ELSEVIER

Effect of nutritional state on Hsp60 levels in the rotifer *Brachionus plicatilis* following toxicant exposure

C.E. Wheelock [a], T.A. Baumgartner [b], J.W. Newman [a], M.F. Wolfe [b], R.S. Tjeerdema [c],*

[a] *Department of Entomology, University of California, One Shields Avenue, Davis, CA 95616, USA*
[b] *Institute of Marine Sciences, University of California, Santa Cruz, CA 95064, USA*
[c] *Department of Environmental Toxicology, University of California, One Shields Avenue, Davis, CA 95616, USA*

Abstract

The nutritional state of an organism can affect the results of toxicity testing. Here we exemplified this fact by examining the effect of nutritional deprivation on heat shock protein 60 (hsp60) production in the rotifer *Brachionus plicatilis* following exposure to two proven inducers of hsp60, a water-accommodated fraction of crude oil (WAF) and a dispersed oil preparation (DO). Both DO and WAF exposures of unfed rotifers resulted in significantly greater hsp60 levels than that of fed DO and WAF exposed rotifers at 8 h: 870 and 3100% of control, respectively. Results clearly demonstrate that a poor nutritional state potentiates stress protein induction upon exposure to water-soluble petroleum products. It is therefore critical to define the organismal nutritional status when reporting toxic responses. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Nutrition; Stress protein; Rotifer; Crude oil; Chemical dispersant

Toxicity studies are extensively used to evaluate the effects of a given toxicant upon organismal health. However, many tests do not appear to significantly evaluate the effects of exposure conditions upon the overall test outcome. While time consuming, failure to identify and optimize critical experimental parameters can lead to variability that can mask or amplify the effects of the toxicant being studied. Of particular importance is the nutritional state of the organism. It has been demonstrated that food limitation can increase organism sensitivity to toxicants (Kooijman and Metz, 1984), which in turn can significantly affect experimental outcome (Cecchine and Snell, 1999). This study was therefore designed to examine the effect of nutritional state upon a common sub-lethal toxicity marker, levels of the 60 kDa heat shock protein (hsp60), in the euryhaline rotifer *Brachionus plicatilis*. The results showed that a compromised nutritional regimen dramatically enhances the induction of hsp60 in the event of toxicant exposure.

* Corresponding author. Tel.: +1-530-754-5192; fax: +1-530-752-3394

E-mail address: rstjeerdema@ucdavis.edu (R.S. Tjeerdema).

Organisms must be able to adapt to a constantly changing external environment for survival. One mechanism cells use to respond to adverse environmental conditions is the synthesis of stress proteins, which are thought to act as molecular chaperones and assist in maintaining protein integrity (Becker and Craig, 1994). Stress protein induction has been assessed for a wide variety of aquatic organisms and stressors (Sanders, 1993). In particular, *B. plicatilis* has been demonstrated to be a good system for both toxicity assays and stress protein studies (Cochrane et al., 1991; Snell and Janssen, 1995; Wheelock et al., 1999).

Previously, hsp60 production was shown to increase in *B. plicatilis* exposed to aqueous crude or chemically dispersed oil (Wheelock et al., 1998, 1999), two forms simulating possible conditions following an oil spill. The first was a water-accommodated fraction of crude oil consisting of the dissolved aqueous fraction of Prudhoe Bay crude oil (PBCO; EPA Standard, RT Corp, Laramie, WY). The second was dispersed oil representing the fraction of oil introduced into the water column following treatment of an oil spill with the chemical dispersing agent Corexit 9527® (Exxon Chemical Co). Corexit 9527® consists of 48% nonionic surfactants (ethoxylated sorbitan monooleate, ethoxylated sorbitan trioleate, and sorbitan monooleate), 35% anionic surfactant (sodium dioctyl sulfosuccinate), and 17% hydrocarbon solvent (ethylene glycol monobutyl ether). With dispersed oil, a larger fraction of dissolved components is found in the water column, increasing the bioavailability to nearby microalgae (Wolfe et al., 1999) and subsequently the rest of the food chain.

The acute toxicity of pollutants to rotifers is better understood than the sub-lethal effects indicated by changes in stress protein levels (Ferrando and Andreu-Moliner, 1992; Snell and Janssen, 1995). In addition, nutritional stress also induces stress protein production (Sanders, 1993; Bernhardt et al., 1997) and reduces rotifer fecundity and subsequently population viability (Cecchine and Snell, 1999). However, little is known of the combined influence of nutritional state and response to xenobiotic exposures. Therefore, the objective of this investigation was to determine the influence of nutritional state on the production of hsp60, a sub-lethal endpoint, in the euryhaline rotifer *B. plicatilis* when exposed to aqueous soluble fractions of crude oil and dispersed oil.

