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# Tolerance to biodegraded crude oil in marine invertebrate embryos and larvae is associated with expression of a multixenobiotic resistance transporter

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**Abstract**

The toxicity of water-soluble fractions of biodegraded crude oil (BWSF) to embryos and larvae of two marine invertebrates, the white sea urchin (*Lytechinus anamesus*) and the fat innkeeper (*Urechis caupo*), was studied. Santa Barbara Channel crude oil was artificially weathered and subjected to biodegradation using a mixed microbe culture obtained from natural oil seep sites. The degradation culture inoculated with seep sediment microbes accumulated 43.7 μg/l water-soluble hydrocarbons. In contrast water-soluble fractions from the non-degraded cultures (NWSF) only accumulated 3.05 μg/l. BWSF proved deleterious to *Lytechinus* embryo development at low concentrations (EC50 = 0.33 mg/l) but was essentially non-toxic to *Urechis* embryos/larvae up to 3.0 mg/l. An established mechanism for handling of a wide array of xenobiotics in *Urechis* embryos is the multixenobiotoic resistance transporter multixenobiotic response (MXR, also known as multidrug resistance, MDR). This mechanism is primarily mediated by ATP-dependent, efflux pumps that extrude a wide array of xenobiotic compounds. In this study, we show that *Lytechinus* larvae do not appear to express MXR efflux protein nor MXR mediated dye efflux capacity. In contrast, BWSF acts as a competitive inhibitor of MXR transport-mediated dye efflux in *Urechis* larvae. These results suggest that MXR may be an important mechanism for extrusion of the by-products of crude oil degradation by microbes, and that the level of its expression may determine the susceptibility of organisms to degraded oil hydrocarbons.


*Keywords:* Embryos; Larvae; Biodegraded oil; Lytechinus; MDR; MXR; Oil seep; Sea urchin; Urechis; P-gp

## 1. Introduction

Crude oil enters the marine environment from a number of natural and anthropogenic sources. It is introduced as an indirect result of crude oil production and transport in addition to catastrophic spills. Crude oil is relatively unstable

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in the marine environment and it immediately undergoes a process termed 'weathering' during which it adheres to sediments, mixes with seawater, loses volatile constituents, and photo-degrades (Boehm, 1987; Payne et al., 1992; Venkatsewaran et al., 1995). The weathering process results in release of low boiling point aromatic and saturated hydrocarbons (Galt et al., 1991), and prolonged mixing with seawater results in release of water-soluble fractions that have been shown to be especially toxic to fish and invertebrate embryos from pristine environments (Davis et al., 1981; Middaugh and Whiting, 1995). Furthermore, exposure to ultraviolet radiation from sunlight is known to increase the toxicity of oil hydrocarbons (Cleveland et al., 2000; Pelletier et al., 1997). Finally, weathered crude oil is susceptible to biodegradation by various naturally occurring, hydrocarbon degrading microbes (Galt et al., 1991).

At sites of natural seepage, such as Coal Oil Point in the Santa Barbara Channel, naturally occurring populations of sediment-associated, oil metabolizing microbes have been described (Spies and Davis, 1979). Since several marine bacteria readily metabolize hydrocarbons from weathered oil, they are often employed in cleansing oil-contaminated sites (Mueller et al., 1992; Bragg et al., 1994). Fertilizers have been used to enhance the growth of crude oil degrading microbes at several sites contaminated with oil from the *Exxon Valdez* in 1989. Fertilized plots demonstrated reductions in deposited oil and a loss of extractable organic matter from the remaining oil (Claxton et al., 1991). Similarly, microbial degradation at sites of natural oil seepage causes a decrease in sediment crude oil along with measurable organic enrichment (Spies, 1987).

Numerous studies have documented the adverse effects of catastrophic crude oil spills on marine environments. Recently the toxicity of biodegraded water-soluble fractions (BWSF) of crude oil has been demonstrated in embryos and larvae of several marine species. The increased water solubility of degraded crude oil is thought to increase oil toxicity to fish (*Clupea pallasi* ) embryos by as much as ten fold (Middaugh et al., 1998). Specific toxicity of water-soluble constituents of biologically degraded oil, rather than water soluble constituents of oil itself, has been demonstrated in two estuarine crustacean species *Palaeomonetes pugio* and *Mysidopsis bahia* (Shelton et al., 1999). Paradoxically, sediments surrounding sites of natural seepage support elevated numbers of several invertebrate species (Steichen et al., 1996) and, therefore, it has been suggested that organisms living near sites of natural seepage have evolved specific mechanisms for coping with constant exposure to potentially toxic by-products of oil exposure (Spies et al., 1982, 1996).

This is supported by the observation that many marine invertebrates are known to live and reproduce in other environments rich in potentially toxic chemicals (Kurelec, 1997). Chemical concentrations in tissues of these organisms are often maintained at levels below those in the surrounding environment. A primary molecular detoxification mechanism, the multixenobiotic response (MXR), has been strongly implicated in these systems. This phenomenon is similar to multidrug resistance (MDR) in mammalian cells, which is associated with the expression of a membrane transport 'P-glycoprotein' (P-gp) belonging to the ABC transporter protein family. P-gp is thought to confer drug resistance by acting as an energy dependent efflux pump (Horio et al., 1988). Overexpression of the *MDR*1 gene product, and associated proteins, has been linked to the resistance of cancer cells to chemotherapeutic agents (Roninson, 1992). In normal tissues the MDR transport proteins have been implicated in transport of lipids, metabolites, ions and both endogenous and exogenous toxins (Shinkel, 1997).

