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Effects of Biologically Degraded Oil on Marine Invertebrate and Vertebrate Embryos and Larvae

Final Technical Summary

Final Study Report

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

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Table of Contents

FINAL TECHNICAL SUMMARY

STUDY TITLE: Effects of Biologically Degraded Oil on Marine Invertebrate and Vertebrate Embryos and Larvae

REPORT TITLE: Effects of Biologically Degraded Oil on Marine Invertebrate and Vertebrate Embryos and Larvae

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KEYWORDS: multixenobiotic resistance protein transporter, biodegraded crude oil, invertebrate embryo, fluorescence excitation

BACKGROUND: A by-product of microbial degradation of crude oil is neutral water soluble hydrocarbons (PAHs). Due to the hydrophobic nature of many PAHs and other oil constituents, they are able to freely cross the plasma membranes of cells and perturb cell function. This process can have profound implications to biota that are repeatedly subjected to PAH exposure, such as in the Santa Barbara channel near sites of natural oil seeps and non-catastrophic releases associated with oil production or transport. To defend against exposure to such compounds, some marine organisms possess mechanisms that bind to PAH compounds and transport them out of cells. This study investigates one such mechanism present in marine invertebrates, the multixenobiotic resistance transport protein (MXR), which is analogous to the mammalian multidrug resistances transporter MDR. Organisms that express less MXR transporter will likely be more susceptible to oil-derived hydrocarbons.

OBJECTIVES:

The objectives of this study were to: **1)** determine the susceptibility of embryonic and larval stages of selected marine invertebrates and vertebrates to the water-soluble fraction of biodegraded crude oil (BWSF) from the Santa Barbara Channel; **2)** determine the basis for species sensitivity to BWSF; **3)** identify biomarkers in these animals for predicting both exposure and effect of Santa Barbara Channel BWSF.

DESCRIPTION:

(1) Crude oil was obtained from a Santa Barbara channel oil platform and artificially weathered and biodegraded in the lab using microbes enriched for their oil degrading ability;

(2) Concentration of biodegraded water soluble fraction (BWSF) of crude oil was quantified using fluorescence excitation;

(3) Multixenobiotic resistance protein (MXR) levels were quantified in invertebrate tissues; and

(4) Embryos were exposed to increasing concentrations of BWSF.

SIGNIFICANT CONCLUSIONS:

The tolerance to degraded oil hydrocarbons in select phyla can be attributed to the presence of a multi-xenobiotic membrane transporter (MXR) which is homologous to the mammalian multi-drug resistance transporter (MDR1) common in cancer cells treated with chemotherapeutics. The degraded Santa Barbara Channel oil is a substrate for the MXR transporter in echiuroid and mollusk embryos and they are able to effect an efflux of these compounds. This was demonstrated using dye efflux assays based on mammalian cell technology, as well as the use of mammalian cell lines which over and under express the MDR/MXR transporter and show differential sensitivity to degraded crude oil.

STUDY RESULTS:

This study has shown that organisms which do not express the MXR transporter are at high risk when exposed acutely or chronically to degraded crude oil. Sites which contain high proportions of species which do not express the MXR transporter (e.g. selected echinoderms, some fish) should be considered “high risk” sites with respect to oil production activities.

STUDY PRODUCTS:

This study will result in two peer-reviewed publications that are presently in the final stages of preparation for submission. The first will include the methods of preparation of biologically degraded water soluble crude oil (BWSF), the responses of early life stages of different phyla to BWSF exposure, and the linking of the presence of the multi-xenobiotic resistance transporter (MXR) in species which are more resistant to BWSF constituents. The second paper will characterize the MXR transporter in the Echiurid worm *Urechis caupo*, and will show substrate specificity and how BWSF can serve as a substrate for this membrane transport protein. These publications will provide direct evidence that the MXR transporter in organisms in the Santa Barbara Channel provides a direct mechanism for adaptation to hydrocarbon-enriched environments. This is turn indicates that organisms lacking transport activity will be more susceptible to oil-derived PAH exposure due to oil exploration activities.

FINAL STUDY REPORT

Background

The removal of volatile compounds through weathering of crude oil results in the release of low boiling point aromatic and saturated hydrocarbons. It has been thought that those components hold the greatest toxicity to marine life (Capuzzo, 1987; Galt *et al*., 1991; Payne *et al*., 1991; Venkateswaran *et al*., 1995). Although biodegradation of crude oil can be considered a component of the weathering process, the process continues well after initial weathering and the elimination of volatile compounds has occurred. Known results of this continued microbial degradation include a measurable decrease in sediment crude oil along with a measurable organic enrichment in those sediments (Spies, 1987).

As petroleum-degrading microbes are well suited to metabolizing hydrocarbons of weathered oil, enhancing such microbial biodegradation has successfully been used as an effective means of cleansing oiled sites (Mueller *et al*., 1992; Bragg *et al*., 1994). Fertilizers were employed after the *Exxon Valdez* oil spill in 1989 to augment the growth of microbial beach populations. Not only was there an enhanced reduction in deposited oil from the fertilized plots, there was also an enhanced loss of extractable organic matter from the remaining weathered oil (Claxton *et al.,* 1991).

