OCS Study
MMS 99-0017

Environmental Degradation and Identification of Toxic Compounds of Petroleum and Associated Materials

Final Technical Summary

Final Study Report

U.S. Department of the Interior
Minerals Management Service
Pacific OCS Region

OCS Study
MMS 99-0017

Environmental Degradation and Identification of Toxic Compounds of Petroleum and Associated Materials

Final Technical Summary

Final Technical Report

Authors

Richard M. Higashi
Donald G. Crosby
Principal Investigors

Prepared under MMS Cooperative
Agreement No. 14-35-0001-30471
by
Southern California Educational Intiative
Marine Science Institute
University of California
Santa Barbara, CA 93106

U.S. Department of the Interior
Minerals Management Service
Pacific OCS Region

Camarillo
January 1994

Disclaimer

This report has been reviewed by the Pacific Outer Continental Shelf Region, Minerals Management Service, U.S. Department of the Interior and approved for publication. The opinions, findings, conclusions, or recommendations in this report are those of the authors, and do not necessarily reflect the views and policies of the Minerals Management Service. Mention of trade names or commercial products does not constitute an endorsement or recommendation for use. This report has not been edited for conformity with Minerals Management Service editorial standards.

Table of Contents

FINAL TECHNICAL SUMMARY

STUDY TITLE: Southern California Educational Initiative, Study 5: Environmental Degradation and Identification of Toxic Compounds of Petroleum Associated Materials

REPORT TITLE: Environmental Degradation and Identification of Toxic Compounds of Petroleum Associated Materials

CONTRACT NUMBER: 14-35-0001-30471

SPONSORING OCS REGION: Pacific

APPLICABLE PLANNING AREA: Southern California

FISCAL YEAR(S) OF PROJECT FUNDING: FY89, FY90, FY91

COMPLETION DATE OF THE REPORT: January 1994

COST(S): FY 89 - $38,000, FY 90 - $41,803, FY 91 - $41,803

CUMULATIVE PROJECT COST: $121,606

PROJECT MANAGER: Richard M. Higashi

AFFILIATION: University of California, Davis

ADDRESS: Crocker Nuclear Laboratory, University of California, Davis CA

PRINCIPAL INVESTIGATORS: Richard M. Higashi and Donald G. Crosby

BACKGROUND: Produced water is a very complex mixture consisting of non-polar and polar organic substances, inorganic cations and anions, as well as combinations of these diverse chemical categories (NRC, 1985; Boesch and Rabalias, 1987). Produced water is known to be an important source of hydrocarbon and metal pollution (Armstrong *et al.*, 1979; Boehm, 1987; Neff, 1987), but little is currently known about the composition of other major categories of produced water constituents (e.g., polar organic compounds and inorganic anions), and even less is known about the fate and transport of these substances in marine environments. Clearly, the complexity of produced water presents a formidable obstacle to understanding its effects near discharge sites. One strategy for dealing with these obstacles is to reduce the complexity of the problem by focusing on the properties of interest in produced water and also by keying in on a particular discharge of produced water.

OBJECTIVES: In this project, the property of chief interest was the bioeffects of produced water at a Carpinteria, California discharge site. The overall goal of this project was to examine what constituents of produced water harbor the biological activity (expressed as toxicity), and once identified, how environmental chemistry of these constituent(s) might interact with the observed biological effects.

DESCRIPTION: Despite the focus on toxicity, the chemical complexity of the produced water still posed a serious obstacle. This is because toxicity is an unusually broad category of bioactivity that may be caused by any of a host of compounds, with elemental compositions spanning the periodic table. This meant that the common investigative strategy of combining

traditional chemical analyses with existing toxicologic literature was unlikely to meet our overall goal.

We conducted toxicant characterization using chemically non-degrading steps (see Higashi *et al.*, 1992a) – an approach similar to that used successfully for decades in biochemistry for identification of bioactive components in complex mixtures. The approach we used is as follows. Produced water samples were collected into teflon-capped glass bottles from a tap in the discharge pipe at Carpinteria, California. There was no headspace in the samples and they were shipped, refrigerated, to our laboratory. Produced water samples were separated into volatile and nonvolatile fractions by lyophilization (freeze-drying). The nonvolatile fraction was subjected to sequential organic solvent extration that generated three fractions: non-polar (containing hydrocarbons), intermediate polar (containing many other organic compounds such as phenolics), and water-only soluble (containing metal ions, polar organic, and anionic constituents). The non-polar and intermediate polar fractions were further fractionated using high-pressure liquid chromatography (HPLC). The water-only soluble was further extracted by an ion-exchange resin (Chelex resin) designed for trapping divalent ions, finally leaving a polar fraction that contained polar organic inorganic anion, and other non-polyvalent cationic substances.

STUDY RESULTS / CONCLUSIONS: Toxicity tests (Cherr *et al.*, 1990) of all fractions repeatedly indicated that the majority of toxicity was removed by Chelex, but the elemental analysis by ICP-atomic emission spectroscopy performed on these fractions indicated that the more “classical” toxic elements sometimes present in Carpinteria produced water (such as Cu, As, and Cr) were not consistently the culprits. In fact, among the transition metals detected (Al, As, Cd, Co, Cu, Cr, Fe, Mn, Mo, Pb, Sn, Zn), none appeared to be of sufficiently high concentration, either alone or in additive combination, to fully account for the respective mussel toxicity, discounting any synergistic effects.

The only elements that were consistently in high concentration were the alkali earth elements Ba and Sr. The Sr levels found in our produced water samples were slightly elevated from the range normally found in seawater as well as formation water (Collins, 1975), but the Ba levels we found were about three orders of magnitude higher than in seawater, and was in the range typical of formation water (Collins, 1975). Considering the produced water fractionation procedure, the Ba in Chelex-retained, water-only soluble residue must be of a soluble form and not present as barite.

We found that the majority of toxicity to mussel embryos was found in the "divalent cation" fraction. However, the toxicity pattern according to the sea urchin sperm toxicity survey, appears to be very different, due to polar organic substances. These results, if widely true of produced water, suggests that analytical and modeling approaches traditionally used for produced water research - that is, those that key on hydrocarbons and metals – may not be relevant to standard chronic toxicity indicators such as the mussel embryo system.

STUDY PRODUCTS:

Cherr, G.N. and R.M. Higashi. 1990. Comprehensive approaches to isolation of conventional and nonconventional toxicants. Proceedings of the Southern California Academy of Sciences, 99th Annual Meeting. CSU Dominguez Hills, Carson, CA. [abstract]

Fan, T.W-M., R.M. Higashi, G. Garman, and G.N. Cherr. 1990. *In vivo* NMR spectroscopoy and imaging assessment of chronic toxicant effects on reproductive potential of mussels. Proceedings of the 11th Annual Society of Environmental Toxicology and Chemistry, Arlington, VA. pp. 103. [abstract]

Fan, T.W-M., R.M. Higashi, G.N. Cherr, and M.C. Pillai. 1991. Aqueous petroleum wastes perturb reproduction in mussels as monitored *in vivo* by NMR spectroscopy and imaging. Proceedings of the 12th Annual Society of Environmental and Chemistry Meeting, Seattle, WA. pp. 221. [abstract]

Fan, T.W-M., R.M. Higashi, G.N. Cherr, and M.C. Pillai. 1992. Produced water perturbs reproduction in mussels as monitored *in vivo* by NMR spectroscopy and imaging. Proceedings of the 1992 International Produced Water Symposium. pp. 6. [abstract]

Fan, T.W-M., R.M. Higashi, G.N. Cherr, and M.C. Pillai. 1992. Use of noninvasive NMR spectroscopy and imaging for assessing produced water effects on mussel reproduction. In: *Produced Water: Technological / Environmental Issues and Solutions*, J.P. Ray and F.R. Engelhardt, eds. Plenum Press, New York. pp. 403-414.

Higashi, R.M., G.N. Cherr, C.A. Bergens, and T.W-M. Fan. 1992. An approach to toxicant isolation from a produced water source in the Santa Barbara Channel. In: *Produced Water: Technological / Environmental Issues and Solutions*, J.P. Ray and F.R. Engelhardt, eds. Plenum Press, New York. pp. 223-233.

Higashi, R.M., G.N. Cherr, C.A. Bergens, T.W-M. Fan, D.G. Crosby. 1991. Toxicant isolation from aqueous petroleum wastes from the Santa Barbara Channel. Proceedings of the 12th Annual Society of Environmental and Chemistry Meeting, Seattle, WA. pp. 193. [abstract]

Higashi, R.M., G.N. Cherr, C.A. Bergens, T.W-M. Fan, D.G. Crosby. 1991. Toxicant isolation from a produced water source in the Santa Barbara Channel. Proceedings of the 1992 International Produced Water Symposium. pp. 32. [abstract]

FINAL STUDY REPORT

I. GENERAL INTRODUCTION

Produced water is a very complex mixture consisting of non-polar and polar organic substances, inorganic cations and anions, and combinations of these diverse chemical categories (NRC, 1985; Boesch and Rabalais, 1987). Produced water is known to be important sources of hydrocarbon and heavy metal pollution (Armstrong *et al.*, 1979; Boehm, 1987; Neff, 1987), but little is currently known about the composition of other major categories of produced water constituents (e.g., polar organic compounds and inorganic anions), and even less is known about the fate and transport of these substances in marine environments. Clearly, the complexity of produced water presents a formidable obstacle to understanding the distribution and effects of produced water near discharge sites. One strategy for dealing with these obstacles is to reduce the complexity of the problem by focusing on the properties of interest in produced water, and also by keying on a particular discharge of produced water.

In this project the properties of chief interest included the bioeffects of produced water at a Carpinteria, California discharge site; this was in coordination with studies by other research groups of the Southern California Educational Initiative (SCEI), supported by the Minerals Management Service of the U.S. Department of the Interior. The overall goal of this project was to examine what constituents of produced water harbor the biological activity (expressed as toxicity), and once identified, how environmental chemistry of these constituent(s) might interact with the observed biological effects.

Despite this focus on toxicity, the chemical complexity of the produced water still posed a serious obstacle. This is because toxicity is an unusually broad category of bioactivity that can be caused by any of a host of compounds, with elemental compositions spanning the periodic table. This meant that the common investigative strategy of combining traditional chemical analyses with existing toxicologic literature was unlikely to meet our overall goal.

Therefore, we conducted toxicant characterization using chemically non-degrading steps (see Higashi *et al.*, 1992a) – an approach similar to one used successfully for decades in biochemistry for identification of bioactive components in complex mixtures. This approach was markedly different from other types of toxicant identification schemes currently in use (e.g., Mount and Anderson-Carnahan, 1988). The research was conducted with four additionally unique thrusts: (a) specific attention was paid to the presence and potential biological activity of the unstudied polar compounds; (b) analytical methods were consistently chosen for their broad detection capability (low bias in chemical species detection); (c) analytical methods were developed interactively with chemical fractionation and toxicity testing requirements; and (d) this project was coordinated with both mechanistic and field projects. In particular, we worked very closely with the SCEI project of Cherr *et al.*[1] on the characterization and mechanisms of biological activity, and with field studies of other SCEI

[1] Assessment of Chronic Toxicity of Petroleum and Produced Water on Reproduction and Develoment in Marine Organisms, Gary N. Cherr, T. W-M. Fan and R.M. Higashi, PIs

projects such as Schmitt and Osenberg[2] with the aim of uncovering chemical and biological markers of relevance in the field.

