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It's All in the Genes: How Genotype Can Impact Upon Response to Contaminant Exposure and the Implications for Biomonitoring in Aquatic Systems

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1. Introduction

"Owing to this struggle for life, any variation, however slight and from whatever cause proceeding, if it be in any degree profitable to an individual of any species, in its infinitely complex relations to other organic beings and to external nature, will tend to the preservation of that individual, and will generally be inherited by its offspring."

– Charles Darwin, *On the origin of species by means of natural selection*

In 1859, Charles Darwin and Alfred Russell Wallace independently proposed the theory of evolution by natural selection. This theory propounded that the inter-generational success of all living organisms is restricted by their range of environmental tolerances and organismal requirements. In the current post-genomics era the impact of environmental stressors upon the *genetic structure* of populations and species continues to be a fascination from both theoretical and practical perspectives. From a theoretical perspective stressors can give deep insight into processes causing genetic subdivision. Practically, stressors can influence genetic diversity and this can provide us with information on the health of an ecosystem. While stressors in the environment can be both anthropogenic as well as natural in origin, in this review we focus on man-made stressors.

Stressors of anthropogenic origin can include physical modifications such as habitat alteration, biological agents such as introduced species, and chemical agents such as industrial pollutants. Of these, chemical pollution is arguably the stressor with the most immediate impact on the health of our biota. In the nearly three hundred years since the industrial revolution, environmental pollution has been on the increase, while the health of our environment has simultaneously suffered. Chemical pollution such as that from urban run-off, industrial discharge and pest control agents to name a few has been shown to have widespread detrimental and long-lasting effects on natural populations. Our aquatic environments are under particular threat as our rivers, estuaries, and oceans are often

ecological sinks or dumping grounds for anthropogenic chemicals (e.g. Birch, 2000; Dunk et al., 2008; Jones, 2010).

Aquatic systems are the life-blood of many habitats, but are also subject to intense anthropogenic use for a variety of purposes including industry, transport as well as recreation. Thus, it is crucial to understand and monitor the health of aquatic organisms and aquatic ecosystems. For example, the British Petroleum (BP) *Deepwater Horizon* oil spill of April 2010 in the Gulf of Mexico saw approximately five million barrels-worth of crude oil lost into the ocean within the span of two months (Lubchenco et al., 2010), with an estimated 779 million litres spilt in total (Atlas & Hazan, 2011). Despite being the largest oil spill in United States history, several media reports suggested that based on observed wildlife mortalities the incident had only a moderate immediate environmental impact. It is likely, however, that immediate impact estimates represent less than 5% of the true biological effect (Williams et al., 2011). Incidences such as the BP oil spill highlight the immense gap in our knowledge about the extent and impact that anthropogenic stressors can have on our biota, especially in our aquatic environments.

The aim of this chapter is to discuss how genetic approaches linked with the tools of molecular biology can contribute to the fields of ecotoxicology and evolutionary toxicology. In the field of ecotoxicology, the importance of organismal genotype on the response of those organisms upon exposure to a contaminant has often been underexplored and its importance underestimated, particularly in the selection of suitable test organisms and sentinel species in biomonitoring programs. In this review, the term "biomonitoring" will be used as a collective representation of all terms relating to the detection and assessment of biological responses to environmental stressors. In the first section of this review we consider research on the effects of anthropogenic contaminants on the genetic structure of natural populations and then consider how the genotype of an organism can affect response to toxicant and contaminant exposure. In particular, we focus upon research that examines aquatic systems and aquatic sentinel species. We also explore the current genetic and molecular methodologies that are being utilised to assess the relationships between the underlying genetic structure of populations and the response to contaminant exposure. The focus of the second section of this chapter is traditional biomonitoring practices and the utilisation of sentinel species for biomonitoring. In this second section, we present a case study of ongoing research into the genetics and molecular biology of contaminant exposure response in an important local estuarine sentinel species in eastern Australia. We conclude that current molecular tools can assist in designing experimental and testing approaches to negotiate the influence of genotype in ecotoxicology.

2. Ecotoxicology and genetics

Ecotoxicology is the study of the effects of toxicants and contaminants, both natural and anthropogenic in origin, on the health of individuals and populations. Ecotoxicological studies involve the testing of model organisms with toxicants and contaminants of interest against reference populations or controls. Such studies are important for identifying substances that are potentially harmful to the health of both humans and other organisms, as well as determining the impact of toxicant and contaminant stressors upon natural ecosystems. The goal of this section is to highlight the need for objective methodologies to assess the impact of toxicant and contaminant exposure in biological systems.

In ecotoxicological studies, measurements of the effects of toxicants and contaminants on biological systems are typically estimated through organismal-level endpoints such as mortality, reproduction and growth. For example, reproduction and development of the ramshorn snail, *Planorbarius corneus*, is known to be impacted by exposure to sewage treatment effluent (Clarke et al., 2009). *P. corneus* is an egg-laying freshwater mollusc with a seasonal breeding pattern that is commonly found in European river systems. Snails collected from a clean waterway over two separate years were exposed to varying concentrations of effluent from sewage treatment facilities for 12-14 weeks in the laboratory and the reproductive output (measured as the number of eggs produced), egg quality (measured as egg mass), as well as gonad development analysed. Although exposure of *P. corneus* to sewage treatment effluent resulted in greater female fecundity with increased egg production among effluent-exposed animals, egg quality was found to be significantly decreased, suggesting a physiological trade-off between reproductive output and offspring viability (Clarke et al., 2009). Additionally, while the development of female gonadal tissue appeared unaffected by exposure to effluent, spermatogenesis in first generation offspring was found to be decreased; this is indicative of exposure to endocrine-disrupting chemicals common in many wastewater effluents (Clarke et al., 2009). While these observed differences in organismal endpoints are likely due to toxicant and contaminant exposure, results may also be confounded by seasonal environmental differences affecting the parental and grandparental generations in the sampling locality from which test organisms were derived. It has been demonstrated that individual fitness can also be affected by "carry-over effects" dating back to the parental and grandparental generations (Hercus & Hoffman, 2000), and this may have contributed to differences between the experimental groups in this study (Clarke et al., 2009).