Studies (including our laboratory) have demonstrated that a by-product(s) of microbial degradation of artificially weathered Alaska North Slope crude oil is a ten fold increase in neutral water soluble hydrocarbons that exhibits significantly high toxicity to developing atherinid and clupeoid fish embryos (Middaugh et al., 1996, 1998). Biodegradation of crude oil occurs in regions of natural seepage (e.g. Coal Oil Point) as well as in regions of oil production and transport where elevated populations of crude oil-degrading microbes are purported to exist (Spies, 1987). It can be assumed that the process of oil biodegradation at these sites is an ongoing process and that the products of that biodegradation are chronically present. The fact that the biodegradation of crude oil is now known to produce water soluble fractions that may be toxic to marine life, means there are profound implications to the biota in the Santa Barbara Channel near sites of natural oil seeps and non-catastrophic release (associated with oil production or transport). In particular, we have been interested in how select species of marine organisms tolerate chronic exposure to microbially degraded oil constituents.

Due to the hydrophobic nature of many PAHs and other moderately hydrophobic oil constituents, they are able to freely cross the plasma membranes of cells and perturb cell function. To defend against exposure to such compounds, some marine organisms possess mechanisms to bind these compounds at the plasma membrane and transport them out of cells (Epel, 1998). One such mechanism involves the binding of these compounds at the plasma membrane and subsequent transportation out of cells due to the presence of a multixenobiotic resistance transport protein (MXR), a member of the ABC (ATP Binding Cassette) transporter protein superfamily homologous to the mammalian multidrug resistance transporter (aka. MDR, p-glycoprotein). Organisms that express less MXR transporter will likely be more susceptible to oil-derived hydrocarbons. MXR transporters in marine organisms function as a "first line of defense" against exposure to organic compounds in the marine environment (Epel, 1998) Organically rich sediments, such as mudflats of estuaries and bays, contain an array of natural and anthropogenic cytotoxic compounds. Although neither their chemical

identities nor biological effects are well known, low to moderately hydrophobic compounds present in these environments are often toxic. Furthermore, natural products from degradation or metabolism of marine organisms (e.g. dinoflagellates and algal toxins) may also be toxic. Due to the hydrophobic nature of some of these toxicants, they are able to freely cross the plasma membranes of cells and perturb cell function.

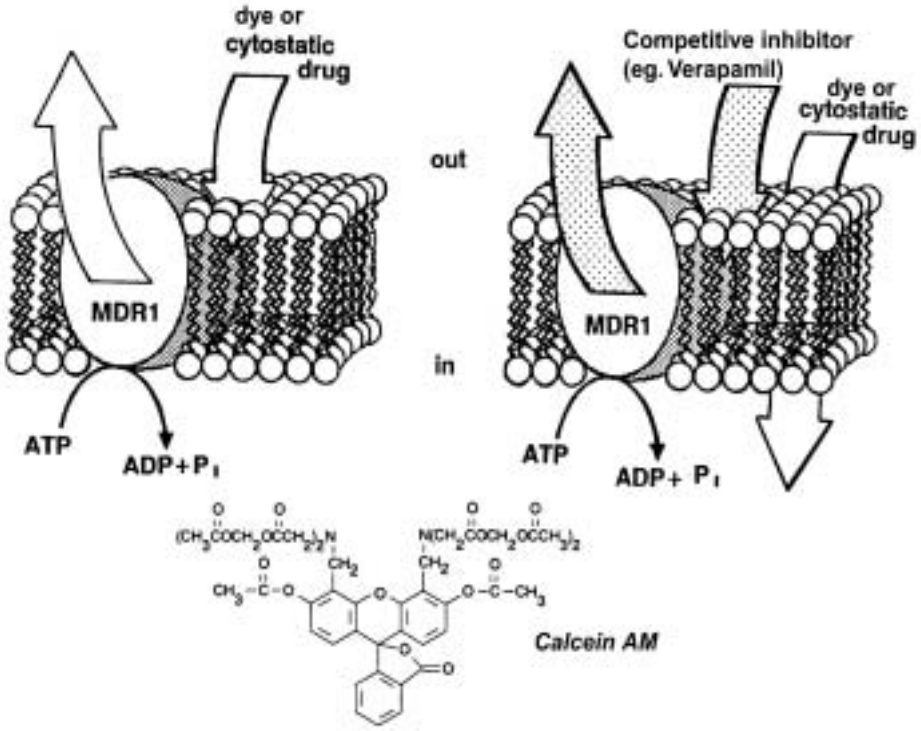


Figure 1: How MXR or the MDR transporter keeps hydrophobic contaminants from entering the cell. Dye, cytostatic drug, or PAH-like compounds enter the membrane and are bound by the transporter which then transports the compound out of the cell in an ATP-dependent manner. If a competitive inhibitor (in higher concentration) is present (e.g. verapamil), the dye or toxicant enters the cell as the transporter is "busy" transporting verapamil. In the case of Calcein –AM dye used for transporter activity analyses, esterases cleave the methoxy ester and thus the Calcein which enters the cytoplasm cannot be transported out of the cell.

