行政院國家科學委員會專題研究計畫　期中進度報告

建立分子生物指標監測水生態系統之砷暴露(1/2)

計畫類別：個別型計畫
計畫編號：NSC93-2313-B-002-075-
執行期間：93 年 08 月 01 日至 94 年 07 月 31 日
執行單位：國立臺灣大學生物環境系統工程學系暨研究所

計畫主持人：廖秀娟

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報告類型：精簡報告
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處理方式：本計畫可公開查詢

中　華　民　國 94 年 5 月 30 日

行政院國家科學委員會補助專題研究計畫 □ 成 果 報 告
▓期中進度報告

（計畫名稱）

建立分子生物指標監測水生態系統之砷暴露(1/2)

計畫類別：▓ 個別型計畫　　□ 整合型計畫

計畫編號：93-2313-B-002-075-

執行期間：　　2004 年　8　月　　1 日至　2005　年 7　月　31　日

計畫主持人：廖秀娟

共同主持人：

計畫參與人員：　李悅芬、游展維、曾月怡、周芷宣、許翔雁、鄭權

成果報告類型(依經費核定清單規定繳交)：▓精簡報告　□完整報告

本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份

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□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列

管計畫及下列情形者外，得立即公開查詢

□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

執行單位：國立台灣大學 生物環境系統工程學系

中　華　民　國　94 年　5 月　30　日

建立分子生物指標監測水生態系統之砷暴露(1/2)

(93-2313-B-002-075-)

期中精簡進度報告

(一)中文摘要

砷污染對人類健康及環境造成潛在的風險。當生物體暴露於砷時，會造成多種不同的毒性效應，例如：立即性的死亡，或緩慢的引發癌症效應。本研究計畫之主要目標，將建立分子生物指標用來監測水生態系統之砷暴露。此外，砷於不同的魚組織的濃度分布，以及其生化效應將被探討。

本研究計畫將分為二年執行，將延續前一年的研究成果，繼續水生態系統中砷暴露之分子生化效應之相關研究。研究成果將有助於建立水生態系統中砷之監測及警示系統，並有助於更準確的風險評估。

關鍵詞： 砷污染、生化效應、分子生物指標

(二)英文摘要

Arsenic contaminants cause a high potential risk to human health and the environment. Exposure of humans and animals to arsenic results in a variety of toxic effects ranging from immediate mortality to slow process of carcinogenesis. The primary goal of this proposal is to develop molecular biomarkers for monitoring arsenic exposure in aquatic ecosystems. In addition, the tissue distribution of arsenic and the corresponding biochemical effects in tilapia fish will be investigated.

This 2-year grant proposal will continue the work from previous year to study arsenic induced biochemical effects in aquatic organisms. The studies employed in this proposal will reflect the specific toxic effect of arsenic and reveal fundamental relationships between chemical structures and biological activity. The molecular biomarker developed in this study may serve an early warning system for the detection and monitoring arsenic exposure in aquatic ecosystems. This may contribute to a better accurate risk assessment.

Keyword: arsenic exposure, biomarker, biochemical effect

(三) 研究背景及目的

Water contaminants cause a high potential risk to human health and the environment. Exposure of humans and animals to certain metal derivatives results in a variety of toxic effects ranging from immediate mortality to slow process of carcinogenesis. Therefore, the environmental metal toxicity is becoming a problem of significant concerns for human and ecosystems. Increased awareness of metal contamination in aquatic ecosystems and identification of metals as carcinogens has generated public and scientific concern regarding human health and ecological risks. However, our ability to assess these risks is limited by poor understanding of (1) the relationship between metal external/internal concentrations and distribution to target sties of receptors, (2) environmental factors that control redox speciation of metals, and (3) biochemical effects of metal and mechanism of metal toxicity as well as corresponding mechanisms of cellular defense.