A fundamental drawback of many ecotoxicological studies is that the results of toxicant and contaminant exposure analyses are indicative only of the health of the individuals being assessed rather than representing the response of the population or species as a whole (Bickham, 2011). In many studies large discrepancies are reported between individuals, test groups, treatments and replicates (e.g. Angus, 1983; Croteau & Luoma, 2009). This is particularly true for many behavioural traits such as exposure recovery or toxicant avoidance (Blomberg et al., 2003; Smith et al., 2010), making population-level and species-level inferences unreliable. For example, a test of phenol tolerance in natural populations of the mosquitofish *Gambusia affinis* identified differential tolerance between individuals (Angus, 1983). *G. affinis* were sampled from both industrially contaminated and clean rivers in the United States and exposed to a single concentration of phenol using a standard 48-hour toxicity assay, with the rate of mortality as the endpoint. The study identified differential tolerance to phenol among the test organisms, with some individuals resistant to phenol toxicity, some showing signs of resistance following acclimation in the presence of sublethal concentrations, while other individuals showed no resistance (Angus, 1983). This timeless study included wild-caught fish, which has unique experimental advantages and disadvantages. The primary advantage is that the results are directly relevant to the population tested. The disadvantage is that the observed differences in phenol sensitivity may be due to differences in the underlying genetics, age and health of the test organisms.

Life-history characteristics such as the developmental stage of the test organisms can also affect the observed response to toxicant exposure. This is particularly important if the overall health of an ecosystem is to be determined. For example, ecotoxicological studies

frequently utilise organisms at specific life or developmental stages, such as embryos or juveniles, as they demonstrate greater sensitivity than mature organisms (Ansari et al., 2010; Hutchinson et al., 1998; Pollino et al., 2002). Thus, a life-history bias in environmental monitoring studies of sensitive taxa may indicate a contamination event. Mhadhbi et al. (2010) examined the effects of acute metal and polycyclic aromatic hydrocarbon (PAH) toxicity on the early life stages of a species of turbot fish, *Psetta maxima*. Fertilized eggs were exposed to a range of environmentally and biologically relevant concentrations of metals and PAHs over a six-day period, then exposure success as measured by embryo survival and developmental malformations in post-hatch fish larvae were compared. It was determined that embryos were more tolerant to metals and PAHs than hatched larvae, which showed pericardial oedema as well as skeletal abnormalities leading to deformed larvae (Mhadhbi et al., 2010).

In this section we have argued that ecotoxicological studies are important in identifying potentially harmful substances in the environment. Traditional ecotoxicological studies and biomonitoring programs typically make use of organismal-based endpoints such as percentage mortality or reproductive output to assess the impact of toxicant and contaminant exposure. However, these endpoints vary greatly between the populations assessed and this can result in data with poor reproducibility and large standard errors (Wirgin & Waldman, 2004). Moreover, toxicant and contaminant concentrations used in many standard toxicity studies are often necessarily high as to induce observable and measurable organismal-level effects. In the following section we show that differences in response to stressors of anthropogenic origin can be influenced by the underlying genetic makeup of the species, and this also needs to be controlled if ecotoxicological studies are to be robust and reproducible.

2.1 Genotype and differential tolerance to toxicant exposure

Differential genetic tolerance in response to stressors of anthropogenic origin has been demonstrated in a wide variety of organisms. This is not surprising. Most species and populations show heritable genetic variability that was arguably first noticed and discussed by Charles Darwin in 1859. What is perhaps more surprising is that the importance of Darwin's first two chapters has not been fully embraced in the broad cross-section of ecotoxicological studies. Perhaps this is because human mediated contamination arguably falls between Chapter 1 "Variation under domestication" and Chapter 2 "Variation under Nature". Low levels of contamination can change the gene frequencies in a population in the same way that sublethal concentrations of pesticides and insecticides cause resistance in insects (Garrett-Jones & Gramiccia, 1954; Livadas & Georgopoulos, 1953). In this section we demonstrate that the genetic makeup of populations and species influences response to stressors, and this needs to be controlled in ecotoxicological studies (Wirgin & Waldman, 2004).

In ecotoxicology, a common approach to examine the relationship between toxicants or stressors and organisms is to measure biologically significant endpoints such as post-exposure mortality, developmental processes, or reproductive output. Most often, the genetic structure of the organisms of interest is not examined. Yet this is likely to be important when documenting the environmental impact of a specific anthropogenic contamination event. Allelic frequencies within a population can, for example, be examined

in field studies and compared against control populations post hoc. For instance, the link between allozymatic genotype and differential survivorship has been demonstrated in the gammarid amphipod *Hyalella azteca* exposed to metals (Duan et al., 2001). One-month old laboratory cultured amphipods were exposed to acute concentrations of cadmium and zinc solutions in a 72-hour test. Mortality was measured as lack of response when the animal was probed; dead or moribund animals were periodically removed and preserved for subsequent allozymatic analysis, and remaining live animals were also sacrificed at the end of the test period. Exposure to both cadmium and zinc resulted in relatively high mortality; surviving cadmium-exposed *H. azteca* also displayed behavioural changes (hyperactivity) after 48 hours of exposure (Duan et al., 2001). Allozymatic analysis found that of the six genotypes identified, animals harbouring genotype AC had a significantly higher rate of survival than all other genotypes following cadmium exposure, whereas genotype CC displayed greater tolerance following zinc exposure (Duan et al., 2001). These findings also suggest differential modes of selection for different stressors, such that genotypes more resistant to a particular stressor may skew the genetic structure of the population in favour of those individuals harbouring the resistant allele.

A complementary experimental approach is to directly examine the relationship between specific genetic types and the response to toxicant and contaminant exposure. The knowledge that organisms harbouring different genotypes can demonstrate differential tolerance and susceptibility to toxicant and contaminant exposure has bearing upon the choice of test individuals for ecotoxicological studies. Studies have identified differential tolerance and susceptibility of specific genotypes to toxicant and contaminant exposure (Roelofs et al., 2009; Snyder & Hendricks, 1997). Differential survival across three different mitochondrial lineages has been demonstrated in the marine harpacticoid copepod *Microarthridion littorale* exposed to a mixture of pesticides (Schizas et al., 2001). Three naturally occurring mitochondrial lineages were previously identified and found to occur in approximately equal frequencies at a South Carolina reserve in the United States. *M. littorale* were sampled from this locality, and animals exposed to a pesticide mixture for a period of 24 hours. Survivorship was defined as those living individuals still displaying normal swimming ability. Following exposure, *M. littorale* were harvested and haplotyped to identify mitochondrial lineage. Of the three lineages identified and tested, it was found that lineage I displayed the highest rate of survival, significantly exceeding even that of the test controls, while lineage II showed greatest susceptibility to the pesticide mixture (Schizas et al., 2001). However, while the results of this study suggest a possible link between mitochondrial DNA and toxicant exposure response, they do not demonstrate direct causality; the role of nuclear DNA in toxicity response and the interaction between nuclear and mitochondrial genes still needs to be examined.

