CHAPTER I

INTRODUCTION

1.1 General Introduction

Thailand has long been realized the importance of shrimp industry to the Thai economy especially the *P. monodon*. Much effort has been put into research and development in this area. Technologies have been continuously developed especially in area such as shrimp culture in the closed system, brood stock production and selective program. All these make Thailand the leader in shrimp export industry.

Currently, disease outbreak has caused serious problem to shrimp production industry. The culture of *P.monodon*, a major shrimp production of Thailiand, is now being replaced by other shrimp specises such as white shrimp, Litopenaeus vannamei. The replacement is hoping that all severe problems associated with *P.monodon* culture could be avoided. In addition, the main advantage of L. vannamei over *P.monodon* is its complete domestication; it grows easier in various pond conditions and brood stock can be produced in captivity.

A large quantity of larvae and brood stock of white shrimps have been imported uncontrollably from foreign countries within the last few years, creating a great concern on the possibility that new pathogens might come with this exotic species and cause new fatal diseases to local shrimps. Furthermore, this alien species will be released inevitably to natural habitat and causes adverse effect to the local shrimp population.

Although, replacing *P.monodon* with new shrimp species may help to maintain the shrimp production but it makes no guarantee that disease spread between species and severe pandemic would not take place in the future. For the sustainability of shrimp culture production, research and development on the improvement of the health and diagnostic tools for the shrimp conditions are still required. To gain the

ability to assess the health and stress condition of the shrimp, studies on molecular responses of the stress responsive genes in shrimp are needed.

Stress conditions have become one of the main molecular studies in infected shrimps. There are evidences indicating that the massive losses of shrimp production by diseases are initially stimulated by stresses. These stress proteins cover all the products of stress inducible genes such as cytochrome P450, metallothioneins, heat-shock proteins, the heme biosynthesis pathway, heme oxygenase, p53, superoxide dismutase. Many organisms are able to synthesize stress proteins, which offer some protection from cellular damage. The environmental stresses, which can induce these proteins, include, trace metal exposure, organic pollutants, changes in temperature and osmolarity. Various numbers of stress responsive molecules have been reported to increase their expression levels several folds during stresses. These potential molecules can be used as biomarkers for the shrimp health condition and for the detecting the cause of stresses.

Among stressful factors, oxidative stress has gained more attention because of it involvement in pathogenic infection and apoptosis. Oxidative stress occurs when redox homeostasis within the cell is altered. In general, oxidative stress is caused by the intracellular accumulation of reactive oxygen species (ROS) or a disturbance of the cellular redox state. The oxidative defenses consist of both nonenzymatic (glutathione, thioredoxin) and enzymatic (superoxide dismutase, peroxidases, catalase) detoxification mechanisms, which destroy ROS or restore the redox balance. Oxidative stress can cause molecular damage to proteins, DNA, membranes, lipids etc. Bacterial and viral infection also induces the generation of ROS, which may be responsible for the induction of oxidative stress and apoptosis.

Another factor, which involves changes of salinity in the water, is osmotic stress. It leads to efflux or influx of water from or into the cell: hyperosmotic stress causes shrinking, hypoosmotic stress causes swelling. The cellular responses to this type of stress deal with the activity of water channels and electrolyte transporters, and the accumulation of osmolytes as well as the protection of proteins and subcellular structures. It has been reported that osmotic stress can cause the organisms to suffer from the lower growth rate and high risk of death. Knowledge of the molecular

mechanisms by which cells respond to changes in osmolarity would not only be useful in understanding osmotic stress response, but also cellular responses to other mechanical forces on the cell.

Within abiotic environmental stresses, handling stress is one of unavoidable factors in animal farms. Farmed shrimps are routinely exposed to a variety of possible handling stress such as netting, holding, transporting, air exposure and confinement. No culture technique can be applied perfectly to every species of cultured shrimps because different animals respond to this stress with different levels of physiological and developmental changes. It has been recognized that protective mechanisms against diseases are impaired by stress, and, consequently, shrimp diseases have been associated with its harmful effects. It will be very helpful if the occurrence of stress coincides with the presence of a pathogen can be earlier detected.

1.2 *Penaeus monodon*

1.2.1 Biology

Penaeid shrimp belongs to the animal kingdom in Phylum Arthropoda, Subphylum Crustacea, Class Malacostraca, Order Decapoda, Superfamily Penaeoidea, Family Penaeidae, Genus Penaeus, Subgenus Penaeus and Species monodon. This group of animal is characterised by the presence of paired appendages and a protective cuticle or exoskeleton that covers the whole animal. The exterior of penaeid shrimp is distinguished by a cephalothorax with a characteristic hard rostrum, and by a segmented abdomen (Fig 1.1). Appendages of the cephalothorax vary in appearance and function. In the head region, antennules and antennae perform sensory functions. The mandibles and the two pairs of maxillae form the jaw-like structures. In the thorax region, the maxillipeds are the first three pairs of appendages and five pairs are the walking legs (pereiopods). On the abdomen found five pairs of swimming legs (pleopods).
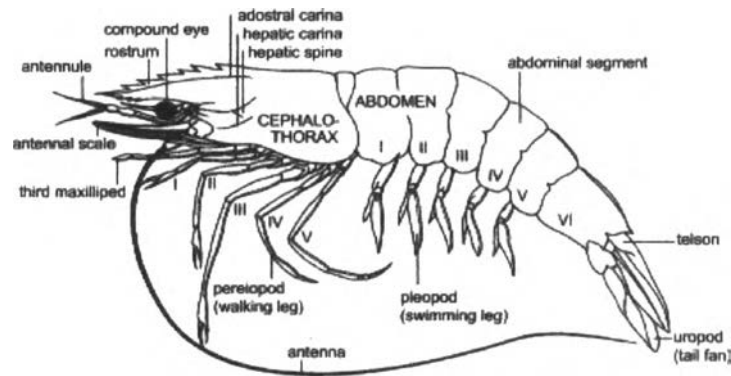
Figure1.1 External morphology of *Penaeus monodon* (Primavera, 1990).

Life cycle includes several distinct stages that are found in a variety of habitats (Figure 1.2). Juveniles prefer brackish shore areas and mangrove estuaries in their natural environment. Most of the adults migrate to deeper offshore areas at higher salinities, where mating and reproduction takes place. The first larval stage, which is the nauplius, develop into the protozoeae, metamorphose into myses and have many of the characteristics of adult shrimp and develop into megalopas, the stage commonly called postlarvae.
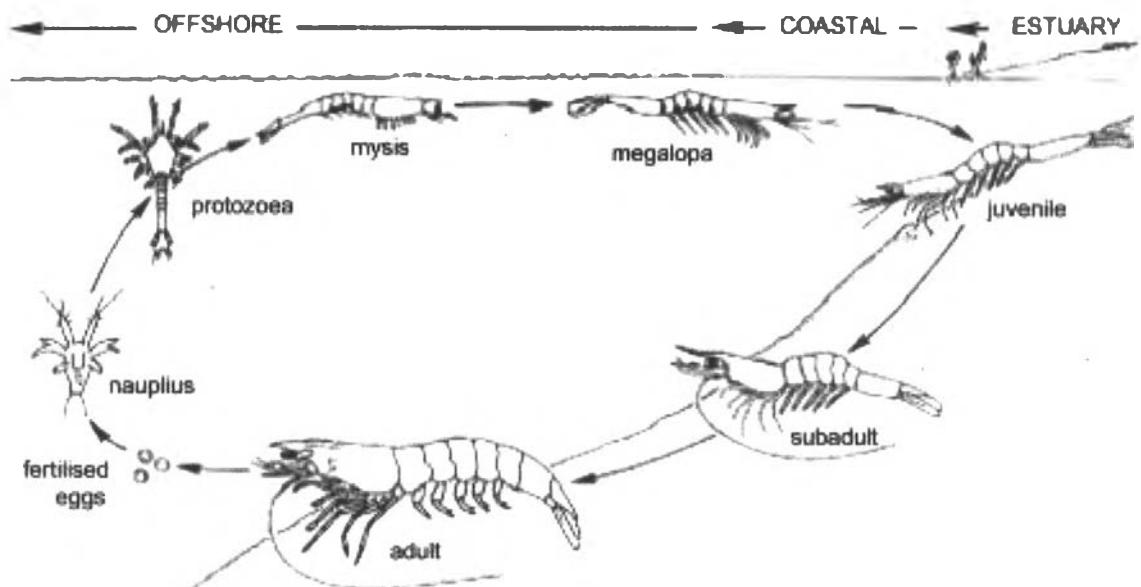

