XENOBIOTIC METABOLISM AND MARKERS OF GENOTOXICITY IN REGULATORY NON-VERTEBRATE ECOTOXICOLOGICAL TEST SPECIES

by

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ABSTRACT

Genotoxicants are well known and important environmental contaminants. However, current ecotoxicological testing strategies generally focus on apical and reproductive endpoints, with little consideration given to subtle sub-lethal effects such as genotoxicity. Moreover, even less information is available on the responses of lower animals and plants to such substances. In this study, the sub-lethal responses of the water flea *Daphnia magna*, and the unicellular alga *Chlamydomonas reinhardtii* were investigated following exposure to known genotoxic substances. These studies were aimed at providing fundamental information on the responses of these two species thus addressing their relevance as potential alternatives to the use of vertebrates (e.g. fish) for the ecotoxicological testing of the effects of such compounds.

Methods were developed to allow the Comet assay to be applied to algal and daphnid cells. Statistically significant increases in DNA strand breaks were detected following exposure of algal cells to selected direct- and indirect-acting genotoxicants. An apparent relatively low sensitivity was not elevated by using a wall-free mutant. In contrast, the DNA damage responses in *D. magna* did not achieve statistical significance.

Methods were also developed to allow the xenobiotic biotransformation capability (via 7-ethoxyresorufin-*O*-deethylase or EROD activity) of both organisms to be measured. *C. reinhardtii* and *D. magna* possessed low, but measurable, basal EROD activity (0.03 pmol/minute/10^6 algal cells and 7 fmol/minute/daphnid). In *C. reinhardtii*, a 48h exposure to β-naphthoflavone (0.2 and 1nM) did not significantly

induce activity, and in *D. magna,* EROD activity was not significantly affected by the presence of dimethylsulfoxide (up to 0.1%) or methanol (up to 0.05%).

In addition to the analysis of effects at the sub-cellular level, gene expression analysis using *D. magna* oligonucleotide microarrays was also undertaken to assess the effect of these gentoxicants, on DNA repair, at the transcriptomic level. The microarrays revealed unique expression profiles for adults and neonates and significantly higher expression of some DNA repair genes in adults. Following exposure to a mixture of sodium dichromate and benzo[a]pyrene, a greater number of DNA repair genes showed up-regulation in adults compared to neonates. The majority of modulated genes implicated sodium dichromate as the primary stressor (e.g. glutathione-S-transferases, peroxiredoxins, ferritins), and potential reproductive and population level effects were identified.

In all this study has highlighted the potential use of *C. reinhardtii* and *D. magna* in genotoxicity testing, using the methods developed herein. These studies revealed that both species are able to activate and respond to genotoxicants, although there were some clear differences in terms of sensitivity to the compounds applied. Furthermore, novel gene expression profiling in *D. magna* also offers a potential complementary measurement of genotoxicant exposure, via the assessment of DNA repair capacity. This methodology could be applied in addition to standard mutation and DNA damage assays to provide a battery approach to non-vertebrate genotoxicity testing.

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LIST OF ABBREVIATIONS

Name	Abbreviation	Definition/Function
20E	20-hydroxyecdysone	Steroid hormone that controls reproduction and development in insects
2-AAF	2-acetylaminofluorene	Carcinogenic and mutagenic derivative of fluorene
2-AF	2-aminofluorene	A carcinogenic and mutagenic compound used in research
3-OH BaP	3-hydroxybenzo[a]pyrene	Metabolite of BaP
5,10-MTHF	Methenyltetrahydrofolate	Involved in photoreactivation
7-ER	7-ethoxyresorufin	Substrate metabolised by CYP1A
8-OH-dG	8-hydroxy-deoxyguanosine	Oxidative damage to DNA
9-OH BaP	9- hydroxybenzo[a]pyrene	Metabolite of BaP
AhR	Aryl hydrocarbon receptor	Induction of cytochrome P450 1A occurs via this receptor
AP Site	Apurinic/apyrimidinic site	Produced in BER following removal of a damaged base.
APE1	Apurinic endonuclease 1	Cleaves the phosphodiester backbone immediately 5' to an AP site
ARNT	Aryl hydrocarbon receptor nuclear translator	Ahr binds to ARNT to activate gene transcription
ATP	Adenosine-triphosphate	Unit of energy in cells
BaP	Benzo[a]pyrene	Polycyclic aromatic hydrocarbon, pro-genotoxicant (requires metabolic activation)
BaP 4,5-diol	Benzo[a]pyrene-4,5-dihydrodiol	Metabolite of BaP
BaP 7,8-diol	Benzo[a]pyrene-7,8-dihydrodiol	Metabolite of BaP
BaP 9,10-diol	Benzo[a]pyrene-9,10-dihydrodiol	Metabolite of BaP
BaP quinones	Benzo[a]pyrene quinones	Metabolite of BaP
BBM	Bold's basal medium	Algal culture medium
BCA	Bicinchoninic acid	Assay to measure protein
BER	Base excision repair	Process of removing damaged bases from DNA

bp	Base pair	Two nucleotides on complementary DNA or RNA strands connected by hydrogen bonds
BRCA1/2	Breast cancer susceptibility gene 1/2	Tumour suppressor genes. Also involved in homologous recombination.
BSA	Bovine serum albumin	Protein standard used in assays measuring protein levels
C. reinhardtii	*Chlamydomonas reinhardtii*	Freshwater unicellular green alga
Cd	Cadmium	
cDNA	Complementary DNA	Synthesised from an RNA template using reverse transcriptase.
CNS	Central nervous system	The brain and spinal cord. Coordinates the activity of the whole body.
CNS buffer	Hypotonic citrate buffer	Buffer used for isolation of *D. magna* nuclei
CPDs	Cyclobutane pyrimidine dimers	UV-induced DNA damage
Cr	Chromium	
Cr (V)	Pentavalent chromium	
Cr(III)	Trivalent chromium	
Cr(VI)	Hexavalent chromium	
cRNA	Complementary RNA	RNA derived from cDNA
CT	Threshold cycle	The PCR cycle at which an increase in reporter fluorescence above the baseline signal is detected
Cu	Copper	
Cy3	Cyanine 3	An orange, reactive water-soluble fluorescent dye (excitation 550nm, emission 570nm)
CYP	Cytochrome P450	Catalyse phase 1 reactions
D. magna	*Daphnia magna*	Water flea (small crustacean)
D. pulex	*Daphnia pulex*	Water flea (smaller than *D. Magna*)
DBH-like-1	Dopamine β hydroxylase-like 1	An enzyme that converts dopamine into noradrenaline
DDB(1/2)	Damaged DNA Binding protein	Postulated to be involved in a general cellular response to DNA damage

D-DDB1	Damage-specific DNA binding protein 1 (127kda)	Involved in nucleotide excision repair
DGC	*Daphnia* genomics consortium	An international network of researchers working to make *Daphnia* a model system for ecology, evolution and environmental sciences
dH_2O	De-ionised water	Purified water
Dhb1	*Daphnia magna* haemoglobin	
DMSO	Dimethylsulfoxide	Used to solubilise hydrophobic substances
DNA	Deoxyribonucleic acid	A double-stranded polymer containing four bases tethered to a sugar-phosphate backbone. Contains genomic information.
DNA-PKcs	DNA-dependent protein kinase catalytic subunit	
dNTP	Deoxyribonucleotide triphosphate	A generic term to describe dATP, dCTP, dGTP and dTTP
DSB	Double strand break	A type of damage to DNA
DTT	Dithiothreitol	A reducing agent used in polymerase chain reaction
EC_{50}	Effective concentration 50	The concentration of a chemical required to exhert an effect on 50% of the population
EPA	Environmental protection agency	A US agency with a mission to protect human health and the environment
EROD	Ethoxyresorufin-*O*-deethylase	Activity indicative of CYP1A-like activity
ESTs	Expressed sequence tags	A short sequence of a trnascribed cDNA sequence
FADH2	Flavin adenine dinucleotide dihydrogen	The reduced form of FAD having accepted two hydrogen atoms
FDR	False discovery rate	Predicts the number that would be identified by chance
Fe	Iron	
FELS	Fish early life stage	Test to observe early life stage toxicity of compounds to fish
FEN1	Flap structure-specific endonuclease 1	Structure specific nuclease involved in long patch base excision repair
FM/4	Algal culture medium	

FPG	Formamidopyrimidine DNA glycosylase	Cleaves 8-oxo-dG (oxidative damage) from DNA
GDS	Genotoxic disease syndrome	The notion that genotoxic chemicals can cause a variety of irreversible toxicities, not just cancer.
GO	Gene ontology	Describes gene products according to their associated biological processes, cellular components or molecular functions using a controlled vocabulary
GPX	Glutathione peroxidase	A peroxidase that provides protection from oxidative stress
GSH	Reduced glutathione	A major protectant from oxidative stress
GST	Glutathione-S-transferase	Conjugate reduced glutathione to electrophilic centres on a wide variety substrates via a sulfhydryl group
H2A	Histone 2A	Involved in the repair of double strand breaks
H_2O_2	Hydrogen peroxide	A highly reactive oxygen species
Hb	Haemoglobin	Iron-containing oxygen transport protein in red blood cells
Hg^{III}	Mercury	
Hg^{0}	Elemental mercury	
His	Histidine	An amino acid
HR	Homologous recombination	Double strand break repair
Hsp70	Heat shock protein 70	Their expression increases in response to heat or other stress, and they are named according to their molecular weight.
IARC	International Agency for Research on Cancer	Coordinates and conducts research into the causes of human cancer, mechanisms of carcinogenesis and develop strategies for prevention and control.
ITPase	Inosine triphosphate pyrophosphatase	Controls levels of potentially mutagenic nucleotide ditp
LD_{50}	Lethal dose 50	The dose of a chemical required to kill 50% of a population.

Lig3	Ligase 3	Involved in DNA repair - links breaks in DNA by forming two covalent phosphodiester bonds between the 3' hydroxyl end and the 5' phosphate end of two nucleotides.
Lig3/XRCC1	Ligase 3/X-ray repair complementing defective repair in Chinese hamster cells 1	Involved in base excision repair
LMPA	Low melting point agarose	Used for recovery of intact DNA fragments following electrophoresis
MGMT	Methylguanine DNA methyltransferase	Elicits direct DNA repair
MMR	Mismatch repair	Repairs mismatches induced during replication and by genotoxicants
MMS	Methyl methanesulfonte	An alkylating agent and genotoxicant
MNNG	*N*-methyl-*N*-nitro-*N*-nitroso-guanidine	A direct acting genotoxicant (radiomimetic)
mRNA	Messenger ribonucleic acid	An exact copy of the coding regions of DNA that is transported for translation.
M/R/N complex	Mre11/Rad50/NBS1 complex	Involved in homologous recombination and non-homologous end-joining
NAD(P)H	Nicotinamide adenine dinucleotide hydorgen (phosphate)	A coenzyme involved in redox reactions
NEIL-1	Nei endonuclease VIII-like 1	Involved in the removal of oxidised pyrimidines in the process of BER.
NER	Nucleotide excision repair	Main repair system for removing chemical or radiation induced bulky DNA lesions, and protein-DNA adducts.
NHEJ	Non-homologous end-joining	Double strand break repair
Ni	Nickel	
NMPA	Normal melting point agarose	
N-OH-2-AAF	N-hydroxy-2-acetyl amino fluorene	N-hydroxy metabolite of 2-AAF (genotoxic)
NQO	4-Nitroquinoline oxide	Direct acting genotoxicant
NTP	Nucleotide triphosphate	A nucleoside with three phosphates
O^6MeGua	O^6-methylguanine	Methylated guanine

OD	Optical density	A measure of the transmittance of an optical element for a given length and wavelength.
OECD	Organisation for Economic Cooperation and Development	Involved in the international harmonisation and validation of test methods to evaluate effects of chemicals.
OGG1	8-oxoguanine glycosylase 1	Cleaves 8-oxo-dG from DNA
OH radicals	Hydroxyl radicals	Highly reactive, short-lived species
OPT	*Ortho*-phthalaldehyde	A reagent for amino acid analysis
p73-like	Delta-n p63 (p73-like protein)	A nuclear transcription factor related to p53, and its role is to promote cell cycle arrest and/or apoptosis
PAHs	Poycyclic aromatic hydrocarbons	Environmental pollutant, metabolised to genotoxic products
PBO	Piperonyl butoxide	A potent cytochrome P450 inhibitor
PBS	Phosphate buffered saline	
PCA	Principal components analysis	A multivariate statistical technique that is used to explore and simplify complex data sets.
PCBs	Polychlorinated biphenyls	Persistent organic pollutants that contain 1-10 chlorine atoms attached to biphenyl.
PCNA	Proliferating cell nuclear antigen	Acts as a processivity factor for DNA polymerase delta in eukaryotes.
PCR	Polymerase Chain Reaction	Technique that enables the amplification of small amounts of DNA
PEC	Predicted environmental concentration	The expected concentration of a compound in the environment.
PER	Photoenzymatic repair	Mechanism for repairing photoproducts.
phr1-1 and phr1-2	Photoreactivation-deficient mutants	Mutants of *Chlamydomonas reinhardtii* deficient in photoreactivation.
PIC1/2/3	Pre-incision complex 1/2/3	An unwound section of DNA around the lesion formed in nucleotide excision repair.
PMT	Photomultiplier tube voltage	Adjusted to reduce saturation of pixels on microarray images.

PNEC	Predicted no-effect concentration	
PO_4-EDTA assay buffer	Phosphate-ethylenediaminetetraacetic acid assay buffer	Buffer used in an assay to determine levels of reduced glutathione in cells.
Pol β	Polymerase β	A DNA polymerase enzyme that catalyses the polymerisation of deoxyribonucleotides
Polδ/ε	Polymerase δ/ε	Fills the gap produced in nucleotide excision repair
Prxs	Peroxiredoxins	Oxidative stress protection
RAD23		Encodes a protein involved in nucleotide excision repair
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals	A European Union regulation concerning the registration, evaluation, authorisation and restriction of chemicals
RFC	Replication factor C	A five subunit protein complex required for DNA replication.
RFC/PCNA-Pol δ/ε complex	Replication Factor C/Proliferating cell nuclear antigen-polymerase delta/epsilon complex	Involved in base excision repair
RNA	Ribonucleic acid	
ROS	Reactive oxygen species	Formed by incomplete one-electron reduction of oxygen, and can cause oxidative stress
RPA	Replication protein A	Involved in nucleotide excision repair
RT	Room temperature	
RT-PCR	Real-time PCR	A technique to amplify and simultaneously quantify a DNA product.
S9	9000*g* supernatants	Microsomal fraction used to assess cytochrome P450 activity.
SCGE	Single-cell gel electrophoresis	Microgel electrophoresis technique that detects DNA damage individual cells (Comet assay).
SDS	Sodium dodecylsulfate	Anionic surfactant
SOD	Superoxide dismutase	An antioxidant enzyme
SSA	Single strand annealing	A process of homologous recombination.
SSC	Sodium chloride-sodium citrate	
SSH	Suppression Subtractive Hybridisation	A technique to develop subtracted cDNA libraries

T0	Time zero	Time at the start of the exposure
T24	24 hour exposure time	
T6	6 hour exposure time	
TBT	Tributyl tin	Moderately to highly persistent organic pollutant.
TBTCl	Tributyl tin chloride	Environmental pollutant
TCA	Trichloroacetic acid	
Three R's	Reduction, refinement and replacement	The reduction, refinement and replacement of animal experimentation.
TRCF	Transcription-repair coupling factor	Involved in recognition of DNA damage by proxy.
TREX1	3' repair exonuclease 1	A non-processive exonuclease
TRI	Toxics release inventory	A publicly available environmental protection agency database containing information on toxic chemical releases and waste management in the USA.
TrxPs	Thioredoxin peroxidases	Conserved proteins that protect against oxidative stress
UV	Ultraviolet radiation	Electromagnetic radiation with a wavelength in the range 10-100nm
VTG(1)	Vitellogenin (1)	The major egg yolk protein in many organisms
WRc	Water Research centre	Provides consultancy in water, waste and the environment
XP	Xeroderma pigmentosum	Photosensitivity syndrome characterised by a very high incidence of light-induced skin cancer
XPA/B/C/D/E/G	Xeroderma pigmentosum group A/B/C/D/E/G	Involved in nucleotide excision repair
XPC-TFIIH	Xeroderma Pigmentosum C-transcription factor II H	Involved in nucleotide excision repair
XPF-ERCC1	Xeroderma pigmentosum group F-excision repair cross-complementing rodent repair deficiency complementation group 1	Involved in nucleotide excision repair
XRCC1	X-ray cross complementing group 1	Involved in single strand break repair
XRCC4	X-ray cross complementing group 4	Involved in non-homologous end joining

Zn	Zinc	
β-HgS	Mercury sulfide	
βNF	β-naphthoflavone	Inducer of CYP1A

CHAPTER ONE:

GENERAL INTRODUCTION

1.1 Introduction

Aquatic pollution is commonplace, and chemicals that damage DNA are known environmental contaminants, often occurring in complex mixtures at unknown concentrations. These so called genotoxicants can be taken up by aquatic organisms where they can damage DNA, either directly or following bioactivation. Numerous methods already exist for determining the genotoxic potential of a chemical, but many have disadvantages associated with them, not least the lack of characterised and validated methods and test organisms. Current ecotoxicological testing strategies are generally focussed on apical and reproductive endpoints perceived to be of high ecological relevance, and little consideration is given to sublethal effects of contaminants. Moreover, many of the methods employed for assessing environmental genotoxicity have been developed in fish, and such studies are relatively time consuming, complex and expensive. In contrast, there is a drive to develop new methodologies, which are rapid, inexpensive and relatively simple, particularly for the assessment of complex mixtures. In this respect, invertebrates (e.g. mussels, *Daphnia*, echinoderms) and plants (e.g. unicellular algae, e.g. *Selenastrum capricornutum*, duckweed, *Lemna*) offer several advantages over vertebrates and also adhere to the spirit of the three Rs of animal testing (reduction, refinement and replacement).

Consequently, the focus of this project was to on gaining an understanding of the response of specific plant and invertebrate species that are important in regulatory ecotoxicology. In particular, studies were undertaken to investigate biotransformation capability (phase I and phase II metabolism) and DNA repair mechanisms (such as excision repair and photoreactivation) after exposure to well known genotoxins, with

the ultimate aim of assessing the relevance of such organisms for the development of small scale, high-throughput screens indicative of exposure to genotoxicants, especially those present in complex mixtures. In particular, the use of species already employed in regulatory environmental testing guidelines would facilitate the application of methods for assessing genotoxicity in existing ecotoxicological test methods. For these purposes, two species that are commonly used for environmental regulatory testing purposes, *Daphnia magna* (water flea) and *Chlamydamonas reinhardtii* (unicellular green alga) were investigated.

1.2 Pollution in the Aquatic Environment

Environmental pollution is a major concern in today's society, with the impact of wastewater discharge from agricultural, industrial and domestic sources representing a particular challenge. Most commonly, effluents are complex mixtures of chemicals that vary in both quality and quantity (Mitchell *et al*., 2002), with individual chemicals often being present at very low concentrations (Dizer *et al.*, 2002). The large number and variety of chemicals present in these mixtures, together with potential synergistic effects, poses great challenges for studying effluents with a view to identifying the types of chemicals present, and their individual/collective toxicity.

Many contaminants entering the aquatic environment are lipophilic and can be readily taken up by aquatic organisms or absorbed by particulate matter (Kirso and Irha, 1998). Examples of such compounds include polycyclic aromatic hydrocarbons (PAHs; Kirso and Irha, 1998), polychlorinated biphenyls (PCBs; Mantis *et al.,* 2005) and phthalates (Schwarzbauer and Heim, 2005). Therefore, the impact of aquatic

contaminants on ecosystems is dependent upon factors including uptake and biotransformation by different species, and effects can be seen at the cellular level, through to the individual, population and ultimately the ecosystem as a whole.

Research in this area has gained much attention in recent years, with particular interest surrounding the presence of genotoxic agents in the environment. Results from the U.S. Environmental Protection Agency's (EPAs) Toxic Release Inventory (TRI) indicate that known mutagenic and genotoxic chemicals are readily found in surface waters, and it has been reported that one third of the toxicants in discharges are rodent carcinogens (Claxton *et al.,* 1998). Moreover, 800 metric tonnes of chemicals released into surface waters are known to be class 1, 2A or 2B carcinogens as classified by the International Agency for Research on Cancer (IARC) (Ohe *et al*., 2004). It is therefore clear that the assessment of complex mixtures for their genotoxic potential is an extremely important consideration for environmental pollution monitoring, especially when considering the implication of these compounds in processes such as carcinogenesis, inherited disease and teratogenesis (Mitchelmore and Chipman, 1998). Consequently, it is important to develop reliable and effective methods for detecting endpoints indicative of exposure to genotoxicants, in particular using test methods that are simple, rapid and cost effective.

1.3 Current Testing Strategies in Ecotoxicology

The Existing Chemicals Regulation is used to assess chemicals released on to the market prior to 1982, and of these, only selected priority substances are risk assessed using available information of variable quantity and reliability (Ahlers *et al.,* 2008).

Following the introduction of the chemicals act in the early 1980s, chemicals that entered the market after 1981 had to have associated information regarding their potential human and environmental risks (Ahlers *et al.,* 2008). Ecotoxicological testing is designed to determine risks that chemicals pose to ecosystems, with population, community and ecosystem effects being of main concern. The data required from manufacturers is dependent upon the amount of chemical produced, and thresholds have been set with different and increasing testing requirements. Table 1.1 shows the principal ecotoxicity testing requirements. In addition to these, for pesticides, additional tests such as acute oral toxicity, bioaccumulation in fish and acute toxicity to honeybees and other beneficial organisms have to be conducted (Walker *et al.,* 2006).

The difference in assessment between new and existing substances, as well as other drawbacks such as inflexible test requirements, led to the proposition of a uniform regulatory strategy for both new and existing chemicals for the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), which came into force in June 2007.

1.3.1 REACH Proposals for Industrial Chemicals

REACH has been introduced to create one system for both new and existing substances and has several aims and objectives, two of the most important being protecting human health and the environment from the risks of chemicals, whilst promoting the reduction of animal testing. Through REACH, more data about chemicals will be available, enabling improved hazard and risk management, with the responsibility to perform risk assessments being passed to industry (REACH, 2006).

Table 1.1 The principal ecotoxicity testing requirements for chemicals, along with situations requiring modification, according to the amount (tonnes; t) produced each year (Walker et al., 2006).

Tonnes of chemical	**Ecotoxicity test(s) required**	**Situations requiring modification**
≥1t	Short-term toxicity test on *Daphnia*	Long-term toxicity test e.g. if water solubility <1mgl^{-1}
≥10t	Growth inhibition study on Algae	Not required for substances with low water solubility/large molecular size
	Short term toxicity test on fish	Long-term toxicity tests e.g. low water solubility
≥100t	Long-term toxicity test on *Daphnia*	Not required in some cases e.g. if substance is unlikely to cross biological membranes
	Long-term toxicity test on fish	Similar exclusions as for *Daphnia*
	Fish early life stage (FELS) toxicity test	Similar exclusions as for *Daphnia*
	Fish short-term toxicity test – embryo and sac fry stages	Similar exclusions as for *Daphnia*
	Fish juvenile growth tests	Similar exclusions as for *Daphnia*
	Short-term toxicity tests on earthworms	Not necessary if exposure to soil is unlikely
≥1000t	Long-term toxicity test on earthworms and other soil invertebrates	Not necessary if exposure to soil is unlikely
	Long-term toxicity test on sediment organisms	May be required if other tests indicate a need
	Long-term or reproductive toxicity test on birds	Not necessary if exposure to birds unlikely

Under REACH, a large number of existing substances, approximately 30000, must be registered by 2018, and this has raised concerns regarding the numbers of animals that will be required for experimentation to fulfil the requirements. REACH does have an obligation to adhere to the three Rs of animal testing: Reduction, Refinement and Replacement (Ahlers *et al.,* 2008), and an element of the system is to promote data sharing for vertebrate studies (REACH, 2007). However, to comply with the three Rs, it is vital that minimising and refining animal experimentation, as well as sourcing alternatives to current animal test species, is prioritised. Intelligent testing strategies are important in this context as they aim to reduce the numbers of fish and amphibians used, as well as substituting them for tests with non-vertebrates (Hutchinson, 2008).

1.3.2 Absence and Benefits of Sub-lethal Endpoints

The main focus of current ecotoxicity tests is on apical (whole organism) endpoints, such as lethality and reproduction, which can predict adverse effects of chemicals at the population level (Hutchinson *et al.,* 2006). Moreover, they reflect the 'Darwinian fitness' of an organism (growth, fecundity and fertility; Jha, 2004). For example, in daphnids the endpoints of immobilisation, to generate an EC_{50} at 48h (OECD Guideline 202) and reproductive output (OECD Guideline 211) are utilised, while for algae the endpoint is growth inhibition (OECD Guideline 201). Generally, using the information gathered from these tests, a predicted no-effect concentration (PNEC) is derived, which is designed to represent a 'safe' exposure level. This is then compared to the predicted environmental concentration (PEC) in order to assess risk (Ahlers *et al.,* 2008). The PEC/PNEC ratio has been widely used, but is crude and may overestimate the environmental risk of a chemical. Moreover, the chemicals may have other effects

not tested for under current strategies, such as genotoxicity, that may, for example, lead to population decline (Walker, 2006).

Apical endpoints have limitations in the information they provide, specifically in relation to modes of action of chemicals, the elucidation of which requires information on biomarker responses (Hutchinson *et al.,* 2006). A biomarker can broadly be defined as a change in a biological response (molecular, cellular and physiological) that can be related to the toxic effects of or exposure to environmental contaminants (Hutchinson *et al.,* 2006; van der Oost *et al.,* 2003). One area, with respect to the aquatic environment, where there has been a particular focus on biomarkers is endocrine disrupting chemicals. Research in this field elucidated vitellogenin as a biomarker for exposure to such chemicals, and the use of biomarkers has been invaluable in extrapolating laboratory and field data to the effects of endocrine disruptors on fish (Hutchinson *et al.,* 2006). Furthermore, van der Oost *et al.,* (2003) concluded that biomarkers have potential for inclusion in routine monitoring and environmental risk assessment following further work to fully understand and interpret biomarker responses. Handy *et al.,* (2003) also proposed the use of biomarkers for regulatory purposes, based on a weight of evidence approach, and also in detecting chronic exposure to pollutants. It is clear then that the continued development of biomarkers may prove important for the detection of exposure to compounds with other sub-lethal effects, such as genetic toxicity. In addition, the development of biomarkers in non-vertebrates may prove vital for REACH in limiting the number of vertebrate animals used in testing, and for adhering to the Water Framework Directive, which focuses on ecological protection of waters, thus requires information on the quality of the biological community (Kaika, 2003).

1.4 Genotoxicity

1.4.1 Mechanisms of Action of Genotoxic Agents

When screening for genotoxic contaminants in the environment it is important to understand the mechanisms by which these compounds can damage DNA. Genotoxic agents can be divided into four main categories:

(1) Chemicals that act directly on DNA

(2) Chemicals whose metabolites damage DNA

(3) Chemicals whose action causes secondary effects (e.g. reactive products of lipid peroxidation (malondialdehyde) that can damage DNA

(4) Chemicals that inhibit DNA synthesis and repair

Although these categories represent the four basic mechanisms of DNA damage, numerous chemicals act via a combination of these. Examples of genotoxic chemicals and their modes of action are given in Table 1.2.

The most common manifestations of DNA damage are single or double strand breaks, base modifications or deletions, and intra- or inter-strand DNA-DNA or DNA-protein cross-links (Lee and Steinert, 2003). The proportion of each type is dependent upon the genotoxic chemical and the context of the DNA sequence (Moustacchi, 2000). Methods exist to detect a range of damage to DNA, and identifying these modifications to DNA provides us with an indicator of either exposure to genotoxins, or the genotoxic potential of effluents. In the context of the aquatic environment, identifying the mechanisms of action of genotoxic agents is complicated by the fact that exposure of aquatic organisms to these chemicals usually occurs in the form of complex mixtures, thus the methods employed to detect these chemicals must be highly sensitive to DNA

Table 1.2 Classes and Examples of Genotoxic Chemicals. (CYPs – Cytochrome P450s; ROS – Reactive Oxygen Species.) Adapted from Lee and Steinert, (2003).

Group of Chemicals	**Example(s) of Chemical**
Direct acting (do not require metabolic activation).	Alkylating agents (e.g. N-methyl-N-nitro-N-nitroso-guanidine (MNNG), N-methyl-N-nitrosourea, ethyl methanesulfonate, methyl methanesulfonte; MMS). Hydrogen peroxide 4-amino biphenyl
Indirect acting (require metabolic activation, often CYP-mediated).	Polycyclic aromatic hydrocarbons (PAHs) e.g. Benzo[a]pyrene (metabolised to arene oxide, a very reactive electrophile). Nitropyrenes
Indirect acting (stimulate metabolic generation of ROS).	Quinones Aromatic nitro compounds (e.g. Nitrofurantoin - undergoes redox cycling). Certain metals (e.g. Chromium).
Inhibit DNA synthesis	Hydroxyurea (Inhibits the conversion of ribonucleotides to deoxyribonucleotides; Young *et al.,* 1967) Cytosine arabinoside (Incorporated into DNA leading to chain termination; Momparler, 2006)

	Ethidium bromide (Intercalating agent) 5-fluorouracil (Metabolites inhibit thymidylate synthase resulting in a nucleotide pool imbalance and act as false bases that are incorporated into DNA; Matuo *et al.,* 2009) 2,4-diamino-5-(3',4'-dichlorophenyl)-6-methyl pyrimidine
Inhibit DNA repair	Aphidicolin (Inhibits DNA polymerase; DiGuiseppe *et al.,* 1990) Novobiocin (Inhibits DNA topoisomerases and excision repair of photo-induced DNA damage; Downes *et al.,* 1985) Several Heavy Metals (e.g. Arsenic – inhibits DNA ligase; Colognato *et al.,* 2007).
Multiple mechanisms of action	Ionic mercury (Binds directly to DNA to produce strand breaks and DNA-DNA crosslinks; inhibits DNA repair). Chromium (Directly damages DNA producing DNA strand breaks and DNA-protein crosslinks; can induce cellular production of ROS). Menadione (Induces necrosis by rapid production of ROS; metabolised to semiquinones which bind to DNA causing strand damage and cross-linking).

damage. Furthermore, numerous pro-genotoxicants exist, thus there is a need for metabolic activation of these compounds to be considered in order to truly assess their potential to damage DNA. In addition, a number of factors can have a large effect on the activation and detoxification of these chemicals such as seasonal variation in diet, environmental conditions and hormonal status. Consequently, assessing the exposure of aquatic organisms to genotoxins is especially challenging (Mitchelmore and Chipman, 1998).

1.4.2 Role of Genotoxicity in Carcinogenesis and Inherited Disease

The importance of having a good understanding of exposure levels of genotoxicants in the environment is highlighted by the implication of these agents in processes including carcinogenesis, teratogenicity, and inherited disease via mutations in germ-cells (Mitchelmore and Chipman, 1998).

Carcinogenesis is a complex, multistage process that has been broken down into at least three steps: initiation, promotion and progression (Weinberg, 1989). Initiation results from exposure of normal cells to genotoxic carcinogens, causing damage to DNA, and producing an 'initiated cell'. This cell has a proliferative advantage over normal cells and thus undergoes greater proliferation, increasing the potential for additional DNA damage, such as mutations, which accumulate as the population of cells expands. This is the second stage, tumour promotion (Loeb and Harris, 2008). The third stage, progression, is characterised by the development of irreversible, aneuploid malignant neoplasms, which occur in a variety of cancers including leukemia, lymphoma, multistage hepatocarcinogenesis, and are related to increased growth rate, biochemical changes within the malignant cell, invasiveness and the ability to metastasise (Pitot and

Dragan, 1991). In addition to these three distinct stages, it is noteworthy that a number of biological processes are de-regulated in cancer (e.g. epigenetic factors (methylation), genetic factors (loss of heterozygosity)), and endogenous mechanisms of cancer exist, including the production of free-radicals that can damage DNA (Halliwell and Aruoma, 1991), errors in replication (Loeb *et al.,* 1974), and the depurination of DNA, producing apurinic sites, which has been shown to occur at a physiological significant rate *in vivo* (Lindahl and Nyberg, 1972).

Mutagenic compounds can also induce heritable defects, by altering DNA in germ cells. These modifications can be 'microlesions' such as small deletions, splicing mutations, or single base pair missense or nonsense mutations, or 'gross lesions' including complex rearrangements and gross insertions, deletions or duplications (Elespuru and Sankaranarayanan, 2007).

The use of cancer and tumorigenesis as endpoints for exposure to genotoxic chemicals is common, but it is not often applicable to lower organisms (Kurelec, 1993), as although tumours have been reported in lower organisms, including molluscs and echinoderms, which are recorded in the Registry of Tumours in Lower Animals (RTLA; Harshbarger, 1974) there remains a lack of study in this area. Thus DNA and chromosomal aberrations observed after exposure to genotoxic agents need to be correlated with alternative endpoints.

1.4.3 Genotoxic Disease Syndrome

Exposure to genotoxicants can result, not only in tumours, but in DNA adducts, gene mutations, chromosomal aberrations and sister chromatid exchanges. There are a

variety of ways the mutation can manifest, including impairments in enzyme function, general metabolism, immunoresponse and reproduction, altered protein turnover, protein adducts, inhibition of growth and faster ageing. These consequences of a mutagenic event, when taken together, have been given the name the Genotoxic Disease Syndrome (GDS), as proposed by Kurelec (1993). GDS represents the concept that genotoxic chemicals can cause a variety of irreversible toxicities, and recognition of GDS may be of great importance in environmental genotoxic risk assessment. GDS more appropriately describes the biological and ecological risks from 'carcinogens' in the environment, and identifying the endpoints associated with it should improve the perception of risks, resulting in more suitable regulatory measures to compounds that cause symptoms of genetic disease (Kurelec, 1993).

1.5 Genotoxicity Testing in Aquatic Organisms

As previously stated, detection of genotoxicants in complex mixtures, for example industrial process effluents, requires assays that are rapid, sensitive and inexpensive (White *et al*., 1996). Generally, laboratory based genotoxicity assays are time consuming, and relatively expensive, with current higher animal based systems (e.g. fish) requiring extensive exposure systems, making them impractical for use assessing effluents or application in the field. Numerous short-term bioassays have been developed for the detection of genotoxic chemicals, using mutation as an endpoint, such as the Ames test, the SOS Chromotest in *Escherichia coli,* and the *Salmonella umu*-test (Oda *et al.,* 2004, Ohe *et al.,* 2004). Advantages of these assays include the routine use of the SOS Chromotest and *umu*-test for monitoring water samples, same day results, minimal

advance preparation, and the development of microplate versions allowing high-throughput screening (Ohe *et al*., 2004; Oda *et al*., 2004). There are, however, some disadvantages, most obviously that they are all *in vitro*, cell based mutagenicity tests. Specifically, with regards to the Ames test, despite being the most widely used *in vitro* test for the detection of gene mutations (Parry, 2000) and its reported applicability to both pharmaceutical and environmental samples (Oda et al., 2004) including surface waters (Ohe et al., 2004), there are some reservations when using this assay as an initial screening test, particularly when the test material is industrial effluent of unknown composition. Firstly, this assay uses a strain of *Salmonella typhimurium* that has a mutation that inhibits the synthesis of Histidine (His), such that exposure to a genotoxic chemical induces a reverse mutation allowing the synthesis of His and growth of the bacteria. The presence of His in the test sample would produce false positive results and affect the interpretation of the findings, and when testing complex mixtures of unknown composition, contamination by His is a possibility. In addition, the Ames test does not detect all mutagenic chemicals. Some clastogens such as inorganic arsenic compounds do not give a positive result in the Ames test (Parry, 2000), nor do compounds that induce DNA or chromosomal damage without mutagenesis. In addition, *in vitro* mutagenicity test systems do not offer the biological complexity associated with testing using integrated organ systems. This is only achievable using *in vivo* test systems such as those employing vertebrates, invertebrates and plants. Taking into account the limitations of existing genotoxicity screens, the current study was concerned with gaining a greater understanding of the genotoxic response of some commonly used aquatic invertebrate and plant ecotoxicological test species, with a view to probing them as potential test organisms for the assessment of the *in vivo* genotoxic effects of

environmental contaminants. This work also served to provide information on how representative of higher organisms these species groups are. Specifically, little information is available on biotransformation capability and this was also a major focus of the current work.

1.6 Alternative Testing Methodologies: Small Scale Screens Using Unicellular Algae and Invertebrates

1.6.1 Unicellular Alage as a Test Species

Algae are commonly used as bio-indicators in the environmental monitoring of water quality (Lin *et al*., 2005; Sauser *et al*., 1997), with microalgae being used extensively in ecotoxicity studies in recent years (Chen *et al*., 1997). They have great diversity in fresh and salt waters (Warshawsky *et al*., 1995) and are the main primary producers, thus they are ecologically important in the aquatic food chain (Geis *et al*., 2000). Their close contact with the aquatic environment means that they are exposed to a large number of contaminants, and many unicellular algae have a large surface area, which is a significant factor when considering the uptake of chemicals (Sauser *et al*., 1998). Moreover, a number of test substances have shown greater toxicity to plants than animals, including metals such as chromium (Cr) and cadmium (Cd), industrial effluents, such as those from oil refineries and paper mills, and organic compounds including nitrobenzene, phenol and dinitrotoluene (Lewis, 1995).

Chlamydomonas reinhardtii is a freshwater unicellular green alga (Figure 1.1), whose cells are spherical or ellipsodical, approximately 10μm in length, with two equal length flagella at the anterior end of the cell (Merchant *et al.,* 2007). The cell wall is

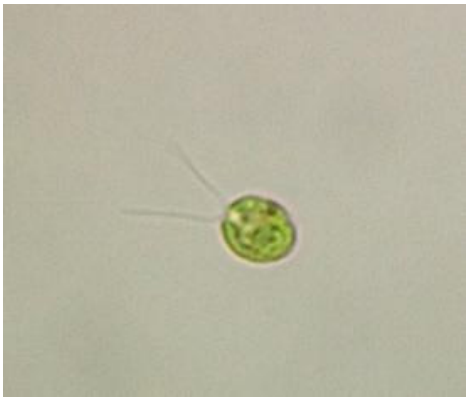

Figure 1.1 Image of a *Chlamydomonas reinhardtii* cell from culture maintained in our lab

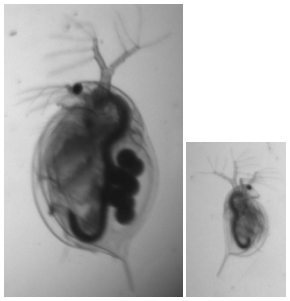

Figure 1.2 Image of a female adult *Daphnia magna* (left) and neonate daphnid (<24 hours old, right) from culture maintained in our lab

multilayered, comprising an insoluble hydroxyproline (Hyp)-rich glycoprotein framework and several Hyp-containing glycoproteins that are chaotrope-soluble (Voigt and Frank, 2003). *C. reinhardtii* is the species most commonly used in ecotoxicology laboratories due to its rapid growth and short generation time. *C. reinhardtii* has been used as a model for plant cell biology and physiology for many years, as a model for research into a wide range of processes including photosynthesis due to its heterotrophic ability to use alternative carbon sources for nutrition (Misumi *et al.,* 2008), metal homeostasis (Hanikenne *et al.,* 2005), DNA repair (Vlcek *et al.,* 2008) and flagella research (e.g. Pan and Snell, 2005).

More recently the genome of *C. reinhardtii* has been fully sequenced (Merchant *et al.,* 2007). It is 1 x 10^8 base pairs and is haploid with 16 or more chromosomes (Harris, 2001) and only one copy of each gene, thus making it an excellent system in which to study mutations (Entrez genome project: *Chlamydomonas reinhardtii*). Studies have been published in which *C. reinhardtii* has been used as a model to understand cell cycle regulation (Bisova *et al.,* 2005), gene expression profiling following Cd exposure (Simon *et al.,* 2008), as well as microarray analysis to investigate copper exposure on gene expression profiles (Jamers *et al.,* 2006). Algae present an advantage when studying the effects of genotoxic agents as they are rapidly dividing and have all essential functions that contribute to DNA damage by these compounds in one cell, so a sum of processes that reflect the effects of genotoxicants in algae can be observed (Erbes *et al*., 1997). Despite this, there are still relatively few published studies that have investigated genotoxic endpoints in unicellular algae.

1.6.2 *Daphnia magna* as a Test Species

Daphnia magna are small, freshwater, herbivorous crustaceans of the order Cladocera (Figure 1.2), and are an important, environmentally sensitive member of the aquatic food chain. They are one of the dominant consumers of algae and are themselves predated on by both fish and invertebrates (Baudo, 1987; Dodson and Hanazato, 1995; Tatarazako and Oda, 2007). This critical role as an intermediate stage in the food-chain means that any effect on *D. magna* could result in community or ecosystem responses (Flaherty and Dodson, 2005).

Under favourable conditions, the *D. magna* lifecycle is via cyclic parthenogenesis, where females reproduce asexually without males to produce broods of genetically identical female offspring, thus providing rapid population expansion (Figure 1.3; Olmstead and Leblanc, 2002; Tatarazako and Oda, 2007). Environmental stressors, such as decreased food, shortened day length and high population density, can induce sexual reproduction. Males develop and sexual reproduction occurs following which resting eggs are produced. These are surrounded by a protective ephippium that enables survival in harsh, unfavourable and extreme conditions, such as drought or freezing temperatures (Olmstead and Leblanc, 2002; 2003), thus allowing the survival of the population at times of environmental stress (Oda *et al.,* 2005).

