IDENTIFICATION OF COPPER-INDUCIBLE GENES IN *Pistia stratiotes*

by

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PENGENALPASTIAN GEN-GEN ARUHAN KUPRUM PADA *Pistia stratiotes*:

oleh

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Tesis yang diserahkan untuk memenuhi keperluan
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other plants to that of *Pistia stratiotes* using CLUSTALW program

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LIST OF ABBREVIATION

ACP	Annealing Control Primer
Al	Aluminium
As	Arsenic
ATP	Adenosine triphosphate
BCP	1-Bromo-3-Chloropropane
bp	base pair
BSA	Bovine Serum Albumin
Cd	Cadmium
cDNA	Complementary DNA
Cl	Chloride
Co	Cobalt
Cr	Chromium
Cu	Copper
DEG	Differentially expressed gene
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylene diaminetetraacetic acid
Fe	Ferum
GSP	Gene specific primer
Hg	Mercury
HSP	Heat shock protein

HNO_3	Nitric acid
IPTG	Isopropyl-β-D-thiogalactopyranoside
K	Kalium/potassium
kb	Kilobase pair
kPa	kilo Pascal
kDa	Kilo Dalton
LB	Luria-Bertani
Mg	Magnesium
$MgCl_2$	Magnesium chloride
M-MLV-RT	Molony murine leukemia virus reverse transcriptase
Mn	Mangan
MOPS	3-[N-Mopholino]propanesulphonic acid
mRNA	Messenger RNA
MT	Metallothionein
Na	Natrium/sodium
NaCl	Natrium/Sodium chloride
NCBI	National Center of Biotechnology Information
Ni	Nickel
OD	Optical density
OECD	Organization for Economic Cooperation and Development
Pb	Plumbum/lead
PCR	Polymerase chain reaction
PTMs	Potentially toxic metals
ppm	part per million
RLM-RACE	RNA ligase-mediated and rapid amplification of 5’ cDNA end

RNA	Ribonucleic acid
RT	Reverse transcriptase
SDS	Sodium dodecyl sulfate
TBE	Tris-borate-EDTA
T_m	Melting temperature
Tris-Cl	Tris-chloride
UV	Ultra violet
v/v	Volume/volume
w/v	Weight/volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Zn	Zinc

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ABSTRACT

In this study, a widely used plant to investigate freshwater pollution, *Pistia stratiotes* was utilized as a model system to determine the rate of copper uptake and to identify the candidate gene(s) that was specifically induced in response to copper challenge. The plant was exposed to various concentrations of copper (0, 1, 5, 10, 25, 50, 75, 100, 150 and 250 µg/ml) for 24 hours. Generally, it was found that the amount of copper absorbed by *Pistia stratiotes* increased as the concentrations of the heavy metal exposed to the plant increased. Interestingly, the content of copper uptake by root was higher than to that of shoot. ACP-based RT-PCR method was used to identify copper-inducible genes. Three copper-inducible candidate gene fragments were identified and were found to be up-regulated in *Pistia stratiotes* exposed to copper in dose- and time-dependent manner. These findings strongly indicate that all three identified genes were positively induced in copper-treated *Pistia stratiotes*. Using RLM-RACE, a full length copper-inducible gene of fragment 1 was successfully cloned. Sequence analysis revealed that the full length gene shared 56% and 71% identity at nucleotide and amino acid levels, respectively, with *Elaeis guineensis* asparagine synthase-related protein.

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ABSTRAK

Dalam kajian ini, Kiambang (*Pistia stratiotes)* iaitu sejenis tumbuhan akuatik yang telah digunakan secara meluas untuk mengkaji kesan pencemaran pada persekitaran akuatik telah digunakan sebagai model sistem untuk mengkaji kadar penyerapan kuprum dan mengenalpasti gen-gen aruhan kuprum. Kadar pengambilan kuprum didalam kiambang dilakukan dengan mengeramkan kiambang didalam berbagai kepekatan kuprum (0, 1, 5, 10, 25, 50, 75, 100, 150 and 250 µg/ml) selama 24 jam. Tumbuhan tersebut didapati mampu menyerap kuprum pada kesemua kepekatan yang digunakan. Akar didapati menyerap kuprum lebih banyak berbanding dengan daun. Dengan menggunakan RT-PCR berasaskan ACP, tiga fragmen gen telah dikenalpasti sebagai fragmen gen aruhan kuprum di dalam *Pistia stratiotes*. Seperti ditunjukkan melalui analisa pengekspresan gen, gen-gen ini dirangsang didalam kiambang yang didedahkan kepada kuprum bergantung kepada dos dan masa. Penemuan ini menunjukan bahawa ketiga-tiga gen yang dikenalpasti telah diaruhkan secara positif oleh kuprum di dalam *Pistia stratiotes*. Menggunakan teknik RLM-RACE, jujukan penuh gen aruhan kuprum, fragmen 1 telah berjaya diklonkan. Hasil analisis jujukan penuh gen mempamerkan identiti yang tinggi dengan *Elaeis guineensis* asparagine synthase-related protein, iaitu 56% pada peringkat nukleotida dan 71% pada peringkat asid amino.