In this section, we have discussed how the genotype of an individual has significant bearing upon the potential response to toxicant and contaminant exposure. However, examining the impact of toxicant and contaminant exposure on organisms based on differences at single genetic loci can be considered the equivalent of measuring the water in the oceans one drop at a time. In the next section, we introduce the emerging field of *evolutionary toxicology* and review how this field of study is attempting to answer questions on how toxicants and contaminants are impacting the genetics of biological systems at the genomic and population levels.

2.2 Evolutionary toxicology

Here, we introduce the concept of evolutionary toxicology as an experimental strategy to investigate the influence of toxicant and contaminant exposure on natural populations. A central goal of evolutionary toxicology is to examine both the direct and indirect effects of toxicant and contaminant exposure on the genetic structure of natural populations (Bickham et al., 2000; Matson et al., 2006; Theodorakis et al., 2001). A chief advantage of evolutionary toxicology over traditional ecotoxicological studies is that the utilisation of molecular and population genetics methodologies means these studies are based on fundamental evolutionary concepts. As a consequence they are able to provide a more holistic representation of an entire population or species, rather than just specific individuals (Bickham, 2011).

Broadly speaking, direct effects (genotoxic effects) are those where exposure to the toxicant or contaminant cause direct damage to genetic material (e.g. insertion / deletion events, base changes, chromosomal rearrangements). Indirect (non-genotoxic) effects are non-mutagenic in nature but cause stress to exposed individuals and can result in downstream population-level effects such as altered reproductive success, survivorship or altered gene expression patterns (Bickham, 2011; Rose & Anderson, 2005).

The identification of a genetic basis for organismal and behavioural traits allows predictions to be made across the entire species and potentially between species. As an example, reburial behaviour is a normal stress response and hazard avoidance mechanism in many shellfish species, and is often indicative of organism health. An investigation into recovery behaviour following metal exposure in the New Zealand freshwater clam, *Sphaerium novaezelandiae* found that this behavioural response is genotype-dependent within this species (Phillips & Hickey, 2010).

Bickham (2011) divides the direct and indirect effects of toxicant and contaminant exposure into four key genetic outcomes which he refers to as the "four cornerstones of evolutionary toxicology". While these four cornerstones represent a useful basis for discussion they are by no means exhaustive of the causative effects of toxicant and contaminant exposure upon organisms. These four cornerstones as described by Bickham are:

1. contaminant-induced increases in mutation rates, resulting in changes in genetic frequencies in impacted populations (e.g. Matson et al., 2006; Theodorakis et al., 2001);
2. contaminant-induced selection at loci affecting survival success, resulting in changes in genetic frequencies in the population (e.g. Cohen, 2002; Theodorakis & Shugart, 1997);
3. alterations to patterns of genetic distribution and migration, leading to demographic shifts (e.g. Maes et al., 2005; Matson et al., 2006); and
4. genome-wide changes in genetic diversity (e.g. Armendariz et al., 2004; Connon et al., 2008; Poynton et al., 2008; for reviews on evolutionary toxicology, see: Bickham, 2011; Theodorakis & Wirgin, 2002; van Straalen & Timmermans, 2002).

In the following sections, we examine how these four cornerstones provide a practical guideline for the design of ecotoxicology and evolutionary toxicology studies, as well as review some of the molecular techniques currently being applied to research in each of these areas. A key advantage of molecular techniques in biomonitoring is that many of these techniques are more sensitive at detecting exposure effects, even at concentrations below the organismal-level effective concentrations (Poynton et al., 2008). The four genetic outcomes

proposed are, however, by no means exhaustive, and novel techniques and new technologies will undoubtedly continue to further elucidate how toxicants and contaminants affect biological systems at the genetic level.

2.2.1 Contaminant-induced increases in mutation rates

The first cornerstone of evolutionary toxicology examines the effects of genotoxicant substances on the integrity of genetic material in biological systems (Bickham, 2011). Genotoxicants can increase mutation rates and directly interact with DNA, thereby causing heritable changes to the molecule. Hallmarks of exposure to genotoxicants can include increased base changes, insertion / deletion events, chromosomal rearrangements, or degradation of the DNA molecule (Rinner et al., 2011; Theodorakis et al., 1997, 2001). Patterns of mutation that typically arise from recent exposure events to genotoxicants are pockets of increased mutation rates restricted to individuals within an impacted area and which are not detected in unimpacted environments (Bickham, 2011). However, caution must be taken to ensure that the detected mutation events are the direct result of genotoxicant exposure rather than having arisen through spontaneous mutational events (Yauk et al., 2008).

Increased mutation rates have been correlated with regions of high industrial activity (e.g. Chung et al., 2008; Matson et al., 2005; Theodorakis et al., 1997). Rinner et al. (2011) identified increased rates of mutation associated with contaminant exposure in an invasive fish species in a highly contaminated region in Azerbaijan. The mosquitofish *Gambusia holbrooki* is an invasive fish species introduced to Azerbaijan in the 1930s to control mosquito densities and have since spread throughout the region. Given its relatively recent history in the region *G. holbrooki* populations are expected to be relatively homogeneous, with novel mutational events easily traceable (Rinner et al., 2011). In conjunction with the work by Matson et al. (2006), the genetic diversity at the mitochondrial control region was assessed in *G. holbrooki* sampled from the heavily contaminated Sumgayit region and surrounding reference sites. It was found that fish sampled from the contaminated region possessed four novel haplotypes and heteroplasmies, of which only one was found to occur among fish from reference populations. The other rare heteroplasmies identified were hypothesized to have been contaminant-induced mutational events as they were not found to occur outside of the contaminated region (Rinner et al., 2011).

Increased DNA damage (i.e. strand breakage) such as those induced by exposure to radioactive compounds can affect reproductive success and reduce reproductive fitness. Theodorakis et al. (1997) investigated the extent of DNA damage in the mosquitofish *G. affinis* exposed to radionuclides in the United States. Adult female fish were collected from liquid waste settling ponds as well as clean reference ponds, and liver tissue and blood samples analysed for DNA integrity using gel electrophoresis. In addition, the fecundity of sampled specimens was quantified in terms of embryo number and the occurrence of observable abnormalities. It was found that for both liver and blood samples, the occurrence of double-stranded DNA breakages was significantly higher in *G. affinis* collected from radionuclide-contaminated ponds than in fish sampled from reference ponds. The results were also reflected in both the percentages of abnormal broods and abnormal embryos counted in the sampled fish. Future studies interested in assaying cellular damage as a result of contaminant exposure may also quantify the

concentrations of 8-oxodeoxyguanosine within a cell as it is a measure of oxidative stress (Nadja et al., 2001).