D. magna are a clonal species and therefore ideal for studies looking at genetic responses to environmental stressors, but a lack of genomic tools had limited its use for genetic studies. In response to this, the *Daphnia* Genomics Consortium (DGC) was established to develop, amongst other things, a *Daphnia* genomic toolbox, and *D. pulex,* whose genome was recently fully sequenced (JGI Genome Portal), is reported to have one of the best characterised genomes (*Daphnia* Genomics Consortium). Following

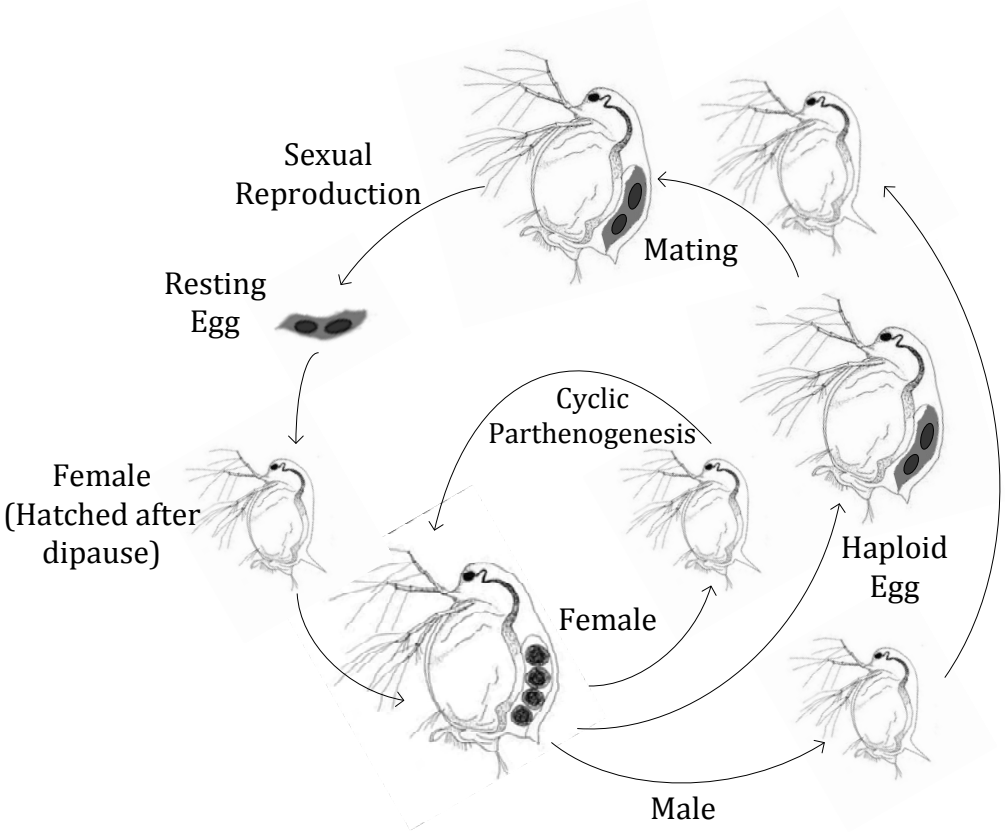

Figure 1.3 Diagram of parthenogenic lifecycle of *Daphnia magna.* Under ideal conditions female daphnids produce genetically identical females asexually. At times of stress males and sexual females develop and sexual reproduction produces resting eggs that hatch once conditions are favourable. Adapted from Ebert, (2005).

completion of the *D. pulex* sequencing project, work is now underway to sequence the *D. magna* genome (wFleaBase; *Daphnia* Genomics Consortium).

It is for these aforementioned reasons, as well as their short lifecycle, small size and ease of culture that *D. magna* are routinely used in ecotoxicity testing. In addition they are an OECD recommended species for the testing of chemicals (OECD Guideline 202).

1.7 The Importance of Understanding Xenobiotic Metabolism

Animals have a number of enzymes capable of undertaking biotransformation, which are concentrated in the liver of vertebrates or the food processing tissues of invertebrates (e.g. the digestive gland of molluscs). These enzymes primarily convert hydrophobic, lipophilic, organic molecules into water-soluble metabolites that can be excreted. Phase I metabolism (oxidation, reduction and peroxidation, mainly by the Cytochrome P450 (CYP450) super family of isozymes) involves the introduction or modification of a functional group including –OH, -COOH, -NO_2 into the compound, to which a large polar moiety such as glutathione, glucuronide or sulphate is added by enzymes during phase II metabolism (Livingstone, 1998).

Biotransformation is known to be a key modulator of the toxicity and bioaccumulation of xenobiotics, since these reactions can, for example, produce metabolites that have different fates to the parent compound, alter elimination rates, or affect the persistence of the chemical in the organism. Moreover, these modifications may significantly affect the toxicity of the compound, either through detoxification, or the production of more toxic metabolites (Kleinow *et al.,* 1987). For example, a large number of pro-genotoxicants exist, which require metabolic activation to a genotoxic

product. These include: PAHs such as benzo[a]pyrene (BaP), and azo dyes, for example chrysoidine. Consequently, the biotransformation capability of a test organism is an important consideration when assessing the toxicity of chemicals in the environment.

Understanding the similarities and differences in the capacity to biotransform between organisms is vital for the design of toxicity tests, modelling the fate of chemicals in ecosystems, and developing biomarkers (Livingstone, 1998). Biotransformation is controlled by a number of factors including the properties of a chemical, as well as species, age and physiological state of the organism, and despite the potential for the capacity to biotransform to have major implications on test results it is often not quantified in ecotoxicology studies (Akkanen and Kukkonen, 2003). It is therefore vital to understand the ability of different aquatic species to metabolise xenobiotics (Chipman and Marsh, 1991), but the capacity to carry out biotransformation is poorly understood for many aquatic organisms. Currently only limited information is available regarding metabolic systems in both unicellular algae and *D. magna*, and the responsiveness of these organisms to genotoxins requiring metabolic activation.

1.7.1 The Role of Cytochrome P450s in Metabolism

1.7.1.1 The Mechanism of Action of Cytochrome P450s

Cytochrome P450s (CYPs) are a haem-containing enzyme superfamily that are one of the most important groups involved in catalysing Phase I reactions of both endogenous (fatty acids, prostaglandins, steroids) and exogenous (carcinogens, drugs, chemical pollutants) substrates (Buhler and Wang-Buhler, 1998). The overall catalysis results from the transfer of an oxygen atom from O_2 into the substrate, and the enzyme

receives electrons from NADPH via electron transfer proteins that are often coupled to CYPs inside the cell membrane (Figure 1.4; Mansuy, 1998).

$$RH + O_2 + 2e^- + 2H^+ (NADPH) \longrightarrow ROH + H_2O$$

There are at least four distinct steps involved in the reaction:

i) Binding of the substrate with the enzyme (introduces a conformational change)

ii) Electron donation (from NADPH (via the reductase) reduces the iron in the enzyme-substrate complex from the ferric to the ferrous state)

iii) Addition of oxygen and rearrangement

iv) Donation of a second electron from NADPH, the complex rearranges, one atom of oxygen binds to the substrate and the product is released (Timbrell, 2004).

1.7.1.2 The Cytochrome P450 Superfamily

CYP genes occur in almost all living systems, having been identified in bacteria, yeast, fungi, plants and vertebrate and invertebrate animals (Buhler and Wang-Buhler, 1998; Stegeman and Livingstone, 1998). Following phylogenetic analysis it is thought that all CYPs diversified from one common ancestor present prior to the evolution of eukaryotes (Nelson and Strobel, 1987). CYPs play a key role in a range of processes, from gene regulation and endocrinology, to carcinogenesis and environmental toxicology (Stegeman and Livingstone, 1998), thus it is important to understand their existence and roles in aquatic organisms.

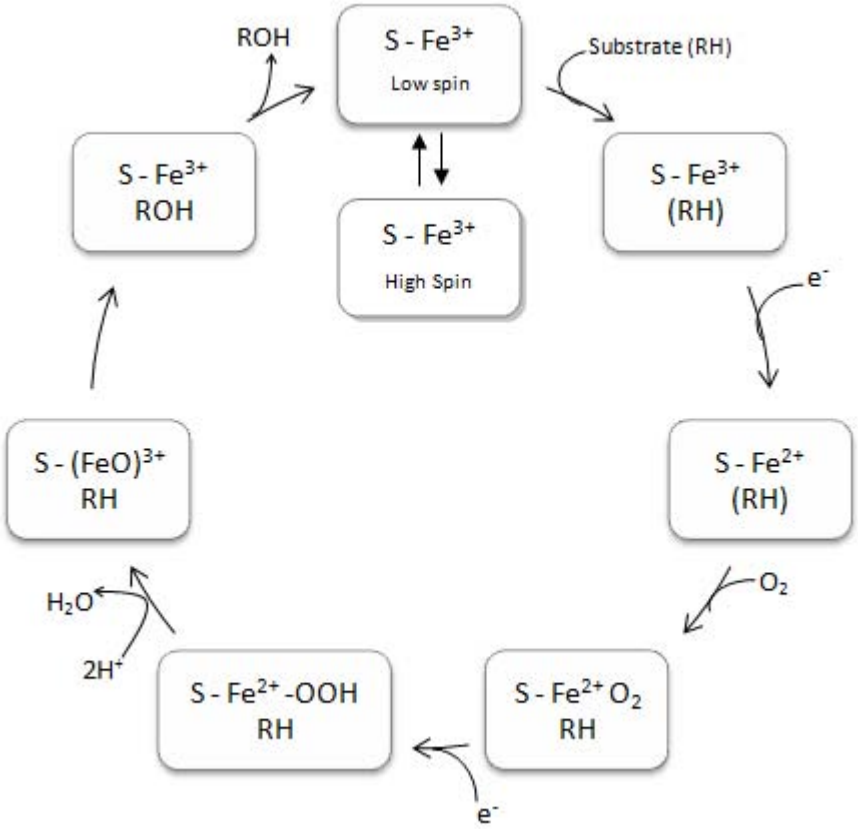

Figure 1.4 Catalytic cycle of cytochrome P450s (CYPs). Adapted from Klaassen, (2001) and Mansuy, (1998).

Mammals have 18 CYP gene families (Nebert and Dalton, 2006) that are divided into subfamilies, based on sequence identity. CYP genes are assigned to a family, such as CYP1 if they have 40% or more overall sequence identity, and separation into subfamilies, CYP1A1-CYP1An, requires at least 55% sequence identity (Snyder, 2000). For a lot of the subfamilies of CYP2, CYP3 and CYP4 there is a large variation in the number of members for different mammals. Extrapolation of genes in these families between rodents and humans is not possible due to species differences and the lack of orthologues for the most part, leading to potentially very different substrate specificities for enzymes of the same name (Nebert and Dalton, 2006).

Members of the CYP1 to CYP4 families have overlapping substrate specificities, and it is these enzymes that are most directly associated with environmentally-induced cancers since they are involved primarily in the metabolism of xenobiotics. Moreover, a number of allelic variants of these genes exist, which can alter metabolism leading to an intermediate or poor metabolism phenotype when compared to the 'normal' or efficient metabolism phenotype (Nebert and Dalton, 2006). An ultrarapid metabolism phenotype has also been observed for debrisoquine, which is metabolised by CYP2D6, where a 12-fold amplified variant was discovered by Johansson *et al.* (1993). Variants of genes involved in the biotransformation of xenobiotics will affect the detoxication and activation of compounds, potentially affecting their toxicity or genotoxicity.

Mammalian CYPs and their involvement in the metabolism of both endogenous and exogenous substrates are well characterised, but although there are reports of CYPs in aquatic organisms, less is known regarding their involvement in xenobiotic metabolism for some species.

1.7.1.3 Identification of CYPs in Aquatic Organisms

Multiple CYP forms have been identified in numerous freshwater and marine fish, primarily in the liver, but also in other tissues at lower concentrations (Buhler and Wang-Buhler, 1998). Moving through the ecosystem, reports have been made of CYP-like activity in marine invertebrates, including arthropods (crustaceans), echinoderms, annelids (polychaetes) and molluscs (James and Boyle, 1998). For example, tributyl tin (TBT) has been shown to be metabolised by CYPs in the hepatopancreas of crabs, *Callinectes sapidus* and *Libinia emarginata*, to β- and δ-hydroxybutyldibutyltin metabolites (Oberdorster *et al.,* 1998). Attention has also focussed on aquatic plants such as some unicellular algae, and CYPs have been identified in species including *C. reinhardtii* and *Skeletonema cosatum* (Nelson, 2006). Clearly then, CYPs also play a role in metabolic processes in aquatic organisms. Characterisation of the CYPs has revealed roles in endogenous pathways, such as ergosterol biosynthesis in plants (Nelson, 2006) and arachadonic acid metabolism in invertebrates (Peters *et al.,* 1998), but confusion surrounds the role of these enzymes in xenobiotic metabolism.

1.7.1.4 The Importance of Isoenzymes of CYPs in the Metabolism of Xenobiotics

Members of the CYP1, CYP2 and CYP3 subfamilies are of key importance in the biotransformation of xenobiotics (Timbrell, 2004). In the context of the marine environment, CYP1A1 is the best-studied member of the CYP superfamily predominantly as it is inducible by dioxins, PAHs and PCBs, all of which are environmentally important contaminants. Importantly, these enzymes are also implicated in the bioactivation of numerous genotoxins including BaP (CYP1A1), 2-AAF (CYP1A2). Forms that are identical or related to members of these groups have been

reported in fish, including Rainbow Trout (Buhler and Wang-Buhler, 1998) and the Atlantic Salmon *Salvo salar* (Olsvik *et al.,* 2007), and in invertebrates such as molluscs (Livingstone, 1998). Further evidence that isozymes of these families are present in certain invertebrates include the reported metabolism of PCBs by crustaceans, suggesting that CYP1 and CYP2-like proteins are present (Snyder, 2000), while unique forms have been identified in some organisms, such as CYP2L in the Caribbean spiny lobster *P. Argus* and CYP10 in the snail *Lymnaea stagnalis* (Livingstone, 1998). Metabolism of compounds including BaP has also been reported in both marine and freshwater algal species (Kirso and Irha, 1998), implying that CYP1A-like enzymes are present in these organisms. The presence of CYP1 and CYP2-like proteins in invertebrates and algae is suggestive of the ability of these organisms to bioactivate xenobiotics, which is of great importance when investigating the potential of such species to be used as sentinel species for assessing the impact of genotoxins.

More detail regarding current knowledge of CYPs in *C. reinhardtii* and *D. magna* is presented in Chapter 4, Section 4.1.

1.8 DNA Repair Mechanisms

Damage to DNA can cause perturbation of the steady-state equilibrium within cells, and lead to activation or an increase in pathways that regulate cell division and growth, or coordinate DNA replication. Importantly when assessing the effect of genotoxic contaminants, one must consider repair mechanisms. There are four types of pathway known to be activated in response to DNA damage in mammalian cells: DNA repair, DNA damage checkpoints, transcription, and apoptosis (Sancar *et al.,* 2004). DNA

repair first requires recognition of the DNA damage, which can occur via one of several different mechanisms, as outlined in Table 1.3 (Sancar *et al.,* 2004).

There are five mechanisms of DNA repair: direct repair, base excision repair (BER), NER, double-strand break repair, and repair of inter-strand cross links.

Direct repair is elicited via two enzymes: photolyase and methylguanine DNA methyltransferase (MGMT). Photolyase occurs in many, but not all species and exists in two forms, one that repairs 6-4 photoproducts (using photons of blue light as energy) and another that repairs UV-induced cyclobutane pyrimidine dimers (CPDs). The mechanism of action of this protein is shown in Figure 1.5. MGMT is nearly universally distributed and its role is to remove the O^6-methyl group from O^6-methylguanine (O^6-MeGua). This protein has been linked with cancer, following studies that showed mice that lacked this enzyme had a heightened susceptibility for tumours (Sancar *et al.,* 2004).

The second process, BER, involves the removal of damaged bases from DNA, and the mechanism in mammalian cells is outlined in Figure 1.6 (Sancar *et al.,* 2004).

NER is the main repair system for removing chemical or radiation induced bulky DNA lesions, and protein-DNA adducts (Figure 1.7), and defects in this system lead to xeroderma pigmentosum (XP), a photosensitivity syndrome characterised by a very high incidence of light-induced skin cancer (Sancar *et al.,* 2004). In addition, damaged DNA binding protein (DDB) plays a role in NER, although it is not required for repair. This protein has the highest affinity for damaged DNA of all identified damaged-DNA binding proteins to date, and it is known that DDB interacts with a number of cellular and viral proteins involved in, amongst other processes, transcriptional regulation. The current consensus is that this protein may be involved in a general cellular response to DNA

Table 1.3 Mechanisms for the recognition of DNA damage in eukaryotes.

Damage Recognition	**Protein/Repair Pathway**	**Mechanism**
Direct recognition	Photolyase; DNA glycosylase	Complimentarily between damage and associated protein
Multi-step recognition	Xeroderma pigmentosum C (XPC) in mammalian nucleotide excision repair (NER); Replication Factor C (RFC) in eukaryotic DNA replication	XPC; proliferating cell nuclear antigen (PCNA) loaded onto DNA by RFC, which then dissociates, enabling action of PCNA as a DNA polymerase clamp
Recognition by proxy	Transcription-repair coupling factor (TRCF)	Binds stalled RNA polymerase, recruits DNA damage-recognition complex, facilitates dissociation of UvrA2 from the complex accelerating the formation of the UvrB1-DNA complex
Recognition of repair intermediates	DNA excision repair	Repair involves excision of damaged base producing intermediates such as single-strand breaks, which are recognised by other systems for repair

damage. Mutations in the DDB2 gene result in a mild form of xeroderma pigmentosum E (XPE; Sancar *et al.,* 2004).

Double strand breaks (DSBs), which can be produced by reactive oxygen species (ROS) or chemicals that produce ROS (e.g. radiomimetic chemicals, which produce DSBs through ROS or energy deposition), ionising radiation (low and high linear energy transfer (LET)), or as a result of V(D)J recombination are repaired by one of two processes: homologous recombination (HR) or non-homologous end-joining (NHEJ; Figure 1.8). Genetic information has revealed that HR is used for the repair of collapsed replication forks, while NHEJ is vital for V(D)J recombination, and is also thought to be the primary repair pathway for DSBs induced by ionising radiation and radiomimetic agents (Sancar *et al.,* 2004).

Ultimately, the different repair pathways may share components such as enzymes, and more than one pathway may be responsible for repairing particular damages. For example, NER and cross-link repair are thought to share the XPF-ERCC1 complex, while O^6MeGua can be repaired by direct repair, BER or NER. This may lead to competition resulting in hindered function, or cooperation to remove the lesion (Sancar *et al.,* 2004).

1.8.1 Repair Mechanisms Identified in *C. reinhardtii*

In *C. reinhardtii*, direct-repair of DNA is well established, and photoreactivation of UV-induced damage is the best characterised repair pathway. Photolyase activity has been demonstrated in both the chloroplast and nucleus, and two allelic photoreactivation-deficient mutants have been isolated, *phr1-1* and *phr1-2* (Vlcek *et al.,* 2008). Petersen *et al.* (1999) isolated the *PHR2* gene, and further characterisation

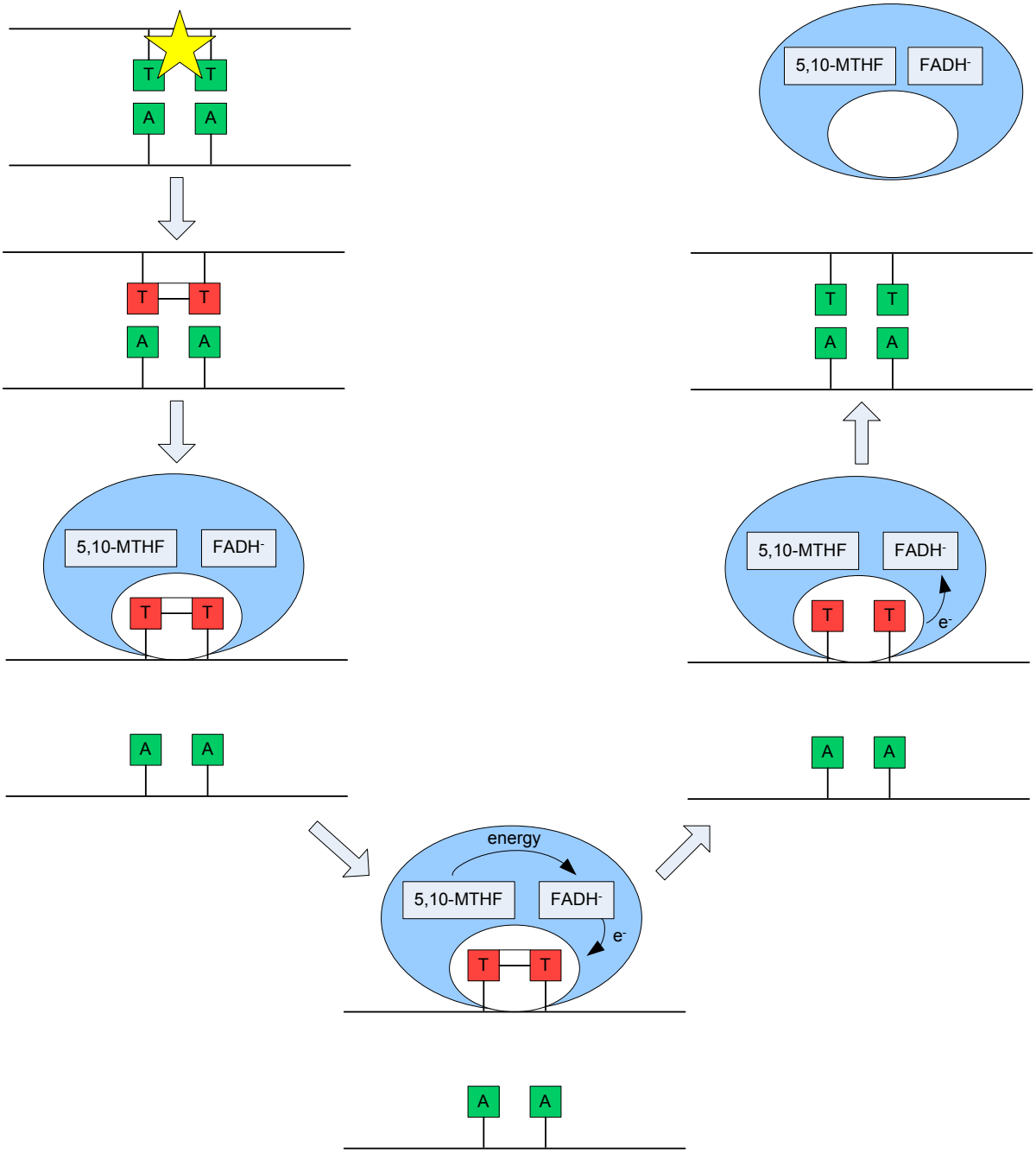

Figure 1.5 The process of photoreactivation. Photolyase binds to the portion of DNA that contains a pyrimidine dimer and flips the dimer into the active site. Methenyltetrahydrofolate (5,10-MTHF) absorbs a photon of light and the excitation energy is transferred to the catalytic cofactor $FADH^-$. An electron is transferred from $FADH^-$ in its excited state to the pyrimidine dimer, which splits, and the electron is returned to regenerate $FADH^-$. Photolyase then dissociates from the repaired DNA (Sancar *et al.,* 2004).

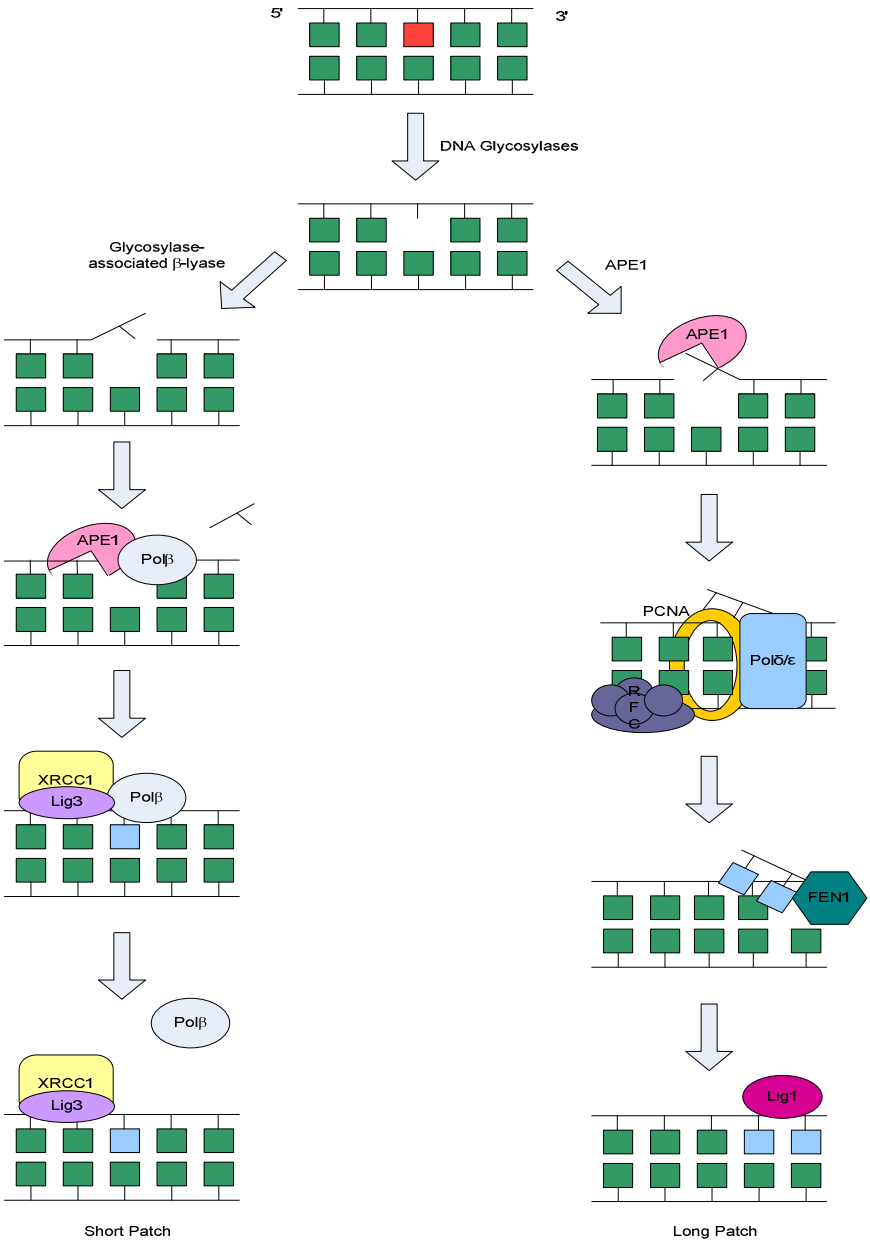

Figure 1.6 The process of Base Excision Repair (BER). DNA glycosylase removes the damaged base producing an apurinic/apyrimidinic (AP) site. AP endonuclease (APE1) cleaves the 5' bond, recruits polymerase β (Pol β) to fill the gap, which is then ligated by the Ligase 3/X-ray repair complementing defective repair in Chinese hamster cells 1 (Lig3/XRCC1) complex. If the AP site is 2-10 nucleotides long (long patch), which can be produced via hydrolytic glycosylases or spontaneous hydrolysis, the site is repaired by the replication factor C/proliferating cell nuclear antigen-polymerase δ/ε (RFC/PCNA-Pol δ/ε) complex producing a flap structure that is cleaved by flap structure-specific endonuclease 1 (FEN1), and ligation is carried out by Ligase 1 (Sancar *et al.* 2004).

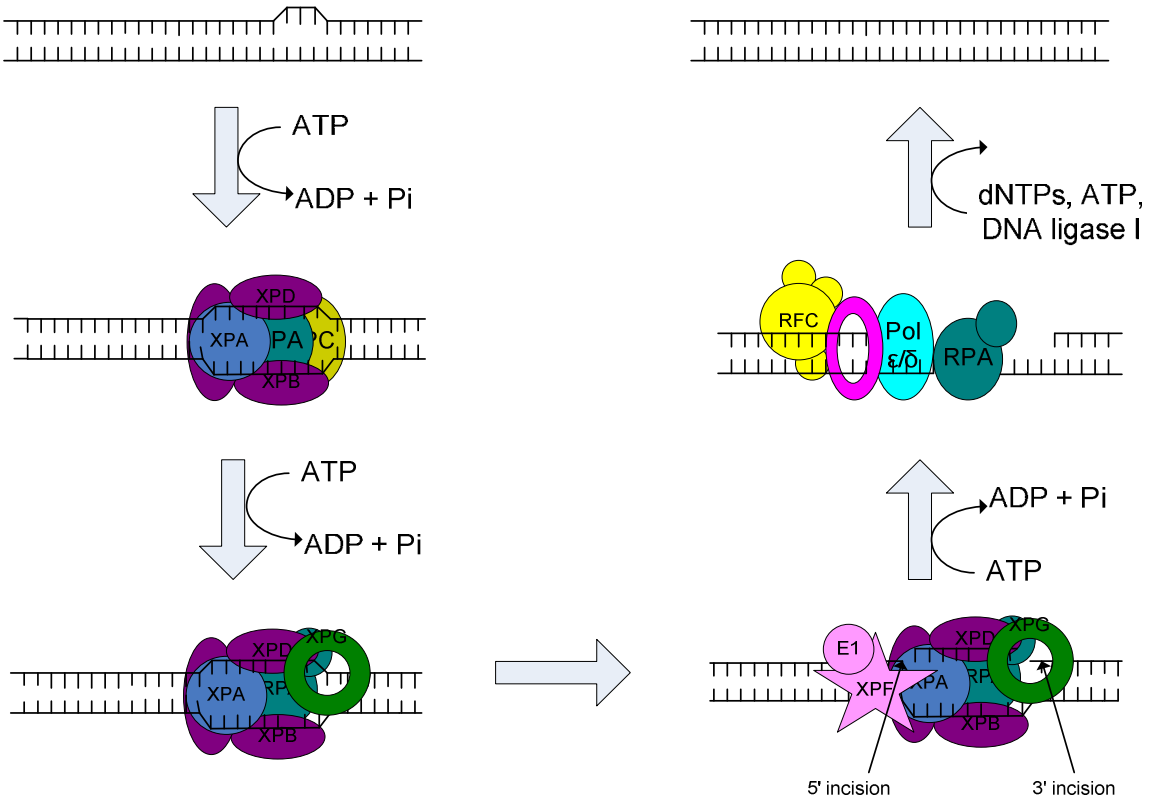

Figure 1.7 The process of Nucleotide Excision Repair (NER) in human cells. The DNA repair factors replication protein A (RPA), xeroderma pigmentosum (XP) A (XPA), and XPC-transcription factor II H (XPC-TFIIH) recognise DNA damage and assemble at the site in a random order where they form a complex. If the site contains a lesion the helicases XPB and XPD hydrolyse ATP, which causes unwinding of duplex by approximately 25 basepairs (bp) around the lesion to form a stable pre-incision complex 1 (PIC1). XPC is replaced by XPG to form a more stable PIC2. XPF-excision repair cross-complementing rodent repair deficiency complementation group 1 (XPF-ERCC1) is then recruited to the damage site to form PIC3. The damaged strand is incised by XPG 3' from the damage site at the 6th phosphodiester bond (± 3), and by XPF-ERCC1 5' to the damage site at the 20th phosphodiester bond (± 5). An oligomer 24-32bp long is released and the gap is filled by polymerase δ/ε (Polδ/ε) aided by the replication accessory proteins proliferating cell nuclear antigen (PCNA) and replication factor C (RFC; Sancar *et al.,* 2004).

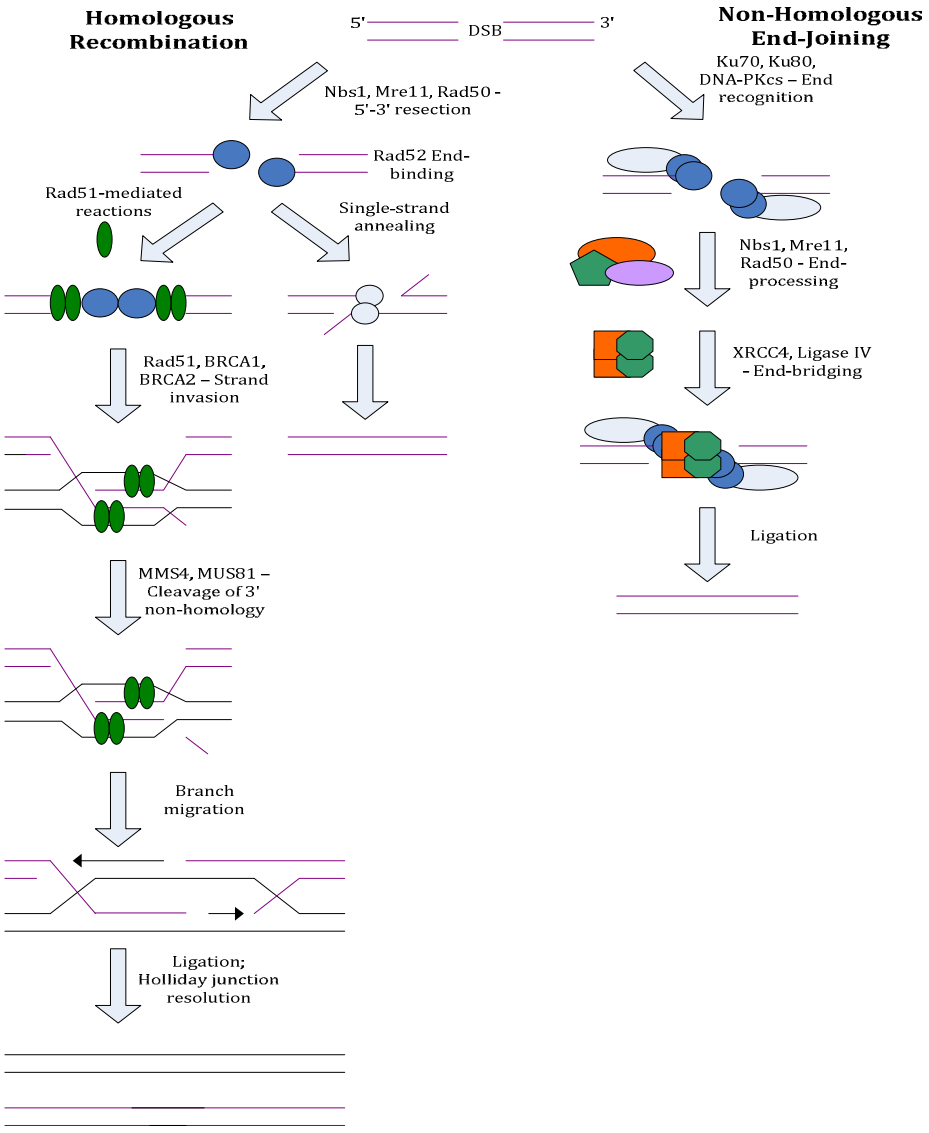

Figure 1.8 Double-strand break (DSB) in eukaryotes: homologous recombination (HR) or non-homologous end-joining; NHEJ. The balance between these varies with cell cycle: HR is upregulated in S and G2 phase while NHEJ is predominant in G1 phase (Shrivastav *et al.,* 2008). HR can be Rad51-mediated, which proceeds through three steps: strand invasion, branch migration and the formation of a Holliday junction. The Holliday junction consists of two recombining duplexes covalently joined by single strand crossovers. This intermediate is cleaved by structure-specific exonucleases (resolvases) such as MUS81-MMS4 heterodimer producing two separate duplexes, which allows information lost in the broken duplex to be retrieved from an homologous duplex. It is known that Rad52, Rad54, Rad55, Rad57, BRCA1 and BRCA2 are also involved in HR, although their exact roles are unclear, and the Mre11/Rad50/NBS1 (M/R/N) may process the DSB termini prior to strand invasion. Alternatively single-strand annealing (SSA) proceeds in which a 5' to 3' exonuclease (potentially the M/R/N complex) digests the duplex to reveal regions with some homology on both sides of the break, which are paired and the non-homologous ends trimmed and ligated. In NHEJ a Ku hetrodimer binds the two ends of a DSB and recruits DNA-PKcs and ligase 4-X-ray repair complementing defective repair in Chinese hamster cells 4 (XRCC4) heterodimer to ligate the two termini of the duplex. It is known that the M/R/E complex can also be involved in NHEJ, especially when this pathway is used to for V(D)J recombination (Sancar *et al.,* 2004).

revealed it to be the structural gene for both the nuclear and chloroplast-targeted photolyase, and that activity of these proteins requires the *PHR1* gene product. In all, five genes predicted to be DNA photolyases, most likely class II, have been identified in *C. reinhardtii,* including the two previously characterised by Small *et al.,* (1995) and Petersen *et al.,* (1999). Moreover, a homologue of the MGMT gene has been identified in *Chlamydomonas*, but not in other plants (Vlcek *et al.,* 2008).

There is little information available regarding BER in *Chlamydomonas,* but the major AP endonuclease was partially purified and characterised by Frost and Small (Vlcek *et al.,* 2008), and exonuclease activity on single-stranded, linear DNA was demonstrated by Tait and Harris (1977a; b).

C. reinhardtii has been shown to possess homologues of some of the eukaryotic genes required for NER, such as those postulated to code for proteins including RAD23, XPA (contrary to higher plants), XPD, DNA repair protein Rad10, and DDB1 and DDB2. A number of UV-sensitive mutants of *C. reinhardtii* have been identified, such as *uvs1,* whose excision of CPDs is blocked, and *uvs3, uvs4, uvs6 and uvs7* who have slower CPD excision than the wild-type (Vlcek *et al.,* 2008). Despite this, only one excision repair gene and its protein product has been studied in more detail: *REX1*, which is required for the excision of CPDs (Cenkici *et al.,* 2003).

Mismatch repair has been postulated to occur in *C. reinhardtii,* specifically involving the gene mutated in the *uvs14* mutant since this strain showed a higher mutation frequency to MNNG and UV exposures (Vlcek *et al.,* 1997).

Recombinational repair has been demonstrated in *C. reinhardtii* with the involvement of the genes *UVSE1, UVSE5, UVSE6 and UVS10*, identified from mutant strains (Vlcek *et al.,* 2008). Moreover, Sarkar *et al.* (2005) identified the involvement of

C. reinhardtii Trxh 1 in the pathways implicated in the repair of abasic sites and/or single strand breaks induced by MMS.

1.8.2 DNA Repair Pathways Identified in *Daphnia sp.*

Daphnids have been shown to have high levels of shielding against DNA damage, which can include absorption by UV-blocking compounds such as melanin and tissues at the surface, and physical shielding by the exoskeleton via reflection and refraction. Daphnids have also been shown to repair DNA damage induced by UV radiation by both photoenzymatic repair (PER) and NER, and these processes have been shown to be temperature-dependent. For example, PER has been shown to be more effective at 10°C than 20°C in a number of daphnid species, possibly reflecting the allocation of energy reserves towards reproduction (and thus population survival) rather than repair, to take advantage of optimum conditions, while NER shows variation both with temperature and with species (Connelly *et al.,* 2008).

1.9 Aims of the Current Study

The main aim of the current study was to investigate the hypothesis that the commonly used ecotoxicological test species (namely in *C. reinhardtii* and *D. magna*) respond to exposure to a range of model genotoxic test compounds with different modes of action. The response of these organisms was assessed in terms of the sensitivity regarding genetic damage, biotransformation capability, and DNA repair mechanisms, with a view to testing the hypothesis that these organisms have the ability to reflect the mammalian activation of model genotoxicants. Moreover, the information gathered was

used to assess the hypothesis that these organisms could be used to develop higher throughput *in vivo* bioassays to test for genotoxic agents in surface waters, which could be coupled to existing ecotoxicological tests as early warning or detection systems. *C. reinhardtii* and *D. magna* are desirable species for a number of reasons. They can be maintained relatively simply and cultured in high numbers to enable high throughput screening and reproducibility of data. *C. reinhardtii* is one of the best characterised species of unicellular green algae and *D. magna* are an OECD approved species widely employed in ecotoxicity testing. Furthermore, employing lower trophic organisms in this research has the potential to reduce the numbers of vertebrate animals used in ecotoxicity assessment.

CHAPTER TWO:

GENERAL METHODS

2.1 Chemicals

All chemicals were purchased from Sigma-Aldrich Chemical company (UK) unless otherwise stated.

2.2 Test Organism Culture and Maintenance

2.2.1 *Chlamydomonas reinhardtii*

Wild type *C. reinhardtii* (derived from a stock cultured at AstraZeneca, Brixham UK) were maintained in freshwater algal culture medium FM/4 (containing 25.5mM $NaNO_3$, 12.2mM $MgCl_2$, 4.41mM $CaCl_2$, 14.7mM $MgSO_4$, 1.37mM K_2HPO_4, 0.186mM H_3BO_3, 0.416mM $MnCl_2$, 0.16mM $FeCl_3$, 0.3mM Na_2EDTA, 3.27µM $ZnCl_2$, 1.43µM $CoCl_2$, 7.26µM Na_2MoO_4, 0.012µM $CuCl_2$, 15mM $NaHCO_3$; Miller *et al.,* 1978) in a shaking incubator (70rpm) held at 20°±1°C, under a 12 h:12 h light:dark cycle. Light was provided initially by a high output Marine Blue 420 Actinic bulb (Arcadia, Redhill, UK), and later by a Gro-Lux photosynthetic tube (Sylvania, Danvers, US), illuminating with blue and red light (440 - 490nm and 625 - 750nm respectively).

Cell wall-free *C. reinhardtii* were provided courtesy of Dr Saul Purton and were the CW15.J3 strain, mating type minus; derived from a stock cultured at University College London, UK, originally established by Jacqueline Girard-Bascou, Institut de Biologie Physico-Chemique, France. These were maintained under identical culture conditions as used for the wild type, except that light was provided by Gro-Lux photosynthetic tube throughout.

2.2.2 *Daphnia magna*

Daphnia magna (Clone Type 5 – IRCHA; derived from a culture at the University of Reading, UK, originally from the Water Research Centre (WRc), Medmenham, UK) were maintained at a density of 20 animals per 1200ml of OECD-recommended ISO test media, aerated for a minimum of 24 hours before use (containing 11.76gl^{-1} $CaCl_2.2H_2O$, 4.93gl^{-1} $MgSO_4.7H_2O$, 2.59gl^{-1} $NaHCO_3$, 0.23gl^{-1} KCl; OECD Guideline 202, Annex 3), modified to contain selenium (0.002mgl^{-1}; required for cuticle production, Elendt and Bias, 1990; referred to as modified ISO media from here on), and held at 20°C±1°C under a 16 h:8 h light:dark photoperiod. Culture medium was renewed twice weekly and supplemented with an organic seaweed extract Marinure, which is a standard organic extract (Wilfred-Smith Ltd, Northants, UK; 3ml <day 7, 4ml day 8 onwards, OD (1:10) 0.8). Daphnids were fed daily with *Chlorella vulgaris* (increasing from 0.5-1mg carbon per day with 100% extra given on weekends) supplemented with baker's yeast (100mgl^{-1}). All cultures were initiated with third or fourth brood neonates <24h old. These conditions maintained the daphnids in the parthenogenetic reproductive cycle.

2.2.3 *Chlorella vulgaris* (Food for *D. magna*)

C. vulgaris (derived from a stock cultured at the Culture Collection of Algae and Protozoa (CCAP) Argyll, Scotland) were maintained in Bold's Basal Medium (BBM) (0.431mM K_2HPO_4, 1.29mM KH_2PO_4, 0.304mM $MgSO_4.7H_2O$, 2.94mM $NaNO_3$, 0.17mM $CaCl_2.2H_2O$, 0.428mM NaCl, 0.428mM EDTA-Na_4, 1.38mM KOH, 44.8µM $FeSO_4.7H_2O$, H_2SO_4, 0.462mM H_3BO_3, 4.9µM $ZnSO_4.7H_2O$, 1.17µM $MnCl_2.4H_2O$, 1.1µM $CuSO_4.5H_2O$, 0.275µM $Co(NO_3)_2.6H_2O$, 0.79µM $Na_2MoO_4.2H_2O$) in a fermenter vessel and held at

20°C±1°C, under a 16-h light and 8-h dark cycle. Light was provided by a Gro-Lux photosynthetic tube (Sylvania, Danvers, US), illuminating with blue and red light (440 - 490nm and 625 - 750nm respectively). To provide a stock for food, cells were collected by centrifugation (2250 x g, 30 minutes, 20°C), and the pellet re-suspended in a volume of water that gave an OD (1:10) of 0.8 at 440nm.

2.3 Growth Curve for *C. reinhardtii*

As an indicator of population growth (Lee *et al.,* 2002), the absorbance of the wild-type culture was recorded twice daily at 660nm using an Uvikon spectrophotometer (Kontron Instruments, Milton Keynes, UK). Cells from selected samples were counted using a haemocytometer to relate absorbance values to cell population.