All of these points will be addressed in more detail at the appropriate places in this final report. This report is structured chronologically in order to best convey the rationale of the activities of this project.

II. YEAR 1: JUNE 1989 to MAY 1990

IIA. Rationale

The initial thrust of this project was on characterization of the toxic material in produced water, in conjunction with the SCEI project of Cherr *et al.* As discussed earlier, this was necessary prior to any environmental chemistry research due to the complexity of produced water. The toxicant characterization involved toxicant detection by marine test organisms, together with the development of fractionation of produced water and toxicant detection by chemical means. The latter two tasks were the principal responsibility of this project.

From the outset, we began preparations for the detection of unexpected or unknown chemicals in produced water. This seemed prudent, based on our previous experience with other "well-studied" effluents (cf. Higashi *et al.*, 1992a), as well as our literature survey, which indicated that produced water typically contains large amounts of unidentified chemicals, such as the polar organic compounds. Thus, we investigated various sample preparation and fractionation methods together with the development of chemical profile monitoring by direct mass-spectrometry.

IIB. Methods

Samples

Four produced water samples were obtained from an onshore spigot at the Carpinteria facility, via the SCEI project of Schmitt and Osenberg. This particular spigot was identified by the plant manager as yielding a produced water sample most similar to the actual discharge. This facility's marine discharge was the study site for several SCEI projects such as that of Schmitt and Osenberg. Each sample was collected into two four-liter amber glass bottles with no headspace and capped with a Teflon-lined cap. The samples were then shipped chilled by overnight courier to the Bodega Marine Laboratory. Refractive indices of produced water were measured upon arrival using an Atago S-10 refractometer.

Lyophilization

Samples were adjusted to receiving water (seawater) pH 8, gravity-filtered on Whatman 8 μm cellulosic filters and divided into10mL and 500 mL aliquots. The 10 mL aliquots were frozen in borosilicate glass vials, and the 500 mL aliquots were frozen in Teflon beakers, both at a temperature of 77 K by immersion of the containers into liquid nitrogen, then lyophilized at <20 millitorr using either a commercial lyophilizer (Virtis 25 SL) or on a glass condenser

[2] Effects of Produced Water on the Settlement of Some Marine Invertebrates, Russell J. Schmitt and Craig W. Osenberg, PIs

system we devised that employed two low dead volume -70°C cold traps in tandem (see **Figure 1**). The volatile fraction was trapped on the first (glass) cold finger while the second (steel) cold finger was necessary to isolate the first finger from vacuum pump back-vapors, as well as to provide an additional condensation pump to prevent produced water sample meltback. The ice-bound volatile sample trapped on the glass cold finger was slipped off as a single piece by allowing the finger to warm to about 0°C, then stored in solid form at -70°C until use. Pieces of the ice were thawed in airtight vials with minimal headspace to generate aqueous samples for chemical analysis and toxicity testing. Lyophilized residue was stored desiccated at -70°C until use.

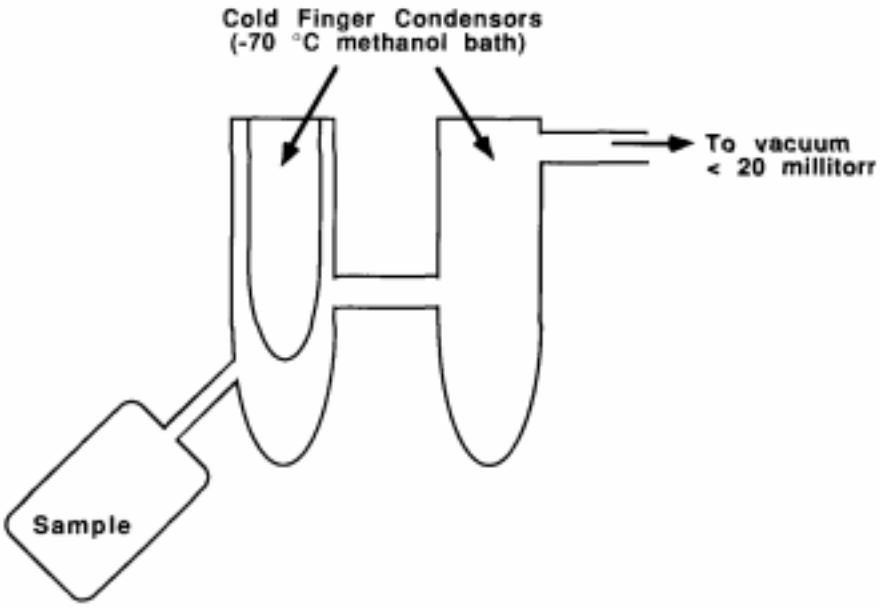

Figure 1. Schematic of the tandem condenser apparatus constructed for lyophilization of produced water for this project. The sample was held in a Teflon beaker and the first (left) condenser was constructed of borosilicate glass, the second of steel. Temperature in the condensers was maintained using freon cold probes immersed in the methanol bath. The left condenser served to trap the ice and volatiles from the sample for further testing, while the right condenser helped to maintain the pressure differential between the sample and the first condenser. In addition, the right condenser minimized any back-streaming of volatiles from the vacuum system, preventing contamination of the left (ice sample) condenser.

Toxicity Tests

Purple sea urchins, *Strongylocentrotus purpuratus*, were selected from a continuous culture maintained at the Bodega Marine Laboratory. Sea urchins were spawned by the injection of KCl into their coelemic cavity. Toxicity assays were conducted on the gametes following a literature procedure (Cherr *et al.*, 1987). Described briefly, this test consisted of incubating sperm in various concentrations of produced water and its fractions and quantifying their ability to initiate fertilization of the eggs at 15°C.

Chemical Analyses

Organic chemical analyses consisted mostly of gas-liquid chromatography with flame ionization or mass spectrometric detection (GC-FID or GC-MS), direct mass spectrometry by

fast-atom bombardment (FAB-MS), FAB-MS coupled with mass-analyzed ion kinetic energy spectrometry (FAB-MIKES), and supercritical-fluid chromatography with flame ionization detection (SFC-FID). Conditions for these methods are described briefly as follows. GC-FID was performed on a Varian (Walnut Creek, CA) 3300 gas chromatograph fitted with a J&W (Rancho Cordova, CA) 0.18 mm i.d. x 40 m DB-1 (0.4 μm coat) open tubular column. GC-FID used H_2 carrier gas velocity at 60 cm/s, injector temperature of 260°C, detector at 320°C, column at 60°C held for 2 min, ramped to 150°C at 20°C/min, and continued onto 300°C at 6°C/min, with the splitless injector held for the first 1.5 min following injection of a 0.50 μl sample. GC-MS used a Hewlett-Packard (Palo Alto, CA) 2890 gas chromatograph interfaced to a VG (Altrincham, UK) Trio-2 quadrupole mass spectrometer, which used the same conditions as GC-FID except that He was used as carrier gas at 30 cm/s, quadrupole mass analyzer at 80°C, tuned to resolution better than 0.96 mmu. FAB-MS used a VG (Manchester, UK) ZAB-2HS reverse-geometry double-focusing mass spectrometer operating in positive ion mode with electron multiplier detection, Xe fast-atom source operating at > 8 kV @ 1 mA, glycerol sample matrix, accelerating potential of 8 kV, magnet scan mode, and tuned to resolution > 1000. FAB-MIKES used similar conditions as FAB-MS, except that He collision gas was introduced in the second field-free region at 10^{-6} torr, ESA scan mode. SFC-FID used a Lee Scientific (Salt Lake City, UT) SFC system, which consisted of a Hewlett-Packard 2890 gas chromatograph with FID and a modified ISCO (Lincoln, NB) SFC syringe pump, a 0.050 mm i.d. x 30 m DB-1 open tubular SFC column, and a variety of pressure and temperature programs.

Inorganic chemical analyses were, at this stage, conducted by the SCEI project of Flegal[3]. Therefore, their methods are not described here.

All common reagents were obtained from Fisher Scientific, Inc., as were all common solvents, which were of HPLC grade. N-methyl pyrrolidinone of HPLC grade was obtained from Aldrich Chemical Co.

IIC. Results and Discussion

The sample preservation procedure, lyophilization (freeze-drying), also served as the first fractionation step. This step proved to be considerably more difficult than originally anticipated, as produced water typically had a very low eutectic ("freezing") point despite the low refractive index that indicated a salinity of about 19 ppt. We surmised that the source of the low eutectic point was not due to inorganic salts, but may have been due to high levels of polar organic compounds. Therefore, lyophilization of produced water required the construction of a cascade condenser system, shown schematically in **Figure 1**; the figure legend and methods section describes this apparatus in more detail. This type of design also facilitated the chemically clean recovery of the ice (volatile) fraction, a provision that commercial lyophilizers are not designed to do.

Four produced water samples were received in the first year of this project. The first two samples were used primarily for the development of the lyophilization method and tests of analytical methods. The next two samples were processed, using the tandem lyophilizer, into

[3] Characterization and Fate of Produced Water Discharged in Shallow Coastal Waters, A. Russell Flegal, PI

ice fractions containing volatile constituents plus all the water, as well as a fine white powder residue containing everything else.

Table 1 shows data from an initial survey using the sea urchin sperm cell test, which indicates that toxicity is not expressed in the particulate fraction, and that toxicity is split between the volatile and residue fractions. The table also shows a common, but sometimes puzzling phenomenon: at first glance, the total toxicity appears to increase because each fraction contains the same toxicity as the parent material. There could be several reasons for this: (a) it is possible that the total toxicity has actually increased due to chemical alteration during sample manipulation; (b) because the toxic mechanisms are different from each other, the two fractions are not additive in toxicity when mixed together; (c) because the toxic mechanisms of the fractions are similar to each other, there is antagonism in toxicity; (d) the shapes of the dose-response curve (e.g., the EC_{50} point) are such that it is independent of the LOEC; (e) some or all of the above. Resolution between these reasons would first require toxicant identification followed by studies on the mechanism of action - precisely the goals of this project and that of Cherr, *et al.* Thus, we put aside this phenomenon and temporarily concluded that chemical analyses of both fractions should be pursued.

Table 1. Effects of produced water and its initial fractions on sea urchin sperm fertilizing capacity.

