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Mytilus trossulus hsp70 as a biomarker for arsenic exposure in the marine environment: Laboratory and real-world results

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Abstract

The highly conserved heat shock protein 70 (hsp70) is induced by heat and chemical toxins, particularly heavy metals such as arsenic (As). The use of *Mytilus trossulus* (bay mussel) hsp70 as a 'screening' biomarker for marine heavy metals contamination was assessed. Some studies have found high hsp70 sensitivity to heavy metals, while others have found the opposite. Few studies have realistically used low heavy metals exposures, and fewer have used real-world contamination exposures. Clean sub-tidal mussels from the Puget Sound, Washington State (WA), USA, were acclimatized for 2 weeks and exposed for 24 h to As-spiked seawater ($n=9$) or to contaminated seawater from an arsenical pesticide plant in Tacoma, WA ($n=10$) followed by a Western blot for hsp70. Hsp70 inductions were insignificant at 10 μg l^{-1} As^{III}, but were strong at 100 μg l^{-1} ($p<0.05$) and 1000 μg l^{-1} ($p<0.01$), with the induction threshold estimated at 30–50 μg l^{-1} As^{III}. Hsp70 induction roughly correlated with arsenical toxicity, with $As^{III} > As^{V} > (CH_3)_2As^{V}$. Altogether, the inter-individual variability of hsp70 levels tends to mask inductions at low As concentrations, making it a crude toxicity biomarker. In addressing this problem, the following options could prove promising: (1) pre- or post-stressing specimens for greater hsp70 sensitivity, (2) use of internal protein controls such as actin, (3) use of hsp70-reporter gene constructs, and (4) detection with hsp60, heme oxygenase-1, metallothionein, CYP450, MXR or GPx.

Keywords: *Heat shock protein (hsp) 70, arsenic, arsenite, arsenate, mussels,* Mytilus trossulus.

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Introduction

US shoreline industrial facilities routinely commission independent laboratories to conduct Environmental Site Assessments (ESAs) to meet Environmental Protection Agency (EPA) and National Pollution Discharge Elimination System requirements. Water, soil, sediment and tissue specimens contaminated with heavy metals are analysed using spectroscopic methods, typically AAS ± HG, GFAA ± HG, ICP-MS or AAF-MS. A suite of heavy metals including arsenic (As), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), mercury (Hg), nickel (Ni), lead (Pb) and zinc (Zn) may be analysed in the 0.01 μg l^{-1} sensitivity range, with QA/QC procedures requiring additional blanks, spikes and replicates. A Phase I ESA for a small site routinely totals US$20 000–100 000, while a Superfund-class site may total in the millions of dollars.

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Often, only a small percentage of the samples will show contamination by two or three specific metals. In the process, significant resources are lost analysing samples that will turn out to be free from contamination.

Hsp70 is strongly induced in eukaryotes by a variety of organic and inorganic toxins, including heavy metals (Feder & Hofmann 1999). The hypothesis in the present study is that hsp70 expression in *Mytlius trossulus* (bay mussel) could serve as an integrated indicator of toxicity. Samples that induce hsp70 expression could be 'flagged' for analysis by more precise but more costly and quantitative spectroscopic techniques, making ESAs less costly and more effective. Indeed, Xenometrix (DPI) (Boulder, Co, USA) has commercially developed the 'CAT-Tox(L)' HepG2 cell line toxicity assay, which includes the hsp70 promoter and which has been recently used for assessing the physiological response to heavy metals such as As (Tully et al. 2000, Tchounwou et al. 2003). Similarly, several hsp70-reporter gene cell line constructs using bioluminescence, CAT or lacZ have been developed for research purposes.

The EPA currently recognizes the use of whole-organism exposure bioassays, in which the toxicity of water samples is assessed from a model organism's physiological response over a period of days (Goulden 1999). The EPA has approved an alga, *Daphnia*, and the fathead minnow as model organisms, as well as mortality, scope-for-growth, tissue pathologies and reproductive failure as assessment parameters (EPA 2003). The EPA has not yet approved the use of more sensitive protein biomarkers. However, cytochrome P450 (CYP1A) assays detecting PAH and PCB contamination have been recently developed and validated in the field by the National Oceanic and Atmospheric Administration and academic scientists (reviewed in Hahn 2002).