2.4 Compound Exposure

2.4.1 *C. reinhardtii*

2.4.1.1 Comet Assay

Concentrations of compounds finally used were determined using the maximum tolerated dose (MTD) approach, where the MTD is defined as "the highest dose that elicits a secific toxic effect but not life threatening impairment in the test animal". (Hutchinson *et al.,* 2009). In this case, the maximum tolerated concentration (MTC) was used, as this has been shown to better reflect chemical exposure via immersion (Winter *et al.,* 2008) A high concentration that did not induce toxicity, and then a second concentration 5-10x lower was used. Exposure was undertaken in 3 replicate flasks per exposure, as follows. Briefly, 50ml of algal cells in mid log phase (day 5) were collected

by low speed centrifugation (300 x *g*, 10 minutes, 4°C), and the pellet re-suspended in 20ml of culture medium. For treatment of algal cells with genotoxic chemicals, 1.98ml of cell suspension was incubated with 20µl (in 100% dimethyl sulfoxide; DMSO) of stock solutions of Gurr chrysoidine Y (BDH Chemicals Ltd, Poole, England; final concentrations 0.1µM and 10µM), NQO (final concentrations 1nM and 5nM) or *N-OH*-2-AAF (a gift from Dr S.S. Thorgeirsson, National Cancer Institute, Bethesda, MD; final concentrations 0.05µM and 5µM). In all cases a DMSO vehicle control group and untreated control were also prepared, and exposure cultures maintained under the same culture conditions as described previously, for a further 24h.

2.4.1.2 Assessment of Induction of Ethoxyresorufin-O-deethylase (EROD) Activity

Exposure was undertaken in 3 replicate flasks as follows. Algal cultures (50ml, day 3) were incubated with β-naphthoflavone (βnF, in 100% DMSO, final concentrations 0.2 and 1nM) for 48h prior to measurement of EROD activity (see Section 2.7). A DMSO vehicle control group (0.1% v/v) and untreated control were also prepared, and exposure and control cultures were maintained under the same culture conditions as described previously.

2.4.2 *D. magna*

2.4.2.1 Establishment of Non-Toxic Doses

To determine appropriate concentrations of genotoxic chemicals for the comet assay and microarray studies, an initial range finding study was conducted, again using the MTC approach as described above. Groups of 10 third brood neonates (<24h old) were

transferred to 100ml fresh modified ISO media and exposed to increasing concentrations of each genotoxic chemical for 24h (n=2 test beakers per concentration). The genotoxicants tested and the final concentrations used are given in Table 2.1. A dH_2O or DMSO vehicle control was prepared where appropriate, and for each replicate an untreated culture was included. Exposure cultures were maintained under the same culture conditions as described previously, but without feeding or supplements. Water chemistry (pH, conductivity and hardness) of the medium was recorded at the time of preparation, and was within the OECD recommended ranges of pH 6-8 and hardness between 140 and 250mgl^{-1} (as $CaCO_3$). Conductivity was within the range 360-480μScm^{-1}. After 24h, any mortality was recorded and daphnids were assessed for their rate of movement. Any slowed movement as compared to the untreated control was deemed to be a toxic response. To determine appropriate combination dose levels for the mixture of sodium dichromate and BaP, daphnids were subsequently exposed to concentrations that had not induced mortality or slowed movement in the initial range finding study using the same experimental design, and toxicity assessed as described above.

2.4.2.2 Compound Exposure in *D. magna*

For the comet assay, assessment of EROD activity, and microarray studies compound exposure was undertaken as follows. Briefly, neonates (3rd brood, <24h old), or adults (7 days old) were collected and transferred to fresh modified ISO media. For treatment of daphnids with genotoxic chemicals, stock solutions were prepared and added to the medium to achieve the final concentrations. In all cases, a dH_2O control group was prepared, and a DMSO vehicle control where appropriate, and exposure

Table 2.1 Concentrations of genotoxic agents used in the range finding study for *Daphnia magna* neonates (<24h) to determine non-lethal exposure concentrations subsequently used in Comet assay or microarray studies, as indicated.

Experiment	**Genotoxic agent**	**Final exposure concentrations**
Comet Assay	Chrysoidine	0.1, 0.5, 1, 2, 3μM
	Sodium dichromate	0.25, 0.5, 0.75, 1, 2, 3, 4μM
	Sodium dichromate–BaP mixture	0.25μM-0.01μM, 0.5μM-0.05μM, 0.75μM-0.1μM, 1μM-0.2μM
Oligonucleotide Microarray	Sodium dichromate	0.25, 0.5, 0.75, 1, 2, 3, 4μM
	Benzo[a]pyrene (BaP)	0.01, 0.05, 0.1, 0.2, 0.5, 1μM
	Sodium dichromate–BaP mixture	0.25μM-0.01μM, 0.5μM-0.05μM, 0.75μM-0.1μM, 1μM-0.2μM
cDNA Microarray	Sodium dichromate	0.003, 0.01, 0.07, 0.7μM
	BaP	0.002, 0.01, 0.04, 0.2μM
	Sodium dichromate–BaP mixture	0.07μM-0.04μM, 0.01μM-0.01μM, 0.003μM-0.002μM

cultures were maintained under the same culture conditions as described previously, but without feeding or supplements. Final concentrations, numbers of daphnids, exposure times and the number of replicates are given in Table 2.2. Water chemistry (pH, conductivity and hardness) of the medium was recorded at the time of preparation, and was within the OECD recommended ranges of pH 6-8, hardness between 140 and 250mgl^{-1} (as $CaCO_3$) , and conductivity was within the range 360-480µScm^{-1}.

2.5 Assessment of Cell Viability in *C. reinhardtii*

Cell viability was assessed in all control and exposed samples using a colony-forming assay, based on the method of Hayashi *et al.,* (2004). Briefly, algal cell suspension was appropriately diluted with culture medium (FM/4) and 100µl transferred to the surface of solidified 0.6% bacteriological agar (Oxoid, Cambridge, UK) prepared with FM/4 algal culture medium, in a sterile 9cm plastic culture dish. This was immediately overlaid with 3.5ml of culture medium supplemented with 0.6% agar at 37°C. The mixture was spread evenly and kept at room temperature for 1h to solidify. The plates were then incubated at 20°±1°C under a 12 h:12 h light:dark cycle for 5-7 days prior to colony counting.

2.6 Alkaline Comet Assay

The Comet assay methodology, originally described by Singh *et al.,* (1988), was modified for application to both *C. reinhardtii* and *D. magna*. Details of the modifications are presented in Chapter 3, Section 3.2. A brief overview of the Comet assay

Table 2.2 Exposure conditions for Comet assay, ethoxyresorufin-*O*-deethylase (EROD) activity determination and microarray studies in *Daphnia magna.*

Experiment	**Age/Number of daphnids per exposure**	**Compound**	**Final Concentration**	**Volume Added**	**Duration**	**Replicate Experiments**
Comet Assay	10 neonates (<24h)	Chrysoidine	0.1, 0.5, 1, and 2µM	100µl in 100ml	24h	3
		Sodium Dichromate	0.25, 0.5, 0.75 and 1µM			
		Sodium dichromate-BaP mixture	0.25µM-0.01µM, 0.5µM-0.05µM, 0.75µM-0.1µM			
EROD activity determination	20 adults (7 days)	DMSO	0.002%, 0.02% and 0.1%	20µl to 100ml	24h	3 (2 replicate beakers per experiment)
		Methanol	0.001%, 0.01%, 0.05%			

cDNA microarray	140 neonates (<24h)	Sodium dichromate-BaP mixture	0.01 + 0.01µM 0.003 + 0.002µM	20µl to 1l	24h	1
Oligonucleotide microarray	100 neonates (<24h) 20 adults (7 days)	Sodium dichromate-BaP mixture	0.25µM-0.01µM 0.75µM-0.1µM	20µl to 1l	6h or 24h	4

methodology, excluding any modifications is presented here.

2.6.1 Assay Method

Twin frosted microscope slides (VWR International, Leuven) were prepared in advance by coating with 0.5% normal melting point agarose (NMPA) in phosphate buffered saline (PBS). For the assay, an aliquot of cells was mixed with 0.5% low melting point agarose (LMPA) in PBS. The solution was spread homogeneously on the slide with a glass cover slip. Slides were then held at 4°C for 30 minutes on a tray to allow the agarose to solidify, following which the cover slips were carefully removed. The slides were then submerged in lysis buffer in a Coplin jar, in the dark, to lyse the cell and nuclear membranes. Subsequently, slides were transferred to a horizontal electrophoresis tank (Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) in which freshly made electrophoresis buffer (0.3M NaOH, 1mM Na_2EDTA, pH 13, pre-chilled to 4˚C) was contained, and left at room temperature in the dark to allow DNA unwinding. The slides were then subjected to electrophoresis (Pharmacia LKB, Cambridge, UK) in the dark. Following electrophoresis, slides were neutralised with three 5 minute washes of neutralisation buffer (0.4M Tris Base, pH 7.5), and then stained with 60µl SYBR Gold solution (1 in 1000 dilution of a 10000x stock, obtained from Molecular Probes, Invitrogen, UK).

2.6.2 Microscopic Comet Evaluation

The prepared slides were subsequently examined at 340x (*C. reinhardtii*) or 200x (*D. magna*) magnification using a fluorescence microscope equipped with a 450-490nm

filter (Zeiss Axiovert 10, Germany), and scored using image analysis software (Comet IV, Perceptive Instruments, UK). One slide was prepared from each replicate (n=3) and 100 cells were scored per slide. A number of parameters exist for measuring DNA strand breaks (e.g. tail length, various tail moment measurements, % tail DNA) of which olive tail moment (OTM) and % tail DNA are the most common (Kumavarel and Jha, 2006). It has been reported that % tail DNA is the most appropriate parameter as it has a linear relationship with the frequency of breaks in the DNA and is largely unaffected by threshold settings (Collins, 2004). Moreover, it is considered more meaningful and comparable between laboratories, since OTM is measured in arbitrary units and different values are produced by different image analysis software (Kumavarel and Jha, 2006). Thus % tail DNA was used for analysis.

2.7 Fluorescence Detection of Ethoxyresorufin-*O*-deethylase Activity

Ethoxyresorufin-*O*-deethylase (EROD) activity was measured in intact algal cells or whole adult daphnids (day 7) by directly measuring their ability to convert added 7-ethoxyresorufin (7-ER), *in vivo.* The method used to detect the resultant resorufin was a fluorometric stop assay method based on that of Burke and Mayer, (1974). Briefly, 2ml of algal cell suspension in culture medium (see above under the 'test compound exposure' section) or 20 adult daphnids in modified ISO media were incubated with 7-ER (8µM) and dicumarol (10µM; Jaquet *et al.,* 1997), which stabilises the reaction product resorufin, by preventing further biotransformation by cytosolic diaphorase (Kern *et al.,* 1997). Incubation was undertaken for 1, 2, 3, 4 or 5h (20°C ± 1°C), to establish the optimum time course for the measurement of EROD activity. Control incubations for algal cells were as described, but kept on ice to minimise enzymatic

activity. Algae were removed from suspension at each time point by centrifugation (300 x *g*, 4°C, 10 min), and 750µl of the resultant supernatant, or 750µl of the daphnia medium (containing excreted resorufin) was added to 250µl of the de-conjugation enzyme mixture ß-glucuronidase/arylsulphatase from *Helix pomatia* (1 in 666 dilution of stock, in 100mM sodium acetate solution; pH 4.5; Roche, UK), and incubated at 37°C for a further 2h. Following this, each incubate was transferred to a fluorescence cuvette, 1ml of 100% ethanol added to stop the reaction, and the fluorescence measured using a Perkin Elmer Luminescence Spectrometer LS5OB (Perkin-Elmer Limited, Beaconsfield, UK) with excitation set at 530nm; and emission measured at 590nm.

2.8 Determination of Total Reduced Glutathione Levels in *C. reinhardtii*

Reduced glutathione (GSH) levels were determined using a fluorimetric method based on that described by Hissin and Hilf, (1976), subsequently adapted for use in samples of tissues by Winter *et al.,* (2005). Briefly, cells were collected by centrifugation (300 x *g*, 10 minutes, 4˚C), the supernatant discarded and the pellet resuspended in 135µl lysis buffer (0.1% Triton-X-100 in PO_4-EDTA assay buffer; 100mM NaH_2PO_4 and 5mM Na_2EDTA, pH 8). Protein mass in cell extracts was quantified using the Bradford method (first described by Bradford, 1976) and the bicinchoninic acid (BCA) assay (first described by Smith *et al.,* 1985; in Walker *et al.,* 1996), BioRad, München, Germany), with BSA used as the protein standard. Next, trichloroacetic acid (TCA; 50%, 15µl) was added to the algal lysate to precipitate the protein, and the lysate spun at 12 000 x *g*, 4˚C, for 10 minutes. In the meantime a GSH standard curve was prepared in 3ml fluorescence cuvettes by diluting GSH in PO_4-EDTA assay buffer to a final volume of 1.8ml and

concentrations across the range 0-2µg. Next, the resultant cellular supernatant (100µl) was added to 1.8ml PO_4-EDTA assay buffer in another 3ml fluorescence cuvette. To each cuvette (samples and standards), 100µl of 5% TCA and 100µl of *o*-phthalaldehyde (OPT 1 $mgml^{-1}$ in 100% methanol) was added, and the cuvette shaken and left to stand for 15 minutes. Following this, fluorescence was measured using a Perkin-Elmer Luminescence Spectrometer (Model LS5OB, Perkin-Elmer, Beaconsfield, Buckinghamshire, UK) set at an excitation of 350nm with emission measurement at 420nm.

2.9 Microarray Analysis of Gene Expression Changes

We conducted a pilot study using a custom cDNA microarray designed and made by Amanda Callaghan's group at the University of Reading, UK. The microarray was constructed using cDNA fragments from three sources: daphnids (24-48h old) exposed to five different stressors (ibuprofen, cadmium, lufenuron, pH and calcium) generated using suppression subtractive hybridisation (SSH), expressed sequence tags (ESTs; 10,272 clones) from a cDNA library from unexposed mixed-age organisms from a 3-4 week old culture (Watanabe *et al.,* 2005), and SSH generation of 1143 clones from 15 adults carrying eggs and 75 juveniles (Soetaert *et al.,* 2006; Heckmann *et al.,* 2008). Slides were pin-printed using an Omnigrid 100 (Genomics solutions, USA) on Corning CMT-UltraGAPS glass slides (Corning, UK). Each microarray contained 48 blocks in a 17 x 18 block format. DMSO (final concentration of 50%) was added to the purified PCR products before printing. Positive control spots (*D. magna* genomic DNA and four Spot Report System PCR products form *Arabidopsis thaliana*: CAB, RCA, RBCL and LPT4; Stratagene, USA) and negative controls (salmon sperm DNA, human and mouse Cot-1, polyA RNA and yeast tRNA were included along with blank spots (50% DMSO) as a

qualitative way of assessing hybridisation efficiency and providing grid orientation. Following printing, slides were UV cross-linked (150mJ/cm^2) and baked at 80˚C for 2h. Slides were stored in the dark under a vacuum at room temperature (Heckmann *et al.,* 2008).

The follow-up study utilised an oligonucleotide microarray available from Ecoarray through Agilent. To construct the array sequences from publically accessible databases (GenBank Nucleotide database (*D. magna,* n=12082; Genome Project ID 13036 (n=11964); Gene Expression Omnibus (GEO) database (accession number GPL2888, all sequences)) were assembled and non-overlapping, unique sequences (singlets and contigs) to prevent cross hybridisation were identified. These sequences were annotated with gene name and ontology (function) by Blast searches and a total of 4936 contigs were assembled. Agilent's E-Array program was used to design the probes (60-bases in length). Details of the number of annotated contigs and probes designed are as follows:

- 2255 contigs were annotated and represented by one probe for the forward strand
- 247 contigs were annotated and represented by one reverse complement for the probe
- 2434 were un-annotated and represented by one sense and one antisense probe.
- In total, 7370 probes were designed.

The microarrays were manufactured for EcoArray by Agilent Technologies in an "8-pack" format, which consists of 8 individual 15K arrays per slide. Within each 15K array

each of the 7370 probes is represented twice. The probes were synthesised *in situ* using Agilent's inkjet based "SurePrint" technology and the arrays were manufactured on superior quality 1" x 3" (24.2mm x 76.2mm) treated glass slides (EcoArray).

2.9.1 Tissue Preparation and RNA Extraction

For the cDNA microarrays (pilot study), following exposure, neonates were preserved, as outlined by Heckmann *et al.,* (2007). Briefly, neonates were transferred immediately to 2.5ml RNA*later*® and left for 15 min at room temperature to allow full absorption into the tissues before being transferred to 1.4ml fresh RNA*later*® and stored at –80°C. Total RNA was extracted by Chris Hill, University of Reading, UK using the RNeasy Mini kit (QIAGEN, Crawley, UK) followed by DNAse treatment using DNA-*free*™ (Ambion, Applied Biosystems, Warrington, UK) following manufacturer's instructions, to remove any genomic DNA traces. Concentrations of RNA were determined by spectrophotometry using Gene-Quant Pro (Biochrom, Cambridge, UK) and the integrity of the RNA was confirmed using a BioAnalyzer 2100 (Agilent Technologies, Stockport, UK).

An overview of the extraction, labelling and hybridisation process for the oligonucleotide microarrays, is given in Figure 2.1. Daphnids were immediately transferred into 0.5ml RLT lysis buffer (RNeasy Mini kit, QIAGEN, Crawley, UK) with the addition of 5µl β-mercaptoethanol to irreversibly denature RNAses by reducing disulfide bonds, as recommended by QIAGEN. Total RNA was extracted using the RNeasy Mini kit (QIAGEN, Crawley, UK) according to the manufacturer's instructions. Following DNase treatment using DNA-*free*™ (Ambion, Applied Biosystems, Warrington, UK) to remove

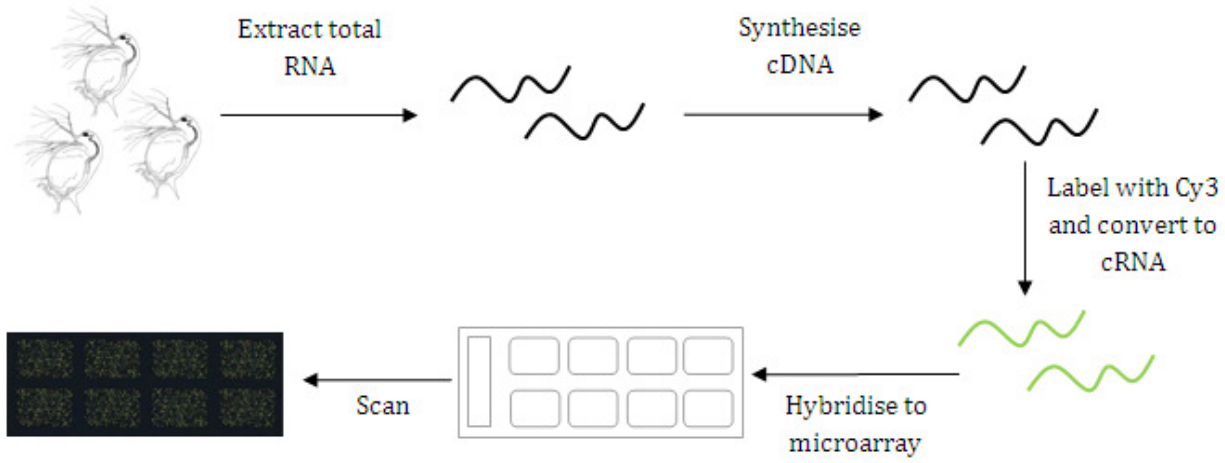

Figure 2.1 Overview of the extraction, labelling and hybridisation process for the *Daphnia magna* oligonucleotide microarrays. Total RNA was extracted from a pool of 100 daphnids from which cDNA was synthesised. This was labelled with Cy3 dye and converted to cRNA. The labelled cRNA was hybridised to the array, and following washing the array was scanned.

any traces of genomic DNA and then concentrated using a speed vac (Eppendorf, UK), as initial measurements revealed the concentration of eluted RNA was too low for subsequent cDNA synthesis. RNA concentration and purity was determined using the NanoDrop ND-1000 UV-VIS Spectrophotometer version 3.2.1 (Nanodrop®, USA). The percentage purity of the RNA samples was assessed by the ratio A260/A280 and was >1.8 for all samples, indicating little protein contamination.

2.9.2 Production of cDNA/cRNA and Subsequent Labelling

For the cDNA microarrays, cDNA synthesis and labelling was carried out by Christopher Hill, University of Reading, UK using the SuperScript™ Plus Indirect cDNA labelling System (Invitrogen, Paisley, UK) following manufacturer's instructions. Briefly, cDNA was synthesised from 20µg total RNA using anchored oligo$(dT)_{20}$ primer (2µl; 2.5µg/µl), made up to a final volume of 18µl with DEPC-treated water and incubated at 70˚C for 5mins, and on ice for a minimum of 1min. To this reaction mixture was added 5X first strand buffer (6µl), 0.1M dithiothreitol (DTT; 1.5µl), dNTP mix (including amino-modified nucleotides; 1.5µl), RNaseOUT (Recombinant RNase Inhibitor to safeguard against ribonuclease degradation of target RNA; 1µl; 40U/µl) and SuperScript™ III Reverse Transcriptase (2µl; 400U/µl), giving a final volume of 30µl. Following gentle mixing the tubes were incubated at 46˚C for 3h. Following this, 15µl 1 N NaOH was immediately added to each reaction tube and incubated at 70˚C for 10mins to degrade any remaining RNA. The pH was neutralised by the addition of 15µl 1 N HCl. The resultant amino-modified cDNA was purified to remove any unincorporated nucleotides using the Purification Module of the kit following manufacturer's instructions and then labelled with Alexa Fluor® dyes (mono-reactive N-hydroxysuccinimide (NHS)-ester

fluorescent dyes) using a two colour reference design (Alexa Fluor® 647 and Alexa Fluor® 555 for experimental and reference pool samples, respectively). Briefly, 2X Coupling Buffer (5µl) and Alexa Fluor® dye (2µl in DMSO) were added to dried cDNA, thoroughly mixed and incubated in the dark at room temperature for 2h. Following this, the labelled cDNA was purified to remove any uncoupled dye using the PureLink™ PCR Purification System following manufacturer's instructions.

For the oligonucleotide microarrays, RNA was reverse-transcribed, labelled and the cDNA converted to cRNA using the Agilent Low RNA Input Linear Amplification Kit with Spike-ins (Agilent Technologies, USA) for controls, following manufacturer's instructions. The labelling was carried out according to the manufacturer's instructions with some modifications. All reagents were supplied by Agilent in the Agilent Low RNA Input Linear Amplification Kit and Spike-in Kit. Briefly, 300-1000ng of extracted total RNA was spiked with the Agilent one-colour spike mix (serially diluted; 3-5µl depending on RNA concentration) and T7 Promoter Primer (1.2µl), and made up to a total volume of 11.5µl using nuclease-free water. The reaction mixture was incubated at 65˚C for 10min to denature the primer and then placed on ice for 5mins. To this mixture 5X First Strand Buffer (4µl), 0.1M DTT (2µl), 10mM dNTP mix (1µl), MMLV-RT (1µl) and RNaseOUT (0.5µl) were added, mixed by pipetting and incubated at 40˚C for 2h, 65˚C for 15min, and on ice for 5min. Following cDNA synthesis, an additional step to purify the cDNA using the QIAquick PCR Purification Kit (QIAGEN, Crawley, UK) was included to remove any unincorporated dNTPs, as initial labelling reactions did not incorporate sufficient amounts of dye (<6pmol Cy3/µg cRNA). The cDNA was subsequently labelled with Cy3 and converted to cRNA using 4X transcription buffer (20µl), 0.1M DTT (6µl), NTP mix (8µl), 50% PEG (6.4µl), RNaseOUT (0.5µl), inorganic pyrophosphatase (0.6µl)

T7 RNA polymerase (0.8µl), and Cy3 (2.4µl). Following gentle mixing the samples were incubated at 40˚C for 2h. Uncoupled Cy3 was removed using the RNeasy mini kit (Qiagen, Crawley, UK) to purify the cRNA and labelling efficiency was determined by NanoDrop ND-1000 UV-VIS Spectrophotometer, version 3.2.1 (Nanodrop®, USA). A yield of 1.65µg cRNA, and a specific activity (Cy3 dye incorporation) of >6pmol Cy3/µg cRNA was considered sufficient.

2.9.3 Hybridisation onto the *D. magna* Oligonucleotide Arrays

Hybridisation on to the cDNA microarrays was conducted by Christopher Hill, University of Reading, UK. A reference pool design was used in which samples from the treated groups were hybridised against a common reference pool sample. Slides were pre-hybridised in a solution containing 50% (v/v) de-ionised formamide, 5X sodium chloride-sodium citrate (SSC), 0.1% SDS and 1% BSA (w/v) in a Techne HB-a Hybridiser (Techne Ltd, Stone, UK) at 42°C for 1h. Following this slides were washed in MilliQ water and 100% isopropanol, and then dried immediately in a Christ RVC 2-25 centrifuge (Martin Christ GmBH, Germany) at 235 x g, for 2min at 30°C. Hybridisation probes (final volume 45µl) containing 22.5µl de-ionised formamide, 5 x SSC, labelled cDNA mix (exposed and reference pool samples) and a hybridisation block mix composed of 0.1% SDS, 0.5mgml^{-1} polyA RNA (Sigma-Aldrich, Warrington, UK), 0.5mgml^{-1} yeast tRNA, 0.5mgml^{-1} salmon sperm DNA, and 25µgml^{-1} human and 25µgml^{-1} mouse *Cot-1* DNA (Invitrogen, Paisley, UK) were prepared and heated at 96°C for 5min to denature the probes. Probes were cooled to room temperature (RT), mixed and hybridised to individual microarray slides under a 25x60 I LifterSlip™ (Implen, Southend on Sea, UK).

One hybridisation was performed for each slide, and one slide was prepared per treatment or control (three slides total). The slides were then placed in an airtight plastic box and incubated at 42°C for 16h in a Techne HB-1 Hybridiser (Techne Ltd, Stone, UK). Following this slides were washed with the following buffers: 2x SSC (at RT, to allow LifterSlip to detach), 0.1x SSC and 0.1% SDS (at RT for 2 x 5min), 0.1x SSC (at RT for 2 x 5min), 0.05x SSC (at RT) and isopropanol (at RT) and then dried immediately in a Christ RVC 2-25 centrifuge (Martin Christ GmBH, Germany) at 235 x g, for 2min at 30°C.

Hybridisation samples for the oligonucleotide microarrays were prepared by mixing 600ng labelled cRNA with 10x blocking agent (5µl), 25x fragmentation buffer (1µl) and made up to a final volume of 25µl with nuclease-free water (all from Agilent Technologies, USA). Samples were mixed and incubated at 60°C for exactly 30 min to fragment the RNA. The reaction was stopped by adding 2x GEx hybridisation buffer HI-RPM (25µl), the samples mixed, placed on ice, and randomly assigned to arrays. The samples were loaded into individual wells of a gasket slide held in the base of a hybridisation chamber (Agilent Technologies, USA), an 8x15K array was placed on top of the gasket slide, the chamber reassembled and clamped, and placed in an Agilent hybridisation oven at 65°C for 17 h (Figure 2.1). Following this, slides were separated from the gasket slide and washed with the following buffers (all from Agilent Technologies, USA): wash buffer 1 (containing EDTA, at RT for 1 min); wash buffer 2 (containing EDTA, at 37°C for 1 min); acetonitrile (at RT for <10 s); and stabilisation and drying solution (containing an ozone scavenger in acetonitrile), at RT for 30 seconds.

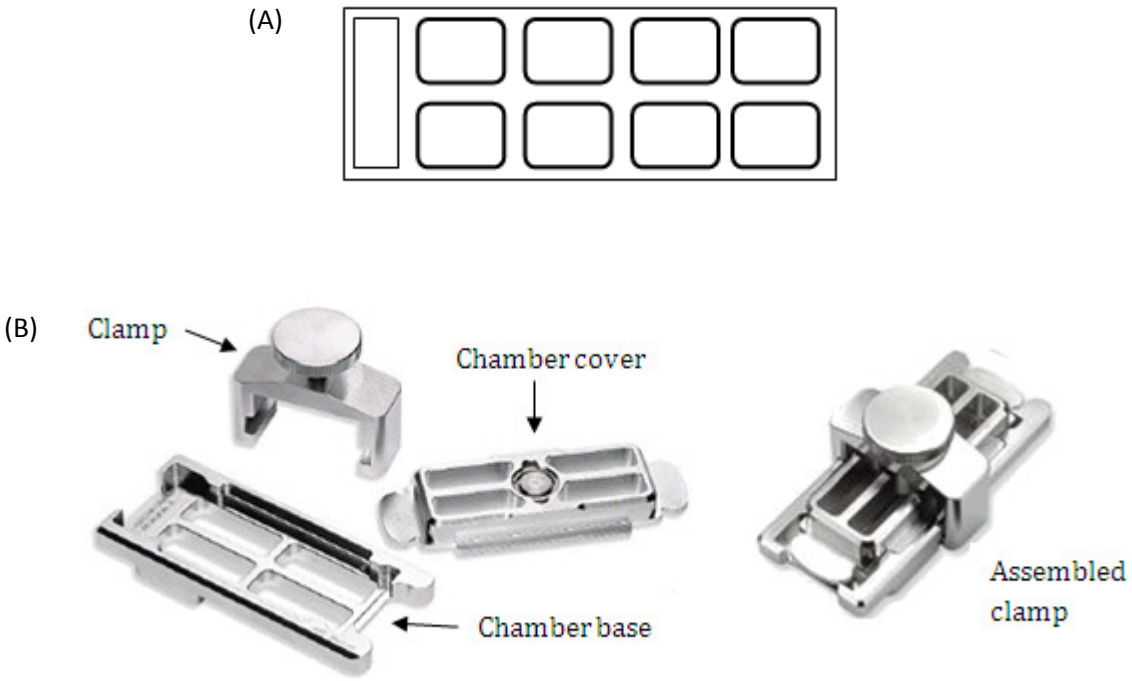

Figure 2.2 (A) Diagram of an 8-pack gasket slide used for loading samples onto Agilent 8-pack arrays, and (B) the individual components and an assembled Agilent hybridisation chamber to hold the microarray and gasket slide securely together (Agilent Technologies, USA). To load the samples, the gasket slide is placed in the hybridisation chamber base, the samples are transferred by pipette into the 8 individual wells of the gasket slide, and then the microarray is placed on top of the gasket slide. The chamber cover is placed on top of the microarray slide in the chamber base and the chamber is clamped together.

2.9.4 Scanning and Data Analysis

The cDNA microarrays were scanned at the University of Reading using a GenePix 4200A Scanner (Axon Instruments, Inverurie, UK) at 100% laser power and with a photomultiplier tube voltage (PMT) optimised for each array slide to ensure minimum saturated spots were obtained. The data were normalised by Christopher Hill and Viacheslav Bolshakov using Lowess block normalisation per slide to the median of ratios (signal and control channels) using spots with a saturation value of less than 50% for at least one channel, and a signal to noise ratio of 3 or greater.

Oligonucleotide microarrays were scanned immediately following drying at 532nm using the GenePix 4000B Scanner (Axon Instruments, Inverurie, UK) at 5 microns resolution. The PMT was set to 550V and the images were analysed using the Genepix pro software version 7 (Axon Instruments, USA) for spot identification and quantification of the fluorescent signal intensities. Spots with low intensity or poor morphology were automatically flagged, and all spots were manually checked for quality. Flagged spots were removed from analysis in GeneSpring, Median signal and control channels (F532) were used for analysis in GeneSpring 7. Data were kindly normalised by Olga Hrydziuszko, University of Birmingham, UK. Data were filtered to remove low or saturated intensity (flagged) spots, and rows that contained more than 20% flagged spots were deleted (7631 rows remained from 15744). Data were log2 transformed and quantile normalisation was conducted followed by de-noising.

2.9.5 Real-Time PCR Confirmation of Oligonucleotide Microarrays

Quantitative real-time PCR (RT-PCR) was conducted, with assistance from Miss Vanja Dakic, to validate relative mRNA expression changes of two selected gene fragments, following 24h exposure of adult daphnids at the high concentration of the sodium dichromate-BaP mixture (0.75µM-0.1µM).

2.9.5.1 Primer Design, Validation, and Product Sequencing

Primers were designed using Primer3 (Primer3) and synthesised by AltaBioscience (Birmingham, UK). Primer sequences for the genes are given in Table 2.3. To synthesise cDNA, 1µl of random primers (300ng, Promega, UK) was added to 500ng of RNA extracted from the different targets (made up to a final volume of 15µl with dH_2O), and incubated at 65°C for 5min. The mixture was then placed on ice before the addition of 0.5µl 25mM dNTPs (Bioline, London, UK), 5µl of 5x first strand buffer, 2µl 0.1mM DTT, and 0.5µl Superscript II reverse transcriptase (all from Superscript II kit, Invitrogen, Paisley, UK). The samples were run on a thermocycler with the following program: 25°C for 10min, 42°C for 90min, and 70°C for 15min. The cDNA was quantified by NanoDrop ND-1000 UV-VIS Spectrophotometer, version 3.2.1 (NanoDrop®, USA).

Primers were validated using PCR, using the Bioline PCR kit (Bioline, London, UK), 1µl of each primer, and cDNA, made up to a final volume of 50µl. The genes of interest were amplified on a thermocycler using the following program: 95°C for 5min, 35 cycles of 95°C for 30s, 60°C for 30s and 72°C for 30s, and 72°C for 5min. PCR products were run on a 2.5% agarose gel and visualised by ethidium bromide staining. Fragment size was approximated using a 100bp ladder (New England Biolabs, Ipswich,

Table 2.3 Primer sequences for Real-Time PCR validation of the oligonucleotide microarray

Gene Fragment	**Left Primer**	**Reverse Primer**	**Primer Efficiency**
Vitellogenin (BAD05137)	TGAGCACTCGTCTGATGGTC	CGGAGTTTGTCACCCAAAGT	94-104%
P73-like (AAT72302)	GCCTGGGCATTTGAACTTTA	ATGGAAGTGATCAGCCTTGG	93-95%
CG8121-PA (XP_624224)	ACGGGTAGCGTGGTACAAA A	CCAGGCTTGGTCATTCCTAA	90-93%

UK).

PCR products were sequenced on a capillary sequencer ABI3730 (Functional Genomics and Proteomics Unit, Birmingham, UK) and sequences subjected to blast searches (FleaBase) to identify genes with high homology.

2.9.5.2 Real Time PCR

RT-PCR of selected genes was conducted on the ABI Prism 7000 (Applied Biosystems, USA) using the SYBR Green SensiMix (Quantace, Watford, UK). Each reaction was run in triplicate and contained 250ng μl^{-1} cDNA template, 1µl of each primer in a final volume of 25µl. Products were amplified and detected using a dissociation protocol, with cycling parameters of 95°C for 30s (denaturing step) and 60°C for 30s (combined annealing and extension). Melting curves were plotted using the ABI Prism 7000 SDS software to ensure only a single product was amplified and no primer dimers were formed. PCR efficiencies of amplicons were calculated using absolute fluorescence values for each well by performing linear regression on the Log (fluorescence) per cycle (LinRegPCR programme; Ramakers *et al.,* 2003). Threshold cycle (CT) values were recorded in the linear phase of amplification by setting a baseline where no amplification was occurring and efficiencies were filtered to be >1.45, averaged for each gene and used to normalise CT values. The internal reference gene (CG8121-PA) was validated using the delta-delta CT method (Livak and Schmittgen, 2001) to determine that it was not affected by treatment, before being used to further normalise the data. The data were then analysed using the delta-delta CT method of relative quantification.

2.10 Statistical Analyses

For the growth curve data the statistical significance of the R^2 values obtained, for both cell number and absorbance values was determined. To identify significant differences between absorbances recorded for the algal growth curve under the marine blue actinic bulb and the Gro-Lux tube, data were assessed first by a one way ANOVA ($P<0.05$) followed by a two-tailed Student's t-test ($P<0.05$).

To determine significant differences between treatment and control groups in the Comet assay, measurements of percentage tail DNA of 100 comets per slide (n=3 slides) were recorded, and the median value used for statistical analysis as recommended by Duez *et al.,* (2003). Statistically significant differences were identified using a one way ANOVA ($P<0.05$), followed by a two-tailed Student's t test ($P<0.05$) to compare control and treated samples.

For the EROD assay, linear regression analysis was used to investigate the significance of the linear relationship with time. A one way ANOVA ($P<0.05$) was used to identify any statistically significant differences between basal EROD activity and that measured following exposure to βNF and DMSO (*C. reinhardtii*), and DMSO and methanol (*D. magna*).

For the oligonucleotide microarrays statistically significant differences between gene expression changes were identified by a parametric Welsh t-test with a P value cut-off of 0.05. A Benjamini and Hochberg False Discovery Rate (FDR) multiple testing correction (Benjamini and Hotchberg, 1995) was applied, which predicted that approximately 5-10% of the identified genes would pass the test by chance. Fold changes were determined by the mean expression ratio of test:control. Principal

components analysis (PCA) was used to identify relationships between differentially expressed genes, carried out in GeneSpring, and the Pearson correlation was applied. Hierarchical clustering was applied using GeneSpring to further investigate differential gene expression between adult and neonate daphnids, using the 'centroid' clustering method, in which the distance between two clusters is the distance between the averages of the data points under one branch and the averages of the data points under another.

Statistically significant differences between CT values for control and treated samples obtained by RT-PCR were determined using a one way ANOVA ($P<0.05$).

CHAPTER THREE: DNA STRAND BREAKS AS A MARKER OF GENOTOXICANT EXPOSURE: METHOD DEVELOPMENT AND UTILISATION IN *C. REINHARDTII* AND *D. MAGNA*

3.1 Introduction

As described in section 1.2, genotoxicants are common contaminants in the aquatic environment. Despite this, current testing strategies do not routinely assess the genotoxic potential of chemicals or effluents (see section 1.3) even though the technology to do so is relatively well validated in higher organisms. There are a number of techniques available for detecting the effects of genotoxic agents. These include the micronucleus assay for clastogens and anuegens, the ^{32}P post-labelling assay for DNA adducts, single-cell gel electrophoresis (SCGE) for DNA strand breaks, and (indirectly) microarray analysis of gene expression changes (e.g. for DNA repair assessment). Of these, alkaline SCGE, or Comet assay (Singh *et al.,* 1988) is a well-established, sensitive method for detecting DNA strand breaks in eukaryotic cells from any tissue, (Collins, 2004; Parry, 2000), including from aquatic organisms (Mitchelmore and Chipman, 1998).

A diagram of the most commonly employed methodology for the Comet assay, a modification published by Singh *et al.* (1988) based on the original protocol developed by Östling and Johanson (1984), can be seen in Figure 3.1. Briefly, cells are embedded in a layer of agarose on a glass microscope slide pre-coated with agarose, and following solidification are lysed using a buffer containing high salt and detergent. This removes membranes, cytoplasm and nucleoplasm, and causes disruption of nucleosomes and solubilisation of histones. This leaves the nucleoid, which contains a scaffold of RNA, proteins, and negatively supercoiled DNA. The nucleoids are then electrophoresed at alkaline pH (>10), which increases the range of damage that can be detected, but not the sensitivity compared to lower pHs (Collins, 2004). A DNA stain is added following

neutralisation that allows visualisation of the DNA in the gel, the resultant image resembling a comet with a distinct head and tail region. The proportion of DNA in the tail, therefore, relates to the degree of DNA fragmentation, as smaller fragments travel further than intact DNA during electrophoresis (Collins *et al.,* 1997). A modification that is commonly made to the assay is the inclusion of a step to digest nucleoids using an enzyme specific for a particular type of damage. A number of enzymes are employed, such as formamidopyrimidine DNA glycosylase (FPG) to detect 8-hydroxy-deoxyguanosine (8-OH-dG) and other altered purines, endonuclease III, which detects oxidised pyrimidines, and T4 endonuclease V to recognise cyclobutane pyrimidine dimers (CPDs; Collins, 2004).

Although originally developed for mammalian cells, the Comet assay has more recently been applied in aquatic organisms. Most of these studies have been in fish (e.g. Winter *et al*., 2004; Diekmann *et al.,* 2004, Mitchelmore and Chipman, 1998), and invertebrates (e.g. mussels, Emmanouil *et al.,* 2006; Canty *et al.,* 2009, and echinoderms, Taban *et al.,* 2004; Canty *et al.,* 2009), with limited analyses in some unicellular algae (e.g. *Rhodomonas* sp., Sastre *et al.,* 2001; *Euglena gracilis*, Watanabe and Suzuki, 2002; Aoyama *et al.,* 2003; Li *et al.,* 2009 and *Closterium ehrenbergii*, Ciniglia *et al.,* 2005). In addition, previous attempts have been made to measure DNA strand breaks in both *C. reinhardtii* (Erbes *et al.,* 1998) and *D. magna* (den Besten and Tuk, 2000). However, very limited information is available regarding the methodology used to study *D. magna,* and in unicellular algae there have been conflicting reports of sensitivity (e.g. Aoyama *et al.,* 2003, Erbes *et al.,* 1998). Consequently, there is a need to optimise the methodology, and gain a better understanding of the applicability of the Comet assay to these organisms,

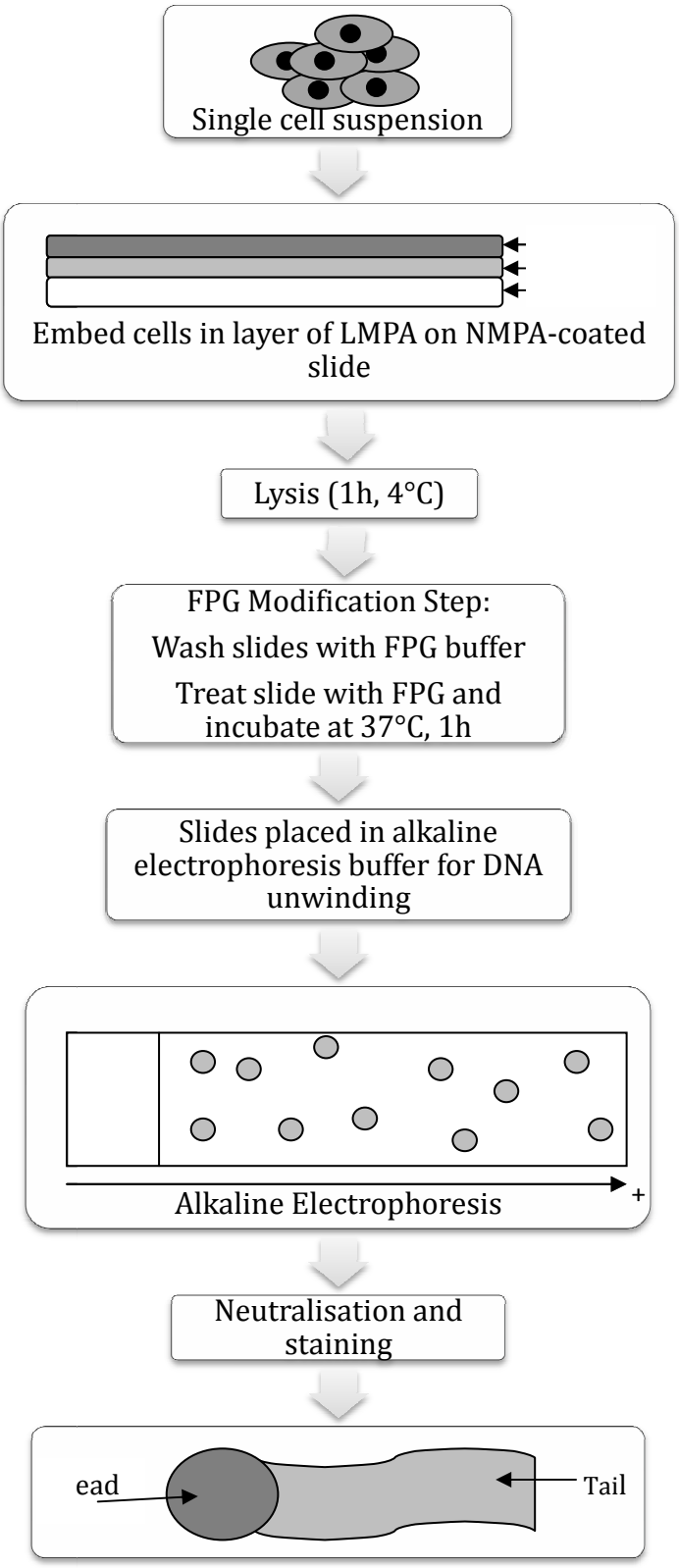

Figure 3.1 Diagrammatic representation of the most commonly employed methodology for alkaline single cell gel electrophoresis (Comet assay) published by Singh *et al.* (1988) based on the original protocol developed by Östling and Johannson (1984). A common modification step to include enzyme digestion, not included in the original protocol, is indicated.

and their sensitivity to a wider range of direct acting as well as pro-genotoxic compounds.