Recent studies have emphasized the importance of MDR-like proteins in aquatic systems (Epel, 1998; Bard, 2000). Kurelec and his colleagues (Kurelec, 1997 for review) have described verapamil sensitive, MXR proteins in several adult bivalve and sponge species, including *Anodonta* sp. (Kurelec and Pivceviac, 1989). Minier and his colleagues found that oysters from polluted sites have higher frequencies of expression of MXR proteins than those from sites with less pollutants (Minier et al., 1993). The MXR transporter protein also appears to be important for juvenile and adult forms of the echiuroid worm, *Urechis*

*caupo*, which live in habitats naturally rich in environmental toxins (Toomey and Epel, 1993). In this study we investigate the toxicity of BWSF of crude oil on embryos from two marine invertebrate model species, *U. caupo* and *Lytechinus anamesus* two species that differ dramatically in their MXR mediated ability to actively extrude toxic organic compounds.

## 2. Materials and methods

### 2.1. Animals

Adult *L. anamesus* were maintained year round at the Bodega Marine Laboratory (BML) in continuous flow through, sand filtered Bodega Bay sea water (13 °C ± 2°) with weekly feedings of locally collected kelp. *Lytechinus* were spawned by injection of 0.25–0.5 ml of 0.5 M KCl into the body cavity via the peristomial membrane. Gametes were collected into chilled 0.7 μm filtered Bodega Bay seawater (FSW) and kept on ice until use. Adult *U. caupo* were collected from mudflats at Bodega Harbor and Pillar Point, CA, and maintained in tanks with flow through seawater and several inches of mud and sand. Gametes were collected within several weeks of collection according to Gould (1967).

### 2.2. Crude oil weathering and degradation

Santa Barbara Channel crude oil was collected into clean 4 l amber bottles at the Gaviota platform in the Santa Barbara Channel and shipped overnight to the BML at 4 °C. The crude oil was artificially weathered according to the method of Middaugh et al. (1996). The oil was autoclaved at 374 °C for 1 h, distilled at 1 atm, and then stored in aliquots at − 20 °C. This oil is subsequently referred to as artificially weathered oil.

Sediment samples were collected from oil seep sites 60 ft below the surface of the Santa Barbara channel at Coal Oil Point. About 100–200 ml of surface sediment was directly scraped into 250 ml, Nalgene polypropylene bottles. Excess seawater was poured off and samples were put on ice and transported to the BML.

A reduced scale adaptation of the method of Middaugh et al. (1996) was used for microbial enrichment and subsequent biodegradation of weathered crude oil. An enrichment culture, containing 3 l of 0.7 μm filtered, full strength (32–34 ppt) Bodega Bay seawater (FSW) fertilized with Bushnell and Haas nutrients (DIFCO) was autoclaved, cooled to room temperature (18 °C) and then inoculated with 0.5% artificially weathered crude oil and 15 ml of fresh seep sediment. The enrichment culture was vigorously aerated with 0.2 μM filtered air in a fume hood at room temperature (17–20 °C). After 14 days the culture was passed over a 8.0 μm paper (Whatman) filter to remove large particles and emulsified oil, and the resulting filtrate was stored in aliquots containing 10% DMSO at − 70 °C.

Final degradation cultures were prepared identically to the enrichment cultures except they were inoculated with 50 ml of the enrichment culture. A negative control (for the effect of the enrichment culture microbes) was prepared by inoculating an identically prepared biodegradation vessel with 50 ml of FSW containing Bushnell and Haas nutrients and 10% DMSO. Incubation was conducted in the same way used for the enrichment culture.

After 14 days, BWSF of crude oil were isolated by sequential filtration of the degradation culture inoculated with 'enrichment' microbes. Large particles and remaining oil particles were removed by sequential filtration through a 5.0 μM followed by 2.0 μm cellulose acetate filters (Millipore) and stored at − 70 °C in aliquots. 'Non-degraded' water-soluble fractions (NWSF) were prepared from the control culture in the same way. Aliquots of BWSF and NWSF for larval exposures were further filtered to 0.45 μM prior to use with embryos. Smaller aliquots were sterile filtered to 0.22 μm for use with cell cultures.

Approximate concentrations of solubilized crude oil hydrocarbons in BWSF and NWSF were determined by fluorescence excitation scanning (Photon Technologies Inc., spectrofluorometer, South Brunswick, NJ) using a modification of the methods of De-Alda Villaizan et al. (1995). A standard curve ($r^2 = 0.96$) for crude oil hydrocarbons was prepared by sequentially dissolving a known quantity of artificially weathered crude oil

in methylene chloride, DMSO and finally FSW and measuring fluorescence (353 ηm) at peak excitation for oil hydrocarbons (290 ηm). All standard values were adjusted for the fluorescence of a DMSO/Methylene chloride control.