Among heavy metals, arsenic has driven special attention to researchers. Arsenic has been classified as human carcinogens. Water contamination by arsenic poses a high potential risk to human health and the environment. To better assist accurate risk assessment posed by arsenic, it is important to study the biochemical effects of these metals in aquatic ecosystems. Arsenic, a known human carcinogen and teratogen (Kitchin, K. T., 2001), is a widespread environmental contaminant that enters water, food, and air from both natural sources and human activities (Hartwig, 1995). Weathering of arsenic-containing rocks, metal processing, burning of fossil fuels and using arsenic pesticides all contribute to arsenic contamination in freshwater systems. This

contamination is a source of concern because of arsenic's potent toxicity. At high levels, arsenic can be lethal to many freshwater organisms. At the lower concentrations typical of most contaminated aquatic ecosystems, arsenic produces more subtle effects that can threaten the survival of aquatic life (Del Razo, et al., 2001). Relatively small amounts of arsenic also pose cancer and other health risks to humans.

Arsenic was ranked first on the Agency for Toxic Substances and Disease Registry / Environmental Protection Agency "Top 20 Hazardous Substances Priority List" in 2001. Arsenic also tops the U.S. Environmental Protection Agency's list of chemicals of concern at toxic waste sites. Exposure to arsenic in drinking water represents a significant health problem for people around the world, including Taiwan. In 1997, the World Health Organization recommended that arsenic in drinking water be recognized as a major public health issue that should be addressed on an emergency basis. In nature, As is found as oxides or sulfur compounds and it is mainly distributed throughout the environment by water. Among the chemicals species of As present in the environment, inorganic arsenic (iAs) is generally considered the most hazardous (Moore et al., 1997; Yamauchi and Flwler, 1994). The characteristics of As salts' reactivity and toxicity depend on their oxidative states, the trivalent form being the most reactive (Del Razo et al., 2001). In humans, exposure to arsenic has been linked to increased risk of cancer, heart disease, diabetes and reproductive disorders (Garcia-Vargas and Cebrian, 1996). However, most studies linking arsenic with human disease have involved people exposed to very high levels - in the workplace, for example, or in parts of Taiwan, Pakistan and other areas of the world where levels of arsenic in drinking water are unusually elevated. In Bangladesh, India, an estimated 70 million people have been slowly poisoned by well water tainted by arsenic from natural sources deep within the ground (Garcia-Vargas and Cebrian, 1996).

The toxicity of As compounds highly depends on the oxidation state and chemical composition of the arsenical (Moore et al., 1997; Yamauchi and Flwler, 1994). As^{III} appears to be the most toxic form regardless of being inorganic or organic form. As^{III} toxicity could be carried out either directly, by attacking –SH groups, or indirectly, through the generation of reactive oxygen species (ROS) (Chen et al., 1998). The toxicity of iAs^{V} appears to be mediated through its ability to substitute phosphate groups, affecting enzymes that depend on this group for its activity.

Arsenic is an important xenobiotic in aquatic ecosystems. Because fish are an important food resource and a major ecosystem component, it is important to assess the biochemical effects of arsenic in fish, determine acceptable levels of arsenic in the aquatic environment, and develop new methods for detecting deleterious effects of arsenic at an early stage.

In environmental mixture, the often-unpredictable antagonistic, synergistic or additive effects between pollutants can occur that make complete chemical analysis complicated. Chemical analyses of complex mixtures are expensive and time consuming and they can not reflect or predict the ultimate bioavailability of a chemical. In this respect, lethality tests have long been used to supplement chemical analyses. However, in majoring acute toxicity at high doses, these tests have little or no predictive value for chronic exposure at lower dose levels making that lethality is too coarse a measure for toxicity (Basu et al., 2002). In contrast, changes at biochemical level are usually the first detectable responses to environmental pollution. This

approach examines the effect at the site of the interaction of chemicals with the different macromolecules in the cell. When the target molecule is part of the defense, repair or detoxification mechanism of the cell, it becomes a direct and specific marker of exposure and effect (biomarker). Therefore, biomarkers may be used in an early warning system to predict biological effects for a wide range of environmental contaminants such as heavy metals (Basu et al., 2002).

It has been suggested that HSPs can be used as biomarkers of environmental pollution (Basu et al., 2002). Since hsp70 genes are highly conserved among organisms (Morimoto, 1994), we hypothesized that hsp70 gene expression will be induced in response to arsenic exposure in fish tilapia (*Oreochromis mossambicus*). Therefore, we propose that expression of heat shock protein 70 is sensitive to levels of arsenic well below those that kill organisms outright. We will measure protein and messenger RNA in the fish and use this measurement to provide a reliable molecular biomarker of arsenic exposure. Additionally, we also study the level of oxidative stress induced by As(III) by measuring GSH in fish tissue. We expect that the stress protein gene expression and oxidative stress response can be used as useful endpoints, or biomarkers, for detecting arsenical stress in ecosystems.