Experimentally, rotifers were obtained from Southern California Edison (Long Beach, CA) and cultured according to conditions described by Wheelock et al. (1999). Rotifers were fed 50 ml of the golden brown algae *Isochrysis galbana* at a density of 3–4 million cells per ml daily unless stated otherwise. Crude oil exposure media (EM) was prepared by stirring 4.0 g of PBCO with or without 40 μl Corexit 9527® in 2.0 kg of 34‰ seawater using recently published standardized procedures (Wolfe et al., 1999; Singer et al., 2000). Oil and water mixtures yielded the water-accommodated fraction (WAF), while inclusion of dispersant produced aqueous fractions termed dispersed oil (DO). While water-accomodated fractions contain a complex hydrocarbon mixture, the suggested chemical characterizations for toxicity testing has been defined as the total hydrocarbon content (THC), equal to the sum of volatile hydrocarbons and the total C_{10}–C_{36} petroleum hydrocarbon profile (TPH) (Singer et al., 2000). The volatiles, commonly referred to as BETX, and are primarily the sum of benzene, ethyl benzene's, toluene and xylene's. Using the EPA PBCO standard reference material at the current loading rate of 2 g/l, the chemical composition of the WAF and DO fractions are ∼ 2 and 100 mg/l THC, respectively (Singer et al., 2001). The resulting mixtures were diluted to 25% EM with 34‰ seawater for the rotifer exposures. Neither WAF and DO dilutions nor the 100% WAF reduced rotifer fecundity. A 50% DO dilution, however, did affect rotifer fecundity in a salinity dependent manner (Wheelock et al., 1999).

Rotifer exposures were run in triplicate using the following scheme: rotifers (∼ 7500) were fed on their normal feeding schedule 12 h prior to exposure. It was determined that rotifers cleared all algae from the cultures after 12 h. The rotifers were then filtered into 500 ml of 34‰ exposure media (either 25% WAF or 25% DO), with 'Fed' samples receiving 20 ml of algae in seawater (∼

3–4 million cells per ml), and 'Not Fed' receiving only 20 ml of 34‰ seawater. Rotifers were exposed for either 8 or 24 h. Following exposure, rotifers were vacuum filtered and frozen at −80 °C. Upon thawing, the samples were homogenized and analyzed using Western blots as previously described (Wheelock et al., 1999). A rabbit anti-hsp60 antibody (Stress-Gen, Victoria, BC) was used for the primary antibody, followed by a goat anti-rabbit secondary antibody (Amersham, Buckinghamshire, England) and visualized with a chemiluminescent tag (ECL, Amersham). Hsp60 levels in individual experiments were normalized to percent of control due to significant inter-gel variation, with each separate blot containing both experimental and control data as shown in Fig. 1.
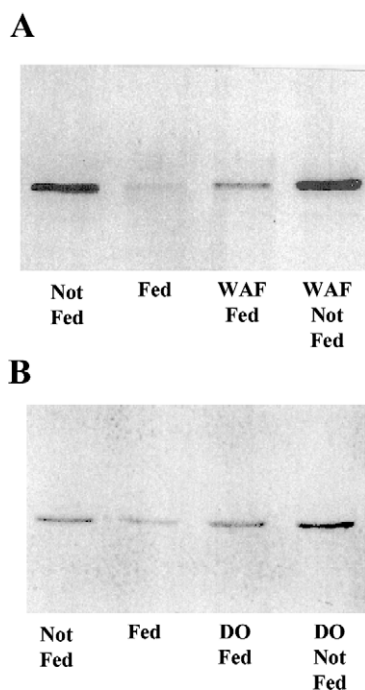

Fig. 1. Autoradiographs of the hsp60 response of rotifers after 24 h of exposure to 25% dilutions of either a water-accommodated fraction (WAF) of crude oil (A) or a dispersed oil (DO) preparation (B). Rotifers were either fed on their normal feeding regimen (Fed) or given an equivalent volume of seawater (Not Fed). Only intra-gel comparisons are valid due to the high degree of inter–gel variability. The 8 h exposures yielded similar patterns, with different intensities as displayed in Figs. 2 and 3.
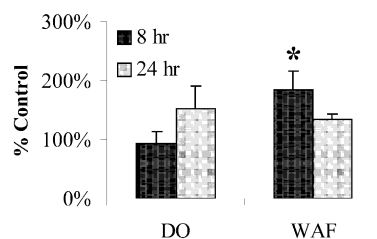

Fig. 2. Effect of chemically dispersed oil (DO) and a water-accommodated fraction (WAF) of crude oil upon hsp60 levels in the marine rotifer *Brachionus plicatilis*. Rotifers were exposed to a 25% solution of the DO or WAF preparation diluted in 34‰ seawater for either 8 or 24 h and fed a normal diet of the golden brown algae *Isochrysis galbana*. Following exposure, rotifers were filtered and frozen at −80 °C. Hsp60 responses measured from Western blots are represented as percent of fed controls (* indicates statistical difference from control, $P < 0.05$; $n = 3$).

