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Hsp60-Induced Tolerance in the Rotifer *Brachionus plicatilis* Exposed to Multiple Environmental Contaminants

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Abstract. Hsp60 induction was selected as a sublethal endpoint of toxicity for *Brachionus plicatilis* exposed to a water accommodated fraction (WAF) of Prudhoe Bay crude oil (PBCO), a PBCO/dispersant (Corexit 9527®) fraction and Corexit 9527® alone. To examine the effect of multiple stressors, exposures modeled San Francisco Bay, where copper levels are approximately 5 µg/L, salinity is 22‰, significant oil transport and refining occurs, and petroleum releases have occurred historically. Rotifers were exposed to copper at 5 µg/L for 24 h, followed by one of the oil/dispersant preparations for 24 h. Batch-cultured rotifers were used in this study to model wild populations instead of cysts. SDS-PAGE with Western Blotting using hsp60-specific antibodies and chemiluminescent detection were used to isolate, identify, and measure induced hsp60 as a percentage of control values. Both PBCO/dispersant and dispersant alone preparations induced significant levels of hsp60. However, hsp60 expression was reduced to that of controls at high WAF concentrations, suggesting interference with protein synthesis. Rotifers that had been preexposed to copper maintained elevated levels of hsp60 upon treatment with WAF at all concentrations. Results suggest that induction of hsp60 by chronic low-level exposure may serve as a protective mechanism against subsequent or multiple stressors and that hsp60 levels are not additive for the toxicants tested in this study, giving no dose-response relationship. The methods employed in this study could be useful for quantifying hsp60 levels in wild rotifer populations.

Over 24 million barrels of oil are released into the marine environment annually, of which tanker accidents account for approximately 12.5% (Clark 1989; National Resource Council 1989). Thus, oil spill clean-up measures have been established for emergency response. Dispersing agents have proven useful for oil-spill treatment, but their toxicity is a concern. An extensive body of literature evaluating dispersant toxicity has been developed over the years (National Resource Council 1989; Tjeerdema *et al.* 1989; Singer *et al.* 1994, 1995a, 1995b), yet there is little information published on the sublethal effects of oil and dispersants. To the best of our knowledge, the effects of these agents on the expression of heat shock proteins has not been previously reported.

Due to experimental complexity, bioassays have classically focused on the effects of a single stressor. However, organisms are generally exposed to multiple stressors *in situ.* For example, coastal species are subject to the influx of waters from different anthropogenic sources containing a variety of contaminants. Therefore, much of the available EC_{50} or LC_{50} data for specific toxicants are difficult and inappropriate to apply toward most environmental samples. An advantage to using stress proteins for environmental monitoring is their ability to integrate the total stress an organism is experiencing (Sanders 1993), whether natural or anthropogenic. Stress proteins, including the 60-kD heat shock protein (hsp60), are a group of highly conserved proteins induced in response to a variety of environmental agents.

Rotifers have a demonstrated sensitivity to petroleum products (Rogerson *et al.* 1982; Snell and Persoone 1989a, 1989b; Ferrando and Andreu-Moliner 1992). Cochrane *et al.* (1991) showed that the euryhaline rotifer *Brachionus plicatilis* produces the stress protein hsp60 in response to copper and tributyltin. They found a maximal increase in hsp60 concentration at a copper dose that is 5% of the LC_{50}. In addition, the induction of the gene responsible for hsp60 in *B. plicatilis* has been demonstrated at the level of mRNA abundance (Cochrane *et al.* 1994).

Most studies in the literature involving rotifers use synchronized cysts to reduce variability among the organisms (Cochrane *et al.* 1991; Snell and Janssen 1995). However, cysts were not used in this study so that field variability could be monitored. The methods employed in this study were designed for use with natural or wild populations. It would be expected that these populations would have a much wider variation in their constitutive levels of hsp60 given their varying physiology and age. Therefore this study examined the utility of monitoring hsp60 levels in wild populations.

The San Francisco Bay Delta region is a system that is in dynamic flux, with a constant input of anthropogenic contaminants. Refineries present in the area introduce petroleum

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hydrocarbons and increase the potential for a spill. Background copper levels range from 2–10 µg/L (Flegal *et al.* 1991). The South Bay in particular has a copper concentration of 5 µg/L, with a salinity of 22‰ (Cayan and Peterson 1993). The environmental model used in this study exposes rotifers to low levels of copper followed by a simulated oil spill and dispersant clean-up and measures hsp60 levels in response to this combination of stressors.