Mammalian MDR proteins have been well studied and are involved in resistance of humans to chemotherapeutic drugs (reviewed by Ambudkar *et al*., 1999). The P-glycoprotein in mammalian systems, as well as the MXR transporter in invertebrate systems, is up regulated upon exposure to chemotherapeutic drugs and possibly xenobiotics. In invertebrates, a MXR-like transporter has been described for sponges, bivalves, arthropods and echiuroid worms (Epel, 1998). Limited studies suggest the MXR transporter may be absent in echinoderms (embryo and larval stages) (Holland-Toomey and Epel, 1993). In clams, individuals from polluted sites show increased transporter activity (Kurelec *et al*., 1996). In oysters and mussels, increased expression of MXR transporter mRNA was observed in animals from polluted sites (Minier *et al*., 1993), although it was not known if actual transporter activity was increased. MXR protein levels can be induced in mussel hemocytes in the laboratory by exposure to the alkaloid vincristine (Minier and Moore, 1996). An indicator of expression of MXR transport glycoproteins can often be the appearance of the non-glycosylated nascent MXR polypeptide chain which appears on Western blots as a lower molecular weight polypeptide (Chin *et al*., 1990). We were able to study MXR transport activity using two different fluorescent dyes as well as a commercially available antibody directed against the highly conserved ATPase region on the mammalian MDR1 protein.

Objectives

The objectives of this study were to: **1)** determine the susceptibility of embryonic and larval stages of selected marine invertebrates and vertebrates to the water-soluble fraction of biodegraded crude oil (BWSF) from the Santa Barbara Channel; **2)** determine the basis for species sensitivity to BWSF; **3)** identify biomarkers in these animals for predicting both exposure and effect of Santa Barbara Channel BWSF.

Description

Degradation of crude oil

Crude oil that was obtained from a Santa Barbara Channel oil platform, shipped to BML at 4ºC, and artificially weathered and biodegraded using microbes enriched for their oil degrading ability. The crude oil was artificially weathered by autoclaving at 374ºC, distilled at 1 atm, and stored in aliquots at -70ºC (ISO/DIS 8708, 1994). The enriched microbe population was obtained from a seep sediment starter that was collected from Coal Oil Point in collaboration with UCSB researchers (Keith Seydel). Agar plates containing Bushnell-Haas nutrients, sea water, and 0.2% weathered oil were inoculated with the seep starter and allowed to incubate for 3 days. Further purification of oil degrading microbe colonies was accomplished by serial inoculations (2x) of growing colonies to new culture plates. Selective enrichment steps (using crude oil agar plates) were successful since they resulted in dramatic increases in oil degrading capabilities and yielded a more uniform (primarily gram negative) bacterial population.

Biodegraded oil was obtained utilizing the artificially weathered oil and our enriched microbe population. Solutions of seawater containing 0.2% weathered crude oil (w/v) were incubated for 14 days at 15ºC with or without enriched cultures of microbes from Coal Oil Point. While complete emulsification of the crude oil/microbe mixture was achieved in ~10 days, the weathered crude oil/sea water mixture without microbes showed virtually no emulsification. Following incubation, both microbially degraded and non-degraded oil-water mixtures were serially filtered through 0.7 µm and 0.45 µm filters to remove particulate oil materials and microbes, aliquoted, and stored at -70ºC. Aliquots were lyophilized for biological experiments as well as additional chemical fractionations. All fractions were filtered to 0.22 µm at the time experiments were initiated. Flasks containing only artificial sea water, Bushnell-Haas nutrients, and weathered oil were aerated and used as non-biodegraded controls. Aeration was utilized to insure that any volatile by-products of degradation were removed from the water. The resultant seawater from the flasks containing microbes, the BWSF, possessed a distinctive yellow coloration and a much reduced pH (pH =5-5.7). The nondegraded flasks (minus the microbes) contained what was denoted as the nondegraded water soluble fraction (NWSF), a clear liquid with a pH of approximately 7.8. Based on the appearance of the different flasks, the crude oil-microbe treatment was almost completely emulsified as compared to the treatment without microbes.

Quantification of BWSF

The concentration of BWSF in seawater following degradation was quantified using fluorescence excitation at 290 nm and emission at 353 nm. A known concentration (W/V) of crude oil was dissolved in methylene chloride and diluted into dimethyl sulfoxide (DMSO) at increasing dilution. A standard curve was established (R^2=0.96) for dilutions of the crude oil and established as the standard in which BWSF concentrations were extrapolated from.

Multixenobiotic Resistance Protein (MXR) Measurements

MXR protein levels were quantified in invertebrate tissues on Western blots following SDS-PAGE using a commercial antibody raised against mammalian MDR-1 (C219, Centocor). MXR transporter efflux activity of embryos, larvae, and cells, before and after exposure to BWSF, were performed using the rhodamine dye exclusion assay (Holland-Toomey and Epel, 1993), or calcein-AM dye accumulation (Hollo et al, 1994). For rhodamine dye efflux, Rhodamine B (1 µM final concentration) was added and embryos incubated in the dark at 14^{o}C for 30 min in the presence or absence of the dye efflux inhibitor verapamil (20 µM) or BWSF. Fluorescence measurements in response to various treatments were made using a temperature controlled, stirred cell Photon Technology International (PTI) spectrofluorometer (546 nm excitation and 590 nm emission). PTI quantitative fluorescence software was used to determine the fluorescence of up to 500 embryos/larvae at one time. This software enabled determination of the emission intensity of samples over time as a direct measure of the inhibition of MXR activity. In other experiments, the methoxy ester form of Calcein, Calcein-AM, was used. In this case, Calcein-AM crosses cell membranes and is a substrate for MDR1-like transporters (Hollo et al 1994). The methoxy ester form of Calcein is not fluorescent. Once inside the cell, endogenous esterases cleave the methox ester to yield fluorescent Calein salt that cannot cross the cell membrane and is thus trapped inside of cells. In this way, dye accumulation in cells and embryos can be determined without concern over the dye coming out of the cells (as can occur with Rhodamine B) during the experiment.

