Induction of hsp70 genes by copper (II) sulfate in *Mytilus trossulus*

**Abstract**

**Introduction**

The presence of metals in aquatic ecosystems from interactions between water and the environment continues to be a pressing issue. Heavy metals, such as copper (Cu) may enter an aquatic ecosystem from natural and anthropogenic sources, including sewage, storm runoff, landfill leaching, atmospheric deposits and shipping and harboring activities (Rainbow, 2002). However, unlike organic pollutants, heavy metals cannot be removed by natural processes, such as decomposition. Instead, they become part of the water-sediment system and accumulate in the aquatic-food chain (Vukovic et al., 2011). Aquatic species are especially vulnerable to changes within their environment, particularly endemic local and migratory species that have limited geographical distribution (Harley et al., 2009). In response to such environmental stressors, organisms respond at the cellular level by synthesizing stress proteins, such as heat shock proteins (HSP) or non-heat inducing metallothioneins (MT) (Karouna –Renier and Zehr, 2003). HSP’s are present at low concentrations under normal conditions, and play essential roles in maintaining cell homeostasis by acting as molecular chaperons. Molecular chaperones assist with the folding, assembly and transport of other proteins (Lewis et al., 2001, Metzger et al., 2012).

The most highly conserved family of HSP’s is the 70kDA family (HSP70) (Bajaszar and Dekonenko, 2010; Mohanarao et al., 2013). The activation of HSP70 gene transcription is mediated through the stress-induced binding of a pre-existing heat shock factor (HSF) to a heat shock element (HSE) on the promoter of the gene (Liu et al., 1994, Sherman and Goldberg, 2001). Activation of this gene is due to a multitude of environmental factors ranging from temperature to heavy metals (Rainbow, 2002; Rajeshkumar et al., 2013). Copper is one of many heavy metals found along the Puget Sound (Schell and Nevissi, 1977). It generates free radicals and oxidizes cellular components through its redox activity (Urani et al., 2001; Seok et al., 2006). And as such, has been shown to modulate the intracellular signal transduction pathway of multiple regulatory genes, such as cytochrome P-450, MT and HSP70, in liver cells (Song and Freedman, 2005). Up-regulation of HSP70 in aquatic invertebrates has resulted in their use as bio indicators of toxic metals in marine environments (Szefer et al., 2002).

 The blue mussel, *Mytilus trossulus* (*M. trossulus*), is a sessile, filter feeding shellfish that often accumulates trace amounts of heavy metals in its soft tissue, resulting in its use as a good bio indicator of pollution (Rainbow, 1995a; Fischer, 1988; Rainbow, 2002b). Few studies have examined the combined effects of multiple environmental factors on the induction of HSP70. Analyzing the effects of these stressors provides a more complete look at how organisms are affected in their natural environment (Anderson et al., 1998). Washington State generates over $100 million in profits from the sales of shellfish every year, and this value continues to be on the rise (Status of the Fisheries Report, 2008). Concern over the transmission of such toxins to human consumers has caused the need to understand the various mechanisms associated with the toxicology of metals to increase (Roesijadi, 1992).

 The objective of the present study was to characterize stress-induced HSP-70 gene expression in *M. trossulus* when exposed to varying concentrations of copper and *p*CO2 levels. I predicted that as ambient *p*CO2 levels and Cu concentrations increased, HSP-70 gene expression would be up-regulated as a result of copper’s oxidative abilities. This study provides insight into the adaptability of bivalves to heavy metal contamination, through examination of HSP-70 expression rate.

**Materials and Methods**

*Organisms*

 Specimens of *M. trossulus* – 3-5.7 cm shell length – were collected from ­­­­­­­­­\_\_\_\_\_\_ and further acclimated to aerated sea water for **\_\_\_\_** days at 12⁰C.

