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Petroleum Pollution and Mutation in Mangroves

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Chlorophyll-deficiency has often been used as a sensitive genetic end-point in plant mutation research. The frequency of trees heterozygous for nuclear chlorophyll-deficient mutations was determined for mangrove populations growing along the southwest coast of Puerto Rico. The frequency of heterozygotes was strongly correlated with the concentration of polycyclic aromatic hydrocarbons in the underlying sediment and with both acute and chronic petroleum pollution. Although epidemiological studies can seldom prove causation, a strong correlation is certainly compatible with a cause–effect relationship. Our results suggest that the biota of oil-polluted habitats may be experiencing increased mutation.

The total discharge of petroleum into marine environments is estimated as between 1.7 and 8.8 million metric tons per annum (National Academy of Science, 1985). Although many petroleum hydrocarbons, especially the metabolites of polycyclic aromatic hydrocarbons (PAH), are highly mutagenic in laboratory tests, documentation of the long-term genetic impact of these widespread environmental contaminants on the biota of any ecosystem is lacking (National Academy of Sciences, 1985). Red mangrove (*Rhizophora mangle*) has life cycle characteristics that allow for the easy detection of recessive lethal chlorophyll-deficient mutations and the measurement of mutation rates in natural populations (Lowenfeld & Klekowski, 1992; Klekowski & Godfrey, 1989; Klekowski *et al.*, 1993). We have found a strong correlation between the incidence of such recessive lethals in coastal mangrove populations in Puerto Rico and the load of PAHs in the underlying sediments. The increased mutation in mangroves raises the question as to whether genetic deterioration is also occurring in other (less genetically tractable) members of the biota of petroleum-contaminated habitats.

Although petroleum hydrocarbons are rapidly degraded when released into most tropical marine waters (Botello & Castro-Gessner, 1980), we have previously documented the persistence of petroleum hydrocarbons once incorporated into the sediments of the tropical intertidal zone (Corredor *et al.*, 1990). Plant species living in this environment, such as the red mangrove, may, consequently, suffer chronic exposure to these xenobiotics. As these plants are known to translocate aromatic hydrocarbons into their tissues (Getter *et al.*, 1985), mutagenesis in mangroves exposed to oil spills is a possibility.

In mammals, the activation of PAHs to mutagens is catalyzed initially by NADPH-dependent cytochrome P-450 reductase (Cyt P-450) (Thakker *et al.*, 1985). Cyt P-450 has been documented in numerous plant species, but with a much narrower specificity for substrates than rat liver Cyt P-450 (Higashi, 1988). In plants, the *in vitro* activation of PAHs to mutagens active in microbial assays has been documented, whereas the *in vivo* activation of PAHs by plant mutagen assays is inconclusive (Veleminsky & Gichner, 1988).

Methods

Sediments were sampled within the mangrove fringe by means of a coring device equipped with 22 mm diameter polycarbonate core liners to a depth of 10 cm. Core samples (three from each site, except for Bahía Sucia where a single core was sectioned at 2 cm intervals to 12 cm) were homogenized and refluxed in a Soxhlet extraction apparatus with methanol and KOH. Methanolic digests were extracted with pentane and concentrated in a rotary evaporator at 40°C. Concentrated extracts were further purified and separated into aliphatic and aromatic fractions using 1% deactivated silica/alumina columns and hexane and a methylene chloride/hexane mix (1:4), respectively, as eluants. Aliphatic fractions were evaporated to dryness and reconstituted to 100 μl with hexane and analysed by gas chromatography using a splitless injector, a flame ionization detector and a 5% phenyl methyl silicone 30 m capillary column. Octacosane in hexane was used to calibrate detector response. Individual n-alkanes were identified by co-elution with a simulated distillation mixture ranging from C-12 to C-40. Aromatic fractions were quantified as the chrysene equivalents using a Hitachi F2000 fluorescence spectrophotometer. Samples were reconstituted in hexane and their fluorescence at 360 nm was quantified using an excitation wavelength of 310 nm (Corredor *et al.*, 1990;

Corredor, 1989; Intergovernmental Oceanographic Commission, 1982).

Because of vivipary, trees heterozygous for chlorophyll-deficient alleles are relatively easy to detect in the mangrove species *Rhizophora mangle* (Klekowski & Godfrey, 1989). There are a large number (~300) of nuclear gene loci in vascular plants that may have alleles causing the albino or chlorophyll-deficient phenotype (Klekowski, 1992); this class of mutations is one of the most common mutant phenotypes in vascular plants. It was previously shown that the frequency of chlorophyll-deficient heterozygotes in a red mangrove population can be used to estimate mutation rates in natural populations (Lowenfeld & Klekowski, 1992).