Sample	Lowest Effective Concentration (LOEC) (%)
Whole Produced Water	3.0
Filtered Produced Water	4.0
Ice (volatile) Fraction	4.0
Residue (nonvolatile) Fraction	4.0

Despite the difficulty in dealing with produced water, lyophilization appeared to be a fractionation step worth implementing since it highly concentrates the sample and is widely regarded as a means to minimize chemical alteration, partially addressing reason (a) above for the apparent toxicity increase. Most importantly, it rigorously separated volatile from nonvolatile constituents. This in turn clarified which analytical techniques were – and were not – useful for these initial fractions. Specifically, the detection and identification of compounds in the ice (volatile) trap can depend entirely on the nonspecific identification technique of GC-MS. In contrast, it is also clear that the nonvolatile residue cannot depend on GC-MS, and must turn to less conventional means of nonspecific detection and analysis.

At this point, it was clear that the volatile fraction can be analyzed by GC-MS and was likely to reflect classes of compounds commonly analyzed by petroleum researchers. The ice fraction, as it contains only volatile constituents, was analyzed by directly injecting the thawed ice into the GC-FID instrument, the result of which is shown in **Figure 2**. Most of the peaks appeared early, indicating that these constituents were indeed volatile; for the reader's reference, peaks less than 10 min retention time on this system were constituents of volatility similar to that of organic solvents and small hydrocarbons.

The elemental (e.g., transition metal) composition of the dry residue may be addressed adequately by many existing methods in petroleum research. However, this was the topic of another SCEI project (Flegal) and we did not address the inorganic compounds at this time. Our main concern was, a host of other compounds in produced water that were not analyzable by the typical GC or elemental analysis methods, even though the organisms in the toxicity test may have been "seeing" them. Thus, we placed an early emphasis on developing methodology for broad detection of chemicals, in particular the traditionally ignored constituents of produced water.

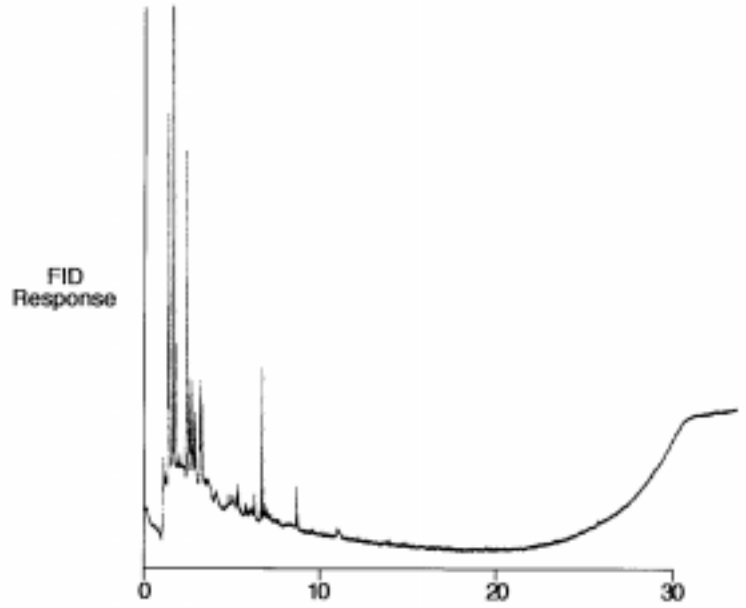

Figure 2. GC-FID of ice fraction from second produced water sample, by direct injection. Note that only highly volatile constituents are present in the sample.

To implement this direction, two general analytical approaches were selected for development. It was decided that direct mass spectrometry by fast-atom bombardment (FAB-MS), linked to structure characterization via mass-analyzed kinetic energy spectrometry (FAB-MIKES), would be useful in detection and identification across most classes of nonvolatile compounds, particularly in conjunction with toxicologic evaluations. The potential utility of this technique, especially toward the nonvolatile and more polar compounds, had been demonstrated in petroleum research (Tibbetts *et al.*, 1985).

Figures 3, **4**, and **5** show the FAB-MS spectrum of nonvolatile residue from the second, third, and fourth produced water samples. In addition to its broad detection capability and ability to work with polar compounds, FAB-MS is a "soft" ionization MS technique. This means that each of the peaks in **Figures 3**, **4**, and **5** probably represent the molecular ions of different compounds, allowing the spectral pattern to directly reflect the composition. Comparison among these figures illustrate that the nonvolatile organic constituents of Carpinteria produced water varied only slightly among these samples.

Another method that we began to examine in the first year for analysis of the nonvolatile fraction was the new technique of SFC-FID. Its principle advantage was that it had the resolution and "universal" detection of GC-FID but was capable of analyzing many nonvolatile compounds that would otherwise require liquid chromatography (Borra *et al.*, 1989), which lacked the practical resolution and broad detection modes of SFC. SFC instrumentation was made available for this project courtesy of Dr. Andrew Clifford (Dept. of Nutrition, University of California - Davis).

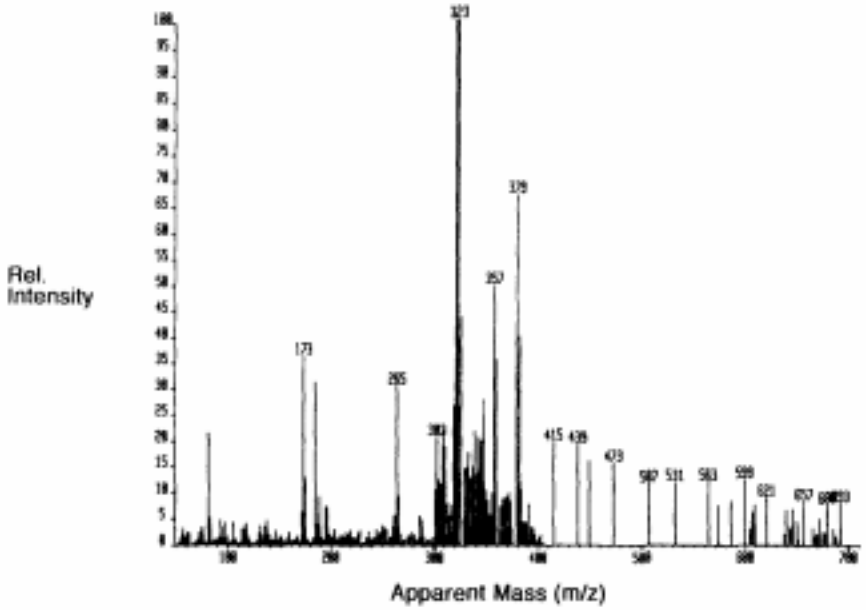

Figure 3. FAB-MS spectrum of lyophilized residueof the second produced water sample. This fraction contains the nonvolatile constituents of the sample. As FAB-MS is a relatively non-specific analysis, plus it is a very "soft" ionization method that produces mostly molecular ions (i.e., one peak per compound), this mass spectrum may be considered to directly reflect the chemical composition present in this sample. This figure should be compared with the following two figures to obtain a feel for the variability of produced water organic constituents.

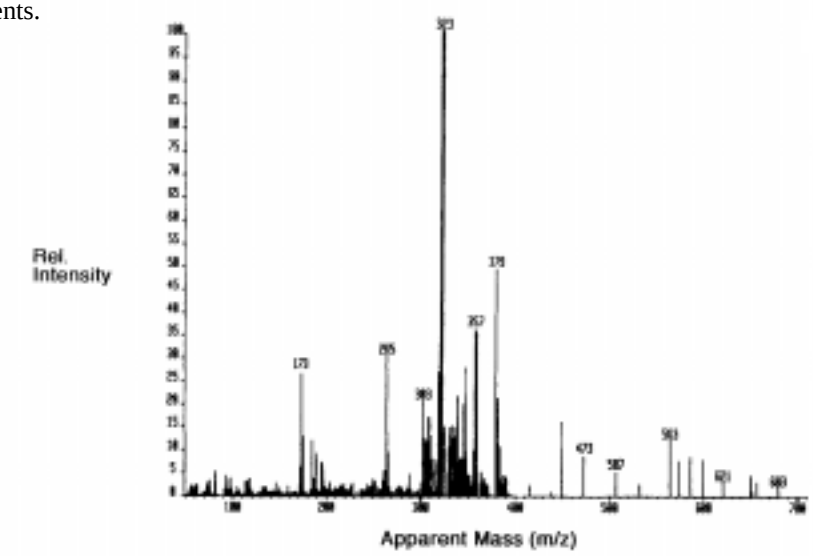

Figure 4. FAB-MS spectrum of lyophilized residue of the third produced water sample. Note the considerable similarity to **Figure 3**.

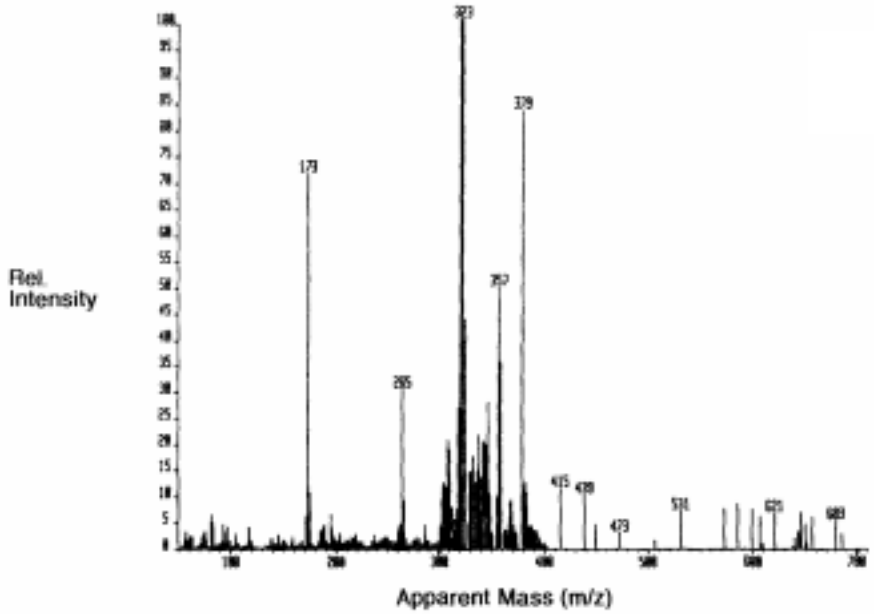

Figure 5. FAB-MS spectrum of lyophilized residue of the fourth produced water sample. As with **Figure 4**, note the similarity between spectra of the different produced water samples.

III. YEAR 2: JUNE 1990 to MAY 1991

IIIA. Rationale

The activities of Year 1 produced the basic tools necessary to begin more comprehensive chemical fractionation and toxicity testing of the initial fractions. Toxicity testing of additional samples, as reported by the project of Cherr *et al.* (1991), revealed variability in the toxicity of the ice (volatile) fraction, but the lyophilized residue (nonvolatile) fraction consistently represented the majority of toxicity. This was consistent with the chemical profiles of the ice fractions being variable, though the residue was much less variated (see **Figures 3-5**). This also illustrated the utility of implementing non-specific chemical detection early on in the research. Therefore, both the biological and chemical measurements guided us to devote the main effort of toxicant characterization on the residue. The next steps consisted of organic solvent extractions to remove the more non-polar compounds, coupled with further separation of these extracts by high-pressure liquid chromatography (HPLC).