Materials and Methods

Organisms

B. plicatilis, from Southern California Edison, Long Beach, were batch-cultured at 16°C and 34‰ or 22‰ using a 24-h photoperiod (8 h dark/16 h light) and fed golden brown algae (*Isochrysis galbana*). Rotifers were cultured in 2.8-L Fernbach flasks (Fisher Scientific, Pittsburgh, PA) with F/2 nutrients (Fritz Industries, Dallas, TX). Algae (*I. galbana*) were cultured under the same conditions and fed to the rotifers at the end of their log growth phase, 3–4 million cells/ml. Algae at these culturing conditions and density were fed to rotifers in all exposures. Cultures were renewed every 7 days by transferring 1 L of old culture to a new flask and adding 800 ml of fresh seawater at the appropriate salinity and 260 µl of F/2 nutrients. Rotifers were fed 50 ml of algae daily. Typical rotifer cultures reached a density of 40 rotifers/ml before harvesting, exhibiting a doubling time of 7 days. All cultures were aerated with Whisper 700 pumps (Willinger Bros., Oakland, NJ).

Seawater

Seawater (34‰) filtered to 0.2 µm was obtained from the UCSC Long Marine Laboratory, stored at 16°C, and refiltered prior to use. Seawater (22‰) was made by diluting 34‰ with an appropriate volume of Nanopure® water (Barnstead Corp., Dubuque, Iowa), and all salinities were verified by refractometer (S/Mill, Atago, Japan).

Exposure Media Preparations

Water-accommodated fractions (WAF) of oil were prepared according to Wolfe *et al.* (1998). Briefly, 2,000 g of seawater was added to an aspirator bottle affixed with a Teflon stopper, upon which 4 g of Prudhoe Bay Crude Oil (PBCO; R.T. Corp., Laramie, WY) were added. The dispersed oil mixtures (DO) were prepared similarly, but Corexit 9527® (Exxon Chemical Corp., Houston, TX) was introduced at a concentration of 10 µg/g of oil. Finally, dispersant-only mixtures (DISP) were prepared by mixing 2,000 g of seawater and 40 µg of Corexit 9527®. The bottles were maintained at 20°C, covered with aluminum foil, and electromagnetically stirred (LTE Scientific, Greenfield, England) for 24 h at 200 rpm. After 20-min settling, the resulting aqueous phases were termed 100% exposure media (EM) for the exposure preparations and were later diluted with seawater to the desired concentrations.

Toxicity Rangefinding

Toxic threshold studies examined fecundity in exposed populations. Rotifer population growth was monitored over time to determine the level of EM that did not cause a significant reduction in population growth levels after 96 h. A student's *t* test was used to determine statistical significance (Sokal and Rohlf 1987). Preliminary exposures were done at 90% EM, with an initial rotifer density of 10 rotifers/ml in 500 ml of seawater. Rotifers were fed 20 ml of algae/day over 96 h. DO (22‰ and 34‰) preparations inhibited reproduction, requiring a range of lower concentrations (75%, 50%, 25%, and 12.5%) to determine the reproductive toxic threshold.

Characterization of the Heat Shock Response

To identify maximum heat shock response, temperature exposures were conducted at 20, 25, 30, 35, and 40°C. Flasks containing 480 ml culture media (CM, pure seawater), 20 ml of feeding algae, and 65 µl of F/2 nutrients (giving a rotifer concentration of 15 rotifers/ml), were placed in the appropriate temperature water bath for 1 h and allowed to recover at 20°C for 24 h.

To determine the time to induction, a time course was run using the temperature giving maximum response. Flasks, prepared as described above, were placed in 35°C water for 1 h and sampled immediately after the heat shock event, and at 1.5, 3, 6, 12, and 24 h. Controls (24 h) maintained at 20°C were included in all experiments. For time course studies, a sample was taken before the heat shock event to measure constitutive levels of hsp60 and compared against controls run concurrently for 24 h. The 24-h control served to measure hsp60 induction resulting from experimental conditions. A heat-shock (positive) control was also included and consisted of flasks set-up with CM, placed in a 35°C water bath for 2 h, and allowed to recover for 24 h at 20°C.