### 2.3. Larval exposures

Eggs of *Lytechinus* and *Urechis* species were fertilized and fertilization success was assessed as elevation of the fertilization envelope and successful completion of the first cell division. Only batches with 90% or greater fertilization success were used for experiments. Gametes from different individuals were not mixed prior to fertilization, thus, each replicate was the product of a unique male and female pairing. At least three replicates were used for all experiments. After fertilization groups of 150–250 embryos were transferred to 60 ml shell vials (Fisher) containing 20 ml of FSW and concentrations ranging from 0–3 mg/l degraded hydrocarbons (corresponding to 0–7% of BWSF or NWSF). Vials were incubated at 15 °C through embryonic development. *Lytechinus* and *Urechis* larvae were fixed in 0.5% paraformaldehyde and 50–100 larvae per vial were scored for successful development to pluteus or trochophore larval stages, respectively.

### 2.4. Dye efflux assays

To assess whether BWSF could be acting as a competitive inhibitor of the MXR transporter we conducted competitive dye transport assays according to the methods of (Toomey and Epel, 1993). Briefly, unexposed embryos of each species were incubated for 30 min at 15 °C either with 1 μM rhodamine alone, 5 μM rhodamine and 20 μM verapamil or 1 μM rhodamine and 2.2 mg/l BWSF hydrocarbons. After incubation embryos were washed twice by hand centrifugation and resuspended in FSW.

Bulk fluorometry was conducted by placing groups of approximately 1500 larvae into a microcuvette (at a total volume of 1.5 ml) with constant stirring in a PTI Ratiomaster spectrofluorometer. Fluorescence was measured at 550 ηm excitation and 580 ηm emission.

### 2.5. Western blotting

To assess whether or not sea urchin and *Urechis* embryos express P-gp, and regulate the level of its expression in response to BWSF, urchin and *Urechis* embryos were exposed to BWSF continuously through development and then collected for electrophoresis at various developmental stages. Exposures were started after first cleavage in each species, and *Urechis* embryos were exposed to 2.2 mg/l BWSF while sea urchin embryos were exposed to 0.22 mg/l. Concentrations were chosen to correspond to the highest dosage tolerated by each species. Two batches of control and BWSF exposed embryos and larvae from each species were collected into conical tubes from cultures, then chilled on ice for 15 min and gently concentrated by hand centrifugation. Pellets of 100–200 ul of embryos were transferred to microfuge tubes containing an equal volume of ice-cold hypotonic lysis buffer (Morris et al., 1991) modified with the addition of a general protease inhibitor cocktail (Sigma). Embryos were disrupted by brief sonication. Aliquots of embryo homogenates were mixed with equal volumes of 2 × sample buffer and solubilized by boiling for 5 min. Protein concentrations of homogenates were determined using a standard BCA protein assay (Pierce). Solubilized homogenates (15 μg of total protein/sample) were loaded onto 10% SDS-polyacrylamide gels and run at 155 V for 90 min at room temperature. Proteins were transferred onto nitrocellulose for 3 h at 115 V in a cooled chamber. Blots were blocked overnight in 4% non-fat milk in Tris-buffered saline (10 mM Tris base, 150 mM NaCl, pH 7.4) MXR protein was labeled by incubating blots with the mouse monoclonal antibody C219 (Fujirebio Diagnostics) at 1 μg/ml, followed by the horseradish peroxidase (HRP) conjugated goat anti-mouse (1:5000) secondary (Sigma). HRP labeled MXR protein was visualized by reaction with chemiluminescent reagent (Pierce) according to manufacturers instructions and exposure to photographic film (Biomax MR, Kodak). Since *Urechis* embryos are known to express P-gp (Toomey and Epel, 1993), they served as a positive control.

### 2.6. GC-MS analysis of BWSF and NWSF

Samples were prepared for analysis by gas chromatography-mass spectroscopy (GC-MS) using C-18 and silica columns (Alltech), using an adaptation of the methods of (Vines et al., 2000). Columns were first conditioned by rinsing with the appropriate solvent (methanol for C-18 columns and hexane for Silica columns) followed by FSW. BWSF and NWSF corresponding to approximately 300 mg/l of each sample was loaded onto each and withdrawn, by vacuum filtration into a clean flask. The filtrate was retained for toxicity testing in embryos. The columns were then washed with $ddH_2O$ followed by methanol (C-18) or hexane (silica). Columns were eluted with 0.5–1 ml of hexane (C-18) or methanol (silica) onto a clean flask in ice. Samples were sent to the Facility for Advanced Instrumentation, UC Davis for analysis on a Varion Saturn 4 GC-ion trap-MS instrument. A 30 m (0.25 mm thickness) capillary column was used with the following temperature program: 60 °C, 1 min, increased by 10 °C/min–300 °C and maintained at 300 °C for 10 min. Peaks were compared with those from the National Institute of Standards and Technology (NIST) library of known compounds to give approximations of their identity based on probability based matching algorithms. Only structures with purity fit values above 700 are listed.

### 2.7. Data analysis

EC50 values were calculated by logit-log analysis of toxicity data. Differences between species in toxicity and dye extrusion were calculated by using *t*-test of means on normalized (log transformed) data. Analyses were done using SIGMA STAT (version 2.03) software.

