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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.

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 unability to degrade radionuclide and chlorinated compound or the degradation may produce toxic

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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.

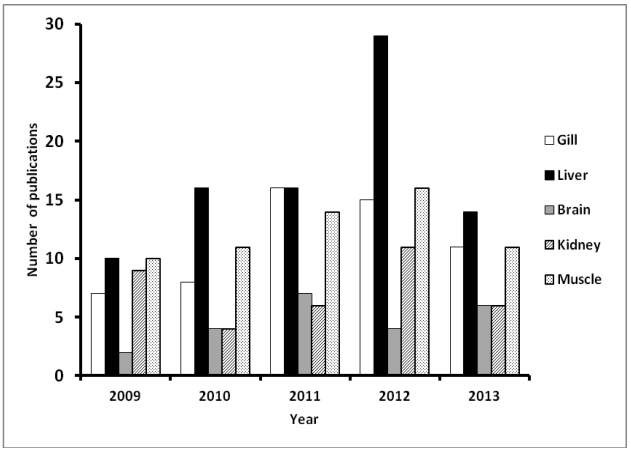

Figure 1. Number of papers published in last 5 years. The research was carried out on Scopus by using five research queries, respectively: (Gill) "Fish gill" and "Heavy metal toxicity,"(Liver) "Fish liver" and "Heavy metal toxicity,"(Brain) "Fish brain" and "Heavy metal toxicity,"(Kidney) "Fish kidney" and "Heavy metal toxicity,"(Muscle) "Fish muscle" and "Heavy metal toxicity." (Scopus, Febuary 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 abudance 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 thet 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_{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, agigatted 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 avoidence behavior, but not at a very high concentration (Giattina *et al*., 1982; Hansen *et al.*, 1999). The induction of avoidence 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

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 Sampth (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 spectraphotometry (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 Jezierska 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; Patnaik *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 mucocyte. 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 swellon 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 (μm^2) 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 andit 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

Enzyme	Species sources	Compound (Toxicity value)	Related references
Cholinesterase (ChE)	*Tilapia mossambica*	Sewage water (Lowering to 26 and 30% of activity)	Al-Ghais, 2013
	Electrophorus electricus	Cu, Ag, Hg (IC_{50}; 1.212, 0.1185 and 0.097 mgL^{-1}, respectively)	Shukor *et al.*, 2013
	Periophtalmodon schlosseri	Cu, Hg, Cr and As (IC50; 0.088, 0.371, 0.112 and 0.141 $mgLl^{-1}$)	Sabullah *et al.*, 2013
	Human serum	Mercury chloride (IC_{50}; 0.88 µM)	Mahmod *et al.*, 2001
	Pomatoschistus microps	Cu (more than 50% inhibition treat by 200 µg/L)	Vieira *et al.*, 2009
	Enchytraeus albidus	Cu (IC_{50}; 0.0579 µM)	Howcroft *et al.*, 2011
	Daphnia magna	Zn (Significant inhibition at 25 mg/L compared to control ($p<0.05$))	Diamantino *et al.*, 2003
	Carcinus maenas	Cr and Cu (49% and 46% inhibition at the concentration of 10 mg/L for Cr and 15 mg/L for Cu, respectively)	Elumalai *et al.*, 2002
	Human blood plasma	As (significant inhibition as the increasing exposure of arsenic $p<0.01$)	Ali *et al.*, 2010
	Sprague-Dawley Rats	As (inhibit more than 50% treated with 20 mg As /kg)	Patlolla and Tchounwou, 2005
	Procambarus clarkii	Cd, Pb and Hg (Significant inhibit activity after 24 and 48 hours exposure)	Devi, and Fingerman, 1995
	Channa striatus	Hg, Cd, Pb, Ni and Zn (higher inhibition; Hg>Cd>Pb>Zn>Ni)	Mat-Jais and Mohamed, 2000
Antioxidant enzyme	*Nucella lapillus*	Cd (GST activity significantly increase beyond concentration at 0.6 mg/L)	Cunha *et al.*, 2007
	Carcinus maenas	Cr and Cu (GST 68% and 61% inhibition at the concentration of 10 mg/L for Cr and 15 mg/L for Cu, respectively)	Elumalai *et al.*, 2002
	Channa punctata	Cd (GST activity significantly increase compared to control ($p<0.001$)	Atif *et al.*, 2005
	Dicentrarchus labrax	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).	Mieiro *et al.*, 2011
	Clarias gariepinus	Plumbum nitrate and zinc chloride (GST, SOD, CAT activity increase after 7 days exposure time)	Saliu and Bawa-Allah, 2012
Glucose oxidase	*Aspergillus niger*	Hg (LOD; 0.5 mg/L)	Samphao *et al.*, 2012
		Cd, Co, Cu and Ni (LOD; 2.4, 2.1, 0.2, 3.3 µM, respectively)	Ghica *et al.*, 2013
Protease	Coriandrum sativum	Hg and Zn (IC_{50}; 3.217 mg/L and 0.727 mg/L, respectively)	Baskaran *et al.*, 2013
	Commercial bromelain	Hg and Cu (IC_{50}; 0.09 to 0.115 mg/L and 0.07 to 0.112 mg/L, respectively)	Masdor and Said, 2011
	Commercial trypsin	Zn and Hg (IC_{50}; 4.8 – 6.7 mg/L and 15.76 – 17.04 mg/L, respectively)	Shukor *et al.*, 2009a
	Commercial papain	Hg, Ag, Pb and Zn (IC_{50}; 0.39, 0.40, 2.16, 2.11 mg l- 1, respectively)	Shukor *et al.*, 2006
Molybdenum reducing enzyme	*Serratia sp.* Strain DRY5	Cu (IC_{50}; 0.099 ±0.013)	Shukor *et al.*, 2009b
Alkaline phosphatase	*Mugil seheli*	Cd and Cu (activity increase after 4 days exposure)	El-Naga *et al.*, 2005
	Cyprinus Carpio	Total 1.25 ppmconcentration of Cd, Pb, Cr and Ni (Activity keep increasing from the 1st day to 32nd days of exposure)	Rajamanickam and Muthuswamy, 2008
	Limnodrillus hoffmeisteri	Cd, Fe and Zn (activity decrease to 92.57, 38.6 and 49.57%, respectively)	Grajeda y Yortega *et al.*, 2011
	Acanthopagrus Latus	Hg (Activity decrease as increase Hg concentration)	Safahieh *et al.*, 2010
Lactate dehydrogenase	*Carcinus maenas*	Cr and Cu (71% inhibition by mixture of both compounds)	Elumalai *et al.*, 2002
	Purfius conchonius	Hg (activty increase in heart and skeletal muscle exposed with 181 µg Hg/L)	Gill *et al.*, 1990
	human hepatocellular carcinoma cell line	As (activity keep increasing from 0.1 to 20 µg/ml concentration after 24 and 48 hours treatment)	Alarifi *et al.*, 2013
	EPC cells from Cyprinus caprio epithelium	Zink chloride (24 hour LDH release IC_{50}; 6.29 mM)	Ní Shúilleabháina *et al.*, 2004
Urease	Jack bean	Cd, Cu, Zn, Ni, Hg (IC_{50}; 0.12,0.013,0.18,0.51 and 0.008, respectively)	Jung *et al.*, 1995
	Soybean	Ag, Hg, Cu (IC_{50}; $2.3x10^{-8}$, 7.1 x 10^{-2}, and 0.0033 mM, respectively)	Kumar and Kayastha, 2010