Petroleum and Dispersant Exposures

Static exposures were conducted in 1-L Erlenmeyer flasks (Fisher Scientific) and prepared as follows: a 50% exposure consisted of 250 ml of exposure media, 230 ml of culture media (CM) or seawater, 20 ml of feeding algae (at a concentration of 3–4 million cells/ml), and 65 µl of F/2 nutrients (giving a total rotifer concentration of 15 rotifers/ml). Remaining concentrations (12.5%, 1.56%, and 0.78% EM) were prepared similarly. Control flasks contained 480 ml CM, 20 ml of feeding algae, and 65 µl of F/2 nutrients. Control and heat-shock flasks contained no EM. Flasks were placed in a cold room (20°C) for 24 h under culturing conditions, after which rotifers were filtered onto an 80-µm nitex screen, transferred to microfuge tubes, and frozen at −80°C.

Copper Exposures

Exposure volumes were all 500 ml, consisting of 460 ml CM, 40 ml of feeding algae (at a concentration of 3–4 million cells/ml), 65 µl of F/2 nutrients, and no EM (giving a rotifer concentration of 15 rotifers/ml). All flasks, except controls and heat-shock, were spiked with 0.5 ml of a 5 mg/l $CuSO_4$ solution (Fisher Scientific) to deliver a final copper concentration of 5 µg/l. After 24-h exposure appropriate volumes of EM and CM were added, for a total volume of 1 L. Additional $CuSO_4$ was added to maintain the exposure concentration at 5 µg/L. A CM-only control, a heat-shock control, and a 24-h and 48-h copper control were run. Exposure conditions and sample processing were the same as described for the oil exposures. Copper concentrations were verified using an inductively coupled plasma mass spectrometer (Element ICP-MS; Finnigan MAT, Bremen, Germany) at the UCSC Marine Analytical Laboratory.

Protein Preparation

Samples were homogenized with a 4710 Series Ultrasonic Homogenizer (Cole-Parmer, Chicago, IL) on ice for 30 s in a homogenization buffer containing 0.3 M sucrose (cat. no. S5-500; Fisher Scientific), 50 mM HEPES (cat. no. H-7523; Sigma Chemical Co., St. Louis, MO), and 1 mM dithiothreitol (DTT; cat. no. 161-0611; Bio-Rad, Hercules, CA). The protease inhibitor aprotinin (cat. no. A-6279; Sigma Chemical) was added in a ratio of 0.5 ml/10 ml of homogenization buffer. Total protein concentration in each sample was determined by the method of Bradford (1976), with bovine serum albumin (BSA; cat. no. A-2153; Sigma Chemical) as a standard and using a Bio-Rad Protein Assay (cat. no. 500-0006; Bio-Rad) on a Lambda 25 UV/VIS Spectrometer (590 nm; Perkin Elmer, Norwalk, CT).

1-D Electrophoresis and Western Blotting

Using the Laemmli (1970) buffer system, samples of equal total protein content (16 µg) were loaded onto a 10% polyacrylamide gel. Gels were run on a Bio-Rad Mini-PROTEAN II Electrophoresis System for 40 min at 200 V (constant) using a PowerPac 300 Power Supply (Bio-Rad) and transferred to a 0.45-µm nitrocellulose membrane (cat. no. 162-0145; Bio-Rad) using the methods of Towbin *et al.* (1979) in a Bio-Rad Mini Trans-Blot Electrophoretic Transfer System. Membranes were blocked in 5% nonfat powdered milk in tris-buffered saline with Tween-20 at pH 8 (TBST; cat. no. P-1379; Sigma Chemical) for 2 h, rinsed in tris-buffered saline with 1% BSA (TBS) at pH 7.2, and probed for 1 h with rabbit anti-hsp60 antibody (cat. no. SPA-804; Stress-Gen, Victoria, BC) in TBS with 1% BSA and 1% nonfat powdered milk (1:3,500 dilution). Membranes were then rinsed in TBST and probed with the secondary anti-rabbit antibody (cat. no. RPN 2108; Amersham, Buckinghamshire, England) in TBS with 1% BSA and 1% nonfat powdered milk for 1 h, a 1:8,500 dilution, followed by another rinse in TBST. A chemiluminescent tag (ECL; cat. no. RPN 2108; Amersham) was added to the antibody for autoradiography detection using Kodak X-Omat™ AR film (Kodak Eastman, Rochester, NY). Quanitation was done using a Model 620 Video Densitometer (Bio-Rad) and all values for hsp60 levels were reported as percent of controls.