The aim of the current chapter, therefore, was to investigate the hypothesis that *C. reinhardtii* and *D. magna* can respond to a number of direct and indirect acting genotoxicants (pro-genotoxic agents that require metabolic activation) that act via different mechanisms, with a view to detecting DNA strand breaks as a sub-lethal marker of exposure to genotoxic agents. In order to do this, modifications first had to be made to the existing methodology for the Comet assay to allow application of this technique to these organisms. Thus, this chapter is divided into two sections, with the first detailing the method development stage, while the second presents the results following application of the modified protocols to detect DNA strand breaks in *C. reinhardtii* and *D. magna* after genotoxicant exposure.

3.2 Development of the Comet Assay Methodology for *C. reinhardtii* and *D. magna*

3.2.1 Comet Assay Method Development in *C. reinhardtii*

3.2.1.1 Modifications to the Assay Protocol

The original Comet assay protocol by Singh *et al.* (1988) was modified as described by Aoyama *et al.* (2003) and Erbes *et al.* (1997) to improve suitability for unicellular algae that possess a cell wall. Specifically, modifications were made to the lysis buffer and lysis conditions, as well as for the times for DNA unwinding and electrophoresis, and the conditions for DNA staining.

Algal cell walls are surrounded by a cell wall composed of three specially structured glycoprotein layers (Erbes *et al.,* 1997). Initially the lysis conditions reported by Erbes *et al.* (1997) to lyse *C. reinhardtii* cells were employed: 10 minutes in modified lysis buffer (300mM NaOH, 30mM Na_2EDTA, 0.1% sodium dodecylsulfate; SDS). This, however, was found to completely destroy the cells and nuclei, and so lysis was tested for shorter time periods using this buffer. This, however, proved unsuccessful and so consequently there was a need to optimise all stages of the Comet assay to ensure optimal conditions.

The conventional lysis buffer used for animal cells, which contains high concentrations of salts and non-ionic detergents (2.5M NaCl, 0.1M Na_2EDTA, 10mM Tris Base, 1% sodium lauryl sarcosinate, 1% Triton-X-100, 10% DMSO), was tested for extended periods up to 1 hour, but sufficient lysis was not achieved. Consequently, an alkaline lysis buffer (Aoyama *et al.,* 2003), which contains ionic detergents (300mM

NaOH, 1mM Na_2EDTA, 0.01% SDS) was tested for 5, 10, 15 and 20 minutes, with a time of 5 minutes proving the most effective. For the wall-free algae, a lysis time of 1 minute was found to be optimal, after testing 1, 2, 5 and 10 minutes. Lysis was conducted at room temperature according to Erbes *et al.* (1997), rather than the conventional 4°C.

Following lysis, the DNA was allowed to unwind in alkaline electrophoresis buffer (300mM NaOH, 1mM Na_2EDTA, pH12.6) pre-chilled to 4°C, before electrophoresing in the same buffer. A range of unwinding and electrophoresis times were investigated with 5 minutes for unwinding and 10 minutes for electrophoresis proving optimal.

The fluorescent stain most commonly used for detecting nucleic acids is ethidium bromide (e.g. Erbes *et al.,* 1997), and this is commonly employed in the Comet assay at a concentration of 20µg/ml. Initial investigations in our study involved the use of this stain up to a concentration of 200µg/ml, but interestingly it was found that staining was insufficient to enable Comet visualisation following electrophoresis. Vegetative haploid cells of *C. reinhardtii* contain 1 x 10^8 base pairs of DNA (Erbes *et al.*, 1997*)* which is less than for mammalian cells, thus it was considered a DNA stain that allowed a more sensitive detection was required for algal Comet visualisation. A number of unsymmetrical cyanine dyes have been developed that provide more sensitive detection of nucleic acids, and it has been reported by Tuma *et al.* (1999) that SYBR Gold is the most sensitive stain available. Upon testing in our system, this stain proved more suitable for staining algal Comets after application at a 1 in 1000 dilution of a 10000x stock.

The final methodology used is described in Chapter 2, Section 2.6.

3.2.2 Comet Assay Method Development in *Daphnia magna*

3.2.2.1 Modifications to the Assay Protocol

The original Comet assay protocol by Singh *et al.* (1988) was modified to improve suitability for *D. magna*. Conditions for *D. magna* used by den Besten and Tuk (2000) were considered during optimisation of the methodology. Specifically, modifications were made to cell collection and isolation, and the lysis conditions.

A single cell suspension was obtained from whole daphnids by den Besten and Tuk (2000) by crushing daphnids and forcing them through a 150µm polyamide gauze. We initially attempted to obtain a single cell suspension from 20 daphnids (<24h old) placed in 500µl culture media and homogenised using a Precellys 24 homogeniser (Stretton Scientific Ltd) at 6400rpm, for 2 pulses of 10s. The suspension was mixed with 100µl molten (37˚C) LMPA, transferred to an NMPA-coated slide and covered with a cover slip before viewing the cell suspension. This method did not homogenise the daphnids sufficiently to produce single cells, so was deemed unsuitable for the Comet assay. Consequently, methods to isolate nuclei were employed.

Nuclei were successfully isolated from cells by adapting a method by Gomez *et al.* (2001). Daphnids were transferred to ice cold hypotonic citrate buffer (CNS buffer; 3.4mM trisodium citrate, 1.5mM spermine tetrahydrochloride, 0.5mM Tris, 0.1% Nonidet P-40 (octyl phenoxylpolyethoxylethanol), pH 7.6; Gomez *et al.,* 2001) and left for 15 minutes to slow movement. Daphnids were then mechanically dissociated by repeated aspiration through a 1ml pipette tip, and it was found that a final volume of 1ml proved optimal for this process. An increasing number of pipetting actions were tested, with 40 being selected for optimal nuclear yield. The solution was clarified to

remove any solid material by filtering through gauze (50µm) and then subjected to low speed centrifugation (400 x *g*, 4°C, 5 minutes). The supernatant was discarded, and a re-suspension volume of 50µl of ice cold CNS buffer was found to be optimal for the Comet assay, as larger volumes affected LMPA solidification.

According to the work of den Besten and Tuk (2000), following this step, cells are lysed for 1 hour, but this proved too long for daphnid nuclei in our investigations. Consequently, a time of 30 minutes was found to be optimal, after testing for increasing times up to 1 hour.

As previously stated, the DNA is then allowed to unwind and electrophoresed in alkaline electrophoresis buffer. Previous unwinding and electrophoresis conditions reported for *D. magna* were 40 minutes and 20 minutes respectively, both conducted at 18°C (den Besten and Tuk, 2000). These steps are most commonly carried out at 4°C thus we optimised DNA unwinding and electrophoresis at this temperature. Times of 10, 15 and 20 minutes were tested in all combinations at 4°C, and a time of 10 minutes was chosen to be the most effective for both DNA unwinding and electrophoresis. Large DNA tails were visible after 10 minutes unwinding and electrophoresis, but much smaller tails were seen at longer electrophoresis times.

The final methodology used is described in Chapter 2, Section 2.6.

3.2.2.1.1 FPG Modified Comet

The FPG modified version of the Comet assay was employed to investigate the oxidative damage induced by exposure to sodium dichromate. Following lysis, slides

were washed three times with FPG buffer before 50µl FPG buffer containing 1U FPG enzyme (Trevigen, Maryland, US) was added to the slide and spread homogeneously by adding a cover slip. Slides were then placed in a covered box at 37°C for 1 hour before being subjected to DNA unwinding, electrophoresis, neutralisation and staining as described previously (Chapter 2, Section 2.6).

3.3 Methods

See Chapter 2, Sections 2.4.1.1 and 2.4.2 for compound exposure in *C. reinhardtii* and *D. magna* respectively, and Section 2.6 for the final Comet assay methodology.

3.4 Results

3.4.1 Cytotoxicity and Cell Viability for *C. reinhardtii* and *D. magna*

3.4.1.1 Assessment of *C. reinhardtii* Cell Viability

Following exposure to the various test chemicals, the viability of the algal cells, including the controls, was analysed using a colony formation assay. The results (Table 3.1) showed that at the concentrations tested, the compounds used did not have a statistically significant impact on cell viability. For wild type cells, mean viability was >71% of the corresponding control value, and for wall-free algae it was >86%, in all experiments undertaken. This is broadly comparable with 90-100% viability observed for *C. reinhardtii* by Erbes *et al.* (1997), and >82% viability recorded for *E. gracilis* (Aoyama *et al.,* 2003).

3.4.1.2 Assessment of *D. magna* Viability Following LD_{50} Studies

The sensitivity of *D. magna* to chrysoidine, sodium dichromate, BaP and a mixture of sodium dichromate and BaP was investigated in order to establish non-lethal concentrations. The results obtained are summarised in Table 3.2 and Table 3.3. After 24h both survival and rate of movement were recorded (slowed movement was regarded as an indicator of toxicity). Consequently the concentrations that showed no

Table 3.1 The number of wild-type and wall-free colonies of *Chlamydomonas reinhardtii* formed from cultures exposed for 24 hours to chrysoidine, 4-nitroquinoline-1-oxide (NQO) and the N-hydroxy metabolite of 2-acetlyaminofluorene (*N-OH*-2-AAF).

Compound		Strain	Mean Number of Colonies (as % of Control)	Standard Deviation (%)
Chrysoidine	0.1µM	Wild-type	98	32.65
		Wall-free	93	25.65
	10µM	Wild-type	94	26.29
		Wall-free	86	11.40
NQO	1nM	Wild-type	85	16.14
		Wall-free	101	8.81
	5nM	Wild-type	71	31.26
		Wall-free	101	4.60
N-OH-2-AAF	0.05µM	Wild-type	95	26.98
		Wall-free	95	5.82
	5µM	Wild-type	123	48.99
		Wall-free	91	0.60

Colonies were grown from an inoculum of algal cells plated on agar prepared in culture medium for 5-7 days prior to counting. Data are presented as the means of the number of colonies as a percentage of the average number of control colonies (1172), or wall-free colonies (957), n=3.

Table 3.2 The viability of neonate daphnids (<24h old) following a 24 hour exposure to increasing concentrations of the azo-dye chrysoidine.

Final concentration of compound	**Percentage Alive**	**Speed of Movement**
Solvent (DMSO) control (0.1% v/v) or untreated control	100%	+++
3μM	63%	+
2μM	100%	+++
1μM	100%	+++
0.5μM	100%	+++
0.1μM	100%	+++

Any mortality was recorded and the rate of movement of live daphnids was compared to those in optimum conditions as an indicator of toxicity. +++ Fast ("normal" speed); ++ Slow; + Very slow movement.

Table 3.3 Viability of neonate (<24h) daphnids following a 24 hour exposure to sodium dichromate and benzo[a]pyrene, individually and in combination, for the Comet assay.

Final Concentration of Compound	Percentage Alive	Speed of Movement
Control (Solvent (DMSO)/Water)	100%	**+++**
Benzo[a]pyrene (µM)		
1	10%	+
0.5	80%	++
0.2	100%	+++
0.1	100%	+++
0.01	100%	+++
0.05	100%	+++
Sodium Dichromate (µM)		
4	0%	-
3	0%	-
2	50%	+/++
1	100%	+++
0.75	100%	+++
0.5	100%	+++
0.25	100%	+++
Benzo[a]pyrene (µM) + Sodium dichromate (µM)		
0.2 + 1	90%	+
0.1 + 0.75	100%	+++
0.05 + 0.5	100%	+++
0.01 + 0.25	100%	+++

Viability was analysed by the number alive and speed of movement as compared to control cultures. Note: +++ Fast ("normal" speed); ++ Slow; + Very slow movement.

toxicity (100% viability) for either the individual or combined exposures were chosen for analysis by Comet assay.

3.4.2 Direct DNA Strand Breaks Induced in *C. reinhardtii* and *D. magna*

3.4.2.1 The Effect of Light on Background DNA Strand Breaks in *C. reinhardtii*

As stated in the general methods chapter, Section 2.2.1, two light sources were employed to culture wild-type algal cells. One was a high output Marine Blue 420 Actinic bulb (Arcadia, Redhill, UK), while the other was a Gro-Lux photosynthetic tube (Sylvania, Danvers, US). DNA strand breaks in algal cells following exposure to each light source measured by Comet assay were compared (Figure 3.2). Using the high output marine blue actinic bulb it was found that prepared samples of cells exhibited relatively high levels of baseline DNA strand breaks when compared to those cultured under the Gro-Lux tube. This difference was supported statistically, with a significant ($P<0.05$) reduction in percentage tail DNA in algal cells after a subsequent two weeks growth under the Gro-Lux tube, and a further significant decrease ($P<0.0005$) after two months, when compared to algal cells grown under the actinic bulb. In addition, an effect on population growth was also observed in association with the different light sources. An optical density (OD) of 0.24 (at 660nm) was recorded on day 5 of culture under the Gro-Lux fluorescent tube, which equates to approximately $2.65x10^6$cells/ml. This was significantly higher ($P<0.0001$) than the OD of 0.099 (660nm) on day 5 (approximately $6.0x10^5$cells/ml) following culture of algae under the high output marine blue actinic bulb. Consequently the Gro-Lux bulb was used for both culturing and exposures, but a comparison of background DNA damage was made between the two light sources. The

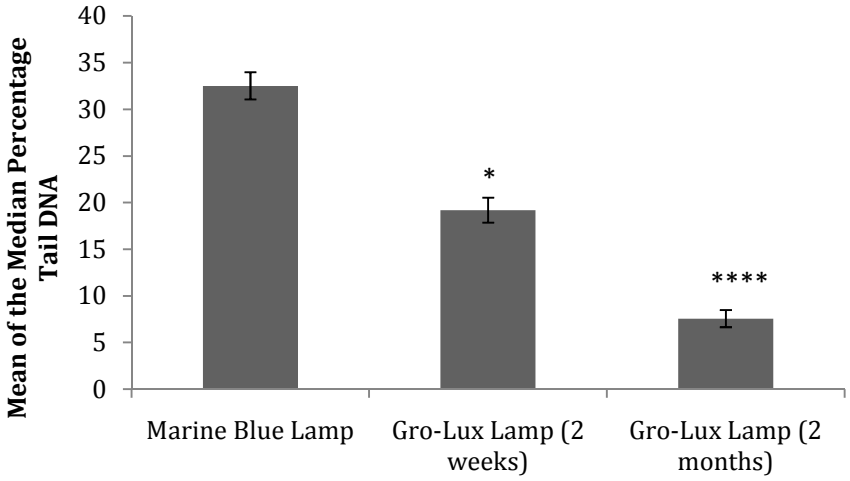

Figure 3.2 The time-dependent decrease in DNA strand breakage as measured by Comet assay induced by light when cultures of *Chlamydomonas reinhardtii* were moved from a high output marine blue 420 actinic bulb to a Gro-Lux fluorescent tube with output close to the natural wavelengths for photosynthesis (440-490nm and 625-750nm). Data are presented as means of median values ± standard deviation, n=3. Statistically significant differences are represented as * (P<0.05) and **** (P<0.0005).

relationship with time for both the absorbance values and cell counts was shown to be linear following linear regression analysis ($P<0.001$, $R^2=0.9889$ and $R^2=0.9591$ respectively).

3.4.2.2 The Effect of Compound Exposure on DNA Strand Breaks in Wild-Type and Wall-Free *C. reinhardtii*

Exposure to both chrysoidine and *N-OH*-2-AAF resulted in statistically significant increases in DNA strand breaks: at 0.1μM ($P<0.05$) and 10μM ($P<0.01$) for chrysoidine; and at 0.05μM ($P<0.05$) and 5μM ($P<0.01$) for *N-OH*-2AAF (Figure 3.3). Although direct comparison of the two concentrations of NQO with the control did not show statistical significance, there was also a linear trend for elevated DNA strand breakage after exposure which was found to be statistically significant following linear regression analysis ($P<0.05$, $R^2=0.9672$). Concentrations of each compound above the highest concentrations used in the study were shown to have cytotoxic effects as assessed by a colony formation assay. The maximum percentage tail DNA values obtained after exposure to the three compounds were: 13.9% following exposure to 10μM chrysoidine, 12.7% for 0.05μM *N-OH*-2-AAF, and 14.1% for 5nM NQO; all compared with a solvent control value of 7.6%.

Although statistically supported concentration-dependent increases in DNA strand breaks were detectable in these cells, the level of damage detected was relatively small when compared with other cells and organisms (see discussion section). Consequently, to investigate if the presence of a cell wall was affecting sensitivity to these compounds by impeding compound uptake, a wall free mutant was employed. The

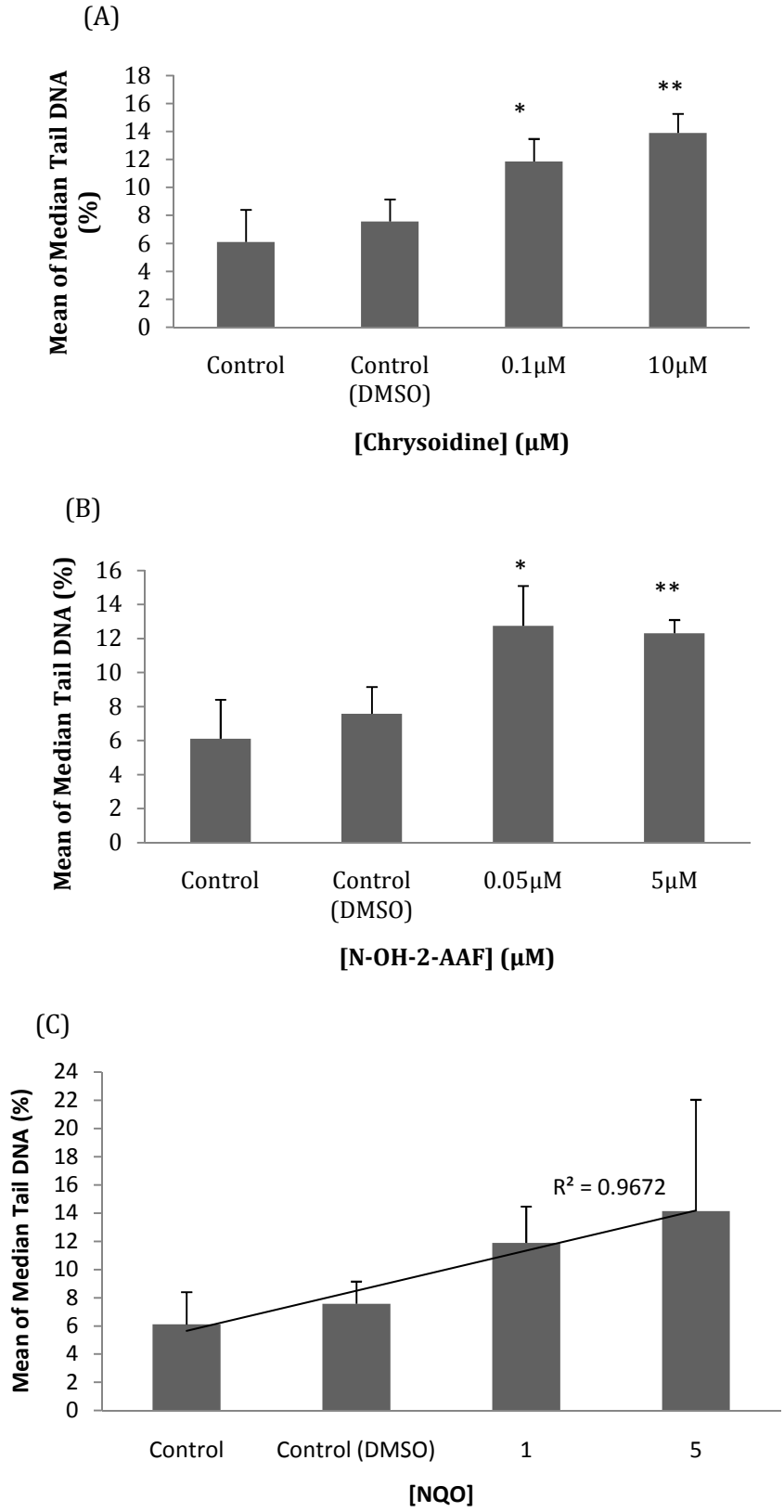

Figure 3.3 DNA strand breaks following exposure to (A) chrysoidine, (B) the N-hydroxy metabolite of 2-acetylaminofluorene (*N*-OH-2-AAF) and (C) 4-nitroquinoline-1-oxide (NQO), all measured in wild-type *Chlamydomonas reinhardtii* and measured by Comet assay. Data are presented as means of median values ± standard deviation, n=3. Statistically significant differences are represented as * ($P<0.05$) and ** ($P<0.01$). Trend analysis for NQO is indicated.

results obtained following the exposure of the wall-free mutant to the same test substances and concentrations, however, revealed no difference compared with the wild type algal cells. Indeed, responses were actually significantly lower than observed for the wild-type cells (Figure 3.4; $P<0.05$ for 0.1µM chrysoidine and 0.05µM *N*-OH-2-AAF, $P<0.01$ for 1nm NQO, and $P<0.001$ for 5µM *N*-OH-2-AAF).

3.4.2.3 Reduced Glutathione (GSH)

In addition to EROD activity, GSH was measured to indicate the degree of protection that might be afforded by this tripeptide. GSH levels were found to be relatively low 0.48nmol GSH/10^6cells (SD=0.19, n=6), when compared with other cell systems (see discussion, section 3.5). Mean GSH levels were also normalised to protein (mg) measured by the Bradford method (14.98nmol GSH/mg protein; SD=6.02, n=6) and the BCA assay (0.46nmol GSH/mg protein; SD=0.18, n=6). It has been suggested that the Bradford assay underestimates protein levels in algal cells (Crossman *et al.*, 2000). The Coomassie Brilliant Blue dye binds to protein, mainly via arginine residues, but also to basic (lysine and histidine) and aromatic (tyrosine, tryptophan and phenylalanine) residues of amino acids. Many species of algae have low levels of the aromatic (tyrosine and tryptophan) and basic (lysine and histidine) amino acids. Thus the dye mainly binds to arginine and phenylalanine, which is thought to account, at least in part, for the low protein levels (Barbarino and Lourenco, 2005). It has also been shown that algal extracts can significantly interfere with the Bradford assay, which may also account for the low estimates of protein (Crossman *et al.,* 2000). Our study is in accord with these findings, obtaining protein levels of 0.47mg protein/ml using the Bradford method compared

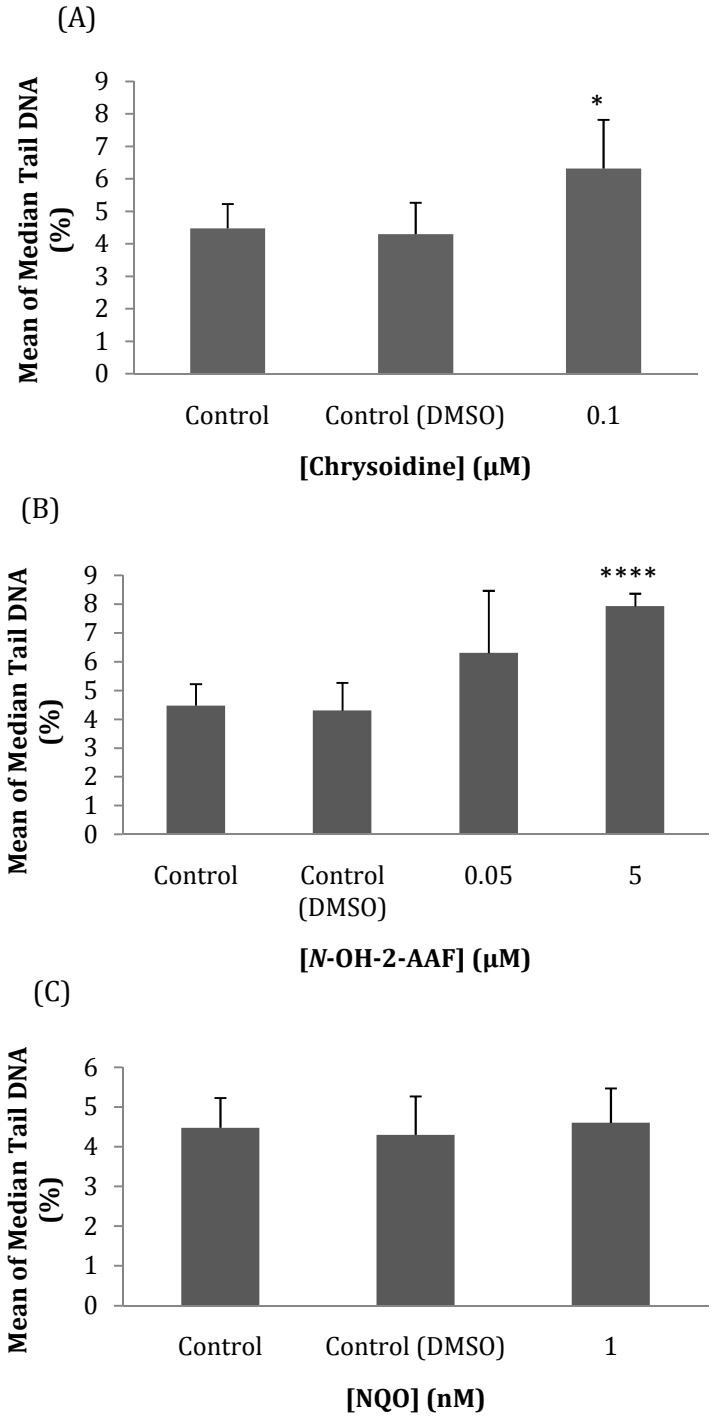

Figure 3.4 DNA strand breaks following exposure to (A) chrysoidine, (B) the N-hydroxy metabolite of 2-acetylaminofluorene (*N*-OH-2-AAF) and (C) 4-nitroquinoline-1-oxide (NQO), all measured in wall-free *Chlamydomonas reinhardtii* and detected by Comet assay. Data are presented as means of median values ± standard deviation, n=3. Statistically significant differences are represented as * ($P<0.05$) and **** ($P<0.0005$).

with 15.8mg protein/ml from the BCA assay. Normalising GSH levels to 10^6 cells gave a similar value as when related to protein levels/10^6 cells measured using the BCA assay (0.48nmol GSH/10^6cells compared with 0.43nmol GSH/mg protein/10^6 cells), thus we felt that cell number was an appropriate method for normalisation.

3.4.2.4 The Effect of Compound Exposure on DNA Strand Breaks in *D. magna*

None of the exposures to any of the compounds tested resulted in statistically significant increases in DNA strand breaks (Figure 3.5, Figure 3.6, and Figure 3.7). Although there appears to be a trend for elevated strand breaks after exposure to chrysoidine up to 1μM and the mixture of sodium dichromate and BaP (up to 0.01μM and 0.25μM respectively) following application of the FPG modified Comet assay, this was not statistically significant, most likely due to high variability and high control values.

The results indicate low sensitivity, and one possible reason for this is that the cells used in the assay were obtained from a homogenate of whole animals, thus a mixture of cell types, were present. Potentially only a small population of the cells present would show a response to the genotoxicants in the form of strand breaks (such as hepatocytes containing CYPs if the compounds require metabolic activation), which may be masked by the large number of non-responding cells. Thus to test this hypothesis, for the FPG and non-FPG data for the combined sodium dichromate and BaP exposures, we divided the Comets into categories of increasing percentage tail DNA to investigate whether the number in the higher categories increased with increasing dose. The results did not reveal such an increase, however, implying that there is not a sub-population of responding cells (Figure 3.8).

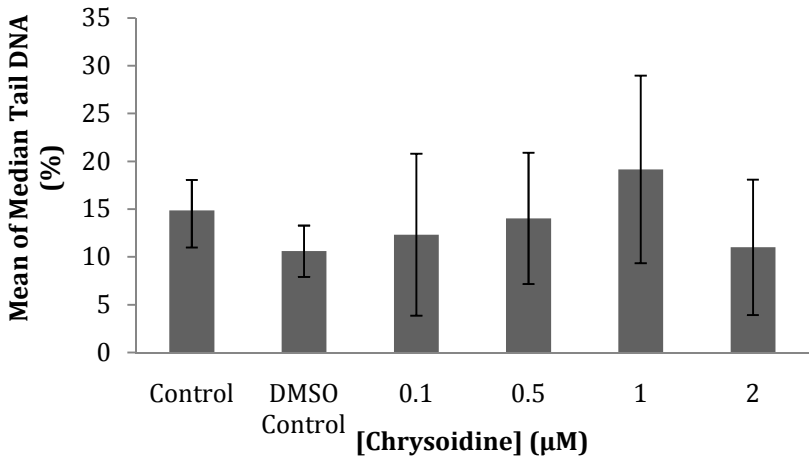

Figure 3.5 DNA strand breaks following exposure to chrysoidine measured in neonate (<24h) *Daphnia magna* as percentage tail DNA by Comet assay. Data are presented as means of median values ± standard deviation, n=3.

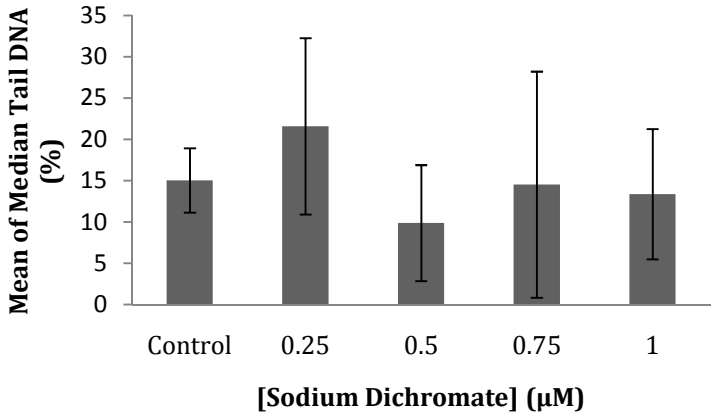

Figure 3.6 DNA strand breaks following exposure to sodium dichromate measured in neonate (<24h) *Daphnia magna* as percentage tail DNA by Comet assay. Data are presented as means of median values ± standard deviation, n=4.

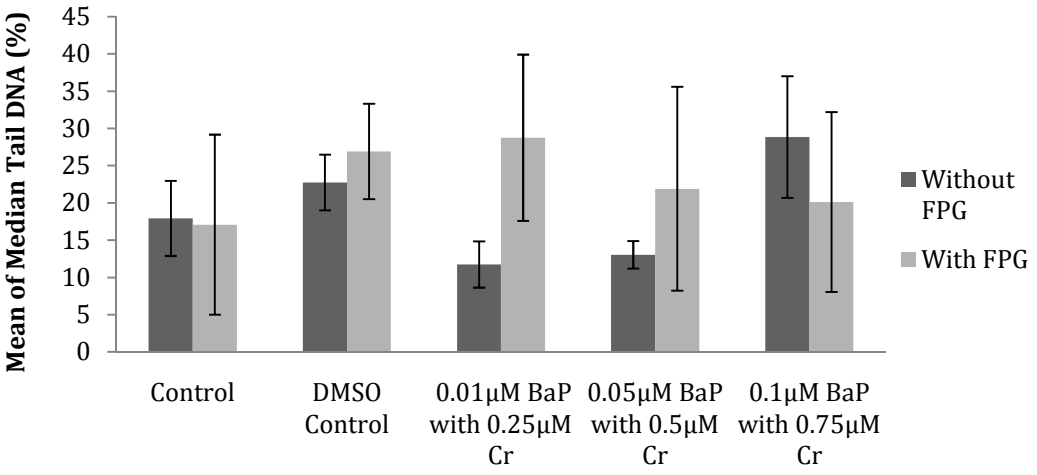

Figure 3.7 DNA strand breaks following a combined exposure to sodium dichromate (Cr) and benzo[a]pyrene (BaP), measured in neonate (<24h) *Daphnia magna* as percentage tail DNA by Comet assay with or without formamidopyrimidine DNA glycosylase (FPG) modification to detect oxidative damage. Data are presented as means of median values ± standard deviation, n=3.

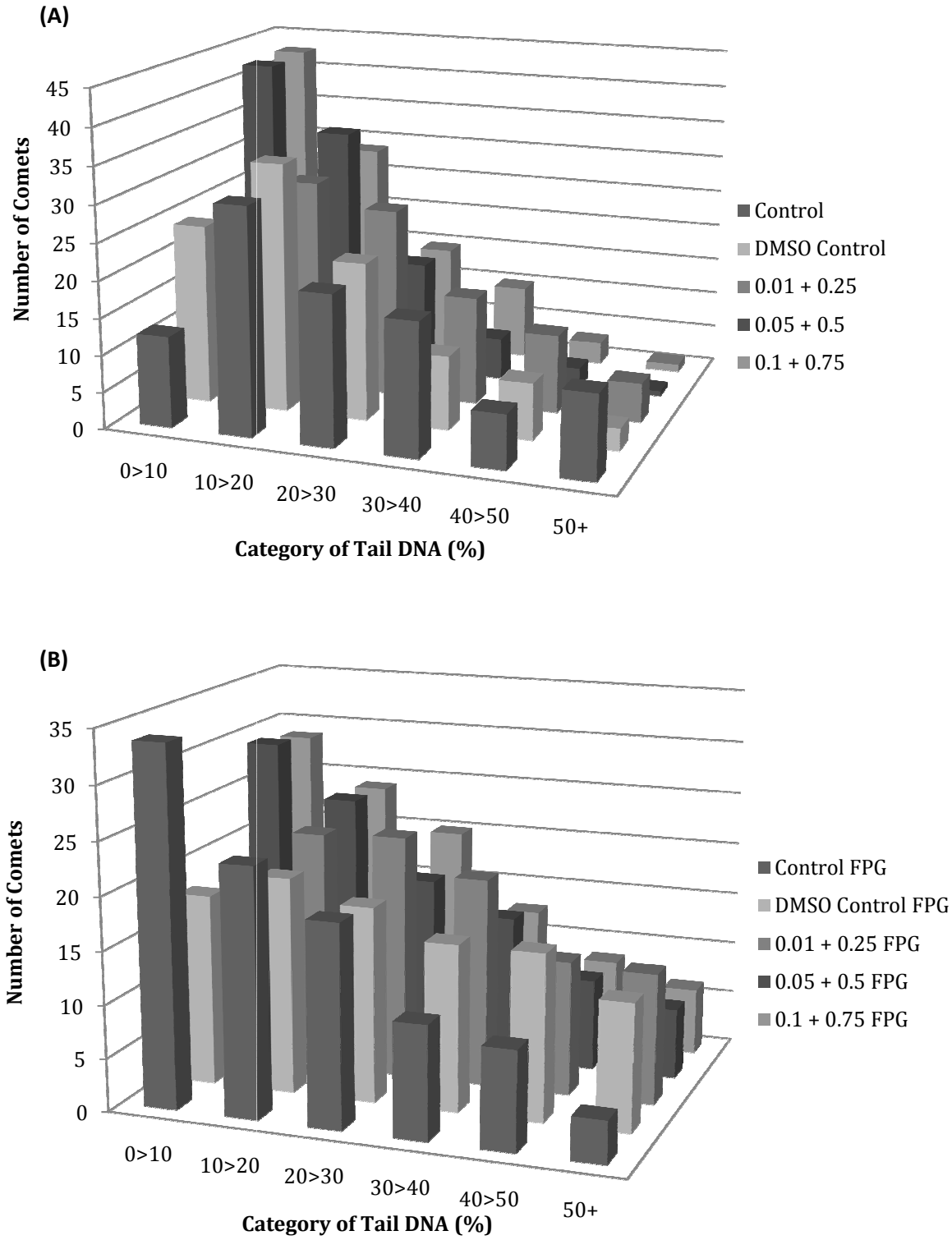

Figure 3.8 The percentage of Comets that fall into categories of increasing percentage tail DNA (0>10%, 10>20%, 20>30%, 30>40%, 40>50% and 50%+) following a combined exposure to sodium dichromate and benzo[a]pyrene (BaP), measured in *Daphnia magna* by Comet assay without (A) or with (B) formamidopyrimidine DNA glycosylase (FPG) modification. Data are presented as the mean number of comets per category of % tail DNA, n=3.

3.5 Discussion

Unicellular algae, including *C. reinhardtii*, and the crustacean *D. magna* are widely used for testing the environmental effects of chemicals and effluents, but current testing methodology focuses on organismal- and population-level endpoints (e.g. lethality and population growth; Walker *et al.,* 2006, OECD guideline 202, OECD guideline 211, Hutchinson *et al.,* 2006). Consequently, there are relatively few published data on the sub-organismal and sub-cellular response of such species. The current study, therefore, aimed to address this by developing methodology for the Comet assay to be applied to *C. reinhardtii* and *D. magna* to enable detection of DNA strand breaks as a marker of exposure to genotoxicants.

We compared the growth and background DNA integrity of *C. reinhardtii* using two light sources of different wavelength characteristics. One was a high output marine blue actinic bulb (Arcadia, Redhill, UK) while the other was a Gro-Lux photosynthetic bulb (Sylvania, Danvers, US). DNA strand breaks in algal cells grown under each light source were detected by Comet assay, and it was noted that algal cells grown under the Marine Blue Actinic bulb were exhibiting higher levels of DNA strand breaks than those grown under the Gro-Lux tube. This led to the suggestion that the different wavelength characteristics of the two bulbs may be having an effect. Ultraviolet radiation (UV), both UVB (280-320nm) and UVA (320-400nm) induce diverse structural damage to DNA (Sastre *et al.,* 2001). The marine blue actinic blub has a UVA component (predominant output is 380-480nm, Figure 3.9) and UVA radiation is known to induce oxidative DNA damage via the generation of reactive oxygen species resulting in the production of the pre-mutagenic oxidised base 8-hydroxy-deoxyguanosine (8-OH-dG; Kvam and Tyrrell,

1997). This damaged base pairs with cytosine and provides a substrate for 8-oxoguanine glycosylase 1 (OGG1), which cleaves 8-OH-dG from DNA (Cooke *et al.,* 2003) resulting in a strand break, which can be detected via the Comet assay. In contrast, the Gro-Lux tube illuminates with light of blue and red radiation only (440-490nm and 625-750nm respectively, Figure 3.10), which are outside of the UV spectrum thus substantially reducing any potential for UV-induced DNA damage.

Further to this, it was observed that algal population growth was much greater under the Gro-Lux tube than the marine blue actinic bulb. It is conceivable that this reduction in population growth was also due, at least in part, to the different wavelengths of light emitted by the two bulbs. Chlorophyll exists in two forms, chlorophyll a and chlorophyll b, which both absorb light in two distinct wavelength bands (400-500nm and 600-700nm; Figure 3.11). The Gro-Lux tube illuminates with light in both these wavelength ranges (440-490nm and 625-750nm), whereas the marine blue actinic bulb predominantly emits light below approximately 480nm. Illumination by the marine blue actinic bulb only may therefore be insufficient to allow photosynthesis and thus growth to occur at its maximum rate. Alternatively, the high levels of DNA damage may induce cell death and thus a reduction in the number of cells in the population. This is reflected by the lower OD and algal cell numbers recorded from cultures grown under this bulb compared to those cultured under Gro-Lux illumination (see Section 3.4.2.1).

Overall, these results indicate a high sensitivity of *C. reinhardtii* to light outside of the wavelengths ultimately used, and serve to emphasise the importance of appropriate lighting conditions when investigating the effects of compounds on DNA integrity (and

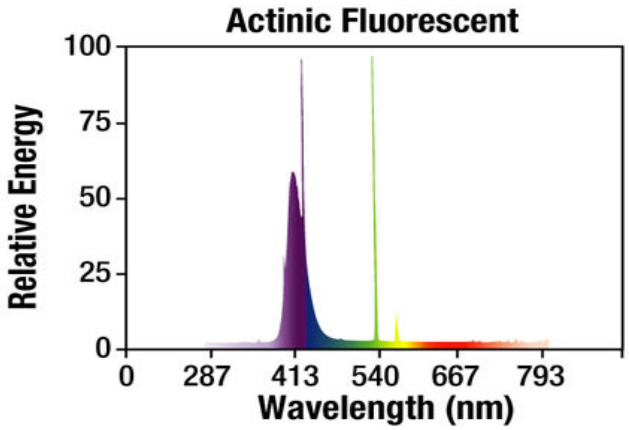

Figure 3.9 Wavelength spectrum for marine blue actinic bulb

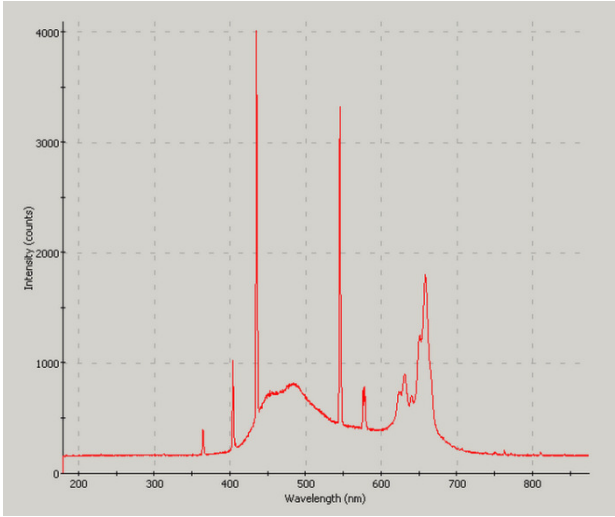

Figure 3.10 Wavelength spectrum for Gro-lux bulb, used for culturing *Chlamydomonas reinhardtii*.

