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**REPORT TITLE**: Bioavailability and Genotoxicity of Produced Water Discharges Associated with Offshore Drilling Operations

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**BACKGROUND**: The major discharges associated with offshore oil and gas production are drilling fluids, drill cuttings, and produced water. During the production of oil and gas, water that is trapped within permeable petroleum-bearing sedimentary rocks is brought to the surface, carrying with it traces of crude oil, drilling fluids and other geological materials (Boesch et al., 1988). This water, called produced water, may contain elevated levels of various inorganic (trace metals) and organic (NAH-PAH) substances of a potentially toxic nature. This report presents results of laboratory and field studies directed by the co-principal investigators.

**OBJECTIVES**: The potential impacts of these discharges on several test organisms were explore through studies of: 1) the bioavailability of dissolved and sediment-bound normal, alkylated and heterocyclic aromatic hydrocarbons to benthic invertebrates and demersal fish, eggs and larvae; 2) the availability of both benthic and pelagic organisms

to metabolize these compounds; and 3) the genotoxicity of the compounds and metabolites in benthic organisms and demersal fish.

**DESCRIPTION**: The study locates at Bayou Rigou, Pass Fourchon (PF) and East Timbalier Island (ETI) were used. The daily discharge of OCS produced water at PF facility is ca. 18,000 bbl/day (Boesch et al., 1988). Bottom sediments in the dean-end portion of PF are typically fine grained. The ETI study focused on a dredged access channel from East Timbalier Bay to ETI. Many discharge points are located in this area. Total daily discharge of both OCS and inland state produced waters at the ETI site is ca. 69,000 bbl/day (Boesch et al., 1988). The dredged channel exhibit slow flow relative to the shallow open bay and bottom sediments are typically fine sands.

Production water discharges from oil-water separation facilities located in nearshore estuarine waterways were analyzed for volatile and semi-volatile organic hydrocarbons by a purge and trap/gas chromatographic technique. Production water samples were extracted for base/neutral, acid extractables by liquid-liquid partitioning (EPA METHOD 625) and analyzed by GC/MS.

Sediment samples collected near the discharge sites were analyzed for NAH-PAH by methanol-hexane extraction with sonication. Extracts were fractionated into a non-polar, semi-polar and polar fraction on silica gel. Final fractions were analyzed by GC/MS. Field collected produced water and surface water samples were serially diluted (0, 25, 50, 75, and 90%) to achieve a concentration gradient for use in the genotoxicity bioassays.

Fish liver microsomes were isolated by differential centrifugation according to standard techniques in the literature (Winston et al., 1989). MFO activities were measured in 0.1M potassium pH phosphate buffer, pH 4, 10 mM glucose-phosphate, 10 mM $MgCl_2$, 0.4 mM NADP and ~ 1-2 mg of microsomal protein in 1 ml. Specific content of cytochrome P450 was measured in dithionite-reduced, CO-liganded microsomes (Omura and Sato, 1964) and quantified from the extinction coefficient (e) 91 mM-1 cm-1. NAD(P)H-cytochrome c (P450) reductase was measured by monitoring reduction of cytochrome c at 550nm (Phillips and Langdon, 1962) and quantified from e = 18.5 mM-1 cm-1 for ferrocytochrome c. Oxidation of the hydroxyl radical scavenger, KMBA, by oxyradicals ethylene production, which can be measured by in the headspace of rubber septa-sealed reaction vessels by gas chromatography (Winston et al., 1984).

P450 contents and reductase activities were determined from field and laboratory samples and correlated with levels and types of xenobiotic compounds founds to be present in the water, the indigenous sediments, the digestive tract and bile as well as the genotoxic endpoints described above. 7-Ethoxyresorufin-o-deethylase (EROD) was determined spectrofluorometrically.

Superoxide dismutase was determined with the SOD-525 Assay of Bioxytech, Cedex, France and carried out as described in the manufacturer's instructions. Catalase activities were determined as described in Claiborne (1985).