Figure1.2 Life cycle of Penaeus monodon.

1.2.2 Diseases and diagnosis

Disease outbreaks have caused serious economic losses in several countries. According to a World Bank Report, global losses due to shrimp diseases were around US $ 3,000 million (Lundin, 1996). Thus, health management is of major importance in aquaculture. Diseases caused by microorganisms are most devastating and, this chapter discusses various microbial diseases of shrimp and strategies for management of microbial diseases.

1.2.2.1 Bacterial diseases

Bacterial diseases cause problems ranging from mass mortalities to growth retardation and sporadic mortalities. *Vibrio* spp are the most important bacterial pathogens of shrimp. *Vibrio* spp are widely distributed in fresh water, estuarine and marine environments. Over 20 species are recognized, some of these are human pathogens (eg. *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*) while some species are pathogens of marine animals including shrimps (eg. *V. harveyi* , *V. spendidus*, *V. penaecida*, *V. anguillarum*, *V. parahaemolyticus*, *V.vulnificus*). *Vibrio* spp are commonly observed in shrimp hatcheries, grow-out ponds and sediments (Otta *et al.*, 1999, 2001). Though most *Vibrio* spp are regarded as opportunistic pathogens, some like *V. harveyi* could be primary pathogens. *V. harveyi* are luminous bacteria found in coastal and marine waters, in association with surface and gut of marine and estuarine organisms and also in shrimp pond water and sediment (Ruby and Nealson, 1978; Yetinson and Shilo,1979; Orndorff and Colwell, 1980; Otta *et al.*, 1999, 2001). They occasionally cause serious mass mortalities in shrimp hatcheries in Asia (Sunaryanto and Mariam, 1986; Tansutapanit and Ruangpan, 1987; Lavilla-Pitogo *et al.*, 1990; Karunasagar *et al.*, 1994). Karunasagar *et al.*, (1994) noted that certain strains of *V. harveyi* isolated from sea water had high LD for *P. monodon* larvae, while isolated from moribund larvae had a low LD50, suggesting that *V. harveyi* strains may vary in virulence. Pizzutto and Hirst (1995) reported that strains of *V. harveyi* virulent to *P. monodon* formed a separate cluster in protein profile and M13 DNA fingerprinting. Presently, no virulence factors have been definitely established in this species, though a number of suggestions have been made eg. extracellular products including proteases,

hemolysins and cytotoxins (Liu *et al.*, 1996), low molecular weight lipopolysaccharide lethal toxin (Monterio and Austin, 1999) and protein toxins T1 and T2 (Harris and Owens, 1999).

Association between *V. harveyi* and bacteriophages was reported by Pasharawipas *et al.*, (1998) in the brown gill syndrome (TBGS) in *P. monodon*. They further noted that lysogenic *V. harveyi* itself could not induce TBGS and luminescence was not critical for shrimp pathogenicity. Oakey and Owens (2000) noted that one of the toxin producing strains of *V. harveyi* (VH642) was lysogenic and carried a myovirus like phage (VHML). Filamentous bacteria such as Leucothrix mucor, *Thiothrix* sp, *Flexibacter* sp, *Flavobacterium*, *Cytophaga* sp may cause infection in penaeid shrimp larvae (Guzmán *et al.*, 2000). Discolouration of gills, low growth and feeding, increased mortality and lethargy are common signs of the disease. The disease is associated with poor water quality. Higher degree of infection may lead to necrosis in gill tissue. The disease can be diagnosed by microscopic examination of gills.

1.2.2.2 Viral diseases

About 20 viruses have been recognized as causative agents of diseases in shrimp. These include members of Parvoviruses, Baculoviruses, Picornaviruses, Toga- like viruses and some of the newly identified virus families. Whitespot syndrome virus (WSSV), a double stranded DNA virus, and Yellow Head Virus (YHV), a single stranded RNA virus, are the most damaging virus to the shrimp farming industry in Thailand and other countries in South-East Asia (Takahashi *et al.*, 1994; Chou *et al.*, 1995; Wongteerasupaya *et al.*, 1995; Lo *et al.*, 1996; Flegel, 1997; Karunasagar *et al.*, 1997; Hsu *et al.*, 1999). They were known to affect most commercially important species of penaeid shrimps (Lightner, 1996) and wild marine shrimps (Lo *et al.*, 1996; Hossain *et al.*, 2001; Chakraborty *et al.*, 2002). Taura syndrome virus (TSV) is the virus that causes serious problem to the shrimp culture in many regions of North, Middle, and South Americas (Lightner, 1996; Lightner *et al.*, 1997). This disease represents a serious problem in the culture of P. vannamei due to the high level of mortality and the economic losses (Lightner *et al.*, 1997). Within the last few decades, increasing numbers of virus have been found in penaid shrimps. Summary of virus found in penaeid shrimps is shown in table 1.1.

Table1.1 Viruses affecting cultured and wild penaeid shrimp(Vijayan, 1998)

Name	Approx. Virion size	Nucleic Acid	Probable Classification	Reference
Baculovirus Penaei (BP)	50-75X300 nm	dsDNA	Baculovirus	Couch, 1974
Baculoviral midgut gland necrosis (BMN)	75X300 nm	dsDNA	Baculovirus	Sano *et al.*, 1981
Infectious hypdermal and hematopoietic necrosis virus (IHHNV)	22 nm	ssDNA	Parvovirus	Lightner *et al.*, 1983
Monodon baculovirus (MBV)	75X300 nm	dsDNA	Baculovirus	Lightner *et al.*, 1983
Hepatopancreatic parvovirus (HPV)	22-24 nm	ssDNA	Parvovorus	Lightner and Redman,1985
Type C baculo virus (TCBV)	75X300 nm	dsDNA	Baculovirus	Brock and Lightner,1990
Lymphoid parvo-like virus (LPV)	25-30 nm	ssDNA	Parvo-like virus	Owens *et al.*, 1991
Iridovirus (IRIDO)	136 nm	dsDNA	Iridovirus	Lightner and Redman,1993
Hemocyte- infecting nonoccluded baculovirus	90X640nm	dsDNA	Baculo-like virus	Owens, 1993
White spot syndrome virus (WSSV)	80-330 nm	dsDNA	Baculovirus	Wongteerasu-paya *et al.*, 1995 Lightner and Redaman, 1998
Reo like virus	SX70nm	dsRNA	Reo-like virus	Tsing and Bonami, 1987
Lymphoid organ vacuolization virus(LOVV)	30XSSnm	ssRNA	Toga-like virus	Bonami *et al.*, 1992
Rhabdovirus of penaeid shrimp (RPS)	75X125nm	ssRNA	Rhabdovirus	Nadala *et al.*, 1992
Yellow head virus (YHV)	44X173nm	ssRNA	Rhabdovirus	Flegel *et al.*, 1995
Taura syndrome virus (TSV)	30-32nm	ssRNA	Picornavirus	Lightner *et al.*,1995