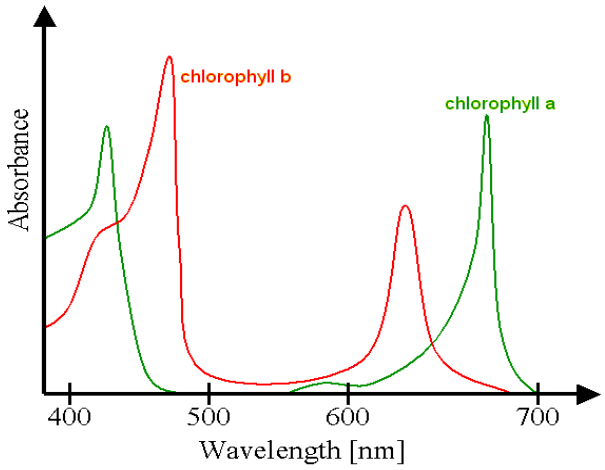

Figure 3.11 Absorbance spectrum of chlorophyll a and chlorophyll b.

providing conditions for optimal photosynthesis). In particular, it is important to ensure that cultures are isolated from exposure to other light sources, including natural sunlight, as this may alter the spectrum of light the algae are exposed to, potentially affecting experimental results. In addition, there are also potential implications regarding the susceptibility of unicellular algae to light-induced genotoxicity in the environment, particularly when considering the associated population-level effects. In support, several studies have shown that UV light can cause decreased growth rates in algae (e.g. Buma *et al.,* 1996; Buma *et al.,* 1997). This, therefore, suggests that UV-light induced DNA damage may cause population level effects, and as such DNA damage in this context could be considered ecologically relevant.

Exposure of *C. reinhardtii* to the pro-genotoxic agent chrysoidine, up to a concentration of 10μM, produced a concentration-dependent, and statistically significant, increase in DNA strand breakage. Exposure to the direct acting genetic toxicant *N-OH*-2-AAF also produced a statistically significant increase in DNA strand breakage at both 0.05μM and 5μM, although in a contrast to chrysoidine, no further increase was observed as the dose was increased from 0.05μM to 5μM. A further direct acting genotoxicant NQO exhibited a trend for elevated percentage tail DNA, which was found to be statistically significant following linear regression analysis, but the increased values of percentage tail DNA following treatment were found not to be statistically significant from those exhibited by the control cells. Interestingly, Erbes *et al.* (1997) also exposed *C. reinhardtii* to 5nM NQO, and did not record significant differences at this concentration.

Although the results show that *C. reinhardtii* is responsive to these direct and indirect (require metabolism) acting genotoxic chemicals, with maximum values of percentage DNA similar for all compounds, the sensitivity to both direct and indirect agents is relatively low. For example, a tail moment of around 8 was recorded following exposure of the rainbow trout cell lines RTG-2 and RTL-W1 for 2h to 5nM NQO (Nehls and Segner, 2001), which is in contrast to a tail moment of 1.5 following a 24h exposure in this study. However Erbes *et al.* (1997) observed a tail moment of approximately 2 following a 24h exposure to 5nM NQO in *C. reinhardtii*, which is more similar to the value obtained in our study. Furthermore, the longest tail moment recorded by Erbes *et al.* (1997) was only approximately 6 following a 24h exposure to 50μM NQO, further supporting the conclusion of low sensitivity of this alga.

We hypothesised that the low sensitivity might relate to hindrance of chemical uptake by the cell wall. Consequently, to investigate this further, a wall-free strain of *C. reinhardtii* was employed, and DNA strand breaks again assessed after exposure under identical conditions. From the data obtained, however, we demonstrated that the lack of a cell wall did not enhance the susceptibility of this species to the genotoxic effects of these agents. This wall-free mutant has previously been used in a number of toxicological studies including: testing anti-tumour agents as an alternative to mammalian cell lines (Maucourt *et al.,* 2002); testing the toxicity of heavy metals as a function of cell growth and volume (Prasad *et al.,* 1998); and investigating the role of the cell wall to heavy metal tolerance (Cain and Allen., 1980). However, the application of this mutant in genotoxicity testing and to the Comet assay has not to our knowledge previously been reported in the literature. Herein we show the utility in emphasising

that the cell wall is not a limiting factor in restricting the response of *C. reinhardtii* to at least some genotoxic chemicals.

Another potential reason for the absence of a large increase in DNA strand breakage following exposure to these well known genotoxicants is that the cells were well protected against reactive intermediates. In this respect, reduced glutathione (GSH) concentrations were ascertained in the same algal cells, as an indication of the level of this major protectant. The mean level of GSH measured in *C. reinhardtii* was 0.48nmol GSH/10^6cells (SD=0.19, n=6). This level of GSH is lower than that reported for many mammalian cell types (e.g. 5nmol/mg protein in L929 cells (Mehlen *et al.,* 1996) compared to 0.46nmol/mg protein in algae) and while GSH has been shown to have a key regulatory role in algae (Irihimovitch and Shapira, 2000) it is not suggestive of a preferential protection in algal cells, although we recognise that additional anti-oxidants may be present.

Despite successful application of the Comet assay to *D. magna* cells, as illustrated by the Comet images in Figure 3.12, the results do not indicate a clear response to the genotoxic agents investigated. No statistically significant increases were observed for any of the compounds, nor does there appear to be any obvious trend for increasing tail DNA with increasing concentration. There was high variability in all samples, which may account, at least in part, for the lack of statistical significance and obvious dose response. The background level of strand breaks was also relatively high (e.g. compared to *C. reinhardtii*; approximately 15% in *D. magna* compared to 5% in algae) and it is possible that the method of nuclei isolation may have contributed to this. However, this technique was optimised with this in mind, and homogenisation was limited to ensure

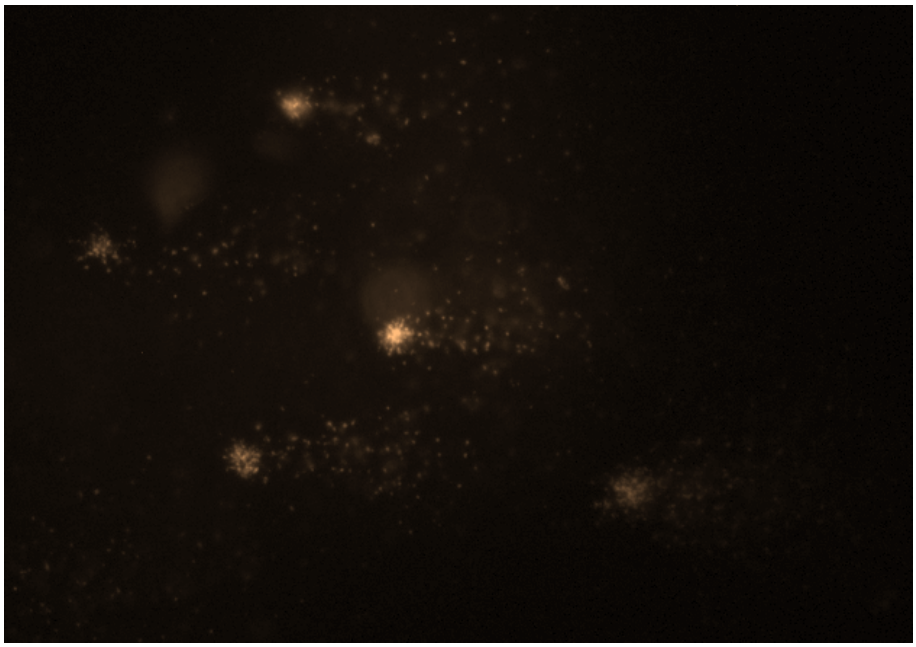

Figure 3.12 Images of Comets observed following exposure of *Daphnia magna* neonates (<24h old) to 1µM chrysoidine for 24 hours. Viewed at 360x magnification.

minimum damage and maximum yield. Furthermore, the method of preparation was consistent throughout and would still enable damage to be detected should the treatments be effective.

Experiments undertaken *in vivo* have been shown to result in more variability in strand break levels compared with *in vitro* exposures. For example, Wilson *et al.* (1998) observed much less marked increases in DNA strand breaks from *in vivo* exposures in mussels, observing that damage was spread between the different categories, compared with mussel cells *in vitro*, which showed a significant concentration dependent increase in strand breaks. High variability between individual animals (mussels) has also been observed (Nacci *et al.,* 1996) and since our Comet analyses were conducted on pooled samples, this may be contributing to the variability. It would therefore be ideal to conduct Comet assay analyses of DNA damage in individual animals, but the small size of neonate daphnids, and thus yield of nuclei, currently limits this approach.

Another potential explanation for the variability is the presence of a mixture of cell type, since the comet assay is traditionally applied to cells of the same type (Cotelle and Ferad, 1999). Moreover, the mixture of cells may vary in their responsiveness to genotoxicants, and if only a small number of cells are responding to genotoxicant exposure, as may be the case, particularly for those requiring metabolic activation, this response may be masked if the majority of nuclei present on the Comet slide are from non-responding cells. Consequently, to further investigate this we looked at the distribution of Comets within the samples, dividing them into categories of increasing percentage tail DNA, with the hypothesis that the number of Comets in the higher categories would increase with an increase in dose. From the resultant graphs, however,

it did not appear that there was a subset of responding cells. This could be attributed, at least for BaP, to low metabolic activation (see Chapter 4, Section 4.4 for more detail). BaP requires oxidation by the CYP enzyme system to a genotoxic product, thus a low activation of BaP may translate to a low level of DNA damage. In crustaceans the hepatopancreas contains the highest concentrations of CYPs (James and Boyle, 1998), thus it might be expected that following exposure to chemicals requiring metabolic activation higher levels of strand breaks would be detected in these cells. It would therefore be interesting to analyse levels of DNA damage in these cells only, if this were practically feasible.

To test the *D. magna* system further with respect to specific oxidative damage we investigated the effect of BaP and sodium dichromate. Sodium dichromate is a hexavalent chromium compound (Cr(VI)) and compounds of this type have been shown to be carcinogenic in both *in vitro* and *in vivo* studies, with IARC having classified Cr(VI) as a group 1 carcinogen (Lee *et al.,* 2005). Cr(VI) compounds are genotoxic and can cause numerous DNA lesions including strand breaks, chromium-DNA adducts, abasic sites, oxidative base damage, including 8-OH-dG, and DNA-DNA and DNA-protein cross links *in vitro* (Emmanouil *et al.,* 2006; Lee *et al*., 2005). The FPG modified Comet assay detects oxidised purines, particularly 8-OH-dG (Collins, 2004) since excision of the oxidised base by FPG results in a strand break, which is detectable using this assay. Thus this version of the assay was employed to investigate whether there was an increase in the amount of tail DNA following a combined exposure of sodium dichromate and BaP. The results do appear to show an increase in percentage tail DNA compared to that detected using the standard assay for increasing combination doses up to 0.05μM BaP

plus 0.5μM sodium dichromate (Figure 3.7), but this trend was not supported statistically, most likely due to the high variability in the samples.

There are currently three published applications of the Comet assay using *D. magna* in the literature (den Besten and Tuk, 2000; Park and Choi, 2007; Lee *et al.*, 2009).

In the study by den Besten and Tuk (2000), responses were reported following a 48h exposure of neonates (<24h) to NQO (LOEC 56μg/l), ethyl methane sulphonate (LOEC 100mg/l), hydrogen peroxide (LOEC 5.6mg/l) and lindane (>1.8mg/l). Responses were also reported in offspring from continuously exposed adults, following a 48h exposure to NQO, cadmium chloride and potassium dichromate. It was reported that Comet tails were measured using a micrometer, however, no information regarding the length of the tails for Comets from control or treated daphnids was provided, thus making comparisons to our study difficult. In addition, the extent to which the sensitivity of this organism to such chemicals, and the utility of the Comet assay in *D. magna* can be interpreted from this study is limited, as comparisons of tail length to those measured in other organisms cannot be made. However, the longer exposure periods used in this published study do support the idea that this species is relatively insensitive to the genotoxic effects of some chemicals. Indeed, it may be pertinent to use a longer exposure period with our test compounds to assess whether a significant induction in DNA strand breaks may be attainable at these concentrations.

The studies by Park and Choi (2007) and Lee *et al.,* (2009) used tail moment as the parameter for DNA damage. Following exposure to bisphenol A, statistically significant, but small, increases in tail moment were observed at 0.009 and 0.088μM relative to the

control (Park and Choi, 2007), but no effect on strand breaks was observed following exposure to nonylphenol (0.005, 0.045, 0.454μM). Lee *et al.,* (2009) observed a statistically significant increase in tail moment following exposure to 15nm CeO_2 particles compared to the control, but no differences were seen following exposure to SiO_2 and TiO_2 particles. The data for tail moment obtained in our study indicates a trend for an increase in tail moment with increasing dose, up to 0.5μM for sodium dichromate, but this was not supported statistically due to high variability (Appendix 1, Figure 1). The published study did not look at % tail DNA, which has been reported to be the most useful parameter to measure DNA damage using the Comet assay (Collins, 2004, see Chapter 2, Section 2.6.2 for more detail). Moreover, Collins (2004) reported that tail moment does not have a linear relationship with dose, and does not provide an indication as to the appearance of the comet, thus it would be beneficial to investigate whether the small differences observed by Lee *et al.,* (2009) were also present upon analysis of the data using % tail DNA as the parameter. In all, these published data support the relative insensitivity of the Comet assay for measuring the effects of genotoxic chemicals in *D. magna,* and ultimately, these published studies together with our data indicate a relatively low sensitivity of the Comet assay when applied to *D. magna*.

In conclusion, this study has shown that DNA damage induced by both direct- and indirect-acting genotoxic agents in *C. reinhardtii* can be measured using the Comet assay. However, there is a comparatively low sensitivity, the reason for which appears not to be related to existence of the cell wall or to a high GSH protection. High variability in the results obtained using nuclei from *D. magna* cells indicates that the Comet assay does not allow sensitive enough detection of strand breaks in this organism, at least using the

method developed here. Sensitivity is paramount when developing assays for environmental pollutants since contaminants are likely to be present at low concentrations, thus the lack of sensitivity demonstrated in applying the Comet assay to *D. magna* in particular raises questions regarding its applicability in this capacity.

In all, the Comet assay methodology was adapted for use with both algal and daphnid cells. The data obtained for *C. reinhardtii* have added to our understanding of the genotoxic response of this important unicellular alga, and sensitivity issues aside, suggest that this species may be suitable for the measurement of DNA strand breaks as an endpoint as part of a suite of ecotoxicological test methods, although its low sensitivity may necessitate the use of higher test substance concentrations than are needed with other test organisms. In relation to *D. magna*, research into using a single daphnid for the assay, or indeed a single cell type, may enable more sensitive detection of DNA damage in this organism.

One component that may relate to sensitivity (for pro-genotoxic agents) is that of limited metabolic activation, and as such the capacity for *C. reinhardtii* and *D. magna* to bioactivate such chemicals is considered in the next chapter.

CHAPTER FOUR: THE CAPABILITIES OF *C. REINHARDTII* AND *D. MAGNA* TO BIOACTIVATE XENOBIOTICS AND THE IMPORTANCE OF THIS FOR ECOTOXICITY TESTING

4.1 Introduction

Biotransformation is an important consideration for ecotoxicity testing as it is known to be a key modulator of the toxicity and bioaccumulation of xenobiotics (see Chapter 1, Section 1.7). Specifically, when considering the effects of genotoxicants, many pro-genotoxicants exist, which require metabolic activation to DNA-damaging products. To design toxicity tests effectively, to elucidate biomarkers, or to model the chemical fate of compounds, it is essential that qualitative and quantitative data on biotransformation pathways be obtained (Livingstone, 1998). It is, therefore, important that chemical detoxification systems are characterised in species of interest, both because of their use in aquatic toxicology, and to establish whether their response to xenobiotics is representative of other organisms.

As discussed in section 1.7.1, Cytochrome P450s (CYPs) are the most important enzyme groups involved in the catalysis of phase I reactions (Buhler and Wang-Buhler, 1998). They play a key role in a range of processes including carcinogenesis and environmental toxicology (Stegeman and Livingstone, 1998), thus it is important to understand the existence of CYPs and their roles in aquatic organisms. Many isoforms appear to be relatively highly conserved across taxa (Gonzalez and Nebert, 1990), with forms identified in some species of algae (Thies *et al.,* 1996; Barque *et al.,* 2002), and invertebrates (Snyder, 2000). However, limited information is available regarding the involvement of CYPs in xenobiotic metabolism in these organisms.

4.1.1 Role of CYPs in Plants

The presence of CYPs in plants is relatively well documented. Reports have been made of the involvement of these enzymes in a large number of vital reactions in plant secondary metabolism (hydroxylation, epoxidation, oxygenation and heteroatom dealkylation reactions; Bolwell *et al.,* 1994), the response of plants to foreign compounds (Thies *et al*., 1996), and *N*- and *O*-dealkylation reactions of xenobiotics (Thies and Grimme, 1996). Moreover, peroxidases and flavin monooxygenases (FMOs), which have been shown to be involved in the activation of a number of pro-mutagens including 2-aminofluorene (2-AF), are widely distributed and highly abundant in plants (Chiapella *et al.,* 2000).

It is therefore apparent that plant species in general possess phase I metabolic capabilities and thus the ability to bioactivate pro-genotoxins, but the situation regarding phase I metabolism and CYPs in unicellular algae specifically is less clear. Of the few published studies (summarised in Table 4.1), xenobiotic metabolism has been recorded for some algal species, although the extent of the metabolism reported has been variable. When specifically considering CYPs, Thies *et al.* (1996) reported findings of diverse CYPs in *Chlorella* spp.; the sequenced genome of *C. reinhardtii* has revealed 39 CYP genes (Nelson, 2006); and *Euglena gracilis* cell lines exhibited CYPs immunologically related to those found in mammals (Barque *et al.,* 2002). The relative contribution of these CYPs to endogenous versus xenobiotic metabolism, however, is not clear, although Sauser *et al.,* (1998) have demonstrated a CYP system in *Selenastrum capricornutum*. This alga was able to activate 2-AF, and pre-treatment with CYP inducers such as Aroclor enhanced this activation.

Table 4.1 Xenobiotic biotransformation capabilities of different species of algae reported in the literature

Algal Species	Compound	Metabolism/Enzyme System	Study
Chlamydomonas spp.	Lindane, naphthalene, phenol	Biotransformation	Semple *et al.,* (1999)
	4-chloro-3,5-dinitrobenzoic acid	De-halogenation via meta cleavage produces 2-hydroxymuconic semi-aldehyde. (Grown in light, and in dark on acetate.)	
	Iso-octane-extracted polycyclic aromatic hydrocarbons (PAHs) from diesel particulate exhaust	Able to remove up to 95% of the PAHs following suitable adaptation (mechanisms unclear). Minimal removal before adaptation.	
Chlorella spp.	Lindane, chlordimeform	Biotransformation	Semple *et al.,* (1999)
	Azo dyes (Eriochrome blueSE and blackT), aniline	Decolourised and used as carbon and nitrogen sources (dependent on chemical structure). Proposed metabolic scheme outlined in Figure 4.1	
Cyanobacteria, eukaryotic microalgae	Naphthalene	Proposed metabolic scheme outlined in Figure 4.2	Semple *et al.,* (1999)

Selenastrum capricornutum	Benzo[a]pyrene (BaP)	Dioxygenase enzyme system implied (cis-dihydrodiols produced) followed by conjugation and excretion under photoautotrophic conditions (gold light).	Warshawsky *et al.* (1995) Schoeny *et al.,* 1988
	Pro-mutagens	At least two enzyme systems shown to activate	Sauser *et al.* (1998)
	2-Aminofluorene (2-AF)	Cytochrome P450 (CYP) system: pre-treatment with known CYP inducers increased mutagenicity, with CYP inhibitor decreased activation.	
Galdieria sulphuraria, Chlorella fusca var. *fusca, Selenastrum minutum* and *Navicula pelliculosa*	Mercury (Hg^{III})	Biotransform Hg^{III} into mercury sulfide (β-HgS) and Hg^{0} Aerated and pH controlled conditions	Kelly *et al.* (2007)

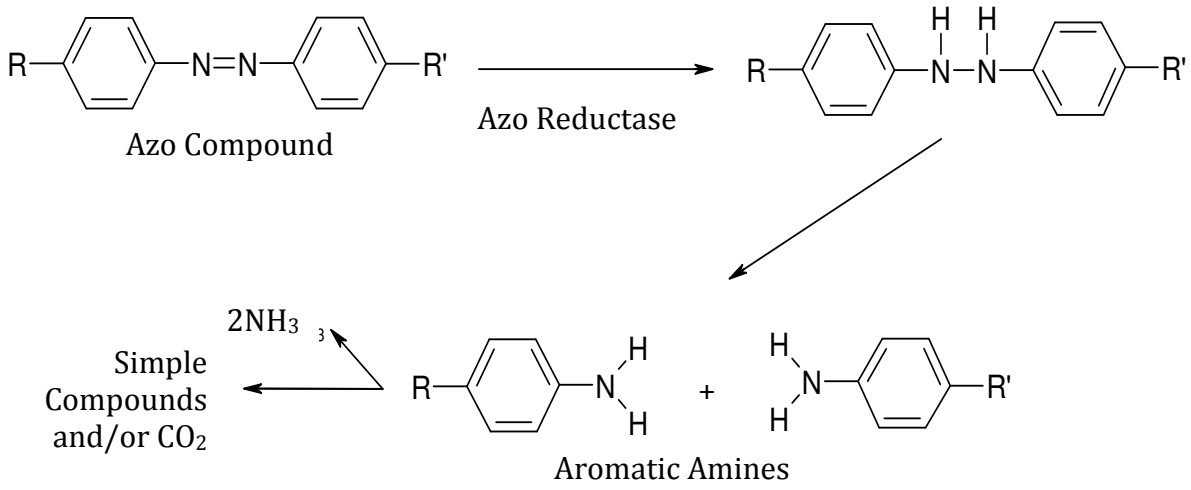

Figure 4.1 Degradation of Azo dyes by eukaryotic algae. Adapted from Semple *et al.,* (1999)

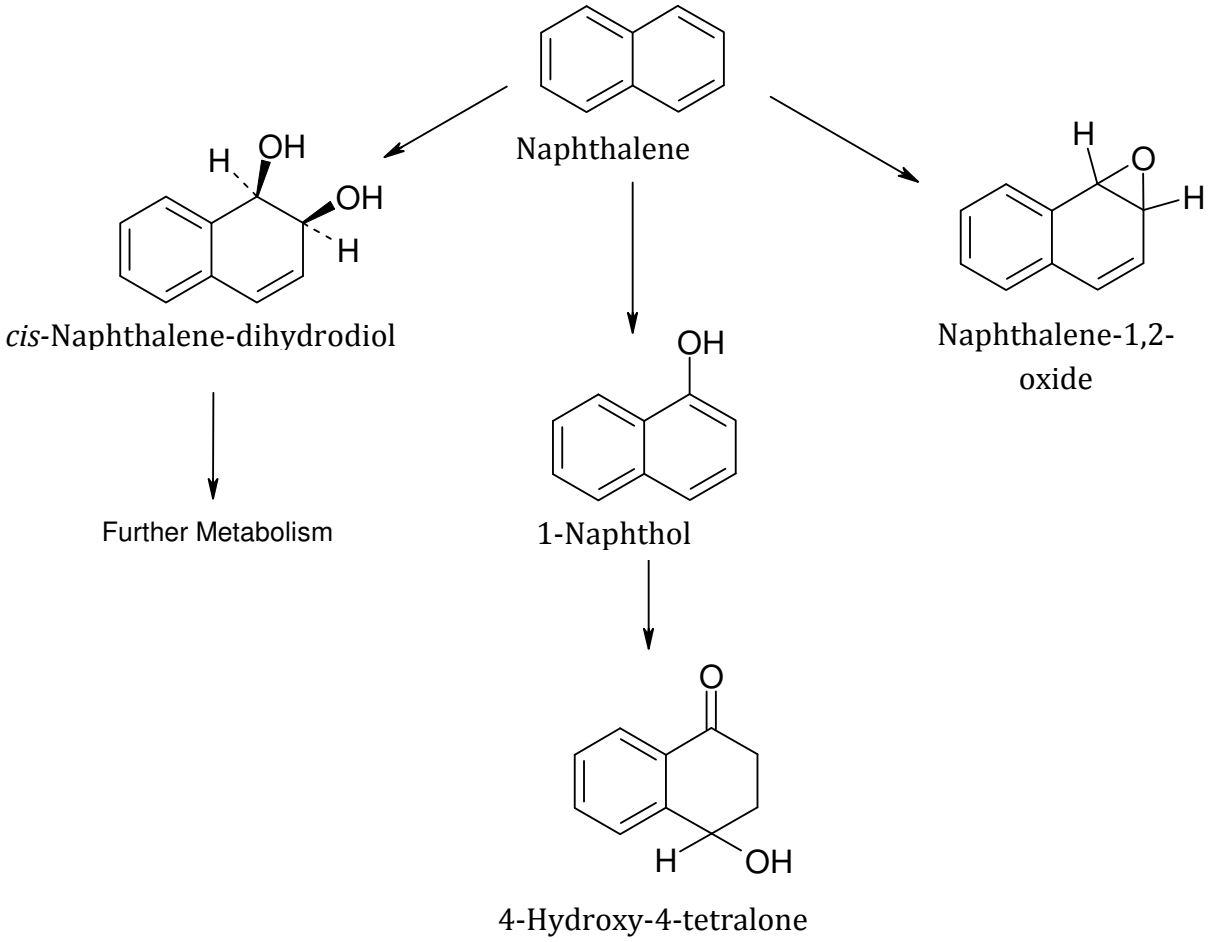

Figure 4.2 Biotransformation of Naphthalene by algae. Adapted from Semple *et al.,* (1999)

Regarding Phase II metabolism, there is some evidence that algae can sulphate compounds, as sulphated polysaccharides have been isolated from a number of species of green algae including *Enteromorpha compressa*, *Monostroma nitidum*, *Caulerpa brachypus*, *and Chaetomorpha crassa* (Lee, 2004).

4.1.2 The Role of CYPs in *Daphnia magna*

With regard to *D. magna,* the role of CYPs in endogenous pathways is relatively well documented. Following sequencing of the *D. pulex* genome, a number of CYPs have been identified, mainly from the CYP2, 3 and 4 families (FleaBase). In crustaceans and insects, the steroid hormone 20-hydroxyecdysone (20E) controls reproduction and development and is synthesised from cholesterol via CYP mediated reactions. In insects, this occurs via the Halloween genes spook-CYP307A1, phantom-CYP306A1, disembodied-CYP302A1, shadow-CYP315A1 and shade-CYP314A1, and orthologues of these genes have been identified in the genome of *D. pulex* (Rewitz and Gilbert, 2008). In addition, moulting involves the steroid hormone ecdysone, and this has been shown to be regulated by the CYP ecdysone 20-monooxygenase (Oberdorster *et al.,* 1998; James, 1998). A gene with sequence similarity to CYP4C1, known to be involved in the metabolism of insect hormones and synthetic insecticides in *Blaberus discoidalis* (tropical cockroach; Uniprot KB), has been identified in the *Daphnia* database (FleaBase). CYP3A2, the gene for testosterone-6-β-hydroxylase, has been identified in *D. pulex* (FleaBase) and it is thought that five distinct CYP forms hydroxylate testosterone at different positions (Baldwin and Le Blanc, 1994). Eicosanoids, which in mammals are derived from arachidonic acid, are cell signalling molecules derived from dietary fatty acids and are known to be involved in regulating the immune system and reproduction.

In *D. magna* transcriptomic analysis has revealed that they play a key role in reproduction (Heckmann *et al.,* 2008) and a gene similar to arachidonic acid epoxygenase (CYP2J2), which metabolises arachidonic acid via the NADH-dependent olefin epoxidation pathway in humans (Wu *et al.,* 1996), has been found in *D. pulex* (FleaBase). Another pathway in daphnids for which a CYP has been identified is the regulation of vitellogenesis by methyl farnesoate, which suppresses the expression of vitellogenin 1 (VTG1) by binding upstream to juvenile hormone-responsive elements (Tokishita *et al.,* 2006). Analysis of the *D. pulex* genome also revealed a gene similar to CYP15A1 (FleaBase), known to catalyse the epoxidation of methyl farnesoate to juvenile hormone III in the cockroach *Corpora allata* (Helvig *et al.,* 2004).

Biotransformation of xenobiotics by *Daphnia* has been studied and there are reports in the literature of pyrene metabolism (Akkanen and Kukkonen, 2003; Ikenaka *et al.,* 2006), and toxaphene (an insecticide) metabolism (Kashian, 2004). Further evidence that *D. magna* possess several forms of CYPs has come from treatment with the inhibitor piperonyl butoxide (PBO), which decreased the metabolism of pyrene (Akkanen and Kukkonen, 2003) and toxaphene (Kashian, 2004), and significantly inhibited testosterone hydroxylase activities (Baldwin and Le Blanc, 1994). Moreover, members of the CYP4 family in *D. pulex* have been identified, including CYP4C3, 4B1, and variation in expression was postulated to be linked with environmental concentrations of xenobiotics (David *et al.,* 2003) suggesting the potential for induction.

4.1.3 Measurement of CYPs Involved in Xenobiotic Metabolism

When specifically considering the role of CYPs in xenobiotic metabolism, CYP families 1, 2 and 3 are particularly important (Timbrell, 2004). Of these, CYP1A is an important subfamily as members are implicated in the bioactivation of numerous genotoxicants (e.g. BaP and 2-AAF) and are inducible by environmental contaminants. Activity indicative of CYP1A-like isoforms can be determined by measuring ethoxyresorufin-*O*-deethylase (EROD) activity, which involves the dealkylation of 7-ethoxyresorufin (7-ER) to resorufin, a fluorescent product. Traditionally, EROD activity is measured in microsomal or 9000*g* supernatants (S9) fractions from homogenised tissues (Burke and Mayer, 1974), but methods have been developed to measure this activity in cells from culture (Behrens *et al.,* 1998; Kennedy *et al.,* 1995; Hahn *et al.,* 1995, 1996). While EROD activity is well documented for several fish species (e.g. chub, Devaux *et al.,* 1998, Winter *et al.,* 2005; rainbow trout, Laville *et al.,* 2004), only a few published studies have measured this activity in algae (*Chlorella fusca,* Thies and Grimme, 1994; *Chlorella sorokiniana*, Thies and Grimme, 1996; *Cladophora* sp., Pflugmacher *et al.,* 2000; a *Euglena gracilis* cell line, Barque *et al.,* 2002) and to our knowledge there are almost no published data on EROD activity in *D. magna.* In an isolated study, Sturm and Hansen (1999) could not detect any enzymatic-*O*-dealkylation in *D. magna*. There is a need, therefore, to investigate and better characterise the xenobiotic metabolic capabilities of *C. reinhardtii* and *D. magna* in order to fully understand their sensitivity to xenobiotics, and ability to activate pro-genotoxicants, particularly as these are two of the most widely used species for the regulatory assessment of ecotoxicological impact.

The activity of CYP1A subfamily members can be induced, and this has the potential to affect activation and subsequent toxic effects of certain, environmentally-relevant xenobiotics. β-naphthoflavone (βNF) is a known CYP1A inducer, which acts through the aryl hydrocarbon receptor (AhR), and induction of EROD activity using this compound has been studied in a number of aquatic organisms, most extensively in fish (e.g. rainbow trout, Ronisz and Forlin, 1998; Atlantic salmon, Grøsvik, *et al.,* 1997; striped bass, Washburn *et al.,* 1996). The environmental pollutant tributyltin chloride (TBTCl) has been shown to have a dose-dependent inhibition of EROD activity in *Chladophora* sp. (Pflugmacher *et al.,* 2000), but to our knowledge there are no published studies investigating the inducibility of EROD activity with βNF in algae. Indeed, to date there is no evidence in the literature that algae possess an AhR.

4.1.4 Effects of Solvents on EROD Activity

In addition to the test substance of interest, in toxicity testing, organic carrier solvents are often required to facilitate the dissolution of hydrophobic substances. Two of the most commonly used solvents are dimethylsulfoxide (DMSO), a dipolar aromatic compound (Barbosa *et al.,* 2003) that is able to solubilise a wide range of chemicals; and methanol. Despite the need for their use, there is some concern regarding the potential for these solvents to affect the test results, either by their own toxic action, additive, synergistic or antagonistic interactions between solvent and test substance, or the ability of the solvent to alter the bioavailability of the test substance (Hutchinson *et al*., 2006; Haap *et al.,* 2008). Despite this, the use of solvents is often unavoidable and as such their potential influence on test results should be considered.

DMSO has been shown to have no significant effects on algal growth after 96 hours (El Jay, 1996), and was reported to be a suitable carrier solvent by Okumura *et al.,* (2001), with EC_{50} values for nine different species of microalgae in the range 3800-23000 ppm. The limits for the use of DMSO in *D.* magna to avoid solvent toxicity have been reported to be between $6.8x10^{-3}$ and $1.1x10^{-2}gl^{-1}$ (0.08-0.14mM), with no effects on reproduction or growth occurring at these concentrations (Barbosa *et al.,* 2003). EC_{50} values for methanol were also determined by Okumura *et al.,* (2001) and reported to be in the range 140-28000 ppm. From this it was concluded that methanol was not a suitable carrier solvent. In *D. magna* the 48 hour LC_{50} for methanol has been reported to be $10gl^{-1}$ (0.31M; Genoni, 1997).

In addition to the consideration of general toxicity, there are growing numbers of reports of the effects of solvents on CYP activities and the inhibitory effects of DMSO and methanol on several CYP forms from human cells have been reported (Table 4.2). There is, therefore, potential for activation of compounds such as BaP, which require a carrier solvent, to be reduced under test conditions, thus underestimating their environmental impact. Moreover, the substrate for measuring EROD activity, 7-ER also requires a solvent vehicle, which may impact on the measured activity. As a wide range of environmentally relevant pro-genotoxic agents require metabolic activation by CYP isozymes, here EROD activity was measured in both *C. reinhardtii* and *D. magna* as an indicator of activity similar to mammalian CYP1A. This isoform is involved in the oxidative activation of many (pro) carcinogens including chrysoidine and 2-AAF, which are relevant to our study. Following on from this, the lack of information regarding the effect of CYP inducers in algae prompted investigation of the potential for βNF to modulate EROD activity in *C. reinhardtii*. Finally, given the lack of information regarding

Table 4.2 The inhibitory effects of the commonly employed carrier solvents dimethylsulfoxide (DMSO) and methanol on several forms of Cytochrome P450 (CYP).

CYP	Solvent	Percentage (v/v)	Cell Type	Reference
2C19	DMSO	0.1%;	Human lymphoblastoid cells	Busby *et al.* (1999)
	Methanol	>3%	Human liver microsomes	Chauret *et al.* (1998)
	DMSO	0.2-5%	Human Hepatocytes	Easterbrook *et al.* (2001)
	DMSO	1% and higher		
2D6	DMSO	0.1%;	Human lymphoblastoid cells	Busby *et al.* (1999)
	Methanol	>1%		
3A4	DMSO	0.1%;	Human lymphoblastoid cells	Busby *et al.* (1999)
	Methanol	>3%	Human liver microsomes	Chauret *et al.* (1998)
	DMSO	0.2-5%	Human Hepatocytes	Easterbrook *et al.* (2001)
	DMSO	1% and higher		
2C8	DMSO	0.2-5%	Human liver microsomes	Chauret *et al.* (1998)

2C9	DMSO	0.2-5%	Human liver microsomes	Chauret *et al.* (1998)
	DMSO	1 and 2%	Human Hepatocytes	Easterbrook *et al.* (2001)
	Methanol	2%		
2E1	DMSO	0.2-5%	Human liver microsomes	Chauret *et al.* (1998)
	DMSO	1-2%	Human Hepatocytes	Easterbrook *et al.* (2001)
	Methanol	1-2%		
1A1	DMSO	>1%	Human lymphoblastoid cells	Busby *et al.* (1999)
	Methanol	>1%		

the effects of DMSO and methanol on CYP activity in *D. magna*, and the reports of their inhibitory effects on CYPs, we investigated the effects of these solvents on EROD activity in this organism. We hypothesise that, despite anticipated differences in the forms of CYPs present in these organisms in comparison to mammals, equivalent xenobiotic oxidation reactions and hence metabolic activation of various carcinogens is possible.

4.2 Methods

See Chapter 2, Section 2.4.1.2 and 2.4.2 for compound exposure in *C. reinhardtii* and *D. magna* respectively, and Section 2.7 for methodology of EROD activity measurement.

4.3 Results

4.3.1 EROD Activity in *C. reinhardtii*

As stated previously, chrysoidine is a pro-genotoxicant, and as such requires CYP1A-mediated biotransformation before exerting a genotoxic effect. As the algae appeared to be responsive to the genotoxic effects of this compound, measurement of the xenobiotic metabolising activity of these cells under normal conditions was assessed using an *in vivo* EROD assay. The data obtained suggest that *C. reinhardtii* possess measurable CYP1A-like activity, as there was a time-dependent increase in the level of resorufin released into the medium following incubation with 7- ethoxyresorufin (7-ER; Figure 4.3). Based on amount of product formed, the substrate remained in excess over the incubation time period, and the relationship with time was shown to be close to linearity following linear regression analysis ($P<0.05$, $R^2=0.8295$). In addition, we looked at the ability of this activity to be induced using the well-known inducer of CYP1A1, namely βNF. Cultures were exposed to 0.2 and 1nM βNF for 48h and then EROD activity measured *in vivo.* Typically higher concentrations than these have been employed to investigate induction of CYP1A activity in fish (e.g. CYP1A1 in trout liver cells has been shown to be induced by pre-treatment with 0.05μg βNF/ml (0.2μM) for 48h; Weimer *et al.,* 2000), but interestingly concentrations above the values finally used in our study showed evidence of toxicity in *C. reinhardtii*. It has since been shown that

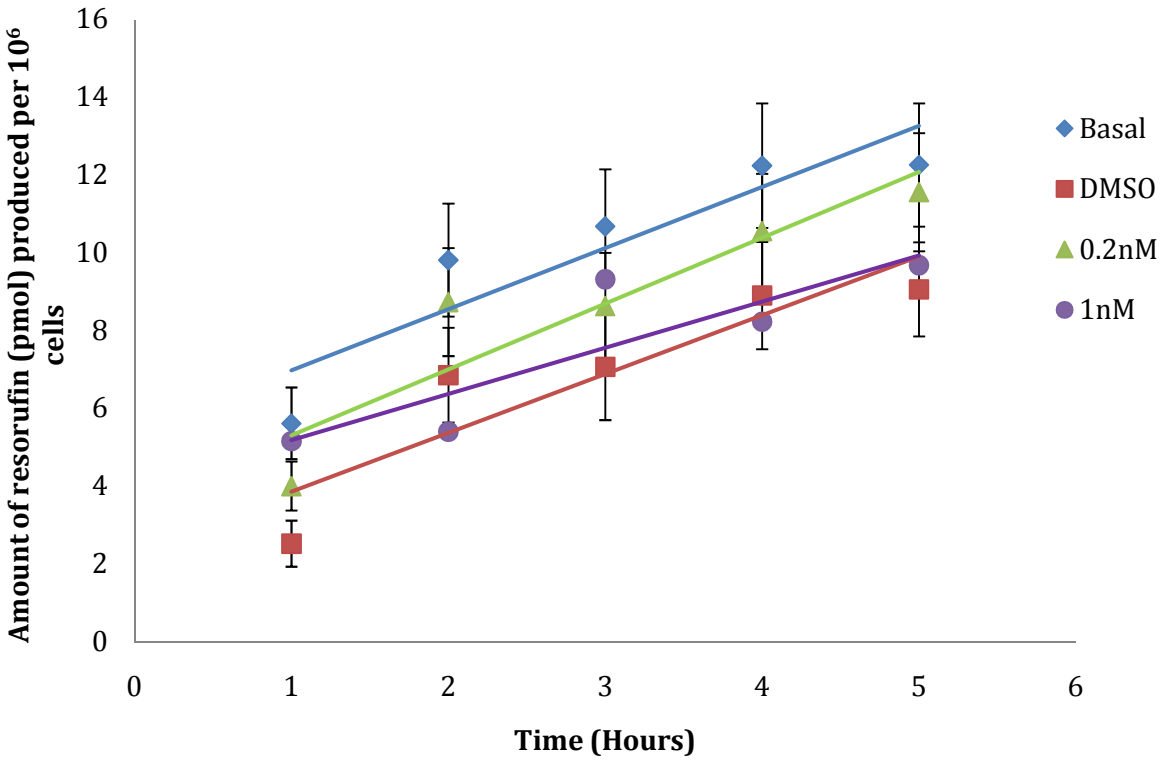

Figure 4.3 Amount of resorufin produced from 7-ethoxyresorufin over time (ethoxyresorufin-O-deethylase (EROD) assay) by *Chlamydomonas reinhardtii* in unexposed cultures, those exposed to dimethylsulfoxide (DMSO; % v/v), or to β-naphthoflavone (βNF; 0.2 and 1nM). Data presented as n=3 replicate culture flasks per exposure ± standard error of the mean (SEM).

concentrations of 0.1 and 1nM were shown to cause a significant induction of EROD activity in rainbow trout and zebrafish respectively (Jönsson *et al.,* 2009), thus induction of EROD activity could reasonably be anticipated in our study. A value of 11.6pmol resorufin/10^6 cells was obtained after 5h from the culture pre-exposed to 0.2nM βNF, in comparison to 9.1pmol resorufin/10^6 cells for the DMSO control, although this difference was not supported statistically. This slight induction of activity was not enhanced by increasing the concentration to 1nM (a value of 9.6pmol resorufin/10^6cells was obtained following 5h). Incidentally the solvent DMSO was found to have a small, but not statistically significant, inhibitory effect on the basal activity (without any DMSO). The relationship between product formed and time for the DMSO and βNF exposed cultures (as with control, untreated incubations) was found to be close to linearity following linear regression analysis ($P<0.05$; DMSO $R^2=0.8208$ and βNF $R^2=0.8512$).

4.3.2 EROD Activity Measured in *D. magna*

As previously stated, we were interested in detecting EROD activity in *D. magna* as an indicator of CYP1A-like activity. Measurement of the xenobiotic metabolising activity of daphnids under normal conditions was obtained using an *in vivo* EROD assay (see Chapter 2, Section 2.7). The data obtained suggest that *D. magna* possess measurable CYP1A-like activity, as there was a time-dependent increase in the level of resorufin generated following incubation with 7-ER, and the relationship with time was shown to be close to linearity following linear regression analysis ($P>0.05$, $R^2=0.872$; Figure 4.4). In addition, the ability of this activity to be affected by two commonly used

solvents, DMSO and methanol, was investigated. Daphnids were exposed to 3 concentrations of either solvent for 24h and then EROD activity was measured *in vivo.* In these studies, the absolute level of activity did vary between different cultures of daphnids, for example a maximal response of approximately 2.4pmol resorufin/daphnid compared to a maximal production of approximately 1.2pmol resorufin/daphnid (basal activity; Figure 4.5 and Figure 4.6). This relatively low activity made the analysis of the inhibitory effect of the solvents insensitive. Nevertheless, no concentration-dependent inhibitory effect of DMSO or methanol was detected. The relationship with time was shown to be linear following linear regression analysis for both untreated and solvent-exposed daphnids except for 0.001% methanol and 0.02% DMSO (Basal $P<0.01$, $R^2=0.9244$; 0.01% methanol $P<0.05$, $R^2=0.8737$; 0.05% methanol $P<0.05$, $R^2=0.8385$; 0.002% DMSO $P<0.01$, $R^2=0.9379$; 0.1% DMSO $P<0.05$, $R^2=0.8979$; Figure 4.5 and Figure 4.6).