1.0 Introduction

For many years, the environment has been constantly polluted by waste product. One of the waste products that threaten the environment is heavy metals which mainly affect the water ecosystem. Increased heavy metal usage in recent years in the industrial areas has been a major concern since heavy metals are released into the environment through industrial waste products. The presence and accumulation of these heavy metals in the environment may directly or indirectly endanger the entire ecosystem. The inability of the fresh water environment to disperse heavy metals effectively, places this habitat under considerable threat (Leatherland, 1998).

Heavy metals that were dumped into aquatic system either cannot be degraded or decomposed (Sham Sani, 1988). As a by product, heavy metal will accumulate in the sediments and organisms in the fresh water environment (Admiraal *et al.,* 1993; Rincon-Leon *et al.,* 1988). The transfer of accumulated heavy metals in the lower trophic organisms to the higher trophic organism through the food chain leads to an adverse effect, not only to the entire ecosystem but also to human that sit at the top of the food chain pyramid (Bires *et al.,* 1995; Roesijadi, 1993).

The Department of Environment (2000) reported that 5,464 water samples from the Malaysian coastal area that were analyzed were found to contain arsenic (0.1%), cadmium (1.7%), cromium (34.7%), copper (11.1%), mercury (8.7%), and lead (14.6%). These figures are way above the water quality standard of the marine interim (DOE, 2000). Yearly report from Ministry of Science, Technology and Environment (2001) showed an increase to 13 polluted rivers compared to only 12 rivers in 2000. It was reported that

an increase level of pollution in marine waters is closely related to the effluent released from factories that were dumped in rivers and finally carried into the sea (DOE, 2000).

There are two methods in detecting heavy metals in aquatic environment which are through ecological research and chemical analysis. The ecological research involves the structure of the community in a certain area. This method is done through observing the distribution and diversity of certain species that are able to survive when exposed to environmental pollutants such as heavy metal (Calow, 1994; Henry & Atchison, 1991). The weakness of ecological approach is that it is not quantitative and therefore, the identity and quantity of the pollutant cannot be determined (Bayne *et al.,* 1985). On the other hand, chemical analysis involves measuring the level of metal substance in water, sediment and organism tissue in the selected area using atomic absorption spectrophotometer. However, this technique does not show the correlation between the metal substance levels in the environment with availability of biological metal substance inside the organism (Waldichuk 1985). Furthermore, this technique does not give any indication towards determining the harmful effect on the organism and also the effect at the molecular level (Besten 1998; Cajaraville *et al.,* 2000). As a result, this technique sometimes does not give accurate information about the level of pollution in the affected area (Gray, 1992).

Nowadays, biomonitoring technique using bioindicator organism is the alternative method to chemical and ecological technique, and is more efficient and sensitive (Cajaraville *et al.,* 2000). Bioindicator organism refers to living organism that is used to identify pollution level through comparing induced

bioindicator characteristic after being exposed to pollutant (Glickman, 1991). Adam (1990) defined bioindicator as multiple measures on the health of organism, population, or community level which include several levels of biological organization and time scales of response.

Bioindicator organism has the capability of synthesizing bioindicator under stress such as the presence of heavy metal in aquatic environment (Rainbow, 1995). This approach not only shows the community structure in the research area but also shows the effect of pollution towards the environment quality and able to predict the relationship between the toxicity and the organism (Depledge & Fossi, 1994). The evaluation of early changes in indicator organisms will allow the prevention of long term effects of the pollution at the population and community level (Bolognesi *et al.,* 1996). In principle, the detection and quantification of these sub-organismal responses could be developed as early warning system and specific indicators of environmental stress especially in aquatic ecosystem (Perceval *et al.,* 2004).

1.1 Objectives of study

Malaysia is fast becoming an industrial country and as a result, many of her rivers have become polluted due to the wastes poured out into her rivers. According to Malaysia Environment Quality Report 2004, the Department of Environment has recorded 17,991 water pollution point sources comprising mainly sewage treatment plants (54%), manufacturing industries (38%), animal farms (5%) and agro-based industries (3%).