1.2.2.3 Diagnosis

Apart from physical observation such as deformed shape, abnormal swimming, or reduced feeding and growth rates, shrimps can be diagnosed by a number of techniques, including histochemical, immunological, and DNA-based techniques. Advanced techniques for specific virus detection and diagnosis have also been developed. Detection of shrimp pathogens especially virus using PCR has become a standard procedure. Various numbers of PCR diagnostic kits specific to major virus such as WSSV, YHV, and TSV have been developed (Nelson and Lightner, 2001). A kit based on DNA dot hybridization probe for the rapid, on-site detection of MBV is being developed by Dr. S.N. Chen, (National University of Taiwan, Taipei, Taiwan) and Vickers *et al.*, (1992). DIG-labeled DNA probes that provide a very sensitive method for the detection of MBV in fixed tissue sections via *in situ* hybridization are available commercially from DiagXotics Inc. (27 Cannon Rd., Wilton, CT 06897, USA). These advanced techniques are very sensitive. Only a few particles of virus can be detected. However, these methods provide no information on health condition of the shrimp.

1.3 Stress

Seyle (1950) first defined stress as the sum of all the physiological responses by which an animal tries to maintain or re-establish a normal metabolism in the face of a physical or chemical force. Stress and resistance to a stressor are energy draining processes (Schreck, 1982; Barton and Schreck, 1987) and while responding to stress an organism should have less energy available to devote to other life functions (Schreck 1990). Stress can be a state of threatened homeostasis, which is reestablished by a complex repertoire of physiological and behavioral adaptive responses of the organism (Chrousos, 1998).

Basically, stress is the force that causes one physiology to adapt from the usual self-costing energy from them. Stress in human is different to those of animals. Stress in animals result from environmental or other factors that extend the animal's physiological processes beyond the normal range. Stress represents the response of the animal following exposure to stressors. The potential stressors are grouped as being environmental, physical and biological (Barton and Iwama, 1991).

In aquatic animals, especially those brought up in an aquaculturing environment, they face many types of stressor such as viral and bacterial infection, temperature, crowdedness, pH or toxicant, etc. Stressors lead to different type of stress. Stresses of interest in aquatic animals are oxidative stress, osmotic stress and handling stress.

1.3.1 Oxidative stress

Activated forms of oxygen are important in the biosynthesis of complex organic molecules, in the polymerization of cell wall constituents, in the detoxification of xenobiotic chemicals and in the defense against pathogens. If the production of activated oxygen exceeds the capacity of cells to detoxify it, deleterious degenerative reactions occur. Thus, the mechanisms of control and damage the potential reactions of activated oxygen are important. Oxidative stress is an unavoidable by-product of the aerobic lifestyle. It is caused by exposure to reactive oxygen intermediates, such as superoxide anion ($O_2^{\circ-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO°), which can damage proteins, nucleic acids, and cell membranes. The maintenance of active growth and metabolism of the animals and overall environmental stress tolerance depends on the balance between the production and the scavenging of activated oxygen. Oxidative stress occurs when there is an elevated concentration of intracellular reactive oxygen species (ROS) in a steady state condition where the balance between reactive oxygen species and antioxidants is tripped towards over abundance of ROS. Antioxidant enzymes (AOE) and related proteins are substances that when present at low concentrations compared with those of an oxidizable substrate significantly delay or prevent oxidation of that substrate (Halliwell and Gutteridge, 1989). Since formation of ROS is a great threat to aerobic cells, the antioxidative defence systems are essential. The damaging effects of ROS are normally kept under control by endogenous antioxidant systems including glutathione, ascorbic acid, and enzymes such as superoxide dismutase (SOD), glutathione peroxidase, and catalase.

1.3.1.1 Sources of oxidative stress

Mitochondria are a major source of ROS, which is produced by the electron transport chain on the inner mitochondrial membrane, and the rate of production is dependent on mitochondrial potential. ROS are generally very reactive molecules possessing an unpaired electron. They are produced continuously in cells either as by-products of metabolism, or by leakage from mitochondrial respiration. NADPH oxidase on the plasma membrane, and cytoplasmic enzymes such as xanthine oxidase and nitric oxide synthase, can all generate superoxide anion (O_2^{o-}) (Boveris, 1984; Chance *et al.*, 1979).

1.3.1.2 Mechanisms of oxidative cell damage

Oxidative stress can damage cells by endogenous oxidants and during phagocytosis. In the process of cell damaged by endogenous oxidants, molecular oxygen passively diffuses into cells and is converted to O_2^{o-} and H_2O_2 by the direct oxidation of flavoproteins, including NADH dehydrogenase II (NdhII). In the plausible contribution of reactive oxygen species to cell damage during phagocytosis, O_2^{o-} is generated by NADPH oxidase on the phagolysosomal membrane (Storz and Imlay, 1999). In biological systems, the reactions of activated oxygen with organic substrates are very complex due to the surface properties of membranes, electrical charges, binding properties of macromolecules, and compartmentalisation of enzymes, substrates and catalysts. Thus, ROS can damage cells by oxidizing membrane phospholipids, proteins, and nucleic acids in various sites even within a single cell differed in the nature and extent of reactions with oxygen.

1.3.1.2.1 Oxidative damage to lipids

Lipid peroxidation is probably the most extensively investigated process induced by free radicals. Singlet oxygen can react readily with unsaturated fatty acids producing a complex mixture of hydroperoxides. Oxidation of unsaturated fatty acids by singlet oxygen produces distinctly different products than the hydroxyl radical (Bradley and Minn, 1992). The lipid bilayer membrane is composed of a mixture of

phospholipids and glycolipids. Their abundant presence at sites where ROS formed renders them easily accessible endogenous targets, rapidly affected by free radicals, especially the group of polyunsaturated fatty acids (PUFAs), which is highly susceptible to reactions with free radicals. The initiation reaction between an unsaturated fatty acid (e.g. linoleate) and the hydroxyl radical involves the abstraction of an H atom from the methylvinyl group on the fatty acid (Frankel, 1985). Peroxidation of lipids in fatty acids may lead to a radical chain reaction (fig.1.3). Because of these chain reactions, one substrate radical (R^o) may result in the formation of many equivalents of lipid peroxides (LOOH) (De Zwart et al., 1999).
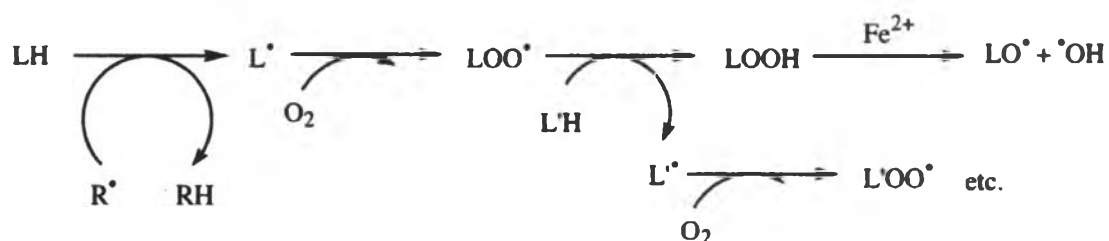

Figure 1.3 Schematic proceed of lipid peroxidation, chain reactions resulting in the formation of many lipid peroxide radicals.

