Mini Review

Heavy metal biomarker: Fish behavior, cellular alteration, enzymatic reaction and proteomics approaches


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Abstract

Due to the latest industrial development, many dangerous chemicals have been released directly or indirectly which resulted in the polluted water bodies. Water rehabilitation is an alternative way to restore the quality of water, followed by the environmental management to control the waste discharge to ensure the balance of the degradation rates or detoxifying by environmental factors. However, this process consumed a lot of time and cost. Besides, most of the metal ions, especially copper which is capable to bioaccumulate in aquatic organism and at the elevated level may cause physiological and biochemical alteration which leads to mortality. Environmental monitoring is the initial step presupposed evaluating the potential toxicity of effluent gushing at its purpose to discharge, avoiding the determining effects of contaminant in water bodies. Due to the high sensitivity of the aquatic life towards dissolving toxicant, the fish has been utilized as the biological measurement (Biomarker) to indicate the existence of toxicant exposure and/or the impact towards the evaluation of molecular, cellular to physiological level. Thus, this paper gives an overview of the manipulation of fish as a biomarker of heavy metals through behavior response, hepatocyte alteration, enzymatic reaction and proteomic studies which have proven to be very useful in the environmental pollution monitoring.

Keywords

Heavy metals, Copper, Behavior, Cellular, Enzyme, Proteomics

Introduction

In the most recent decades, most of the country are undergoing a rapid industrial development, urbanization, construction, mining activities and deforestation. These activities may leads to the environmental problem such as land, air and water pollution. Water pollution is a major problem across the globe with the presence of harmful contaminants in the environment that had increased much concerns because of the green revolution (Skouras et al., 2003). Spain and New Delhi was reported to be contained with urban sewage, industrial liquid waste and liquid flows off from agricultural and industrial activities (Nagdeve, 2004; Moreno et al., 2006). Previous studies reported several rivers in Malaysia which has been polluted with pesticides and fertilizer residues from over-application of agricultural activities and heavy metals from domestic waste of industrial factory (Leong et al., 2007; Abbas Alkarkhi et al., 2008; Yap and Pang, 2011). Juru river, Penang is an example of the most polluted river in Malaysia due to the rapid development of the economy along with urbanization (Al-Shami et al., 2011).

The other common hazardous material which caused negative impacts to the aquatic ecosystem are phenol (Busca et al., 2008), azodyes (Hong et al., 2007), acrylamide (Sathesh Prabu et al., 2007), automobile lubricant (Lopes and Bidoia, 2009), which can be eliminated through the remediation of either physical, chemical or biological. Remediation explanation was well reviewed by Wang et al. (2012). Bioremediation is a method capable of degrading environmental pollutants into a nontoxic compound by living organisms through enzymatic and metabolic action influence by the type of medium, pH, oxygen, temperature and nutrition available (Vidali, 2001). However, although bioremediation have many benefits such as lower cost, reduced site disruption and permanent removal of the waste, this method still have some limitation and controversial, such as numerous pollutants, especially heavy metals, and the inability to degrade radionuclide and chlorinated compound or the degradation may produce toxic...
metabolites (Boopathy, 2000; Shukor et al., 2006). Thus, preventive step is crucial for this situation.

Biomarker was considered as the reliable method to evaluate the biological response towards environmental risk so that preventive measures can be taken. This method has the advantage to elucidate the stress level through bioassay on the organism at various stages from biomolecular, histologically to physiological alteration caused by contaminant exposure. For example, the principle of bioassay is to measure the inhibition level of the cells biochemical characteristics in order to determine toxicant (Krawczyński, 2000). The application of cholinesterase (ChE) as a biomarker for metal and organochlorine compound in Kootenai River was also given as an example (Kruse and Scarnecchia, 2002). Biomarker for monitoring environmental quality in aquatic ecosystem had raised a great deal and promising tool of interest caused by its economical method, early warning signal, adequate in uncovering overall considered toxicities of complex mixtures and measurement precision (Mayeux, 2004; Paustenbach and Galbraith, 2006; Sarkar, 2006). In this review, we describe various fish biomarker and its significance as a diagnostic tool for determining fish health status and aquatic pollution monitoring. This review also focuses more on heavy metal; copper, especially as their adverse effect and pollutant source was higher compared to the others.

Fish as a biomarker tool

Fish has turned into a favorite subject biomarker research caused by its sensitivity to temperature changes, natural surroundings and water quality deterioration and additionally aquatic contamination antagonistically influence the fish health, which might bring mortalities and ecosystem degradation (Skouras et al., 2003). Fish biomarker including the assessment of biomolecular, cellular and physiological alteration that were utilized for monitoring the biological effect of toxicant especially metal exposure. In the last 5 years, an increasing interest towards biomarkers of heavy metals have been recorded as observed in Figure 1, where the number patterns of papers published in these fields within the last 5 years was reported. The interest in biomarkers for heavy metals impact was defined parallels to the development of biomonitoring program, according to the test subject either had been exposed in the past or currently exposed to environmental stimuli. Fish liver shows the highest popularity in the study of heavy metals toxicity with the number of 85 papers published followed by muscle, gill, kidney and brain with the number of 62, 57, 36 and 23 papers, respectively.

Figure 1. Number of papers published in last 5 years. The research was carried out on Scopus by using five research queries, respectively: (Gill) “Fish gill” and “Heavy metal toxicity,”(Liver) “Fish liver” and “Heavy metal toxicity,”(Brain) “Fish brain” and “Heavy metal toxicity,”(Kidney) “Fish kidney” and “Heavy metal toxicity,”(Muscle) “Fish muscle” and “Heavy metal toxicity.” (Scopus, February 2014)

The entrance of heavy metals from direct contact may cause inhalation to be bioaccumulated into the liver before it is being distributed to the body tissues of fish and caused metabolism abnormalities such homeostatic imbalance, enzyme inhibition and retarded of growth development at the elevated level (Cohn et al., 1992; Ali et al., 2003; Canli and Atli, 2003; Flora et al., 2008; Sarosiek et al., 2009; Lauer et al., 2012). Vutukuru et al. (2005) reported that heavy metal such as copper decreased the respiratory and metabolic rate of freshwater fish, Esomus danricus understudied. The significant decreasing in the number of glycogen, tissue oxygen consumption and piruvate level of the whole body of Cyprinus carpio was determined due to the stress by copper exposure (Reddy et al., 2008). Toxicity effects of pesticides on fish has been reported to give response to biochemical, cellular and proteome of the test body (Kruse and Scarnecchia, 2002; Matos et al., 2007; Biales et al., 2001; Anzolin et al., 2012; Sukumaran et al., 2013). Thus, more study can be conducted from this finding based on fish biomarker which can help on estimating the toxicity level through the observation of fish behavior, cellular alteration, enzymatic, and proteome response.