ESAs and similar assessments use only well-defined and thoroughly validated assays that meet EPA QA/QC benchmarks, which generally accept no greater than $\pm 5\%$ variability in reproduction. In this regard, this study assessed the use of *M. trossulus* hsp70 as a integrated biomarker of toxicity by exposing healthy mussels to two conditions: (1) laboratory exposures: nine exposure sets and one control set designed to simulate the hsp70 expression at a range of realistically low As concentrations; and (2) Atofina exposures: three exposure sets and one control set designed to simulate hsp70 expression when exposed to contaminated water from a real-world industrial facility. This study addresses the following questions:

- Is hsp70 induced by As at realistically low, real-world concentrations?
- Is the hsp70-As dose–response curve at these concentrations linear?
- Are there complications in hsp70 expression in mussels?
- Is the hsp70 response to As species correlated with known toxicities?
- Can hsp70 provide an integrated index of toxicity in the field?

Materials and methods

Mussel and water sample collection

From 1939 to 1971, the Atofina plant manufactured Penite, a potent herbicide containing sodium arsenite ($NaAsO_2 = As^{III}$). Dumped batches settled into the site's three aquifers, elevating As to as high as 910 $\mu g\, l^{-1}$ in recent years. Tidal flux floods the aquifers and later withdraws As-laden water through shoreline seeps (Mariner et al. 1996). As contamination along the shores has been confirmed by water sample

and tissue analysis of mussels and crabs conducted on behalf of the Washington State Department of Ecology, as well as a study performed in 2000 by La Porte (2001). Six litres of clean seawater and 550 mussels were collected on 30 August 2003 by scuba-diving to a colony at 15 m depth, 15°C, 90 m off the environmentally clean shores of Browns Point, Puget Sound, WA, USA. Six litres of seawater from the Atofina tidal drain were also collected into polyethylene bottles. Mussels were transported to the University of Chicago overnight on ice-chilled towels soaked in seawater to prevent thermal shock (physical handling shock does not induce hsp70 expression, as demonstrated in rainbow trout; Washburn et al. 2002). Mussels were incubated in 13°C artificial seawater matched to Commencement Bay salinity at 22 ppt and sustained on baker's yeast, aerators, and a sub-sand filter, for 2 weeks before As exposure, with a 90% survival rate.

Arsenic exposures

After 2 weeks' acclimation, 280 mature mussels of 4–5 cm shell length and no barnacle growth were segregated into 14 tanks, each containing an aerator and seawater at 13°C from the original aquarium (As exposures) or from Browns Point (Atofina exposures). The Atofina exposures used Browns Point water as a control and Atofina tidal drain water as treatment (Figures 1 and 5). Samples were analysed for As content by hydride generation-atomic absorption spectroscopy with HCl digestion with a Varian SpectrAA-220/VGA-77 (EPA Method 7061A), with the following results: Browns Point: 1–2 $\mu g\,l^{-1}$ As; Atofina 1: about 30 $\mu g\,l^{-1}$ As, Atofina 2: about 50 $\mu g\,l^{-1}$ As, Atofina 3, about 60 $\mu g\,l^{-1}$ As. Laboratory exposures used a series of As spikes as treatment: (1) sodium arsenite (As^{III}), $NaAsO_2$ (CAS 7784-46-5) from Fisher Scientific, (2) sodium arsenate (As^{V}), Na_2HAsO_4-$7H_2O$ (CAS 10048-95-0) from Sigma-Aldrich, and (3) cacodylic acid ($(CH_3)_2As^{V}$), $Na(CH_3)_2AsO_2$ (CAS 124-65-2) from Sigma-Aldrich, measured by precision analytical balance and prepared by serial dilution. Following 24-h exposure, nine mussels were selected from each exposure set and frozen at −80°C.