Some of the advantages of lyophilizing samples were previously discussed above. The next few steps of chemical fractionation demonstrated additional advantages. As compared with the common practices of liquid:liquid or solid-phase extractions of aqueous samples, the high degree of concentration achieved by lyophilization prior to solvent extraction helped achieve higher efficiency. The putative sample stabilization allowed for method development by repeating extractions on actual samples, while the dry powder nature of the residue allowed usage of the full range of solvents, including water-miscible solvents for extraction. The latter two were simply not possible with existing extraction methods.

IIIB. Additional Methods

We continued using methods described for Year 1. The following methods were added to the research in Year 2.

Sequential Solvent Extraction

Please consult **Figure 6** for a schematic representation of the following procedure. Dry, white, powdery produced water residues resulting from lyophilization were subjected to three sequential non-polar solvent extractions by placing the sample in a borosilicate flask containing methylene chloride ($MeCl_2$) at 50 mL/g residue, and micro-agitation of the flask in a 100-watt sonic bath for 30 min. The extracted residue in each case was retained on 0.45 μm teflon filters. After the final filtration, traces of solvent were removed using a stream of N_2 flowed through the filter. The dried residue from $MeCl_2$ extraction was then subjected to acetonitrile (MeCN) extraction in a manner identical to the $MeCl_2$ extraction. The left-over residue was found to be only soluble in water. The $MeCl_2$ and MeCN soluble extracts were concentrated to near dryness on a rotary evaporator, transferred to small glass vials with the aid of the respective extraction solvents, and concentrated to dryness under a N_2 stream and stored desiccated at -20°C until use.

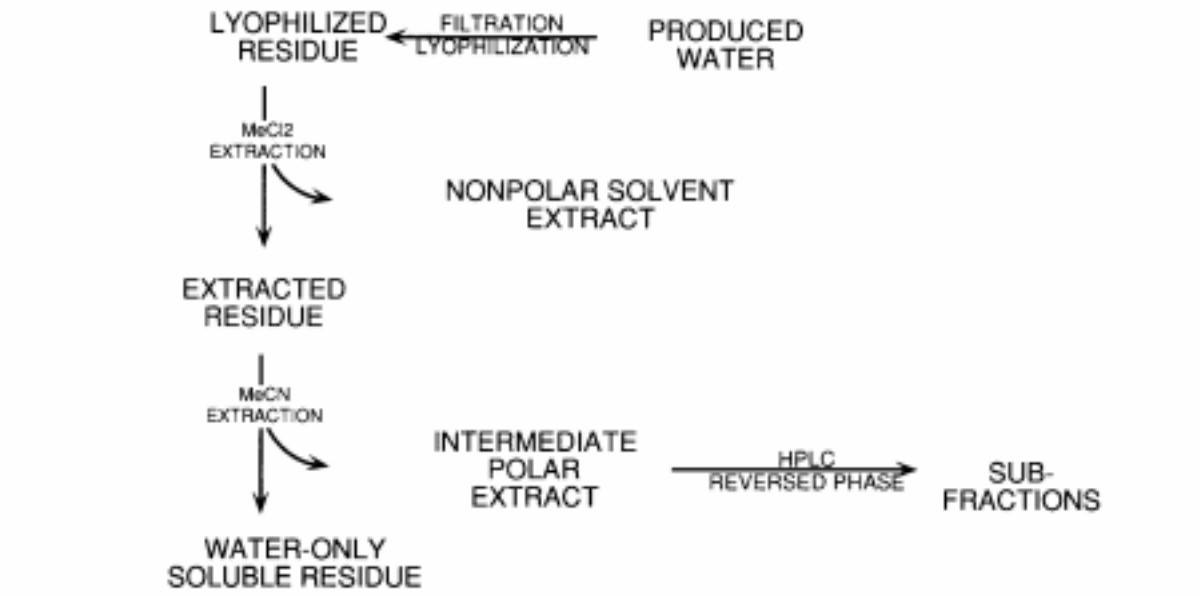

Figure 6. Fractionation scheme at the end of Year 2 research.

Preparative Chromatography

Semi-preparative high-pressure liquid chromatography (HPLC) was used to further fractionate the MeCN extract. The procedure employed an ISCO (Lincoln, NB) ternary gradient HPLC system with 10 mm x 250 mm 5 μm spherical particle C_{18} column and ISCO V_4 variable-wavelength UV-visible detection. Chromatographic conditions were 95:5:0 (water:MeCN:isopropanol) held for 10 min, then a linear gradient to 5:90:5 over the next 40 min, and held at 5:90:5 for the next 30 min. The flow was held constant over the entire run at 2 mL/min, and the void time of 5 min. The UV-visible detector was set to 220 nm for

detection during method development, but was turned off during actual sample collection to avoid photodegradation of sample. Fractions were concentrated by rotary evaporation with excess MeCN to azeotrope off the water, then transferred to a small vial for final concentration by N_2 stream to dryness, and stored desiccated at -20°C until use.

IIIC. Results and Discussion

Figure 6 outlines the fractionation scheme at this point in the project. The solvent extraction procedures were developed by maximizing the recovery of constituents detected by GC-FID analyses of the extracts. **Figures 7a** and **7b** show the GC-FID tracings of the $MeCl_2$ and MeCN extracts, respectively. Note that the two tracings are dissimilar, for the most part containing peaks exclusive of each other. This indicates that the solvent extraction procedures yield relatively sharp chemical distinctions between the fractions, which is desirable for subsequent operations. Other common solvent combinations - using hexane, acetone, and methanol - did not appear to produce such a distinction.

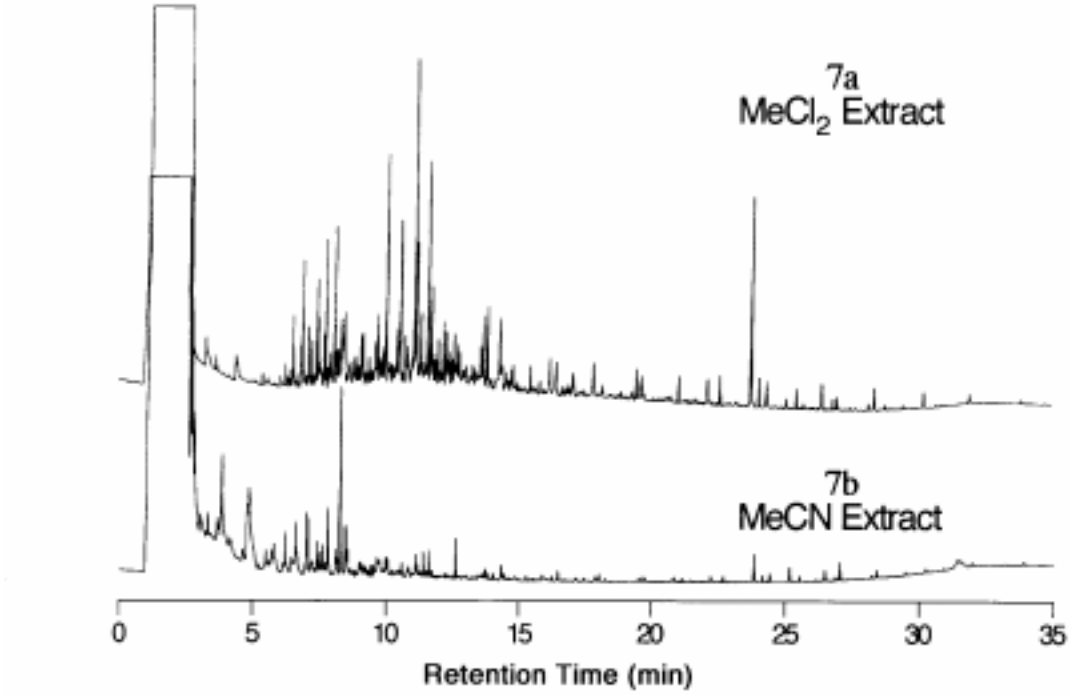

Figure 7. GC-FID chromatograms of the methylene chloride and acetonitrile extracts of lyophilized produced water.

The sharp chemical separation was also reflected in the toxicity assays of the solvent fractions, as shown in the top half of **Table 2**. Again using the sea urchin sperm cell test, the $MeCl_2$ extract exhibited no significant toxicity (EC_{50} » 20%, based on amount of fraction in the original produced water), while the MeCN extract contained toxicity equivalent to the original sample (EC_{50} = 3.9%). Thus, the MeCN extract was subjected to further separation by preparative reversed-phase high-pressure liquid chromatography (HPLC).

Table 2. Effects of produced water fractions on sea urchin sperm fertilizing capacity.

Sample	Effective Concentration (EC_{50}) (%)
Whole Produced Water	4.0
$MeCl_2$ Extract	» 20.0
MeCN Extract	3.9
HPLC Polar Fraction	1.2
HPLC Intermediate Polar Fraction	» 20.0
HPLC Non-polar Fraction	7.8

Figure 8 illustrates this HPLC tracing, obtained using photometric detection at low wavelength to reduce chemical detection bias. Three fractions were collected, bypassing the detector to avoid photodegradation upon collection, representing polar (collected from 7-11 min, with minimal retention on the column), intermediate polar (11-50 min), and non-polar (50-70 min) constituents. **Table 2**, reveals that the non-polar fraction contained slight toxicity to the sea urchin sperm cell test (EC_{50} = 7.8%), the intermediate polarity fraction had no detectable toxicity (EC_{50} » 20%), and the polar fraction contained by far the majority of toxicity (EC_{50} = 1.2%).