2.2.2 Contaminant-induced selection at loci affecting survival success

The second of the four cornerstones of evolutionary toxicology describes changes in genetic diversity or allelic frequencies within populations as a result of selection acting upon loci affecting the survivorship of organisms (Bickham, 2011). Exposure to toxicants or contaminants typically causes stress in organisms, which in turn can affect any number of downstream organismal endpoints including survivorship and reproductive success. In the words of Charles Darwin, "*… if variations useful to any organic being do occur, assuredly individuals thus characterised will have the best chance of being preserved in the struggle for life; and … will tend to produce offspring similarly characterised*" (Darwin, 1859). Thus, individuals harbouring favourable genetic variations will likely be subject to positive selection, whereas those less tolerant are selected against, consequentially shifting the genetic structure of the population and / or species.

Where loci involved in the response to a particular stressor are known, it is possible to assess the effects of selection by quantifying allele frequencies or genetic diversity at those loci. Cohen (2002) examined the patterns of amino acid substitutions in the major histocompatibility complexes (*Mhc*) in the estuarine teleost fish *Fundulus heteroclitus* exposed to polychlorinated biphenyls (PCBs). *Mhc*s are a large class of proteins involved in vertebrate immune response by binding and presenting antigens to T-cells for further processing. High genetic variability in *Mhc* genes is maintained by pathogen and antigen-driven balancing selection (Robinson et al., 2003). In this study, direct sequencing of the *Mhc* class II B genes was performed and referenced against the mitochondrial hypervariable control region. It was found that population-specific amino acid replacements were correlated with contaminant exposure (Cohen, 2002). Additionally, *F. heteroclitus* sampled from PCB contaminated sites and clean reference localities displayed habitat-specific *Mhc* patterns that were not reflected in the sequences of the mitochondrial reference region (Cohen, 2002). *F. heteroclitus* sampled from the contaminated sites also showed significantly elevated rates of loci-specific non-synonymous amino acid substitutions that were not detected in fish from control sites. Together, these differences in substitution patterns suggest a selective stress response to PCB exposure in *Mhc* proteins (Cohen, 2002).

A major hurdle to detecting selection occurs when the stressor is unknown, or when the specific stress response loci or pathways have not been identified. One approach to overcome this "needle in a haystack" problem is to assay the cellular bioenergetics of impacted organisms. Stress response is often an energetically costly process, whereby cells must either bear toxicant accumulation load costs or induce cellular excretory mechanisms to clear the presence of the stressor. Mitochondria are responsible for the majority of ATP production in the cell, and thus measuring mitochondrial bioenergetics can be indicative of exposure to toxicant or contaminant stress. Cells undergoing stress response may either up-regulate ATP production to cope with the presence of the stressor, or toxicant exposure may inhibit mitochondrial function. Mitochondrial bioenergetics can be measured in a number of ways, including oxygen consumption and transmembrane potential (da Silva et al., 1998; Toro et al., 2003; Vijayavel et al., 2007). For example, Toro et al. (2003) assessed the effects of exposure to organic pollutants on bioenergetic responses in the giant mussels, *Choromytilus*

chorus sampled from off the coast of Chile. *C. chorus* were assayed for contaminant clearance rate, oxygen consumption, and scope for growth, which is indicative of the energy budget dedicated to growth and reproduction. The study found that animals sampled from the heavily polluted locality demonstrated both lower rates of contaminant clearance from tissues, higher rates of oxygen consumption, as well as a negative energy budget for growth and reproduction (Toro et al., 2003). On the other hand, *C. chorus* from the unimpacted site showed higher clearance rates and lower oxygen requirement, with a strongly positive scope for growth, thus indicating high energy expenditure associated with toxicant exposure response (Toro et al., 2003). An alternative, or perhaps complementary, approach would be the measurement of metabolic rates in specific tissues such as permeabilised muscle fibres using a respirometer such as the OXYGRAPH-2K (Oroboros Instruments, Innsbruck, Austria) (Pichaud et al., 2011). Contaminants and toxicants can have tissue-specific effects; thus the measurement of metabolic efficiency in mitochondria-rich tissues such as muscle fibres allows for targeted assessment of the effect of environmental contaminant exposure (Pichaud et al., 2011).

2.2.3 Alterations to patterns of genetic distribution and migration

The third cornerstone of evolutionary toxicology examines the effect of stressors on gene flow and migration that lead to changes to inter-population genetic structure (Bickham, 2011). Migration and gene flow between populations are fundamental to the demographic and evolutionary stability of populations, particularly in cases where population sizes are small. Stressors such as anthropogenic toxicants or contaminants can affect the direction of movement between populations and hence the distribution of genotypes and allele frequencies (Maes et al., 2005; Matson et al., 2006).

Assessment of genetic diversity through functional enzymatic assays can be indicative of the fitness costs incurred by toxicant and contaminant stressors. Maes et al. (2005) assayed allozymatic and microsatellite genetic diversity in the catadromous yellow eel *Anguilla anguilla* from metal contaminated rivers in Belgium, where populations have been on the decline over the past two decades. Adult *A. anguilla* were sampled from three interconnected contaminated river basins, and muscle and liver tissue assayed for metal accumulation and genetic diversity. *A. anguilla* collected from the most polluted river basin showed the highest levels of metal accumulation and showed the least heterozygosity at all allozymatic loci investigated, which was not reflected in the microsatellite analysis. This suggests a genotypic shift toward specific homozygote classes and / or a greater ability to clear metal accumulation by homozygotes in the most contaminated system (Maes et al., 2005). Future studies may also consider how mitochondrial gene expression changes with toxicant exposure as upregulation of genes encoding mitochondrial subunits has been shown to occur with mild mitochondrial dysfunction (Ballard et al., 2010).

Population genetics methodologies have also been applied to assess genetic diversity and patterns of gene flow between populations. Matson et al. (2006) examined the effects of chronic chemical contaminant exposure on population genetic structure and genetic diversity in marsh frogs from Azerbaijan. *Rana ridibunda* were collected from several localities within a region of known contamination as well as from two clean reference sites surrounding this region. Genetic diversity at the mitochondrial control region was measured and the genetic distances between populations calculated to determine migration

patterns between populations. This study identified a significant difference in the level of genetic diversity between the contaminated and reference regions as indicated by both haplotypic and nucleotide diversity, where overall mitochondrial diversity among *R. ridibunda* from the contaminated region was significantly lower (Matson et al., 2006). Analysis of genetic differentiation and gene flow between populations indicated that the rate of migration into the contaminated region far exceeded that of outward movement, suggesting *R. ridibunda* in the contaminated region have both decreased fitness and reproductive success, and that inward migration provided a compensatory effect for decreased reproductive success of the impacted population.