Statistical Analysis

Data was transformed by taking the natural log (ln) of the treatment value divided by the control measurement. A two-way ANOVA was then run using replicate and oil/dispersant preparation concentrations as the two effects. Control data were used to establish that the treatment had an effect on hsp60 levels. In all cases the two-way ANOVA showed a high level of significance. Given that the overall F test was significant, a protected least significant difference (PLSD) was used to examine the significance level between each treatment and control value as well as between individual treatments (Sokal and Rohlf 1987).

Results

Establishment of Chronic Dose

The concentration of exposure media that did not significantly effect rotifer population growth was determined for the different oil/dispersant preparations at both salinities. It was not possible to obtain an LC_{50} for the different oil/dispersant preparations used in this study. None of the concentrations examined (up to 90% exposure media) proved to be lethal. Therefore the growth of the population over 96 h was used as an indicator of toxicity. Even at 90% EM the rotifer populations continued to grow, increasing three to five times inoculation levels over the 96-h static exposure. However, this proved to be a significant inhibition of reproduction ($p < 0.05$) at both salinities. Exposures with lower concentrations indicated 25% DO results did not vary from controls at 34‰ ($p > 0.01$) and 50% DO at 22‰ ($p > 0.01$). All 22‰ exposures were therefore conducted with a maximum of 50% EM.

Optimization of Heat Treatment

Induction of heat shock response occurred under a range of temperatures (20–40°C). Both salinities responded similarly to the heat challenge with increasing hsp60 up to 35°C and significant mortality at 40°C. Maximal response at both 22‰ and 34‰ was seen at 35°C, significantly increasing over control values ($p < 0.05$). Heat shock (positive) controls (35°C) in all samples resulted in a hsp60 response significantly greater than control levels ($p < 0.05$). For all experiments, the heat shock controls increased three- to fourfold over control values.

Time to Induction

Results of the time course studies showed a significant increase ($p < 0.05$) in hsp60 up to 24 h following the initial heat shock event at both 34‰ and 22‰. The 24-h controls at 22‰ and 34‰ gave a significant increase over initial hsp60 levels ($p < 0.05$). However, this elevation could still be discriminated from the heat-shocked rotifers. All results are presented as percent of the 24-h control. Thus the level of stress resulting from the exposure conditions is accounted for in the presentation of the data.

Effect of Salinity

A set of rangefinding exposures was conducted at 34‰ over a narrower concentration range (25% EM to 3.12% EM) to examine the effect of salinity on the heat shock response. Results from these exposures proved to be nearly identical to those at 22‰ demonstrating that the salinities tested did not significantly change hsp60 response. Given that 22‰ and 34‰ responded similarly, further studies were conducted at only 22‰.

Effect of Water Accommodated Fractions (WAF)

Results from the WAF exposures varied with concentration (Figure 1). The exposures produced levels of hsp60 that were twice that of control values at lower WAF concentrations. However, at the higher concentrations examined, the levels of hsp60 were not statistically different from controls. While differences in hsp60 levels between high and low WAF exposures were observed, any statistically significant trend was masked by the large standard error in the results. Exposures