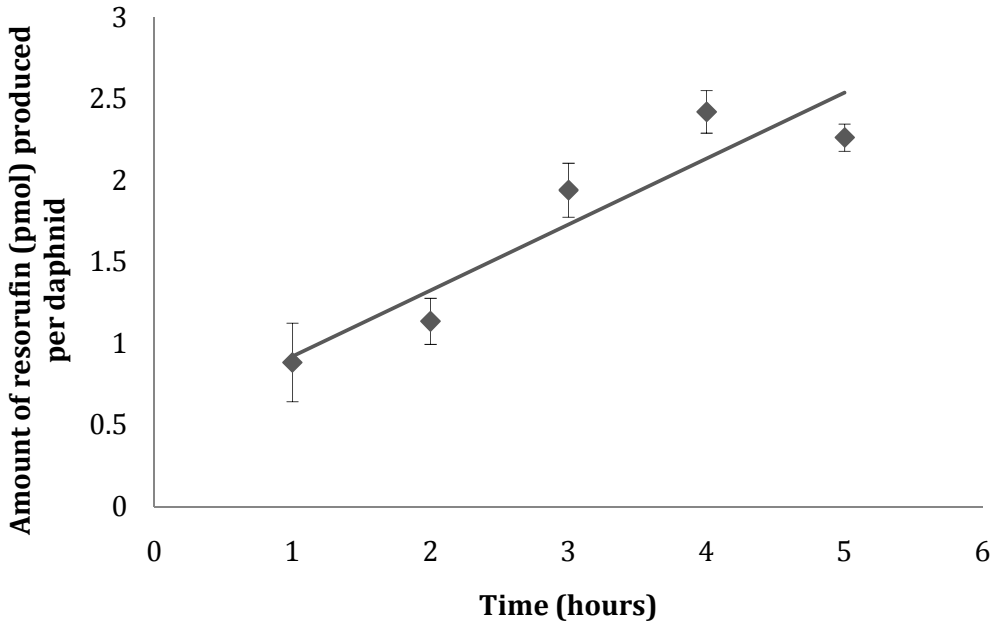

Figure 4.4 Amount of resorufin produced from 7-ethoxyresorufin over time (ethoxyresorufin-O-deethylase (EROD) assay) by adult (day 7) *Daphnia magna* in unexposed cultures. Data presented as n=3 replicate beakers each containing 20 daphnids ±standard error of the mean (SEM).

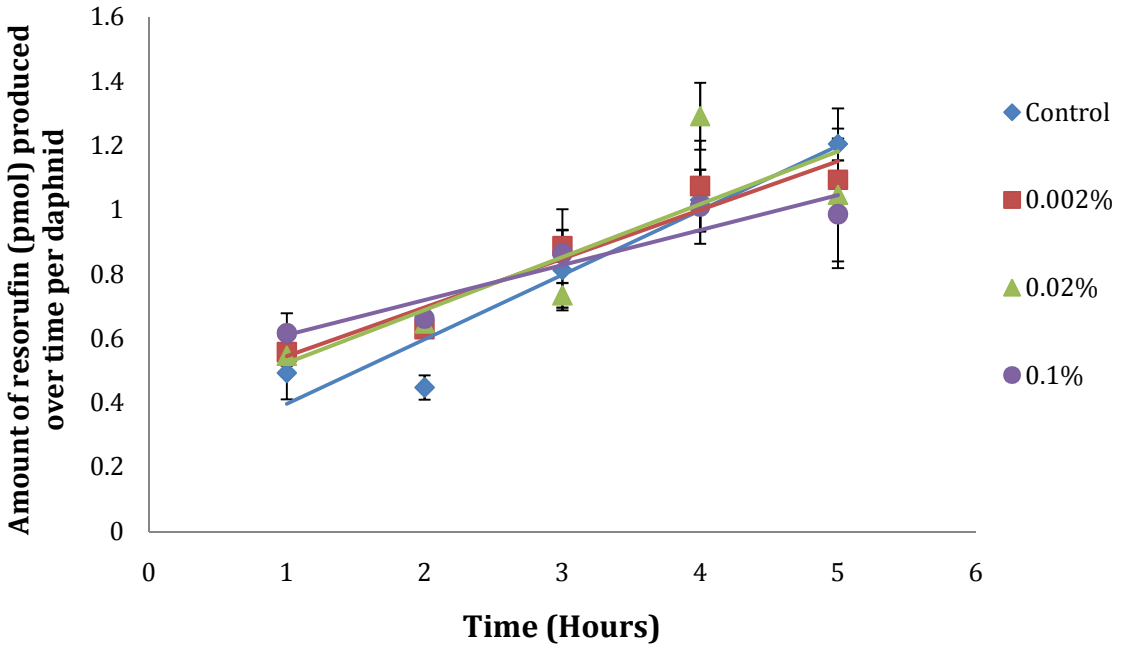

Figure 4.5 Amount of resorufin produced from 7-ethoxyresorufin over time (ethoxyresorufin-O-deethylase (EROD) assay) by adult (day 7) *Daphnia magna* in unexposed cultures, or those exposed to dimethylsulfoxide (DMSO) at 0.002%, 0.02% or 0.1%. Data presented as n=3 replicate beakers each containing 20 daphnids ± standard error of the mean (SEM).

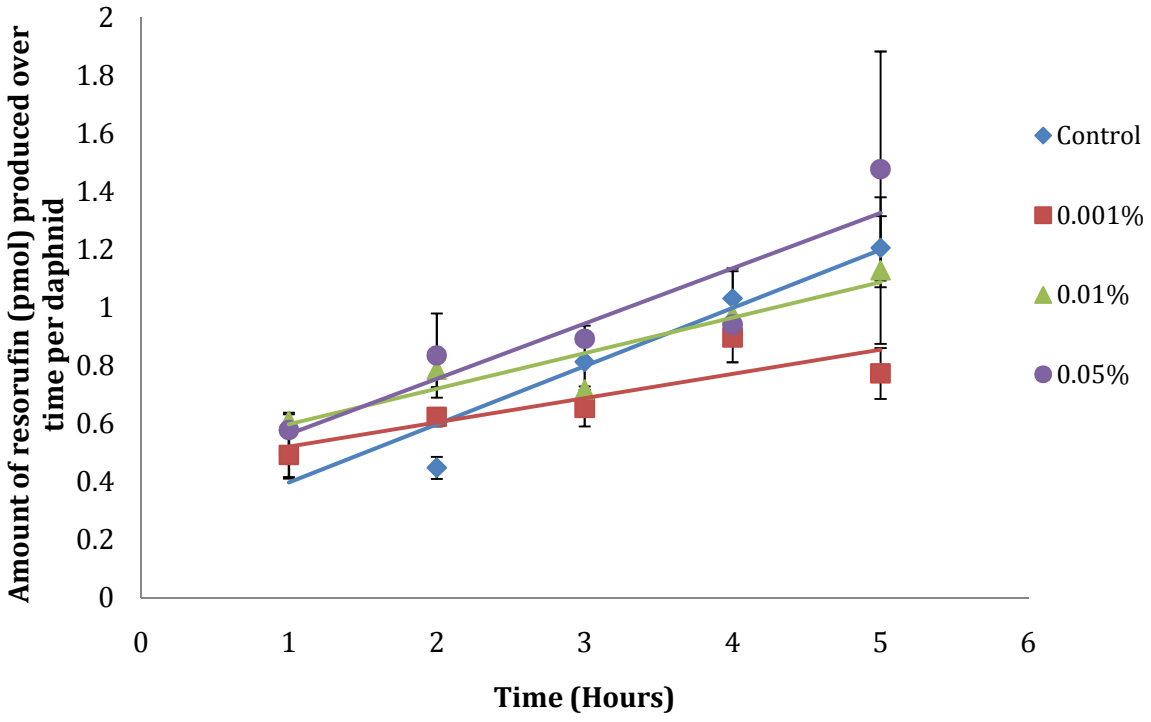

Figure 4.6 Amount of resorufin produced from 7-ethoxyresorufin over time (ethoxyresorufin-O-deethylase (EROD) assay) by adult (day 7) *Daphnia magna* in unexposed cultures, or those exposed to methanol at 0.001%, 0.01% or 0.05%. Data presented as n=3 replicate beakers each containing 20 daphnids ± standard error of the mean (SEM).

4.4 Discussion

Despite the widespread use of *C. reinhardtii* and *D. magna* in ecotoxicity testing, there is limited information available regarding the capability of these organisms to metabolise xenobiotics. A great number of pro-genotoxicants exist, thus to assess the potential genotoxic effects of these compounds in these species, and to elucidate biomarkers for such compounds, a more complete characterisation of biotransformation pathways in these species is essential. The current study aimed to go some way towards this by investigating the presence of CYP1A-like activity in *C. reinhardtii* and *D. magna,* since CYP1A is involved in the metabolism of many of xenobiotics that are of particular environmental concern (Brown *et al.,* 2008).

Suggestive of the presence of xenobiotic metabolic capability in both *C. reinhardtii* and *D. magna*, with similar characteristics to mammalian CYP1A-mediated activity, there was an increase in the production of resorufin over time, implying EROD activity in these cells. The level of activity detected (e.g. 0.03pmol/minute/10^6 cells for *C. reinhardtii* and 7fmol/minute/daphnid for *D. magna*) however, was much lower compared to values reported for other organisms, such as for striped bass, which has been shown to have a specific activity of 1.23pmol/min/mg microsomal protein (Washburn *et al.,* 1996).

Exposure of *C. reinhardtii* to the pro-genotoxin chrysoidine, up to a concentration of 10μM, produced a dose-dependent, and statistically significant, increase in DNA strand breakage (see Chapter 3, Section 3.3.2.2). These results suggested that *C. reinhardtii* possesses the metabolic capability to convert chrysoidine into the ultimate genotoxic metabolite. In mammals, phase I metabolism of this azo dye is mainly via

CYP1A2-mediated monooxygenation (Vaziri *et al.,* 2001). It is not known if the same pathway is present in this algal species, as although previous studies have demonstrated EROD (CYP1A-like) activity in the microalga *Chlorella fusca* (Thies and Grimme, 1994), CYP1A1 and CYP1A2 are thought to have diverged after the emergence of mammals, a hypothesis supported by the identification of only one CYP1A gene in fish. This gene does, however, have the catalytic properties of both CYP1A1 and CYP1A2 (Leaver and George, 2000). The low EROD activity measured in *C. reinhardtii* (0.03pmol/minute/10^6 cells) is in accordance with the findings of Warshawsky *et al.* (1995), who observed a low level of metabolism of BaP (metabolised by CYP1A1 and 1B1 in mammals) by *C. reinhardtii*. After three days, 96.8% of the parent compound remained in the pellets, 92.2% in the media. The other percentage was converted to the metabolites dihydrodiols, phenols and quinones, although in this case EROD activity was not determined. The low activity in our study was not related to a lack of substrate uptake as we also found activity to be equivalently low in 9000*g* supernatants of algal extracts, in which EROD activity was measured at a rate of 0.03pmol/minute/mg protein (data not shown in results section). It should be noted that the levels of protein in the algal microsomes were measured using the BCA assay as the Bradford method has been reported to underestimate the amount of protein in algal cells (Crossman *et al.,* 2000). For more detail see Chapter 3, Section 3.3.2.3.

The activity of EROD remained low even after conditions of sustained exposure to PAHs (based on our findings with βNF), which showed only a small increase in resorufin from 9.1pmol/10^6cells to 11.6pmol/10^6 cells after a 5h incubation with the substrate (0.2nM). Interestingly, our study with βNF revealed that pre-incubation of *C. reinhardtii* with 0.1% (v/v) DMSO for 48h results in a slight inhibition of the basal EROD activity:

the amount of resorufin produced after 5h decreased from 12.3pmol/10^6 cells to 9.1pmol/10^6 cells. This is interesting as DMSO has been reported to be a suitable solvent to use in algae since it did not affect growth after 96h (El Jay, 1996), but to our knowledge there have not been any investigations into its effects on CYP activity. Also of interest was that initial investigations revealed doses above those used in the study showed toxicity to *C. reinhardtii*. Concentrations of βNF in the micromolar range are routinely used in other organisms to induce EROD or CYP1A activity, thus *C. reinhardtii* at least are highly sensitive to this compound. This has potential implications for the sensitivity of this alga to PAHs in the environment. It is known that the concentration of PAHs varies depending on the level of industry and petroleum contamination, with levels having been recorded in the range of 5ng/g of soil in undeveloped areas to 1.79 x 10^6 ng/g at an oil refinery. Moreover, in marine sediments concentrations exceeding 10^5 ng/g in urban estuaries have been recorded (Cerniglia, 1992). A study by Law *et al.,* (1997) recorded levels of BaP in unfiltered water in the range <1 to 909ngl^{-1}. Thus possible population effects may result from toxicity of PAHs to algae, which in turn could impact on the ecosystem as a whole.

Our studies with *D. magna* involved exposure to the PAH BaP, which requires metabolic activation catalysed by CYP1A1 and CYP1B1 in mammals, and chrysoidine (metabolised by CYP1A2 in mammals), thus we wanted to determine whether this organism was capable of activating these compounds. Analysis of the effects of these pro-genotoxicants on DNA using the Comet assay did not reveal statistically significant increases in percentage tail DNA. Although factors such as the high variability in all samples is likely to have affected the sensitivity of the assay, the lack of a dose-dependent increase in strand breaks for either compound could potentially be

attributed, at least in part, to a lack of activation (for more detail see Chapter 3). Consequently, to investigate the presence (or indeed absence) of CYP1A-like activity in *D. magna,* EROD activity was measured in unexposed adult daphnids using a novel *in vivo* assessment method.

Low levels of EROD activity (maximum production of 2.4pmol resorufin/daphnid) were measured in adult daphnids, which, to our knowledge is the first report of EROD activity (albeit a low activity) in *D. magna*, since a previous attempt by Sturm and Hansen (1999) in microsomes from neonate daphnids revealed no detectable EROD activity. The low EROD activity measured is in accord with other data for CYP1A-mediated xenobiotic metabolism in *D. magna.* Baldwin and Le-Blanc (1994) reported a lack of induction of CYP activity by βNF, a known inducer of CYP1A1, and a study involving BaP showed that 80% of the parent compound was detected following a 24h incubation (Akkanen and Kukkonen, 2003). It could therefore be hypothesised that *D. magna* have limited xenobiotic metabolic capability. In contrast to this, two studies have shown that *D. magna* are able to metabolise the PAH pyrene (Akkanen and Kukkonen, 2003; Ikenaka *et al.,* 2006), thus it may be the case that *D. magna* possess an alternative enzyme to CYP1A1 that is able to metabolise xenobiotics. In support of this, a gene with homology to the novel human CYP2S1, which has been postulated to be important for extrahepatic xenobiotic metabolism (Rylander *et al.,* 2001) has been identified in the *D. pulex* genome, but a search of the database did not reveal any genes with similarity to CYP1A1. Moreover, as detailed in the introduction, CYP1A is inducible via the AhR, which binds to the aryl hydrocarbon receptor nuclear translator (ARNT) to activate gene transcription. A gene with homology to the AhR has been identified in the *D. pulex* genome (Fleabase) and a homolog of ARNT has also been identified in *D. magna*, but its

role has been postulated to be in the hypoxia response, due to the presence of recognition sequences for hypoxia-inducible factor-1 (Tokishita *et al.,* 2006). In crustaceans it has been shown that substrates of members of the CYP2 and CYP3 families, such as testosterone, are metabolised more rapidly than compounds normally substrates for CYP1, such as ethoxyresorufin. Moreover, CYP2 and CYP3 family members have been found to be the most abundant CYPs in the hepatopancreas of shrimp, lobster, crayfish and crab (James and Boyle, 1998).

The low level of EROD activity measured in *D. magna* in this study is also similar to that measured in a number of other invertebrates. For example, in sea anemone columnar microsomes from *A. xanthogrammica* and *A. elegantissima*, activities of 0.8pmol/min/mg protein and 2.28pmol/minute/mg protein were measured respectively (Heffernan and Winston, 1998). Cheah, *et al.,* (1995) recorded an EROD activity of 1.4pmol/minute/mg protein in microsomes from the digestive gland of *Octopus pallidus*, and in hepatopancreas microsomes from crayfish, a rate of 0.7pmol/minute/mg protein was measured (Escartin and Porte, 1996). Since these activities were measured in microsomes from specific tissues, it is likely that a higher concentration of CYPs relative to protein levels would be present, compared to *in vivo* studies in whole organisms. Our study was conducted in whole organisms, which has the advantage of allowing uptake of the substrate, and excretion of the metabolite to be taken into consideration, thus providing a more realistic representation of the processes that occur in addition to metabolism.

In addition to measuring a low basal level of EROD activity in daphnids, we investigated the effect of the pre-exposure to solvents on this activity. We tested up to

0.1% DMSO and methanol up to 0.05% (pre-incubated for 24h) in our study, but neither solvent affected basal EROD activity. Inhibition of CYP1A1 has previously been observed following exposure to 1% and 3% DMSO and methanol (see Table 4.2). A concentration of 0.1% DMSO has been reported to inhibit the activity of a number of recombinant CYPs (Busby *et al.,* 1999), and CYP2E1 in intact human hepatocytes (Easterbrook *et al.,* 2001). This concentration showed some inhibition of EROD activity in our study with *C. reinhardtii,* but this was not evident with *D. magna*. Inhibition of CYPs by methanol is less widely reported than for DMSO, and reported inhibition of two CYPs, CYP2E1 and CYP2C9, required higher doses (greater than 1%; Easterbrook *et al.,* 2001) compared to a minimum of 0.2% for inhibition by DMSO (Chauret *et al.,* 1998). In our study, the highest dose tested for methanol was 0.05%, which may be too low to see an effect on CYP activity, especially when considering the low basal level of activity in *D. magna* when compared to other organisms. Furthermore, the length of pre-incubation with the solvents may be of importance. In this study daphnids were pre-exposed for 24h, but the slight decrease in basal EROD activity observed in *C. reinhardtii* followed a 48 hour pre-incubation with 0.1% DMSO.

Although the assay was not highly sensitive due to low basal activity in both *C. reinhardtii* and *D. magna*, EROD activity was still evident in the presence of the solvents. Since these solvents are commonly employed in toxicology testing, it is important to know that, for these solvents at least, EROD activity in *D. magna* and *C. reinhardtii* is not markedly affected by their presence. The concentrations above 1% that have been reported to inhibit CYP activity are high and lower doses, such as those used in our study, would be preferable for use in toxicity testing to minimise the effect of the solvent. OECD guidelines recommend that solvents should be used at a concentration

below 0.1ml/l, which provides a reasonable working concentration for most solvents (OECD, 2000), and minimising concentrations was further reiterated by Hutchinson *et al*., (2006) after a review of evidence for the effects of solvents on ecotoxicological test species.

In all, in this chapter we have demonstrated that both *C. reinhardtii* and *D. magna* possess low, but measurable CYP1A-like activity. In *C. reinhardtii* the response to chrysoidine in the form of strand breaks measured by the Comet assay, coupled with measurable EROD activity indicates this alga has a degree of metabolic activation capacity, a lack of induction following prolonged exposure to βNF in algae implies a relative lack of inducibility. In answer to the question can these organisms activate xenobiotics?, the evidence for *D. magna* is equivocal, with low, but measurable EROD activity, combined with insensitive genetic toxicity to the pro-genotoxicants chrysoidine and BaP as measured by the Comet assay. For *C. reinhardtii*, however, results from the Comet assay with chrysoidine and measurable EROD activity indicates that this alga is likely to be able to activate xenobiotics via this pathway, although this alga was also relatively insensitive to the inducer of EROD activity (βNF). This is perhaps unsurprising given the lack of evidence for AhR in algae, but is of great importance, both from an environmental perspective and when considering the sensitivity of algae to pro-genotoxicants. The low EROD activity and lack of induction in this alga suggests that compounds such as BaP may be highly persistent in the environment. This is in agreement with work by Warshawsky *et al.,* (1995) who showed that 96% of BaP remained following incubation for three days with *C. reinhardtii,* however a study by Liebe and Fock (1992) (in Semple *et al.,* 1999) showed that this alga was able to remove some iso-octane-extracted PAHs from the media (95.2% of 9H-Fluorene-9-one and

85.2% 1,2-Dimethyl phenanthrene), although the underlying mechanisms were unclear. Considering this and the response to chrysoidine measured by Comet assay in our study, it is clear that *C. reinhardtii* can activate xenobiotics, but potentially predominantly via systems other than CYP1A. The study by Warshawsky *et al.,* (1995) highlighted that some species of algae were able to metabolise BaP such as *Selenastrum capricornutum*, and other algal species have also been shown to metabolise xenobiotics. It is apparent then, that individually one species of algae is not able to metabolise all pollutants in a mixture, but collectively algae may be able to degrade a substantial proportion, and may represent an important route for xenobiotic degradation in the environment.

Given the sensitivity issues regarding detecting DNA strand breaks in *D. magna* using the Comet assay, and the low level of EROD activity in this organism, we sought to identify an alternative biomarker for exposure to genotoxicants. Modulation of gene expression represents one of the first changes to occur following toxicant exposure, and thus may provide an early indicator of exposure of *D. magna* to genotoxicants. This is considered in the next chapter.

CHAPTER FIVE: TOWARDS THE IDENTIFICATION OF A GENE EXPRESSION PROFILE INDICATIVE OF EXPOSURE TO GENOTOXICANTS IN *DAPHNIA MAGNA*

5.1 Introduction

In recent years there has been an increase in the development and application of genomic techniques in terms of the biology, bioinformatics and the technology. These provide large amounts of data on the biochemical and molecular status of an organism (Ankley *et al.,* 2006). The combination of genomics with mammalian toxicology, toxicogenomics, has become an important field. Toxicogenomics brings together information from genomics and bioinformatics to enable mechanisms of toxicity to be identified and characterised (Williams *et al.,* 2003), and has at least three main goals: 1) to understand the relationship between environmental exposure and adverse effects; 2) to identify useful biomarkers of exposure and of effect; and 3) to elucidate molecular mechanisms of toxicity (Waters and Fostel, 2004; Watanabe *et al.,* 2007).

Recently, ecotoxicogenomics has developed, which is the application of toxicogenomics to ecotoxicology, enabling the impact of compounds on individual aquatic organisms, to be monitored (Iguchi *et al*., 2007). This provides scope for environmental monitoring of contaminants, with a view to identifying the types of pollutant present in the aquatic environment, particularly when mixtures are present. Thus better information regarding the toxic effects of chemicals can be acquired, ultimately protecting both environmental and human health (Poynton *et al.,* 2007). Moreover, the potential utility of this technique in a regulatory context has been recognised. The REACH program (discussed in Chapter 1, Section 1.3.1) and emerging contaminants are expected to increase the need for regulatory testing (Ankley *et al.,* 2006; REACH, 2006), and applications of microarrays to ecologically relevant species used in regulatory assessments are increasing (e.g. flounder: Williams *et al.,* 2003;

fathead minnow: Miracle *et al.,* 2003; rainbow trout: Tilton *et al.,* 2005; zebrafish: van der Ven *et al.,* 2005). Furthermore, there has also been an increase in the toxicity testing of complex mixtures from the environment, such as effluents and ambient waters, to evaluate the remediation and treatment of sites, or for compliance monitoring. The scope for toxicogenomics to provide information regarding mechanisms of action, either anticipated or unanticipated, as well as the potential for understanding additive or synergistic effects in mixtures, could allow for screening of numerous chemicals in a shorter time frame and using fewer animals than is currently possible (Ankley *et al.,* 2006).

Toxicogenomics has enabled responses to be studied at the gene level, aiding the elucidation of mechanisms of action of chemicals, since different mechanisms can produce specific gene expression patterns (Ankley *et al.,* 2006). Moreover, the ability to combine gene expression profiles with the resultant phenotypes, such as impaired growth or reproduction, can provide an understanding of the genes that control these processes, and ultimately the survival of an organism, population and indeed ecosystem (Heckmann *et al.,* 2008).

Cells commonly respond at the level of gene expression before the phenotype can be seen, as a compensatory mechanism for stresses caused by toxicant exposure (Watanabe *et al.,* 2007). Alternatively, expression changes can be the direct result of the presence of the chemical, such as when considering the effects of hormone analogues, which on binding to a transcription factor will modulate the expression of genes under its control (Ankley *et al.,* 2006). Some genes respond to specific stresses, while others, including cytokines, growth factors and oncogenes, seem to represent a more general response to stress. Many stress-responsive genes are regulated at the level of mRNA,

thus monitoring mRNA levels under different conditions can provide information regarding the cellular processes being affected (Amundson *et al.,* 2001).

DNA microarrays, which consist of small amounts of either oligonucleotides or cDNA spotted on to glass microscope slides, provide a way of monitoring these changes in gene expression (Waters and Fostel, 2004). They enable a large number of genes to be studied in one analysis (Bartosiewicz *et al.,* 2000) and provide a high throughput means of screening the numerous variables that are required to efficiently examine gene expression patterns (Iguchi *et al.,* 2007). Microarray analysis can provide many clues to understanding the molecular pathways by which toxicants act (Watanabe *et al.,* 2007). In particular, arrays can provide valuable information as to the specific modes of action of chemicals, as shown by Soetaert *et al.,* (2006) who constructed a reproduction-related array to identify propriconazole-induced reproductive toxicity.

Information about the mechanisms of action of chemicals is invaluable, but is often only obtained for individual compounds. In the environment compounds never occur singly, rather they are present in complex mixtures with many other chemicals, thus the potential for interactions is high. Compound interactions are of great concern from a toxicological perspective, since they may influence the overall toxicity and therefore organism effects. When investigating mixture toxicity, two models are usually considered: concentration addition or independent action, depending on the similarity of the mechanisms of action of the individual chemicals (Cedergreen and Streibig, 2005; Vandenbrouk *et al.,* 2009). Interactions are often not this straightforward, however, as a compound may act as an agonist for one pathway, and an antagonist for another, further complicating the results (Cedergreen and Streibig, 2005; Barata *et al.,* 2006; Vandenbrouk *et al.,* 2009). The effects of mixtures on organisms are difficult to predict,

thus it is important that an un-biased assessment method is utilised. Multi-endpoint techniques enable in-depth information regarding compound interactions to be obtained, and microarrays represent one example, as they provide an overview of many endpoints and mechanisms (Vandenbrouk *et al.,* 2009). Although mixture toxicity responses in aquatic organisms have only been addressed in a limited number of microarray studies (e.g. Finne *et al.,* 2007; Geoghegan *et al.,* 2008; Vandenbrouk *et al.,* 2009), all highlighted the potential of this technique for investigating mixture toxicity.

Microarray studies investigating the effects of single compounds are more common, and have been applied to an ever-expanding number of aquatic species. These include fish (Gunnarsson *et al.,* 2007; Ju *et al.,* 2007; Martyniuk *et al.,* 2007; Williams *et al.,* 2007), algae (Zhang *et al.,* 2004; Jamers *et al.,* 2006; dos Santos Ferreria *et al.,* 2007), shrimp *Litopenaeus vannamei* (Robalino *et al.,* 2007), and *D. magna* (Soetaert *et al.*, 2006, 2007; Iguchi *et al*., 2006; Poynton *et al.,* 2007, 2008a; 2008b; Watanabe *et al.,* 2007; Heckmann *et al.*, 2008). *Daphnia* spp. has long been established in ecotoxicity testing, but, as discussed in section 1.3.2, standard tests rely on whole-organism responses only, giving little mechanistic insight. In recent years, *Daphnia* spp. has emerged as a key model invertebrate for genomics studies (Heckmann *et al.,* 2008). The genome of *D. magna* is still being characterised, thus the availability of sequence information is limited. Despite this, the small number of applications of microarrays to this species shows that this is an appropriate technique, in spite of the poor genome characterisation (Watanabe *et al.,* 2007). In addition, their parthenogenic lifecycle means that genetic variability should be low in this organism (Heckmann *et al.,* 2008), making it ideal for genetic studies. With limited understanding of the mechanisms of chemical toxicity in invertebrates, ecotoxicogenomics applied to *D. magna* has great

potential to provide this information (Watanabe *et al.,* 2007). For example, Poynton *et al.,* (2007) identified potential zinc-induced effects on moulting and subsequently reproduction, while Soetaert *et al.,* (2006) implicated fenarimol in the induction of haemoglobin.

Despite the ecological importance of *D. magna* and its use in regulatory testing, the genomic resources available for this species are limited. Since the first analysis of expressed sequence tags (ESTs) in *D. magna* by Watanabe *et al.,* (2005), several studies utilising custom *D. magna* microarrays have been published (Soetaert *et al.,* 2006, 2007; Iguchi *et al*., 2006; Poynton *et al.,* 2007; Heckmann *et al.,* 2007; Watanabe *et al.,* 2007; Vandenbrouk *et al.,* 2009). Despite this, the *D. magna* genome is not fully sequenced, although sequencing is underway through the *Daphnia* Genomics Consortium (*Daphnia* Genomics Consortium). The genome of a related species *D. pulex* has been fully sequenced (JGI Genome Portal), however, and this has proven useful for identifying genes of interest in *D. magna*. In late 2007 a *D. magna* oligonucleotide microarray was released by Ecoarray (Agilent Technologies, USA), which is still the only commercial *D. magna* microarray available.

The aim of the current study was to employ *D. magna* microarrays as a method of identifying subtle molecular level responses indicative of exposure to genotoxic agents. A number of studies have shown that different types of chemicals can produce specific gene expression profiles. For example, a study by Poynton *et al.* (2007) identified unique gene expression profiles following exposure of *D. magna* to different metals, and Amin *et al.,* (2001) identified distinct gene expression profiles for two classes of xenobiotics, barbiturates and peroxisome proliferators. A unique expression profile for peroxisome proliferators was also reported by Hamadeh *et al.,* (2002), as well as for phenobarbitol-

like enzyme inducers. In relation to genotoxicants, differential gene expression patterns have been obtained for genotoxic and non-genotoxic carcinogens in rat liver (Ellinger-Ziegelbauer *et al.,* 2004, 2005, 2008, 2009) and HepG2 cells (van Delft *et al.,* 2004). Furthermore, Bartosiewicz *et al.,* (2001) identified fingerprints of gene regulation following exposure of mice to cadmium chloride, BaP and trichloroethylene. These studies provide evidence that gene expression profiles can be used to discriminate between compounds with different mechanisms of action.

Based on this, two genotoxicants with different mechanisms of action were selected to investigate whether unique gene expression profiles could be detected for these compounds when presented in a mixture.

Sodium dichromate is a Cr(VI) compound that is widely used in industry for applications including catalysis and an alloying metal for the production of stainless steel (Lee *et al.,* 2005). It is known that Cr (VI) dissolves easily in the aquatic environment (Lin, 2002) and can readily cross cell membranes via a surface anion transport system (Nudler *et al.,* 2009). Once in the cell Cr(VI) is reduced to lower oxidation states by physiological reducing agents such as reduced glutathione (GSH), NAD(P)H, some pentoses and $FADH_2$ (Shi and Dalal, 1990; Cervantes *et al.,* 2001) as well as CYPs, vitamins C (ascorbate) and B_{12} and the mitochondrial respiratory chain (Stearns *et al.,* 1994; Tsou and Yang, 1996; Cervantes *et al.,* 2001). These lower oxidation states are the reactive Cr(V) and Cr(IV) and stable Cr(III) (Snow, 1994). During the reduction of Cr(VI) ROS are produced (Cervantes *et al.,* 2001), and oxidative DNA damage is thought to be the main type of insult induced by Cr(VI) (Aiyar *et al.,* 1991; Itoh *et al.,* 1995; Cervantes *et al.,* 2001), although DNA strand breaks, Cr-DNA adducts, and DNA-DNA and DNA-protein cross links, and mutations are also produced (Cupo *et al.,*

1985; Bridgewater *et al.,* 1994; Snow, 1994; Xu *et al.,* 1994; Hodges *et al.,* 2001; Lee *et al.,* 2005). Of the metabolites of Cr(VI), Cr(III) is regarded as the main one in cells and has been reported to be more genotoxic than Cr(VI) in cell-free genotoxicity assays. Moreover, Cr(III) cannot enter or leave cells through membrane transport systems. Cr(III) can bind with DNA to produce adducts and DNA-DNA cross-links (Tsou and Yang, 1996), has been reported to affect DNA transcription and replication (Snow, 1994), and can induce mutations (Tsou and Yang, 1996). It has also been proposed that enzyme activities can be affected by reaction of sulfhydryl and carboxyl groups with Cr(III) (Cervantes *et al.,* 2001), and displacement of magnesium ions by low concentrations of Cr(III) can affect the activity of DNA polymerase, amongst others and increase the bypass of oxidative DNA lesions (Snow, 1994). Cr(V) complexes can be formed by reduction of Cr(VI), and these can react with H_2O_2 leading to the production of OH radicals, which can result in modifications to DNA (Shi and Dalal, 1990).

BaP is a polycyclic aromatic hydrocarbon (PAH), which represent a group of potent environmental carcinogens, found in combustion and petroleum products from power factories and vehicle exhaust fumes (Jenstrom and Graslund, 1994). BaP has been shown to be metabolically activated to a number of genotoxic metabolites by liver microsome extracts from, for example, rodents and fish: 3-hydroxybenzo[a]pyrene (3-OH BaP), 9-hydroxybenzo[a]pyrene (9-OH BaP), benzo[a]pyrene-4,5-dihydrodiol (BaP 4,5-diol), benzo[a]pyrene-7,8-dihydrodiol (BaP 7,8-diol), benzo[a]pyrene-9,10-dihydrodiol (BaP 9,10-diol), and BaP quinones. Of these, 3-OH BaP is the major metabolite in a number of species (Miranda *et al.,* 2006). These metabolites, particularly the diol-epoxides, react with DNA to form adducts, specifically with the exocyclic amino groups of guanine and adenine. Adducts can also be formed by the one electron oxidation of BaP, but these are

often unstable and apurinic (AP) sites are produced following cleavage of the N-glycosidic bond (Banasiewicz *et al.,* 2004). BaP metabolites have also been shown to bind to rat liver microsomal and cytosolic proteins (Miranda *et al.,* 2006), globin (Melikian *et al.,* 1996) and albumin (Helleberg and Tornqvist, 2000). BaP quinones can undergo redox cycling, resulting in the production of ROS, which can lead to oxidative stress and lipid peroxidation, and contribute to carcinogenesis. Moreover, one electron oxidation of BaP can be induced leading to the production of protein-binding intermediates (Miranda *et al.,* 2006). We measured a low level of EROD (CYP1A-like) activity in adult *D. magna* (see Chapter 4), but despite this it has been shown that *D. magna* can metabolise the PAH pyrene (Akkanen and Kukkonen, 2003; Ikenaka *et al.,* 2006; for more detail see Chapter 4), thus *D. magna* do potentially have the ability to activate BaP.

These two chemicals together therefore represent mixtures that might affect a range of DNA repair processes, cell cycle check points and oxidative stress response genes. We conducted a preliminary study in which a custom cDNA microarray was employed to provide an insight into the gene expression changes. This study highlighted potential key genes reflective of exposure to these chemicals, but was limited by the lack of DNA damage and repair associated genes on the array. Thus we followed this up with a more detailed study utilising Agilent oligonucleotide microarrays, which had a selection of DNA damage and oxidative stress response genes present. Furthermore, this study aimed to investigate the differences in gene expression changes between neonate and adult daphnids, particularly in response to genotoxicant exposure.

5.2 Methods

See Chapter 2, Section 2.4.2 for compound exposure, and Chapter 2, Section 2.9 for details of RNA extraction, labelling reactions, microarray hybridisations, scanning and data analysis.

5.3 Results

5.3.1 Establishing Sub-Lethal Concentrations of Genotoxicants for Analysis of Gene Expression Responses

The sensitivity of *D. magna* to sodium dichromate and BaP was investigated in order to establish non-lethal concentrations. The toxicity of the chemicals was first established individually and then in combination. The results of the range finding study can be seen in Table 5.1 and Table 5.2. After 24h both survival and rate of movement were recorded (reduced motility was regarded as an indicator of toxicity). Consequently the combined concentrations of 0.003μM sodium dichromate with 0.002μM BaP ("low dose"), and 0.01μM sodium dichromate with 0.01μM BaP ("high dose"), were selected for the cDNA microarray pilot study, and slightly different concentrations of 0.25μM sodium dichromate with 0.01μM BaP ("low dose"), and 0.75μM sodium dichromate with 0.1μM BaP ("high dose") were chosen for the oligonucleotide microarray study. These pilot studies were all conducted in neonates (<24h old), but the selected concentrations also proved to be sub-lethal in adults (7 days old).

Table 5.1 Viability of neonate (<24h) daphnids following exposure to sodium dichromate and benzo[a]pyrene, individually and in combination, for the cDNA microarray study.

Concentration	Percentage Alive	Speed of Movement
Control (Solvent (DMSO)/Water)	100%	+++
Sodium dichromate (µM)		
0.7	75%	++
0.07	100%	+++
0.01	100%	+++
0.003	100%	+++
Benzo[a]pyrene (µM)		
0.2	100%	+
0.04	100%	+++
0.01	100%	+++
0.002	100%	+++
Sodium dichromate (µM) + Benzo[a]pyrene (µM)		
0.07 + 0.04	50%	+
0.01 + 0.01	100%	+++
0.003 + 0.002	100%	+++

Viability was analysed by the number alive and speed of movement as compared to control cultures. Note: +++ Fast ("normal" speed); ++ Slow; + Very slow movement. N=2 test beakers per concentration, 10 neonate daphnids (<24h) per beaker.

Table 5.2 Viability of neonate (<24h) daphnids following exposure to sodium dichromate and benzo[a]pyrene, individually and in combination, for the oligonucleotide microarray study.

Concentration	Percentage Alive	Speed of Movement
Control (Solvent (DMSO)/Water)	100	**+++**
Benzo[a]pyrene (µM)		
1	10	+
0.5	80	++
0.2	100	+++
0.1	100	+++
0.01	100	+++
0.05	100	+++
Sodium Dichromate (µM)		
4	0	-
3	0	-
2	50	+/++
1	100	+++
0.75	100	+++
0.5	100	+++
0.25	100	+++
Benzo[a]pyrene (µM) + Sodium dichromate (µM)		
0.2 + 1	90	+
0.1 + 0.75	100	+++
0.05 + 0.5	100	+++
0.01 + 0.25	100	+++

Viability was analysed by the number alive and speed of movement as compared to control cultures. Note: +++ Fast ("normal" speed); ++ Slow; + Very slow movement. N=2 test beakers per concentration, 10 neonate daphnids (<24h) per beaker.

An initial pilot study was conducted in collaboration with Amanda Callaghan and Christopher Hill at the University of Reading using a cDNA custom microarray, which led to a more detailed study employing a commercially available oligonucleotide array from Agilent with improved annotation and a wider range of genes.

5.3.2 Pilot Study Using cDNA Microarray: The Effect of Compound Exposure on Gene Regulation

The custom made cDNA array contained 12K probes constructed from redundant cDNA libraries, thus more than one probe could represent a single gene. Comparing both treatments to the control, based on a 2-fold cut off, 737 genes showed apparent differential regulation at the high dose, and 859 genes were either up or down regulated at the low dose. As this was a pilot study, replicates were not included, thus we were unable to perform statistical analysis on the data. We have used the apparently modulated genes as indicators of effects which were followed up with a more detailed study. A large number of the modulated genes on the array were un-annotated, thus these sequences were subjected to blast homology searches (BLASTX) to identify those that had similarity to sequences in the public databases. From these, 250 individual genes could be identified as being modulated, as some of the clones were found several times. An overview of the differentially expressed genes is given in Appendix 2. Categories of the differentially expressed genes were identified according to their function, and the numbers of genes in each category over both combination dose levels are shown in Figure 5.1.

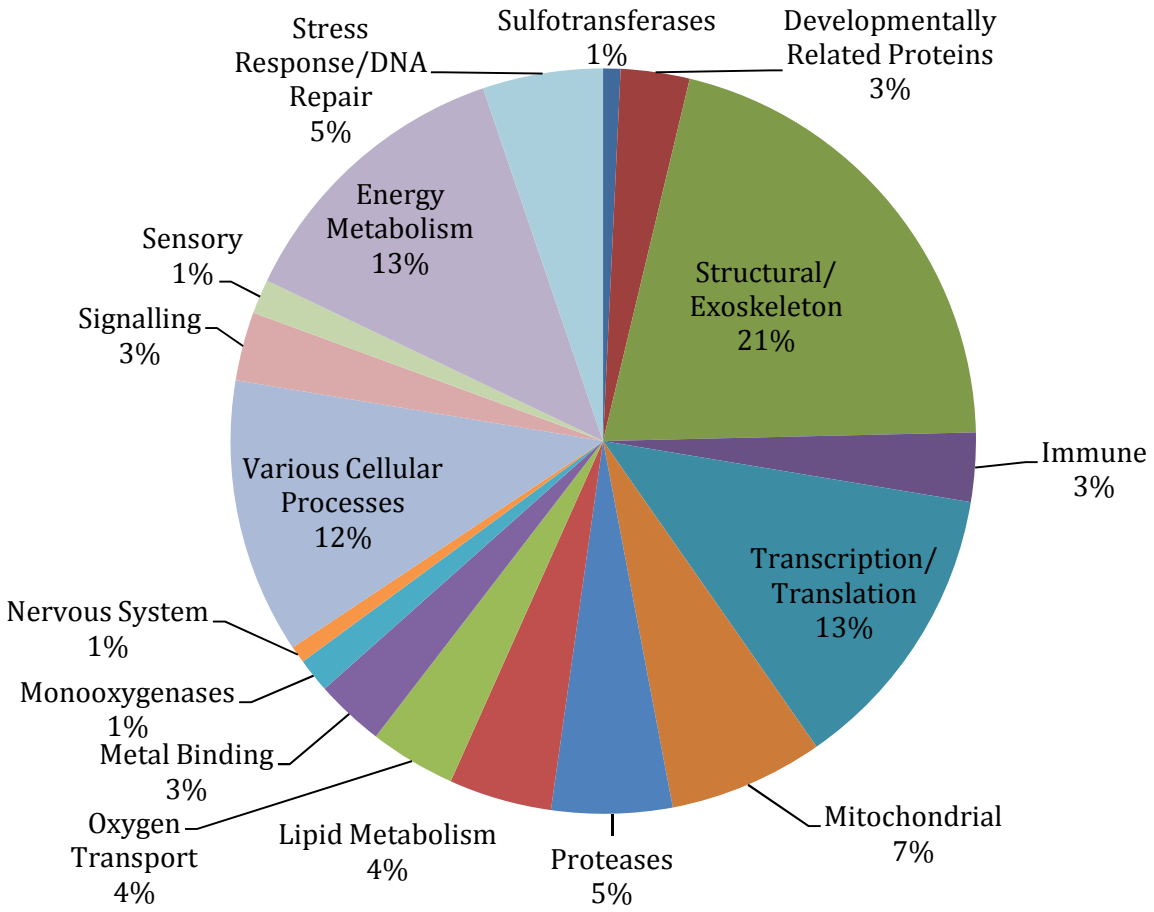

Figure 5.1 Pie chart showing the categories of apparently differentially expressed genes identified over both combination dose levels, indicative of the types of genes that may be changing in response to exposure to the mixture of benzo[a]pyrene and sodium dichromate. As some of the clones were found several times, duplicated clones were counted once.