2.2.4 Genome-wide changes in genetic diversity

In recent years, genome-wide "omic" approaches have become increasingly commonplace in evolutionary toxicology (Snape et al., 2004; Van Aggelen et al., 2010). Omic approaches encompass whole genome sequencing studies, transcriptomic studies, as well as proteomic expression studies, with the latter two being most commonly applied in ecotoxicology and evolutionary toxicology (e.g. Iguchi et al., 2007; Poynton et al., 2008; Zhou et al., 2010). Although some toxicants may impact exclusively upon specific loci or cellular machinery, organismal response and recovery to toxicant exposure is likely to be a genome-wide process; hence, the ability to encompass and measure total cellular response to toxicant exposure is crucial in evolutionary toxicology (Bickham, 2011). An additional characteristic of both transcriptomic and proteomic profiling worth considering is that results are not only reflective of the underlying genetic structure of the organism, but are also indicative of the functionality of differences in the genetic structure, such that non-functional genetic changes (e.g. silent mutations in non-coding regions) are generally not detected. Thus, it can be considered that transcriptomic and proteomic analyses may be able to better identify biologically significant genetic changes induced by toxicant exposure.

Transcriptomic analyses examine genome-wide gene expression and are commonly used to compare differences in transcript profiles between organisms exposed and unexposed to toxicants and contaminants. A further application of transcriptomics in biomonitoring is the potential to identify novel informative genetic markers of exposure by pinpointing genes and loci that are differentially expressed. The use of transcriptomics to create toxicant exposure expression profiles and for biomarker discovery has been well documented in the widely used model organism *Daphnia magna* (e.g. Vandegehuchte et al., 2010; Watanabe et al., 2008). *D. magna* has demonstrated high sensitivity to a wide range of toxicants and contaminants, and distinct transcriptomic profiles have been generated for a number of metals and organic toxicants such as copper, zinc, lead and PAHs (Poynton et al., 2008). A major advantage of transcriptomics lies in the high detection sensitivity of these assays. Unlike standard toxicology tests that often require high doses of toxicants to induce a measurable effect in the test organism, gene expression patterns can differ significantly even with exposures to very low toxicant concentrations. In fact, distinct expression differences have been detected at concentrations as low as one-twentieth of the 50% effective concentration (EC50) value (Poynton et al., 2008). Moreover, it was found that higher exposure concentrations often resulted in the loss of toxicant-specific expression patterns in favour of a generalised stress responses (Poynton et al., 2008), making transcriptomic profiling a powerful and sensitive tool for biomonitoring. However, in addition to the high

cost a disadvantage of transcriptomics is the presence of transcriptional "noise" as a result of stochastic gene expression, and these random differences in transcript levels between samples and even individual cells must necessarily be accounted for when conducting transcriptomic analyses, particularly in the case of low abundance transcripts (Raj & van Oudenaarden, 2008).

Similarly, proteomic studies have been utilised to generate genome-wide protein expression profiles. Zhou et al. (2010) conducted protein expression assays on farm-reared abalone *Haliotis diversicolor supertexta* exposed to select endocrine disrupting compounds. *H. diversicolor supertexta* were exposed to low concentrations of two plasticiser compounds (1% of the previously established 50% lethal concentration (LC50) value) and hepatopancreatic tissue assayed for protein expression differences. The study identified 27-35 differentially expressed proteins in *H. diversicolor supertexta* exposed to the plasticisers when compared against the untreated control; nearly 20 of the identified proteins were up-regulated proteins, and 9-16 proteins were down-regulated (Zhou et al., 2010). Mass spectrometry identified many of the up-regulated proteins to be associated with general stress response, while several of the down-regulated proteins were involved in metabolism. In addition, the study also identified a number of compound-specific differentially expressed proteins.

3. Environmental monitoring

In this section we discuss the advantages of environmental monitoring to determine the health of natural systems and then consider the use of an amphipod crustacean to monitor the health of estuarine sediments in eastern Australian waterways. Environmental monitoring or detecting the presence of toxicants and contaminants falls under the two broad categories of *bioindication* and *biomonitoring*. In this section we focus on the latter.

Bioindication is a qualitative assessment of the impact of environmental stressors (Holt & Miller, 2011). An example is the destabilisation of lysosomes in the oyster, *Crassostrea virginica* exposed to environmental stressors (Ringwood et al., 1998). Lysosomes are considered to be sensitive organelles to contaminant-induced stress as they are involved in a host of cellular repair and defence functions. In this study, *C. virginica* exposed to high salinities as well as copper solutions displayed higher levels of lysosomal destabilisation than control organisms. These results were also congruent with field studies of *C. virginica* deployed in contaminated estuarine sediments (Ringwood et al., 1998).

Biomonitoring is the process of quantitatively determining the impact of environmental stressors on biological systems (Holt & Miller, 2011). Kreitsberg et al. (2010) measured the effects of PAH exposure in flounder following an oil spill off the coast of Estonia. In this study, *Platichthys flesus trachurus* were sampled at two-month intervals beginning five months post-spill and liver samples taken to examine PAH accumulation. Samples were analysed for PAH concentrations by high-performance liquid chromatography. Biologically high concentrations of PAHs were detected in the liver tissue of *P. flesus trachurus* caught five months after the spill, which was found to decrease over time (Kreitsberg et al., 2010).

The chief purpose of biomonitoring is the measurement and tracking of chemical substances in biological systems with the purpose of monitoring and assessing exposure to stressors, particularly those of anthropogenic origin. The assessment of toxicants and contaminants in biological systems through biomonitoring provides an environmentally relevant indication

of the impact of environmental stressors within a given habitat or ecosystem. Direct quantification of contaminants such as sediment and water using chemistry-based ecotoxicological methodologies may not give any indication of biological or ecological relevance. Furthermore, chemical and physical analyses are also unable to assess ecological processes such as population shifts, nor assess levels of biodiversity within an ecosystem.