*Experimental Design*

Mussels were randomly assigned to 4 treatment groups (n = 10) in 208L of continuously aerated seawater, for 14 days. One control group was exposed to 400ppm *p*CO2 (normal). Group two was exposed to 1500ppm *p*CO2 levels (high – pH: 7.5). A third group received a dose of copper (II) sulfate (5mg/L), and normal *p*CO2levels. The fourth group was subjected to a combined copper and high *p*CO2 treatment. All treatments were held at 12⁰C for the duration of the experiment.

Gill tissue samples were extracted every 7 days, with baseline samples taken on day 0. Samples were weighed and stored at -80⁰C for RNA extraction and quantitative PCR analysis.

*RNA isolation*

RNA from all gill tissue samples were homogenized using TriReagent (Molecular Research Center, Inc., Cincinnati, OH). After complete homogenization, chloroform was used to prevent any salt or DNA carryover. RNA was precipitated, washed and quantified using Nanodrop spectrophotometer. RNA was then reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega, Madison, WI).

*Gene expression*

Hsp70 primers were designed using NCBI Primer-Blast and Primer 3 software (hsp70fw: AAGCATCACAAGAGCCAGGT; hsp70rv: GCAGCCTTGTCTAGTTTGGC). Quantitative PCR was carried out using ­­­­­\_\_\_\_\_\_. Each 25µL reaction contained SsoFast EvaGreen super mix (12.5 µL), 0.5 µL of each primer (10 µM), and ultra-pure water (10.5 µL). PCR conditions included initial denaturation at 95⁰C for 10 minutes, followed by 40 cycles of 95⁰C for 15s, 55⁰C for 15s, and 72⁰C for 15s, followed by 95⁰C for 10s. Fluorescence was measured at the end of the extension step. Melting curve analysis was preformed following qPCR. The temperature was increased from 65⁰C to 95⁰C, with fluorescence measured every 0.5⁰C for 5s.

*Statistical Analysis*

A two-way ANOVA was carried out in order to analyze significant differences between Hsp70 gene expression after copper exposure, and the combined effects of copper and high *p*CO2 exposure. Significance levels were set at p<0.5.

**Results**

400ppm/copper = 32.1

1500ppm/copper = 55.13

Graph comparing average rates of expression

Table displaying p-value/standard deviations/ average levels

**Also need description of melting curve and confidence that products are specific.**

**Discussion (Assuming my prediction is correct/ results from running qPCR this week)**

 Mussels, as filter-feeding bivalves, are important players in marine ecosystems and our economy. These organisms are present in aquatic environments world-wide, and as such, are subjected to changing environmental conditions, including variations in water temperature, pH levels and toxic discharge, making them good bio indicators of a healthy environment (Guo et al., 2012). Such stresses can induce cellular oxidative stress, in the form of reactive oxygen species; heat shock proteins play a vital role in protecting mussel cells from their damage (Davies, 1995). The present study determined the synergistic and individual effects of copper and high *p*CO2 on Hsp70 gene expression.

 Mussels were chronically exposed to copper (5mg/L) over the course of this study. Gill tissue samples revealed a marked increase in Hsp70 gene expression as a result of exposure. Highest concentrations of heavy metals have been found in exposed gill tissue, resulting in its examination in this study (Seok et al., 2006). The combination of a high *p*CO2 and copper exposure also revealed a significant increase in mRNA expression. *p*CO2 levels are representative of projected environmental change, whereas copper concentrations are similar to those used in other studies that have been shown to induce hsp70 expression in marine bivalves ( Anderson et al., 2011; Metzger et al., 2012). In the present study, the presence of mortality in the laboratory suggests that the chosen copper concentrations were toxic lethal doses.

 Our results are consistent with studies using similar copper concentrations. In the zebra mussel, *Dreissana polymopha*, Hsp70 gene expression was found to be elevated after exposure for 24h (Clatyon et al., 2000). *Macoma nasuta* exposed to sediment from the northern San Francisco Bay, contaminated with copper, found high levels of Hsp70 expression in sampled gill tissues (Werner et al., 2004). A study on *Crassostrea gigas,* revealed a significant increase in Hsp70 mRNA expression in gill tissues when exposed to 30mg/L of copper (Metzger et al., 20120). The rise seen in hsp70 transcriptional rates could be mechanistically similar to cadmium-induced hsp70 activation due to binding of a downstream promoter region (Wu et al., 1986). In the current study, rises in hsp70 mRNA levels after 7 days of exposure could indicate the adaptability of the organism to copper sulfate exposure. The presence of such high levels of mRNA could potentially make hsp70 a good biomarker of heavy metal induced oxidative stress and the need for a reduction in heavy metal pollution.