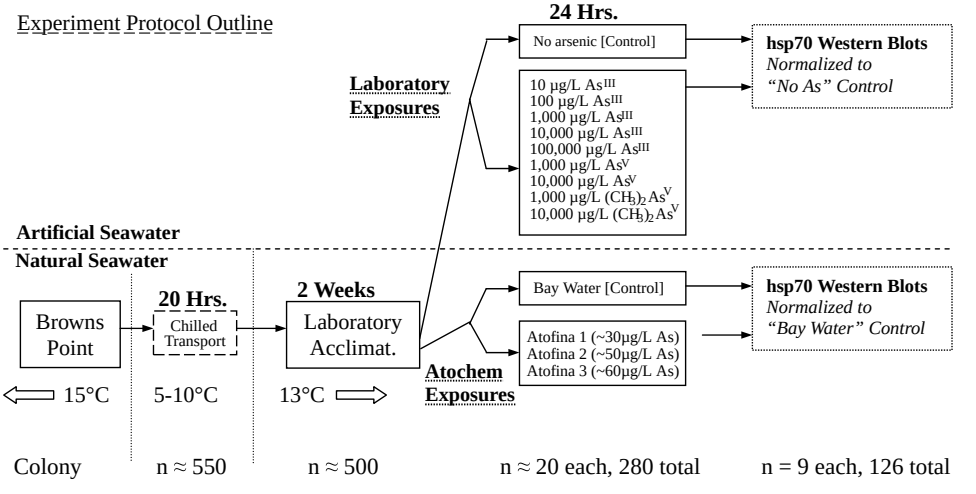

Figure 1. Outline of the experiment protocol. The number of healthy mussels at each stage is indicated as '*n*'.

Hsp70 blotting, detection and analysis

Thawing gills were excised, weighed and homogenized with a motorized Teflon/Pyrex pellet-pestle using 1 mg tissue to 5 ml Sigma protease inhibitor (AEBSF, EDTA, Bestatin, E-64, leupeptin, and aprotinin) in Tris-HCl. Gills were selected since they are the mussel tissue that expresses hsp70 most strongly after heavy metals exposure (Sanders et al. 1994, Lyons et al. 2003). Homogenates were incubated at 100°C for 5 min, centrifuged at 14 000*g* for 15 min, and the supernatant aliquoted. Protein concentrations were normalized to 200 μg ml^{-1} using the Sigma BCA Protein Assay (standard absorbance linear regression: $r^2 = 0.998$, BSA protein standard, assay range: 20–100 μg ml^{-1} at 562 nm). Fifty microlitres of sample were combined with 20 μl Laemmli buffer, vortexed, and incubated at 100°C for 5 min. Thirty microlitres tissue homogenate were added to 30-μl wells of 4–15% Tris-HCl polyacrylamide gels. To correct for inter-gel variability, a tenth lane was loaded with 20 μl of a mussel tissue homogenate standard (Inter-Blot Control). MAB reactivity was confirmed with Stress Gen Recombinant Rat hsp70(Hsp72) Protein Standard (SPP-758), while molecular weight was confirmed with Broad-Range Kaleidoscope Prestained Standards. Gels were run in Biorad Ready Gel Cells for 60 min at 200 V in BioRad SDS-PAGE Running Buffer.

Blots were performed with a BioRad Mini Trans-Blot Electrophoretic Transfer Cell for 60 min at 100 V using GE Osmonics Nitrocellulose Membrane in buffer containing 25 mM Tris base, 192 mM glycine, 20% methanol. After transfer, the membranes were washed three times with 1 × PBS + 0.1% Tween-20, then incubated for 1 h in 5% NFDM and 0.02% thimersal in 1 × PBS blocking solution. Blots were washed three times, incubated for 2 h in Affinity Bioreagents Heat Shock Protein 70 Rat IgG MAB (MA3-001, Hybridoma 7.10) at 1:500 dilution in 30% NFDM and 1 × PBS. The 7.10 MAB cross-reacts with hsp70-1/2/3 and Hsc70, with verification of cross-reactivity in mussels by Robert et al. (1997). Vijayan et al. (1997) demonstrated Hsc70 in trout is not induced by As as high as 3746 μg l^{-1}, even while hsp70 is strongly induced, indicating that cross-reactivity would not distort the stress response. Membranes were washed three times in 1 × PBS, incubated for 1 h in Jackson ImmunoResearch Laboratories' peroxidase-conjugated AffiniPure goat anti-rat IgG (H + L) secondary antibody at 1:2000 dilution. Membranes were washed three times in 1 × PBS + 0.3% Tween-20, then three times in 1 × PBS + 0.1% Tween-20.

Membranes were developed for 20 min in Pierce's SuperSignal West Pico horseradish peroxidase substrate, and recorded with a Apha-Innotech FluoroChem CCD camera. Background-subtracted IODs were determined with Amersham's Image-Quant TL imaging software, then normalized in Microsoft Excel to the appropriate controls: (1) inter-blot control, for inter-gel/inter-blot variability, (2) laboratory control for the laboratory exposures, and (3) Browns Point control for the Atofina exposures. Two-tailed, unpaired *t*-tests assuming unequal variance (heteroscedastic), with $\alpha = 0.05$ (= critical *p* value) were performed in Excel.

Results

Hsp70 levels from 24-h As^{III} exposures are shown in Figure 2.
Hsp70 levels from 24-h As^{III}, As^{V}, $(CH_3)_2As^{V}$ 24-h exposures are shown in Figure 3.
Hsp70 levels from 24-h Atofina exposures are shown in Figure 4.