Overall, more genes were down regulated at the low dose (626 down vs 233 up), and a greater number showed up regulation at the high dose (403 up vs 334 down), indicative of a potential effect of exposure concentration on gene expression. More specifically, genes in some categories (e.g. oxygen binding and transport, and exoskeleton-related proteins) showed more frequent up regulation at the relatively high dose, whilst down regulation of developmentally-related proteins and cell structural proteins was more evident at the relatively low dose. Furthermore, genes in these categories seem to show a shift in expression between doses, for example oxygen binding and transport proteins showed more down regulation at the low dose (78%), but 100% up regulation at the high dose (Table 5.3).

A limited number of genes involved in the response to stress and DNA repair were up-regulated following exposure, namely glutathione peroxidase (GPX), glutathione-S-transferase (GST), putative defense protein, oxidative stress protein, Heat shock protein 70 and Xeroderma Pigmentosum group A (XPA). This indicates that responses specific to exposure to oxidative stressors and genotoxicants are responsive and detectable in *D. magna.*

With a view to elucidating an expression 'fingerprint' for exposure to stressors we compared the apparently differentially expressed genes from our study with those from published *D. magna* array studies. This highlighted some common changes in gene expression following exposure to various other chemical stressors (Table 5.4), the majority of which seem to be common to metal exposure, potentially implicating sodium dichromate as the dominant stressor in our study. The apparent up-regulation of genes

Table 5.3 The shift in apparent differential expression of some gene categories following treatment with the low and high dose of the sodium dichromate-benzo[a]pyrene mixture. For comparison, some categories that did not show a shift in expression have been included.

Category	**Low Dose**		**High Dose**	
	Up	**Down**	**Up**	**Down**
Oxygen binding and Transport	22%	78%	100%	0%
Exoskeleton-related proteins	29%	71%	77%	23%
Lipid metabolism	100%	0%	80%	20%
Developmentally-related proteins	0%	100%	67%	33%
Cell Structural Proteins	27%	73%	59%	35%
Transcription and Translation	91%	9%	90%	10%
Mitochondrial	60%	40%	61%	39%

Table 5.4 Genes showing a common trend for differential expression in *Daphnia magna* following treatment in our study compared to published microarray gene expression data for *D. magna*.

Exp. Change	**Gene**	**Exposure**	**Study**
Up	Ferritin	Sodium dichromate-BaP mixture (0.01µM–0.01µM)	Current study
		Cd ($1/10^{th}$ LC_{50})	Poynton *et al.,* (2007)
		Cu ($1/10^{th}$ LC_{50})	Poynton *et al.,* (2007)
Down	Cuticle Protein	Sodium dichromate-BaP mixture (0.003µM–0.002µM)	Current study
		Zn	Poynton *et al.,* (2007)
		Propriconazole (1µg/ml, 8 days)	Soetaert *et al.,* (2006)
		Ni (0.5mg/L; 2mg/L)	Vandenbrouk *et al.,* (2009)
Up	Trypsin	Sodium dichromate-BaP mixture (0.01µM–0.01µM)	Current study
		Sodium dichromate-BaP mixture (0.003µM–0.002µM)	Current study
		Zn	Poynton *et al.,* (2007)
		Fenarimol (anti-ecdysteroidal fungicide)	Soetaert *et al.,* (2007)

Down	Vitellogenin fused with superoxide dismutase	Sodium dichromate-BaP mixture (0.003µM–0.002µM) Cd Ni	Current study Poynton *et al.* (2007) Vandenbrouk *et al.,* (2009)
Up	Haemoglobin (Dhb1)	Sodium dichromate-BaP mixture (0.01µM–0.01µM) Fenarimol (1µg/ml; 48h)	Current study Soetaert *et al.,* (2007)
Down	NADH Dehydrogenase	Sodium dichromate-BaP mixture (0.003µM–0.002µM) Cd	Current study Poynton *et al.,* (2007)
Up	Sulfotransferase	Sodium dichromate-BaP mixture (0.003µM–0.002µM) Cu Ibuprofen	Current study Poynton *et al.,* (2007) Heckmann *et al.,* (2008)
Up	Fatty acid binding protein 3	Sodium dichromate-BaP mixture (0.003µM–0.002µM) Ibuprofen	Current study Heckmann *et al.,* (2008)

common between our study and that of Soetaert *et al.,* (2007), in which the fungicide fenarimol was used, and Heckmann *et al.,* (2008), who exposed daphnids to the non-steroidal anti-inflammatory drug ibuprofen are interesting as the compounds do not have any obvious similarities with the chemicals used in our study, thus these genes potentially reflect a more generalised stress response following toxicant exposure.

5.3.3 Oligonucleotide Microarray: Gene Expression

Adult and neonate daphnids were exposed for 6h or 24h to two combination dose levels of sodium dichromate and BaP. To identify expression changes due to treatment, RNA from exposed daphnids was compared to RNA from control samples. Comparisons were also made between the control samples from adults and neonates to facilitate identification of age-specific responses. Three to four biological microarray replicates were utilised.

Of the 15000 gene fragments (duplicated) on the array, differentially expressed genes were identified by comparing control samples between adults and neonates, and also control and treated samples (based on a 2-fold cut-off). Statistically significant differences were determined by a parametric Welch t-test with a P value cut-off of 0.05. A Benjamini and Hochberg False Discovery Rate (FDR) multiple testing correction (Benjamini and Hotchberg, 1995) was applied, which predicted that approximately 5% of the identified genes would pass the test by chance. In total, of the 4,208 genes tested, 2113 gene fragments showed statistically significant differential expression between adults and neonates (FDR <0.05). Of these genes 362 had higher expression and 516 had lower expression (2-fold cut-off) in adults compared to neonates, of which 199 of the

up-regulated genes and 88 of the down-regulated genes could be identified with known proteins. An overview of all genes identified with known proteins that showed statistically significant differential expression between un-treated adults and un-treated neonates is presented in Appendix 3, Table 1 and Table 2.

Regarding expression changes as a result of treatment, all treated and control samples for adult daphnids and neonate daphnids were tested separately for statistically significant differences (parametric Welch t-test, P value cut-off 0.1, Benjamini and Hochberg FDR multiple testing correction). The differential expression of 106 genes in adults was statistically significant (FDR <0.1), while for neonates, 34 genes showed statistically significant differential expression (FDR <0.1). The number of genes that were apparently differentially expressed as a result of treatment based on a 2-fold cut-off at each concentration and time point for neonates and adults is given in Table 5.5.

An overview of all the differentially expressed genes identified with known proteins following treatment is given in Appendix 3, Table 3. Those genes identified with known proteins that showed statistically significant differential expression following treatment are given in Appendix 3, Table 4.

Table 5.5 The number of apparently differentially expressed genes in adult and neonate *Daphnia magna* as a result of treatment at each concentration and time point. The number of genes that could be identified with known proteins is also indicated.

Time point	Concentration	Number of genes	Number of genes (identified)	Number of genes up/down	Number of identified genes up/down
Adults					
6h	Low	538	187	286/252	141/46
	High	919	627	552/367	409/218
24h	Low	459	256	169/290	53/203
	High	471	168	295/176	146/22
Neonates					
6h	Low	374	147	173/201	61/86
	High	729	291	360/369	174/117
24h	Low	343	68	244/99	37/31
	High	597	229	325/272	118/111

Apparently differentially expressed genes are based on a 2-fold cut-off. Low = low dose of the sodium dichromate-BaP mixture (0.25µM-0.01µM) and High = high dose of the sodium dichromate-BaP mixture (0.75µM-0.1µM).

5.3.3.1 Principal Components Analysis

The transcripts that were detectable were subjected to principal components analysis (PCA) using features embedded within GeneSpring. PCA is a multivariate statistical technique that is used to explore and simplify complex data sets, which was invented by Karl Pearson in 1901 (Raychaudhuri *et al.,* 2000). By far the greatest separation of data using this method was observed for adult and neonate samples, according to age, providing validation for using this method. Distinct clustering of neonate and adult samples was observed with the PC1 axis accounting for 21.11% of the variation and 16.98% explained by PC2 (Figure 5.2).

Given that global gene expression separated according to the life stage of the daphnids, it would not be expected that this data would also separate according to treatment, since the main observed difference would be that of age. As a result, selected gene lists (those shown to respond to treatment) for adults and neonates were analysed by PCA, which indicated that a subset of genes were useful for discriminating between treated and un-treated daphnids.

The PCA scores plot for the genes that responded to treatment in adult daphnids shows some separation, with the treated samples (low and high concentrations of the mixture) and the control samples (time zero and time-matched) clustering separately (Figure 5.3). PC1 explains 26.76% of the variation, while PC2 accounts for 13.98%. For the neonate data, no separation of the data according to treatment was seen along the PC1 and PC2 axes (Figure 5.4), however, when PC2 was plotted against PC3 there was some separation of control samples (time zero and time matched) from treated samples

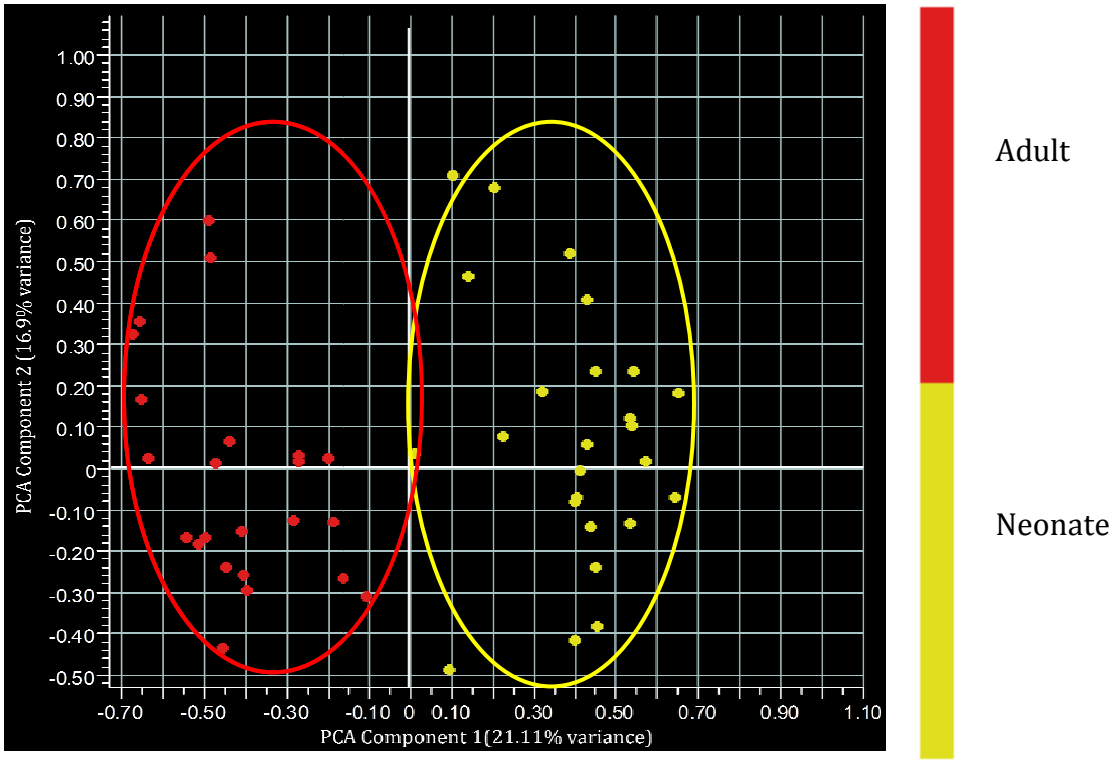

Figure 5.2 Principal components analysis scores plot from analysis of all treated and control samples from the microarray study. The plot shows separation of adult and neonate microarray data according to age along the PC1 and PC2 axes.

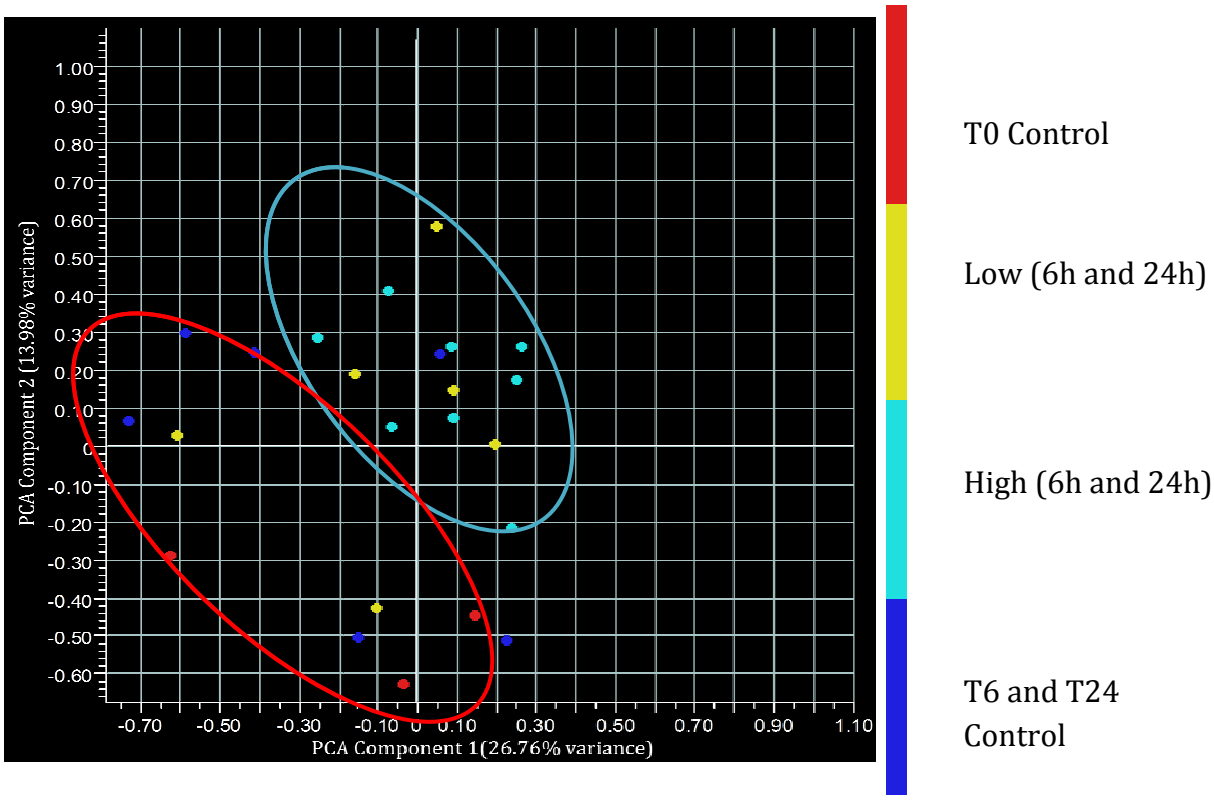

Figure 5.3 Principal components analysis (PCA) scores plot for all genes that responded to treatment in adult *Daphnia magna* from the microarray study for both mixture concentrations and both time points. The plot shows separation of samples according to treatment along PC1 and PC2 axes, with control and treated samples clustering separately (groups encircled in red and blue respectively). Abbreviations used are T0: time zero, T6: 6 hour time point, T24: 24 hour time point, Low: low concentration of the mixture, High: high concentration of the mixture.

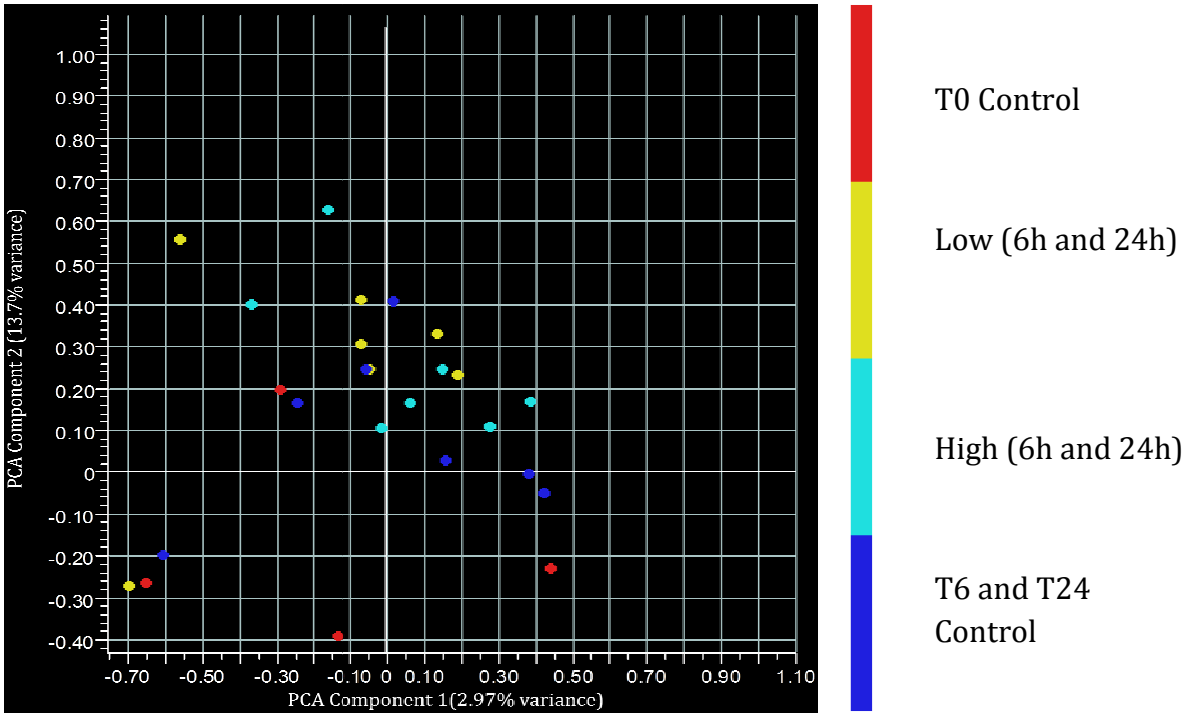

Figure 5.4 Principal components analysis (PCA) scores plot for all genes that responded to treatment in neonate *Daphnia magna* from the microarray study for both mixture concentrations and both time points. The plot shows a lack of separation of samples according to treatment along PC1 and PC2 axes. Abbreviations used are T0: time zero, T6: 6 hour time point, T24: 24 hour time point, Low: low concentration of the mixture, High: high concentration of the mixture.

(low and high concentrations of the mixture; Figure 5.5). PC2 accounts for 13.2% of the variation and PC3 explains only 10.17%.

5.3.3.2 Hierarchical Clustering

Hierarchical clustering revealed unique expression profiles generated by neonate and adult daphnids, corroborating the separation observed using PCA. A Gene tree was generated in Genespring (Agilent Technologies) in which genes were clustered horizontally according to the similarity in their change in expression across the two age groups. Genes with similar function are clustered together (Figure 5.6).

5.3.3.3 Differential Gene Expression According to Age

Comparison of the significantly differentially expressed genes between adults and neonates revealed 362 genes that were more highly expressed in adults than neonates (2-fold cut-off, FDR<0.05). Genes associated with DNA repair and oxidative stress protection showed significantly higher expression in adults. Figure 5.7 shows a comparison of the expression levels of a selection of DNA repair genes identified to be statistically significantly higher expressed in adults compared to neonates in control samples only. The P values are given in Table 5.6. Regarding oxidative stress protection, glutathione peroxidase (GPX) and glutathione-S-transferase (GST) showed significantly higher expression in adults (P value <0.05 and <5E-05 respectively).

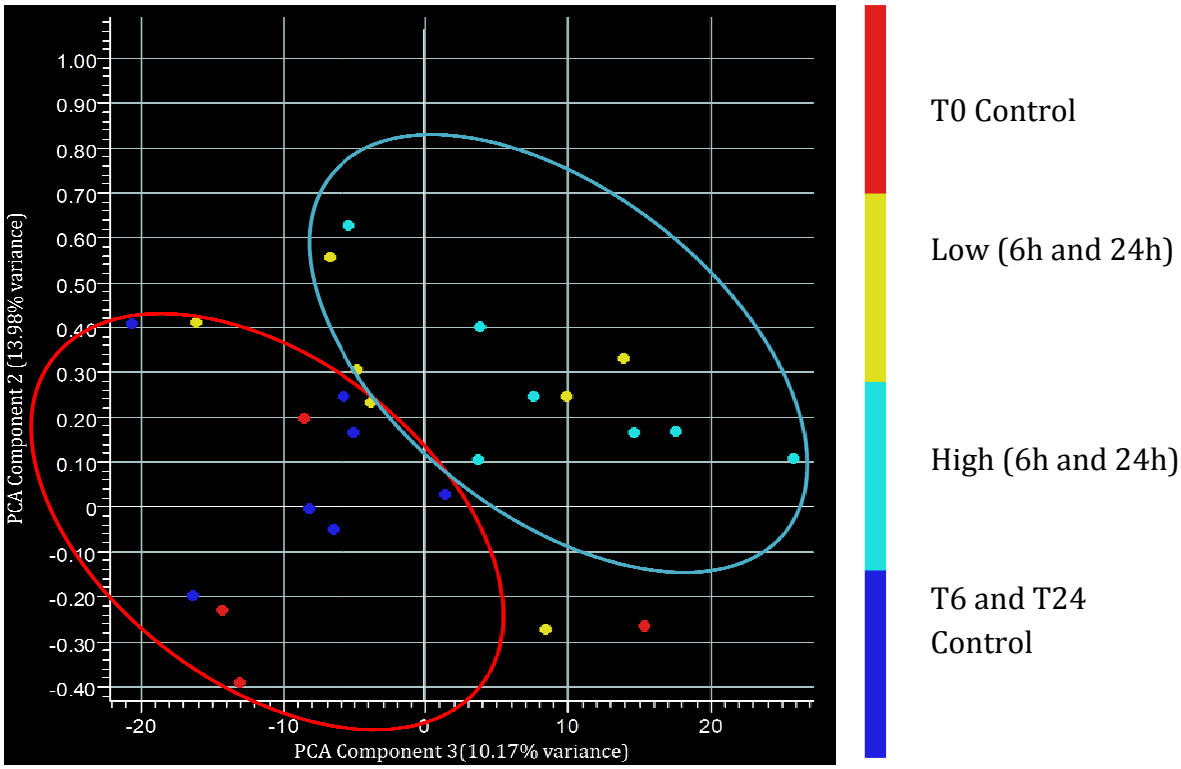

Figure 5.5 Principal components analysis (PCA) scores plot for all genes that responded to treatment in neonate *Daphnia magna* from the microarray study for both mixture concentrations and both time points. The plot shows separation of samples according to treatment along PC2 and PC3 axes, with control and treated samples clustering separately (groups encircled in red and blue respectively). Abbreviations used are T0: time zero, T6: 6 hour time point, T24: 24 hour time point, Low: low concentration of the mixture, High: high concentration of the mixture.

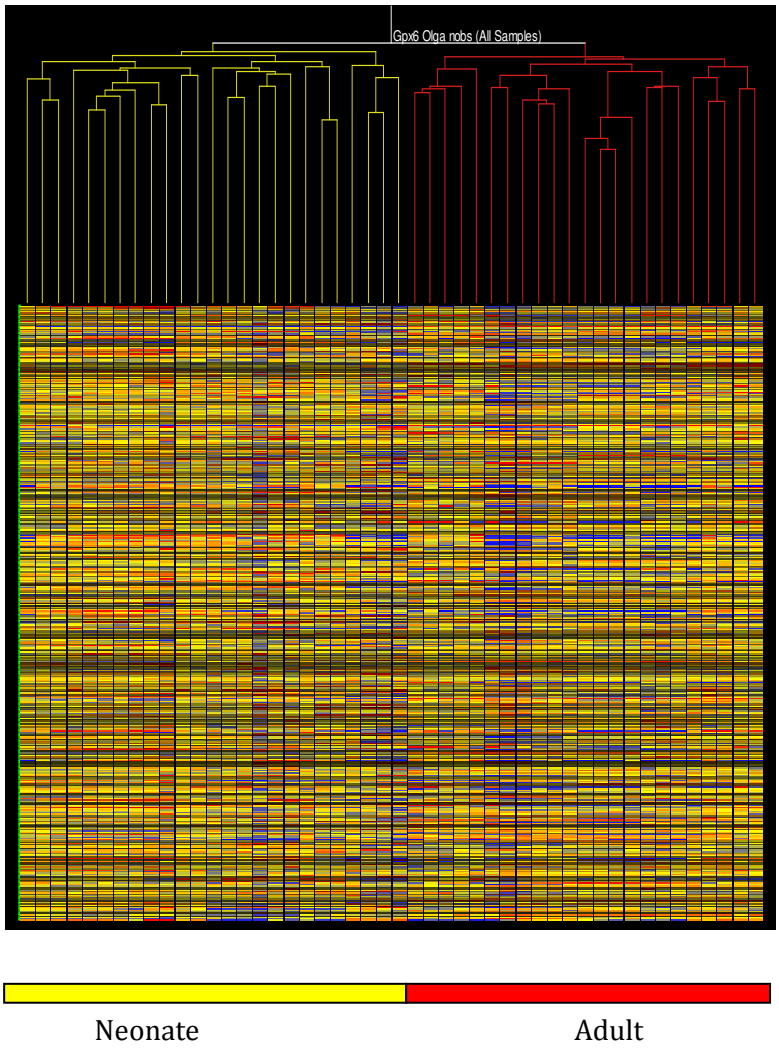

Figure 5.6 Gene tree generated in GeneSpring (Agilent Technologies) using the Pearson correlation showing relationships between expressed genes and the age of the daphnids. The gene tree is coloured as a gradient with respect to expression level, with red indicating up-regulation and blue down-regulation. For clarity, individual genes have not been labelled.

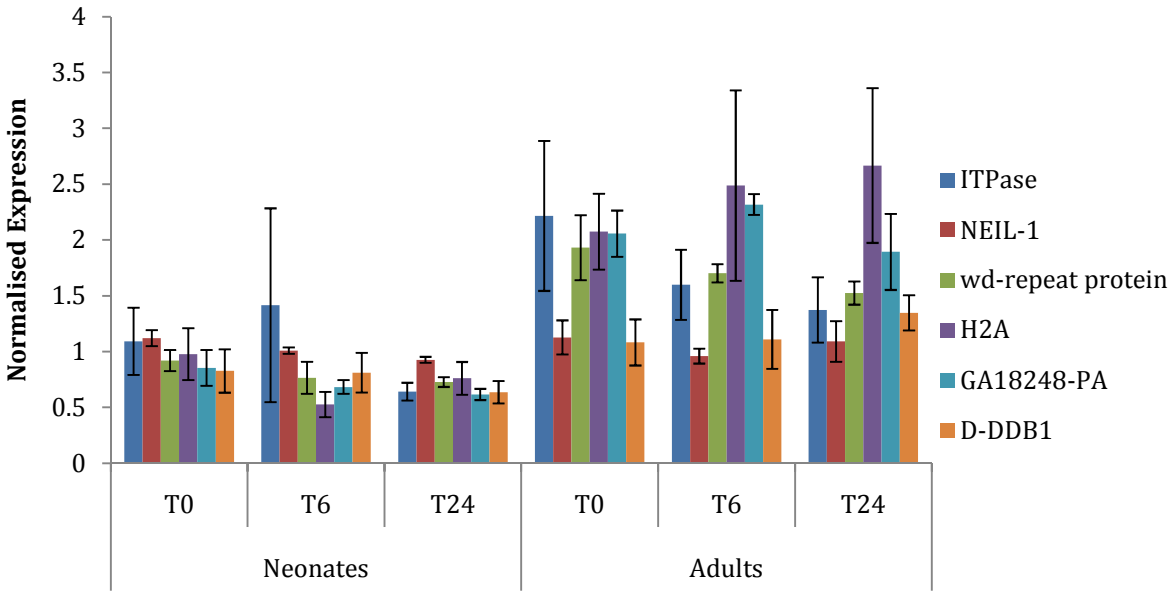

Figure 5.7 Genes involved in DNA repair that showed statistically significantly higher expression in control adult daphnids compared to control neonates (t test, equal variances not assumed, P<0.01). Abbreviations used are ITPase: Inosine triphosphate pyrophosphatase, NEIL-1: nei endonuclease VIII-like 1, H2A: Histone 2A, D-DDB1: Damage-specific DNA binding protein 1 (127kDa), T0: Time 0 hours, T6: 6h, T24: 24h. Data are presented as means of median normalised expression values, n=3-4 biological replicates, 20 adults pooled per sample, ± standard error of the mean.

Table 5.6 Genes involved in DNA repair that were shown to have significantly higher expression in un-treated adult daphnids compared to un-treated neonates.

Accession #	Gene	P value
XM_661952	GA18248-PA	4.19E-09
AC119986	Damage-specific DNA binding protein 1 (127kDa; D-DDB1)	2.59E-08
X56335	Histone 2A (H2A)	2.66E-07
CR726589	Wd-repeat protein	9.93E-07
NM_018672	Nei endonuclease VIII-like 1 (NEIL-1)	0.002
DQ217309	Inosine triphosphate pyrophosphatase (ITPase)	0.007

Statistically significant differences were identified by Welch T test assuming unequal variances.

A major category that showed significantly higher expression (2-fold cut-off) in adults was cell cycle-associated genes, including the cyclins a1, b3 and b4 (FDR<0.05; Appendix 3, Table 1). Genes coding for proteins involved in protein folding and protein degradation also showed significantly higher expression in adults, such as the heat shock proteins (hsp) 70 and 90, and proteasome subunits (FDR<0.05). Genes encoding variants of vitellogenin (VTG), which is the major egg protein in *D. magna* (Kato *et al.,* 2004), such as VTG-like protein, vitelline membrane outer layer 1 protein and VTG fused with SOD showed significantly higher expression in adults (FDR<0.05), with VTG fused with SOD showing expression of up to 216 fold higher than in neonates. The monooxygenase CYP3A and genes for haemoglobin also showed significantly higher expression in adult daphnids (FDR<0.05).

In neonates 516 genes were significantly more highly expressed by a factor of 2-fold or more (t test), primarily associated with cuticle formation and degradation, mainly cuticle proteins and chitinase enzymes (FDR<0.05; Appendix 3, Table 2).

Overall, adults appear to have a greater capacity to repair DNA (at least based on gene expression level) and respond to oxidative stress, and show greater levels of cell cycling, protein folding and degradation. In neonates, genes involved in cuticle formation and degradation are expressed at a higher level.

5.3.3.4 Differential Gene Expression According to Treatment

5.3.3.4.1 Adults

Differential gene expression was observed in adults following exposure to both the low and high concentrations of the BaP-sodium dichromate mixture at both time

points (compared to the time-matched control). As highlighted previously, the majority of the changing genes are based on a 2-fold cut off, as variability in the data limited the number of statistically significant changes that could be identified.

Following a 6h exposure to both concentrations, a greater number of genes showed differential expression than at the 24h time-point, compared to the time-matched controls (see Table 5.5). Gene expression changes were observed in a number of categories at both time points, particularly oxygen binding and transport, cuticle proteins, developmental proteins, and metabolism, including enzymes involved in glycolysis.

After a 6h exposure, genes in additional groups to those mentioned above also showed modulation, such as those associated with cell cycle (e.g. cyclin b3 and b4). A greater number of stress response genes were differentially expressed after a 6h exposure, detailed in Table 5.7. Some specific genes involved in DNA repair showed up-regulation in response to treatment in adults. Following a 6h exposure, the p73-like protein showed the most notable increase in expression which was significant at the high concentration ($P<0.05$) compared to the 6h control. NEIL-1 also showed a statistically significant increase in expression at the low dose ($P<0.05$) compared to the time-matched control. The genes for the DNA repair proteins D-DDB1, RFC, RAD23, TREX-1 and 6-4 photolyase also showed an apparent increase in expression following a 6h treatment with the low and high dose compared to the time-matched control (Figure 5.8). However, these were not statistically significant based on the number of samples analysed.

Table 5.7 Stress-related genes showing a higher level of expression in adults following a 6h exposure to either the high or low concentration (conc.) of the sodium dichromate-benzo[a]pyrene mixture compared to the 6h control.

Accession#	**Gene**	**Conc.**	**Exp. change**
AY174095	Delta class glutathione-S-transferase	High	4.755
AJ720637	Hsp40 homolog, subfamily A, member 2	High	2.3
NM_001031375	Hsp40 homolog, subfamily B, member 12	High	2.073
AC117255	Glutathione peroxidise	High	2.027
AC024213	Glutathione-S-transferase family member	Low	2.979
	(GST-11)	High	9.196
BC054309	Peroxiredoxin 6		2.59

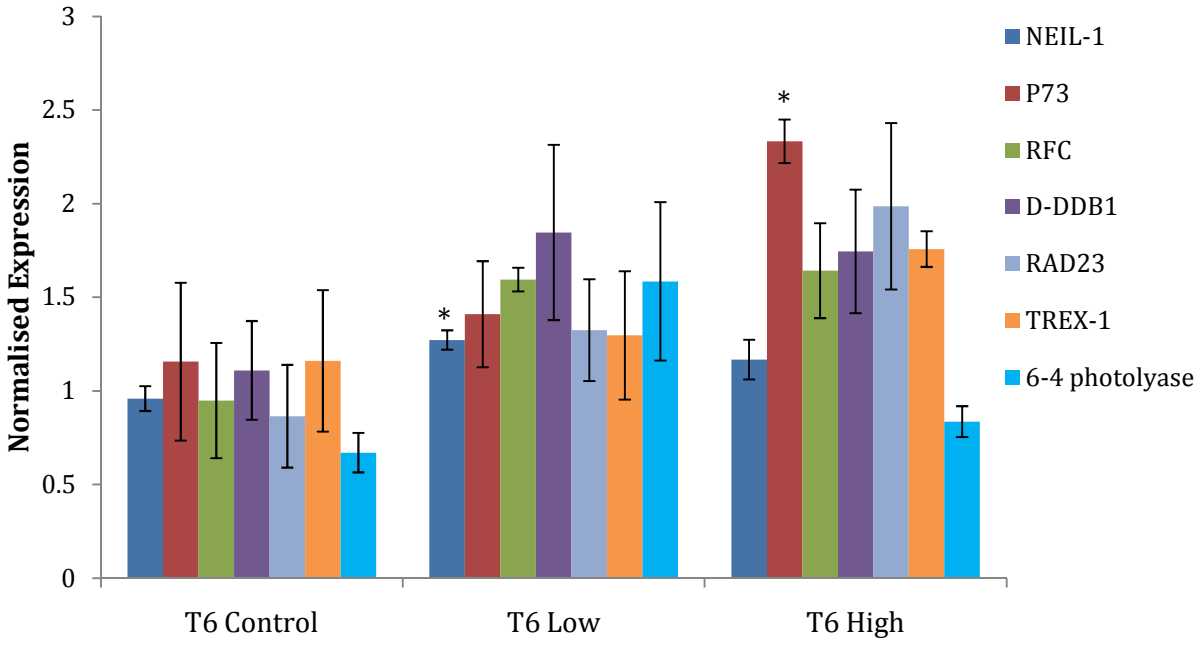

Figure 5.8 Genes involved in DNA repair that showed increased expression in response to a 6h exposure to the low or high dose of the sodium dichromate-BaP mixture as compared to the 6h control in adult daphnids. Abbreviations used are NEIL-1: nei endonuclease VIII-like 1, p73-like: Delta-n p63 (p73-like protein), RFC: replication-factor-C 40kD subunit, D-DDB1: Damage-specific DNA binding protein 1 (127kDa), TREX-1 (three prime repair exonuclease 1); T6: 6h time point, Low: low dose of the mixture, High: high dose of the mixture. A * denotes a statistically significant increase in expression from the control, $P<0.05$ (T test, equal variances not assumed). Data are presented as means of median normalised expression values, n=3-4 biological replicates, 20 adults pooled per sample, ± standard error of the mean.

Genes involved in the response to oxidative stress showed up-regulation following exposure in adult daphnids. After 24h the gene for glutathione-*S*-transferase 11 was up-regulated following exposure to the both the high and low concentrations, and glutathione peroxidase in response to the low concentration. Genes for cuticle proteins and chitinases showed a clear down regulation at the low dose, which was statistically significant for two chitinase genes (compared to the time zero control, FDR<0.1). A shift to up-regulation of these genes was seen at the high concentration after 24h, while in comparison, a general trend for down regulation was seen at the 6h time point for both concentrations of the mixture.

Genes encoding for proteins involved in the synthesis of haemoglobin (e.g. 5-aminolevulinic acid synthase), as well as those encoding haemoglobin proteins show a clear trend for up-regulation following treatment at both time-points, although more genes in this category differed in expression at the high concentration. The gene for *D. magna* haemoglobin (U67067) showed a significant up-regulation following a 24h exposure to the high concentration compared to the time-matched control (FDR<0.1). In addition, ferritins were up-regulated following a 6h exposure to both concentrations (Table 5.8) and genes involved in lipid metabolism such as 3-hydroxyacyl-CoA dehydrogenase and fatty acid binding proteins (carrier proteins for fatty acids) showed up-regulation at both doses and time-points (Table 5.9).

Table 5.8 Differential regulation of metal binding genes following exposure to either the low or high dose of the sodium dichromate-benzo[a]pyrene mixture for 6 hours or 24 hours in adults and neonates.

Accession Number	**Gene**	**Age**	**Time/Concentration**	**Expression Change**
AJ292556	Ferritin	Neonate	6h low/high	Up (18/10)
			24h low	Up (3)
		Adult	24h low	Down
AJ245734	Ferritin 3-like protein	Neonate	24h low/high	Up (2/3*)
		Adult	6h low/high	Up (2)
			24h low	Down

A * denotes a significant change with Benjamini and Hochberg multiple testing correction, false discovery rate (FDR) <0.1, 2-fold cut-off. Low = low dose of the sodium dichromate-BaP mixture (0.25µM-0.01µM) and high = high dose of the sodium dichromate-BaP mixture (0.75µM-0.1µM).

Table 5.9 Up-regulation of genes involved in lipid metabolism in adult and neonate daphnids following exposure to the low or high concentration of the sodium dichromate-benzo[a]pyrene mixture for 6 hours or 24 hours, compared to the time-matched controls.

Accession Number	Gene	Fold Change	Time/ Conc.
Neonates			
AC125538	Apolipoprotein d	2.784	6h L
		2.3	6h H
AC091073	Probable long chain fatty acid CoA ligase	2.56	6h H
XM_600800	Sphingosine-1-phosphate lyase 1	2.082	6h H
		2.1	24h H
AL592494	Methylmalonyl CoA epimerase	2.336	24h L
		2.6	24h H
AL591363	Acyl-Coenzyme A binding domain containing 5 isoform a	2.3*	24h H
Adults			
XM_420490	3-hydroxyacyl-CoA dehydrogenase	2.159	6h H
AK112532	Acyl-CoA synthetase short-chain family 3	2.152	6h L
		3.171	6h H
XM_503900	Fatty acid binding protein	3.046	24h L
		3.116	24h H
AC125538	Apolipoprotein d	3.146	24h H
XM_810384	Fatty acid-binding protein, muscle (M-FABP)	3.101	24h H
BC084493	Lipase	3.093	24h H
AL672066	Liver basic fatty acid binding protein	3.067	24h H

A * denotes a significant change with Benjamini and Hochberg multiple testing correction, false discovery rate (FDR) <0.1, 2-fold cut-off. Abbreviations used: Conc. (concentration); H = high dose of the sodium dichromate-BaP mixture (0.75μM-0.1μM); L = low dose of the sodium dichromate-BaP mixture (0.25μM-0.01μM).

5.3.3.4.2 Neonates

In neonates, a greater proportion of the differentially expressed genes were in response to treatment with the high concentration.

The monooxygenase CYP3A showed up-regulation in response to treatment with the high dose for 24h compared to the time-matched control, which was supported statistically (FDR<0.1, 2-fold cut-off), and the monooxygenase dopamine β hydroxylase-like 1 (DBH-like-1) was apparently up-regulated following treatment with low dose for 6h.

Up-regulation of genes involved in the response to oxidative stress was observed in neonates, although a smaller range of genes was identified than in adults (Table 5.10). Of these, up-regulation of the gene encoding glutathione-S-transferase 11 was statistically significant following a 24h exposure to the high dose compared to the time matched control (FDR<0.1, 2-fold cut-off).

With respect to genes involved in DNA repair, there is apparent up-regulation of ITPase, H2A and the unknown gene GA18248-PA after 6h at the low dose compared to the time-matched control (Figure 5.9), although the increase in expression is much less marked than that observed for DNA repair-associated genes in adults. The unattributed gene GA18248-PA also showed a 1.4 fold increase in expression after a 24h exposure to the high dose compared to the time-matched control.

With regard to reproduction, there was a trend for down-regulation of genes encoding vitellogenin (VTG), which is the primary egg yolk protein in *D. magna* (Kato *et*

Table 5.10 Stress response genes up-regulated in neonate *Daphnia magna* following exposure for 6h or 24h to the low or high dose as indicated, compared to the time-matched control.

Accession Number	Gene	Fold change	Time/Conc.
AY174095	Delta class glutathione S-transferase	3.1	6h L
		6.1	6h H
AF133268	Glutathione-S-transferase	2	24h H
AC117255	Glutathione peroxidase 3	2.5	6h H
AC024213	Glutathione S-Transferase family member (gst-11)	2.5	6h L
		5.3	6h H
		8	24h L
		13*	24h H
CR937859	Thioredoxin peroxidase I (Peroxiredoxin I)	2	6h H
		2.4	24h L
XM_533241	Peroxiredoxin V	3	24h H
	Peroxiredoxin	2.2	24h L

A * denotes a significant change with Benjamini and Hochberg multiple testing correction, false discovery rate (FDR) <0.1, 2-fold cut-off. Abbreviations used: Conc. = concentration; H = high dose of the sodium dichromate-BaP mixture (0.75µM-0.1µM); L = low dose of the sodium dichromate-BaP mixture (0.25µM-0.01µM).