Biomonitoring can be conducted on both whole organisms as well as biological materials such as specific tissues, bodily fluids or cells. In biomonitoring, assessing the effects of toxicants and contaminants on whole organisms is most common; in this review we focus on environmental biomonitoring. Traditionally, two broad organismal measures of sensitivity or tolerance of biota toward toxicants and contaminants are commonly applied in biomonitoring (Mandaville, 2002). One is a measure of organism sensitivity, and the second is a snapshot assessment of the quality of a particular environment. A major shortcoming of both traditional biomonitoring measures is the underlying assumption that related organisms will demonstrate similar responses toward toxicant exposure. This is not always true. It has been documented that sister species can show differential susceptibility to toxicants and contaminants (e.g. King et al., 2004, 2006a).

In the following section, we examine the use of sentinel species in biomonitoring. We then consider the use of an amphipod crustacean in biomonitoring in eastern Australia.

3.1 Sentinel species in biomonitoring

As with ecotoxicological studies, biomonitoring studies and programs typically involve assessing the response of model organisms or *sentinel species* (also known as bioindicators or biomonitors) to toxicant and contaminant exposure. Perhaps the earliest and best known example of a sentinel species is the canary in the coal mine. Even as recently as the late 20th century, it was customary for coal miners to take canaries deep into the mine shafts with them as an early warning system against the build-up of toxic gases. The birds, being more sensitive to the presence of methane and carbon monoxide gas than humans, would fall ill and perish long before gas concentrations became dangerous for the miners, allowing workers to escape the tunnels safely. Environmental sentinel species serve the same function as the canary in the coal mine, providing an early detection system to the presence and effects of toxicants and contaminants in the environment.

A sentinel species can be any ecologically relevant organism that is well characterised, locally abundant and simple to survey and culture. Basic criteria of a good sentinel species are that it should be sensitive to the toxicant or contaminant of interest at both biologically and ecologically relevant concentrations, and do so in a reproducible manner that is reflective of the whole population, species and / or ecosystem. A good sentinel species should also possess readily measurable responses that may include reproductive output, developmental check-points or measures of growth (Holt & Miller, 2011). A limitation of many sentinel species is that the intraspecific variation in the response to a contaminant is not known because the underlying genetic variation in that species has not been quantified. This is of particular concern when a sentinel species is exposed to a range of contaminants such that distinct genetic pathways are differentially affected. In the extreme case, a specific species may be a highly sensitive sentinel with regard to one contaminant but a less sensitive sentinel with regard to a second contaminant.

The use of sentinel species to assess the impact of environmental stressors can give biological insight. Sentinel species essentially serve three major functions:

1. to assist in monitoring changes in the physical and / or chemical environment that can be of either natural (e.g. temperature, precipitation, salinity) or anthropogenic origin (e.g. use of chemical agents, habitat restructuring, industrial contamination);
2. to assist in tracking ecological processes (e.g. changes in population densities); and
3. monitoring the biodiversity of a given habitat or ecosystem (Holt & Miller, 2011).

In the following section, we present a case study of ongoing research applying some of the above methodologies to a sentinel species employed in eastern Australia to assess the health of estuarine sediments and ecosystems.

3.2 Case study: *Melita plumulosa*, the environmental canary of Eastern Australian waterways

In many aquatic systems, benthic organisms such as crustaceans serve as the "environmental canaries" of aquatic environments. These invertebrate species possess many useful qualities that make them ideal sentinel species to monitor the health of waterways. Among the macroscopic bottom-level feeders in a food web, benthic organisms are some of the most abundant members of an ecosystem and facilitate the transfer of energy and nutrients between microbial communities and the higher members of the food web. Most species of aquatic invertebrates feed by filtering or ingesting nutrients from the water or sediment, and thus can accumulate high concentrations of toxicants or contaminants that may be present within a habitat. It has been observed that population disruption or loss of bottom-level organisms and benthic invertebrate species can result in the disruption and even collapse of entire ecosystems (Owens & Dittman, 2003; Pillay et al., 2010; Wallace & Webster, 1996).

Among aquatic invertebrate species, crustaceans of the order Amphipoda are some of the most well characterised and commonly utilised organisms in biomonitoring studies. Amphipods are a highly diverse order of crustaceans found to inhabit nearly all marine and freshwater habitats worldwide and are a major benthic component in terms of both biomass and species diversity. To date, nearly one hundred amphipod families have been described (Lowry et al., 2000). Because of their ecological importance, numerical abundance as well as sensitivity to a variety of anthropogenic toxicants and contaminants, amphipods have long been utilised as sensitive environmental sentinels (e.g. Castro et al., 2006; Roach et al., 2001; Wu & Or, 2005). In particular, amphipods are often utilised to monitor the health of aquatic sediments, which can serve as an ecological sink for many anthropogenic toxicants and contaminants (Chapman & Wang, 2001; De Lange et al., 2006; Simpson et al., 2005). Other aquatic sentinel species include fish, frogs, molluscs, copepods and water fleas (e.g. Matson et al., 2006; Poynton et al., 2008; Rinner et al., 2011; Ringwood et al., 1998; Schizas et al., 2001).

3.2.1 Biology of *Melita plumulosa*

In eastern Australia, the local amphipod species, *Melita plumulosa* (Zeidler's Melita; family: Melitidae) (Fig. 1) is a commonly utilised sentinel species to assess the health of estuarine sediments (Ecotox Services Australasia, 2009). Originally isolated and described by Zeidler

from specimens found in a coastal pool 100 m from the sea in northern New South Wales (Zeidler, 1989), *M. plumulosa* is an epibenthic amphipod endemic to eastern Australian waterways that can be found in the intertidal zone beneath rocks or shell-grit (King et al., 2006b; Lowry et al., 2000). Although the full geographic range of this amphipod has not been definitively established, historically it has been found in many waterways and estuaries between Brisbane and Melbourne along the east coast of Australia (Lowry et al., 2000); recently this amphipod has been sampled from the Pine River in Queensland down to the Yarra River in Victoria (Fig. 2).

In many river systems, *M. plumulosa* are found to co-localise with a sister species, *Melita matilda* (Hyne et al., 2005). In the field, *M. plumulosa* and *M. matilda* appear to cohabit the same environmental niches and share similar morphologies. Under the microscope, these two species can be definitively distinguished by an additional posterodorsal spine on urosomite 1 that is unique to *M. plumulosa* (Fig. 1) (Lowry et al., 2000). Although these two organisms are often found to co-localise and commonly sampled together, a study testing the sensitivities of eight local amphipod species in Australia and New Zealand to anthropogenic toxicants and contaminants demonstrated significantly different sensitivities between these two sister species (King et al., 2006a). Therefore, *M. plumulosa* has been designated as the sentinel species for monitoring sediment contamination in eastern Australia.