## 3. Results

### 3.1. Crude oil degradation

Cultures that were inoculated with Santa Barbara Channel microbes produced visibly greater levels of crude oil emulsification than controls. Little or no oil remained adherent to the sides of the glass culture vessels in the microbe inoculated cultures. After filtration the resultant BWSF and NWSF were acidic, pH 6.5. The addition of these fractions to seawater for toxicity assays had no effect on seawater pH at the concentrations used. Fluorometric determination of soluble hydrocarbon concentrations showed dramatically higher levels of soluble hydrocarbons in BWSF versus NWSF, at 43.7 versus 3.05 mg/l, respectively. These values were based on the standard curve developed for crude oil (see Section 2).

### 3.2. Larval exposures

*Urechis* embryos were insensitive even to relatively high (3 mg/l) concentrations of BWSF (Fig. 1). In contrast, sea urchin embryo development was abnormal ($P < 0.05$) after exposure to BWSF at concentrations as low as 0.1 μg/l (Fig. 1). Abnormalities after exposure to low doses of BWSF consisted of incomplete spicule development and shortened of the arms of pluteus larvae (Fig. 2). Abnormal sea urchin embryo development occurred in a dose dependent fashion both at hatching (not shown) and later at the pluteus larval stage (Fig. 2). At high concentrations of BWSF (2.2 mg/l) sea urchin embryos showed more severe developmental delay and abnormalities including abnormal cell division and poor ciliary movement. Sea urchin embryos that hatched in high concentrations of BWSF were often irregular in shape and observed on the bottom of vials rotating in circles (in contrast to normal circular gastrulae which swim in the water column). Although sea urchin embryos exposed to low doses of BWSF simply appeared to be developmentally delayed relative to their control counterparts we found that, even after being grown for several additional days, these embryos rarely developed completely (not shown).

To compare the relative toxicity of equivalent starting amounts of weathered crude oil, with or without microbial degradation, sea urchin embryos were exposed to NWSF ranging from 0.5 to 5% of the culture volume (2.5–25 mg/l). These doses corresponded to the proportions of BWSF

used in the initial exposures to give final hydrocarbon concentrations of 0.22–2.2 mg/l. At equivalent proportions in seawater, BWSF is more toxic than NWSF (Fig. 3). Calculated EC50 values for BWSF and NWSF were 0.74 and 1.07%, respectively. However, if normalized for hydrocarbon content, NWSF is significantly more toxic than BWSF, with EC50 values of 0.03 and 0.33 mg/l, respectively. Thus, the enhanced toxicity of BWSF may be attributed to the dramatic increase in water-soluble hydrocarbons after microbial degradation of oil.

The toxicity of BWSF was not stage specific. Sea urchin embryos were only slightly less suscep-

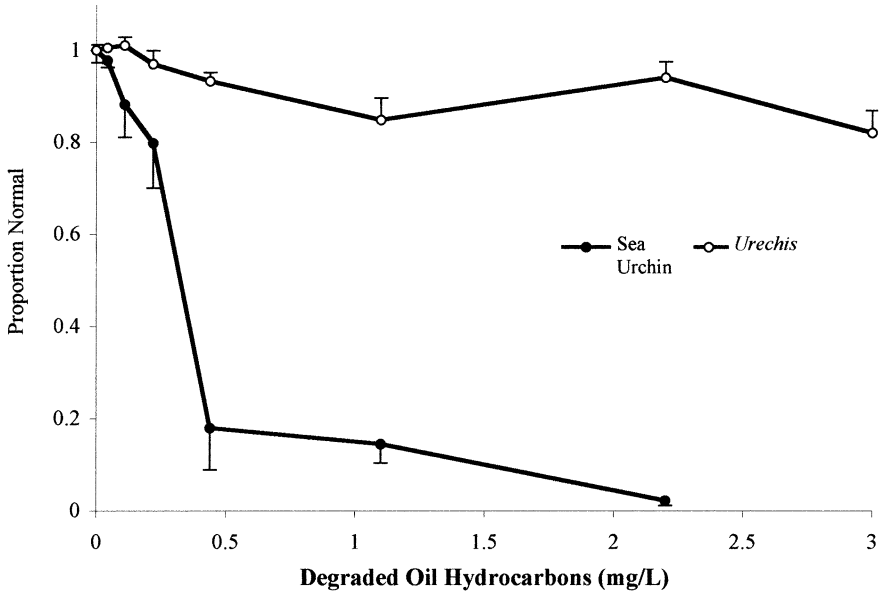


Fig. 1. Effect of continuous exposure to BWSF on development to larval stage in sea urchin and *Urechis* embryos ($n = 3$ batches embryos per species, $\pm$S.E.M.). Embryos were exposed to BWSF continuously through development after completion of the first cleavage. Normality was scored when controls for each group reached the larval stage (trocophore in *Urechis* and pluteus in sea urchins). Normal animals were scored as those that had morphologically developed to the same extent as the controls.