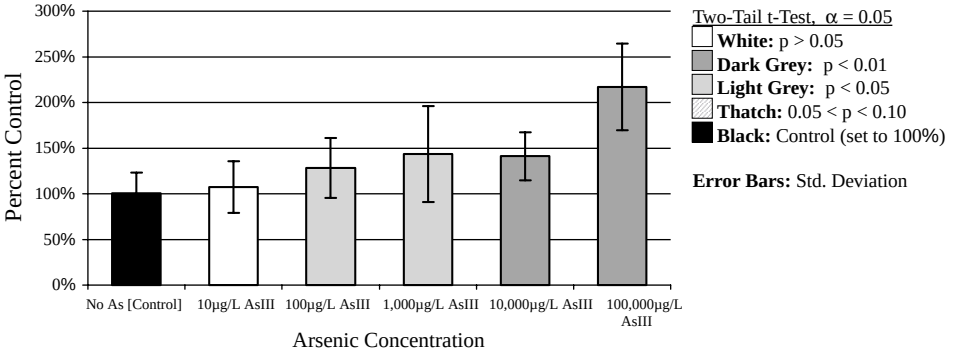

Figure 2. Hsp70 expression levels in mussels exposed to 0, 10, 100, 1000, 10 000 and 100 000 $\mu g\,l^{-1}$ As^{III} for 24 h. The response was statistically significant at 100 $\mu g\,l^{-1}$ (128%, $p=0.045$), 1000 $\mu g\,l^{-1}$ (143%, $p=0.046$) and 10 000 $\mu g\,l^{-1}$ (141%, $p=0.003$), and exceptionally strong at 100 000 $\mu g\,l^{-1}$ (217%, $p=0.00003$). Results are means $\pm$ SD of eight to nine values.

Discussion

Hsp70-arsenic induction threshold and the dose–response curve

An As biomarker should have a linear sensitivity between 1 and 100 $\mu g\,l^{-1}$. As ranges between 1.0 and 1.8 $\mu g\,l^{-1}$ in the world's oceans (Figure 5), and 1.0–1.1 $\mu g\,l^{-1}$ in Commencement Bay. The industrially contaminated Hylebos Waterway contains As at 2.1 $\mu g\,l^{-1}$, while 1999 samples from Atofina's Hylebos shores recorded 3.75, 7.74, 14.1 and 13.8 $\mu g\,l^{-1}$. The EPA limits discharge As levels to 36 $\mu g\,l^{-1}$ As (chronic) or 69 $\mu g\,l^{-1}$ As (acute), while the drinking water As is limited at 50 $\mu g\,l^{-1}$, or 10 $\mu g\,l^{-1}$ starting in January 2006. Several species of fish, amphibians and mammals have shown developmental sensitivity below 100 $\mu g\,l^{-1}$. For example, Dix et al. (1998) found that 30 $\mu g\,l^{-1}$ As^{III} had no effect on mouse blastocyst development, but that at 90 $\mu g\,l^{-1}$, 70% of the embryos were arrested at the blastomere stage; and at 300 $\mu g\,l^{-1}$, 100% were arrested at the four-cell stage, with hsp70-1/3 antisense oligonucleotide inhibition increasing embryonic sensitivity.