Similarly, subjects exposed to *p*CO2 (1500ppm) and copper (5mg/L) experienced higher than normal hsp70 mRNA expression, when compared to our control. The synergistic effect of this environmental stressor resulted in an increased capacity for hsp70 up-regulation. Changes in metabolism due to high CO2 levels could explain the mussel’s elevated ability to counter the oxidative stress from exposure to copper. However, the effects of pH on hsp70 expression still need to be further examined and understood. An interesting approach for future studies could be to examine this hypothesis with a combination of other naturally occurring stressors, including temperature and salinity.

 The results of the present study show that various environmental stressors can synergistically alter the rate of hsp70 expression. The effect of copper sulfate, alone, on mRNA expression was consistent with results found in closely related genus’s. Understanding the eco-toxicological impact of copper on aquatic species is key to determining their usefulness as bio-indicators of an ecosystems health.

**References:**

1. Anderson, AJ., Mackenzie, F. T., Gattuso, J. P., 2011. Effects of ocean acidification on benthic processes, organisms and ecosystems. Ocean Acidification, vol. 8; 122-153.
2. Anderson, Richard C., Lindauer, Kelly R., Prescott, David M., 1996. A gene-sized DNA molecule encoding heat-shock protein 70 in *Oxytricha nova*. Gene, vol. 168; 103-107.
3. Bajaszar, George, Dekonenko, Alexander, 2010. Stress-Induced Hsp70 Gene Expression and Inactivation of *Cryptosporidium parvum* Oocysts in Chlorine-Based Oxidants. Applied and Environmental Microbiology, vol. 76; 1732-1739.
4. Clayton, Maureen E., Steinmann, Roland, Fent, Karl, 2000. Different expression patterns of heat shock proteins hsp60 and hsp70 in zebra mussel (*Dreissena polymorpha*) exposed to copper and tribuutylin. Aquatic Toxicology, vol. 47; 213-226.
5. Davies, K. J., 1995. Oxidative stress: the paradox of aerobic life. Biochemical Society Symposium, vol. 61; 1-31.
6. Dondero, Francesco, Piacentini, Luciana, Banni, Mohamed, Rebelo, Mauro, Burlando, Bruno, Viarengo, Aldo, 2005. Quantitative PCR analysis of two molluscan metallothionein genes unveils differential expression and regulation. Gene 345, 259-270.
7. Fischer, H, 1988. *Mytilus edulis* as a quantitative indicator of dissolved cadmium : Final study and synthesis. Marine Ecology, vol. 48; 163-174.
8. Guo, R., Ebenezer, V., Ki, J.-S., 2012. Transcriptional response of heat shock protein (hsp70) to thermal bisphenol A, and copper stresses in the dinoflagellate *Prorocentrum minimum*. Chemosphere, vol. 89; 512-520.
9. Liu, Bo, Xu, Lei, Ge, Xianping, Xie, Jun, Xu, Pao, Zhou, Qunlan, Pan, Liangkun, Zhang, YuanYuan, 2013. Effects of mannan oligosaccharide on the physiological responses, HSP70 gene expression and disease resistance in Allogynogenetic crucian carp (*Carassius auratus gibelio*) under *Aeromonas hydrophila*infection. Fish and Shellfish Immunology, vol. 34; 1395-1403.
10. Marjorie Aelion, C., Harley, Davis T., Lawson, Andrew B., Cai, Bo, McDermott, Suzanne, 2013. Temporal and spatial variation in residential soil metal concentrations: Implications for exposure assessments. Environmental Pollution, vol. **5**; 1-4.