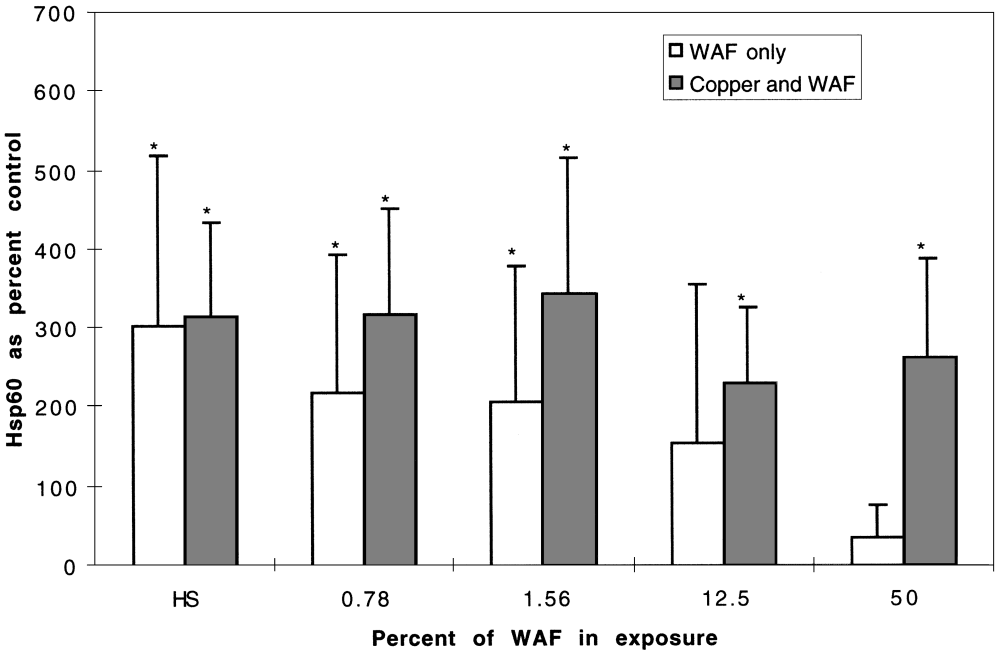

Fig. 1. Comparison of hsp60 response to the water-accommodated fraction preparation (WAF) of Prudhoe Bay crude oil (PBCO) versus WAF with copper preexposure. For WAF-only exposures rotifers were exposed to a range of concentrations of WAF for 24 h at 20°C. For copper and WAF exposures rotifers were exposed to 5 µg/L of copper ($CuSO_4$) for 24 h followed by copper and WAF for an additional 24 h at 20°C. After 24 h, rotifers were filtered and frozen at −80°C. Concentrations are represented as percentage of WAF, and hsp60 response is given as percent of control. HS = heat shock control consisting of a 1 h heat shock at 35°C followed by recovery for 24 h at 20°C (*denotes a statistically significant difference from control with $p < 0.05$, $n = 4$)

with copper (5 µg/L) induced levels of hsp60 two to three times those of control. Samples which were exposed to copper for 24 h maintained those hsp60 levels upon subsequent exposure to WAF at all concentrations. These hsp60 levels in the presence of copper and WAF proved to be statistically different from controls ($p < 0.05$), but not from each other. No dose-response relationship was seen, the heat shock response appeared to be either on or off with no gradation in the response.

Effect of Dispersed Oil (DO)

The DO exposures produced levels of hsp60 that could not be discriminated yet were all significantly greater than controls ($p < 0.05$). Significant variation among the individual exposures was not observed and needless to say, a dose-response relationship was absent. Figure 2 compares the hsp60 levels resulting from DO exposures alone with those resulting from DO and copper. All exposures resulted in hsp60 levels with values three to four times greater than controls. The copper exposures resulted in levels of hsp60 that were not statistically different from levels produced in noncopper exposures.

Effect of Dispersant (DISP)

Results from DISP exposures were statistically indistinguishable from DO exposures and again were all significantly greater than controls ($p < 0.05$). Hsp60 levels produced from DISP exposures alone versus those from DISP and copper are shown in Figure 3. Exposures resulted in a four- to fivefold increase in hsp60 levels over controls. All copper and noncopper DISP exposures induced levels of hsp60 that could not be discriminated from any of the DO exposures or from each other.

Copper Exposures

Copper controls had an average copper concentration 5.4 µg/L greater than unspiked seawater controls as measured by ICP-MS. Copper gave a hsp60 response significantly greater than controls ($p < 0.05$). There was no statistical difference in the hsp60 levels produced after 24 h and 48 h exposure to copper. Copper controls resulted in hsp60 levels three to five times those of controls. A typical autoradiograph demonstrating the response for copper and WAF at 22‰ is displayed in Figure 4.

Discussion

In the event of a petroleum release, chemical-dispersing agents may be employed to increase the solubility of the crude oil. There is concern over the toxicity of dispersed oil, as well as the dispersing agents, to marine organisms. To further address this issue, this study examined the effects of a water-accommodated fraction (WAF) of Prudhoe Bay crude oil, a dispersed oil preparation (DO), and a dispersant alone preparation (DISP) at the cellular level by quantifying changes in hsp60 levels in response to these toxicants. If baseline constitutive levels of hsp60 were established in rotifers in an area at risk, *e.g.*, the San