Quantification of dye efflux with Calcein-AM treated embryos/larvae (0.5 µM) was conducted using the cuvette-based fluorometric method as described above, or using video imaging on an Olympus BX50-WI fixed stage upright microscope using xenon-illumination and a cooled stage. Images of 3-5 individual larvae/field (5-10 fields/treatment) were collected using Universal Imaging MetaMorph software. Verapamil (3 µM) and the highly specific MDR1 inhibitor Reversin (Sharom et al, 1999) were used as positive controls along with BWSF and control seawater.

BWSF Exposures

All experiments were conducted in triplicate and included a seawater control. Embryo exposures were conducted in 20 ml volumes in acid-washed borosilicate glass vials containing 50-100 embryos/ml. Eggs were fertilized, washed, and introduced into increasing concentrations of BWSF or the NWSF. Embryos were cultured to the hatching stage (7-10 days at 12ºC) for Pacific herring (*Clupea pallasi*), to trochophore stage for *Urechis caupo*, pluteus stage for *Lytechinus anamesus*, and veliger stage for *Crassostrea gigas*. Normal morphology and survival were scored for all treatments, with stage-specific morphology based on the seawater controls for each experiment. Reference exposures to NWSF were also conducted in sea urchin.

Study Results

Particularly striking are the dramatic differences in susceptibility observed in larvae from two species native to the California coast, *Lytechinus anamesus* (urchin) and *Urechis caupo* (innkeeper worm). BWSF contains a >10 fold increase in PAH-like compounds based on fluorescence emission (Fig. 2). Our research shows that BWSF exerts dramatic effects even at low concentrations on sea urchin embryos. Continuously exposed urchin embryos exhibit dose-dependent delay in development in concentrations as low as 0.2 ppm BWSF

(corresponding to a 1:400000 dilution of crude oil) in filtered seawater (Fig. 3). NWSF, the non-degraded water soluble fraction of weathered oil, was less toxic than BWSF in sea urchins (data not shown).

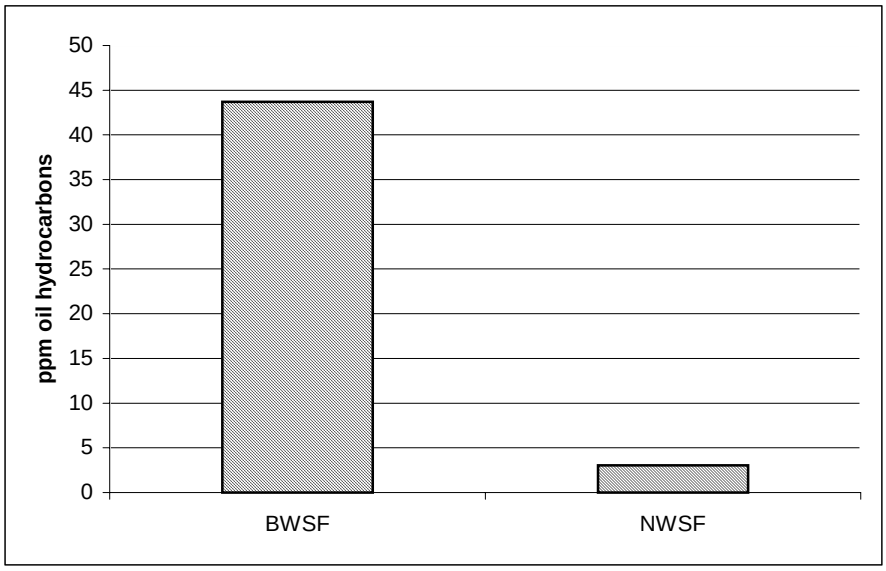
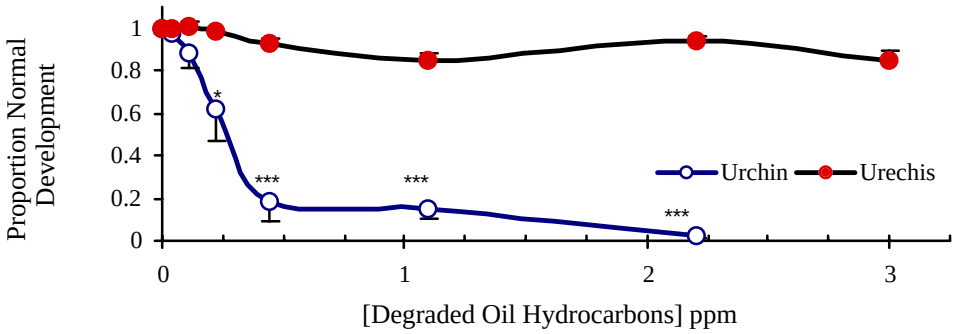


Fig. 2 Concentration of soluble crude oil hydrocarbons in BWSF vs. NWSF. This was based on a crude oil standard curve.



** = P<0.05, ***=P<0.005*

Figure 3. *Lytechinus anamesus* and *Urechis caupo* were exposed to different concentrations of BWSF in seawater post-fertilization and followed through to the pluteus and trochophores stages. BWSF has almost no effect on *Urechis* as compared to *Lytechinus.*

The effects were evident in BWSF exposed embryos not only at the pluteus stage (Fig. 3) but were also manifested earlier in a delay to hatching. In contrast, concentrations of BWSF up to 3 ppm had no effect on *Urechis* trochophore development, even at 96 hrs. of exposure (Fig. 4).