Fish behavior

The studies on fish behaviors provide a lots of knowledge and information because, any behavior alteration can be related to physiological biomarker in aquatic species (Kristiansen et al., 2004; Amiard-Triquet, 2009; Hellou, 2011). For example, the monitoring of behavioral response becomes an
impending option to environmental change, disease, stress and the presence of toxic compound in water, which most of this condition initiates the variation of fish behavior (Petrell and Ang, 2001; Kane et al., 2004; Almazán-Rueda et al., 2004; Gerhardt, 2007). Fish behavior represents the fish physiological response towards the environmental factor. Moreover, the interaction of fish behavior related to the ecology can be easily observed even if it can quantified (Scott and Sloman, 2004) and at the same times effecting the consequences of metal toxicity upon the concentration and species, including size (Vosyliene et al., 2003; Hussain et al., 2011). For instance, the existence of metal ion in the environment mediation increased the mucus like secretion from gill, excessive excretion, anorexia and also the fin movement (Ezeonyejiaku et al., 2011). Chronic metal exposure includes complex physiological alterations in many body systems, involving the increased oxygen consumption, reduced mean swimming velocity or speed, up-regulation of ionic parameter, the decreasing amount of optimal lymphocyte and the increasing level of neutrophils, altered immunity system, the adjustment of Cu-dependent and independent enzyme activities, and abundance of epithelial cells in the gills or intestine (Handy, 2003). Alteration in behavior is considered as a sensitive biomarker to evaluate the toxicant exposure and/or effect (Gerhardt, 2007). Affected fish with behavior alteration toward toxicant especially pesticides or heavy metal has been reported by Patil and David (2010), Ezeonyejiaku et al. (2011) and Javed (2012). The parameter for behavior alteration measurement such as swimming performance, avoidance behavior and feed intake has been implemented.

Swimming performance
Swimming performance is considered as behavior parameters to assess the physiological status of aquatic life to measure the presence and effects of contaminant (Ballesteros et al., 2009; Cailleaud et al., 2011; Almeida et al., 2012). The result clearly showed that the dependence on the concentration of toxic causes the loss of resistance in the fish swims which has been proven by the study of Vieira et al. (2009). In the duration of copper exposure, the concentration level of Na\(^{+}\), K\(^{+}\) and Ca\(^{2+}\) in plasma decreased (Pilgaard et al., 1994; Beaumont et al., 1995) caused by the increasing of total plasma ammonia concentration affecting the swimming speed (Grosell et al., 2002). These compound ions are known to have a number of metabolic and physiological effects that may influence swimming performance by interfering with the metabolic status of the muscle or affect central or peripheral nervous activity, transmission at the neuromuscular junction, excitation/contraction coupling or muscle electrophysiology (Beaumont et al., 1995). The excessive level of ammonium ions are capable of replacing K\(^{+}\) in the exchange of mechanism consequence in depolarization of neuron (Binstock and lecar, 1969) then causes fatigue associated with low contraction force in the skeletal muscle (Sjøgaard, 1991). Swimming performance has been implemented by the previous study to assess the toxicity of the compound by the measurement of swimming velocity (cm s\(^{-1}\)) based on swimming distance and time required to cover it, or through the critical swimming speed of calculation on maximal swimming speed (U\(_{\text{max}}\)) and exhaustion time (Waser et al., 2009; Almeida et al., 2010).

Avoidance behavior
Previous authors recommended that the studies of avoidance behavior can be utilize as a very sensitive indicator of ecotoxicology effects and should be used as a corresponding tool in risk evaluation (West and Ankley, 1998; Kravitz et al., 1999; Moreira-Santos et al., 2008). In this study, avoidance behavior was observed through fish that escape the water, excessive aggressiveness, agitated or shows an unsteady swimming pattern with irregular movement (Ezeonyejiaku et al., 2011). Other reports strengthen the study about low concentration of copper exposure, fish activity that shows avoidance behavior, but not at a very high concentration (Giattina et al., 1982; Hansen et al., 1999). The induction of avoidance caused by irritation of the gill and taste, had also shows the aggressiveness of the fish at the same time. However, the high concentration had displayed a failure to avoid copper concentration which indicated that the detection and avoidance are not functioning properly, confusing the organisms which will causing it to be disoriented (Hansen et al., 1998). Other species such as prawn Palaemon serratus cannot avoid the high concentration of organophosphate fenitrothion (Oliveira et al., 2013). This proved that copper was capable to damage the function of the olfactory system by impairing olfactory epithelial structure and at the same time reducing the neurophysiological response towards the olfactory stimulant (Bjerselius et al., 1993). The avoidance behavior test has been well explained by Lopes et al. (2004) through calculation of the entrance and exited by a number of fish from the test compartment in the evidence test chamber, or from the other data that can be obtained from the computer vision monitoring such study developed by Jian-Yu et al. (2005). There were various chambers that has been developed for the avoidance behavior

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study such as Dornfeld et al. (2009) and Oliveira et al. (2013).

**Feed intake**

Swimming performance and avoidance behavior affects the fish appetite because both of the activities is a part of the main nature determining the survival of the fish such as food obtaining, reproduce, and also avoid adverse conditions (Plaut, 2001). According to Ali et al. (2003) studies showed that the fish which were exposed to different concentration (0.15, 0.3 and 0.5 ppm) to affect their diet, reducing their feed consumption caused by refused feed after exposure about 4-5 hours. Pelgrom et al. (1994) observed the nutritional status of fish influenced by the accumulation of copper at the same times affected the other metal concentration content in the fish. Due to the study by Parveen and Javed, (2010), Fish Catla catla reduced the feed intake after a week exposure of sublethal copper at 19.44 ppm. Common carp also shows a reduction of food intake at 0.8 µM copper exposure which determined after a week of test. Copper exposure caused the increasing level of glucose in blood, which suppressed fish’s appetite and decreased food intake (Colgan, 1973). This process occurs from the hormonal change which turned the system to fooled into receiving the caloric intake more than normal then utilized the of liver glycogen into blood glucose. Moreover, a high energy demand is needed for metabolic coordination in the liver for maintaining the continuance of detoxification process to overcome chemical stress (Moreira et al., 2006; Amiard-Triquet, 2009; Oliveira et al., 2013) which proved by James and Samph (1995) had showed the decreasing level of glycogen in freshwater fish, Heteropneustes fossilis liver exposed to sublethal of copper and ammonia or individually. Study done by Ezeonyejiaku et al. (2011) reported that the mortality response of the fish can be observed at the end of the exposure times, which the fish sank into the bottom of the containers and became motionless. Feed intake can be measured by the calculation of feed conversion ratio which the increasing value correspond to the increasing toxicity level in the medium (Vincent et al., 1996; Ali et al., 2003), or calculation on feed conversion efficiency which the value is lower compared to the control indication of the toxicity affection such as the study done by Javed (2012) which affected the selected fish after being exposed with different type of heavy metals.