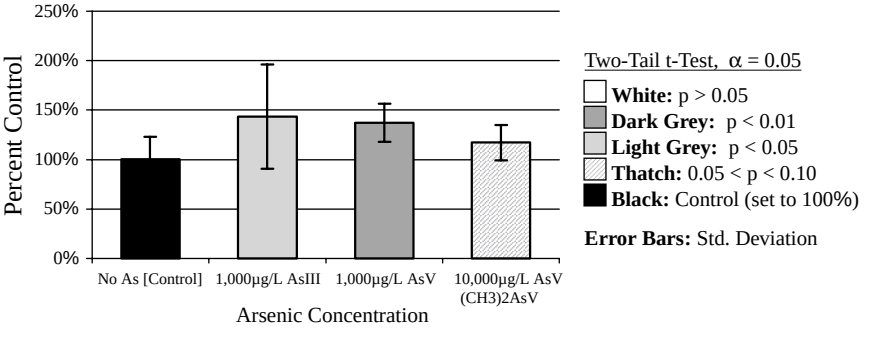

Figure 3. Hsp70 expression in mussels exposed to (1) 0 $\mu g\,l^{-1}$ As, (2) 1000 $\mu g\,l^{-1}$ As^{III}, (3) 1000 $\mu g\,l^{-1}$ As^{V} and (4) 1000 $\mu g\,l^{-1}$ $As^{V}(CH_3)_2$ for 24 h. Mussels exposed to 1000 $\mu g\,l^{-1}$ As^{III} and 1000 $\mu g\,l^{-1}$ As^{V} are expressed as elevated hsp70 (143%, $p=0.046$, and 137%, $p=0.002$, respectively). Mussels exposed to 1000 $\mu g\,l^{-1}$ $(CH_3)_2As^{V}$ did not show a significant response (117%, $p=0.099$). Results are means $\pm$ SD of eight to nine values.

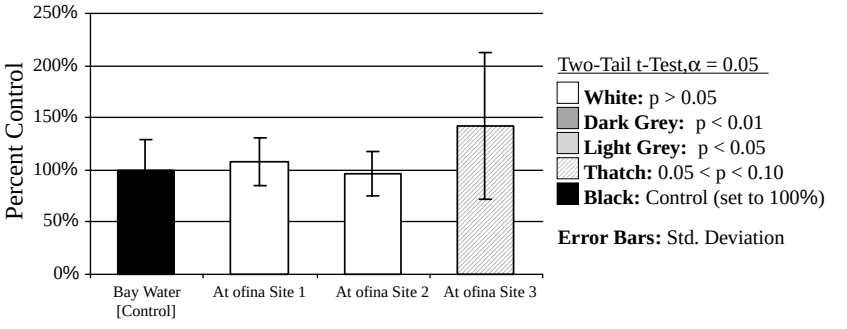

Figure 4. Hsp70 expression in mussels exposed to arsenic-contaminated drain-water from the Atofina facility for 24 h. Among the three exposure sets, only Atofina Site 3 gave a weak hsp70 induction at 142% ($p = 0.064$). Results are means ± SD of eight to nine values.

In this study, significant hsp70 elevation was observed at 100 μg l^{-1} As^{III}, while 10 μg l^{-1} As^{III} showed no elevation (Figure 2). Although there are least 20 *in vitro* studies examining hsp70 induction by As, most studies examined immediate physiological effects by using potent 10, 100 or 300 μM concentrations (749, 7490 and 22 470 μg l^{-1}, respectively; reviewed by Del Razo et al. 2001). No previous study has probed the hsp70-As induction threshold, although Schill (2003) and Bradley et al. (1998) probed the threshold for Cd, and Sanders (1991), Vedel (1995) and Dunlap (1997) have probed the threshold for Cu. Disparities in the organism, metal, concentration and exposure time make these studies difficult to compare, but they indicate that hsp70 can be induced by Cd and Cu in the 1–10 μg l^{-1} range, and by As below 100 μg l^{-1}. The lowest confirmed hsp70 inductions have been at 74.9 μg l^{-1} As by Johnston et al. (1980) and Rossi et al. (2002) (Figure 6). By extrapolation from these results as well as the results of this study, the hsp70-As threshold lies between 10 and 74.9 μg l^{-1}, most likely at 30–50 μg l^{-1} As.

This study found the following potency in hsp70 induction: $As^{III} > As^{V} > (CH_3)_2As^{V}$ (Figure 3). These values correspond with the relative toxicities established in the literature: $(CH_3)_2As^{III}$ and $(CH_3)As^{III} > As^{III} > As^{V} > (CH_3)As^{V}$ and $(CH_3)_2As^{V}$ (Del Razo et al. 2001). Both the 1000 μg l^{-1} As^{III} and 1000 μg l^{-1} As^{V} inductions were statistically significant relative to control ($p < 0.05$), but the inductions were not significantly different from each other ($p = 0.65$). The $(CH_3)_2As^{V}$ induction was less than both As^{III} and As^{V}, but even the difference

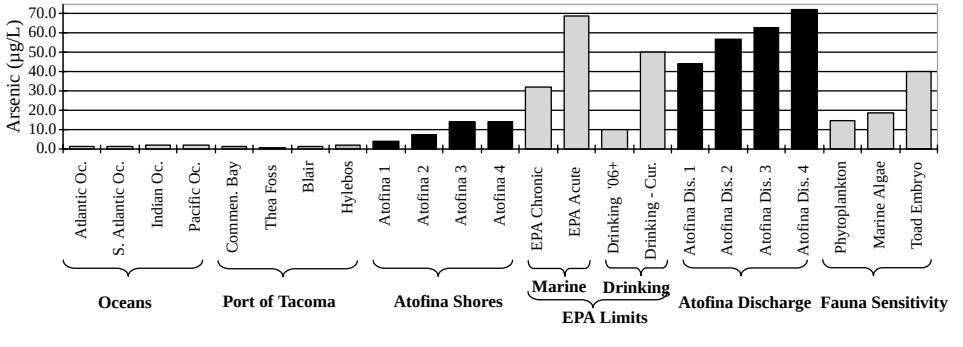

Figure 5. Representative arsenic concentrations relevant to the use of hsp70 as an environmental biomarker.