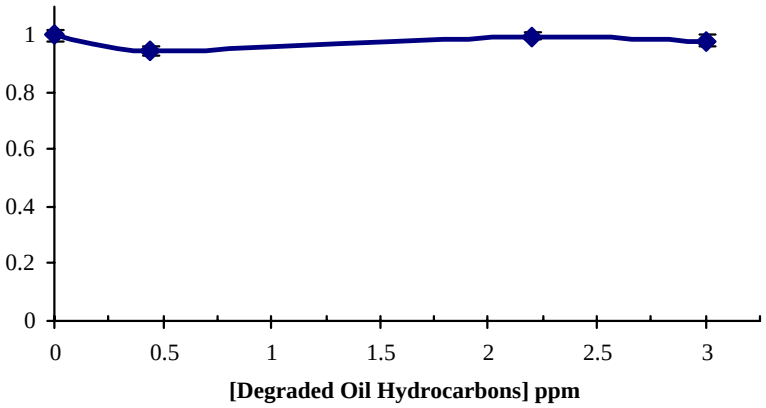


Figure 4. Even when *Urechis* trochophores were incubated with BWSF for up to 96 hrs., the longest period for sea urchin embryo/larval exposures), BWSF (up to 3 ppm) still has no effect on morphology or swimming.

Previous research on stress responses of urchins has suggested that urchins do not express the full complement of stress responsive genes until after gastrulation. To investigate the hypothesis that BWSF exerts all of its effects on early life stages (prior to hatching) we assessed normal development in urchin larvae exposed to BWSF only at or after hatching (Fig. 6). Similar effects were observed to those seen in continuously exposed embryos. We have also investigated the possibility that the difference in susceptibility to BWSF observed between the two species is caused by the different times taken to develop to larval stage at similar temperatures. We exposed *Urechis* to BWSF for 96 hours (corresponding to the time taken for normal urchins to reach the pluteus stage) and found no significant effect of BWSF (Fig. 4).

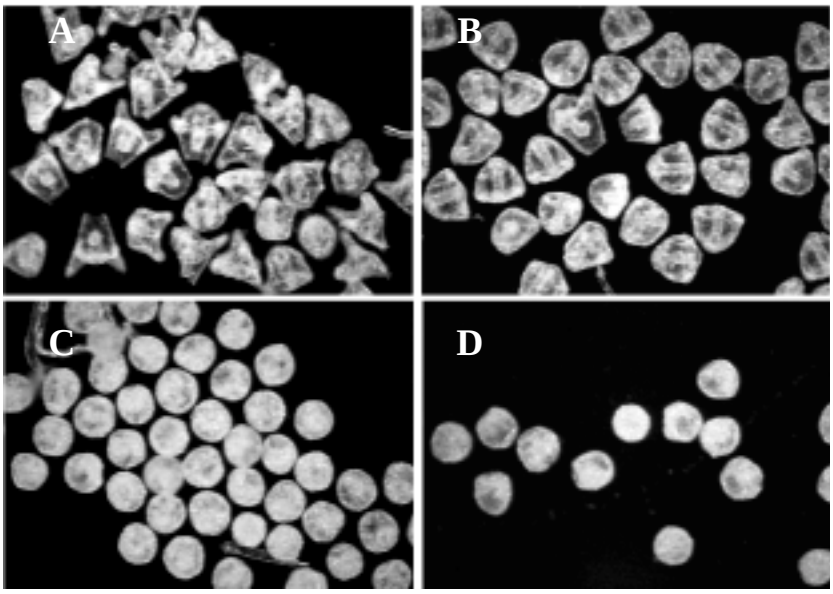


Figure 5. Effects of BWSF exposure on development of plutei at 96 hrs post-fertilization. A. Control embryos incubated in seawater. B. Embryos in 0.44 ppm BWSF in seawater. C. 1.1 ppm BWSF in seawater. D. 2.2 ppm BWSF in seawater. Concentrations of BWSF were based on a fluorescence emission of a crude oil standard curve (see Methodology).

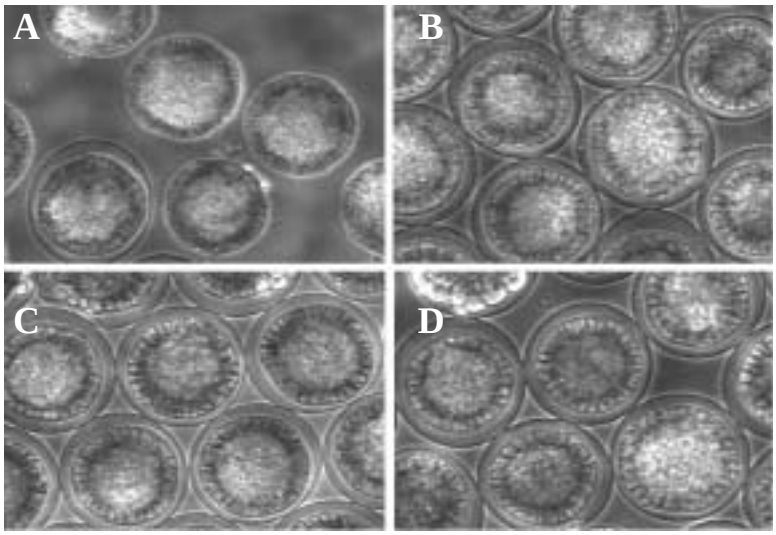


Figure 6. BWSF exposure results in delayed hatching of blastula stage sea urchin embryos ~18 hrs post-fertilization. A. Control embryos incubated in seawater. B. Embryos in 0.44 ppm BWSF in seawater. C. 1.1 ppm BWSF in seawater. D. 2.2 ppm BWSF in seawater. Concentrations of BWSF were based on a fluorescence emission of a crude oil standard curve (see Methodology).