M. plumulosa and can tolerate a wide range of environmental conditions, including sediment particle sizes ranging from silt to gravel, salinities ranging from freshwater to seawater, as well as a wide range of temperatures (Hyne et al., 2005; King et al., 2006b). Under laboratory conditions, the average lifespan of *M. plumulosa* is 8-11 months; gravid females release live young that become sexually mature in four to six weeks (Hyne et al., 2005). Males are readily distinguishable from females by their larger gnathopod 2 as well as having more bristles along the antennae (Hyne et al., 2005). Optimal culture conditions for this amphipod as established by Hyne et al. (2005) are seawater of 25‰ salinity at 25°C ambient temperature on sediment composed of > 96% silt.

The advantages of utilising *M. plumulosa* as a sentinel species are manifold. The key advantage to employing this species for biomonitoring is the sensitivity of this amphipod to a range of sediment-bound contaminants (King et al., 2006a). Many contaminants often bind to and accumulate in aquatic sediments (Birch, 2000), the concentrations of which may not be reflected in the overlying pore water and water column. As this amphipod displays epibenthic behaviour and feeds on detritus by ingesting the sediment (Hyne et al., 2005), it has been demonstrated that *M. plumulosa* is particularly sensitive to sediment-bound contaminants (King et al., 2005, 2006b). Additionally, laboratory cultures of *M. plumulosa* are relatively simple to establish and maintain, as this amphipod has demonstrated robustness to a wide range of environmental conditions, and the optimal culture conditions have been well established (Hyne et al., 2005).

A disadvantage of this organism is the undetermined distribution of this amphipod. Although *M. plumulosa* has been found in many estuaries along the eastern Australian coast (Fig. 2), its established geographic distribution is not global across Australia and is predominantly limited to brackish sediments along the coastal fringe (Lowry et al., 2000). Furthermore, where *M. plumulosa* and *M. matilda* co-localise and in mixed-species laboratory cultures, *M. matilda* often outnumber and outcompete *M. plumulosa* (Hyne et al., 2005),

making sampling time-consuming and correct identification crucial. Therefore, the applicability of this organism as a global sentinel species across Australian waterways is limited. A second disadvantage of *M. plumulosa* is the variability in female fecundity (Gale et al., 2006; Mann & Hyne, 2008). Female fecundity is a commonly assessed organismal endpoint of toxicant exposure in *M. plumulosa*, and is closely linked with dietary fatty acid composition which is crucial to ovary maturation and embryo development in amphipods and other crustacea (Clarke et al., 1985; Hyne et al., 2009; Middleditch et al., 1980). Recently, fine-milled silica has been shown to be a feasible alternative standardised substrate for short term toxicity tests; however, field collected sediments are still necessary for both long term tests and laboratory culture maintenance (Mann et al., 2011).

Fig. 1. The estuarine sentinel species of eastern Australia, the amphipod *Melita plumulosa*. The distinguishing morphological feature of this species is the additional posterodorsal spine on urosomite 1 (circled in red).

3.2.2 Ecotoxicology of *Melita plumulosa*

A key characteristic that makes the amphipod *M. plumulosa* an ideal sentinel species for monitoring and assessing sediment health is that it feeds on detritus by ingesting sediment, and is therefore directly exposed to sediment-bound toxicants and contaminants (Hyne et al., 2005; King et al., 2005; Spadaro et al., 2008). Studies have demonstrated this amphipod to be sensitive to both aqueous and sediment-bound metals and PAHs in both acute and chronic exposures, with juvenile animals displaying greater sensitivity than adult *M. plumulosa* (Gale et al., 2006; Hyne et al., 2005; King et al., 2005, 2006; Spadaro et al., 2008).

A range of toxicology tests have been established using *M. plumulosa*. These include a 42-day (full life-cycle) chronic whole-sediment test using metal-spiked sediments (Gale et al., 2006), a 10-day acute whole-sediment test using both adult and juvenile animals and metal-spiked sediments (King et al., 2006b), and more recently a 13-day reproduction test using both laboratory cultured amphipods as well as *in situ* testing (Mann et al., 2009, 2010, 2011). In all cases genetically heterogeneous amphipods were employed, with large numbers of individuals needed to offset the potential effects of inter-individual variation in toxicant

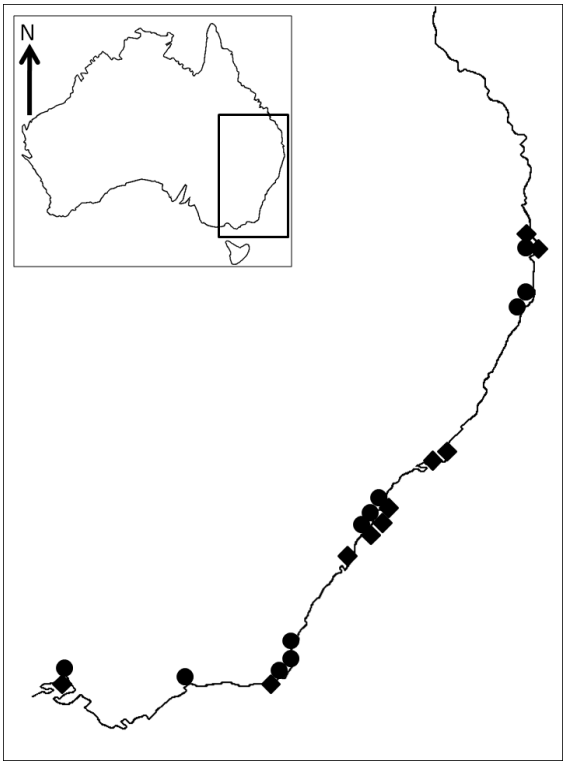

Fig. 2. Known geographic distribution of *Melita plumulosa* along the eastern coast of Australia. Historic sampling sites (1989 – 2000) are represented by circles, and current sampling sites (2000 – present) are represented by diamonds (Historic data courtesy J.K. Lowry and current data courtesy R.M. Mann).

response. Even with the vast numbers of individuals utilised in each of these tests (in excess of 200 and up to 1 000 individuals for the reproduction test (Mann et al., 2009)), a large degree of variance can still be observed both within and between replicates and treatments. Previously, we demonstrated that different genotypes can give rise to differential stress response; therefore, one possible explanation for the large discrepancies observed may be attributed to the use of genetically heterogeneous test populations. An alternate explanation for these results is the differences in age of test animals and hence reproductive potential of the individuals used.