(四) 研究方法與步驟

Subchronic toxicity test

We have conducted subchronic toxicity test for tilapia fish exposed to a range of sub-lethal level of arsenic exposure. Juvenile tilapia (*Oreochromis mossambicus*) was acclimated for 2 weeks prior to the experiments. Fish were kept in glass aquaria with constant aerated freshwater (25 L), under 12:12 light-dark cycle, at water temperatures of 22-25 ^{o}C. Temperature was recorded and water quality determined for DO_2, pH in the beginning of experimental period. Subchronic toxicity tests were performed to determine internal body burden and hsp70 gene expression for fish exposed to a range of sub-lethal level of arsenic exposure. During the experimental period, all groups were fed daily (3% of body weight - BW) and the unfed diet remains were collected and dried to constant weight. The water was changed once a week to maintain arsenic concentrations. Fish were exposed to a range of arsenic: 0, 1, 2, 4 ppm for 4 weeks. For each dose of metal, 10 fish were exposed. Control and each test concentration were conducted in duplicate. After this period, fish were removed at the same time from each aquarium, anaesthetized in benzocaine before killing by transecting the spinal cord. The fish were put on water- dissolved benzocaine (80 mg/l) and were anesthetized in seconds. No side effects were related with this procedure. Liver, muscle, gills, intestine, blood, and kidney were removed, weighed, immediately frozen in liquid nitrogen and stored at -80^{o}C for further analyses. Figure 1 shows where tissue samples were removed from tilapia fish.

The measurement of arsenic concentration in fish tissue is in the process of being determined by using atomic absorption spectrometer.

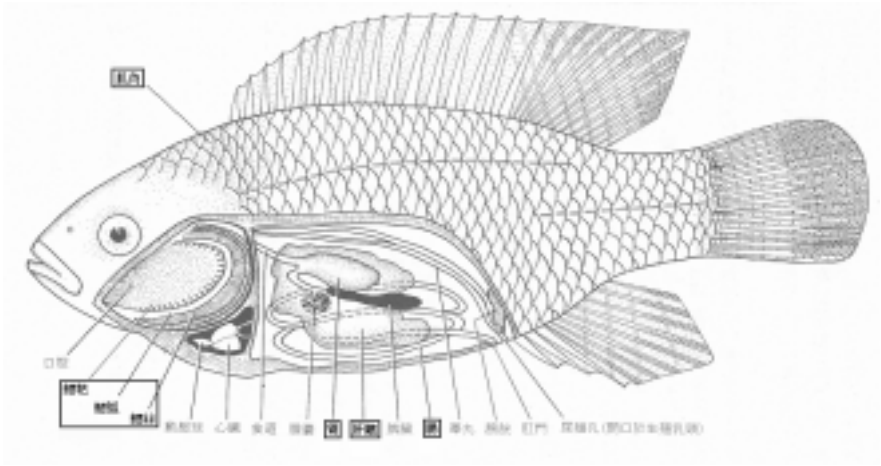

Figure 1. This figure shows where the tissues samples were removed from tilapia fish in current research.

RNAs isolation from fish tissues

Frozen fish tissues (liver, muscle, gills, intestine, blood, and kidney) were thawed and homogenized in TRIzol Reagent (GibcoBRL) using a glass-Teflon homogenizer according to the manufacturer's instructions. Briefly, after 5 min room temperature incubation, chloroform was added for phase separation. The upper aqueous phase which contains RNA was collected, and the RNA was precipitated by mixing with isopropyl alcohol. The RNA pellet was washed once with 75% ethanol, and the RNA pellet was air-dried and finally re-dissolved in RNase-free water.

RNA quantification and separation

The amount and yield of isolated RNAs from various fish tissues were quantified by measuring A_{260} and ratio of A_{260} / A_{280} was used to indicate the quality of RNAs. In addition, the integrity and quality of RNAs were further examined by electrophoresis of RNAs through Agarose Gels Containing Formaldehyde.