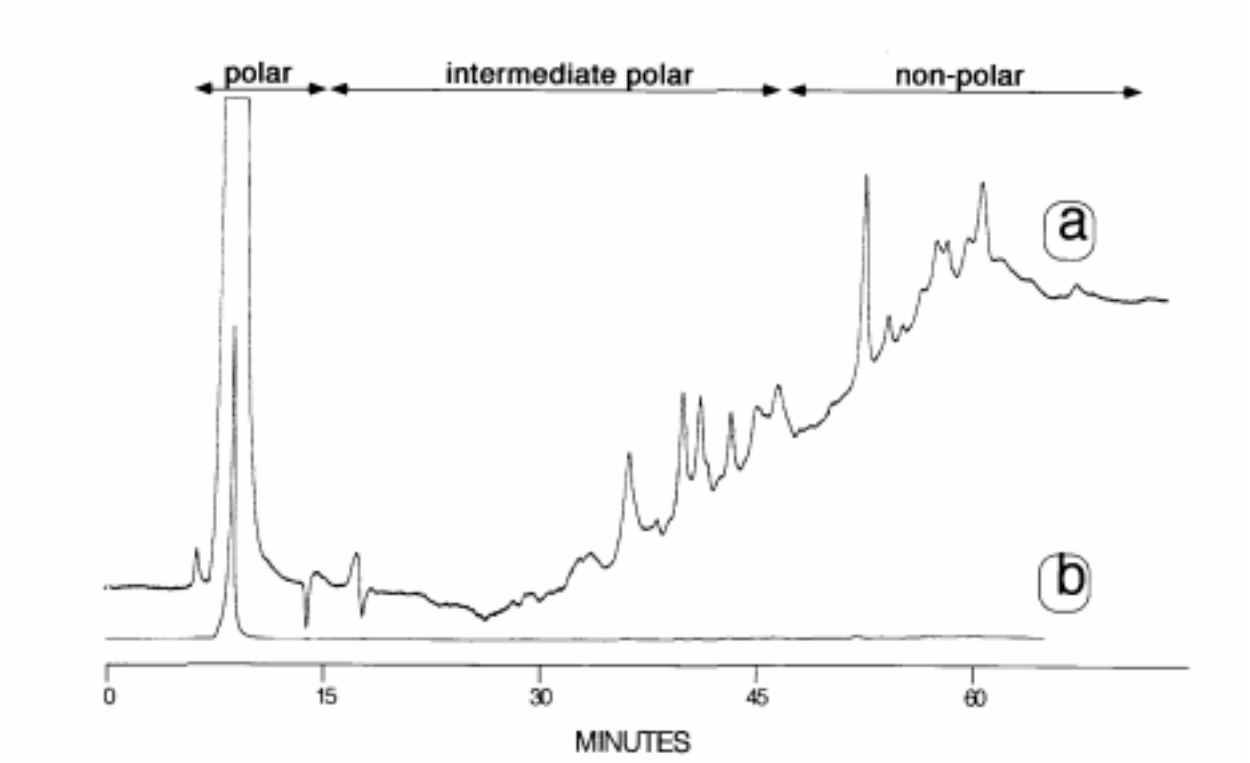

Figure 8. HPLC separation of the MeCN extract of lyophilized produced water. Tracing (a) is as detected at 220 nm, tracing (b) is the same except at higher attenuation to illustrate the relative size of the polar peak. Conditions are stated in the text. Arrows at the top of the figure indicate the regions collected for toxicity assay by the sea urchin sperm cell test.

The detection of toxicity in the polar fraction was interesting because it was not likely to contain volatile hydrocarbons (they were removed upon lyophilization), or metals (they tend

to be left behind by solvent extractions), or non-polar hydrocarbons (which elutes much later on the HPLC).

Figure 9 shows the UV-visible spectrum of the HPLC polar fraction (superimposed on whole produced water for comparison). The hump in the absorbance at ~245 nm suggests the presence of an aromatic structure. The rest of the spectrum is indeterminate.

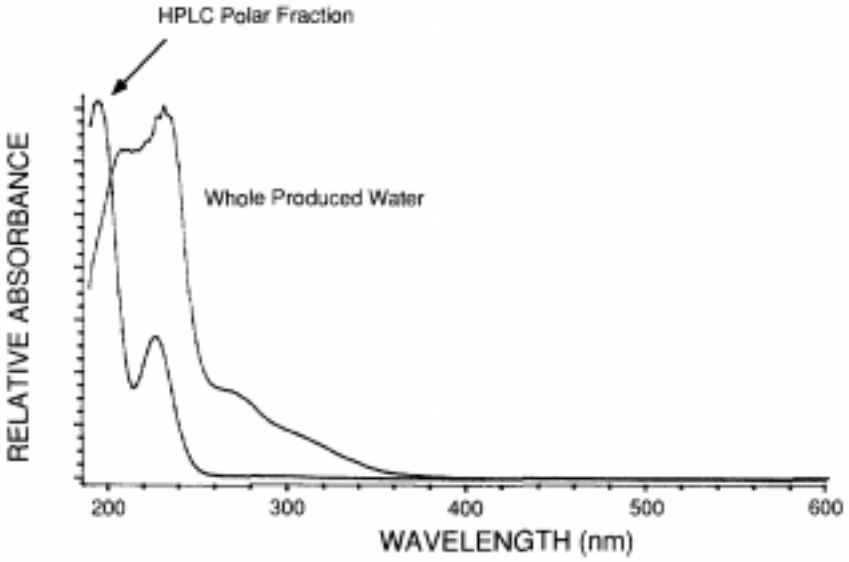

Figure 9. UV-visible spectra of filtered produced water and of the HPLC polar fraction that is toxic in the sea urchin sperm cell test.

Figure 10 shows the GC-MS chromatogram of the HPLC polar (toxic) fraction. This figure indicates that this fraction contained relatively few peaks, with one broad peak from 10-13 min as the most abundant component in the chromatogram. Its poor chromatographic behavior suggess an acid structure. As shown in **Figure 11** its mass spectrum is also very simple. However, it must be noted that GC-MS was in this case, a selective detection method. This is due to a polar fraction from HPLC which was likely to contain compounds that do not gas chromatograph. Naturally, we then turned to FAB-MS analysis of this fraction, but it was marred by interference from HPLC column material "bleed", which may take some effort to compensate. In any case, it was clear that the HPLC polar fraction was a mixture so that further separation of the HPLC polar fraction needed to be implemented before we could obtain direct evidence of the toxic component(s) toward the sea urchin sperm.

Figure 12 shows the GC-MS chromatogram of the HPLC non-polar (slightly toxic) fraction. As shown throughout this research, the different chemical compositions of the HPLC polar (**Figure 10**) and non-polar (**Figure 12**) fractions mirrored the extensive differences in toxicity (**Table 2**). The HPLC non-polar fraction also differed from the HPLC polar fraction in that many compounds in the former can be expected to gas-chromatograph. Thus, **Figure 12** may be a reasonable representation of the HPLC non-polar fraction contents. If so, this fraction

appears to dominated by the large peak at scan #1291, however further identification in this fraction was not pursued due to its low toxicity.

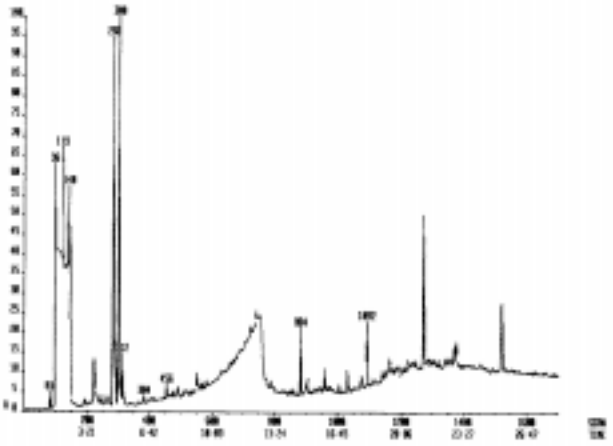

Figure 10. GC-MS total ion chromatogram of the HPLC polar fraction, direct injection. Ordinate is arbitrary, and large peak before scan #150 is due to solvent.

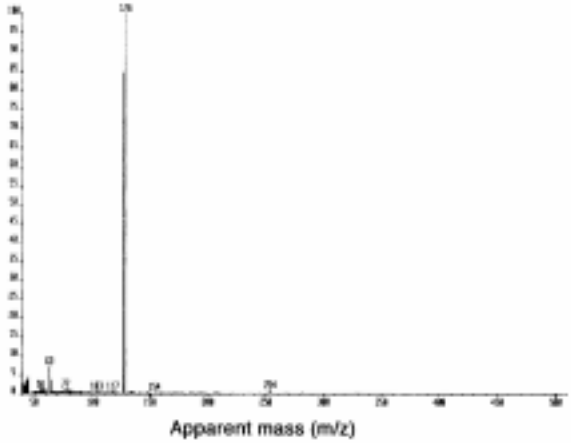

Figure 11. Mass spectrum of scan #753 from **Figure 10** (the broad peak in the chromatogram). Ordinate is arbitrary units.

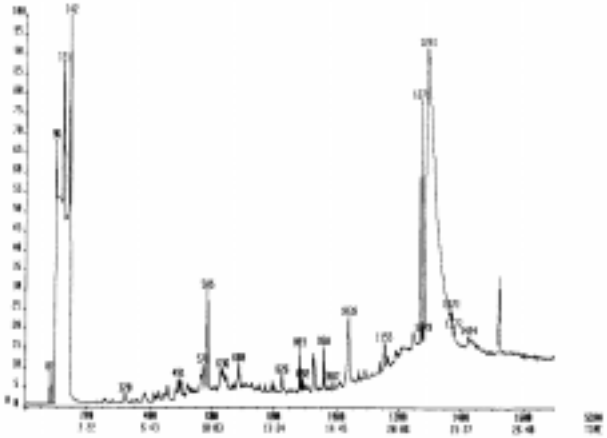

Figure 12. GC-MS total ion chromatogram of the HPLC non-polar fraction, direct injection. Ordinate is arbitrary scale, and peaks before scan #150 are due to solvent.

IV. YEAR 3: JUNE 1991 to MAY 1992

IVA. Rationale

The fractionation-toxicity approach has been developed and tested using the sea urchin sperm cell test, mostly due to its speed and convenience of nearly year-round gamete availability. However, there is ongoing debate about its relevance to environmental effects on organisms (e.g., SETAC Symposium, 1991). More importantly, other SCEI projects such as that of Cherr *et al.* and Osenberg and Schmitt have reported significant effects on mussel embryos and adults (SCEI Annual Report, 1992). Accordingly, the mussel embryo test was used for the remainder of the study for greater interpretive relevance to the other laboratory and field studies being conducted by SCEI researchers. Nevertheless, it should be pointed out that the more involved mussel embryo test would not have been practical in developing the analytical and fractionation methods. In this final year, we essentially found that no changes in fractionation procedures needed to be made after the switch to the mussel embryo test.

All fractionation steps were performed in a fashion that made as few presumptions as possible regarding the classes of constituents that were the sources of toxicity. Chemical stability and the structures relevant under the receiving water conditions were also considered. For example, the pH of produced water was set to 8 (seawater pH) and was not manipulated further to "optimize" organic solvent extraction, as is frequently done; large swings of pH will alter many structures. In any case, it was not known prior to this year's research what types of structures to optimize for. However, as described below, drastic changes in pH occurred at elution from a cation exchange column due to the nature of the technique. But by this time, we knew that the toxic constituent(s) were of a chemical nature not destroyed by low pH.

A major effort in our reseach in Year 3 was, with the project of Cherr and Fan, expended on the incorporation of "in-house" elemental analysis (despite this being the main topic of the SCEI project of Flegal). This was a necessary move - as with all the other analytical methods – because the sample preparation was driven primarily by toxicity testing requirements. The analysis sample size and form was frequently non-standard, requiring methods to be developed interactively with the chemical fractionation and toxicity testing.

Most of the Year 3 results have been reported in a publication (Higashi *et al.*, 1992b).

IVB. Additional Methods

We continued using methods described above for Years 1 and 2. The following methods were added to the research in Year 3. Please note that, for brevity, all fractions are now referred to by roman numerals, as indicated in **Figure 13**.

Toxicity Tests

California mussels, *Mytilus californianus*, were collected from Bodega Bay, CA. Mussels were spawned by immersing them in high concentrations of an algal monoculture, *Isochrysis galbana*. Toxicity assays were conducted on the embryos using a slightly modified procedure of Cherr *et al.* (1990); briefly, this consisted of scoring mussel embryos for the normality of

their shell formation after 48 hours of culture at 15°C in various concentrations of produced water and its fractions.