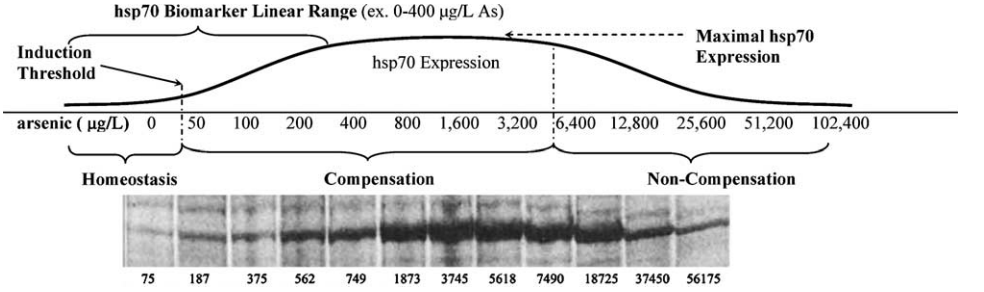

Figure 6. (top) Hsp70–arsenic response curve hypothesized by Eckwert et al. (1997); (bottom) chick fibroblast exposure to As^{III} ($\mu g\ l^{-1}$) for 4 h, as determined by [^{35}S]-methionine labelling; adapted from Johnston et al. (1980).

between 1000 $\mu g\ l^{-1}$ As^{III} and $(CH_3)_2As^V$ was weak ($p = 0.19$). Kim et al. (1983) and Liu et al. (2001) are the previous studies that have examined the relative hsp70-inducing potentials of As^{III} and As^V. Kim et al. (1983) found that hsp70 inductions by As^{III} and As^V are statistically indistinguishable. Liu et al. (2001) found As^{III} to be a more potent inducer of hsp70 than As^V by 3–6-fold.

The present study found a roughly linear hsp70 response on a logarithmic scale to concentrations of As^{III} ranging from 10 to 100 000 $\mu g\ l^{-1}$ (Figure 2). Hsp70-heavy metals studies in bacteria, woodlouse, nematodes, salmon, trout, collembolans, centipedes and human cell lines have also found a dose–response curve with a linear response range followed by a plateau of maximal expression (discussed by Snyder et al. 2001). Johnston et al. (1980) most clearly defined the hsp70-As dose–response curve, with a linear range from 50 to 3500 $\mu g\ l^{-1}$ (Figure 6). Eckwert et al. (1997) divided the dose–response curve into three sections: (1) homeostasis: a state of basal hsp70 expression, (2) compensation: a state of stress accompanied by hsp70 induction, and (3) non-compensation: a state of severe stress and pathological damage blocking hsp70 expression. It is hypothesized that strong hsp70 expression is limited by energy requirements and deleterious, abnormal binding of over-expressed protein. For example, forced expression of *Drosophila* hsp70 at normal temperatures slows cell growth, while over-expression of hsp70 during heat shock reduces growth, development, and survival (Krebs & Feder 1998). Likewise, nutrient-sufficient algae can generate an hsp70 response to Cu stress, while nutrient-starved algae cannot (Lewis et al. 2001).

In this study, the hsp70 signals were highly variable within the exposure sets. Of nine exposure sets, only three had a $p < 0.01$, two had a $p < 0.05$, two had a $p < 0.1$, and three were statistically insignificant. Each set included eight to nine mussels, and magnitudes of induction ranged from 110 to 220%. High inter-individual hsp70 variability has also been observed in 21 previous heavy metals studies. According to Lundebye et al. (1997), the signals at low metals concentration tend to be 'masked by the high degree of interindividual variability in the stress protein levels'. More than sensitivity, specificity or cost, variability of hsp70 expression between individuals presents the most serious challenge to the feasibility of hsp70 a toxicity biomarker.