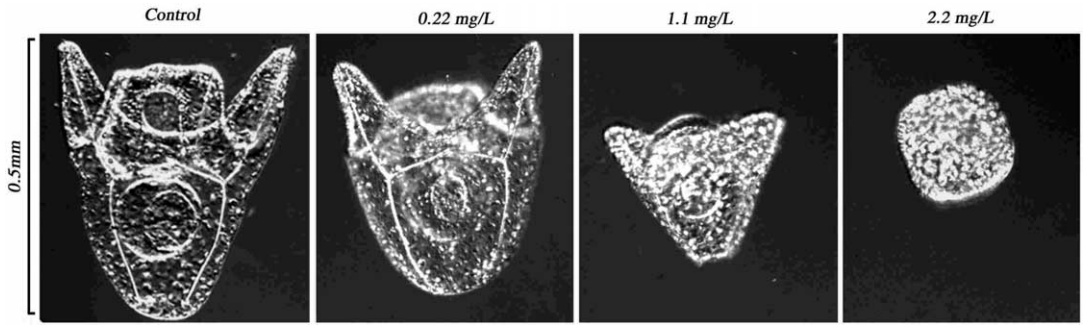


Fig. 2. Light micrographs of sea urchinembryos continuously exposed to BWSF show that BWSF delays development to pluteus. Specifically we observed incomplete development of the arms and spicules of pluteus larvae in what appeared to be otherwise normal larvae. At intermediate concentrations (1.1 mg/l) effects were more pronounced and there was no development of arms. At higher concentrations complete developmental arrest occurred at the early gastrula stage.

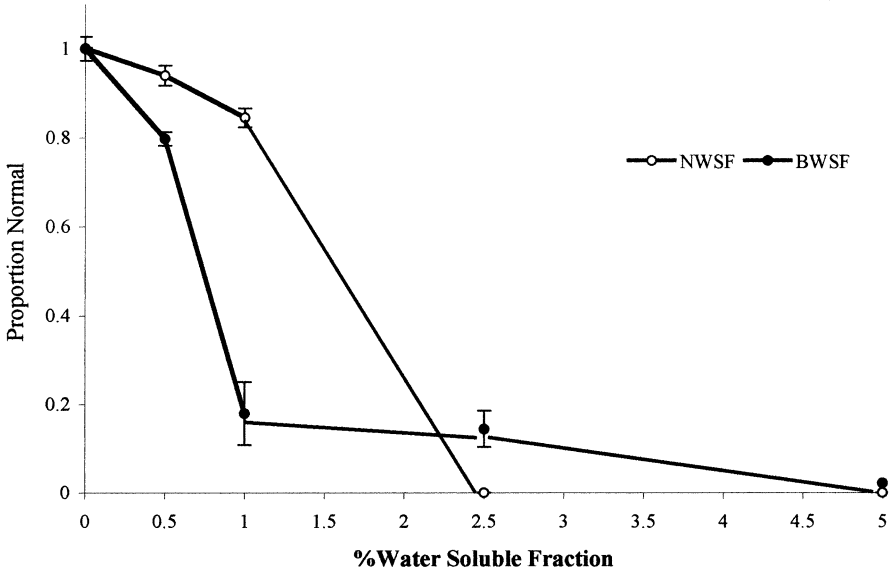


Fig. 3. Relative toxicity of BWSF and NWSF to sea urchin embryos was assessed by continuous exposure of embryos to either toxicant. Normality was assessed as described previously (see Fig. 1).To account for the difference in relative hydrocarbon concentration between the two fractions concentrations are shown as percentage of culture volume. NWSF is more toxic to sea urchin embryos than BWSF, but it contains significantly lower concentrations of soluble hydrocarbons.

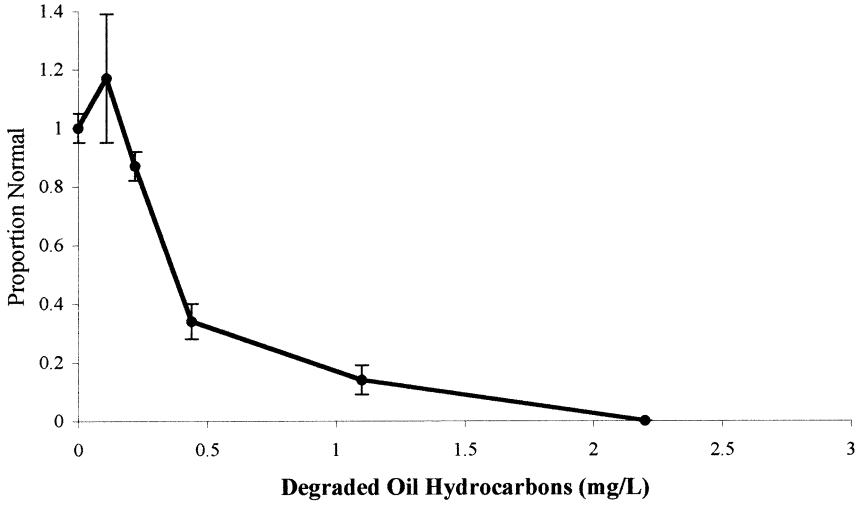


Fig. 4. To assess whether or not the effects of BWSF occurred specifically during early development sea urchin embryos were allowed to develop to hatching (early gastrula) prior to BWSF exposure. Normality was scored as previously described (see Fig. 1). Toxicity of BWSF was only moderately reduced if sea urchin embryos were allowed to develop to hatching in clean FSW prior to BWSF exposure.

tible to the effects of BWSF if allowed to develop to hatching in clean FSW prior to exposure to BWSF (Fig. 4).