**Cellular observation**

**Molecular quantification**

Deposition of pesticides and heavy metals in digested fish protein were measured by previous studies using high performance liquid chromatography (Rao et al., 2010), gas chromatography (Fianko et al., 2011), Inductive couple plasma (Ribeiro et al., 2005) and atomic absorption spectrophotometry (Shukla et al., 2007). Due to fish gill properties, such high permeability and large area of contact with environment had made this organ to become the primary target of be toxicant to be accumulated before being transported into the fish’s body. Study done by Jayakumar and Paul (2006) reported that the exposure of sublethal concentration of cadmium showed that this metal ion was highly accumulated in the gill tissue compared to other organ within the first 10 days until it reached 40th days of exposure, which decreased the cadmium concentration but inversely proportional with the other organ; liver, kidney and muscle, which were increased with the exposure time. This situation has been explained by Jezirowska and Witeska (2007) and Kim et al. (2011) which the metal exposure, such copper was rapidly increased in the early period of exposure and decline at recovery periods while other organ shows higher metal accumulation at the recovery periods. The liver is the main toxicant deposition as it neutralize and detoxify before distributed to another organ or eliminate through excretion. Previous studies shown that the liver can actually accumulate metals higher than other tissues like skin, muscle and gills (Yousafzai et al., 2009; Karayakar et al., 2010; Crafford and Avenant-Oldewage, 2011). However, the utilization of the fish organ as biomarker of toxicant has been implemented to assess the river contamination and fish health status, such as the study by Ribeiro et al. (2005), Qadir and Malik (2011), and the good example from Javed and Usmani (2011) which the accumulation of copper in selected organ of C. punctatus and L. Rohita; collected from fish market was reported to exceed the permissible limit set by WHO/FAO (1989) hence proves the capability to assess the toxicity level and health status.

Hepatosmotic index (HSI) is another parameter of ecotoxicology which the calculation was based on the ratio of liver weight per body weight (Jelodar and Fazli, 2012). HSI was reported to become a useful measurement to assess the pesticides and metal toxicity level, such as the study by Versonnen et al. (2003), Chandra et al. (2004), Abdel-Hameid (2008), and Kaoud et al. (2012). HSI is the measurement on the status of energy stored in a fish. It has been reported on the toxic environment that fish normally has a smaller liver, which reserve less energy in the liver that caused the HSI value to becomes higher than the normal condition such as the study done by
Figueiredo-Fernandes et al., (2007) reported the HSI value of Nile tilapia liver increased with the increasing of the copper sulfate concentrations after 21 days exposure. Jelodar and Fazli (2012) and Lenhardt et al. (2009) studied based on HSI value to evaluate the contamination site for every month using a frog and starlet, respectively, as a biomarker tool. Thus, HSI was considered as a sensitive indicator to provide information on potential water contamination impact (Yang and Baumann, 2006; Lenhardt et al., 2009).

**Microscopic observation**

Several fish organs such as gill, liver, kidney, muscle and brain were selected to visualize the cellular alteration due to pesticides and metal toxicity impact (Rojik et al., 1983; Tort et al., 1996, Rodrigues and Fanta, 1998; Pugazhvendan et al., 2009; Kaoud et al., 2012; Patmaik et al., 2011; Ahmed et al., 2013; Al-Bairuty et al., 2013). Gill is the first direct contact with the external environment and changes in fish gill around the most usually distinguished reactions to environmental toxins (Au et al., 2004). Normal and affected gill tissues was visualized by Campagna et al. (2008) which the affected gill showed structural deformation such as epithelial lifting at secondary lamella, hyperplasia of primary epithelium, fusion of secondary lamella, aneurisms, necrosis and infiltration of inflammatory cells with the disintegrate of epithelial cells of secondary lamellae including mucus secretion and swollen mucocyt. Fish gill defense mechanism and its potential as biomarker has been well explained by Nascimento et al. (2012).

Fish liver and kidney also are an alternative biomarker tool to evaluate toxicity level. At the beginning of toxicity level, morphology of parenchyma cell shows the abnormalities such as cytoplasmic vacuolation along with dilation and congestion of sinusoid depending on duration and toxicant concentration exposure, such as the study by van Dyk et al. (2007) and Younis et al. (2013). At high toxicity level, other abnormalities appeared such as macrophage activity, hyalinization, hemorrhage, binucleai, apoptosis and necrosis development (Cavas et al., 2005; Wolf and Wolfe, 2005; Younis et al., 2013). Ultrastructure visualizations were performed by Gernhöfer et al. (2001) and Abdel-Moneim and Abdel-Mohsen (2010) by monitoring and evaluate the health status of fish in contaminated areas. Normal parenchyma cells which is untreated or unaffected by toxicant showed normal polygonal shape with the normal form of the nuclear envelope, endoplasmic reticulum, spherical shape of mitochondria and cytoplasm, but affected cell showed the clear development of karyorhexis, karyolysis and pyknosis nucleus associated with the clumping of nuclear chromatin, fragmentation of endoplasmic reticulum, enlargement of hepatocyte bile canaliculi, lipid droplet accumulation, vacuolation, increasing number of lysosome, dilation and matrix dense in mitochondria until elimination processes such apoptosis; cell budding formation, and necrosis; cell swollen and ruptured membrane plasm observed (Paris-Palacios et al., 2000; Varanka et al., 2001; Jiraungkoorskul et al., 2007; Abdel-Moneim and Abdel-Mohsen, 2010; Costa et al., 2010; Narayan and Al-Bader, 2011; Salem, 2011). Unlike liver, affected kidney shows an additional impairment such as the damage to the epithelium of some renal tubules and increased Bowman's space in the kidney while affected brain tissues showed the swelling of blood vessels on the ventral surface of the cerebellum, alteration in nerve cell bodies in the telencephalon and the thickness of the mesencephalon layers (Al-Bairuty et al., 2013).

This observation can be measured by semiquantitative analysis, such as the study by Gernhöfer et al. (2001), and Abdel-Moneim and Abdel-Mohsen (2010), or fully quantitative analysis done by Paris-Palacios et al. (2000) by measuring the surface area of clear, dark and nucleus of hepatocyte (µm²) with nucleolus diameter (µm) then compare it with concentration and duration of copper exposure, while Figueiredo-Fernandes (2007) study was based on the calculation of hepatocyte nucleus per mm of hepatic tissue (Hepat. nucl. mm⁻²) and it had been compared with different copper concentration treatment.