With increasing public concern regarding to environmental pollution, there is a growing need to monitor, evaluate, manage and remediate

ecological damage. In order to do so, we need a simple and reliable means of assessing the presence of heavy metal substance. Currently, pollution is monitored using complex procedures, via the use of atomic absorbance spectrophotometer, monitoring of defined species distributions or by measuring the lethal exposure limit of bioindicator species. This testing procedure to determine the amount of heavy metal presence in a particular sample requires very expensive equipment. Even so, studying these parameters gives sparse information and most of these procedures do not directly provide the amount and the effects of heavy metal presence in the target species. In this study, *Pistia stratiotes* was chosen because of its ability to absorb more copper compared to other floating macrophytes (Qian *et al.,* 1999*)* and the widespread availability of the plant in Malaysian freshwaters.

The ability of various pollutants (and their derivatives) to mutually affect their toxic actions complicates the risk assessment based solely on environmental levels (Calabrese, 1991). Deleterious effects on populations are often difficult to detect in feral organisms since many of these effects tend to manifest only after longer periods of time. Such scenarios have triggered the research to establish early-warning signals, or molecular-biomarkers, reflecting the adverse biological responses towards anthropogenic environmental toxins. In an environmental context, biomarkers offer promise as sensitive indicators demonstrating that toxicants have entered organisms, have been distributed between tissues, and are eliciting a toxic effect at critical targets (McCarthy and Shugart, 1990).

Thus, the identification of the molecular changes that occur under heavy metal exposure can help in the current water biomonitoring procedures

and may identify potential diagnostic and prognostic markers that could detect pollution faster and at an earlier stage.

Taking into consideration all these factors, the objectives of this study are:

1. To determine the rate of copper absorption in the root and shoot of *Pistia stratiotes* exposed to various concentrations of copper
2. To identify the candidate gene(s) that was specifically expressed in response to copper challenge in *Pistia stratiotes*
3. To determine the dose response of the selected gene identified in objective (2) and clone the full-length gene

It is hoped that in the long term, *Pistia stratiotes* and the identified gene(s) will be used as molecular biomarker(s) for detecting copper pollution in fresh water environment. The availability of new molecular techniques has opened up exciting possibilities to isolate the stress-adaptive genes and to manipulate gene expression for a better understanding of their mode of action. In future, it is hoped that a powerful diagnostic and prognostic technique to detect copper far below the lethal dose in freshwaters would be developed.

2.0 Literature Review

2.1 Pistia stratiotes

Pistia stratiotes is commonly known as water lettuce and originated from Africa. *Pistia stratiotes* is a free-floating perennial of quiet ponds (Plate 2.1a and Plate 2.1b). It is stoloniferous, forms colonies, and has rosettes up to 15 cm across. It has long, feathery, hanging roots. Its leaves are obovate to spathulate-oblong, truncate to emarginate at the apex, and long-cuneate at the base. Leaves are light green and velvety-hairy with many prominent longitudinal veins. Inflorescences are inconspicuous and up to 1.5 cm long. Flowers are few, unisexual, and enclosed in a leaflike spathe.

Kasselmann (1995) notes that for *Pistia stratiotes* to survive, it requires a wet, temperate habitat. It is usually found in lakes and rivers, however, it can survive in mud. *Pistia stratiotes* can endure temperature extremes of 15° C (59° F) and 35° C (95°). The optimal growth temperature range for the plant is 22-30° C (72-86° F). *Pistia stratiotes* prefers slightly acidic waters (6.5 - 7.2 pH).

Pistia stratiotes reproduces vegetatively and by seed. Vegetative reproduction involves daughter vegetative offshoots of mother plants on short, brittle stolons. Rapid vegetative reproduction allows water lettuce to cover an entire lake, from shore to shore, with a dense mat of connected rosettes in a short period of time (Kasselmann, 1995).

There have been numerous reports of *Pistia stratiotes* ability as a bioaccumulator (Qian *et al.,* 1999; Cecal *et al.,* 2002) which serve as a basis for the hypothesis in this study.

Plate 2.1a. Side view of *Pistia stratiotes*

Plate 2.1b . The top view of *Pistia stratiotes*

Maine *et al.,* (2001) reported that *Pistia stratiotes* has superior performance and higher average relative growth rate when breed in heavy metal induced stress environment compared to *Salvinia herzogii, Hydromistia stolonifera* and *Eichhornia crassipes*. Other than that, water lettuce has been shown to have high tolerance to heavy metals especially copper. Water lettuce can be found in lakes and ponds all over Malaysia, thus, making it viable to be an indicator for heavy metal stress especially at the molecular level.

