAH, particularly BAP, in water, sediments and organisms has been recognized for over 20 years. Yet, dependable, high resolution techniques, such as those utilized in this study, have only recently been developed and a more precise and complete information base can now be developed.

A major effort was made to identify and interpret statistically significant relationships between PNAH and their concentrations in shellfish and between individual PNAH isomers. Certain of the relevant findings from these studies are summarized below:

1. Quantities of a single PNAH present in shellfish cannot be used to predict total PNAH.
2. For each site, different independent variables (individual PNAH) could be identified and used to predict total PNAH in bivalve molluscs. It may be possible to identify multiple site-specific independent variables after a suitable sampling period and subsequently to measure only those key variables for an adequate assessment of total PNAH. Complete analyses could perhaps be made periodically to confirm the continuing validity of the established relationship; deviations may indicate new sources of contamination. Such an approach may result in considerable cost reduction for long-term monitoring programs.
3. The presence or absence of benzo(a)pyrene was not a significant variable for predicting total PNAH at any site. Thus, the concept that BAP can be used as an index of PNAH contamination was not supported by the results of this study. From this and other studies, it seems that the use of BAP for making decisions about the quantities and presence or absence of other PNAH should be abandoned or modified.
4. While it was established that quantitative predictions about total PNAH could not be made on the basis of individual PNAH measurements, the results suggest that certain qualitative relationships existed which may permit first approximations of individual PNAH concentrations. In general, there were no significant differences between individual PNAH with 4 rings, or between those with 5, 6, or 7 rings. Phenanthrene, a 3-ring compound, differed significantly from other PNAH.

Statistical analyses revealed an empirical relationship between individual PNAH concentrations and their respective solubilities. The concentrations in shellfish were greater for the PNAH isomer which had the higher solubility in water. This finding contrasts with the observation that organic/water (e.g., octanol/water) partition coefficients show an inverse relation to water solubility. Because the concentration in the organic phase (shellfish, in this study), C_o, is equal to the product of the partition coefficient (K) and concentration in water (C_w), the data suggested that the ratio of the PNAH concentrations in water would have to be generally greater than the ratio of their reciprocal partition coefficients or their water solubilities. Direct measurements of PNAH concentrations in seawater will be necessary to confirm whether the uptake of PNAH by shellfish can be represented by a simple partition process.

Although there have been numerous reports of apparent correlations between the appearance of abnormal cells in shellfish and their habitation in polluted, primarily oil-contaminated, environments, there have been no published reports of cancer induction in bivalve molluscs by exposure to, or injection of, PNAH. Significant questions about the effects of PNAH on bivalve molluscs and the metabolic capabilities these species have for altering PNAH, remain unanswered. There have been numerous reports that bivalve molluscs cannot metabolize PNAH, yet the evidence presented is by no means definitive, and recent studies indicate that at least some species can metabolize BAP. It remains to be determined if carcinogenic metabolites can be formed by these species. If bivalves are not subject to PNAH-induced carcinogenesis, and the cellular abnormalities are related to a neoplastic process, then other causative agents must be responsible. Assuming the condition is analogous to neoplasia, it seems evident that this disorder in *M. edulis* has greater potential for serving as a model for studying cancer-like diseases in an invertebrate. The cells have many characteristics in common with malignant conditions in mammals, and affected mussels can be obtained easily and on a regular seasonal basis by procedures developed during this study.

Recommendations

Future efforts should be directed towards fully defining the sampling protocols to be used in monitoring studies and toward identifying and evaluating endogenous and exogenous factors that may influence PNAH concentrations under ambient (field) conditions. The latter should include studies of potential sources and measurements of PNAH in water.

It may be productive to conduct similar statistical analyses for PNAH data collected during future studies and from other established biological monitoring programs. Confirmation or extension of the types of relationships identified during this research may eventually lead to simplified monitoring approaches and result in substantial cost reductions.

Further efforts should be made to characterize the large, abnormal, presumably neoplastic, cells; establish culture techniques suitable for maintaining and growing the cells; and identify the causal agent(s) of these disorders in bivalve molluscs.

While required data are not yet available or are incomplete, initial attempts should be made to establish acceptable or unacceptable levels of PNAH in seafood products consumed by humans.

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The complete report, entitled "Polynuclear Aromatic Hydrocarbons and Cellular Proliferative Disorders in Bivalve Molluscs from Oregon Estuaries," (Order No. PB 82-189 523; Cost: $9.00, subject to change) will be available only from:
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