**Enzymatic biomarker**

Enzyme-based biomarker was considered as the most simple estimation for toxicant existence. This method gave multiple advantages such as rapid determination and it is also considered sensitive even the toxicant exist in low concentration, and low technical application. These properties together had mada it to become a highly promising method to be use in pharmacology, agriculture and environmental protection. Various sources of enzyme from bacteria, plant and animal was reported to be a sensitive biomarker with toxicant especially heavy metals (Table 1). Fish is considered as a biomarker tool and a highly sensitive enzyme as sentinel species allows the detection of lower contamination levels. Moreover, several manufactured substances caused an adverse effect in vivo and in vitro. Thus, the combination of in vivo and in vitro study gave multiple information aid standardization of environmental management and treatment to minimize and eliminate the toxicant.
### Table 1. List of enzymes from various sources as biomarker candidate for Ecotoxicology monitoring. Ag, argentum (Silver); As, arsenic; Cd, Cadmium, Cr, Chromium; Co, cobalt; Cu, Copper; Fe, ferum; Hg, Mercury; Pb, plumbum (Lead); Mo, molybdenum; Ni, nickel; Zn, zink

<table>
<thead>
<tr>
<th>Enzyme Cholinesterase (ChE)</th>
<th>Species sources</th>
<th>Compound (Toxicity value)</th>
<th>Related references</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrophorus electricus</td>
<td>Tiliajsonsymbiosa</td>
<td>Cu, Ag, Hg (IC50: 1.212, 0.1185 and 0.097 mg/L, respectively)</td>
<td>Shukor et al., 2013</td>
</tr>
<tr>
<td>Perspexiplaedus doliolii</td>
<td>Tiliajsonsymbiosa</td>
<td>Cu, Hg, Cr and As (IC50: 0.088, 0.371, 0.112 and 0.141 mg/L)</td>
<td>Sabullah et al., 2013</td>
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<tr>
<td>Human serum</td>
<td></td>
<td>Mercury chloride (IC50: 0.88 μM)</td>
<td>Mahmood et al., 2001</td>
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<tr>
<td>Pomegranates usnicaps</td>
<td></td>
<td>Cu (more than 50% inhibition treat by 200 μg/L)</td>
<td>Vieira et al., 2009</td>
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<tr>
<td>Enchytraeus albidus</td>
<td></td>
<td>Cu (IC50: 0.0579 μM)</td>
<td>Howcroft et al., 2011</td>
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<tr>
<td>Daphnia magna</td>
<td></td>
<td>Zn (Significant inhibition at 25 mg/L compared to control (p&lt;0.05))</td>
<td>Diamantino et al., 2003</td>
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<tr>
<td>Carcinus maenas</td>
<td></td>
<td>Cr and Cu (49% and 46% inhibition at the concentration of 10 mg/L for Cr and 15 mg/L for Cu, respectively)</td>
<td>Ellumalai et al., 2002</td>
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<tr>
<td>Human blood plasma</td>
<td></td>
<td>As (significant inhibition as the increasing exposure of arsenic p&lt;0.01)</td>
<td>Ali et al., 2010</td>
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<tr>
<td>Sprague-Dawley Rats</td>
<td></td>
<td>As (inhibit more than 50% treated with 20 mg As/kg)</td>
<td>Patilwa and Tchounwou, 2005</td>
</tr>
<tr>
<td>Procambarus clarkii</td>
<td></td>
<td>Cd, Pb and Hg (Significant inhibitory activity after 24 and 48 hours exposure)</td>
<td>Devi, and Fingerman, 1995</td>
</tr>
<tr>
<td>Chima striae</td>
<td></td>
<td>Hg, Cd, Pb, Ni and Zn (higher inhibition; Hg&gt;Cd&gt;Pb&gt;Zn&gt;Ni)</td>
<td>Met-Has and Mohamed, 2000</td>
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#### Antioxidant enzyme

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<tr>
<th></th>
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<th>Cd (GST activity significantly increase beyond concentration at 0.6 mg/L)</th>
<th>Cunha et al., 2007</th>
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<tbody>
<tr>
<td>Nucella lapillus</td>
<td></td>
<td>Cr and Cu (GST 68% and 61% inhibition at the concentration of 10 mg/L for Cr and 15 mg/L for Cu, respectively)</td>
<td>Ellumalai et al., 2002</td>
</tr>
<tr>
<td>Carcinus maenas</td>
<td></td>
<td>Cd (GST activity significantly increases compared to control (p&lt;0.001))</td>
<td>Atif et al., 2005</td>
</tr>
<tr>
<td>Chara punctata</td>
<td></td>
<td>Hg (CAT and GPX activity low at moderate concentration of Hg2+ site and high activity at high Hg2+ contamination; GST activity increase at moderate site and decrease at high Hg2+ contamination).</td>
<td>Meiro et al., 2011</td>
</tr>
<tr>
<td>Dicentarchus labrux</td>
<td></td>
<td>Plumbum nitrate and zinc chloride (GST, SOD, CAT activity increase after 7 days exposure time)</td>
<td>Salihu and Bawa-Allah, 2012</td>
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#### Glucose oxidase

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<th>Hg (LOD: 0.5 mg/L)</th>
<th>Samphao et al., 2012</th>
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<tr>
<td>Aspergillus niger</td>
<td></td>
<td>Cd, Co, Cu and Ni (LOD: 2.4, 2.1, 0.2, 3.3 μM, respectively)</td>
<td>Ghica et al., 2013</td>
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#### Protease

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<tr>
<th></th>
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<th>Hg and Zn (IC50: 3.217 mg/L and 0.727 mg/L, respectively)</th>
<th>Baskaran et al., 2013</th>
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<tbody>
<tr>
<td>Coriandrum sativum</td>
<td></td>
<td>Hg and Cu (IC50: 0.09 to 0.115 mg/L and 0.07 to 0.112 mg/L, respectively)</td>
<td>Masoder and Said, 2011</td>
</tr>
<tr>
<td>Commercial bromelain</td>
<td></td>
<td>Zn and Hg (IC50: 4.8 - 6.7 mg/L and 15.76 - 17.04 mg/L, respectively)</td>
<td>Shukor et al., 2009a</td>
</tr>
<tr>
<td>Commercial trypsin</td>
<td></td>
<td>Hg, Ag, Pb and Zn (IC50: 0.39, 0.40, 2.16, 2.11 mg l-1, respectively)</td>
<td>Shukor et al., 2006</td>
</tr>
<tr>
<td>Molybdenum reducing enzyme</td>
<td></td>
<td>Cu (IC50: 0.099 ±0.013 )</td>
<td>Shukor et al., 2009b</td>
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#### Alkaline phosphatase