Toxicant exposure combined with a normal feeding regimen produced only mild effects on hsp60 levels in either DO or WAF exposed rotifers (Fig. 2). The 8 h WAF exposure elicited the only statistical increase of hsp60 levels: 150% of control ($P < 0.05$, Students T-test). While nutritional deprivation alone enhanced hsp60 levels (Not Fed ~ 200–300% of Fed; data not shown), the combination of nutritional deprivation with toxicant exposure yielded substantial increases above this elevated background (Fig. 3). Both DO
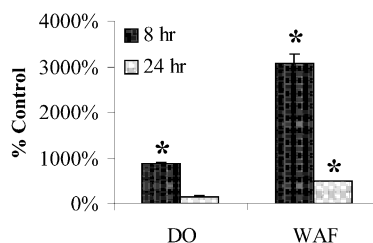

Fig. 3. Effect of nutritional state upon hsp60 levels in the marine rotifer *Brachionus plicatilis* following exposure to chemically dispersed oil (DO) and a water-accommodated fraction (WAF) of crude oil. Rotifers were exposed to a 25% solution of the DO or WAF preparation diluted in 34‰ seawater for either 8 or 24 h and deprived food for the length of the toxicant exposure. Following exposure, rotifers were filtered and frozen at −80 °C. Hsp60 responses measured from Western blots are represented as percent of fed controls exposed to either DO or WAF (* indicates statistical difference from control, $P < 0.05$; $n = 3$).

and WAF exposures of Not Fed rotifers resulted in significantly greater hsp60 levels than that of Fed DO and WAF exposed rotifers at 8 h: 870 and 3100% of control, respectively. These changes were transient, however, and at 24 h, only the WAF treatment maintained hsp60 levels significantly greater than controls (490%). While algae will absorb WAF/DO components during Fed exposures (Wolfe et al., 1999) resulting in both dietary and aqueous exposures, the rotifers had cleared all algae from the system within 24 h. Therefore, Fed rotifers would have accumulated a dose equivalent to the total algal toxicant load (minus metabolism and excretion), plus the toxicant directly absorbed from the water column. Dietary exposure would therefore be expected to further increase Fed rotifer exposure relative to unfed. However, the hsp60 response levels for Fed exposures were approximately an order of magnitude below those of Not Fed rotifers, suggesting that the hypothetical increase in toxicant load resulting from two routes of exposure was not as significant as the effects of nutritional deprivation. These results indicate that nutritional deprivation synergizes the hsp60 response to toxicant exposure in the rotifer. The transient nature of hsp60 levels may be due to either organismal death or changes in protein production or degradation. This trend observed in Fed populations was amplified in Not Fed populations, suggesting a similar underlying mechanism in each group. No acute organismal toxicity was observed in control organisms during these experiments as evaluated by microscopic examination and reproduction rates. If elevation of hsp60 levels were performing a direct protective role in the organism, a constant level of the protein would be expected for the duration of toxicant exposure. Alternatively, hsp60 may simply stabilize newly synthesized proteins. In this case, if the toxicant insult induced metabolic defense systems, once these mechanisms were in place, the need for hsp60 would diminish and proteolytic enzymes would degrade the protein. This scenario provides one possible rationale for the transient behavior of hsp60 observed here.

The toxicants DO and WAF are known inducers of hsp60 in *B. plicatilis* and the level of hsp60 induction seen in this study compares well to reported levels (Wheelock et al., 1999), with Fed WAF exposures resulting in hsp60 levels only slightly greater than control values. Wheelock et al. reported a greater induction of hsp60 levels following exposure to DO than was observed in this study. However, the differences between values reported in this study and those of Wheelock et al. do not differ greatly and fall within the range of the respective standard deviations. Variations in the absolute numbers could results from differences in sample handling and experimental conditions such as the nutritional state of the organisms as evidenced in this study.