At 15 °C *Lytechinus* typically developed to the pluteus stage in approximately 93 h while *Urechis* develop to trochophore was less than 48 h. Thus,

to determine if the increased time of exposure to BWSF in sea urchin embryos had any significant effect on the results, additional experiments were conducted in which *Urechis* embryos were exposed to BWSF for 93 h (Fig. 5). This extended exposure to BWSF still had no significant effect on *Urechis* embryo/larval development.

### 3.3. Dye efflux

*L. anamesus* larvae did not appear to possess functional MXR mediated rhodamine dye extrusion capacity. No significant differences were observed between verapamil or BWSF treated embryos and controls (Fig. 6). In contrast, *Urechis* larvae extruded significantly more dye in the absence of the MXR inhibitor verapamil or BWSF. BWSF seemed to induce relatively large increases in dye accumulation in *Urechis* larvae that were not significantly different from those observed with verapamil ($P < 0.001$).

### 3.4. Western blots

P-gp is expressed relatively homogeneously throughout *Urechis* embryo development and does not appear to be induced by exposure to high (2.2 mg/l) concentrations of BWSF (Fig. 7). In agreement with earlier findings in purple sea urchins (Toomey and Epel, 1993), *Lytechinus* embryos and larvae do not express significant levels of a protein that cross reacts with monoclonal antibody C219. Exposing sea urchins to 0.22 mg/l did not result in expression of a cross-reactive protein.

### 3.5. GC/MS analysis of BWSF/NWSF

Column filtrates were tested for their toxicity to sea urchin embryos. Dramatic reductions in toxicity were observed in sea urchins exposed to proportions of extracted fractions corresponding 1.1 ppm concentration of BWSF. Abnormal development was reduced from 86% in BWSF treated controls to 42% in sea urchins exposed to C-18 extracted fractions. Silica extraction completely removed toxicity at the 1.1 mg/l level. These reductions in toxicity suggest that the columns are removing many of the water-soluble toxic constituents of degraded and non-degraded oil hydro-

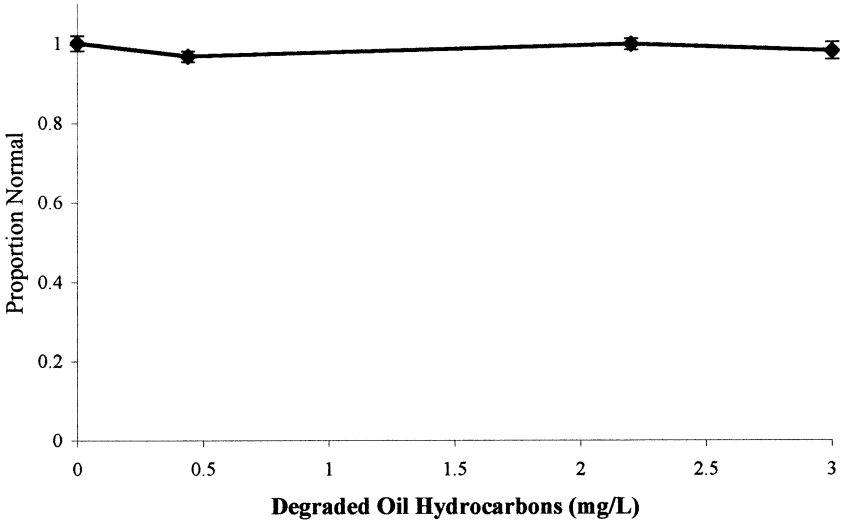


Fig. 5. *Urechis* embryos were exposed to BWSF for 96 h post first cleavage.. Prolonged exposure to BWSF was not deleterious to *Urechis* embryos. *Urechis* embryos continued to swim normally and showed normal development of the apical tuft of cilia even after exposure to high concentrations of BWSF.

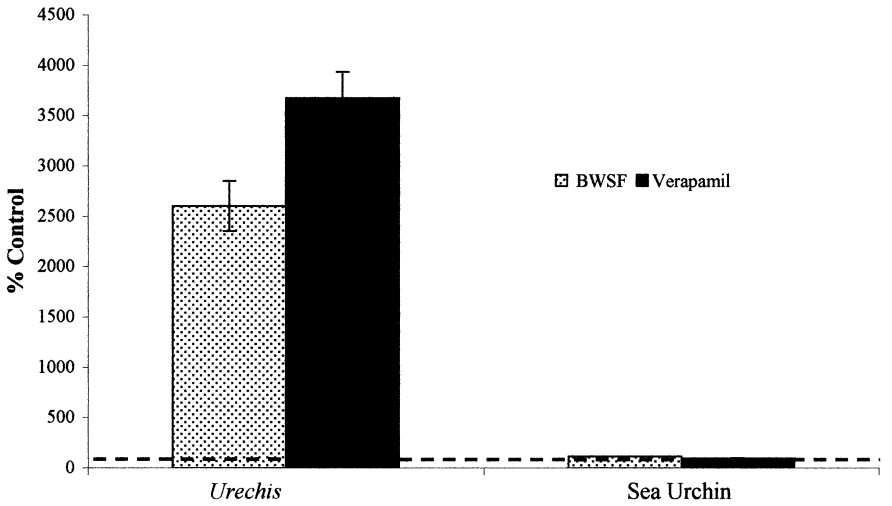


Fig. 6. To assess whether or not BWSF is a P-gp substrate we conducted ompetitive dye efflux experiments. Both BWSF (2.2 mg/l) and Verapamil (20 μM) cause significant increases in rhodamine-B (1 μM) dye accumulation in *Urechis* but not sea urchin embryos. Dotted line indicates control fluorescence level. Increases in fluorescence relative to control levels indicate increased dye accumulation as a consequence of competitive inhibition of rhodamine efflux. Bars represent means of 2–3 experiments ( ± S.D.).