Complications of hsp70 expression in mussels

Mussels are ideal marine biomarker species because they are: stationary, widespread, easy to collect, filter-feeders that accumulate toxins, and have been used in 18 hsp70

toxicity studies between 1988 and 2004 (Viarengo & Canesi 1991). *M. trossulus* is the dominant species in the Puget Sound, and is mildly less stress tolerant than the closely related and better-known *M. edulis* (Hellou & Law 2003).

Inter-tidal mussels are exposed to sunlight and air during low tide, and may experience body temperature elevations as high as 20°C. Consequently, inter-tidal mussels exhibit hsp70 'memory effects,' in which hsp70 levels reflect prior tidal exposure (Buckley et al. 2001). To eliminate these memory effects, mussels were collected by scuba diving to 15 m below the mean low tide line, ensuring that only sub-tidal mussels would be collected. Hsp70 has also be differentially expressed with age in *Drosophila*, chickens, humans and a variety of other organisms (Snoeckx et al. 2001). In view of these findings, only mature mussels of 4–5 cm in shell length and minimal barnacle growth were selected.

Elevated salinity has been shown to increase hsp70 sensitivity to heavy metals. Tedengren et al. (1999) found that hsp70 in *M. edulis* from the highly saline North Sea (28 ppt) was more sensitive to Cu than *M. edulis* from the less saline Baltic Sea (6 ppt). Baltic Sea mussels transplanted and acclimatized to the North Sea developed hsp70 sensitivities to Cu resembling the native North Sea mussels. Similarly, Werner (2004) found that mussels acclimatized to 0.5, 6.5 and 25 ppt saline in the field and the laboratory had differential hsp70 sensitivity to heat shock. Low salinity mussels showed no response ($p < 0.05$), while high salinity mussels showed a robust response ($p < 0.05$), with similar findings by Hamer et al. (2004). The authors speculate that low salinity heightens energy-dependent osmoregulation, thereby dampening the cellular capacity to express hsp70. Interestingly, Woo et al. (2002) found that the non-induced hsp70-2 isoform is sensitive to hypertonicity, and is regulated by flanking sequences for tonicity-responsive enhancer binding protein (TonEBP).

Chemotolerance may influence hsp70 induction by heavy metals in mussels and other organisms. In Köhler et al. (2000), millipedes from a control site and a mine site were exposed for 10 days to litter spiked with Cd, Pb, Cu and Zn, followed by a 10-day recovery. The mine site millipedes did not show hsp70 induction, while control site millipedes showed an average 250% induction, suggesting the development of hsp70 chemotolerance.

Metabolism may also influence hsp70 induction by As. Eukaryotes methylate inorganic As to $AsCH_3$ and $As(CH_3)_2$, but marine fauna such a mussels have alternative pathways that produce arsenosugars such as the non-toxic arsenobetaine, apparently through a bacterial mussel symbiote, *Pseudomonas* sp., which is capable of synthesizing and degrading arsenobetaine (Ritchie et al. 2004). This unique As metabolism in mussels may complicate their use as hsp70 biomarker species. Alternative organisms used in prior hsp70 environmental studies include gastropods, clams, oysters, ciliates, salmon and trout, marine sponge, sea urchins, amphipods, crabs, rotifers, *C. elegans*, green algae, and *Daphnia* (reviewed in Mukhopadhyay et al. 2003).

Hsp70 as a toxicity biomarker in the field

Laboratory assessments of hsp70 have often used one or two chemical toxins, but industrial sites are often contaminated by several heavy metals and organics. In a series of studies between 1997 and 2000 by Köhler, Eckwert, and colleagues have explored links between hsp70 expression Pb, Zn and Cu exposure in fauna from European mining and mill sites. In Köhler et al. (2000), woodlice *Oniscus asellus*, *Porcellio scaber* and millipedes

Julus scandinavius were collected from 28 metal-polluted forest sites and a Pb/Zn opencast mine along the Neckar River in Germany. The authors observed 'high intra-site variability' in hsp70 levels, but applied complex regression models, finding positive correlation of hsp70 and soluble Pb ($p < 0.0001$ *P. scaber*, $p < 0.01$ *J. scandinavicus*), soluble Zn ($p < 0.01$ *J. scandinavicus*), and soluble Cd ($p < 0.05$ *J. scandinavicus*). According to the authors, none of the parameters alone accounted for more than 18% inter-site variability, but, together, they accounted for 96% of inter-site variability.