Overall, clear phylogenetic differences were found with respect to embryo responses to BWSF. Pacific herring and sea urchins were approximately ten times more sensitive to BWSF than *Urechis* and oyster embryos (Figs. 7 and 8).

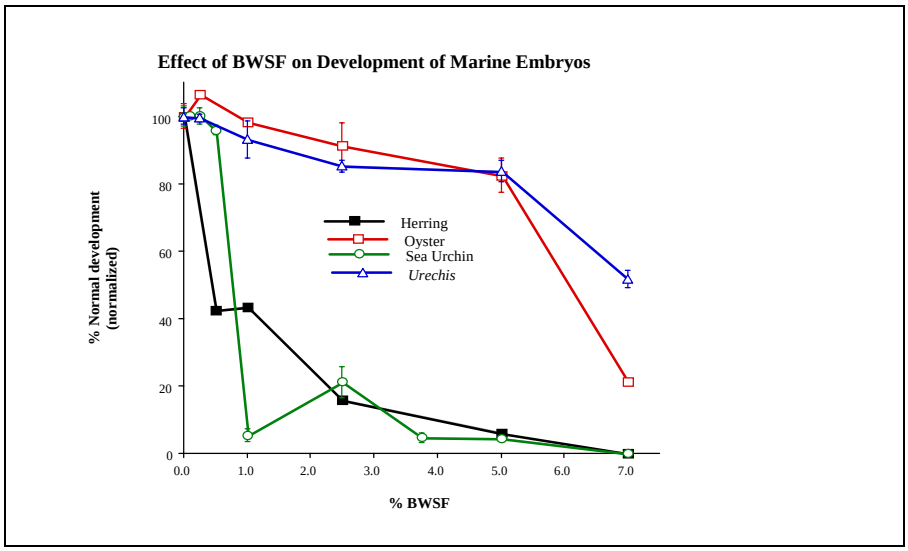


Figure 7: Responses of herring, sea urchin, oyster, and *Urechis* embryos to BWSF fraction. Note that *Urechis* and oysters are an order of magnitude less sensitive than herring and sea urchin embryos. Concentrations here are expressed as percentage BWSF (1% = 0.44 ppm).

Figure 8: Sea Urchin (***L. anamesus*), *Urechis*,** and oyster (*C. gigas*) larvae which were exposed to 2.2 ppm BWSF or control medium immediately after fertilization and cultured to the larval stage. Note the severe abnormalities in the sea urchin pluteus larva, while the *Urechis* trocophore and the oyster veliger larvae are relatively normal at this concentration of BWSF.

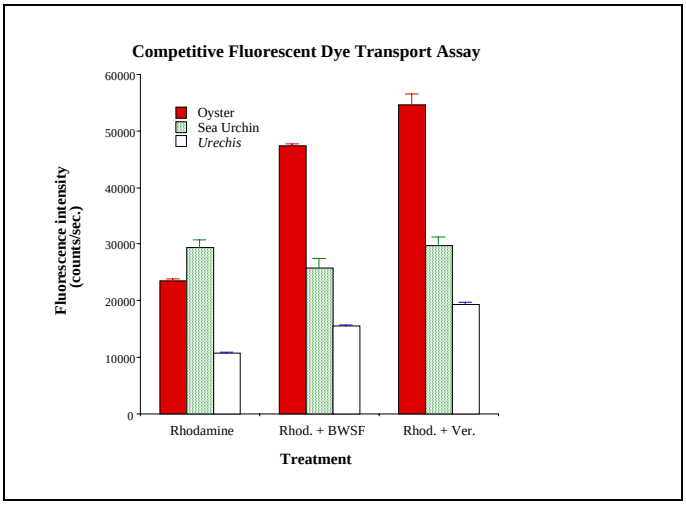


Figure 9: Quantitative fluorescence measurements of rhodamine dye in seawater ("Rhodamine"), seawater + 0.44 ppm BWSF ("Rhod + BWSF"), and a positive control ("Rhod + Ver."), verapamil (20 µM). Note that for *Urechis* and oyster embryos, there is a significant increase in fluorescence in the presence of BWSF and verapamil. There is no change in sea urchins, indicating an absence of MXR transporter.

Our results indicate that BWSF acts as a competitive inhibitor of ATPase mediated dye efflux. Quantitative rhodamine dye efflux experiments show that BWSF exposure causes an increase in dye accumulation in *Urechis* and oyster embryos, but not in sea urchin embryos (Fig. 9). Our data suggest that this efflux is mediated by a homologue of the mammalian multi-drug resistance protein (MXR). Figure 10 shows the expression of multi-xenobiotic resistance protein (MXR) throughout development in both sea urchins and *Urechis*. Consistent with observations in the purple urchin (Holland-Toomey and Epel, 1993), *Lytechinus* do not express MXR at any stage of development. In contrast, *Urechis* express relatively uniform amounts of this protein throughout development, regardless of BWSF exposure. Thus the expression of MXR is consistent with the observed absence of stage specificity in susceptibility of urchins or *Urechis*. Oyster embryos also possess the MXR transporter based on Western blot analysis (data not shown).