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 gluthathione 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 Matkovics, 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 acetylcholinesterase (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; Armentrout *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; Dorts *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 pI 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 approch (2D-PAGE)

Organ (aquatic species)	Protein identity		Treatment method (References)
	Upregulated	Downregulated	
Whole body (*Mytilus edulis*)	• 13 protein induced	• 10 protein repressed	Blue mussels exposed with 70 ppb of copper (Shepard *et al.*, 2000)
Whole body (*Ophelina sp.*)	• Extracellular serine proteinase • Myosin regulatory light chain • Antistasin-like • β-thymosin • Sulfotransferase 1C-like • Protein phosphatase regulatory subunit 3B-B • TEPP protein • Placenta-specific gene 8 protein-like • Paramyosin • Alcohol dehydrogenase • Ribonuclease h1-like •	• Probable protein brick1 • Sodium-dependent phosphate transport protein • Phosphatidylinositol glycan anchor-like	Sampling on sediment contained with high copper level (Neave *et al.*, 2012)
Whole body (*Scytosiphon gracilis*)	• Transferase • tRNA synthetase • Phosphomannomutase • Proteosome, subunit α • ATP synthase, subunit α • Ribulose biphosphate carboxylase large chain • Glyceraldehyde 3-phosphate dehydrogenase 1 • Peptidase/protease • tRNA binding protein • ATP binding protein • Transcriptional regulator • Carbohydrate kinase • RNA binding protein • ABC transporter subunit	• Acetylglutamate kinase • Phosphoenolpyruvate carboxykinase • Dehydrogenase	The marine alga exposed to seawater supplemented with 100 μgL^{-1} copper (Contreras *et al.*, 2010)
Gill (*Oncorhynchus mykiss*)	• Carbonic anhydrase 1 • Alpha-N-acetylgalactosaminidase • MHC class II beta chain • Predicted: similar to NME1-NME2 protein • Histone H3 • Hemopexin-like protein • Putative ribosomal protein L7 protein • Ribosomal protein L30 • Ribosomal protein L6 • KH domain-containing, RNA-binding, signal transduction-associated protein 1 • SNF2 super family	• Transferrin • Serum albumin • ATP synthase subunit delta • Keratine, tyle I cytoskeletal 18 • Type I procollagen alpha 1 chain • Complement factor H precursor • FK506-binding protein 1A • Hypothetical protein HPAG1_0260 • Myeloperoxidase precursor • Myeloid-specific peroxidase • Heterogeneous nuclear ribonucleoprotein A0 • Small nuclear ribonucleoprotein Sm D2 • 40S ribosomal protein S14 • Ribosomal protein S19 • Cold-inducible RNA-binding protein • Serum albumin	Exposed with 50µg/L copper concentration (Eyckmans *et al.*, 2012)
Gill (*Cyprinus carpio*)	• Keratin 8 • Mitochondrial ATP synthase beta subunit • F-actin-capping protein subunit beta • Krt5 protein • Triosephosphate isomerase 1b • Nephrosin precurso • Pancreatic carboxypeptidase A1 • 40S ribosomal protein S9 • Ribosomal protein L8 • Histone H3a • Carbonyl-phospate synthesis L chain, ATP-binding protein • Ribosomal protein L8	• Tropomyosin 3 isoform 2 • Tropomyosin alpha-4 chain • Rho GDP-dissociation inhibitor 2 • Proteasome subunit alpha type-6 • Translationally-controlled tumor protein • Myosin regulatory light chain 2, smooth muscle isoform • Hypothetical protein LOC436656 • Predicted: similar to death-inducer obliterator 1 (DIO-1) (Death-associated transcription factor 1) • (DATF-1) (hDidol)	
Gill (*Carassius auratus gibelio*)	• Transferrin precursor • Stress-70 protein, • Apolipoprotein A-I • beta-globin	• Ubiquitin/actin fusion protein 2 • Predicted: similar to splicing factor, arginine/serine-rich 2 (SC-35) • alpha-globin	
Serum (*Sparus aurata*)	• Cytosolic alanine aminotransferase 2 • gamma-glutamyl-carboxylase • growth hormone receptor • RAG2 • RAG1 • cytochrome c oxidase subunit I	• Wap65 • glutathione S-transferase	Juvenile *Sparus aurata* exposed with 0.5 mg/L of $CuSO_4$ for 14,28 and 52 days (Isani *et al.*, 2011).
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/mL $CuSO_4$ for 24 hours (Kim *et al.*, 2007)