1.3.1.2.2 Oxidative damage to proteins

Oxidative attack on proteins results in site-specific amino acid modifications, fragmentation of the peptide chain, aggregation of cross-linked reaction products, altered electrical charge and increased susceptibility to proteolysis. The amino acids in a peptide differ in their susceptibility to attack, and the various forms of activated oxygen differ in their potential reactivity. Primary, secondary, and tertiary protein structures alter the relative susceptibility of certain amino acids. H_2O_2 can also directly oxidize protein cysteinyl residues, sulphur containing amino acids or thiol groups. Activated oxygen can abstract an H atom from cysteine residues to form a thiyl radical that will cross-link to a second thiyl radical to form disulphide bridges.

Alternatively, oxygen can add to a methionine residue to form methionine sulphoxide derivatives. Reduction of both amino acid residues may be accomplished in microbial systems by thioredoxin and thioredoxin reductase (Farr and Kogama, 1991). Other forms of free radical attack on proteins are not reversible. For example, the oxidation of iron-sulphur centres by superoxide destroys enzymatic function (Gardner and Fridovich, 1991). Many amino acids undergo specific irreversible modifications when a protein is oxidised. For example, tryptophan is readily cross-linked to form bityrosine products (Davies, 1987). Histidine, lysine, proline, arginine, and serine form carbonyl groups on oxidation (Stadtman, 1986). The oxidative degradation of protein is enhanced in the presence of metal cofactors that are capable of redox cycling, such as Fe. Oxidative modification of specific amino acids is one mechanism of marking a protein for proteolysis (Stadtman, 1986). In *E. coli* there are specific proteases that degrade oxidised proteins (Farr and Kogoma, 1991)

1.3.1.2.3 Oxidative damage to DNA

Activated oxygen and agents that generate oxygen free radicals, such as ionising radiation, induce numerous lesions in DNA that cause deletions, mutations and other lethal genetic effects. Characterization of this damage to DNA has indicated that both the sugar and the base moieties are susceptible to oxidation, causing base degradation, single strand breakage, and cross-linking to protein (Imlay and Linn, 1986). Degradation of the base will produce numerous products, including 8-hydroxyguanine, hydroxymethyl urea, urea, thymine glycol, thymine and adenine ring-opened and saturated products. The principle cause of single strand breaks is oxidation of the sugar moiety by the hydroxyl radical (Kamiya, 2004). Cross-linking of DNA to protein is another consequence of hydroxyl radical attack on either DNA or its associated proteins (Oleinick *et al.*, 1986). Treatment with ionising radiation or other hydroxyl radical generating agents causes covalent leakages such as thymine-cysteine addicts, between DNA and protein. DNA is an obvious weak link in a cell's ability to tolerate oxygen free radical attack. As a consequence, the cell has a number of DNA repair enzymes (Beyer *et al.*, 1991).

1.3.1.3 Defense mechanisms against oxidative stress

Increasing evidence suggests that the cumulative damage caused by ROS contributes to numerous diseases. To protect against the damage caused by oxidative stress, cells possess a number of antioxidant enzymes and repair activities, most of which are expressed at low levels during normal growth. In response to elevated concentrations of ROS, the expression of many antioxidant proteins is induced. Significant enzymes induced by ROS include superoxide dismutase (SOD), glutathione peroxidase, and catalase.

1.3.1.3.1 Superoxide dismutase

Superoxide dismutase (SOD) is an enzyme that catalyses the dismutation of superoxide to hydrogen peroxide and oxygen:

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H+ \rightarrow H_2O_2 + O_2$$

Since SOD is present in all aerobic organisms and most subcellular compartments that generate activated oxygen, it has been assumed that SOD has a central role in the defence against oxidative stress (Beyer, *et al.*, 1991; Bowler *et al.*, 1992; Scandalias, 1993). There are 3 types of SOD classified on the basis of the metal cofactor: the copper/zinc (Cu/Zn-SOD), the manganese (Mn-SOD) and the iron (Fe-SOD) isozymes (Bannister *et al.*, 1987). These isozymes can be identified on the basis of their sensitivity to KCN and H_2O_2. The Mn-SOD is resistant to inhibitors such as KCN and H_2O_2, whereas the Cu/Zn-SOD is sensitive to both inhibitors and Fe-SOD is resistant to only KCN and sensitive to H_2O_2. The subcellular distribution of these isozymes is also distinctive. The Mn-SOD is found in the mitochondria of eukaryotic cells; some Cu/Zn-SOD isozymes are found in the cytosol, others in the chloroplasts of higher plants. The Fe-SOD isozymes are seldom detected in plants, but when detected, Fe-SOD is usually associated with the chloroplast compartment (Bowler *et al.*, 1992). The prokaryotic Mn-SOD and Fe-SOD, and the eukaryotic Cu/Zn-SOD enzymes are dimers, whereas the Mn-SOD of mitochondria is tetramers (Scandalias, 1993). All forms of the SOD are nuclear-encoded and are targeted to their respective subcellular compartments by an amino terminal targeting sequence. Several forms of SOD have been cloned from a variety of animals.

Manganese superoxide dismutase (Mn-SOD) is considered to be one of the most important intracellular antioxidant enzymes. Mn-SOD is a homotetramer with manganese at its active sites, and has a molecular weight of 88 kDa (Fridovich and Freeman, 1986) and is localized to mitochondria. Its promoter area contains binding sites for several transcription factors. One of the most important transcription factors is NF-κ B (Das et al., 1995). Mn-SOD is induced by changes in the cellular redox state, inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) (Wong and Goeddel, 1988; Tsan et al., 1990; Visner et al., 1990), interleukin-1 (IL-1) (Masuda et al., 1988), interleukin-6 (IL-6) (Tsan et al., 1992; Warner et al., 1996), interferon gamma (IFN-γ) (Harris et al., 1991), lipopolysaccharide (LPS) (Clerch et al., 1996), high oxygen tension in hyperoxia exposed animals (Freeman et al., 1986; Ho et al., 1996), chronic ozone exposure (Weller et al., 1998), and also by exposure to cytotoxic drugs (Akashi et al., 1996; Das et al., 1998), asbestos fibers (Mossman et al., 1986) and oxidants such as H_2O_2, thioredoxin, and peroxynitrite (Das et al., 1997; Warner et al., 1996; Jackson et al., 1998). Animals with enhanced levels of AOEs, as well as the animals exposed to sublethal hyperoxia, become tolerant to lethal dose of O_2 (Crapo et al., 1980). Mn-SOD constitutes approximately 10-15% of the total SOD activity in most tissues (Tsan, 2001). However, Mn-SOD has been shown to be essential for the survival of animals.