2.2 Effects of heavy metals

The lack of ability of the fresh water environment to dissolve the pollutants effectively places this habitat under considerable risk (Lippmann, 2000; Haslam, 1990). Even in tiny amounts, some of these pollutants can cause serious damage. For instance, heavy metal pollution in fresh water has been shown to produce retardation in growth and development of plants and fish (Dufus, 1980).

Heavy metal pollutants are not essential for plants, and excessive amounts can cause growth inhibition, as well as reduced photosynthesis, mitosis, and water absorption (Demayo *et al.* 1982). Most of the pollutants are toxic to all phyla of aquatic biota, though affects are modified significantly by various biological and abiotic variables (Wong *et al*. 1978). For example, wastes from lead mining activities have severely reduced or eliminated populations of fish and aquatic invertebrates, either directly through lethal toxicity or indirectly through toxicity to prey species (Demayo *et al.* 1982).

2.3 Effects of heavy metals on the environment

Increasing environmental pollution by heavy metal contaminant has been reported in recent years in almost every developing country in South East Asia (Hardoy *et al.,* 1992). The main source of heavy metal pollution comes from discharge of untreated and semi treated effluents from metal-related industries such as manufacturing of batteries, circuit boards, electroplating, textile dyes and plastic fabrication (Wong *et al.,* 2001a).

Heavy metals have long been recognized as one of the most important pollutants in coastal waters (Johnson *et al.,* 2000). It is caused by their toxicity and capacity to accumulate in marine organisms, however at low concentrations, some is essential in many physiological processes for plant, animal and human health (Basile *et al.,* 2005). In trace amount, several of these ions are required for metabolism, growth and development (Basile *et al.,* 2005). Although a number of heavy metals are nutritionally important in low concentration, many can be cytotoxic when cells are confronted with an excess of these vital ions (Basile *et al.,* 2005).

Heavy metals are a long-term problem, unlike organic pollutant in which heavy metals are not biodegradable and will enter the food chain through a number of pathways causing progressive toxic actions due to the accumulation in different organs during a life span and long term exposure to contaminated environments (Machynlleth, 1998). Austin (1998) also mention about the capability of vertebrates and invertebrate to accumulate heavy metals from aquatic environment. For instance, cadmium, copper, lead and zinc have been detected using atomic absorbance spectrometry in gill,

muscle, vertebrate and viscera of rabbitfish (*Siganus oramin*) from polluted waters around Hong Kong (Zhou *et al.,* 1998).

2.4 Effects of Heavy Metals on Human

In general, some heavy metals such as zinc and copper are needed by the human body in low concentrations, but some other heavy metals may cause harm to human life such as destroying the immune system, disrupting the reproductive systems and affecting the development in children (Machynlleth, 1998). Certain toxic heavy metals such as cadmium, mercury and lead are immunotoxic to the human immune system (Shenker *et al.,* 1998). There is evidence that mercury interferes with the function of lymphocytes. Mercury induces lymphocytes proliferation, increased level of immunoglobin, autoantibody production and immune-complex deposits (Kim and Sharma, 2003).

The main characteristics of chronic lead toxicity are sterility both in males and females, and abnormal fetal development (Johnson. 1998). Methylmercury is also reported to counteract the cardioprotective effects and to damage developing fetuses and young children (Burger and Gochfeld, 2005). Maternal exposure can threaten the fetus because chemicals can be transferred across the placenta to the developing fetus (Burger and Gochfeld, 2005).

In high concentration, most heavy metals seem to affect the hematopoietic system, as observed by the decrease in circulatory erythrocytes or change in the number of blast cells in the kidney (Zeeman and Anderson, 1995). Lead has been reported in inhibiting heme synthesis and in

decreasing red cell survival in carcinogenicity and nucleic acid destabilization (Pyatt *et al.,* 2005).

Acute exposure of copper and zinc will can cause fever, vomiting, nausea, stomach cramps and diarrhea have been reported (WHO, 1996). At long term, exposure to heavy metals such as copper, cadmium, chromium, zinc, mercury and lead caused carcinogenic effects (Pyatt *et al.,* 2005).