**SIGNIFICANT CONCLUSIONS**: EROD was induced in *Fundulus grandis* by 3-methylcholanthrene (3MC), ethylmethanesulphonate (EMS), and cyclophosphamid (CP). Field-exposed organisms yielded much lower EROD values than experimentally induced levels reported in microsomes of other fish species. Clear patterns of EROD induction were not evident in fish samples from contaminated areas. Liver microsomes of all fish, regardless of the area from which they were sampled, exhibited highly variable EROD activities, which in some cases showed overlap. Obvious correlations were not evident between antioxidant enzyme activities and distance from point sources of offshore contamination, contaminated coastal sites vs. reference sites, species, collection time, depth of habitat, sediment-dwelling fish vs. demersal fish and between offshore site. Microsomal P450 was found to be mostly denatured or highly contaminated with other interfering CO-binding chromaphores. EROD, P450 reductase and CO-binding data should be reconciled before tenable conclusions of the effects of pollutant outfall can be made. Mutagen mobilization into co-solvent extracts were detectable by the *Salmonella typhimurium* assay, with tester strain varying in sensitivity. Promutagens were detectable in all extracts, via activation with Arochlor-induced rat liver S9. Mutagenicity varied among co-solvent concentrations, perhaps reflecting mobilization of different arrays of compounds. The *umu* gene induction assay, which uses *S. typhimurium* 1535/psk1002, did not detect mutagens in the extracts whether assays were conducted in the presence or absence of fish liver S9 or microsomes. Isomer specific analyses of bioaccumulated petroleum aromatic hydrocarbons in tissues of aquatic organisms yields information on the relative bioavailability of these contaminants, which rapidly enter biological foodwebs in aquatic systems. This suggests that contaminated sediments remain as bioavailable toxic substances even after discharges are suspended.

**STUDY RESULTS**: Laboratory studies indicated induction of ethoxyresorufin O-deethylase (EROD) in the marine work, *Nereis virens* exposed to petroleum-contaminated sediment from Pass Fourchon, and oil production site in coastal Louisiana. EROD activity was detected in microsomal fractions extracted from excised gut tissue. EROD in worms exposed in 20% sediment was ~14-fold higher than controls after 7 days; after 14 days the increase as ~20-fold over controls. In 50% sediment as 27-fold induction of EROD was seen after 7 days, and after 14 days induction was ~!132-fold. These data could not be reproduced in two subsequent experiments, thus, our data needs to be reconciled with those of Fries and Lee (1984), who showed EROD induction in gut tissues of *N. virens* when exposed to benzo(a)pyrene via food. Western blots of the worm gut microsomes probed with rabbit anti-trout CYP1A1 polyclonal antibody did not indicate the presence of proteins with common epitopes of CYP1A1 proteins. Laboratory challenge experiments were conducted to evaluate EROD induction in adults, larvae, and embryos of the killifish exposed to CP and EMS, two benchmark mutagens with known genotoxic effects on *Fundulus*, and 3MC, a classical MFO inducer and model carcinogen in mammalian studies. 3MC exposure induced EROD to 2.8, 1.9, and 2.2 nmol/min/mg in embryos, larvae, and adults, respectively. EMS induced EROD to 1.50, 1.60, and 0.57 nmol/min/mg. CP induced to 1.25, 1.20, and 0.18 nmol/min/mg.

Bottom-dwelling feral fishes were periodically collected from offshore petroleum production platforms. S9 and microsomal fractions were prepared from pooled or individual livers, and assayed for EROD activity. Field exposed fishes yielded much lower EROD values than in microsomes from experimentally induced fish reported in the literature. S9 preparations were prone to inhibitory effects and a larger base of field data is needed to validate the use of EROD for detecting exposure history and qualitatively demonstrating bioavailabity of dissolved and sediment-bound chemicals. Collected trips to offshore and coastal marsh areas yielded fish specimens from both contaminated and control (pristine) locations. Generally, microsomes from the livers of both control fish and fish from contaminated sites showed very low or no detectable EROD activity. Clear patterns of EROD activities were not evident in fish sampled from contaminated areas. The data point to some recognized problems in biomarker research; fish from large areas display temporal and spatial variability; biochemical responses of fishes are variable as a function of species, sex, gonadal status, season, temperature, and nutrition. Field studies may result in disconcerting data, even though controlled laboratory studies are very predictable.

Oxygen radicals (ROS) are produced as a normal part of all aerobic life. Metals and organic chemicals increase ROS production in biological systems with accompanying protein degradation, enzyme inactivation, lipid peroxidation, DNA damage and cell death. In the few cases where antioxidant enzymes correlate with distance from a platform an inverse relationship was noted. This might relate to depletion of antioxidant defenses under conditions of high xenobiotic exposure. Obvious correlations were not evident between catalase or SOD activity and distance from offshore platforms, contaminated coastal vs. reference sites, species, collection time, depth of habitat and sediment-dwelling fish vs. demersal fish. Microsomal flavoprotein reductases are rate-limiting components of NAD(P)H-dependent MFO and also active loci of ROS production. NAD(P)H-dependent cytochrome c reduction was studied to indicate ranges of intra and inter species activities and the effects of field site environments on those activities. The specific content of cytochromes P450 were also measured to determine the efficacy of microsomal preparations for use in FMO studies. NAD(P)H-cytochrome c reductase activities were measured in liver microsomes of fish sampled from various offshore sites. NADH-cytochrome c reductase ranged from below detectable limits in Bay Whiff caught at ST 52 to ~1000 nmol/min/mg protein in catfish caught at ST 34. NADH-dependent activities were consistently higher in catfish (870±170 nmol/min/mg, n=7), irrespective of the distance from the platform at which they were caught. Fringed flounder had the next highest activities with NADH (83±44 nmol/min/mg, n=6). Fringed flounder from ST 36, 37, 52, 53 and 54 had notably lower NADH-cytochrome c reductase activities than those caught from ST 34. NADPH-dependent activities ranged from 0.2 nmol/min/mg in Bay Whiff caught at ST 53 and 55 to 300 nmol/min/mg in catfish caught at ST 34. Microsomal P450 was found to be mostly denatured or highly contaminated with other interfering CO-binding chromaphores. EROD, P450 reductase and CO-binding data must be reconciled before tenable conclusions of exposure effects can be made. The large variability among the different species, lack of correlation between MFO and field parameters, and