Semi-quantitative RT-PCR

RT-PCR is performed using Ready-To-Go^{TM} RT-PCR beads (Amersham Pharmacia Biotech). Briefly, total RNA is diluted to 1 μg/μl in RNase-free water, mixed with 0.5 μg of pd $(T)_{12-18}$ and 47 μl of RNase-free water to a final volume of 49 μl in a reaction tube containing RT-PCR bead. The reaction is incubated at 42°C for 30 min, followed by 95°C for 5 min to inactivate the reverse transcriptase and completely denature the template. Forward / reverse primers to the amplification of HSP70 will be designed from the literatures. One of the gene specific primer sets (5' and 3'primers for HSP70) is added to give a final volume of 50 μl. The number of PCR cycle is varied to determine the optimal number that would allow detection of the appropriate messages, while still keeping amplification for these genes in the log phase. Five μl aliquots of the PCR reaction are collected after 18, 20, 22, 24, 26, 28 and 30 cycles to determine the linear range of the reaction, in two independent experiments. Control amplifications are done either without RT or without RNA. As a control, a DNA fragment of the constitutively expressed L18 ribosomal protein gene is amplified using the L18 primers. Following PCR amplification, the reaction products are run at

100 V on a 1% agarose gel with 0.5 μg/ml ethidium bromide. The HSP70 and L18 reactions are analyzed in the same slot on a 2% agarose gel. The total band volumes of amplified products are calculated by gel-documentation software (Kodark). All glass- and plastic ware are treated with diethyl pyrocarbonate and autoclaved.

GSH assay

For GSH assay, we exposed fish to As(III) for 1 and 3 days. GSH_T was measured according to the method of Baker et al. (1990). Fresh fish tissues (liver, muscle, gills, intestine, blood, and kidney) were washed in 1×PBS. The sample was resuspended in 5% 5-sulfosalycyclic acid (SSA), sonicated, and centrifuged at 10 000×*g* for 2 min. The supernatant was then collected for use in the assay. For the GSH_T assay, 10 μl aliquots of each sample were placed in a 96-well plate with 200 μl reaction buffer (100 mM NaH_2PO_4, 1 mM EDTA (pH 7.5), 0.15 mM DTNB, 0.2 mM NADPH, 1 U/ml glutathione reductase). Rate of absorbance change of samples was measured at 405 nm at 25 °C over 2 min. Concentrations of GSH_T were determined using standard curves of GSH in 5% SSA.

(五) 結果與討論

Subchronic toxicity test

During the exposure period, death of the fish was not observed indicating that arsenite treatment to fish was within sublethal concentration. Table 1 and Table 2 show the measurement of fish weight and size during arsenic exposure period.

Table 1. Measurement of the weight of fish samples

Exposure period	Arsenic exposure concentration (ppm)						
	0	1		2		4	
NO.	0	1	1R	2	2R	4	4R
Week1	20.8	17.4	22.3	32.9	49.2	30.8	36.5
Week2	32.6	17.4	22.1	13.5	20.6	18.7	35.7
Week3	-	21.2	25.4	25.6	16.1	15.4	24.4
Week4	-	28.2	19.5	22.0	21.2	18.4	14.3

(unit：g)

Table 2. Measurement of the size (length) of fish samples

Exposure period	Arsenic exposure concentration (ppm)						
	0	1		2		4	
NO.	0	1	1R	2	2R	4	4R
Week1	10.2	10.2	12.3	14.0	14.5	12.5	14.2
Week2	13.1	10.3	11.2	9.8	11.3	10.5	13.5
Week3	-	11.0	11.3	11.7	10.0	10.0	11.7
Week4	-	12.5	11.1	11.2	11.0	10.2	9.7

(unit：cm)

RNAs isolation from fish tissues

Liver, muscle, gills, intestine, blood, and kidney were removed from fish after As(III) exposure. Tissue samples were weighed and immediately frozen in liquid nitrogen and stored at -80^{o}C for further analyses. The amount and quality of isolated RNAs from various fish tissues were analyzed. The representative RNA gel was shown in Figure 2.