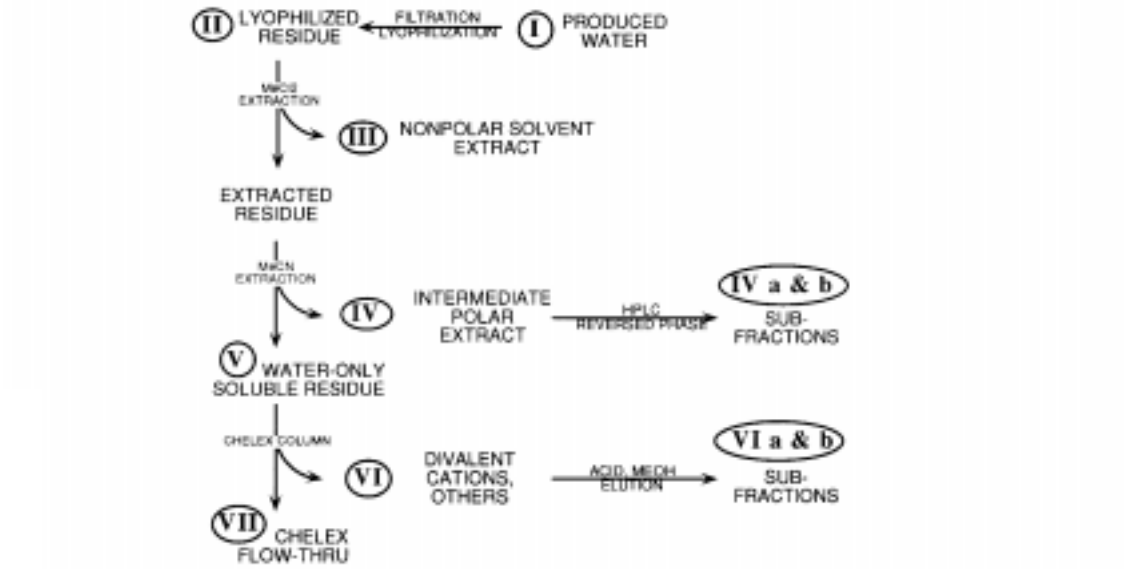

Figure 13. Fractionation scheme at the end of Year 3 research.

Various fraction-dependent considerations had to be met for the toxicity tests. The fractions generated are shown in **Figure 13** and the various fractions are referred to by the roman numerals from this figure. For the organic solvent-based (III, IV, and VIb) and HPLC fractions (IVa and IVb), a dispersant was used, as the constituents were expected to have low solubility in water. We found that a solvent of exceptionally broad polarity and solvation range, N-methyl pyrrolidinone (NMP), exhibits low toxicity (NOEC > 100 ppm) in these marine tests (Cherr, unpublished data). Thus for these fractions, the dried samples (see sample generation procedures below) were first dissolved in small volumes of NMP then dispersed into seawater. The highest concentration of NMP used in a given test (highest among all tests = 50 ppm) served as control. Blank HPLC runs were included as additional controls for IVa and IVb. The water soluble dry-residue fractions (II and V) were first dissolved in distilled water, then they, along with the aqueous fractions (volatile fraction, I, VIa, and VII), were salinity-adjusted with appropriate volumes of 2x seawater for toxicity testing. Seawater served as controls for measurements of these fractions; the exception was VIa which was highly saline after neutralization, which precluded salinity adjustment by addition of seawater. As a result 1N HCl was used as control to detect any false-positive "toxicity" due to Ca^{2+} deficiency from the lack of seawater. All seawater used in this study was obtained from Bodega Bay, CA and was 0.45 μm filtered.

Fractionation

Chelex-100 polyvalent cation exchange resin (Bio-Rad Laboratories, Richmond, CA), mesh 100-200, was used for fractionation of the water-only soluble fraction (V). The resin was prepared by rinsing the resin in pH 7.2 unbuffered HPLC-grade (17.8 megaOhm) water until the pH of the eluate was close to pH 7, and this material was stored hydrated until use. For fractionation, 1 mL of hydrated Chelex was used per "10 mL" of V (i.e., the amount of V that

was generated from 10 mL of I). A typical sample preparation constituted "20 mL" of V. Using volumes for this sample size, 2 mL hydrated Chelex was gravity packed into a polypropylene microcolumn (0.8 cm i.d. x 6 cm) fitted with a polyethylene 10 μm frit. The column was rinsed with three bed volumes of distilled water (3 x 2 mL) and allowed to flow by gravity until the meniscus of the water reached the top of the Chelex bed ("eluted"). The approximate flow rate was 1 mL/min. V was dissolved in 20 mL of distilled water, added to the column, and eluted; constituents retained on the column was VI and the resulting eluant was VII.

VI was further separated into two fractions by the following procedure. VIa was generated by eluting the column with 3 x 2 mL of 1N HCl, which was then neutralized with NaOH to generate the aqueous sample for toxicity testing. VIb was generated by eluting the VIa-devoid Chelex column with 3 x 2 mL of 1:1 MeOH:1N HCl, neutralized with NaOH, rotary-evaporated with excess MeOH to azeotrope off the water, transferred using MeOH to a small vial to eliminate excess salt, concentrated by N_2 stream to dryness, and stored desiccated at -20°C until use.

Chemical Analysis

Atomic spectroscopy for element analysis used either ARL (Applied Research Laboratories, Inc., Dearborn, MI) 3510 inductively-coupled Ar plasma emission ("ICP-AES") or Varian AA-1475 absorption with Varian VGA-76 hydride-generator instruments. Samples for atomic spectroscopy (I, II, IV, IVa, V, VIa, VII) were analyzed by a digestion procedure using 20% $HClO_4$ + 50% HNO_3 (Agemian and Thomson, 1980) and measurement by ICP-AES (Buchanan and Hannaker, 1984). For analysis of As, the same digestion procedure was coupled with measurement by hydride generation-AA (Brumbaugh and Walther, 1989). NIST-traceable standards were used to determine the response curves for each element.

IVC. Results and Discussion

The fractionation scheme completed by the end of Year 3, shown in **Figure 13**, is described as follows. Produced water samples (I) were separated into volatile and nonvolatile (II) fractions by lyophilization (freeze-drying), then II was subjected to sequential organic solvent extraction that generated non-polar (III, containing hydrocarbons), intermediate polar (IV, containing many other organic compounds such as phenolics), and water-only soluble (V, containing metal ions, polar organic, and anionic constituents) fractions. Further fractionation of IV was achieved using HPLC, while V was extracted by an ion-exchange resin (Chelex resin) designed for trapping polyvalent cations (fraction VI), finally leaving a polar fraction (VII) that contained polar organic, inorganic anion, and other (non-polyvalent) cationic substances. VI was further separated into two fractions by elution with acid (VIa) and acidic MeOH (VIb).

The main findings are determined by the relative toxicity of each fraction. These data for mussel embryo toxicity are summarized in **Table 3**, which shows that the lyophilization step removed a small portion of toxicity, but the majority remained with the residue (II). For illustration of the trend, the data in **Table 3** are taken from two to three replicates of a single produced water sample. As lyophilization represents rigorous removal of volatile constituents

(e.g., even water is removed), it follows from this data that relatively nonvolatile constituents accounted for the majority of toxicity.

Table 3. Toxicity of produced water fractions to mussel embryos.

	FRACTION	EC_{50} (%)
I	Whole Produced Water	2.12
II	Lyophilized Residue of **I**	2.86
III	$MeCl_2$ Extract of **II**	~15
IV	MeCN Extract of **II**	»20
IVa	Polar HPLC Fraction of **IV**	»20
IVb	Non-polar HPLC Fraction of **IV**	»20
V	Water-only Soluble Residue of **II**	2.65
VIa	Chelex-Retained **V**, acid eluted	2.87
VIb	Chelex-Retained **V**, acidic MeOH eluted	4.63
VII	Chelex-Flow Thru of **V**	»20

Extensive organic solvent extraction of II, likewise removed only a small part of the toxicity to mussel embryos (**Table 3**); the fractions generated from II, which were III and IV, contained little or no measurable toxicity. However, significant toxicity, similarly found in the previous sea urchin sperm toxicity test, existed in IV (the MeCN extract in **Table 2**). Further fractionation of IV by HPLC was undertaken in the same fashion as described previously for Year 2. The fractions were collected, as shown in **Figure 8**, except this time, the intermediate polar and non-polar fractions were combined for the mussel embryo test, resulting in a single fraction called IVb. Not surprisingly, due to the low toxicity of IV towards mussel embryos, its subfract ions IVa and IVb also exhibited no measurable toxicity (**Table 3**). GC-MS analyses of fractions III and IV (the corresponding GC-FID tracings were previously shown in **Figure 7a** and **7b**) revealed relatively low (<1 ppb) levels of non-aromatic and polynuclear aromatic hydrocarbons, and appeared to contain similar levels of phenolic compounds as well as possible structures related to indenes and indolines. The final MeCN extract of the residue contained no detectable GC peaks (data not shown) and almost no absorbance down to 200 nm (data not shown), indicating that further solvent extraction would not have been fruitful.

The remaining residue (V) proved to be soluble in only water (methanol, ethanol, isopropanol, acetone, tetrahydrofuran, N-methyl pyrrolidinone, dimethylsulfoxide, dimethylformamide, ethyl ether, and hexane were tested) and still contained the majority of the toxicity to mussel embryos (**Table 3**).

At this point, the likelihood of organic compounds contributing to toxicity had diminished, so we turned to atomic spectroscopy to examine selected element composition of the fractions. The analyte list consisted mostly of transition and B-type metal elements, as many of them are aquatic toxicants and some of these have been reported to exist at significant levels in produced water (cf. Boesch and Rabalais, 1987). The results are shown in **Table 4**. For the particular sample shown, V did not appear to contain much of any elements of known toxicity, even though there were higher levels of Ba and Sr. Other produced water samples periodically showed slightly more elevated levels of toxic As or Cu, but these elements did

not appear to correlate with produced water toxicity. Note that some of the As was lost upon lyophilization, which was a consistent trend across many samples. This suggested that volatile forms of As may be a routine contributor to the moderate toxicity exhibited by the volatile fraction.

Table 4. Elemental analysis of a sample produced water and its fractions (Sampled: July 30, 1991).

	Concentration (ppm)						
Element	**I**	**II**	**IV**	**IVa**	**V**	**VIa**	**VII**
Ag	<0.01						
Al	0.07						
As*	0.0067	0.0045	<0.001	<0.001	<0.001		
Ba	13		1.6	<0.002	18	12	0.004
Cd	<0.01						
Co	0.05		<0.01	<0.01	0.04		
Cu	0.0044		<0.005	<0.0005	<0.005	0.012	<0.005
Cr	0.10		<0.075	<0.075	0.08		
Fe	0.46						
Mn	0.45		0.03	0.03	0.05		
Mo	0.02		<0.02	<0.02	0.08		
Ni	<0.02						
Pb	<0.10		<0.10	<0.10	<0.10		
Sn	0.01						
Sr	13		<0.01	<0.01	10	7.5	<0.010
Zn	0.05		0.06	0.06	0.01	0.015	<0.005
B	55						
Ca	189						
K	44						
Mg	35						
Na	5445						
P	20						
S	21						

Blank spaces = not determined, usually due to toxicologically insignificant levels in the parent fraction
*By hydride generation atomic absorption spectrometry
Fractions III, IVb, and VIb are "organic" fractions and were not analyzed for elements.