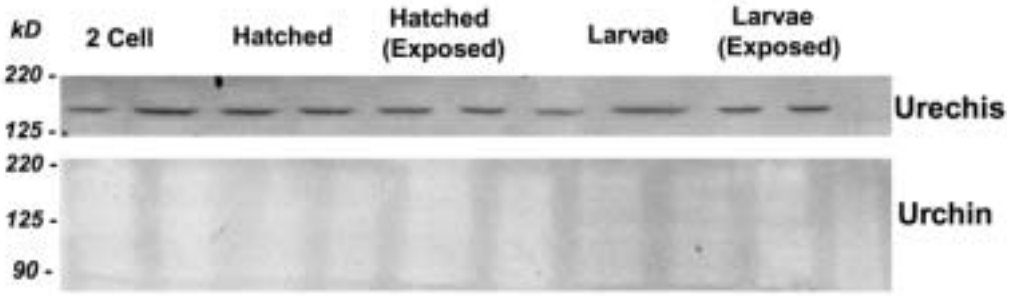


Fig. 10. Western blot analyses of *Urechis* and sea urchin embryos using a commercial monoclonal (C219) antibody raised against mammalian MDR1. All stages of *Urechis* embryos contain the transporter, but it is absent from sea urchin embryos. All lanes contain equal amounts of protein (30 µg) and blots were developed using a chemiluminescent method which is the most sensitive method of detection available.

To confirm that the dye efflux results observed in sea urchins and *Urechis* were not due to species-specific differences in membrane permeability to dye we developed a dye efflux assay using calcein-AM (acetoxymethyl ester). The AM form of calcein is membrane permeable, while the free form is not. Moreover only the free form is fluorescent. Because the

AM group is rapidly cleaved off by intracellular esterases the assay measures only the fluorescence of calcein trapped within the cells.

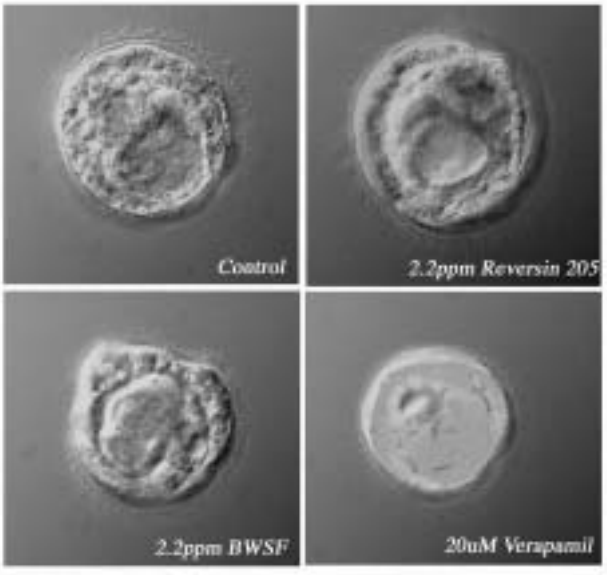


Fig. 11. *Urechis* trochophore larvae incubated with Calcein-AM (O.5 µM) in seawater (control), Reversin 205, BWSF, and verapamil. In seawater, the Calcein dye is efficiently removed via efflux. Reversin, at a similar concentration as BWSF is a potent inhibitor of dye efflux as has been reported in mammalian cells. BWSF at the lowest concentration of biological effects is also a competitive inhibitor of dye efflux. Verapamil in excess is a potent inhibitor as been observed previously.

The results are identical to those previously reported using the dye rhodamine. In addition, we have included a highly specific inhibitor of MDR1 in mammalian cells, "Reversin" (Sharom et al., 1999). This peptide is even more effective than equivalent concentrations of verapamil (Fig. 11, 12).

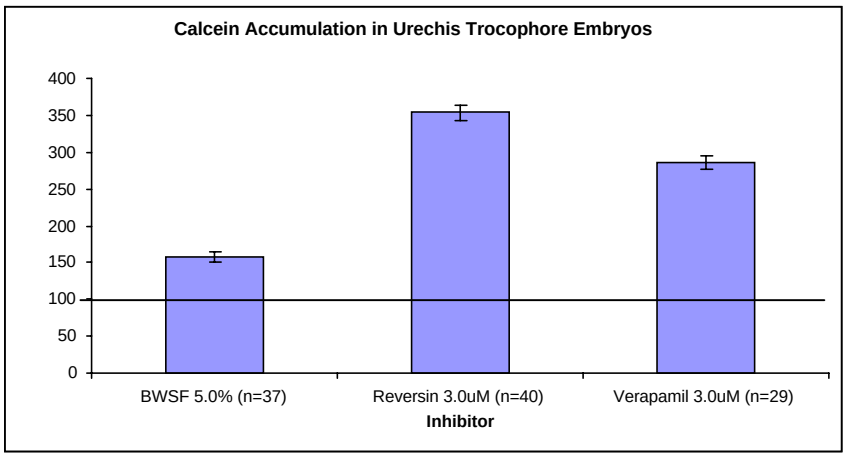


Fig. 12. Calcein dye accumulation in *Urechis* trochophores as compared with seawater controls. BWSF (2.2 ppm) induces a 57% increase in dye accumulation, whereas Reversin (at 2.4 ppm) results in a >200% increase. Verapamil at similar concentrations results in a 150% increase in dye accumulation.