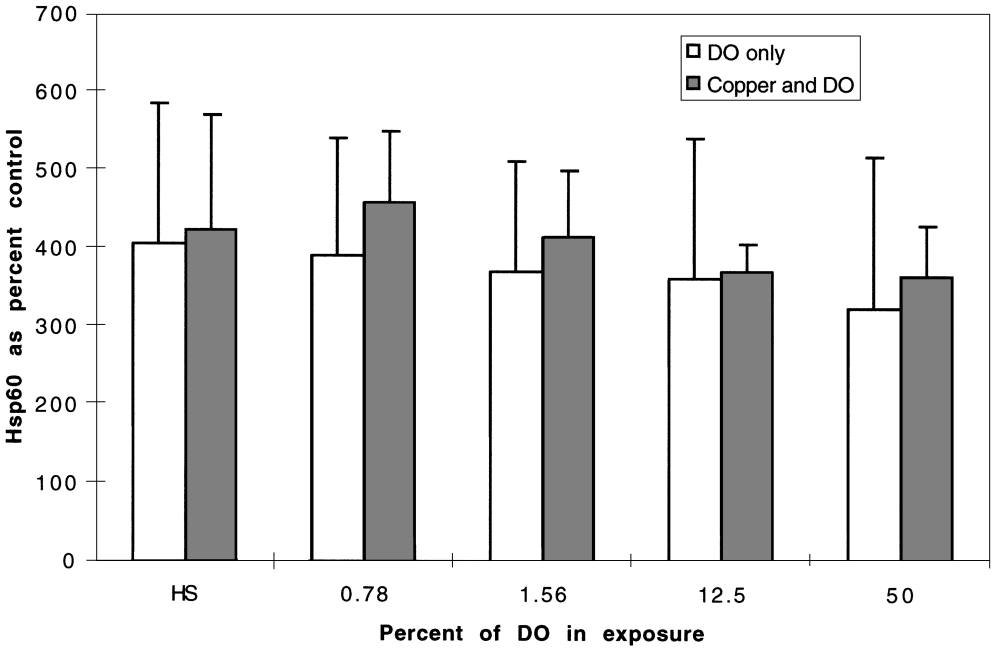

Fig. 2. Comparison of hsp60 response to a preparation of Prudhoe Bay crude oil and the chemical dispersant Corexit 9527® (DO) versus DO with copper preexposure. For DO-only exposures rotifers were exposed to a range of concentrations of DO for 24 h at 20°C. For copper and DO exposures rotifers were exposed to 5 µg/L of copper ($CuSO_4$) for 24 h followed by copper and DO for an additional 24 h at 20°C. After 24 h, rotifers were filtered and frozen at −80°C. Concentrations are represented as percentage of DO, and hsp60 response is given as percent of control. HS = heat shock control consisting of a 1 h heat shock at 35°C followed by recovery for 24 h at 20°C (all points significantly greater than control with $p < 0.05$, $n = 4$)

Francisco Bay, then changes in those levels could be monitored following a petroleum release. However, this would require working with wild populations of rotifers instead of physiologically similar rotifers hatched from cysts. Toward this end, this work used batch cultured rotifers in an attempt to model natural variation to asses the possibility of use of wild populations in monitoring.

In *B. plicatilis*, both salinities produced a similar response of increasing production of hsp60 with increasing temperature. This is in agreement with results published by Cochrane *et al.* (1991). Once the temperature of maximal hsp60 induction without significant mortality was determined, it was possible to monitor the response over time. The time-course study showed that hsp60 levels steadily increased following the stress event. The study was not extended past 24 h to avoid measuring stress due to lack of food and waste product build-up. Of particular interest is the speed of onset of the stress response. Immediately following the stress event, significantly elevated levels of hsp60 were measured. Due to the extremely large standard deviations in individual values, it was not possible to obtain any significance between individual time points. However, all time points up to 24 h were significantly elevated over controls. Given that hsp60 levels remained elevated throughout the period, 24 h was chosen to achieve maximal induction of hsp60 while avoiding other sources of stress. The 24-h controls demonstrated that there was a significant level of stress associated with the exposure conditions. Several sources of inherent stress exist, including physical manipulation of the rotifers, waste product accumulation, and change in aeration. However assuming this underlying stress did not affect response to exposures and that these stresses were relative, presentation of results as percent of controls allowed for the extraction of meaningful data.

Since DO was the only exposure media capable of inhibiting rotifer growth rate, it appeared to be the most toxic of the three preparations. The definition of toxicity therefore became important. In this study, toxicity refers solely to something that is proteotoxic or capable of affecting protein synthesis. Therefore while DO appeared to be the most toxic in terms of ability to inhibit growth, its proteotoxic capability, as indicated by hsp60 induction, had to be examined along with the other preparations.