2.5 Heavy Metals

In its strict sense the term “heavy metals” includes only elements with densities above 5 g/cm^3, but frequently, biologists use this term for a vast range of metals and metalloids which are toxic to plants such as amongst others, copper, iron, manganese, zinc, nickel and arsenic (Hopkin, 1989). A total of 59 types of heavy metals can be found in the environment and some of them are toxic to the environment (Novotny, 1995).

All heavy metals including the essential heavy metal micronutrients are beneficial to living organism at certain concentration. However they can become toxic to aquatic organisms as well as humans if the exposure levels are sufficiently high. Zinc acts as an essential cofactor for a wide variety of metalloproteins and enzymes, and is not normally toxic except at extremely high concentrations. Copper on the other hand is an essential enzyme cofactor but is also a potent cellular toxin at higher concentration. In contrast, metals such as cadmium are highly toxic and without nutritional value (Burdon, 1999). Table 2.1 categorizes several heavy metals based on their roles, which are essential, probably essential for human and toxic pollutants. Around 100 or more mg/day of macronutrients are necessary for the effective

Table 2.1 The role of heavy metals in humans

Elements	Role
Cr, Co, Cu, Fe, Mn, Zn	Essentials micronutrients (no more than a few mg/day)
Ni, Sn, V	Possibly essential micronutrients
Al, As, Cd, Pb, Hg (Some may possibly be essential)	Toxic pollutant

and efficient functioning of the body, however, for micronutrients, only a few mg/day is required (Siegel, 1998).

The essential micronutrients like copper, iron and zinc are found in enzymes capable of carrying oxygen as hemoglobin, whereas cobalt is needed by the human body to make vitamin B_{12} to form hemoglobin (Austin, 1998).

Some of the heavy metals that are grouped into possibly essential metals are nickel and tin which are usually found in some industries such as textiles, chemical industry and oil refining. Nørum *et al.,* (2005) reported that nickel and tin are required during reproductive cycle of spiny scallops (*Chlamys hastate*). It was suggested that the limit of 2 µg/L for nickel in marine water should pose minimal risk to aquatic animals and human (Connell and Hawker, 1992). However, a high level of nickel is capable of causing promutagenic lesions such as DNA strand breaks (Kasprzak, 1997).

The third group of heavy metals is the ones that serve as toxic pollutants to the environment and living organisms. For example, mercury which presents potential hazard even at low concentrations due to enrichment in the food chain (Forstner and Wittmann, 1979). This toxic pollutant can cause weakening of muscles, loss of vision, lack of coordination, attacks the central nervous system, speech and hearing impairments, impairments of the cerebral functions and eventual paralysis which in numerous cases resulted in coma and death. Damage caused by mercury in brain was found mainly in the cerebellum and sensory pathways with lesions in the cerebral cortex (Forstner and Wittman, 1979; Machynlleth, 1998).

2.6 Copper

Copper is usually needed and used in many industry-related activities, which include acting as heat and electrical conductor, component in water pipes, roof coverings, household goods and chemical equipment (WHO, 1996). Copper can easily form complexes with organic compounds, which are quite stable in the environment (Zhou *et al.,* 1998). It is estimated around 3.2 million tonnes of copper are released to the environment from 1910-1990 (WHO, 1996).

Copper is essential to life and is found in all body tissues. Overall the human body needs about 80 µg of copper per kg of body weight (Piscator, 1979). Copper deficiency can lead to a variety of abnormalities, including anemia, skeletal defects, degeneration of the nervous system, reproductive failure, pronounced cardiovascular lesions, elevated cholesterol, impaired immunity, and defects in the pigmentation and structure of the hair (Smith, 1974; National Research Council, 1980).

However, at high concentrations, copper is also capable of causing toxic effects. The ingestion of excess copper can cause gastrointestinal problems and the exacerbation of vibriosis in human (Austin, 1998; Siegel, 1998). Copper is also reported to be highly toxic against sperms (Wong *et al.,* 2001) and may affect spermatogenesis with regard to motility, production, maturation and fertilizing capacity of the spermatozoa (Skandhan, 1992). In addition, copper is also hazardous to cells as it can lead to the formation of hydroxyl radicals through Fenton-type reaction, which is highly damaging towards cell components such as lipids, DNA and proteins (Burdon, 1999).

The suggested safe level of copper in drinking water by U.S. EPA 1986 for humans is 1 mg/L compared with 0.018 mg/L for acute aquatic life criteria (Cooney, 1995), whereas the lethal oral dose for adults lies between 50-500 mg of copper salt per kg of body weight (WHO, 1996).