11. Metzger, David C., Pratt, Paul, Roberts, Steven B., 2012. Characterizing the effects of heavy metal and *Vibrio* exposure on Hsp70 expression in *Crassostrea gigas* gill tissue. Journal of Shellfish Research, vol. 31; 627-630.
12. Mohanarao, Jagan G., Mukherjee, Ayan, Banerjee, Dipak, Gohain, Moloya, Dass, Gulshan, Brahma Biswajit, 2013. Hsp70 family genes and Hsp27 expression in response to heat and cold stress *in vitro* in peripheral blood mononuclear cells of goat (*Capra hircus*). Small Ruminant Research, vol. **13**; 1-5.
13. Karouna-Renier, Natalie K., Zehr, Jonathan P.,2003. Short-term exposure to chronically toxic copper concentrations induce HSP70 proteins in midge larvae (*Chrionomus tentans*). Science of the Total Environment, vol. 98; 8-18.
14. Lewis, S., Donkin, M.E., Depledge, M.H., 2001. Hsp70 expression in *Enteromorphis intestinalis* (Chlorophyta) exposed to environmental stressors. Aquatic Toxicology, vol. 51; 277-291.
15. Rainbow, Philip S., 2002. Trace metal concentrations in aquatic invertebrates: why and so what?. Environmental Pollution, vol. 120; 497-507.
16. Rainbow, Philip S., 1995. Biomonitoring of heavy metal availability in the marine environment. Marine Pollution Bulletin, vol. 31; 183-192.
17. Rosejiadi, G., 1992. Metallothioneins in metal regulation and toxicity in aquatic animals. Aquatic Toxicology, vol. 22; 81-113.
18. Schell, William R., Nevissi, Ahmad, 1977. Heavy metals from waste disposal in central Puget Sound. Environmental Science Technology, vol. 11; 887-893.
19. Seok, Seung-Hyoek, Park, Jong-Hwan, Baek, Min-Won, Lee, Hui-Young, Kim, Dong-Jae, Uhm, Hyung-Min, 2006. Specific activation of the human HSP70 promoter by copper sulfate in mosaic transgenic zebrafish. Journal of Biotechnology, vol. 126; 406-413.
20. Sherman, Michael Y., Goldberg, Alfred L., 2001. Cellular defenses against unfolded proteins: A cell biologist thinks about neurodegenerative diseases. Neuron, vol. 29; 15-32.
21. Song, Min O., Freedman, Jonathan H., 2005.Expression of copper responsive genes in HepG2 cells. Molecular and Cellular Biochemistry, vol. 279; 141-147.
22. Szefer, P., Frelak, K., Szefer, K., Lee, Ch-B., Kim, B.-S., Warzocha, J., Zdrojewska, Ciesielski, T, 2002. Distribution and relationships of trace metals in soft tissue, byssus and shells of *Mytilus edulis trossulus* from the southern Baltic. Environmental Pollution, vol. 120; 423-444.
23. Urani, C., Melchioretto, P., Morazzoni, F., Canevali, C., Camatini, M., 2001. Copper and zinc uptake and hsp70 expression in HepG2 cells. Toxicology in Vitro, vol. 15; 497-502.
24. Vukovic, Zivorad, Radenkovic, Mirjana, Stankovic, Srboljub J., Vukovic, Dubravka, 2011. Distribution and acclimation of heavy metals in the water and sediments of the River Sava. Journal of the Serbian Chemical Society, vol. 76; 795-803.
25. Werner, Ingeborg, Teh, Swee J., Datta, Seema, Lu, XiaoQiao, young, Thomas, M.m 2004. Biomarker responses in *Macoma nasuta* (Bivalvia) exposed to sediments from northern San Francisco Bay. Marine Environmental Research, vol. 58; 299-304.
26. Wu, Barbara J., Kingston, Robert E., Morimoto, Richard I., 1986. Human hsp70 promoter contains at least two distinct regulatory domains. Biochemistry, vol. 83; 629-633.