In order to test whether divalent cations were generally involved in toxicity, V was passed through a bed of hydrated Chelex resin; then the toxicity of the flow-thru (VII) was tested. Chelex resin is a divinylbenzene/styrene resin with imidodiacetate functionality. The resulting dramatic reduction in toxicity is shown at the bottom of **Table 3**. However, it could not be immediately concluded that simple divalent cations were the source of toxicity in V, due to a pale yellow material which was strongly adsorbed in a narrow band at the top of the resin bed. This observation made it clear that at least two more major fractions, the colorless divalent cations and the yellow material, needed to be resolved for toxicity testing.

The divalent cations were first eluted from the Chelex column using 1N HCl, which generated VIa. **Table 4** shows that even the strongly-adsorbed Zn^{2+} ion (Bio-Rad, 1988) was eluted with good recovery, and **Table 3** shows that the majority of toxicity resided in this fraction. For the pale yellow band, it was found that acidic MeOH was necessary for elution. Acidic

MeOH, in fact, eluted the band very rapidly, while the band remained at the top of the column with either aqueous acid or MeOH alone. The resulting eluate, VIb, harbored significant toxicity as well, as seen in **Table 3**. VIb, when titrated to basic condition, evolved an ammoniacal odor, consistent with our initial suspicion that the yellow band was composed of organic material.

To gain a qualitative sense of the relative toxicity among fractions, we converted the data from **Table 3** to relative "toxicity units"; these data are shown graphically in **Figure 14**. Clearly, the majority of toxicity to mussel embryos lay in II, which persisted in V upon further fractionation. The final fractionation found equivalent toxicity in the "divalent cations" sub-fraction VIa. There was also substantial toxicity expressed in VIb. In contrast, our earlier work using the sea urchin sperm cell test indicated that fraction IV (MeCN extract), and more specifically, sub-fraction IVa (HPLC polar fraction), appeared to harbor the largest portion of the toxicity (**Table 2**).

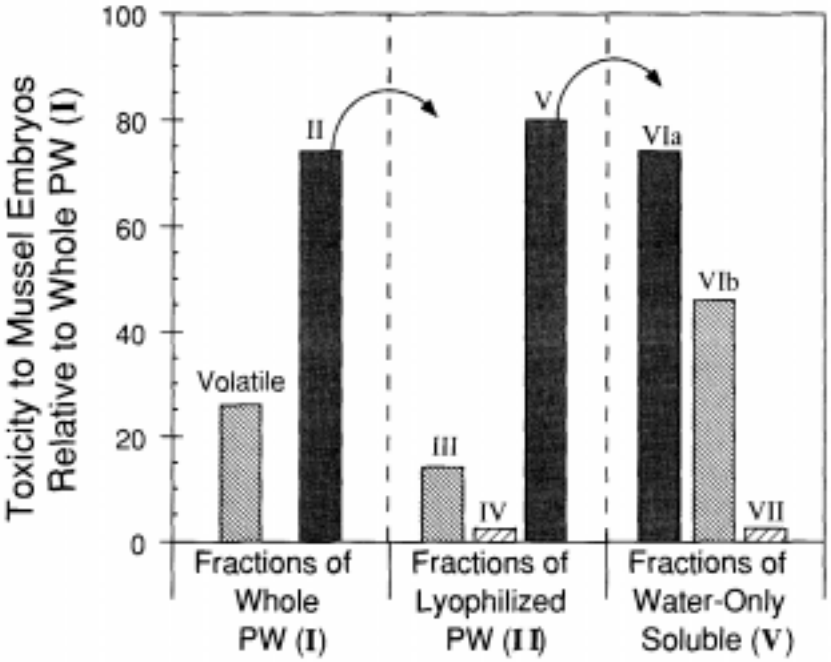

Figure 14. Graphical representation of toxicity data from **Table 3**, converted to relative "toxicity units". Roman numerals refer to fractions described in **Figure 13** and **Table 3**. The full scale of the ordinate represents toxicity of whole produced water. The bars in each box represent toxicity from sub-fractions of the indicated fraction, and the arrows denote the fraction that was used to generate the sub-fractions in the next box. See the text for cautionary notes on interpretation of this type of graph.

It should be noted that **Figure 14**, while useful qualitatively, illustrates some problems inherent in this type of representation in conjunction with toxicity testing. For instance, based on the tallest bars, the conclusion can be drawn (as we did above) that "all" of the toxicity of II, expressed through V, is retained in VIa. On the other hand, there is substantial additional toxicity in VIb. Thus it appears that the total toxicity units has "grown" past the original level of toxicity (VIa + VIb > II or V). This phenomenon was discussed earlier with regard to **Table 1**. There can be several explanations for this. One explanation for this discrepancy is

that the non-linear shapes of the toxicity curves invalidate such simple additivity of toxicity units, emphasizing the limitations of representing toxicity data as single numbers such as EC_{50}. A related explanation is that the toxicity of VIa masked the action of VIb when they were together. On the other hand, it is possible that there was actual antagonism of toxicity between VIa and VIb. Finally, it is conceivable that the fractionation process has altered some previously nontoxic constituent, which is now expressing toxicity.

For the acidic eluate from Chelex, VIa, the fraction seems unlikely to contain constituents other than polyvalent cation elements, considering the properties of the fraction and how it was isolated. Temporarily assuming that a single, polyvalent cationic toxicant was involved, (in order to arrive at the observed level of chronic toxicity in VIa [EC_{50} = 2.87%]), even the more toxic metals such as Cu would have to be present in produced water at the low ppm to high ppb range. However, among the metals, only the alkali earth elements Ba and Sr in our analyte list were routinely present at or above that concentration range (e.g., **Table 4**).

It appears that little is known about aquatic toxicity of Ba and Sr. However we found the Ba can exert acute toxic effects to amphipods (Vincent *et al.*, 1986) and algae (Jernelov *et al.*, 1976) at the levels found in our produced water samples. Sr was reported to have low acute toxicity to an aquatic nematode in comparison with other metals (Williams and Dusenbery, 1990). To the best of our knowledge, there is no previous report of chronic toxicity levels of Ba or Sr to marine life.

The Sr levels found in our produced water samples were slightly elevated from the range normally found in seawater as well as formation water (Collins, 1975). The Ba levels we found were about three orders of magnitude higher than in seawater, and were in the range typical of formation water (Collins, 1975). In addition, marine phytoplankton (Chow and Goldberg, 1960; Martin and Knauer, 1973), corals (Lea *et al.*, 1989), planktonic forams (Lea and Boyle, 1991), and clams (Sadiq *et al.*, 1990) are well-known to accumulate Ba. Considering the produced water fractionation procedure (**Figure 13**), the Ba in VIa must be of a soluble form and not present as barite. Thus, the best candidate in our analyte list for the toxicant in VIa is currently Ba ion.

Of course, it is possible that several of the detected toxicants, each at well below toxic levels, can collectively express the observed toxicity. Relating to that issue is synergism, in this study, the differences in toxicity between fractions was large. This suggests that synergism between fractions was not likely, however there is the possibility of synergism within a fraction such as VIa. Finally, some other polyvalent element not on our analyte list for **Table 4** could account for the toxicity. For example, Hg was occasionally found in produced water at levels that may have causes he observed toxicity (Collins, 1975; Middleditch, 1984). However, our analyte list covered most other metals typically found in produced water (Collins, 1975; Boesch and Rabalais, 1987). Interestingly, some correlation to toxicity in VIa was revealed by UV-visible spectrophotometry, shown in **Figure 15**. The feature to note is the peak centered around 326 nm – the peak area of which appears to correlate qualitatively with toxicity of the fractions.

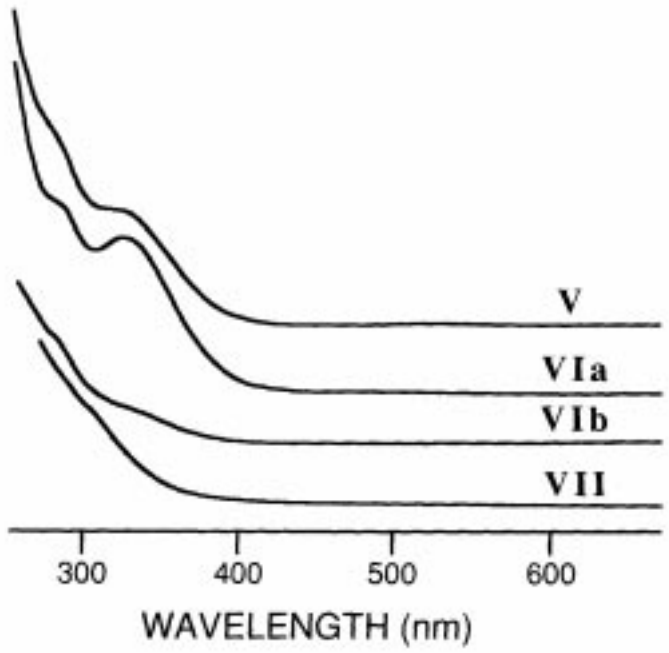

Figure 15. UV-visible spectra of selected fractions. The spectra are offset vertically for clarity, but all spectra are presented on the same ordinate scale to allow direct comparison of absorbances. Note the peaks at ca. 300 and 326 nm in V, which is more pronounced in VIa, probably in part due to removal of the interference from VIb and VII.

For the acidic MeOH eluate from Chelex, VIb, **Figure 15** shows that this fraction does not contain significant chromophores, and as mentioned previously, possibly contains a polar amine compound. Its behavior on the Chelex column is inadequately but best explained if VIb contained an organic quaternary amine. There is very little information in the literature concerning polar organic compounds in produced water, but various phenols appear to be present (Lysyj, 1982). Amino acids are polar nitrogenous compounds found in petroleum associated waters (Degens *et al.*, 1964), but these would be components of IV or VII. One study reports that produced water from North Sea operations consists of largely phenolate, propionate, and butyrate salts, with 20-30 ppm of nitrogen as NH_{4+} salts (Somerville *et al.*, 1987). Another report indicates that NH_3 ("unionized") accounted for the majority of toxicity to *Ceriodaphnia* and fathead minnows in produced water from a simulated *in situ* oil shale retort (Hill, 1986). In our case, barring any unusual ligand chemistry, it is very unlikely that ammonium would be present at high levels after adjustment to pH 8, lyophilization, and extraction by MeCN, followed by an even more unlikely strong retention on Chelex column, strong enough that it resists elution by 1N HCl.