2.6.1 Metabolic function and toxicity of copper in plant

Copper is required for plant nutrition only in trace amounts and at higher concentrations can be toxic to cells. Critical deficiency levels are in the range of 1-5 mg/kg plant dry mass and the threshold for toxicity is above 20-30 mg/kg dry mass (Marschner, 1995). Some hyperaccumulators may accumulate up to 1000 mg Cu/kg in leaves (Morrison *et al.,* 1981).

Copper is a redox-active metal with an electrochemical potential of -260 mV. Thus, it is not surprising that copper is an essential component of many electron carriers. For example, copper is present in plastocyanin (photosynthesis), cytochrome c oxidase (respiration), laccases, superoxide dismutase, ascorbate oxidase (antioxidative defence), and, is also involved in the control of hormone metabolism as ethylene receptor (Rodriguez *et al.,* 1999).

For normal plant growth, maintenance of metal homeostasis is important. Excess uptake of redox active element causes oxidative destruction. Intracellular free copper ions can react with water to produce free radical hydroxyls, which in turn reacts to cause membrane lipid peroxidation (De Vos *et al.*, 1993, Luna *et al.* 1994), cleavage of sugar phosphate backbone of nucleic acids (Chubatsu and Meneghini, 1993) and protein denaturation resulting from the formation of disulfide linkages between Cys

residue (Stohs and Bagchi, 1995). In addition, copper can displace other divalent cations coordinated with macromolecules, causing their inactivation or malfunction (Lidon and Henriques, 1993). Copper also inhibits other cellular proteins, such as cell wall expansins (McQueen-Mason and Cosgrove, 1995).

Copper is normally found only as protein-bound forms in cells, since free ion may generate oxidative stress and causes serious damage to organic molecules. Thus, the reactivity of copper that makes it so useful in redox reaction also makes it toxic. Free copper ions readily oxidize thiol bonds within proteins, causing disruption of their secondary structure.

The principal mechanism of copper toxicity involves the Fenton-reaction, characterized by metal-catalyzed production of hydroxyl radicals from superoxide and hydrogen peroxide (Elstner *et al.,* 1988; Briat and Lebrun, 1999). This process has been demonstrated in isolated chloroplasts (Sandmann and Böger, 1980), intact algal cells (Sandmann and Böger, 1980) and intact roots (De Vos *et al.,* 1993). Reactive oxygen species destruct biological macromolecules like proteins, lipids, DNA and as a consequence cause cell death by necrosis or apoptosis (programmed cell death) (Dat *et al.,* 2000).

2.6.2 Copper detoxification and tolerance

Since excess uptake of redox active elements in plant can cause a lot of damages, therefore, uptake, transport and distribution within the plant must be stringently controlled. Plants have a range of potential mechanisms at the cellular level that might be involved in the detoxification and, thus, tolerance to heavy metal stress. These all appear to be involved primarily in avoiding the

build-up of toxic concentrations at sensitive sites within the cell and, thus, preventing the damaging effects. For example, there is little evidence that tolerant species or ecotypes showing an enhanced oxidative defense but rather tolerant plants show enhanced avoidance and homeostatic mechanisms to prevent the onset of stress (De Vos *et al.,* 1991; Dietz *et al.,* 1999).

2.6.2.1 Plasma Membrane

The plant plasma membrane may be regarded as the first 'living' structure that is a target for heavy metal toxicity. Plasma membrane function may be rapidly affected by heavy metals as seen by an increased leakage from cells in the presence of high concentrations of metals, particularly copper. Rapid potassium ion efflux has been widely interpreted as a symptom of toxicity resulting from copper-induced oxidative damage to the plasma membrane (De Vos *et al.,* 1991, 1993; Murphy and Taiz, 1997). Similarly, others concluded that damage to the cell membrane, monitored by ion leakage, was the primary cause of copper toxicity in roots of *Silene vulgaris* (De Vos *et al.,* 1991), *Mimulus guttatus* (Strange and Macnair, 1991), and wheat (Quartacci *et al.,* 2001). Certainly direct effects of copper treatments on lipid composition of membranes have been reported (Hernandez and Cooke, 1997; Quartacci *et al.,* 2001) which may have a direct effect on membrane permeability. In *Nitella*, copper-induced changes in cell permeability were attributed to non-selective increase in conductance and inhibition of the light-stimulated H^+-ATPase pump (Demidchik *et al.,* 1997).

Thus, tolerance may involve the protection of plasma membrane integrity against heavy metal damage that would produce increased leakage of solutes from cells (De Vos *et al*., 1991; Strange and Macnair, 1991; Meharg, 1993). One of the factors that may be involved in the maintenance of plasma membrane integrity in the presence of heavy metals could be via enhancing membrane repair after damage (Salt *et al*., 1998).