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<tr>
<th></th>
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<th>Cd and Cu (activity increase after 4 days exposure)</th>
<th>El-Naga et al., 2005</th>
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<tr>
<td>Mugil seheli</td>
<td></td>
<td>Total 1.25 ppm concentration of Cd, Pb, Cr and Ni (Activity keep increasing from the 1st day to 32nd days of exposure)</td>
<td>Rajamanickam and Muthuswamy, 2008</td>
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#### Lactate dehydrogenase

<table>
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<tr>
<th></th>
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<th>Cr and Cu (71% inhibition by mixture of both compounds)</th>
<th>Ellumalai et al., 2002</th>
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<tr>
<td>Parofi ch寅onlta</td>
<td></td>
<td>Hg (activity increase in heart and skeletal muscle exposed with 181 μg Hg/L)</td>
<td>Gill et al., 1999</td>
</tr>
<tr>
<td>Human hepatocellular carcinoma cell line</td>
<td></td>
<td>As (activity increase from 0.1 to 20 μg/mL)</td>
<td>Alarifi et al., 2013</td>
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<td>EPC cells from Cyprinus carpio epithelium</td>
<td></td>
<td>Zinc chloride (24 hour LDH release IC50: 0.29 mM)</td>
<td>Ni Shotilambalasingh et al., 2004</td>
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#### Urease

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<tr>
<th></th>
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<th>Cd, Cu, Zn, Ni, Hg (IC50: 0.120,0.018,0.180,0.051 and 0.008, respectively)</th>
<th>Jung et al., 1995</th>
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<tbody>
<tr>
<td>Soybean</td>
<td></td>
<td>Ag, Hg, Cu (IC50: 2.3x10^-6, 7.4 x 10^-6, and 0.0033 mM, respectively)</td>
<td>Kumar and Kayastha, 2010</td>
</tr>
</tbody>
</table>
exposure based on the mode of action and reaction with biological system

In vivo assay

In vivo test was implemented by previous study to evaluate environmental risk, such as the effects metal ion on aquatic organism. (Singh et al., 2012; Saliu and Bawa-Allah, 2012; Han et al., 2013). Fish has been purposely exposed with toxicant either at different concentration or duration exposure time, and at the same time inducing the oxidative stress by generating highly reactive oxygen species (ROS) leading to cell death program (Kehrer, 2000; Do Lago et al., 2011). The entrance of toxicant may activate the function of detoxifying enzyme to neutralize the xenobiotic to non-toxic compound, and antioxidant enzyme plays an important role as a protective mechanism and hemostasis balance to remove ROS from either free and no free radicals (Tripathi and Gaur, 2004). Previous report mentioned the metabolic enzyme such as aspirate amino transferases (ASAT), alanine amino transferases (ALAT), superoxide dismutase (SOD), and glutathione s-transferase (GST) had significantly increase in the activity induced by the presence of toxicant especially heavy metals (Singh et al., 2012; Saliu and Bawa-Allah, 2012; Han et al., 2013). However, it depends on the targeted organ and species suchas the study done by Crupkin and Menone (2013) mentioned that GST activity in liver had significantly decreased while increase in gill and brain of fish Australoheros facetus after exposed with cadmium. Chourpagar and Kulkarni (2012) reported GST from freshwater crab activity was decreased as the exposure periods increased by copper sulfate. A previous report mentioned the metabolic enzyme such as aspirate amino transferases (ASAT), alanine amino transferases (ALAT), superoxide dismutase (SOD), and glutathione s-transferase (GST) had significantly increase in the activity induced by the presence of toxicant especially heavy metals (Singh et al., 2012; Saliu and Bawa-Allah, 2012; Han et al., 2013). However, it depends on the targeted organ and species suchas the study done by Crupkin and Menone (2013) mentioned that GST activity in liver had significantly decreased while increase in gill and brain of fish Australoheros facetus after exposed with cadmium. Chourpagar and Kulkarni (2012) reported GST from freshwater crab activity was decreased as the exposure periods increased by copper sulfate. A Biomarker of oxidative stress based on antioxidant enzyme such SOD, GST, catalase (CAT), and glutathione peroxidase (GPX) have been utilized to determine the toxicity level of metal ion in aquatic organism and has been proved to be sensitive (Radi and Matkovic, 1988; Lopes et al., 2001; Farombi et al., 2007).

Vutukuru et al. (2006) reported that the activity of SOD and CAT of Esomus danricus were decreased after being exposed to copper at different concentration and increasing exposure times. A similar result was reported by Zikić et al. (2001) which Carassius auratus gibelio Bloch SOD and CAT activity, had decreased for the first 4 days, then increase in the 7th day higher compared to control while transaminase keep increasing as the length of exposure times increase. Different result reported by Saliu and Bawa-Allah (2012) which the measurement of both SOD and CAT activity from juvenile Clarias gariepinus had increased for the first 7 days and 14 days respectively. after being exposed with Zn salt and Pb, then decreased onwards the exposure days. Vieira et al. (2009) report based on the exposure of estuarine fish Pomatoschistus microps with different concentration of copper and mercury ranging from 0 to 400 µg/L and 0 to 50 µg/L, respectively, which enzyme such as acetylcolinesterase (AChE) and 7-ethoxyresorufin-O-deethylase (EROD) activity had decreased, while GST, GPX, SOD, CAT and lactate dehydrogenase (LDH) associated with the increasing concentration of metal ion. AChE is capable to increase in activity at the low concentration of metal ion such as iron caused by up-regulated of cholinesterase (ChE) gene to produce more ChE to degrade the accumulation of acetylthiocholine in the synaptic cleft (Bainy et al., 2006; Sant’Anna et al., 2011), de Lima et al. (2013) proved the increasing activity of ChE after exposure 0.06 mg/L of copper on Danio rerio. Study of ChE was very significant caused by the relationship of this enzyme with the swimming activity of fish (Vieira et al., 2009; Tilton et al., 2011).