Results

We studied the relationship between sediment hydrocarbon loads and mangrove albinism at sample sites along the southwestern coast of Puerto Rico. The easternmost sample site is Guayanilla Bay, the location of a major petrochemical complex, thermoelectric power plant and transshipment port. An extensive body of scientific literature addresses the environmental impact of this petrochemical complex on the marine environment of Guayanilla Bay (Lopez, 1979a). Lopez (1979b) surveyed the sediments of Guayanilla Bay in 1976 and 1977 and demonstrated widespread contamination by petroleum hydrocarbons. Persistence of such pollution through 1987 has been documented (Corredor, 1989). Two sites were studied in Guayanilla Bay; one site is located along the western shore of the bay and the other is within a small artificial embayment known as the thermal cove. The former site is well removed from the immediate influence of the petrochemical complex. Although it is probably the recipient of occasional surface slicks generated at the complex given the prevailing circulation patterns (Goldman, 1979), relatively low levels of petroleum hydrocarbons were documented in these mangrove sediments by Lopez (1979b). Repeated operational oil spills have been documented within the thermal cove (US EPA, 1971) making this a chronically polluted site.

Three sites were sampled in the vicinity of the village of La Parguera, west of Guayanilla Bay. Montalva Bay is a large open embayment fringed entirely by mangroves. Other than the exhaust from small fishing vessels, mostly outboard powered, there are no significant sources of petroleum pollution at Montalva. Phosphorescent Bay is regularly visited by tourist boats, both diesel and outboard powered, to view the bioluminescence. The mangrove channels west of La Parguera are visited occasionally by tourist boats and fishermen.

Bahía Sucia, at the southwestern extreme of the island of Puerto Rico, is the site of two massive oil spills. The tanker *Argea Prima* released 69 000 bbl of crude to the area in 1962. In 1973 approximately 24 000 bbl of oil washed ashore at Bahía Sucia from the tanker *Zoe Colocotronis*. Large numbers of individual red mangrove trees and seedlings were killed outright in the most heavily contaminated areas (Nadeau & Berquist, 1977). The extent of oil contamination was surveyed within 24 h of the spill (Nadeau & Berquist, 1977) and again in 1977 (Page *et al.*, 1979; Internal Report, 1977), in 1978–79 (Gilfillan *et al.*, 1981) and in 1989 (Corredor *et al.*, 1990). Three years after the spill, soil samples from the mangrove community had 79–80 000 ppm of extractable hydrocarbons (Page *et al.*, 1979). Some areas of Bahía Sucia are still devoid of mangrove growth. Recent observations (Corredor *et al.*, 1990) indicated that substantial concentrations of hydrocarbons occur at discrete depths below the sediment surface in correspondence to the two massive oil spills documented for the area within the last three decades. Finally, samples were collected along the unimpacted western shore of the Cabo Rojo peninsula bordering the open waters of the Mona Passage.

Several criteria may be used to differentiate between biogenic and petrogenic hydrocarbons (National Academy of Sciences, 1985; Gruenfiled & Frank, 1977). Aliphatic fractions of petrogenic hydrocarbons are particularly characterized by the presence of a complex mixture of branched alkanes, cycloalkanes and isoprenoid alkanes which normally appear as a continuous envelope known as the unresolved complex mixture (UCM) in chromatograms of the aliphatic fraction. Although controversy still exists regarding the occurrence of biogenic PAHs, consensus is that biosynthesis of such compounds is rare. On the other hand, petrogenic hydrocarbons exhibit a characteristically high PAH content.

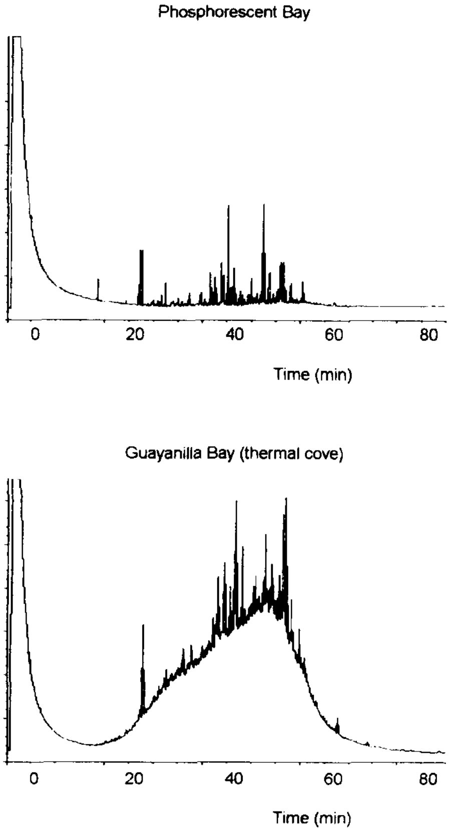

Fig. 1 Chromatograms of the aliphatic fractions of cores from Phosphorescent Bay and Guayanilla (thermal cove). Abscissa—chromatographic retention time; ordinate—relative detector response.