An alternative strategy for controlling intracellular metal levels at the plasma membrane involves the active efflux of metal ions, although there is very little direct evidence for such a process in plants. However, in bacteria, efflux pumping is the basis of most toxic ion resistance systems, involving transporters such as P-type ATPases or cation/H^+ antiporters (Silver and Ji, 1994; Silver, 1996). Efflux pumping systems have been identified for copper, cadmium, zinc, cobalt, and nickel (Silver, 1996).

Another group of transporters that appear to be involved in copper homeostasis by a copper export system are the heavy metal CPx-ATPases, a branch of the P-type ATPases (Solioz and Vulpe, 1996; Williams *et al*., 2000). Defects in these ATPases have been linked to two human disorders, Menkes disease and Wilson disease that result from defective copper export, and thus, the accumulation of copper in some tissues (Solioz and Vulpe, 1996).

2.6.2.2 Intracellular proteins

The roles of protein that are involved in heavy metal detoxification are discussed in Section 2.9.

2.7 Toxicity Test

Toxicity test is important in providing qualitative and quantitative data on the adverse or toxic effects of chemicals on aquatic organisms which in turn can be used to assess the potential for, or degree of, damage to an aquatic ecosystem (Cooney, 1995). The purpose of toxicity test is to determine the strength of the chemical from the degree of response elicited in the test organisms, but not to estimate the concentration of the chemical that is toxic to those organisms. Since these effects are not necessarily harmful, a principal function of the tests is to identify chemicals that can cause adverse effects on the organisms. These tests provide a database that can be used to assess the risk associated with a situation in which chemical agent, the organism, and the exposure condition are defined (Rand and Petrocelli, 1985).

There are two types of toxicity test. The first type is acute toxicity test, which is used to determine the concentration of test material that produces a specific adverse effect on a specified percentage of test organisms during short exposure. The second type is chronic toxicity test that uses a long exposure (Cooney, 1995).

In order to determine the relative toxicity effect of a new chemical to aquatic organisms, an acute toxicity test is first conducted to estimate the median lethal concentration (LC_{50}) of the chemical in the water to which test organisms are exposed. LC_{50} is the concentration that causes 50% mortality of a test population over a specific amount of time (Cooney, 1995).

The most commonly used test to measure or evaluate the impact of chemicals towards the environment is the acute toxicity test. For any new

chemical, the primary information in the process of aquatic hazard evaluation is obtained from short term or acute toxicity test (Murthy, 1986).

2.8 The use of living organism to monitor pollution

Currently, majority of the research carried out in the area of pollution monitoring is geared towards the establishment of early-warning signals. This approach is crucial to avoid the damage caused by the pollutants to our ecosystem is way beyond repair if the adverse effects of pollution are detected at the later stage.

Bioindicators and biomarkers can act as a warning system against pollution. The role of these indicators in the environmental assessment is envisaged as determining whether or not, in a specific environment, organisms are physiologically normal. Aquatic organisms, in general are of potential interest as ecologically sensitive targets of environmental stress, and may act as vectors for pollutant contamination of humans via the food chain (Carginale *et al.,* 2002).

There are several compelling reasons for identifying bioindicator species and biomarkers in relation to environmental pollution, which are (1) providing early warning signals of environmental deterioration, (2) assessing environmental pollution or environmental hazards, (3) identifying cause and effect between stressors and biological responses, (4) assessing the integrated responses of organisms to environmental stress due to pollution and (5) determining scientific information for addressing ecological and possibly human health risk issues at contaminated sites (Jamil, 2001; Adams *et al.,* 2003).

2.8.1 Bioindicator

Adam (1990) defined bioindicators as multiple measures on the health of organism, population, or community level which includes several levels of biological organization and time scales of response.

In essence, bioindicator could also be defined as an organism giving information on the environmental conditions of its habitat by its presence or absence or by its behavior (Van Gastel and Van Brummelen, 1994). A study of these organisms is generally linked to the use of mathematical distribution of these organisms in the communities which the bioindicator species belong to (Jamil, 2001). Occurrence of such species in a particular area indicates special habitat conditions and such species are referred as ecological indicators, since they indicate marked conditions of the environment. The evaluation of early changes in indicator organisms could allow the prevention of long term effects of pollution at the community and population level (Bolognesi *et al.,* 1996). In principle, the detection and quantification of these sub-organismal responses could be developed as specific indicators of environmental stress notably for aquatic ecosystem (Perceval *et al.,* 2004).