The results of this study show that, in the case of rotifers, nutritional status can potentiate toxic responses. These results raise the issue of appropriate exposure conditions for toxicity testing. Cecchine and Snell (1999) reported that most toxicity tests performed with rotifers are done under ideal nutritional conditions. These conditions represent an abundant unlimited food supply and therefore, artificially inflate population health and fecundity. Normal field conditions for rotifers would more likely be food limiting, such as those present in oligotrophic waters. The results of this study show that food limitation can greatly alter the effects of toxicity testing, with marginal toxic responses being greatly amplified in nutritionally deprived organisms. To aid in extrapolation of laboratory results to environmental toxicity, this data suggests that it will be more appropriate to conduct toxicity testing under conditions that more accurately reflect those that the organism would experience in its natural environment.

The results of this study can probably be extended to other xenobiotics and organisms, and possibly other toxicological endpoints. These results also suggest the need to monitor other physiological variables in the exposure scenario when evaluating stress proteins, including test organism overcrowding, light stress, waste production, temperature, handling, etc. It is clearly not possible to test and account for all physical and physiological variables. It is therefore, necessary to rigorously define the system employed for the toxicity testing and to qualify the results to that of the system

employed for the analysis. If the appropriate physiological effects are not accounted for, then other effects inherent in the experimental design may overshadow the toxic endpoint being studied, making interpretation ambiguous, and comparability with independent studies difficult. It is also important to define the question being asked in the study. If the project is designed to probe the response of the organism in its natural environment, then experimental parameters may need to be adjusted to accurately reflect the organism's normal surroundings.

Acknowledgements

References

Becker, J, Craig, A., 1994. Heat-shock proteins as molecular chaperones. Euro. J. Biochem. 219, 11–23.

Bernhardt, J., Volker, U., Volker, A., Antelmann, H., Schmid, R., Mach, H., Hecker, M., 1997. Specific and general stress proteins in *Bacillus subtilis*-a two-dimensional protein electrophoresis study. Microbiology 143, 999–1017.

Cecchine, G, Snell, T.W., 1999. Toxicant exposure increases threshold food levels in freshwater rotifer populations. Environ. Toxicol. 14, 523–530.

Cochrane, B.J., Irby, R.B., Snell, T.W., 1991. Effects of copper and tributyltin on stress protein abundance in the rotifer *Brachionus plicatilis*. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 98, 385–390.

Ferrando, M.D., Andreu-Moliner, E., 1992. Acute toxicity of toluene, hexane, xylene, and benzene to the rotifers *Brachionus calyciflorus* and *Brachionus plicatilis*. Bull. Environ. Contam. Toxicol. 49, 266–271.

Koojiman, S.A.L.M., Metz, J.A.J., 1984. On the dynamics of chemically stressed populations: the deduction of population consequences from effects on individuals. Ecotox. Environ. Saf. 8, 254–274.

Sanders, B.M., 1993. Stress proteins in aquatic organisms: an environmental perspective. Crit. Rev. Toxicol. 23, 49–75.

Singer, M.M., Aurand, D., Bragin, G.E., Clark, J.R., Coelho, G.M., Sowby, M.L., Tjeerdema, R.S., 2000. Standardization of the preparation and quantitation of water-accommodated fractions of petroleum for toxicity testing. Mar. Pollut. Bull. 40, 1007–1016.

Singer, M.M., Jacobson, S., Tjeerdema, R.S., Sowby, M.L., 2001. Acute effects of fresh versus weathered oil to marine organisms: California findings. Proceedings of the 2001 International Oil Spill Conference, American Petroleum Institute, Washington, DC, pp. 1263–1268.

Snell, T.W., Janssen, C.R., 1995. Rotifers in ecotoxicology: a review. Hydrobiologia 313, 231–247.

Wheelock, C.E., Wolfe, M.F., Olsen, H.E., Tjeerdema, R.S., 1998. Characterization of hsp60 response in the rotifer, *Brachionus plicatilis*, exposed to multiple environmental contaminants. Mar. Environ. Res. 46, 453–456.

Wheelock, C.E., Wolfe, M.F., Olsen, H.E., Tjeerdema, R.S., Sowby, M.L., 1999. Hsp60-induced tolerance in the rotifer *Brachionus plicatilis* exposed to multiple environmental contaminants. Arch. Environ. Contam. Toxicol. 36, 281–287.

Wolfe, M.F., Olsen, H.E., Gasuad, K.A., Tjeerdema, R.S., Sowby, M.L., 1999. Induction of heat shock protein (hsp)60 in *Isochrysis galbana* exposed to sublethal preparations of dispersant and Prudhoe Bay crude oil. Mar. Environ. Res. 47, 473–489.