Copper zinc superoxide dismutase (Cu/Zn-SOD), is an intracellular enzyme mainly localized in cytosol (Crapo et al., 1992), and although more abundant than Mn-SOD, it is not inducible to the same extent (Shull et al., 1991; Kinnula et al., 1995). Cu/Zn-SOD is a homodimer with molecular weight of 32,5 kDa and contains both copper and zinc at its active sites (Fridovich and Freeman, 1986). Copper is essential for the enzyme's catalytic activity, and zinc imparts stability to the protein structure (Fridovich, 1975). Cu/Zn-SOD is expressed in alveolar epithelial cells, fibroblasts, and capillary endothelial cells in normal rat lung (Coursin et al., 1992; Chang et al., 1995).

Prokaryotic cells and many eukaryotic algae contain only the Mn-SOD and Fe-SOD isozymes, which are believed to be more ancient forms. In the bacteria E. coli, SOD activity is transcriptionally regulated by the SOX RS operon (Farr and Kogoma, 1991). To date it has been shown that SOD activity is increased in cells in response to diverse environmental and xenobiotic stresses. Mn-SOD mRNA levels increase after exposure to hyperoxia in most of the cases, but also decreased mRNA

levels have been reported (Clerch, 2000). Severe hyperoxia leads to decreased Mn-SOD activity in rats (Clerch and Massaro 1993) and baboons (Clerch *et al.*, 1998). When hyperoxia tolerant neonatal rats are compared to non-tolerant adults, the major difference in the AOE profile is the ability of neonate to increase the level of Mn-SOD activity while Mn-SOD activity is decreased in the lungs of the hyperoxia-exposed adult rats due to posttranslational decrease in activity and decrease in Mn-SOD synthesis (Clerch, 2000). Hypoxia decreases Cu/Zn-SOD mRNA expression in alveolar type II epithelial cells and lung fibroblasts *in vitro* (Jackson *et al.*, 1996). In contrast to Mn-SOD, Cu/Zn-SOD can also act as a superoxide reductase and a superoxide oxidase (Liochev and Fridovich, 2000). However, Cu/Zn-SOD is not required for normal development and survival in mice, since Cu/Zn-SOD gene knock out mice develop normally to adulthood and show no apparent evidence of oxidative damage (Tsan, 2001), and overexpression of Cu/Zn-SOD does not alter the expression of Mn-SOD (White *et al.*, 1993) and cannot compensate the deficiency of Mn-SOD *in vivo* (Copin *et al.*, 2000).

Several reviews on superoxide dismutase have recently been published which describe the characteristics of the enzymes, the cloned cDNA sequences and genes, and the effects of overexpression in transgenic animals. Mn-SOD gene knockout mice all died within 10-21 days after birth from cardiomyopathy, metabolic acidosis and neurodegeneration (Li *et al.*, 1995; Lebowitz *et al.*, 1996). On the other hand, only 50 % of Mn-SOD activity is sufficient for normal resistance to hyperoxia (Tsan *et al.*, 1998). Experiments with transgenic mice overexpressing Mn-SOD have given inconclusive results of resistance to oxygen toxicity when measured with survival in hyperoxic conditions (Wispe *et al.*, 1992; Ho *et al.*, 1998). By administrating SOD to reperfused tissues after hyperoxia, the injury was found to be exacerbated. This indicated that the balance of oxidants and antioxidants might play primary role against the development of the cell and tissue injury, (McCord, 1993). There was also initial evidence on the exogenic protective role of SOD-mimics, such as manganic salen compound, EUK-8. EUK-8 has shown to improve pulmonary function in a porcine model of LPS-induced ARDS (Gonzales *et al.*, 1995) and tracheal administration of catalytic antioxidant metalloporphyrin attenuates bleomycin-induced pulmonary fibrosis of mice lung (Oury *et al.*, 2001). Cu/Zn-SOD overexpression is also protective against ischemia/reperfusion injury in brains (Kinouchi *et al.*, 1991), intestine (Deshmukh *et al.*, 1997) and myocardium (Chen *et al.*, 2000).

Overexpression of Cu/Zn-SOD may also be deleterious to the host, probably due to its non-specific peroxidase activity and/or its ability to enhance nitration of tyrosyl residue by peroxynitrite (Tsan, 2001). Polyethylene glycol conjugated (PEG) (Beckman *et al.*, 1988) and liposome-entrapped SOD (Freeman *et al.*, 1985) decrease oxygen toxicity in rats. In human lung Cu/Zn-SOD expression has been found in bronchial epithelium (Kinnula *et al.*, 1994), type II pneumocytes, and alveolar macrophages (Coursin *et al.*, 1996).

1.3.1.3.2 Catalase

Catalase is a heme-containing enzyme that catalyses the dismutation of hydrogen peroxide into water and oxygen. (Fridovich and Freeman, 1986). It is found in all aerobic eukaryotes and is important in the removal of hydrogen peroxide generated in peroxisomes (Kinnula *et al.*, 1995) by oxidases involved in ß-oxidation of fatty acids, the glyoxylate cycle (photorespiration) and purine catabolism. Catalase is a homotetrameric enzyme and all forms of this enzyme excess 220 kDa. In animal models or cell cultures, catalase has been shown to be induced by hyperoxia, oxidants and cytokines (White *et al.*, 1989; Tsan *et al.*, 1990; Shull *et al.*, 1991), but also controversial results have been found (Jornot *et al.*, 1992; Ho *et al.*, 1996; Pietarinen-Runtti, 1998). In human lung, catalase is not elevated by hyperoxia in human tracheal epithelial cells during 12 h exposure *in vivo* (Erzurum *et al.*, 1993) or during 48 h *in vitro* (Pietarinen-Runtti *et al.*, 1998). Exposure of rats to LPS decreases catalase (Clerch *et al.*, 1996). Catalase is the only antioxidant enzyme increased both at the mRNA and at the activity levels during human lung morphogenesis towards term (Asikainen *et al.*, 1998). Individuals with acatalasemia, a rare congenital condition with catalase deficiency in erythrocytes and lower levels of catalase activity in other tissues, seem to be asymptomatic (Ogata, 1991), although an increased incidence of diabetes mellitus has been reported (Goth, 2001). Both polyethylene glycol conjugated catalase (White *et al.*, 1989) and liposome-entrapped catalase (Buckley *et al.*, 1987) protect against oxygen toxicity, but greater benefits are accomplished with the combination of PEG-SOD and PEG-CAT (White *et al.*, 1989) or liposome-entrapped SOD and CAT (Freeman *et al.*, 1985). Careful examination of the structure of beef liver catalase has shown four NADPH binding sites per catalase tetramer (Fita

and Rossmann, 1985), but these sites were not in close association with the hydrogen peroxide reaction centre. Instead, NADPH functions in animal catalase to protect against inactivation by hydrogen peroxide (Kirkman *et al.*, 1987).

1.3.1.3.3 Other enzymes and compounds with antioxidative activity

Recently, the possible importance of thioredoxins and peroxiredoxins has raised a great amount of interest. Thioredoxins are small redox proteins that undergo NADPH-dependent reduction by thioredoxin reductase and in turn reduce oxidized cysteine groups on proteins. The mitochondrial form thioredoxin-1 has been more widely studied and it has been implicated to have a role in various human diseases including cancer (Powis and Montfort, 2001). Peroxiredoxins (I-VI) utilize thioredoxin as the electron donor for antioxidation and they protect cells from ROS insult and regulate the signal transduction pathways to influence cell growth and apoptosis (Butterfield *et al.*, 1999). Both thioredoxins and peroxiredoxins have cell specific expression and distribution in human lung (Kinnula *et al.*, 2001; Soini *et al.*, 2001; Kinnula *et al.*, 2002).