In vitro assay

In vitro assay was conducted by discriminating a component of an organism in order to provide specific detail analysis. For example, ChE isolated from Torpedo californica was a study of the molecular structure including amino acids presented in the catalytic triad of enzyme responsible for substrate degradation (Sussman et al., 1991). From this understanding, other data can be obtain such as the interaction of the enzyme with toxicant which shows high affinity to interact with amino acids present at the active and the allosteric site of ChE. For example, pesticides such as carbamate and organophosphate are capable to bind at the anionic and esteric site of acetylcholinesterase through carbamoylation and phosphorylation (Forget and Bocquene, 1999; Rosenberry et al., 2005) at responsible amino acids such as serine (Ser), histidine (His), glutamic acid (Glu), tyrosine (Try), and Asparagine (Asp) (Main, 1979; Fukuto, 1990; Kovarik et al., 1999; Ma et al., 2010; Thompson et al., 2010). Glusker et al., 1999 mentioned that heavy metal plays important role as an enzyme cofactor by facilitating the substrate to enzyme to form an enzyme substrate, but the heavy metal tend to inhibit the enzyme at the elevated level. Heavy metal was also reported to inhibit cholinesterase activity (de Souza Dahm et al., 2006; Frasco et al., 2008; Sant’Anna et al., 2011). Cation- π attraction of the Imidazole group of His in cholinesterase strongly attract free metal ion such as Zn and Cu (Bhanumathy and Balasubramanian,
1998; Abdelhamid et al., 2007; Rajesh et al., 2009). The negative charge of amino acid such glutamate and aspartate provides an attraction to bind with metal ion lead to structural alteration (Masson et al., 1996; Sarkarati et al., 1999). Metal ion also has an affinity to interact with other amino acid such as cysteine, methionine, phenylalanine, and tryptophan at the active and the allosteric site of protein (Glusker et al., 1999; Armentrount et al., 2013).

Thus, the in vitro assay had gave much information and increase the prediction during the term of toxicity of the compound and suitable treatment will be implemented to overcome this situation. Moreover, in vitro assay is a rapid detection as it reduces the animal testing (Takhar and Mahant, 2011). In vitro detection using acid and alkaline phosphatase (Mazorra et al., 2002; Safahieh et al., 2010) and Glucose 6-phosphate Dehydrogenase (Cankaya et al., 2011; Comakli et al., 2013) extracted from fish has been proven to be another alternative source which is sensitive towards heavy metals contamination. Although the sensitivity varies with other species, this information can be considered as a turning point for the development of biosensor kit for environmental contamination.

Proteomic analysis

Proteomic analysis had gave mass contribution method to determine the answers for the questions about agriculture and aquaculture production, medical field development, and nutrition quality and safety, including halal management, which the related question is based on chemical and physical factor, including contaminant exposure concentration and duration, biological adaptation, treatment, changes in temperature, osmotic stress, oxygen consumption, in addition to the components of developmental pathway, infection and symbioses (Carbonaro, 2004; Tomanek, 2011; Rodrigues et al., 2012; Silvestre et al., 2012).

In the development of biomarker applications, proteomic-based approaches were implemented by the previous study to evaluate the impact of toxicant such as heavy metal towards sentinel fish targeted organ and was considered valuable in the detection of early response to this toxic compound (Wang et al., 2011; Lu et al., 2012). Ecotoxicoproteomic by the combination of gel electrophoresis and mass spectrometry was expected to locate the biochemical mechanism involved in acute or chronic toxicity of heavy metals through the identified protein expression signatures (PES) and pathway impairment. A combination of both studies may strengthen the analysis results (Khoudoli et al., 2004). Selected organ such as gill, liver, and brain was reported to have a significant impact under in vivo heavy metal exposure at the proteomic level (Feng et al., 2003; Wang et al., 2011; Dortts et al., 2011; Eyckmans et al., 2012). The early relationship assessment between PES and the toxicant induction after run with one dimensional electrophoresis (1DE) or two dimensional electrophoresis (2DE) will be interpreted semiquantitatively either upregulations and downregulation of PES. For the examples, the study by Sanders et al. (1994) and Feng et al. (2003) based on 1DE showed upregulation of heat shock protein 70 parallel with copper concentration treatments and other stress protein were detected especially apoptotic factor such study by Kawakami et al. (2006).1DE result was limited to protein molecular weight separation while 2DE gave more detail result in which the protein has been separated according to their pl through isoelectrofocusing (IEF), then the second separation via molecular weight was performed by SDS-PAGE. The optimization of 2DE was well reported by Khoudoli et al. (2004).

There were various pattern types of PES; 1) Protein band or spot maintained their intensities, 2) Protein band or spot intensities kept on increasing (upregulation), and 3) Protein band or spot intensities kept on decreasing (Downregulation). Most of the type one PES are related to structural protein such as the study done by Wang et al. (2011) which actin, keratin and lamin were not affected by mercury. Another report also shows various functional proteins, especially enzyme remains unchanged during the copper treatment such as Fumarate hydratase, 4-Hydroxyphenylpyruvate dioxygenase, glutathione reductase, Uroporphyrinogen decarboxylase, Lactoylglutathione lyase, Homogentisate 1,2-dioxygenase, Serine/threonine kinase 3 and Xanthine dehydrogenase (Chen and Chan, 2011; Chen and Chan, 2012). Type two and three patterns was called the unique protein affected by any type of treatment concentration or/and duration (Tomanek, 2011). However, this unique PES are preferable for further analysis and would be identified using mass spectrometry (MS) such as matrix-assisted laser desorption/ionization-time of flight analysis/MS (MALDITOFF/MS) and liquid chromatography/MS (LC/MS).

Unique PES

An increase on the quantity of cellular component, especially protein, in response to an external effect is called upregulation. Under stress conditions, related protein such antioxidant, detoxifying enzyme, apoptotic and necrotic factor were upregulated in order to maintain the homeostasis or deleterious
Table 2: The effect of copper toxicity on organ proteome level based on proteomic approach (2D-PAGE)