As previously described, the ability of the MXR transporter to rid cells of hydrophobic toxic compounds, is functionally similar to multidrug resistance (MDR) in humans. The biochemical similarity is evidenced by the fact that antibodies to a human MDR-glycoprotein, MDR1, recognize the MXR transporter in both *Urechis* and *C. gigas*. The MDR transport proteins have been implicated in transport of metabolites, ions and both endogenous and exogenous toxins (Gottesman and Pastan, 1988; Horio et al, 1988; Sarkadi et al, 1992). To unequivocally demonstrate that the MXR transporter is responsible for BWSF tolerance, we examined two variations of a human fibroblast cell line, NIH-3T3 cells. One was a non-transfected 3T3 cells that did not possess the MDR gene and thus was unable to express MDR transporters, and the second was an MDR-transfected sub-line of 3T3 cells that does express

the MDR transporter. NIH-3T3 (ATCC) cells were thawed from liquid nitrogen and cultured in standard media (high glucose DMEM (Gibco-BRL) supplemented with 10% fetal bovine serum (Hyclone) and sodium 110mg/liter pyruvate) through 2 to 3 passes, and harvested by trypsin-EDTA treatment. After a two day recovery, cells were then cultured in 30 mm Falcon dishes either in the presence or absence of BWSF at concentrations that ranges from 0.5% to 5% of the culture media for a period of seven days. Differential growth of cell cultures was used as a determinate for whether or not BWSF had an effect on 3T3 cells.

At the start of each experiment, just under 4 x 10^5 cells were added to dishes that contained 0.22, 0.66, 1.2, or 2.2 ppm BWSF. The differential sensitivity of the MDR and non-MDR 3T3 cells to BWSF mirrored that which we observed when comparing echinoderm and echiuroid embryos. Two significant responses to BWSF were demonstrated with the transfected 3T3 cells. First, transfected 3T3 cells were capable of withstanding concentrations that were higher than were the non-transfected cells. Second, the effect of BWSF on cells that utilize an MXR transporter is cumulative over time, that is cells or organisms appear to lose the ability to tolerate MXR substrates over time. This is demonstrated in Fig. 13. After five days of co-incubation, increase in cell number in 0.22, 0.66 or 1.2 ppm BWSF was not significantly different from controls (not shown) or from each other. Only cells in the cultures containing 2.2 ppm BWSF exhibited a significant difference in the 5 day cell number (compared with cultures of controls and the lower BWSF concentrations). Continued exposure to BWSF lowers the effective dosage from 2.2 to 1.2 (Fig. 13). After seven days of co-incubation, cell growth was inhibited significantly in both 2.2 and 1.2 ppm BWSF. Positive cell growth was obtained in the two lower concentrations (0.22 & 0.66 ppm), but not in the 1.2 and 2.2 ppm BWSF exposures. Again, these cells tolerated BWSF concentrations similar to those that can be withstood by *Urechis* and *C. gigas* embryos.

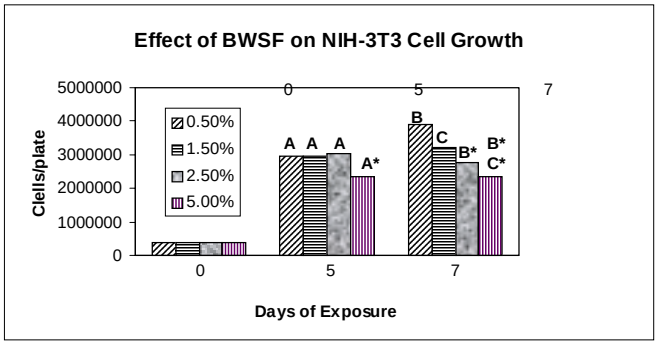


Figure 13. NIH-3T3 G185cells, transfected with the MDR gene, were grown on plates containing either 0.5 (0.22 ppm), 1.5 (0.66 ppm), 2.5 (1.2 ppm), or 5 (2.2 ppm) % BWSF. The numbers of cells in each treatment were scored at 5 and 7 days of exposure.
A-A* = ANOVA ($P<0.05$)
B-B* = ANOVA ($P<0.01$).
C-C* = ANOVA ($P<0.05$).

Conclusions

This study has demonstrated that Santa Barbara Channel crude oil that is biologically degraded with Coal Oil Point sediment microbes, is far more toxic than the water soluble fraction of crude oil. This is due to a dramatic increase (>10 fold) in water soluble and bioavailable hydrocarbons that result from microbial activity. Concentrations of degraded oil as low as 0.2 ppm (equaling a 1:400,000 dilution of crude oil) impacts echinoderm embryo development. Surprisingly, mollusk and echiuroid worm embryos are ten times more tolerant to the degraded oil than echinoderm and fish embryos/larvae.

The tolerance to degraded oil hydrocarbons in select phyla can be attributed to the presence of a multi-xenobiotic membrane transporter (MXR) which is homologous to the mammalian multi-drug resistance transporter (MDR1) common in cancer cells treated with chemotherapeutics. The degraded Santa Barbara Channel oil is a substrate for the MXR transporter in echiuroid and mollusk embryos and they are able to effect an efflux of these compounds. This was demonstrated using dye efflux assays based on mammalian cell technology, as well as the use of mammalian cell lines which over and under express the MDR/MXR transporter and show differential sensitivity to degraded crude oil.

This study has shown that organisms which do not express the MXR transporter are at high risk when exposed acutely or chronically to degraded crude oil. Sites which contain high proportions of species which do not express the MXR transporter (e.g. selected echinoderms, some fish) should be considered "high risk" sites with respect to oil production activities.

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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.