Low molecular weight antioxidant family consists of many compounds, each of which acts as a direct chemical scavenger neutralizing ROS components or indirectly, through transition metal chelation. Most of the antioxidants are reducing agents, which quench ROS through donation of electron(s) to the ROS, neutralizing its activity (Chevion and Chevion, 2000). They are small molecules that can often penetrate into cells, accumulate (at high concentrations) at specific compartments near where oxidative damage might occur, and be generated by the cell (Halliwell and Gutteridge, 1989). Vitamin E, or α-tocopherol, is a lipid soluble antioxidant that can convert O_2^{o-}, OH^o, and lipid peroxyl radicals to less reactive form (Heffner and Repine, 1989). Also β -carotene, a carotenoid metabolic precursor to vitamin A, is a lipid soluble antioxidant.

Water soluble antioxidants include vitamin C, free GSH unrelated to its role in the GSH redox cycle, uric acid, glucose and taurine (Heffner and Repine, 1989). Ascorbate has found to have a gluthione sparing effect (Martensson *et al.*, 1991). Also bilirubin, ubiquinol, flavonoids and dihydrolipoate have been suggested to have antioxidative capacities (Halliwell and Gutteridge, 1990; Stocker *et al.*, 1987; Fridovich, 1999). Possible therapeutic implications of these compounds have been

discussed (Middleton *et al.*, 2000), but they are not currently regarded as valid therapy.

1.3.1.4 Detection of oxidative stress

Reactive free radicals formed within cells can oxidize biomolecules and this may lead to cell death and tissue injury. Establishing the involvement of free radicals in the pathogenesis of a disease, however, is extremely difficult, due to the short lifetimes of these species, but also due to the lack of sufficiently sensitive technology to detect radicals directly in biological systems (Cheeseman and Slater 1993; Pryor, 1986). As a consequence of these analytical problems related to oxidant stress and free radical mechanisms of injury, much of the evidence is circumstantial. Therefore, in many diseases, it is still not clear whether free radicals are the sole cause of the injury or are formed as the result of the disease (Jaeschke, 1995). For this reason, there is a great need for biomarkers of radical damage, which can be used to monitor the involvement of such damage in the pathogenesis of diseases or in the toxicity of xenobiotics.

1.3.2 Osmotic stress

One mechanical stress that can be sensed by all cells is osmotic stress, which is applied when a cell is exposed to a non-isotonic extracellular environment. Exposure to such an environment causes major water and ion fluxes across the cell membrane, changes in cell volume, and thus causes forces to be applied to the cell membrane.

Osmotic stress is a change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of a stimulus indicating an increase or decrease in the concentration of solutes outside the organism or cell. The regulation of salt (ion) balance is fundamental to all life. The structure and function of cells depend closely on their interactions with water and things that are dissolved in water and few factors affect the viability of an organism as extensively as osmoregulation. Osmotic stress may be particularly disruptive to cell function during rapid changes in salinity since the compensatory adjustments of intracellular organic solute concentrations often occur over a period of hours or days,

lagging behind the onset of changes in hemolymph osmolarity (Dall, 1975; Bartberger and Pierce, 1976; Gilles, 1979; Pierce, 1982). During this transient period of acclimation, inorganic ions that perturb enzyme function may be the principal intracellular osmolytes involved in cell volume regulation (Warren and Pierce, 1982). In all organisms, homeostasis is an important mechanism to maintain the stability of their living conditions.

1.3.2.1 Homeostasis

Homeostasis is the property of an open system, especially living organisms, to regulate its internal environment to maintain a stable, constant condition, by means of multiple dynamic equilibrium adjustments, controlled by interrelated regulation mechanisms. The term is most often used in the sense of biological homeostasis. If the system does not succeed in reestablishing its balance, it may ultimately lead the system to stop functioning. Complex systems, such as multicellular organisms, must have homeostasis to maintain stability and to survive. These systems do not only have to endure to survive; they must adapt themselves and evolve to modifications of the environment. Organisms exhibited equilibrium in their physiological state is not necessarily static. To avoid osmotic stress, animals in both aquatic and terrestrial environments must maintain the right concentration of solutes and amount of water in their body fluids. This involves 2 main functions of homeostasis in aquatic organisms; the regulation of the amounts of water and minerals in the body which is known as osmoregulation and the removal of metabolic waste which is known as excretion.

1.3.2.2 Osmoregulation

Osmoregulation is the active regulation of the osmotic pressure of bodily fluids to maintain the homeostasis of the body's water content. Osmotic pressure is a measure of the tendency of water to move into one solution from another by osmosis. The higher the osmotic pressure of a solution the more water wants to go into the solution. There are 2 major types of osmoregulation found in aquatic animals; osmoconformers and osmoregulators. Most marine invertebrates are osmoconformers. They regulate their internal salinity to be equal to the surrounding seawater. These animals keep their body fluids isotonic to the external environments. Therefore, they

do not have to actively adjust their internal osmotic state. Osmoregulators, on the other hand, tightly regulate their body osmolarity, which always stays constant, and are more common in the animal kingdom. Osmoregulators actively control salt concentrations despite the salt concentrations in the environment. In aquatic animals, gill is a morphologically and functionally complex tissue that is the site of numerous, interconnected physiological processes, which are vital to maintaining systemic homeostasis in the face of changing internal (e.g., acidosis) and environmental (e.g., salinity) conditions. Some euryhaline invertebrates such as crab, Callinectes sapidus, behaves both as an osmoregulator when equilibrated in salines in the range of 800 mosM and below and an osmoconformer when equilibrated in salines (Lang, 1987)

1.3.2.3 Osmoregulation in crustaceans

Most crustaceans live in saline water, and numerous studies have demonstrated the importance of osmoregulation as a physiological adaptation to salinity and its variations in adults and also throughout development (Péqueux 1995; Charmantier et al., 2001). For crustaceans with high hemolymph osmolality and ion content, freshwater poses the physiological challenge of constant influx of water and diffusive loss of ions. Few crustacean species have successfully adapted to this medium. Crayfishes are decapod crustaceans that have adapted well to freaswater. They can spend their entire life span, including reproduction and development, in this medium. Under natural conditions, most of them have adopted a stenohaline way of life, although they may survive some degree of experimentally increased salinity (Holdich et al., 1997). The maintenance of their body fluid composition is based on several adaptive mechanisms, including a relatively low permeability of the teguments to prevent water invasion and ion loss, an active uptake of ions by using specialized epithelia of the branchial chambers, and the production of dilute urine through the excretory antennal glands (Barradas et al., 1999). Most studies on crayfish osmoregulation have been conducted in adults or large juveniles, but data available on juveniles are scarce (Wheatly and Gannon, 1995).