Rangefinder exposures conducted at 34‰ gave a similar response to exposures at 22‰. Therefore the brackish salinity (22‰) was used for further tests. This result demonstrates that salinity does not effect the stress response in *B. plicatilis* at salinity ranges observed in San Francisco Bay. A tolerance to variance in salinity was expected given that this species is euryhaline.

In comparing the toxicity of dispersed versus undispersed oil, an unexpected result was observed. It was anticipated that higher concentrations of the oil/dispersant mixtures would result in elevated levels of hsp60. However, the WAF preparation resulted in hsp60 levels that were not significantly different from control at higher concentrations. Only the extremely low levels of WAF produced values of hsp60 significantly greater than controls. Considering that all exposures were done below the 96-h reproductive toxic threshold for all oil/dispersant mixtures at both salinities and the fact that lower concentrations of WAF did induce hsp60 at levels significantly greater than control levels, the oil would be expected to provoke a response

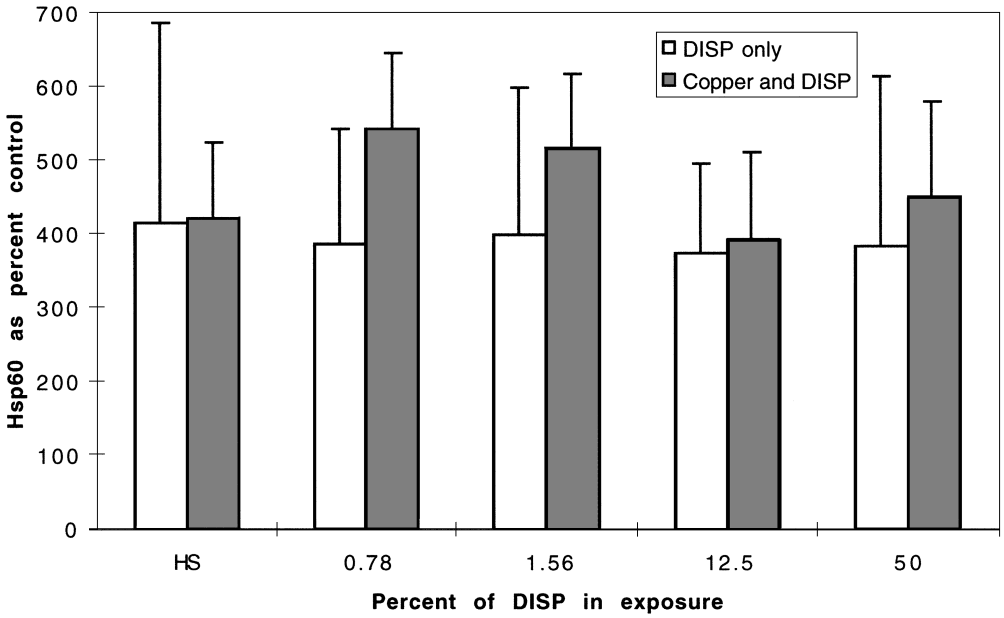

Fig. 3. Comparison of hsp60 response to a chemical dispersant preparation, Corexit 9527® (DISP) versus DISP with copper preexposure. For DISP only exposures rotifers were exposed to a range of concentrations of DISP for 24 h at 20°C. For copper and DISP exposures rotifers were exposed to 5 µg/L of copper ($CuSO_4$) for 24 h followed by copper and DISP for an additional 24 h at 20°C. After 24 h, rotifers were filtered and frozen at −80°C. Concentrations are represented as percentage of DISP, and hsp60 response is given as percent of control. HS = heat shock control consisting of a 1 h heat shock at 35°C followed by recovery for 24 h at 20°C (all points significantly greater than control with $p < 0.05$, $n = 4$)

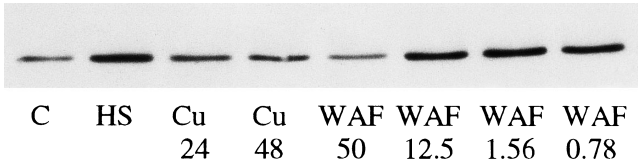

Fig. 4. Autoradiograph demonstrating the hsp60 response for copper (5 µg/L) and the water-accommodated fraction preparation (WAF). Rotifers were exposed to copper (5 µg/L) and a range of concentrations of WAF for 24 h at 20°C. Concentrations are represented as percentage of WAF. HS = heat shock control consisting of a 1 h heat shock at 35°C followed by recovery for 24 h at 20°C. Cu24 = the 24-h copper control, consisting of a 24-h exposure to copper (5 µg/L) at 20°C. Cu48 = the 48-h copper control, consisting of a 48-h exposure to copper (5 µg/L) at 20°C

at higher concentrations. Therefore, the explanation that the WAF was toxic enough to inhibit successful stress protein synthesis appears plausible. Whether this effect is translational, posttranslational, or transcriptional would require additional investigation. Cochrane *et al.* (1994) were able to measure stress related changes in mRNA abundance in *B. plicatilis.*