It has also been demonstrated that the results of laboratory tests on *M. plumulosa* are not always replicable in *in situ* bioassays (Mann et al., 2010; Wilkie et al., 2010). A study by Mann et al. (2010) showed that laboratory tests demonstrated the contaminated field sediment to have high reproductive toxicity and confirmed previously established results; however, amphipods used in *in situ* bioassays displayed no evidence of reduced reproductive success. It was hypothesised that these discrepancies between the laboratory test results and the *in situ* bioassays are likely due to tidal water exchanges that can affect the bioavailability of contaminants in the overlying water as compared to the static

laboratory tests (Mann et al., 2010). Additionally, other environmental fluctuations such as precipitation and the presence of other biota not present in the laboratory tests may also contribute to the bioavailability of contaminants within each test system.

The sensitivity of this amphipod to a range of toxicants and anthropogenic contaminants is not disputed. However, ecotoxicological studies such as these highlight the clear need for methodologies and testing protocols that include an assessment of how genetic variation within a species influences contaminant response. In the following section we briefly discuss what is currently known about the genetics of *M. plumulosa*.

3.2.3 Genetics of *Melita plumulosa*

Over the past decade, the biology and ecology of the amphipod *M. plumulosa* has been well characterised, and much has been established regarding its organismal-level response to toxicant and contaminant exposure. However, there remains a large gap in the knowledge of the genetics of this important sentinel species.

Currently, ongoing research into the population genetics as well as the genetics of the toxicological response in *M. plumulosa* is being conducted. For example, a study of the effects of chronic contaminant exposure on natural populations of this amphipod in eastern Australia identified genetic subdivision between impacted and reference populations, as well as significant life-history trait differences between the two populations (Chung et al., 2008). In this study, amphipods were sampled from one industrially contaminated waterway and one clean reference waterway, and the genetic diversity at one mitochondrial and one nuclear locus assessed. In addition, amphipod size and female fecundity as measured by the number of embryos per gravid female were also determined to assess whether the genetic and organismal data were correlative. The results of this study identified discrepancies in the level of genetic diversity between the two genetic loci examined, with mitochondrial genetic diversity notably higher among amphipods sampled from the contaminated river system than those from the reference waterway; this pattern of genetic diversity was not reflected at the nuclear locus (Chung et al., 2008). Furthermore, life-history trait variations also identified amphipods sampled from the contaminated river as being significantly smaller in size and less fecund than those sampled from the reference waterway, suggesting that chronic exposure to industrial contaminants has impacted both the health and genetic structure of the local amphipod population (Chung et al., 2008). A major limitation of this study is that amphipods from only two waterways were examined; analysis of additional populations and waterways is necessary to determine if the observed trends are indeed indicative of toxicant exposure, or if these data represent an isolated phenomenon.

Further research into the underlying genetic structure of *M. plumulosa* is currently underway, including an assessment of responses to other industrial contaminants prevalent in contaminated waterways in eastern Australia. To address the genetic concerns raised in this chapter, current research on this sentinel species involves the development and use of animals of standardised genetic lineage for toxicity assays. Furthermore, whole-genome assays as well as bioenergetic analyses have also been proposed.

4. Conclusion

The impact of anthropogenic toxicants and contaminants on the health of natural populations is an evolutionarily recent phenomenon given the brief industrial history of the

human race. However, even though the footprints of anthropogenic toxicants and contaminants are relatively fresh, the effects upon biota have been demonstrated to be persistent and severe. Perhaps one saving grace of mankind is our ability and our desire to investigate and understand our impact upon the natural world.

In this chapter we reviewed past and current research on the effects of toxicants and contaminants on the genetic structure of natural populations, with a particular focus on aquatic, estuarine and marine systems. Genetics underpins all biological processes and it is of crucial importance that we understand the impacts that anthropogenic stressors have upon organisms at the molecular level. While it is clear that in this post-genomics era many powerful and informative tools are available to assess the impact of anthropogenic stressors on natural systems, further research must still be undertaken to complete our understanding of the consequences of toxicant and contaminant exposure on natural populations and species.

The four cornerstones of evolutionary toxicology proposed by Bickham (2011) provide a descriptive and practical framework by which the genetic-level effects of toxicant and contaminant exposure can be classified. Furthermore, this framework provides ecotoxicologists and evolutionary toxicologists with a practical and generalised guideline by which the problem of understanding the mechanisms of how anthropogenic stressors impact upon biological systems can be approached.

While we continue to expand our understanding of the mechanisms and impacts anthropogenic stressors have upon biota, biomonitoring is also crucial to keep track of the damage to natural populations occurring in our present. Given our current understanding of genetics and molecular processes, a key consideration is the effect of genotype on an organism's response to stressors. In the past, biomonitoring techniques have typically taken a broad-brush approach because there was an assumption that the mean response of an organism was more or less similar between individuals, as well as being sufficiently representative of that of the total population or species. However, it is now well understood and accepted that there is a significant influence of genotype on individual stress response variations, and this fact cannot be ignored.

Current molecular tools can assist in designing experimental and testing approaches to negotiate the influence of genotype. These may include conducting toxicology tests according to genotype and directly assessing the genetic impact of toxicants and contaminants. Additionally, molecular tools can help us identify the most appropriate sentinel species and individuals to assess the impact of toxicant and contaminant exposure. Nevertheless, it must be remembered that the genome of any organism is a large and often poorly understood minefield with complex molecular interactions. As a consequence it is unlikely that any one methodology will be able to fully account for all the minute levels of variation and interactions within biological systems.

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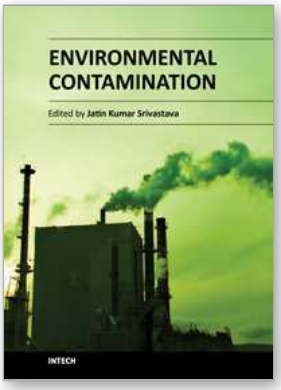

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Nature minimizes the hazards, while man maximizes them. This is not an assumption, but a basic idea of the findings of scientists from all over the world. The last two centuries have witnessed the indiscriminate development and overexploitation of natural resources by man causing alterations and impairment of our own environment. Environmental contamination is the result of the irrational use of resources at the wrong place and at the wrong time. Environmental contamination has changed the lifestyle of people virtually all over the world, and has reduced the extent of life on earth. Today, we are bound to compromises with such environmental conditions, which was not anticipated for the sustenance of humanity and other life forms. Let us find out the problem and its management within this book.

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