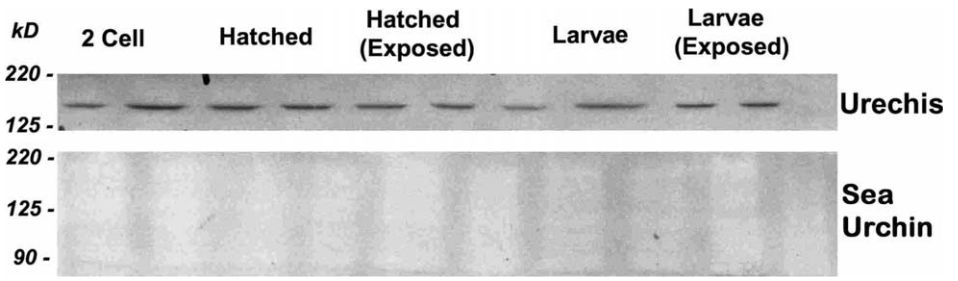


Fig. 7. Western blot of P-gp using monoclonal Ab. C219 (Anti-P-gp) in sea urchin and *Urechis* embryos. Embryos were continuously exposed to either 0.22 mg/l BWSF (sea urchin embryos) or 2.2 mg/l (*Urechis* embryos) BWSF. Sea urchins embryos do not appear to express P-gp at any point during development. In contrast *Urechis* embryos appear to express P-gp continuously.

carbons. Table 1 summarizes the composition and structural homologies (from existing databases) of major peaks found by GC-MS analysis of water-soluble oil fractions. Constituents are listed in descending order of their relative abundance. Silicon atoms associated with silica column extracts were likely contaminants associated with the elution process. However, even after accounting for their contribution to molecular weight of silica extracts of BWSF and NWSF, there were reductions in mean molecular weight of the top seven constituents from 295.9 g/M in NWSF to 234.7 g/M in BWSF.

## 4. Discussion

In this study, we have examined the toxicity of crude oil degraded by microbes similar to those naturally occurring in the Santa Barbara channel. Our results provide further evidence that biodegradation of crude oil may actually result in enhanced oil toxicity. As has been previously reported (Shelton et al., 1999), the mechanism of this enhanced toxicity after biodegradation appears to be as a result of increased accumulation of small, water-soluble hydrocarbon fractions. Consistent with this hypothesis is the observation

that silica extraction of BWSF caused greater reductions in toxicity than C-18 extraction. The complete removal of toxicity by silica columns suggests that most of the toxicity is mediated by smaller, polar constituents. Previous studies have shown significant enrichment of small hydrocarbons during degradation (Middaugh et al., 1996). The low levels of water-soluble hydrocarbons measured in NWSF suggest that although water-soluble fractions of non-degraded oil are highly toxic, they are probably less likely to accumulate at high concentrations without enhanced microbial degradation. Levels of toxicity were observed at concentrations relevant to those previously measured in laboratory and environmental samples. Stuermer et al. (1981) have reported that water soluble fractions of Santa Barbara Channel seep oil prepared in the laboratory contained hydrocarbon concentrations as high as 9.7 mg/l. Previous studies have suggested that environmental concentrations range from 10 μg/l in near-seep water to as high 0.5 mg/l directly at the sediment–water interface (Spies, 1987).

To our knowledge, this is the first report that demonstrates species-specific embryo tolerance to high levels of water-soluble fractions from degraded oil. Over the range of concentrations used, the effects of BWSF on *Urechis* embryos/larvae were negligible. In contrast the effects of BWSf on sea urchin embryos were significant. At low doses (0.22 mg/l) sea urchins develop normal morphology but at a slower pace than their un-exposed counterparts. At higher doses abnormalities are more significant and include aberrant cleavage and severely delayed development. Further experiments are required to clarify if the effects of exposure to low doses of BWSF are reversible.

It is not surprising that animals living in close contact with microbe rich sediments, such as *U. caupo*, have evolved multiple mechanisms for coping with toxic by-products of microbial metabolism. *Urechis* live and reproduce in environments that are rich in microbial byproducts. P-gp in *Urechis* embryos has been previously shown to extrude microbial byproducts (Toomey et al., 1996). *Urechis* also possess multiple mechanisms that confer tolerance to various toxic compounds including those that would not be