1.3.2.4 Protein instability or denaturation by osmotic stress

Cations (Na^+, K^+, Mg^{+2}, and Ca^{+2}) and anion (Cl^-) are all present in seawater and are known to destabilize proteins at excessive concentrations (>1 M) (Somero and Yancey, 1997). *In vitro* results from 3T3 and SV-3T3 (rat) cells in culture showed that the presence of the osmolyte betaine (*N*-trimethylglycine) reduced the production of HSP70 during hypertonic (500 mos$M$) incubation (Petronini *et al.,* 1993). Heat-shock transcription factor (HSF1) can be activated by either hyper- or hypo-osmotic stress in mammalian (HeLa) cells, but does not induce HSP70 mRNA, indicating that it may play a different role in regulating osmo-sensing pathways or osmotic stress proteins (Caruccio *et al.,* 1997). In rat kidney, both the mRNA and protein levels of HSP72 and HSP25/27 increased steeply along the corticopapillary axis in a pattern that matched tissue solute levels in the distal tubule. It was believed that hypertonicity rather than hyperosmolarity was actually responsible for these patterns, because increased HSP synthesis correlates with the addition of relatively membrane-impermeable substances (NaCl) and not with the addition of membrane-permeable substances. Thus, alterations in membrane fluidity or shrinking and swelling may influence HSP expression in some cellular systems without protein denaturation (Beck *et al.,* 2000). Increased molecular chaperone mRNA levels may be required in a particular tissue for signal transduction or osmolyte responses to intracellular ionic changes. It was found in salmon that hyper-osmotic stress could raise the levels of HSP90 mRNA in the branchial lamellae (both *in vitro* and *in vivo*), but not in the kidney (Pan *et al.*, 2000). Because cortisol is believed to govern osmoregulatory capacity in salmon by influencing chloride cell differentiation and ATPase activity in the gill, and HSP90 is known to regulate glucocorticoid receptors. It was hypothesized that HSP90 might be playing a specific role in signal transduction during osmotic stress. There was evidence in lobster that molting hormone (ecdysteroid) titers can influence molecular chaperone gene expression (Chang *et al.,* 1999; Spees *et al.,* unpubl.).

Expression and activity of Na^+-K^+-ATPase is often correlated directly with salinity (Wilson *et al.*, 2002; Singer *et al* .2002; Lin *et al*, 2003; and Hawkings *et al*, 2003). It was reported that Na^+-K^+-ATPase activity and/or expression actually decreases when some euryhaline species are acclimated to seawater (Marshall and Bryson, 1998). In sea bass (*Dicentrarchus labrax*), the expression of Na^+-K^+-ATPase increased (compared with seawater controls) when the fish was acclimated to either

fresh water or 200% seawater, which was suggested that any osmotic stress might affect the expression of this important transport enzyme (Varsamos *et al.*, 2002). Some of these discrepancies may be due to differential expression of Na^+-K^+-ATPase isoforms, because a recent study has demonstrated that, in the rainbow trout gills, Na^+-K^+-ATPase α1b is upregulated in seawater, but Na^+-K^+-ATPase α1a is downregulated (Richards *et al.*, 2003). In blue crab (Callinectes sapidus), hyperosmotic treatments led to a reduction in arginine kinase flux, while the hypo-osmotic treatments led to an enhanced arginine kinase flux.

1.3.3 Handling stress

Health conditions of cultured animals can be affected by either procedural stress (restraint, handling, or novelty) or physical stresses (hunger, thirst, fatigue, injury, or thermal extremes). The key to prevention of stress is good management which means maintaining good water quality, good nutrition, and sanitation. Although, most farms have established procedure for handling their animals, it is still quite difficult to determine the conditions of animals that later lead to disease outbreak and mortality.

Studies to determine the amount of stress on farm animals during routine handling and transport often have highly variable results and are difficult to interpret from an animal welfare standpoint. Rough handling may be more detrimental and stressful to animals with an excitable temperament compared to animals with a more placid temperament. Many apparently conflicting results of different studies may be explained if the varying amounts of procedural stress and physical stress within each study are considered.

It is widely accepted that handling stress can reduce growth in fish. (Pickering, 1990). Getting scraped or bruised during handling can lose their protective slime coating, thereby reducing their natural defense against pathogens. Losses of scales or cuts are even a more dramatic invitation to infection or direct mortality due to injury. Crowding, which includes several stressors as well as physical differences in the rearing environment, has been shown to decrease growth in several salmonid species (McCormick *et al.*, 1998) while a single handling stress of 2-year-old brown trout, *Salmo trutta*, resulted in several physiological changes lasting up to 2 weeks without affecting growth rate (Pickering *et al.*, 1982). A daily acute handling stress for 10

weeks did not decrease growth of juvenile rainbow trout *Oncorhynchus mykiss* (Barton *et al.*, 1987).

Transporting stress can increase the amount and duration of pathogen shedding and thereby result in increased infectiousness, which was well described for salmonella in various animal species (Wierup, 1994). The shedding of pathogens by the transported animals results in contamination of vehicles and other transport-related equipment and areas. Handling and transport can cause a stress response in fish as a result of chasing and capture, removal from the water, confinement at high densities and degradation of water quality.

1.4 Biomarker

Biomarker is a biochemical, physiological, or histological change or aberration in an organism that can be used to estimate either exposure to chemicals or resultant effects (Huggett *et al.*, 1992). They are classified into 3 types: biomarker of exposure, biomarker of effect, and biomarker of susceptibility.

1.4.1 Biomarker of exposure

An endogenous substance, its metabolite or the product of an interaction between a xenobiotic agent and some target molecules or cells can be measured in a compartment within an organism. Some of potential biomarkers of exposure detected in various organisms are shown in Table 1.2.

Table 1.2 Biomarker of exposure

Biomarker of exposure	Chemicals	Organisms (Species)	Effects	Comments	References
Oxidative stress response system	Different prooxidants, pesticides, metals	Many vertebrate species, earthworms, crustaceans, molluscs, insects, spiders	Induction or inhibition	Induction of catalase and Se-dependent glutathione peroxidase, often inhibition of glutathione reductase, slight effects on superoxide dismutase, enhanced levels of reduced glutathione	Dietrich et al., 2002
Stress proteins (SP, hsp)	Toxic metals, various organic compounds	Many vertebrate and invertebrate species - aquatic and terrestrial	Induction	Different forms of *hsp* are induced, depending on a chemical, tested species and preadaptation to stressing factors	Sanders and Martin, 1993
Metallothionein (MT) synthesis and gene expression amplification.	Cd, Pb, Cu, Zn, Ni, Hg, some organic compounds	Many vertebrate species, earthworms, crustaceans, molluscs, insects, spiders	Induction	Dose-dependent manner of induction; species-depenedent differences in MT characteristics - generally high cysteine content, less aromatic aminoacids	Cosson, 2000
Mixed function oxidases (Cytochrome P450) - gene expression and activity.	PCBs, DDT congeners, PAHs	Vertebrates, insects, isopods, crustaceans	Induction (adaptive process)	More sensitive in vertebrates than invertebrates; difficult to measure normal range of activity. In invertebrates also other than CYP1A species-specific isoforms (in insects CYP6, CYP 9, CYP18)	Arinç et al.,2000
Glutathione S-transferase (GST)	Inducers: pinenes, pesticides, metals, xantotoxin,. Inhibitors: sulfamides, phtalimides, atrazine, quitozene	Earthworms, insects, spiders, isopods, mussels	Induction or inhibition	Many naturally existing inducers can mimic effects of xenobiotics. GST inducers act on existing isoforms; with no effects on creation of the new isoenzymes	Radim, 1998
Changes in DNA integrity	Different genotoxic xenobiotics; metals, hydrocarbons, pesticides	Vertebrate species, echinoderms, polychetes, earthworms, insects, mollusks, spiders	Ocurrence	Difficulties in linking with higher –level effects, often lacking reference material, difficulties in subtracting effects of DNA self-repair	Boynton et al., 2003
Scope for growth	Hydrocarbons, pesticides, metals	Crustaceans, isopods, snails, insects	Reduction	Direct effects of pollutants (increased costs of detoxification) or feeding avoidance, resulted in reduced reproductive success	Halldorsson et al., 2005