The DO and DISP mixtures elicited very similar responses at both salinities. There was no dose-response relationship for either preparation. Hsp60 levels were elevated at all exposure concentrations, and there was no statistical difference between them. The question of which preparation is more toxic to the rotifers becomes difficult to answer. The ability to inhibit hsp60 induction may indicate a more toxic response then producing elevated levels of hsp60.

The complications resulting from the effects of the oil and dispersant on hsp60 levels illustrate the importance of developing assays that examine the effects of more than one stressor. The use of copper as a second stressor dramatically affected the results. After the initial 24-h exposure, copper induced hsp60 in all samples. This was expected given the results of Cochrane *et al.* (1991), who demonstrated that *B. plicatilis* produces increased levels of hsp60 in response to sublethal doses of copper. The 24-h LC_{50} for copper in *B. plicatilis* ranges from 63 µg/L (Snell *et al.* 1991) to 130 µg/L (Snell and Persoone 1989a). There was no difference in hsp60 levels following 24-h or 48-h exposure to copper. When oil/dispersant mixtures were added, elevated hsp60 levels in the organism showed a relatively small further increase in hsp60 level that was not significant. The sum of the individual copper exposure and the individual oil/dispersant exposure was greater than the level of hsp60 produced by the combined exposure. This response suggests that the stress response observed was not synergistic or additive in nature.

Comparison of the response between the individual oil/dispersant exposures alone and those which had the copper preexposure treatment was notable. For WAF, the copper preexposure served to elevate hsp60 levels, which remained for the duration of the ensuing WAF exposure. All concentrations of WAF with copper preexposure resulted in hsp60 levels that were significantly elevated over controls in contrast to the WAF alone, where hsp60 levels were not significantly elevated. It is possible that the copper elicited response was sufficient to protect the organism from WAF-induced stress.

The DO and DISP exposures produced no dose-response relationship. Hsp60 levels were elevated at all exposure concentrations, and there was no statistical difference between them. The copper preexposure resulted in significantly increased levels of hsp60 over controls, and the further addition of DO or DISP caused an elevation in hsp60 levels, but it was not

significant. Further investigation should address the possibility of a toxic threshold.

Previous studies with *B. plicatilis* have used cysts instead of batch cultures (Cochrane *et al.* 1991). This study examined the utility of batch cultures for stress protein work. The data obtained showed very large standard errors, thus, although it was still possible to extract significance out of the data, the wide level of variance made most comparisons not statistically significant. All results are presented as percentage of control, thereby accounting for variance between samples. However, smaller standard errors would allow for a more meaningful interpretation of the data. Given that the goal of this work was to investigate whether it would be possible to use hsp60 levels in wild populations of rotifers as an indicator of stress, the lack of variation and sensitivity in the response indicates that this application would not be appropriate. However, Cochrane *et al.* (1991) observed a dose-response relationship of hsp60 levels toward increasing levels of copper. Therefore additional study should examine the dose-response relationship in batch-cultured versus cyst-hatched rotifers.

The level of hsp60 increased in *B. plicatilis* exposed to a range of concentrations of oil and dispersant as well as copper at 5 µg/L. These data suggests that WAF is more toxic than DO and DISP in terms of its ability to induce hsp60, but that both DO and DISP are capable of causing significant proteotoxicity to *B. plicatilis.* However, this interpretation cannot be verified without further study of the effects of WAF on protein synthesis and mRNA levels. Results also indicate that the stress response is a poor indicator of overall stress for the toxicants used in this study given that there is no dose-response relationship for any of the exposures. The possible protective nature of stress proteins was demonstrated by the elevated levels of hsp60 resulting from exposure to WAF following preexposure to copper. It is possible that rotifer populations in a polluted region may adapt to their conditions and subsequently have higher constitutive levels of hsp60 than rotifers living in a more pristine area. For this reason it would be interesting to examine wild populations of rotifers. The techniques employed in this work to measure hsp60 levels in batch cultures could be applied to wild populations.

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