<table>
<thead>
<tr>
<th>Organ (aquatic species)</th>
<th>Protein Identity</th>
<th>Treatment (References)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole body <em>Mysis edulis</em></td>
<td>13 proteins induced</td>
<td>Blue mussels exposed with 70 ppb of copper (Shipherd et al., 2005)</td>
</tr>
<tr>
<td>Whole body <em>Ophiura sp.</em></td>
<td>10 proteins repressed</td>
<td>Sodium-dependent phosphate transport protein (Eyckmans <em>et al.</em>, 2012)</td>
</tr>
<tr>
<td>Whole body <em>Sparus aurata</em></td>
<td>Probable protein brick1</td>
<td>Phosphatidylinositol glycan anchor-like (Neave <em>et al.</em>, 2012)</td>
</tr>
<tr>
<td>Whole body <em>Ctenophoca gracilis</em></td>
<td>Tranferase</td>
<td>Acetylcholinesterase</td>
</tr>
<tr>
<td></td>
<td>dRNA synthetase</td>
<td>Phosphoglycerate carboxylase</td>
</tr>
<tr>
<td></td>
<td>Phosphomannomutase</td>
<td>Dehydrogenase</td>
</tr>
<tr>
<td></td>
<td>Proteosome, subunit α</td>
<td>The marine algae exposed to seawater supplemented with 100 μg/L copper (Contreras <em>et al.</em>, 2010)</td>
</tr>
<tr>
<td>Gill <em>Diacetes auratus</em></td>
<td>ATP synthase subunit β</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATP synthase subunit β</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATP synthase subunit α</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ribulose biphosphate carboxylase large chain</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glyceraldehyde 3-phosphate dehydrogenase 1</td>
<td></td>
</tr>
<tr>
<td>Gill <em>Cyprinus carpio</em></td>
<td>Peptidase/protease</td>
<td></td>
</tr>
<tr>
<td></td>
<td>dRNA binding protein</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATP β binding protein</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Transcriptional regulator</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Carbohydrate kinase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RNA binding protein</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ABC transporter subunit</td>
<td></td>
</tr>
<tr>
<td>Gill <em>Oryzias latipes</em></td>
<td>Carbonic anhydrase 1</td>
<td>Exposed with 50 μg/L copper concentration (Eyckmans <em>et al.</em>, 2012)</td>
</tr>
<tr>
<td></td>
<td>α-lactalbumin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATP synthase subunit δ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Keratin 8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hemoglobin-like protein</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Potrait ribosomal protein L7 protein</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ribosomal protein L6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NHR domain-containing, RNA binding, signal transduction-associated protein 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TRF super family</td>
<td></td>
</tr>
<tr>
<td>Gill <em>Ctenophoca gracilis</em></td>
<td>Keratin 8</td>
<td>Troponycin 3 isoform 2</td>
</tr>
<tr>
<td></td>
<td>Mitochondrial ATP synthase beta subunit</td>
<td>Troponycin alpha-4 chain</td>
</tr>
<tr>
<td></td>
<td>F-actin-capping protein subunit beta</td>
<td>Rho GDP-dissociation inhibitor 2</td>
</tr>
<tr>
<td></td>
<td>Krf5 protein</td>
<td>Proteosome subunit alpha type-6</td>
</tr>
<tr>
<td></td>
<td>Triosephosphate isomerase 1b</td>
<td>Transcriptionally-controlled tumor protein</td>
</tr>
<tr>
<td></td>
<td>Nohrin precursor</td>
<td>Myosin regulatory light chain 2, smooth muscle isoform</td>
</tr>
<tr>
<td></td>
<td>Pancreatic carboxypeptidase A1</td>
<td>Hypothetical protein LOC43696</td>
</tr>
<tr>
<td></td>
<td>40S ribosomal protein S9</td>
<td>Predicted: similar to death-inducer blocker 1 (DIO1)- (Death-associated transcription factor 1)</td>
</tr>
<tr>
<td></td>
<td>Ribosomal protein L4</td>
<td>(DIO1)- (Death-associated transcription factor 1)</td>
</tr>
<tr>
<td></td>
<td>Histone H3a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Carbonyl-phosphate synthesis L chain, ATP-binding protein</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ribosomal protein L8</td>
<td></td>
</tr>
<tr>
<td>Gill <em>Cyprinus carpio</em></td>
<td>Transferin precursor</td>
<td>Ubiquitin–actin fusion protein 2</td>
</tr>
<tr>
<td></td>
<td>Stress-70 protein,</td>
<td>Predicted: similar to splicing factor, arginine–serine-rich 2 (SC-35)</td>
</tr>
<tr>
<td></td>
<td>Apolipoprotein A-1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>beta-globin</td>
<td>alpha-globin</td>
</tr>
</tbody>
</table>

Serum *Sparus aurata*

- Cytosolic alamine aminotransferase 2
- Gamma-glutamyl-cysteine
- Growth hormone receptor
- RAG2
- RAG1
- Cytochrome c oxidase subunit I
- Wap6
- Glutathione S-transferase

Tissues *Oryzias latipes*

- DNA-directed RNA polymerase, beta subunit
- Glucose regulated protein precursor
- Heat shock cognate protein
- Tubulin beta