1.4.2. Biomarker of effect

A measurable biochemical, physiological, behavioural or other alteration within an organism that, depending upon the magnitude, can be recognised as associated with the established or possible health impairment or disease. The examples of biomarker of effect are shown in table 1.3

Table1.3 Biomarker of effect

Biomarker of effect	Chemicals	Organisms (Species)	Effects	Comments	References
Delta-aminolevulinic acid dehydratase	Lead	Mammals, birds, earthworms	Inhibition	Inhibition of ALAD, reduction of hemoglobin content correlate with the mortality rates	Oliveira and Luengo, 2003
Lysosomal membrane integrity and functioning	Non-specific, PAHs, PCBs, heavy metals, plasticisers, pesticides	Earthworms, cladocerans, insects, mollusks	Increased membrane permeability	Much evidence for aquatic than for terrestrial organisms; tests based on reduction of nuclear red retention time documented *in situ* on soil organisms.	Lam and Wu, 2003
Immunotoxic effects (humoral and cell mediated immunity, immunopathology)	Metals, PAHs, organotins, biocides aromatic amines	Vertebrates, earthworms, polychetes, crustaceans insects, mollusks	New forms, increased immune activity	Increased phagocytosis or agglutination formation of new coelomocytes less correlated with the dose of a chemical. Difficult to differentiate between direct and indirect effects and to prove the links between contaminants and a disease	Jennings *et al.*, 1988; Sullivan, 1989; Holsapple, *et al.*, 1991

Details in table 1.4 show the biomarkers which can be either exposure or effect.

Table 1.4 Biomarker of exposure or effect

Biomarker of exposure or effect	Chemicals	Organisms (Species)	Effects	Comments	References
Acetylcholine sterase - activity	Organo-phosphates and carbamates	Insects, mollusks, crustaceans, isopods, earthworms	Inhibition	Higher response to organophosphates than carbamates; correlations with the death rate and other sublethal effects; often resistance appear in insects, gives little information on the risk for soil-dwelling invertebrates	Pfeifer *et al.*, 2005
Peroxidation of lipids	uranium, heavy metals, pesticides	Vertebrates, insects, mollusks, isopods, earthworms	Increased peroxidation	Tests (thiobarbituric acid for malone aldehyde) less clear with invertebrates	Dietrich *et al.*, 2002

1.4.3 Biomarker of susceptibility

Biomarker of susceptibility is an indicator of an inherent or acquired ability of an organism in responses to the challenge of exposure to a specific xenobiotic substance. It must differentiate between the low- and the high-risk groups which have low false positive and low false negative rates, be easy to perform, and be simple to interpret such as acetyltransferases (NAT1 and NAT2, EC 2.3.1.5) meet these needs in many respects. Since the recognition of the NAT2 acetylation polymorphism from its effects on isoniazide toxicity (Weber and Cohen , 1968), it has been studied in relation to several diseases in hope of finding a useful marker of susceptibility. By the use of different substrates, acetylation activity could be separated into NAT1 (*p*-aminobenzoic acid [PABA]) and NAT2 (sulfamethazine [SMZ]) activity. Until recent reports of variations in PABA metabolism (Weber and Vatsis, 1993), NAT1 was thought to be monomorphic while the polymorphism was ascribed to the NAT2 activity. The identification of the genetic basis for the phenotypic variations was identified in 1990 (Ohsako and Deguchi, 1990) for NAT2 and in 1993 (Vatsis and Weber, 1993) for NAT1. Other investigators quickly reported the identification of

ethnic variations (Hickman and Sim, 1991) when the most frequent variant alleles seen in Caucasians differed in Asian and African-American populations.

1.5 Application of biomarkers to Environmental Health and Risk Assessment

Risk assessment is an important tool for setting environmental standard, which limits the exposure to harmful agents with the aim of protecting health. However, it is a new discipline and currently available methods and detailed information on exposure and toxicity are frequently inadequate. Biomarkers have significant potential for strengthening current risk assessments by filling in important gaps between the exposure and disease. Biomarkers are useful tools for understanding the nature and extent of human exposure and risk from environmental toxicants. They can serve as quantitative measures of chemical exposures and biologically effective doses, as well as early warning signals of biologic effect. During the past few years, biomarkers have been applied for monitoring many kinds of stresses associated with both wildlife and cultured animals. A number of studies on aquatic invertebrate biomarkers have been reported (Hyne and Maher, 2003).

Stress proteins, especially HSP70 and HSP60, have been used as biomarkers in a range of algae, invertebrates, fish, and higher vertebrates. Several suggestions are made to improve the utility of stress proteins as a biomarker of exposure e.g., consideration of the kinetics of stress protein induction relative to the pharmacokinetics of pollutant accumulation in the organism of concern, and selection of the type of stress protein for biomonitoring (Lewis *et al.*, 1999).

Molecular biomarker system (MBS) to assess the physiological status of *Palaomenetes pugio* (grass shrimp) challenged with exposure to heat stress, cadmium, atrazine, and the water-accommodating fraction of fuel has been developed. The MBS assayed 9 specific cellular parameters of shrimp that are indicative of a nonstressed or stressed condition: heat-shock protein 60, heat-shock protein 70, αB-crystallin homologue, lipid peroxide, total glutathione level, ubiquitin, mitochondrial manganese superoxide dismutase, metallothionein, and cytochrome P-450 2E homologue. By using these 9 parameters, the MBS can distinguish between the

responses to each stressor, and to the nonstressed control conditions. The MBS was able to determine the structural integrity of the cell as defined by protein turnover, protein chaperoning, and lipid composition via lipid peroxide levels, and the status of key metabolic processes such as cytoskeletal integrity and glutathione redox potential

Various oxidative stress biomarkers in gill, kidney and liver tissues in the Indian freshwater fish *Wallago attu* were investigated from two sites along the river Yamuna, which differ in their extent and type of pollution load. A comparison was made between the biomarker responses and general water chemistry at the two sites. The oxidative stress biomarkers that were analyzed included superoxide dismutase (SOD), catalase (CAT), xanthine oxidase (XOD) and glutathione redox cycle enzymes viz., glutathione peroxidase (GPx), glutathione reductase (GR) and glucose 6-phosphate dehydrogenase (G6PD). Levels of reduced glutathione (GSH) and lipid peroxidation (LPO) were also evaluated. All biomarkers were found to be substantially higher in the fish collected from polluted site (Pandey *et al.*, 2003).

The objectives

To clone and characterize Mn-SOD and Cu/Zn-SOD gene

To determine the expression of stress responsive genes of interest including Mn-SOD, AK, HSP70, HSP90, DAD-I, and TPx during oxidative, osmotic, and handling stresses in *P.monodon*

To verify the expression of stress responsive genes as biomarkers for determination of stresses in *P.monodon*.