Werner and colleagues have studied hsp70 levels in bivalves in relation to contaminants in San Francisco Bay. In Werner et al. (2004), clams incubated in sediment samples exhibited hsp70 expression that correlated with the tissue content of stressors such as Pb ($p < 0.0001$), Zn ($p < 0.01$), Cd ($p < 0.05$), DDT and PAHs. Despite the complexity of hsp70 induction, these crude field results suggest that hsp70 could successfully integrate numerous contaminants to provide a cumulate index of toxicity as suggested by the hypothesis. Similarly, Veldhuizen-Tsoerkan et al. (1991) found a correlation between hsp70 levels and real-world exposure to Cd and PCB contamination. Pedersen and Lundebye (1996) collected shore crabs *Carcinu maenes* from five sites along a Cu/Zn/Cd heavy metals gradient, and found no hsp70 correlation. Mutwakil et al. (1997) found hsp16/hsp70 expression correlation with exposure to metals-contaminated mine tailings using lacZ/hsp promoter transgenic *C. elegans*. Lundebye et al. (1997) found a single hsp60 induction in mussels collected near shipyard contaminated with Zn, Cd and PAHs. Hamer et al. (2004) speculated on a weak correlation between pollution exposure and hsp70 in *M. galloprovincialis*. Aït-Aïssa et al. (2003) used hsp70 promoter-CAT reporter gene construct to assess dose–responses to industrial waste, and found linear responses and correlation of hsp70 with aquatic bioassay results. These field studies have shown that hsp70 expression tends to correlate with contamination in real-world settings, but that hsp70 expression profiles are often obscured by high variability.

Conclusions

This study found that hsp70 induction for a 24-h exposure is weak and insignificant below 100 $\mu g\ l^{-1}$ for As^{III}, while strong signals can be observed above 1000 $\mu g\ l^{-1}$. These results and those of previous studies suggest that the hsp70 induction threshold lies near 30–50 $\mu g\ l^{-1}$ for As^{III}. This study also found that hsp70 expression weakly correlates with the toxicity of the arsenical. However, this author and previous authors have found that high inter-individual variability in the hsp70 signals tends to 'mask' hsp70 inductions at low heavy metals concentrations, limiting its utility as a toxicity indicator in a field that demands sensitivity and reliability.

There are a few options that could increase sensitivity or reduce variability in hsp70 induction by heavy metals. (1) Chemical stress heightens thermal hsp70 sensitivity, and vice versa. Studies in mussels (Veldhuizen-Tsoerkan et al. 1991, Bradley et al. 1998, Tedengren et al. 1999) have successfully used pre- or post-heat stressing to increase hsp70 sensitivity to heavy metals. (2) Protein controls could be used to normalize hsp70 levels. In two mussel studies, actin (Lyons et al. 2003) and an unidentified 46 kDa protein (Roberts et al. 1997) were used as controls against hsp70 variation. (3) Hsp70 ELISAs are reportedly more sensitive to induction than Western blots in mussels, and hsp70-1/3-specific ABs may reduce the signal-to-noise ratio (Pempkowiak et al. 2001, Ireland et al. 2004). (4) Transgenic systems may provide even stricter control against

hsp70 variation. Successful constructs include hGH-hsp70 in NIH/3T3 cells (Fischbach et al. 1993), 16 kDa Hsp-lacZ in *E. coli* (Stringham & Candido 1994), *Drosophila hsp*70 promoter linked to *E. coli* lacZ fused into *C. elegans* (Mutwakil et al. 1997), a human cell hsp70 promoter-CAT reporter gene construct (Aït-Aïssa et al. 2003), a marine bacterium *Vibrio fischeri* bioluminescence operon (luxC,D,A,,E)-dnaK/grpE (hsp70) in *E. coli* (Van Dyk et al. 1994), and an hsp70 promoter-eGFP zebrafish transgenic for studying developmental toxicology (Blechinger et al. 2002). (6) Hsp70 could be accompanied by other hsps. Hsp60 has demonstrated more narrow sensitivities than hsp70, and it is strongly induced by Cu and Zn, but not by As, Cd and Hg (Wagner et al. 1999). (7) Assays that combine several proteins, such as metallothionein, haem oxygenase-1, CYP450, MXR, GPx and the hsps could prove more promising than solitary biomarkers. For example, Blom et al. (1992) exposed *E. coli* to nine organic toxins and found that low molecular weight hsps and carbon-starvation proteins have a unique 'fingerprint' expression pattern for each toxin, with similar work with PCBs and Cu and *M. edulis* by Shepard et al. (2000). Altogether, it clear that mussel hsp70 expression alone is a crude and insufficient biomarker of heavy metals toxicity in marine samples, and requires more sophisticated techniques to control variability.

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