N/A

Fish exposed to 0, 0.1, 1, 5 mg/L CuSO4 for 24 hours (Kim *et al.*, 2007)
<table>
<thead>
<tr>
<th>Liver (Oreochromis aureus)</th>
<th>Liver (Danio rerio)</th>
<th>Liver (Oreochromis niloticus)</th>
<th>Liver (North Ronaldsay sheep)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat shock protein</td>
<td>Heat shock protein</td>
<td>Interleukin-1 alpha precursor</td>
<td>Cathepsin D</td>
</tr>
<tr>
<td>Cytochrome P450</td>
<td>Glucose-6-phosphate dehydrogenase-like</td>
<td>Growth hormone precursor</td>
<td>Heat shock protein Hsp 27</td>
</tr>
<tr>
<td>Catalase</td>
<td>Cytochrome P450 1A</td>
<td>Glutathione transferase</td>
<td>Peroxiredoxin 3</td>
</tr>
<tr>
<td>ATP synthase</td>
<td>GST</td>
<td>Probable short chain</td>
<td>Epoxydehydrolase b</td>
</tr>
<tr>
<td>NADH dehydrogenase</td>
<td>Cu/Zn superoxide dismutase</td>
<td>dehydrogenase/reductase</td>
<td>Ferritin light chain</td>
</tr>
<tr>
<td>Mitochondrial uncoupling protein UCP</td>
<td>Cytochrome c oxidase subunit II</td>
<td>NADH dehydrogenase subunit 4L</td>
<td>Plasma retinol binding protein</td>
</tr>
<tr>
<td>Insulin-like growth factor precursor</td>
<td>Peroxiredoxin-1</td>
<td>Star3 protein</td>
<td>Medionine adenyl transferase</td>
</tr>
<tr>
<td>Vimentin</td>
<td>NADH dehydrogenase subunit 4L</td>
<td>Insulin-like growth factor I</td>
<td>Isocitrate dehydrogenase</td>
</tr>
<tr>
<td>Putative collagen alpha 1</td>
<td>NADH dehydrogenase subunit 4L</td>
<td>Insulin-like growth factor I</td>
<td>Epoxydehydrolase a</td>
</tr>
<tr>
<td>Glutathione S-transferase</td>
<td>NADH dehydrogenase subunit 4L</td>
<td>Interleukin-1 alpha precursor</td>
<td></td>
</tr>
<tr>
<td>Beta hemoglobin A</td>
<td>NADH dehydrogenase subunit 4L</td>
<td>Cytochrome P450</td>
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<td>Metallothionein</td>
<td>NADH dehydrogenase subunit 4L</td>
<td>Small ubiquitin-related modifier 1 precursor</td>
<td></td>
</tr>
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<td>Calmodulin</td>
<td>NADH dehydrogenase subunit 4L</td>
<td>Myoglobin</td>
<td>Myoglobin</td>
</tr>
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<td>Interleukin-1 alpha precursor</td>
<td>NADH dehydrogenase subunit 4L</td>
<td>Zinc finger protein</td>
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<tr>
<td>Transferrin</td>
<td>NADH dehydrogenase subunit 4L</td>
<td>MHC class II beta chain</td>
<td>MHC class II beta chain</td>
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<tr>
<td>Ferritin</td>
<td>NADH dehydrogenase subunit 4L</td>
<td>Procollagen type I alpha 1 chain</td>
<td>Procollagen type I alpha 1 chain</td>
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<tr>
<td>Serotransferrin</td>
<td>NADH dehydrogenase subunit 4L</td>
<td>Igfry protein</td>
<td>Igfry protein</td>
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<tr>
<td>Vimentin</td>
<td>NADH dehydrogenase subunit 4L</td>
<td>Myosin heavy chain</td>
<td>Myosin heavy chain</td>
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<tr>
<td>V-ATPase subunit A</td>
<td>NADH dehydrogenase subunit 4L</td>
<td>Proteasome beta 3 subunit</td>
<td>Proteasome beta 3 subunit</td>
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<tr>
<td>Glycerophosphate dehydrogenase</td>
<td>NADH dehydrogenase subunit 4L</td>
<td>Procollagen type I alpha 1 chain</td>
<td>Procollagen type I alpha 1 chain</td>
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<tr>
<td>Insulin-like growth factor I precursor</td>
<td>NADH dehydrogenase subunit 4L</td>
<td>Aldhdehyde dehydrogenase</td>
<td>Aldhdehyde dehydrogenase</td>
</tr>
<tr>
<td>Fructose-bisphosphate aldolase</td>
<td>NADH dehydrogenase subunit 4L</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Serine/threonine protein kinase</td>
<td>NADH dehydrogenase subunit 4L</td>
<td>Proteasome subunit beta type-9</td>
<td>Proteasome subunit beta type-9</td>
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<td>Aldhdehyde dehydrogenase</td>
<td>NADH dehydrogenase subunit 4L</td>
<td>IGF2BD2</td>
<td>IGF2BD2</td>
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<tr>
<td>Endode</td>
<td>NADH dehydrogenase subunit 4L</td>
<td>Bcl91 protein</td>
<td>Bcl91 protein</td>
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<td>Isocitrate dehydrogenase 2</td>
<td>NADH dehydrogenase subunit 4L</td>
<td>Keratin 18</td>
<td>Keratin 18</td>
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<tr>
<td>Fructose-bisphosphate aldolase</td>
<td>NADH dehydrogenase subunit 4L</td>
<td>Novel protein similar to human thin</td>
<td>Novel protein similar to human thin</td>
</tr>
<tr>
<td>A</td>
<td>NADH dehydrogenase subunit 4L</td>
<td>Cardiac myosin light chain-1</td>
<td>Cardiac myosin light chain-1</td>
</tr>
<tr>
<td>A</td>
<td>NADH dehydrogenase subunit 4L</td>
<td>Apolipoprotein E</td>
<td>Apolipoprotein E</td>
</tr>
</tbody>
</table>

Zebrafish liver cell line exposed with CuCl₂ at the concentration of 25 and 50% of 96h LC₅₀ value (Chen and Chan, 2011).

Tilapia liver cell line exposed with CuCl₂ at the concentration of 20 and 50% of 96h LC₅₀ value (Chen and Chan, 2009).

Copper introduced into sheep diet (Simpson et al., 2006).
processes of affected parenchyma cells. For example, the presence of toxicant in biological system may regulate the synthesis of detoxifying enzyme such as GST and GPx the toxicant and produce ROS at the same time (Sreejai and Jaya, 2010; Hossain et al., 2012). ROS such as radical and non-radical compound activates the expression of antioxidant gene to synthesis SOD and CAT to decrease the intracellular ROS level (Sheehan et al., 2007; Yang et al., 2013). However, the elevated level of ROS may cause damages in intracellular component which leads to the synthesis of apoptotic or necrotic compound such as caspase, cytochrome c, BAX, and BAD (Kawakami et al., 2008).

Downregulation of protein by environmental stimuli are related to the suppression of protein synthesis by inhibition reaction, limitation or time delay for mRNA transcription and translation, degradation, cellular damaged are repaired and harmful agents are neutralized or eliminated (Young et al., 1987; Jensen, 2006; Wan and Liu, 2008; Liu et al., 2013; Sánchez-García et al., 2013). For example, a study done by Tanimoto and Kizaki (2002) mentioned the effect of proteosome inhibitor obstruct Ras/ERK signaling pathway subsequent in the downregulation of Fas ligand expression associate with the inhibiting synthesis of apoptotic and necrotic compound. Another study done by Fernando et al. (2013) showed the downregulation of protein expression such as carbamoyl phosphate synthase 1 (CSP 1) and 78 kDa glucose regulated protein (GRP 78/HSPA5) in hepatocyte which both has been degraded affected by alcoholic steatosis, and both were selected as a biomarker for the early detection of hepatic lipidosis. Toxic metals such as As, Pb, Cd and Cu caused downregulation by anti-apoptotic compound, Bcl-2 where this protein losses their function to maintain the mitochondria membrane permeability leads to structural destruction and the release of cytochrome c to activate the executive enzyme for the cell death program (Mehta et al., 2006; Rana, 2008; Hughes et al., 2011; Siddiqui et al., 2013; Galano et al., 2014). As the toxicant gave a great impact to the proteome level, proteomic approach was utilized as a biomaker to evaluate the toxicity level of those compounds. Table 2 shows the example of proteomic study on varies organ and species toward copper toxicity and provide the capability as a sensitive biomarker for the environmental factor.

**Conclusion**

The study of fish behaviors, cellular alteration, enzymatic reaction and proteomic approach promises the sensitive biomarker method to elucidate heavy metal on concentration, acute and chronic exposure toxicity. But, both methods have their own cons such as time consuming, cost and high technical ability. However, the combination of this method provides an integrative measurement and improves the understanding of the overall biological risk arising from the whole burden of bioavailable contaminants in areas contaminated especially by heavy metals. Thus, this method is supposed to be utilized in the biomonitoring program as a preliminary screening to elucidate other possible pollutant came from agricultural pesticides and fertilizer, industrial waste and civilization sewage.

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