Liver (*Oreochromis aureus*)	• Heat shock protein • Cytochrome P450 • Catalase • ATP synthase • NADH dehydrogenase • Mitochondrial uncoupling protein UCP • Insulin-like growth factor I precursor • Vimentin • Putative collagen alpha 1 • Glutathione S-transferase • Beta hemoglobin A • Metallothionein • Calmodulin • Interleukin-1 alpha precursor • Transferrin • Ferritin • Serotransferrin • Vitellogenin • V-ATPase subunit A • Glyceraldehyde-3-phosphate dehydrogenase • Insulin-like growth factor I precursor	• Proteasome subunit, alpha type • Actin • Zinc finger protein • MHC class II beta chain • Procollagen type I alpha 1 chain • Igfbp5 protein • Myosin heavy chain • Proteasome beta 3 subunit • Procollagen type I alpha 1 chain • Aldehyde dehydrogenase	
liver (*Danio rerio*)	• Heat shock protein • Glucose-6-phosphate dehydrogenase-like • Cytochrome P450 1A • GST • Cu/Zn superoxide dismutase • Cytochrome c oxidase subunit II • Peroxiredoxin-1 • NADH dehydrogenase subunit 4L • Stat3 protein • Zinc finger protein • Transferrin • ATP-binding cassette subfamily B member 8 • V-type ATPase subunit G-like protein • Novel protein similar to human transporter 2, ATP-binding cassette, subfamily B • ATPase • Insulin-like growth factor I • Interleukin-1 beta • Vitellogenin	• Fructose-bisphosphate aldolase C • Serine/threonine protein kinase • Aldehyde dehydrogenase • Enolase • Isocitrate dehydrogenase 2 • Fructose-bisphosphate aldolase A • Proteasome subunit beta type-9 • IGF2BP2 • Bactin1 protein • Keratin 18 • Novel protein similar to human titin • Cardiac myosin light chain-1 • Apolipoprotein E	Zebrafish liver cell line exposed with $CuCl_2$ at the concentration of 25 and 50% of 96h LC_{50} value (Chen and Chan, 2011).
liver (*Oreochromis niloticus*)	• Interleukin-1 alpha precursor • Growth hormone precursor • glutathione transferase • Probable short chain • dehydrogenase/reductase • NADH dehydrogenase • Cytochrome c oxidase subunit II • ATP synthase subunit beta • Zic family member 1 • Insulin-like growth factor-1 • Parvalbumin beta • Metallothionein • Vitellogenin	• Cytochrome P450 • Small ubiquitin-related modi? er 1 precursor • Myoglobin • Zinc finger protein 60 • MHC class II antigen • Proteasome • Actin •	Tilapia liver cell line exposed with $CuCl_2$ at the concentration of 20 and 50% of 96h LC_{50} value (Chen and Chan, 2009).
Liver (North Ronaldsay sheep)	• Cathepsin D • Heat shock protein Hsp 27 • Peroxiredoxin 3 • Epoxide hydrolase b • Ferritin light chain • Plasma retinol binding protein	• Methionine adenosyl transferase • Isocitrate dehydrogenase • Epoxide hydrolase a	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 transcrption 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 supposes 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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