denaturation of MFO catalyst in microsomal preparations are contrary to laboratory conducted exposure studies. Additional problems were fish catches were unpredictable with respect to species, lack of knowledge of sex and gonadal status of the fish and variable conditions of abiotic facts such as fluctuations in oxygen tension.

Mutagen mobilization from sediment into co-solvent extracts was detectable in the Ames assay, with tester strains varying in sensitivity. Promutagens were detectable in all extracts, via activiation with Arochlor-induced rat liver S9. Mutagenicity effects varied among co-solvent concentrations, perhaps reflecting mobilization of different arrays of compounds. Low co-solvent fractions enhanced extraction of water-soluble mutagens but suppress extraction of lipophilic mutagen. In high co-solvent fraction appeared to suppress the extraction of some water-soluble mutagens. TA 102 was the most responsive tester strain with these extracts; TA 98 showed no response to mutagens. The *umu* gene induction assay did not detect mutagens in either the presence or absence of fish liver S9 or microsomes. The ability of microsomes from Lake Champagne and Pass Fourchon catfish to activate 2AA was compared with that of rate liver microsomes. About 2-fold enhancement of *umu* gene induction with the rat microsomes was noted, but no increase was observed over background controls with the fish samples.

Sediment bound contaminants associated with chronic produced water discharges are bioavailable to the filter-feeding bivalve *Crassostrea virginica*. Petroleum-associated aromatic hydrocarbons increased in all experiments in a dose- and time-dependent fashion. The data suggest that contaminated sediments remain as bioavailable toxic substances even after discharges are suspended.

**STUDY PRODUCTS**: Winston, G.W., J.C. Means, L. Riley, S. Dobias, and Z. Yan. 1995. Bioavailabiltiy and genotoxicity of produced water discharges associated with offshore drilling operations, final report. Louisiana Universities Marine Consortium for U.S. Dept. of the Interior, Minerals Management Service, Gulf of Mexico Region, OCS Office, New Orleans, LA. Contract No. 14-35-0001-30470. OCS Study MMS 95-0020. 110 pp.

No journal articles have been completed to date; several are in progress. The following published abstracts were presented at national meetings.

1992 Induction of ethoxy-resorufin O-deethlase (EROD) in the killifish *Fundulus grandis* by selected mutagens. Means, Reily, Yan and Winston. Presented at the 13th annual SETAC meeting, Cincinnati, OH, November 8-12, 1992.

1992 Induction of EROD in the sandworm *Nereis virens* exposed to petroleum contaminated sediment. Reily, Means, Yan and Winston. Presented at the 13th annual SETAC meeting, Cincinnati, OH, November 8-12, 1992.

1994 Field Application of Biomarkers to Petroleum Contaminated Sites in Louisiana. L.A. Reily and J.C. Means. Presented at the Annual Society of Toxicology Meeting, March 13-17, 1994, Dallas, TX.

1994 Co-solvent Mobilization and Modeling of Bioaccumulation Potential of Petroleum-Associated Mutagens from sediments. J.C. Means, L.A. Reily and D.J. McMillin. Presented at the Annual Society of Toxicology Meeting, March 13-17, 1994, Dallas, TX.

1994 Methods for Assessing Bioavailability of Petroleum-Associated Mutagens from Sediments Using Co-Solvent Mobilization. Presented at the 25th Annual Meeting of the Environmental Mutagen Society, Portland, OR, May 7-12, 1994.

1994 The significance of reactive oxygen species in chemical-contaminated marine environments: An overview of studies of the marine mussel, *Mytilus edulis*. G.W. Winston, 15th Conference of the European Society for Comparative Physiology and Biochemistry: Biochemical and Physiological Effects of Pollutants and Toxicological Assessment of Environmental Quality. Genova, Italy, Sept. 20-24, 1994.

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