Furthermore, other advantages of monitoring with the use of bioindicator is that biological communities reflect overall ecological quality and integrate the effects of different stressors, thus providing a broad measure of their impact and an ecological measurement of fluctuating environmental conditions. As a whole, routine monitoring of biological communities is reliable and relatively inexpensive compared to the cost of assessing toxicant pollutants (Georgudaki *et al.,* 2003).

2.8.2 Biomarker

The ability of various pollutants (and their derivatives) to mutually affect their toxic actions complicates the risk assessment based solely on environmental levels (Calabrese, 1991). Deleterious effects on populations are often difficult to detect in feral organisms since many of these effects tend to manifest only after longer periods of time. When the effect finally becomes clear, the destructive process may have gone beyond the point where it can be reversed by remedial actions or risk reduction. Such scenarios have triggered the research to establish early-warning signals, reflecting the adverse biological responses towards anthropogenic environmental toxins (Bucheli and Fent, 1995).

Therefore, one of the strategies to assess the contamination and its potential effect is the use of biomarkers in ecological surveys to verify the bioavailability and presence of relevant concentrations (Bucheli and Fent, 1995). Biomarker is broadly defined as measurement of changes at molecular, biochemical, cellular, or physiological levels in individuals representing a larger group or population. The present or past exposure of the individual to at least one pollutant chemical substance is also revealed (Camatini *et al.,* 1998; Jamil 2001; Corsi *et al.,* 2003). Van Gestel and Van Brummelen (1994) defined biomarker as biochemical, histological and morphological responses to an environmental chemical that are measured inside an organism. Bolognesi *et al.,* (1996) said the goal of biomarker is to detect the early biological events, biochemical or physiological change, resulting from a given exposure that can predict the onset of adverse health effects.

This technique makes use of biological endpoints in living organisms (biomarker) of environmental insults. In a way, biomarkers offer promise as sensitive indicators which can indicate the uptake of toxicants into organisms, distribution of toxicants within tissues, and toxicological effect of toxicants at critical targets. Due to these attributes, biomarkers measured in animals from sites of suspected contamination can be an important and informative component of an environmental monitoring program. In such a monitoring program, the biomarker responses of animals or plants from a suspect site would be compared to those of the same species collected from reference sites (McCarthy and Shugart, 1990).

Measurement of biomarker responses to exposure offers the potential of providing information that cannot be obtain from measurements of chemical concentrations in environmental media or in body burdens. For example, measurement of biomarker responses at the molecular and cellular level can provide evidence that organisms have been exposed to toxicants at levels that exceed normal detoxification and repair capacity (McCarthy and Shugart, 1990). Responses either at the lower level or higher level of biological organization provide information to understand and interpret the relationship between exposure and adverse effect. In particular, responses measured from the lower levels of biological organization such as DNA damage or enzyme activity, often manage to provide sensitive and specific response to particular toxicant. Hence, this kind of biomarkers gives an opportunity to measure and diagnose the type of contaminant or toxicant that particular organism is exposed to (McCarthy and Shugart, 1990). Moreover, the exposure of an organism to toxic chemicals may result in the induction of a

chain reaction of events starting with an initial insult to the DNA and culminating in the appearance of an over pathological disease (Shugart, 1990). Many of these pollutants are chemical carcinogens and mutagens with the capacity to cause various types of DNA damage and if goes un-rectified, may cause more harm to the organism.

Heavy metals can affect all the different classes of biomolecules included within the context of the genome, transcriptome, proteome and metabolome. In the field of marine environmental monitoring, molecular biomarkers (including changes in gene and protein expression and enzyme activities) have been shown to aid the recognition of pollutant exposure and impact (William *et al.,* 2003). In addition, it was widely recognized that metal compounds may have profound effect on gene expression patterns, as demonstrated by the growing number of metal responsive genes that have been identified in different organisms (Hall, 2002; Jonak *et al.,* 2004).

Induction of genes by heavy metals does not only indicate exposure of which the genes can be potentially developed as molecular biomarkers, but it may also indicate the products of the genes that are also responsible in detoxification mechanisms (Laws, 1993).

In time, the biomarkers will thus become a routine, well-characterized and scientifically and legally defensible tool for monitoring and assessing environmental pollution. Based on the magnitude and pattern of the biomarker responses, the environmental species offer the potential of serving as sentinels demonstrating the presence of bioavailable contaminants and the extent of exposure, surrogates indicating potential human exposure and effects and predictors of long-term effects on the health of populations or the