Table 1
Results of GC-MS analysis of C-18 and silica column extracts of BWSF and NWSF

| BWSF C-18 | BWSF Silica | NWSF C-18 | NWSF Silica |
|---|---|---|---|
| $C_{41}H_{72}O_2$; MW 592 | $C_{13}H_{21}NO_3$; MW 239 | $C_{24}H_{38}O_4$; MW 390 | $C_7H_4F_2O$; MW 142 2,5-difluorobenzaldehyde |
| $C_{19}H_{19}N$; MW 261 Butylated hydroxytoluene | $C_8H_{14}O$; MW 126 Ethanone, 1-cyclohexyl | $C_{10}H_{12}O_2$; MW 164 | $C_8H_{14}O$; MW 126 |
| $C_{41}H_{72}O_2$; MW 592 | $C_{10}H_{26}O_2Si_2$; MW 234 | $C_{41}H_{72}O_2$; MW 596 | $C_{17}H_{27}NO_2$; MW 277 Phenol, 2,6-bis(1,1-dimethylethyl)-4-methyl, methylcarbamate |
| $C_{14}H_{22}O$; MW 206 | $C_{14}H_{20}O_2$; MW 220 | $C_{15}H_{24}O$; MW 220 | $C_{16}H_{48}O_6Si_7$; MW 532 |
| $C_{19}H_{19}N$; MW 261 | $C_{15}H_{24}O$; MW 220 Butylated hydroxytoluene | $C_{22}H_{14}O_6$; MW 374 | $C_{13}H_{40}O_5Si_6$; MW 444 |
| | $C_{17}H_{34}O_2$; MW 270 | | $C_{13}H_{24}O_2Si_2$; MW 268 |
| | $C_{22}H_{34}O_4$; MW 362 | | $C_{19}H_{54}O_7Si_7$; MW590 |
| | | | $C_8H_{24}O_4Si_4$; MW 296 Cyclotetrasiloxane, octamethyl |

Predicted structures with purity fit values (to known NIST standards) of greater than 700 are listed.

expected to interact well with MDR transporters (Arp et al., 1995).

Previous studies have demonstrated the importance of several mechanisms, other than MXR/MDR, in the survival of various oil seep-dwelling organisms (Klotz et al., 1983; Spies et al., 1982, 1996). Moreover, several studies have also suggested that MXR transporter expression may be part of a more generalized cellular stress response and that its expression is coordinated with the expression of other stress proteins. In fish and mammalian liver (Cooper et al., 1999; Silverman and Schrenk, 1997) expression of MXR is also associated with expression of mixed function oxidases (MFO). In *Mytilus californianus* (Eufemia and Epel, 2000) and mammalian systems (Chin et al., 1990; Vilaboa et al., 2000) protein denaturing stresses such as metal exposure and heat shock also result in elevated MXR transporter expression. Collectively, these observations suggest that simultaneous induction of several cellular stress tolerance mechanisms may act to confer tolerance to chronic stresses such as exposure to BWSF and related compounds at natural seep sites.

Nonetheless, because drugs appear to bind to MDR transporters while in solution in the phospholipid bilayer (Ueda et al., 1997; Romsicki and Sharom, 1999), MXR represents a first line of defense against environmental toxins (Epel, 1998). Thus, it is likely that elevated levels of MFO (and other detoxication systems), after exposure to petroleum hydrocarbons, occurs only after primary mechanisms of drug extrusion have been overwhelmed. Under these conditions cytotoxicity may be mediated by endogenous compounds that are otherwise maintained at non-toxic concentrations.

Many of the compounds in degraded oil have structures that one would expect to interact well with MDR-like transporters (Ambudkar et al., 1999). Previous studies have demonstrated that oil hydrocarbons can result in enhanced MXR activity (Kurelec, 1995) in marine invertebrates. Our results show that biodegraded hydrocarbons act as competitive inhibitors of MXR mediated efflux in *Urechis* embryos/larvae. These results are not unexpected, given the promiscuity of MDR transporters (Gottesman and Pastan, 1993; Sharom, 1997).

This is the second study that has failed to demonstrate verapamil-sensitive rhodamine dye efflux and P-gp expression (as determined by labeling with C219 antibody) in sea urchin embryos (Toomey and Epel, 1993). Nonetheless, these results are not conclusive. Given the high level of conservation among ABC transporter proteins and their nearly ubiquitous expression (van Veen and Konings, 1997) it seems likely that some form of these proteins should be expressed in the sea urchin embryo/larva. Moreover, MDR-like transporter proteins are known to play important roles during development via efflux of endogenously produced molecules such as differentiation factors (Good and Kuspa, 2000).

In many cases, ABC transporter proteins other than P-gp (Cole et al., 1992) confer drug resistance via active extrusion of drugs. Thus, an alternate hypothesis for the observed species-specific differences in toxicant resistance is the possibility that the protein(s) responsible for efflux of endogenous compounds and xenobiotics in sea urchin embryos differ from those expressed in *Urechis* embryos. As the various families of ABC transporter proteins are known to differ significantly in substrate specificity and efflux kinetics (Cole and Deely, 1998; Ambudkar et al., 1999) it is possible that they may not all be readily detected using standard rhodamine efflux assays (Twentyman et al., 1994).

Characterizing species and stage specific patterns of drug resistance protein expression is necessary in order to compose a more accurate picture of species-specific differences in susceptibility to environmental toxins. In order to address this question it is likely that multiple and/or degenerate probes targeted against various MDR-like transporter proteins and genes will be required. Such an approach has already been successfully employed for the cloning of transporters from model organisms (Allikmets and Dean, 1998) and for the specific identification of various MDR related proteins (Scheffer et al., 2000).

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