In summary, we have found that the majority of toxicity to mussel embryos are found in the "divalent cation" fraction, as summarized in **Table 3** and **Figure 14**. The toxicity pattern according to the sea urchin sperm toxicity survey, however appears to be very different, due to polar organic substances (IVa). These results, if widely true of produced water, suggests that analytical and modeling approaches traditionally used for produced water research – that is, those that key on hydrocarbons and metals – may not be relevant to standard chronic toxicity indicators such as the mussel embryo system. This research has already prompted other SCEI researchers to examine both field and mechanism aspects of Ba in produced water discharges (Cherr and Fan, 1993; Osenberg *et al.*, 1992).

REFERENCES

Agemian, H. and R. Thomson. 1980. Simple semi-automated atomic absorption spectrometric method for the determination of arsenic and selenium in fish tissue. *Analyst* **105**:902-907.

Armstrong, H.W., K. Fucik, J. Anderson, and J.M. Neff. 1979. Effects of oil field brine effluent on sediments and benthic organisms in Trinity Bay, Texas. *Mar. Environ. Res.* **2**:55-69.

Bio-Rad Laboratories. 1988. Chelex 100 and Chelex 20 Chelating Ion Exchange Resin Instruction Manual, Bio-Rad Laboratories, Richmond, CA.

Boehm, P.D. 1987. Transport and transformation processes regarding hydrocarbon and metal pollutants in offshore sedimentary environments. In: *Long-term Environmental Effects of Offshore Oil and Gas Development*, D.F. Boesch & N.N. Rabalais, eds., Elsevier Applied Science, New York, pp. 233-286.

Boesch, D.F. and N.N. Rabalais, eds. 1987. *Long-term Environmental Effects of Offshore Oil and Gas Development*, Elsevier Applied Science, New York, 708pp.

Borra, C., F. Andreolini, and M. Novotny. 1989. Use of capillary supercritical fluid chromatography and microcolumn liquid chromatography for the determination of nonvolatile organics in aqueous environmental samples. *Anal. Chem.* **61**:1208-1210.

Brumbaugh, W.G. and M.J. Walther. 1989. Determination of arsenic and selenium in whole fish by continuous-flow hydride generation atomic absorption spectrophotometry. *J. Assoc. Off. Anal. Chem.* **72**:484-486.

Buchanan, A.S. and P. Hannaker. 1984. Inductively coupled plasma spectrometric determination of minor elements in concentrated brines following precipitation. *Anal. Chem.* **56**:1379-1382.

Chow, T.J. and E.D. Goldberg. 1960. On the marine geochemistry of barium. *Geochim. Cosmochim. Acta* **20**:192-198.

Cherr, G.N., J.M Shenker, C. Lundmark, and K.O. Turner. 1987. Toxic effects of selected bleached kraft mill effluent constituents on the sea urchin sperm cell. *Environmental Toxicology and Chemistry* **6**:561-569.

Cherr, G.N., J. Shoffner-McGee, and J.M. Shenker. 1990. Methods for assessing fertilization and embryonic/larval development in toxicity tests using the California mussel *Mytilus californianus*. *Environmental Toxicology and Chemistry* **9**:1137-1145.

Cherr, G.N., R.M. Higashi, and T.W-M. Fan. 1991. Assessment of chronic toxicity of petroleum and produced water components to marine organisms. In: Southern California Educational Initiative Program Overview of Year 2, Coastal Research Center, Marine Sciences Institute, University of California - Santa Barbara.

Cherr, G.N., R.M. Higashi, J.M. Shenker, and T.W-M. Fan. 1993. Final Report: *Assessment of chronic toxicity of petroleum and produced water components to marine organisms.* Minerals Management Service. MMS-94-0035.

Collins, A.G. 1975. *Geochemistry of Oilfield Waters*. Elsevier Scientific Publishing, Amsterdam, 496 pp.

Degens, E.T., J.M. Hunt, J.H. Reuter, and W.E. Reed. 1964. Data on the distribution of amino acids and oxygen isotopes in petroleum brine waters of various geologic ages. *Sedimentology* **3**:199-225.

Fan, T.W.-M., R.M. Higashi, G.N. Cherr, and M.C. Pillai. 1992. Use of noninvasive NMR spectroscopy and imaging for assessing produced water effects on mussel reproduction. In: *Produced Water: Technological/ Environmental Issues and Solutions*, J.P. Ray and F.R. Engelhardt, eds., Plenum Publishing, New York. pp. 403-414.

Higashi, R.M., G.N. Cherr, J.M. Shenker, J.M. Macdonald, and D.G. Crosby 1992a. A polar high molecular mass constituent of bleached kraft mill effluent is toxic to marine organisms. *Environmental Science and Technology* **26**:2413-2420.

Higashi, R.M., G.N. Cherr, C.A. Bergens, T.W-M. Fan, and D.G. Crosby 1992b. An approach to toxicant isolation from a produced water source in the Santa Barbara Channel. In: *Produced Water: Technological/ Environmental Issues and Solutions*, J.P. Ray and F.R. Engelhardt, eds., Plenum Publishing, New York. pp. 223-233

Hill, S. L. 1986. *Toxicological characterization of water produced during simulated in situ processing of Green River Formation oil shale*. U.S. Dept. of Energy Report # DOE/FE/60177-2390, 29 pp.

Jernelov, A., C.C. Landahl,, T. Larsson, A. L. Martin, O. Westling. 1976. Barium ecology in aquatic systems. Inst. Vatten- Luftvardsforsk., [Publ.] B, B 277, 25 pp.

Lea, D.W. and E.A. Boyle. 1991. Barium in planktonic foraminifera. *Geochimica et Cosmochimica Acta* **55**:3321-3331.

Lea, D.W., G.T. Shen, and E.A. Boyle. 1989. Corraline barium records temporal variability in equatorial Pacific upwelling. *Nature* **340**:373-376.

Lysyj, I. 1982. *Chemical composition of produced water at some offshore oil platforms.* U.S.E.P.A. Report # EPA-600/2-82-034.

Martin, J.H. and G.A. Knauer. 1973. The elemental composition of plankton. *Geochimica et Cosmochimica Acta* **37**:1639-1653.

Middleditch, B.S. 1984. *Ecological effects of produced water discharges from offshore oil and gas production platforms*. Final Report on API Project No. 248. American Petroleum Institute, Washington, D.C., 160 pp.

Mount, D.I. and L. Anderson-Carnahan. 1988. Methods for aquatic toxicity identification evaluations: Phase I toxicity characterization procedures., US EPA-600/3-88/034. pp.1-30.

National Research Council. 1985. *Oil in the Sea: Inputs, Fates, and Effects*. National Academy Press, Washington, D.C., 601pp.

Neff, J.M., N.N. Rabalais, and D.F. Boesch. 1987. Offshore oil and gas development activities potentially causing long-term environmental effects. In: *Long-term Environmental Effects of Offshore Oil and Gas Development*. D.F. Boesch & N.N. Rabalais, eds., Elsevier Applied Science, New York, pp.149-173.

Neff, J.M. 1987. Biological effects of drilling fluids, drill cuttings and produced waters. In: *Long-term Environmental Effects of Offshore Oil and Gas Development*. D.F. Boesch & N.N. Rabalais, eds., Elsevier Applied Science, New York, pp. 469-538.

Osenberg, C.W., R.J. Schmitt, S.J. Holbrook. 1992. Spatial scale of ecological effects associated with an open coast discharge of produced water. In: *Produced Water: Technological/ Environmental Issues and Solutions*, J.P. Ray and F.R. Engelhardt, eds., Plenum Publishing, New York, pp. 387-402.

Sadiq, M., T.H. Zaidi, and H. Al-Mohana. 1990. Barium bioaccumulation in clams collected from different salinity regimes along the Saudi coast of the Arabian Gulf. *Bull. Environ. Contam. Toxicol.* **45**:329-335.

SCEI Annual Report 1992 1992. Southern California Educational Initiative Annual Report of Year 3, Coastal Research Center, Marine Sciences Institute, University of California - Santa Barbara.

SETAC Symposium. 1991. on the West Coast Sea Urchin Sperm Cell Assay, G. Chapman program chair. , 12th Annual Meeting of the Society of Environmental Toxicology and Chemistry, Seattle, WA November, 1991. .

Somerville, H. J., D. Bennett, J.N. Davenport, M.S. Holt, A. Lynes, A. Mahieu, B. McCourt, J.G. Parker, and R.R. Stephenson. 1987. Environmental effect of produced water from North Sea oil operations. *Mar. Pollut. Bull.* **18**:549-558.

Stumm, W. and J.J. Morgan. 1981. *Aquatic Chemistry*, John Wiley & Sons, New York, p.323-417.

Tibbetts, P.J.C., N.J. Haskins, J. Holland, and R. Large 1985. The use of fast atom bombardment FAB: mass spectrometry for monitoring petrochemicals. *Intern. J. Environ. Anal. Chem.* **21**:199-212.

Vincent, M., J. Debord, and B. Penicaut. 1986. Comparative toxicity of metal chlorides and of a synthetic organic molluscicide, N-trityl-morpholine, upon two aquatic amphipod crustaceans, *Gammarus pulex* and *Echinogammarus berilloni*. *Ann. Rech. Vet.* **174**:441-446.

Williams, P.L. and D.B. Dusenbery. 1990. Aquatic toxicity testing using the nematode, *Caenorhabditis elegans*. *Environ. Toxicol. Chem.* **9**:1285-1290.

The Department of the Interior Mission

As the Nation's principal conservation agency, the Department of the Interior has responsibility for most of our nationally owned public lands and natural resources. This includes fostering sound use of our land and water resources; protecting our fish, wildlife, and biological diversity; preserving the environmental and cultural values of our national parks and historical places; and providing for the enjoyment of life through outdoor recreation. The Department assesses our energy and mineral resources and works to ensure that their development is in the best interests of all our people by encouraging stewardship and citizen participation in their care. The Department also has a major responsibility for American Indian reservation communities and for people who live in island territories under U.S. administration.

The Minerals Management Service Mission

As a bureau of the Department of the Interior, the Minerals Management Service's (MMS) primary responsibilities are to manage the mineral resources located on the Nation's Outer Continental Shelf (OCS), collect revenue from the Federal OCS and onshore Federal and Indian lands, and distribute those revenues.

Moreover, in working to meet its responsibilities, the **Offshore Minerals Management Program** administers the OCS competitive leasing program and oversees the safe and environmentally sound exploration and production of our Nation's offshore natural gas, oil and other mineral resources. The MMS **Royalty Management Program** meets its responsibilities by ensuring the efficient, timely and accurate collection and disbursement of revenue from mineral leasing and production due to Indian tribes and allottees, States and the U.S. Treasury.

The MMS strives to fulfill its responsibilities through the general guiding principles of: (1) being responsive to the public's concerns and interests by maintaining a dialogue with all potentially affected parties and (2) carrying out its programs with an emphasis on working to enhance the quality of life for all Americans by lending MMS assistance and expertise to economic development and environmental protection.