TABLE 1

Sediment hydrocarbon content ($\mu g\ g^{-1}$).

Sample	n	Resolved alkanes		UCM		Total aliphatic		PAH		Toal hydrocarbons	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Guayanilla Thermal Cove	3	40.4	14.5	1792.4	1169.3	1832.8	1178.5	58.8	7.8	1891.6	1177.2
Bahía Sucia	6	16.7	30.1	92.7	93.9	109.4	117.0	41.7	40.1	151.1	155.5
Canal Parguera	3	90.2	79.4	188.1	119.0	278.3	129.6	6.0	1.3	284.3	130.9
Phosphorescent Bay	3	45.8	21.2	93.7	55.6	139.5	52.4	1.1	0.5	140.6	52.2
Bahía Montalva	3	37.3	17.2	33.9	20.9	71.2	34.1	0.9	0.6	72.1	33.8
Cabo Rojo West	2	9.5	5.0	25.6	5.1	35.1	0.1	0.6	0.1	35.7	0.2
Guayanilla West	3	34.0	48.8	79.8	70.3	113.8	117.8	0.5	0.1	114.4	117.9

Results of sediment hydrocarbon analyses are presented in Table 1. Representative chromatograms of the aliphatic fractions are presented in Fig. 1. Samples from the thermal cove at Guayanilla exhibited the heaviest hydrocarbon load. The large UCM (Fig. 1) in the aliphatic fraction of these samples clearly denotes their petrogenic origin. Samples from Montalva and Phosphorescent Bays and from the inshore channels at La Parguera carry moderate hydrocarbon loads, although the constant boat traffic at Phosphorescent Bay is reflected by a larger hydrocarbon content. The samples from western Cabo Rojo show the lowest levels of contaminant hydrocarbons. Samples from Bahía Sucia exhibited substantial preference of aromatic over aliphatic hydrocarbons indicating selective preservation of the former in this environment known to have been affected by massive oil spills.

For each site listed in Table 1, the mangrove populations were surveyed for trees heterozygous for recessive chlorophyll-deficient mutations. The frequency of trees heterozygous for such recessive lethal alleles was determined. The Bahía Sucia site had 108 mangroves surveyed and 4 heterozygotes detected, Guayanilla West had 406 mangroves surveyed and 2 heterozygotes, Guayanilla Thermal Cove had 264 mangroves surveyed and 14 heterozygotes, Phosphorescent Bay had 651 mangroves surveyed and 12 heterozygotes, Bahía Montalva had 499 mangroves surveyed and 6 heterozygotes, Canal Parguera had 794 mangroves surveyed and 14 heterozygotes, and Cabo Rojo West had 757 mangroves surveyed and 1 heterozygote detected. Based upon comparative biochemical and ultrastructural analyses of the homozygous mutant phenotypes, the majority of trees appear to be heterozygous for different chlorophyll-deficient mutations. There may be as many as 300 different nuclear loci that can have chlorophyll-deficient alleles (Klekowski, 1992).

The relationship between the mutation frequency in the mangroves and the underlying sediment load of the different hydrocarbon fractions was assessed by means of comparative correlation analysis. Mangrove mutation frequencies were most closely correlated with PAH sediment loads, Fig. 2. The coefficient of determination (r^2) for PAH indicates a 94% covariation of mutation frequency and PAH loads in the sediments from site to site. The close fit observed between PAH load and mutation rate, together with the known mutagenicity of PAHs, strongly suggests a cause–effect relationship. Since PAHs are one of the most ubiquitous of environmental pollutants, the implications of this correlation are not limited to Puerto Rican mangroves, nor, in fact, to mangroves alone. Increased incidence of massive oil spills in recent years may have biotic consequences not previously anticipated.

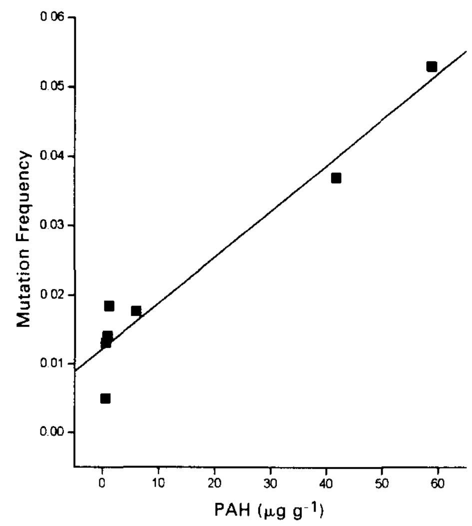

Fig. 2 The relationship between the frequency of mangrove trees heterozygous for recessive, chlorophyll-deficient mutations in a population and the concentration of polycyclic aromatic hydrocarbon (PAH) in the underlying sediment.

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