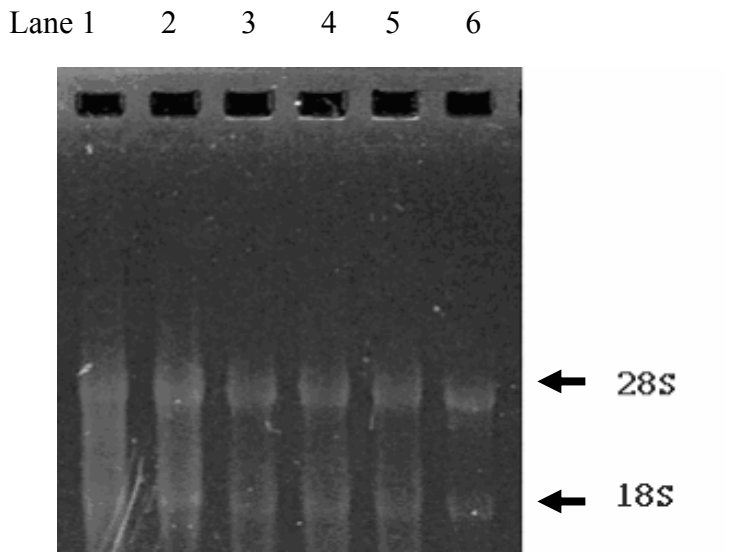

Figure 2. Representative RNAs gel. Purified RNAs from liver, muscle, gills, intestine, blood, and kidney were located in lane 1 ~6, respectively.

GSH assay

We also study the level of oxidative stress induced by As(III) by measuring GSH in fish tissues. We expected the level of oxidative stress can be used as As(III) exposure to fish. Current results showed that GSH content induced by As(III) was tissue- and time-dependent as shown in Figure 3. Moreover, figure 4 showed that GSH content induced by As(III) was dose-dependent.

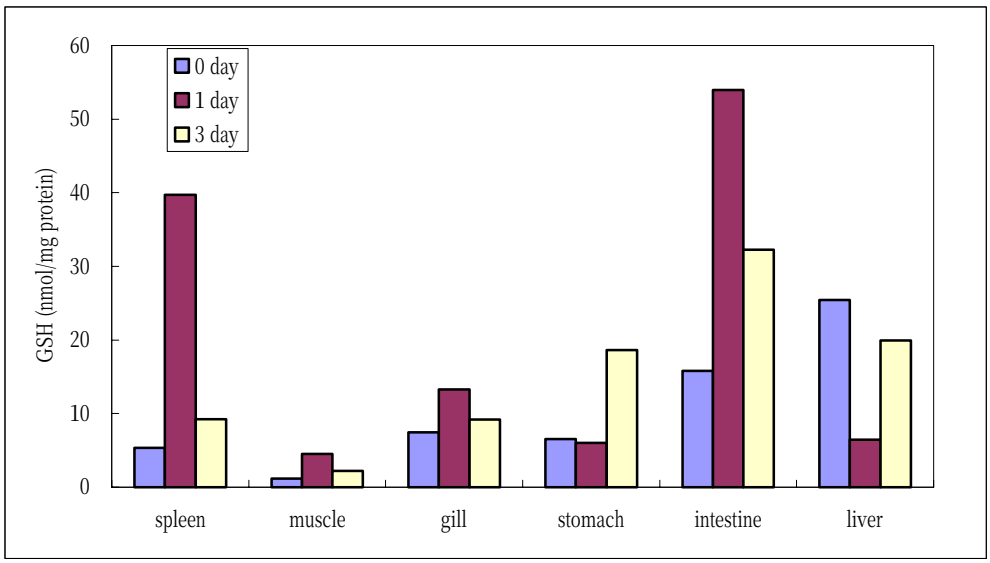

Figure 3. Representative GSH assay result. Data were fish exposed to 1 ppm As(III) for 0, 1, 3 days, respectively.

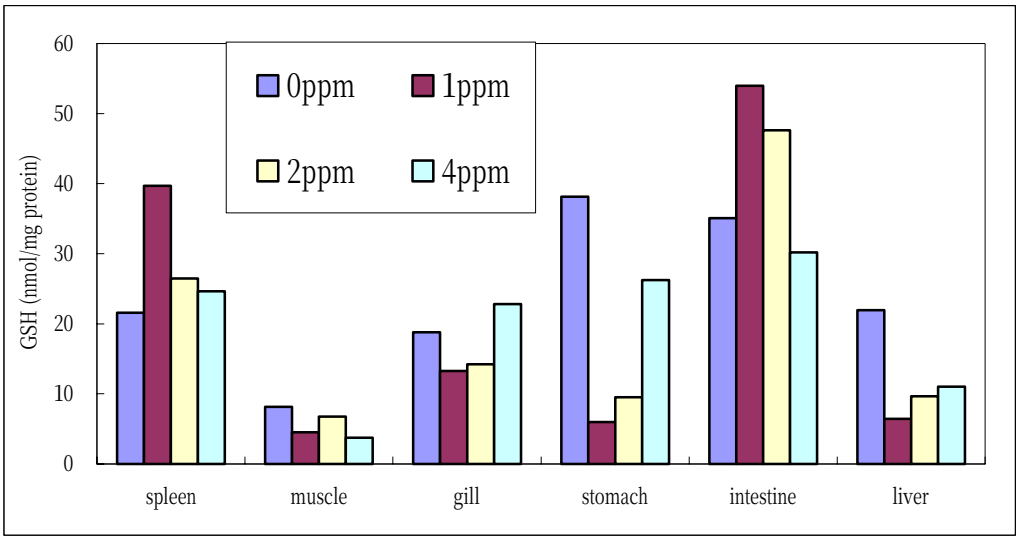

Figure 4. Representative GSH assay result. Data were fish exposed to 1 day with 0, 1, 2, 4 ppm As(III), respectively.

(六) 計畫成果自評

Currently we have successful isolated high yield and quality RNAs for RT-PCR analyses. Qualitation of HSP70 and MT gene expression by using RT-PCR strategies is under way. In addition, we are currently repeating GSH-related experiment. Current results fulfill the expecting results and progress proposed in this project. After completing this project, the results are suitable for publication in SCI journal.

(七) References

Baker, M.A., Cerniglia, G.G. and Zaman, A., 1990. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. *Anal. Biochem.* **190**, pp. 360–365.

Albores, A. et al. (1992) *Chem-Biol. Interact.* 85, 127-140

Bauman, J. W. et al. (1993) *Fundam. Appl., Toxicol*. 21, 15-22

Basu, N. et al., (2002) *Gene* 295, 173-183

Beyersmann, D. (2002) *Toxicol. Lett.* 127, 63-68

Bierkens, J. (2000) *Toxicology* 153, , 61-72

Chen, Y. C., et al. (1998) *J. Cell. Physiol.* 177, 324-397

Del Razo, L., M. et al. (2001) *Toxicol. Appl. Pharmacol.*177, 132-148

Garcia-Vargas, G. G. and Cebrian, M. E. *In toxicology of metals*, pp. 423-438, CRC

Hartwig, A. (1995) *Biometals* 8, 3-11

Honda, K. I. Et al. (1992) *Teratog. Carcinog. Mutag.* 11, 235-244

Kitchin, K. T. (2001) *Toxicol. Appl. Pharmacol.* 172, 249-261

Maitani, T. et al. (1987) *Toxicol. Lett.* 39, 63-70

Moore, M. M. et al. (1997) *Mutat. Res.* 386, 279-290

Morimoto, R. I. *The Biology of Heart Shock Proteins and Molecular Chaperones*, Cold Spring Harbor, NY, 1994; p.1-30.

Ritossa, F. (1996) *Cell Stress Chap.* 1, 97-98

Wijeweera, J. B. et al. (2001) *Toxicol. Sci.* 61, 283-294

(三) 研究方法、進行步驟及執行進度

The 1st year (08/93 – 07/94)

Specific Aim 1

Investigation of the biochemical effects of target sites of mode of arsenic in various tissues of fish.

To accomplish this aim, stress response gene expression (HSP) analysis to the exposure of chronic sub-lethal concentration of arsenic in different fish tissues will be conducted. The stress response will be compared with the internal body burden of arsenic in different fish tissues.

Specific Aim 2

Development of mechanism-based biomarkers for metal mixtures in fish. This aim seeks to establish correlations among metal toxicities and mechanism-based biomarkers and to determine the utility of mechanism-based biomarkers and broader indices of cellular effect to predict associated effects of multiple chemical exposures. This aim will be achieved by quantify the effects of various concentration of arsenic, singly and in combination, on stress response in fish. Dose-response relationship of biomarker and arsenic species will be conducted.

As a primary goal, we plan to identify sensitive biomarkers for arsenic - molecular changes that act as warning system indicating that cells have been exposed, affected or are particularly susceptible to damage by arsenic. Developing biomarkers will contribute to a better understanding of the way arsenic affects living systems and will be useful in monitoring arsenic exposure in people and the environment.

RNA isolation

Frozen tissues are thawed and homogenized in TRIzol Reagent (GibcoBRL) using a glass-Teflon homogenizer according to the manufacturer's instructions. Briefly, after 5 min room temperature incubation (20°C), chloroform is added for phase separation. The upper aqueous phase is collected, and the RNA is precipitated by mixing with isopropyl alcohol. The RNA pellet is washed once with 75% ethanol, and the RNA pellet is air-dried and finally re-dissolved in RNase-free water.

Stress protein analysis

HSP70 protein was analyzed by Western blotting. Whole tissues are homogenized in ice-cold lysis buffer (containing 100 mM Tris-HCl (pH 7.5), 0.1% SDS, 1 mM ethylenediamine tetra acetic acid (EDTA), 1 mM pepstatin A, 1 mM a-toluenesulfonyl fluoride (PMSF), 1 mM leupeptin, and 0.01 mM aprotinin). The tissue lysates are cleared in a microcentrifuge at 16,500g for 3 min at room temperature. Ten microliters of supernatant is taken for protein determination

with the bicinchoninic acid (BCA) assay using bovine serum albumin (BSA) as a reference. A 1:1 solution of supernatant and SDS-sample dilution buffer is boiled for 3 min and then frozen at -80oC until HSP70 quantification.

Levels of HSP70 protein are measured using the discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) method. Proteins from each fish tissues are resolved with a 4% stacking and 12.5% resolving gel on a Mini-Protean II electrophoresis cell (Bio-Rad, CA). Prestained molecular weight markers (Gibco- BRL, Burlington, ON, Canada) and tissue protein samples from control (non-stressed) fish are added to every gel to normalize the data.

Following electro-phoretic separation, the proteins are transferred onto nitrocellulose membranes for immunoblotting. Briefly, membranes were incubated for 1 h with HSP70 primary antibody (HSP 70 antibody will be purchased from commercial). Following the incubation in primary antibody, membranes are incubated in an alkaline phosphatase conjugated secondary antibody for 1 h. Both antibodies were diluted with 2% wt / vol skim milk powder in TTBS (20 mM Tris, 500 mM NaCl, 0.05% v/v Tween-20, pH 7.5).

Statistical analysis

All data are presented as mean ± standard deviation. The levels of HSP70 mRNA in the different tissues are analyzed by ANOVA to test if different heavy metal exposure causes significant quantitative changes in the gene expressions. The level of significance is set at p value < 0.05.

The 2nd year (08/94 – 07/95)

Specific Aim 3

Ecotoxicological assessment of heavy metals contamination in contaminated sites. This aim is to determine the feasibility of biomarkers in real environmental samples. Water samples from arsenic contaminated sites will be collected and the composition of arsenic species will be analyzed. Fish will be exposed to environmental samples and the induction of HSP70 will be analyzed. Experimental procedures are similar to those in the 1st year.

（四）預期完成之工作項目及成果

The 1st year (8/93 – 7/94)：

1. The induction of HSP70 mRNA and protein after arsenic exposure in different fish tissues is determined.
2. Dose-response relationships of arsenic species exposure are established.
3. Relationship of toxicity and biochemical effects is established.
4. Biomarkers of arsenic exposure / effect are developed.
5. This project could at least train two master students and two undergraduate students. The students will learn how cells response to an environmental toxicant arsenic. The students will also learn the knowledge of toxicology and molecular biology as well as many biotechnology techniques.

The2rd year (8/94 – 7/95):

1. The environmental samples are tested for the feasibility of biomarkers.
2. This project could at least train two master students and two undergraduate students. The students will learn how cells response to an environmental toxicant arsenic. The students will also learn the knowledge of Ecotoxicology and Molecular Biology as well as many biotechnology techniques.