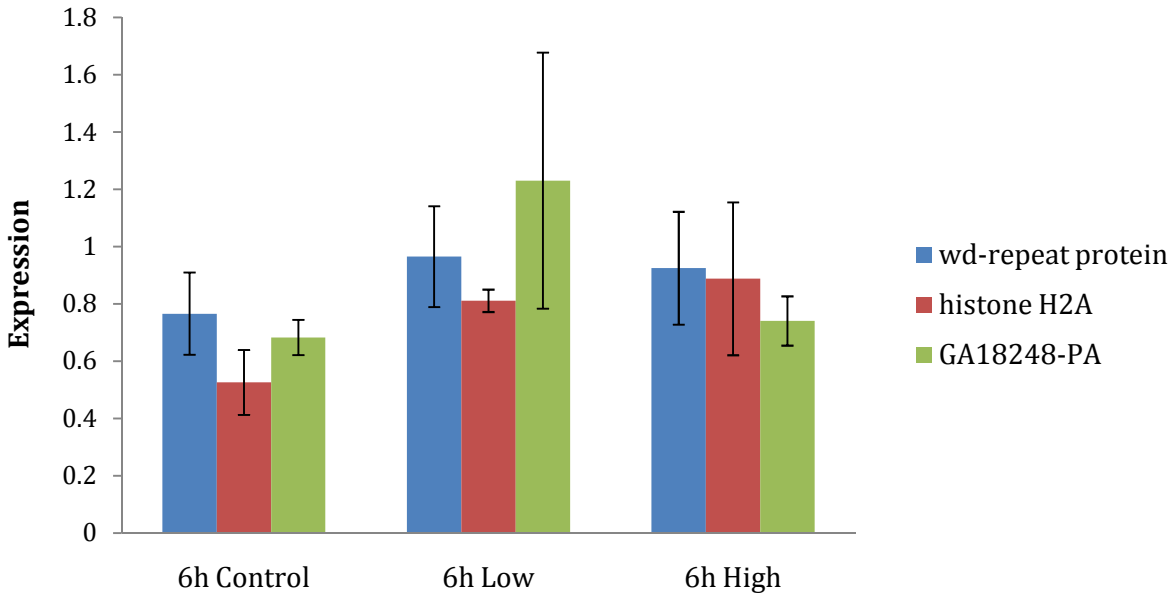

Figure 5.9 Genes involved in DNA repair that showed apparent up-regulation following exposure for 6h in neonate daphnids to the low and high concentration of the sodium dichromate-benzo[a]pyrene mixture. Data are presented as means of median normalised expression values ± standard error of the mean, n=3 biological replicates, 100 neonates pooled per replicate. Abbreviations used: 6h = 6 hour exposure, High = high dose of the sodium dichromate-BaP mixture (0.75µM-0.1µM); Low = low dose of the sodium dichromate-BaP mixture (0.25µM-0.01µM).

al., 2004), at all concentrations and time-points except for a 6h exposure to the low dose, where an up-regulation of VTG fused with SOD was observed. The down- regulation of vitellogenin with treatment was supported statistically at the 24h high dose for the gene for vitelline membrane outer layer 1 protein (FDR<0.1, 2-fold cut-off).

Up-regulation of ferritins followed exposure following exposure to both concentrations and time points (Table 5.8) which was supported statistically for the 24h exposure to the high dose in neonates (FDR<0.1, 2-fold cut-off).

Genes involved in lipid metabolism were up-regulated in response to treatment over both doses and time-points in neonates (Table 5.9), of which, acyl-coenzyme A (binding domain containing 5 isoform A) was statistically significantly more highly expressed following a 24h exposure to the high dose (FDR<0.1, 2-fold cut-off).

A gene encoding haemoglobin showed significant down-regulation on comparison of all treated and control samples (FDR<0.1).

5.3.3.5 Blast2GO Analysis

The nucleotide sequences of the differentially expressed genes (significantly, 2 fold) were submitted to Blast2GO (http://www.blast2go.de; Conesa *et al.,* 2005), which identified the biological processes, molecular functions and cellular component GO terms for the 'known' fragments. The GO terms that were statistically significantly over- and under-represented among lists of differentially expressed genes in un-treated adults compared to un-treated neonates are shown in Appendix 4, Table 1 and Table 2.

In adults, over-represented GO terms included those involved with cell cycle, macromolecule metabolism, protein catabolism, and response to oxidative stress (Appendix 4 Table 1; Figure 1) which supports the conclusion from the gene expression data that adult daphnids have a greater capacity to respond to oxidative stress and that cell cycling and protein folding and degradation occur to a greater extent. For neonates, GO terms associated with chitin metabolism and catabolism were more highly over-represented (Appendix 4, Table 2), again supporting the conclusion from the gene expression data that cuticle formation and degradation occur to a greater extent.

In response to treatment, the GO term glutathione transferase activity was significantly over-represented in treated compared to control samples (FDR=0.07).

5.3.3.6 Real-Time PCR Results

Based on microarray data for gene expression in adult daphnids following a 24h exposure to the highest dose of the BaP-sodium dichromate mixture (0.1μM-0.75μM), four genes that exhibited changes in expression between treated and time-matched control samples were selected for real-time PCR (RT-PCR) analysis; vitellogenin (VTG), p73-like protein, CYP3A and caspase-8. This is useful for validation of microarrays since the array process is complex, and the results can be affected by each step. Validation of changes in gene expression is achieved using an alternative method, of which RT-PCR is currently regarded as the most accurate and reproducible technique (Rajeevan *et al.,* 2001).

The efficiencies of the primers for each gene were calculated and found to be too low for CYP3A and caspase-8 (<90%), thus data are only presented here for VTG and p73-like protein (normalised to the reference gene, CG8121-PA, which did not change with treatment; P value 0.16, one-way ANOVA). The data obtained partially supported the gene expression changes highlighted by microarray analyses.

There is agreement between the RT-PCR results and microarray data, for VTG, as both show up-regulation of the gene, although the change is much greater following RT-PCR analysis (Table 5.11). RT-PCR is reported to be much more sensitive for quantifying mRNA than microarray analyses (Hook *et al.,* 2006), which may explain the greater changes in expression observed, and higher backgrounds in the microarrays may also account for the smaller changes seen using this approach.

We also analysed p73-like protein, however a lack of correspondence with the microarray data was observed. The results from the RT-PCR showed up-regulation for

this gene, whereas no change in expression was observed using microarray analysis. This difference is likely to be due to the fact that p73 is expressed as multiple isoforms that arise either from alternative splicing or alternative use of the promoter (Melino *et al.,* 2002). Disagreement between microarray and RT-PCR results is not uncommon. For example, Poynton *et al.,* (2007) and Hook *et al.,* (2006) observed differential expression of genes using RT-PCR that were found to be unchanged using microarray analysis.

Table 5.11 Comparison of fold changes for the selected gene fragments as determined by real-time polymerase chain reaction and microarray analysis.

Gene Fragment	**Microarray**	**Real-Time PCR**	**P value (Real-Time PCR)**
Vitellogenin (VTG)	1.03	3.17	$<5 \times 10^{-4}$
P73-like	0.8	4.1	$<5 \times 10^{-6}$
CG8121-PA (reference gene)	1	1.7	0.16

Each gene was amplified from the same mRNA extracted from adult daphnids treated for 24h with the highest concentration of the benzo[a]pyrene-sodium dichromate mixture (0.1µM-0.75µM) as used for the microarray study. Three to four biological replicates with three technical replicates of each were run.

5.4 Discussion

We were interested in linking exposure of *D. magna* to genotoxic agents to changes in gene expression, particularly those involved in DNA damage and repair, which may act as biomarkers. In particular, this would be of value for establishing the effects of uncharacterised chemicals or chemical mixtures. Responses of such biomarkers might then act as an alert for more specific studies on DNA damage *per se,* to establish in more detail the nature of the damage and the potential causative agent. Since chemicals never occur in isolation in the aquatic environment, we were particularly interested in applying microarrays to a mixture scenario. The potential for interactions between chemicals in mixtures is high, and compound interactions are of great concern from a toxicological perspective, since they may influence the overall toxicity and therefore organism effects.

Initial experiments provided evidence of exposure to the genotoxicants sodium dichromate and BaP, with toxic concentrations being identified for both compounds. Sub-lethal concentrations of these genotoxicants were established in order to enable analysis of genotoxic effects. Interestingly, non-lethal concentrations of sodium dichromate and BaP alone were toxic to daphnids when given in combination, indicative of additive or synergistic effects.

To identify whether gene-expression changes indicative of exposure to these genotoxicants were detectable in *D. magna,* a preliminary study utilised a custom cDNA microarray from the University of Reading. Exposure to a relatively high and low concentration of the mixture of sodium dichromate and BaP resulted in apparent differential changes of genes involved in a range of processes based on a 2-fold cut-off,

including oxygen binding, exoskeleton-related proteins and developmentally related proteins. Within these categories, there appeared to be a concentration effect on gene expression, with a greater proportion of the modulated genes down-regulated at the low dose, and up-regulated at the high dose. The reason for this apparent effect is not clear. Comparison to published microarray studies in *D. magna* revealed some commonality with gene expression changes observed in our study, such as the up-regulation of genes encoding ferritins and down-regulation of cuticle protein gene (Table 5.4). Specifically, the majority of these genes showed expression changes in response to metals, thus implicating that this sub-set of genes may have the potential to indicate exposure to metals in *D. magna*.

This microarray did present some limitations when considering the effects of genotoxicants on gene expression. We obtained the array largely un-annotated, thus we subjected the sequences of apparently modulated transcripts to BLAST searches to identify genes with high similarity. Of the genes and processes identified to be affected by the genotoxicants, only a small number of genes involved in the stress response were apparently up-regulated in response to treatment. Furthermore, and perhaps most importantly, only one gene involved in DNA repair, XPA, was identified, and we were particularly interested in determining whether there was a link between exposure to genotoxicants and expression changes of such genes. Nevertheless, genes for glutathione peroxidise (GPX), a glutathione-*S*-transferase (GST) and oxidative stress protein, known to be involved in the response to oxidative stress in other organisms (e.g. GST was up-regulated in response to Cd in flounder; Sheader *et al.,* 2006), were apparently up-regulated in response to treatment. Since both compounds can induce oxidative stress, sodium dichromate via the induction of ROS synthesis and BaP following metabolic

activation to quinones, amongst other metabolites (see Section 5.1), this indicates proof of principle that exposure to these compounds is detectable via gene expression changes.

To investigate further the responses to these compounds, we employed a commercially available *D. magna* oligonucleotide microarray from Agilent, which contained genes representative of a wide range of processes. In particular, a greater number of genes involved in DNA repair and the response to oxidative stress were present on the array, making it more suitable for our study. To date only a limited number of microarray studies have been conducted in *D. magna*, primarily involving neonates, although studies by Poynton and colleagues have investigated gene expression changes in adult daphnids (Poynton *et al.,* 2007, 2008a, 2008b). Our study involved both adult and neonate daphnids, with a view not only to identifying expression changes relating to exposure, but also between these age groups, to determine whether age reflects the differential expression of genes in response to toxicants.

5.4.1 Life-Stage Related Differences in Gene Expression

Comparison of gene expression between untreated adults and neonates revealed a switch from cuticle formation and breakdown as the dominant process in neonates to cell cycle regulation and reproduction in adults. This observation was supported by the GO terms found to be over-represented in neonates (e.g. chitin binding and metabolism, structural constituent of the cuticle and extracellular region) and adults (e.g. cell cycle, eggshell formation and insect chorion formation).

In adult daphnids genes mainly involved in cell cycle regulation and reproduction showed higher expression than in neonates. VTG fused with SOD showed the greatest induction of expression compared to neonates of up to 216 fold, which correlates with the timing for the deposition of eggs into the brood chamber at around 5-10 days of age (Ebert, 2005). Genes involved in sex-determination, such as complementary sex determiner, showed higher expression in adults. As discussed in Chapter 1 *Daphnia* spp. is parthenogenic, thus under ideal conditions the population is entirely female. These genes are likely to be more highly expressed in adults to control the sex of the developing embryos. Moreover, the higher expression of cell cycle genes, such as cyclin b3, checkpoint kinase, and cyclin a1, may reflect the development of the daphnid embryos.

Genes encoding proteins involved in protein folding and degradation, such as HSP 70 and 90 and proteasome subunits, showed significantly higher expression in adults. This may be indicative of a higher synthesis and turnover of proteins in adults compared to neonates.

The monooxygenase CYP3A showed significantly higher expression in untreated adult daphnids compared to neonates (FDR<0.05). The CYP3A subfamily members identified in *D. magna* have been shown to be critical for xenobiotic and steroid metabolism. In vertebrates, CYP3A subfamily members have substantial steroid 6β-hydroxylase activity and daphnids also have high levels of this activity (Baldwin, 2007). Higher expression of this gene may indicate a greater ability for adult daphnids to metabolise xenobiotics, as well as steroid hormones, which play a role in moulting, reproduction and maturation regulation (Baldwin and LeBlanc, 1994).

Genes encoding haemoglobin showed significantly higher expression in adult daphnids compared to neonates. It is known that haemoglobin is transferred from the mother's haemolymph to the eggs, and that the levels of haemoglobin increase to their maximum just prior to the first reproduction (Paul *et al.,* 2004), which may explain the higher levels in adults observed in our study, since at 7 days old the daphnids will likely be reproductively mature and producing eggs. Moreover, gas exchange in *Daphnia* occurs to a large extent via diffusion, with the whole carapace shown to be permeable to oxygen to an extent. The importance of oxygen diffusion into daphnids is known to decrease as body size increases (Paul *et al.,* 2004), which may provide an explanation for the lower levels of haemoglobin gene expression in neonates.

In neonates, cuticle formation and breakdown (moulting) appear to be dominant processes occurring. The cuticle is the *Daphnia* exoskeleton and provides protection to tissues, as well as giving support (Andersen *et al.,* 1995; Soetaert *et al.,* 2007). Chitin and cuticle proteins are the most important structural components of this exoskeleton, and proteolytic and chitinase enzymes are required for apolysis of the cuticle and successful completion of the moulting cycle (Soetaert *et al.,* 2007). In neonates, genes coding for cuticle proteins had higher expression than in adults at both time points, with more genes detectable at the 24h time point. In addition, at the 24h time-point a gene encoding a seine protease was also observed to have higher expression, which is likely to reflect the apolysis of the cuticle to enable moulting. Juvenile daphnids develop through four to six juvenile instars, which are discrete stages of development during which growth is accomplished by moulting (Ebert D, 2005). Each of the first three pre-adult instars in *Daphnia* are approximately one day in length (Anderson *et al.,* 1937), which provides a likely explanation for the greater number of genes encoding cuticle

proteins and serine proteases showing high expression at 24h. The present study used neonates less than 24h old, thus at the 24h time point the daphnids are likely to have reached the end of an instar and will be undergoing moulting. The length of the instars may also provide an explanation for the lower expression of these genes in adult daphnids. The first adult instar lasts approximately 2 days, with subsequent instars increasing in length (Anderson *et al.,* 1937). Since the duration of this study was only 24 hours and used daphnids 7 days old, it is likely that the daphnids have not reached the end of an instar and thus are not undergoing moulting. It would be useful to investigate the expression of genes involved in this process at later time points to validate this hypothesis.

The detection of the different dominant processes in adults and neonates, which can be explained by their age and life cycle, provides a useful validation for the array and elucidates important molecular changes occurring during development.

An interesting and important observation from the point of view of our study was that a number of transcripts encoding genes involved in the response to DNA damage and oxidative stress showed sustained higher expression in adults compared to neonates. This may be indicative of a greater capacity for adults to respond to DNA damage and oxidative stress than neonates. Alternatively, it may reflect a higher level of DNA damage and reactive oxygen species as a result of increased metabolic processes, or differences in systems involved in the activation or detoxication of pro-genotoxic agents between adults and neonates. There is insufficient information on daphnids to conclude on this. This finding has implications for ecological and toxicity testing, particularly when considering the effects of genotoxic chemicals. Currently neonates are

used for toxicity testing, with adults only utilised when investigating the reproductive effects of compounds. However, the data obtained from this study indicate that adult daphnids may be more responsive to genotoxicants and thus may be more suitable for genotoxicity testing. In support, the Comet assay in neonates did not reveal increases in strand breaks following exposure to the sodium dichromate-BaP mixture, yet gene expression analysis following exposure indicated the occurrence of DNA damage via the up-regulation of DNA damage responsive genes.

Following treatment, genes involved in a number of processes showed modulated expression in adults, particularly those that are reflective of the mechanisms of action of sodium dichromate and BaP, such as those involved in DNA repair and the response to oxidative stress (see Table 5.6 and Table 5.7).

5.4.2 Evidence of Enhanced Capacity for DNA Repair in Adults Following Chemical Treatment

A number of genes involved in the response to DNA damage showed up-regulation in adults in response to treatment, although there are other genes with GO terms linked to DNA repair are present on the array that did not show a response to treatment (Appendix 3, Table 5).

In adults, an up-regulation of the DNA repair-associated genes p73-like protein, D-DDB1, RFC and NEIL-1 was observed in response to exposure at the 6h time point (Figure 5.8). The p73-like protein showed the most notable increase in expression, which was concentration-dependent. This protein is a nuclear transcription factor

related to p53, and its role is to promote cell cycle arrest and/or apoptosis (Kaghad *et al.,* 1997; Kramer *et al.,* 2005). Under steady-state conditions p73 is maintained at very low levels, but at times of particular types of genotoxic stress, such as cisplatin treatment, this protein is significantly induced (Kramer *et al.,* 2005). Thus, the genotoxic stress induced by exposure to BaP and sodium dichromate appears to induce expression of this gene to enable cells with damaged DNA to undergo cell cycle arrest to facilitate repair.

RFC showed apparent up-regulation at the low concentration compared to the control, which was sustained, but not increased at the high concentration. RFC is involved in base excision repair (BER) and nucleotide excision repair (NER; see Chapter 1 for more detail), of which NER has been demonstrated in *Daphnia* (Connelly *et al.,* 2008). Cr(VI) compounds (of which sodium dichromate is one) are known to induce DNA-protein cross-links (Lee and Steinert, 2003), which are repaired by NER (Sancar *et al.,* 2004), thus the up-regulation of the RFC gene in this study implicates activation of NER in response to sodium dichromate-induced DNA damage.

D-DDB1 is another gene associated with DNA repair that was up-regulated in adults in response to treatment. In humans a number of DNA binding proteins have been identified, such as DDB (XPE), which has shown to be vital for excision repair (Hwang *et al.,* 1999; Takata *et al.,* 2002), with XPE specifically being involved in NER. Furthermore, DDB studies have demonstrated that this protein is induced by treatment with genotoxicants (Takata *et al.,* 2002). In our study D-DDB1 showed up regulation at both mixture concentrations, although slightly higher expression was observed at the low dose, further evidence of the inducibility of DDB proteins following genotoxicant

exposure. It has been reported that DDB recognises DNA lesions induced by reactive oxygen species (ROS), which, coupled with the involvement of DDB proteins in excision repair, implicates NER in the repair of oxidative DNA damage, a type of lesion known to be induced by sodium dichromate. The d-ddb1 gene has been cloned in *Drosophila* and the protein product appeared to have a role in recognising UV-induced DNA damage, and also repair of ROS induced lesions in the CNS (Takata *et al.,* 2002). A search on wfleabase revealed two genes with similarity to DDB1 and DDB1 (XPE) from *D. pulex*, which would be interesting to characterise to further understand the role of this protein in *D. magna*.

The NEIL-1 gene encodes a member of the FPG/Nei family of DNA glycosylases involved in the removal of oxidised pyrimidines in the process of BER (Bandaru *et al.,* 2002). Cr (VI) can induce the production of ROS, which can oxidise DNA bases producing, for example, pyrimidine lesions such as 8-OH-dG (Shi and Dalal, 1990). Thus, the up-regulation of NEIL-1 in our study provides some evidence that BER may occur in *D. magna*, and adds weight to the idea that sodium dichromate is the primary genotoxic stressor in our mixture.

The gene that encodes RAD23 showed apparent up-regulation following a 6h exposure to both the low and high concentration of the mixture compared to the time-matched control. This protein is involved in NER (Dantuma *et al.,* 2009), thus providing further evidence for sodium dichromate lesions being more prominent than those produced by BaP.

TREX-1 showed apparent up-regulation following exposure to the mixture of sodium dichromate and BaP, most notably at the high dose. This gene encodes a protein

that is the major 3'-5' exonuclease in mammalian cells and acts preferentially on single-stranded DNA. This protein is thought to be involved in DNA replication, or gap-filling during DNA repair (Yang *et al.,* 2007). This gene has also been shown to be up-regulated in mouse fibroblasts following exposure to UVC radiation, and in human fibroblasts after BaP exposure (Christmann *et al.,* 2009). Thus up-regulation of this gene may be in response to exposure to BaP in our mixture.

The gene for photolyase was identified as apparently up-regulated following exposure to the low dose for 6h. Photolyase exists in two forms and is involved in the repair of 6-4 photoproducts and UV-induced CPDs (Sancar *et al.,* 2004), thus the role of this protein in the repair of DNA damage induced by sodium dichromate and BaP is unclear.

When comparing the number of up-regulated genes associated with a DNA damage response, the most notable changes were identified at the 6h time-point in adults. A possible explanation for this is that 6h represents a key time-point in the switching on of these genes in response to the genotoxic insult. Since much less induction of these genes was observed after 24h, it could be postulated that, on the whole, the expression of these genes has returned to baseline levels following production of sufficient protein. Alternatively, or in addition, removal or sequestration of the compounds, for example into lipid stores, could be occurring. This highlights the importance, and challenges of selecting the appropriate endpoints for such studies.

In neonates differential expression of genes involved in the DNA damage response was much less notable. Limited up-regulation was observed for ITPase, H2A

and the unattributable transcript GA18248-PA, primarily after a 6h exposure, further implicating this time point is key in the response to DNA damage.

In response to DNA double strand breaks (DSBs) in mammals, H2A molecules adjacent to the site of damage are rapidly phosphorylated. These then form foci, which are important for recruitment of proteins involved in DNA repair (Paull *et al.,* 2000; Celeste *et al.,* 2002; Redon *et al.,* 2003). The expression of this gene was apparently increased following exposure, and since strand breaks can be induced by sodium dichromate (Hodges *et al.,* 2001), this provides further evidence that the DNA damage resulting from exposure is primarily caused by this compound.

The microarray did contain other DNA repair genes that we were unable to analyse as the spots did not pass the quality thresholds for the normalisation. Those that were undetectable are involved in mismatch repair, NER and double strand break repair. In addition, the array does not contain all the genes in the *Daphnia* genome, thus there are likely to be other genes involved in DNA repair that we were unable to investigate.

Overall, up-regulation of DNA repair genes was more apparent in adult daphnids, which adds weight to the conclusion that adults have a greater capacity for repair when considering the significantly higher expression of these genes in un-treated adults.

5.4.3 Evidence of Oxidative Stress

Several genes involved in the response to oxidative stress were up-regulated in both adults and neonates following exposure to the BaP-sodium dichromate mixture, including homologues of glutathione-*S*-transferase (GST), and peroxiredoxins.

The role of GST is to protect cells from oxidative damage by catalysing the conjugation of glutathione (GSH) with electrophilic substances and conjugating the products of lipid peroxidation to GSH. Organisms are known to adapt to oxidative stress by up-regulating anti-oxidant enzymes, since a failure to detoxify excess ROS can lead to oxidative damage such as lipid peroxidation and DNA damage (Barata *et al.,* 2005), and *Daphnia* spp. are known to have have catalase, superoxide dismutase and GST (Barata *et al.,* 2005). Moreover, oxidative stress has been shown to transcriptionally regulate GST (Casalino *et al.,* 2004). One mechanism of action of sodium dichromate is the induction of cellular ROS, thus it could be postulated that GST is responding primarily to exposure to this genotoxicant. Up-regulation of GST has previously been reported in the European flounder *Platichthys flesus* following exposure to Cd (Sheader *et al.,* 2006), rainbow trout in response to Zn (Hogstrand *et al.,* 2002), and Iberian endemic minnows *Leuciscus albournoides* following environmental exposure to copper and selenium (Lopes *et al.,* 2001). Moreover, Poynton *et al.,* (2007) reported an up-regulation of GST homologues in *D. magna* following Cd exposure, which is also known to induce oxidative stress, and since transcripts for this gene were up-regulated at all concentrations and time points in neonates and adults in our study, GST may represent a useful biomarker for oxidative stress.

Another group of genes involved in protection against oxidative stress are the peroxiredoxins (Prxs), or thiol peroxidases. These enzymes are ubiquitous, having been identified in animal, plant and yeast cells, and most eubacteria and archea (Wood *et al.,* 2003), and are expressed at high levels due to their role in detoxifying peroxides and peroxynitrite. In addition, these enzymes have been associated with other cell functions including apoptosis and cell cycle (Abbas *et al.,* 2008). Thioredoxin peroxidises (TrxPs) also belong to this family (Berggren *et al.,* 2001). Genes from this family were up-regulated in our study at all concentration and time-points except one in neonates, and at one concentration and time-point in adults, most likely in response to sodium dichromate-induced oxidative stress. This anti-oxidant system has previously been reported to be induced following exposure to cadmium in *P. flesus* (Sheader *et al.,* 2006) and in response to oxidative stress in mammals (Watson and Jones, 2003). Peroxiredoxin genes have also been shown to be up-regulated in *D. magna* in a study by Poynton *et al.,* (2007) following exposure to Cd, thus these genes may also represent a useful biomarker for oxidative stress.

Expression data for genes involved in metal binding revealed up-regulation of ferritins following exposure in both adults and neonates, with the exception of a 24h exposure to either concentration in adults. A cytosolic heavy-chain ferritin has been identified in *D. pulex* (Poynton *et al.,* 2007). Ferritins are involved in scavenging and storing iron (Fe) and Fe is known to induce transcriptional and translational up-regulation of these proteins (Muller *et al.,* 1991). Moreover, ferritin is regulated by oxidative stress (Poynton *et al.,* 2007), and Muller *et al.,* (1991) proposed that ferritin may be the primary detoxification response to heavy metals in Xenopus cells. Thus the up-regulation of genes for ferritin and ferritin 3-like protein observed in our study may

be part of the response to oxidative stress induced by sodium dichromate (Poynton *et al.,* 2007) and may represent useful biomarkers for this type of insult. More generally, these metal binding proteins may indicate exposure to metals, Cr(VI) in our study, and also Cu and Cd (Poynton *et al.,* 2007), at least in neonates. In adults, however, the ferritin genes presented a more variable response, with a down regulation after a 24h exposure to the low dose, and no differential expression recorded at the highest concentration at this time point. Thus ferritins do not represent an appropriate biomarker for metal exposure in adult daphnids.

5.4.4 Effects on Monooxygenases

The monooxygenase CYP3A was shown to be up-regulated in neonates following a 24h exposure to the high dose. This gene has been implicated in the mono-oxygenation of BaP in channel catfish *Ictalurus punctatus* (James *et al.,* 2005), as well as CYP1A, with the proposed metabolites including 3-OH BaP and BaP-7,8-dihydrodiol (James *et al.,* 2001; 2005). Thus the up-regulation of this gene in our study could be in response to exposure to BaP in the mixture, which to our knowledge is the first report of CYP3A induction in *D. magna*.

5.4.5 Effects on Lipid Metabolism

In this study up-regulation of genes involved in energy metabolism pathways was observed. Genes involved in lipid metabolism, such as 3-hydroxyacyl-CoA dehydrogenase, and fatty acid binding proteins (carrier proteins for fatty acids) were up-regulated in both adults and neonates. In daphnids the lipid fraction is mobilised to

maintain homeostasis during toxicant exposure (De Coen and Janssen, 2003). The main part of the lipid fraction in adults has been suggested to be utilised in egg production (De Coen and Janssen, 2003), thus alteration of the lipid status by toxicants may ultimately lead to population level effects, as there will be less lipids available for reproduction, which may be reduced to allow survival of the mother (De Coen and Janssen, 2003). Furthermore, depletion of lipid levels in neonates may also affect the population dynamics, as it has been shown that effects during early development can have long lasting effects of growth, survival and reproduction, to the second generation at least (De Coen and Janssen, 2003).

5.4.6 Potential Effects on Energy Budgets

In adults induction of ATP synthase was observed, at both concentrations following a 6h exposure, and at the high dose after 24h. In neonates, up-regulation was only observed after a 6h exposure to the high concentration (Appendix 3, Table 2). This induction of ATP synthase indicates that the daphnids, particularly the adults, have a higher energy (ATP) requirement as a result of exposure to the BaP-sodium dichromate mixture. This response was also observed by Soetaert *et al.,* (2006) in response to exposure to the fungicide propriconazole, although this was in neonates. In addition, α-amylase was shown to be up-regulated in adults (24h, high dose) and neonates (6h, low and high dose). This digestive enzyme catalyses the first step of starch and glycogen digestion (Soetaert *et al.,* 2007), and induction of this gene has previously been reported following toxicant exposure (De Coen and Janssen, 1997; Soetaert *et al.,* 2007). This response also points towards a higher energy need of *D. magna* following toxicant

exposure. It has been shown that toxicant exposure can result in an increase in basic energy metabolism leading to energy budgets being reduced (De Coen and Janssen, 2003), which is also implied by the induction of ATP synthase in daphnids in this study. Thus the impact of these agents is not only acutely apparent in relation to DNA damage and oxidative stress, but may also have longer term effects, impacting on the survival of populations by compromising energy budgets.

5.4.7 Effects on Haemoglobin (Hb)

Hb was significantly differentially expressed in adults following a 24h exposure to the high dose of the mixture compared to the time-matched control. It has been shown that induction of Hb is partly regulated by an endocrine pathway in response to cues from the environment (Rider *et al.,* 2005). The mechanism by which BaP or sodium dichromate affect the methyl farnesoate signalling pathway to induce Hb levels is unclear. Induction of Hb levels has previously been reported by Rider *et al.,* (2005) in response to terpenoid hormone analogues, and Soetaert *et al.,* (2007) following exposure to fenarimol.

5.4.8 Effects on Reproduction

Some evidence of potential effects of exposure on reproductive capability has already been highlighted in the form of the depletion of lipid stores. Analysis also revealed another modulated gene with potential to affect reproduction. The chitinase gene was found to be down-regulated in adults following a 6h exposure to the high dose.

As discussed previously, this enzyme is involved in moulting, being implicated in the breakdown of the cuticle (Soetaert *et al.,* 2007). Daphnids need to shed their exoskeleton to release their brood of neonates, thus if the breakdown of the cuticle is decreased or inhibited, reproductive effects may ensue. Indeed, Poynton *et al.,* (2007) correlated a decrease in chitinase activity with chronic reproductive effects following exposure to zinc, thus this gene may represent a useful biomarker for metal exposure.

5.4.9 Conclusions

Overall, the present study has shown that age of daphnids accounts for a large proportion of the differences in gene expression observed. Adult daphnids have significantly higher expression of genes involved in cell cycling and reproduction, while in neonates, cuticle production and degradation-associated genes are more highly expressed. Of importance from the point of view of genotoxicants is the higher expression of genes involved in the response to DNA damage and oxidative stress in adults, implicating a greater capacity to respond to compounds that induce these effects. It may also be concluded that neonates may be more susceptible to DNA damage as gene expression indicated a significantly lower DNA repair capacity. Although many of the genes responding by 2-fold or greater following treatment were not statistically significant, but drawing on the GO terms that were significantly overrepresented and the genes that were identified as statistically significantly modulated following exposure, provide confidence in the changes observed. Moreover, RT-PCR validation of the array provided some support for the gene expression changes observed on the array.

Effects of exposure on gene expression were identified. From a DNA repair perspective, the results primarily indicate an induction of NER in adult daphnids in response to the presence of oxidative lesions and protein-DNA cross-links, most likely introduced by sodium dichromate. A large proportion of the responding genes are predictably involved in the oxidative stress response (such as GST and peroxiredoxins), which together with the DNA repair genes induced, points towards sodium dichromate being the primary stressor in our mixture. Further evidence for this hypothesis is provided by the up-regulation of the metal transport proteins ferritins.

Apparent up-regulation of TREX-1 following a 6h exposure at both concentrations does implicate a role for BaP in the DNA damage response, and although BaP can induce oxidative stress via the production of quinones (Behrend *et al.,* 2003), drawing on the results from Chapter 4, sodium dichromate is still likely to be the main genotoxic stressor in our study. We detected only very low EROD (CYP1A-like) activity in adult daphnids, and to our knowledge there are no published measurements of this activity in neonates. Since BaP requires metabolic activation (by CYP1A1 and CYP1A2 in mammals), it is probable, given the low EROD activity, that this is not occurring to any great extent. Moreover, although quinones are one of several metabolites of BaP, along with phenols, which can convert to quinones, (Miranda *et al.,* 2006), the contribution of these to the induced oxidative stress is likely to be minimal. Some evidence for the metabolic activation of BaP is provided by the up-regulation of CYP3A in neonates. CYP3A activity is detected using substrates such as 7-benzyloxy-(4-trifluoromethyl)-coumarin *O*-debenzylase (BFCOD; Hasselberg *et al.,* 2004; James *et al.,* 2005), and thus would not have been detectable by the EROD assay (Chapter 4). As discussed in Section 5.3.4, this gene has been implicated in the mono-oxygenation of BaP in catfish (James *et*

al., 2005), however to our knowledge there is no information in the literature regarding this activity in daphnids.

With regard to identifying potential biomarkers of exposure, our data highlights glutathione-S-transferase (GST) as the most consistent biomarker for the overall stress response. GST responds to treatment in both adult and neonate daphnids in a concentration and time dependent fashion. It is also one of the most highly and significantly induced genes following treatment for all high dose exposed samples compared to all controls (5.6 fold; FDR<0.1). Moreover, when investigating the more frequent GO terms in the list of genes induced by treatment compared to control samples, glutathione transferase activity was the most overrepresented category (P value 0.00014, FDR 0.07), further consolidating this gene as a potential biomarker for stress response in *D. magna*. When specifically considering biomarkers for genotoxicants in adults, the genes for p73-like protein and NEIL-1 showed statistically significant up-regulation in adults following treatment, and thus may represent potential biomarkers for exposure to DNA damaging compounds.

As discussed in Chapter 3, increases in DNA strand breaks following exposure to the sodium dichromate-BaP mixture, as well as chrysoidine, were not identified in neonates using the Comet assay. In comparison, microarray analysis of gene expression changes in neonate daphnids detected up-regulation of some DNA repair genes following exposure. This provides some evidence that enhanced DNA repair, and therefore DNA damage, is occurring. As discussed in Section 5.3.1, up-regulation was observed after 6h, with almost no changes occurring at the 24h time-point. Since a 24h exposure was chosen for the Comet assay, it could be postulated that possible damage

was occurring, but the repair processes had already been activated, returning the levels of strand breaks to baseline levels (see Chapter 6 for further discussion).

Overall, this study has revealed some interesting and useful information with regard to the responses of *D. magna* to genotoxicants. Our results show that different life stages of *D. magna* can be distinguished, with unique expression profiles being produced for adult and neonate daphnids. Specifically, un-treated adult daphnids appear to have a greater capacity to repair DNA damage and respond to oxidative stress than neonates, and a greater number of genes involved in these responses showed up-regulation following treatment of adults. Despite the complexity of analysing responses to chemical mixtures in terms of attributing gene expression changes to particular compounds, in this study it does seem that the majority of the modulated genes point towards sodium dichromate as the primary stressor. The results also highlight the potential for the identification of reproductive and population level effects following acute exposures. Currently, information on such end points is primarily gathered from chronic exposure studies, thus the ability to detect long-term effects of genotoxicants from an acute study has huge potential for improving the high-throughput nature of toxicity testing. In all, this study has provided evidence that microarrays can be used to study the effects of genotoxicants when presented in a mixture, providing evidence both of the mechanisms of action and potential chronic effects, and has gone some way to identifying unique gene expression profiles for genotoxicants, at least for sodium dichromate and potentially metals in general.

CHAPTER 6:

GENERAL DISCUSSION

Environmental pollution is still a major concern in society today. Anthropogenic compounds enter the environment from a variety of sources including industry, agriculture and sewage treatment works. The US EPA Toxic Release Inventory (TRI) for 2007 reported that 4.1 billion pounds of compounds were disposed of or released into the environment in the USA, of which 12% were primary metals and 5% hazardous waste (EPA TRI, 2007a). These contaminants have the potential to induce detrimental effects on individual organisms that can lead to population and ecosystem responses. The environment is less contaminated now than in previous years, indicated by the EPA's TRI for 2007, which reported a decrease in the total reported disposal or other releases of compounds on- and off-site from more than 2500 million pounds in 1998 to just over 1000 million pounds in 2007 (EPA TRI, 2007b). Despite this, the presence of numerous chemicals in complex mixtures, albeit at relatively low concentrations, is of concern, as knowledge regarding the sublethal effects of mixtures is relatively limited. It is known that mutagenic and genotoxic chemicals are readily found in surface waters (Ohe *et al*., 2004), and these have potential implications in processes such as carcinogenesis, inherited disease and teratogenesis. However, current regulatory testing strategies mainly employ whole organism (apical) endpoints, such as growth inhibition and establishment of LD_{50} and LC_{50} values. Although sub-lethal effects such as genotoxicity are investigated for some chemicals during production, fish and other aquatic organisms are less widely used than mammalian systems (Hayashi *et al.,* 1998). Moreover, the introduction of the REACH programme has increased the need for genotoxicity testing in aquatic organisms to an even greater extent (REACH, 2006). Related to this, laboratory based genotoxicity assays, particularly using vertebrates, are generally time consuming, and relatively expensive, with many requiring large-scale

exposure systems, making them impractical for use in assessing effluents or application in the field. Utilising invertebrate and plant species already employed in toxicity testing would allow more high-throughput assessment of the *in vivo* genotoxic potential of compounds. Moreover, the use of such organisms also offers advantages in terms of the 3Rs of animal testing as it would lead to a reduction in the number of vertebrate animals used in testing, particularly under REACH where the potential for high animal usage is clear.

When considering current toxicity testing methodology, compounds are often tested individually, whereas in the environment they are present in complex mixtures of unknown concentrations, which provides potential for additive and synergistic interactions. These interactions can greatly affect the impact chemicals have on organisms, thus a better understanding of the effects of mixtures and not just individual compounds, is required.

The use of apical endpoints in testing provides limited information with respect to mechanisms of action. Information on biomarkers can provide a route to elucidating mechanisms of action, as well as indicating exposure to particular groups of chemicals. For example, the identification of vitellogenin as a biomarker for exposure to endocrine disruptors has proven invaluable in extrapolating laboratory and field data to the effects of endocrine disruptors on fish (Hutchinson *et al.,* 2006), evidence that the continued development of biomarkers may prove important for the detection of exposure to compounds with other sub-lethal effects, such as genetic toxicity.

Under investigation in the current study were two components. The first was the hypothesis that the organisms under study (*C. reinhardtii* and *D. magna*) have the ability

to activate (where necessary) pro-genotoxicants, and are susceptible to DNA damage by a range of toxicants. The second was the practical application of optimised and potentially novel test systems in these organisms to provide screens for genotoxic agents, either individually or in mixtures, to which they may be exposed in the environment.

DNA strand breaks, either induced directly or as a result of excision repair, represent a sensitive, non-specific biomarker for genotoxic agents, and can be measured using the single cell gel electrophoresis (SCGE) or Comet assay (Collins *et al.,* 2004). Consequently, application of the Comet assay as a technique for routine biomonitoring was investigated in both *C. reinhardtii* and *D. magna* (see Chapter 3).

The data presented in Chapter 3 for *C. reinhardtii* support the hypothesis that this alga is able to absorb, and metabolically activate, certain xenobiotics from the environment, and will show DNA damage following exposure to some genotoxic agents. Following modifications to the original Comet assay methodology by Singh *et al.,* (1988), based on alterations made by Erbes *et al.,* (1997) and Aoyama *et al.,* (2003), we demonstrated that this technique can be used to detect DNA strand breaks in *C. reinhardtii* following exposure to known genotoxicants. Despite the relatively low sensitivity, significant, concentration-dependent increases in strand breaks were detected, expressed as the percentage of DNA in the tail. In relation to the metabolic activation capabilities of this alga, a concentration-dependent increase in strand breaks following exposure to the pro-genotoxicant chrysoidine was identified. This result indicated that *C. reinhardtii* is able to bioactivate xenobiotics, which was confirmed by measurement of EROD activity, indicative of CYP1A-like activity in mammals, in control

cells over a 5h time course (Chapter 4). The rate of 7-ethoxyresorufin metabolism was much less than has been recorded for various higher organisms, such as fish and mammals. Furthermore, when exposed to the known inducer of CYP1A βNF, only a slight, non-significant increase was seen compared to the DMSO control. This low rate of xenobiotic metabolism and low sensitivity to an inducer has important implications when considering the environmental relevance and also the sensitivity of algae to pro-genotoxicants. A potential conclusion that can be drawn from these data is that compounds such as BaP may not be metabolised in algae. A previous study, by Warshawsky *et al.,* (1995), supports this hypothesis, in which it was demonstrated that 96.8% of BaP detected in the *C. reinhardtii* algal pellet was in the parent form following three days incubation, thus implying limited metabolism and excretion was occurring. Whilst this persistence may be detrimental, the fact that metabolism also activates BaP to reactive intermediates suggests a potentially low sensitivity of this alga to the genotoxic effects of this, and other progenotoxic compounds. The insensitivity of *C. reinhardtii* to the CYP1A inducer βNF also has consequences regarding the fate of pro-genotoxicants in the environment. Induction of CYP1A and other enzyme systems increases the bioactivation of compounds metabolised by this route, particularly pro-genotoxicants such as BaP. In fact, BaP can induce its own metabolism by acting as an inducer for CYP1A1, resulting in increased genetic damage. The fact that *C. reinhardtii* showed no significant induction of EROD activity following exposure to βNF potentially means that such insensitivity would be shown to other compounds that act by the same mechanism. In many organisms, inducers of CYP1A1 act via the arylhydrocarbon receptor (AhR), evidence of which has yet to be found in algae. Consequently, the mechanism may not exist for activation of CYP1A-like activity in algae. A lack of

inducibility of CYP1A-like activity in this alga will limit the extent to which compounds such as BaP can be activated, adding to the bioaccumulative potential of these compounds.

It is also important to consider the potential for environmental persistence of chemicals, which is determined by their uptake and breakdown by all the organisms present in the ecosystem. In this study we demonstrated a similar sensitivity of a cell wall-free mutant of *C. reinhardtii* to genotoxicants, as measured by the Comet assay (Chapter 3), compared to that of the wild type, suggesting that the cell wall does not hinder uptake of these chemicals. Although *C. reinhardtii* appears to have limited xenobiotic metabolic capabilities, a study by Warshawsky *et al.,* (1995) showed that another species of algae, *Selenastrum capricornutum,* could take up BaP, and metabolise this compound to a much greater extent, with only 1% of the parent compound remaining in the media, and 41.7% in the pellet. Although this study indicates species differences between algae regarding their xenobiotic uptake and metabolic capabilities, at least for PAHs, the results imply that such chemicals are unlikely to be persistent in the environment. Furthermore, it reinforces the importance of understanding the xenobiotic metabolic capabilities of algae used for toxicity testing.

These results need to be considered from an environmental perspective also. Since algae are able to take up these compounds, but metabolise them to variable degrees, there is great potential for bioaccumulation of these compounds, which is well documented for other compounds such as PCBs (e.g. Kelly *et al.,* 2007). This, in turn, may lead to genotoxic loading in higher organisms, which, when considering fish for example, may present as cancer. For example it has been shown that some heavy metals such as

chromium and cadmium bioaccumulate and are biomagnified in the food chain, thus producing genotoxic effects in plants, animals and humans (Filipic and Hei, 2004; Gichner *et al.,* 2004; Vajpayee *et al.,* 2006).

Although the sensitivity of the Comet assay in *C. reinhardtii* was low compared to other organisms, this technique may be suitable for the measurement of DNA strand breaks as part of a suite of ecotoxicological test methods. As such, this technique could be employed in the first level of a tiered testing approach, whereby compounds are screened for deleterious effects on organisms, as it has been shown that strand breaks as a result of exposure to both direct and indirect acting genotoxicants are detectable. It is possible that the low sensitivity of the assay may necessitate the use of higher test substance concentrations than are needed with other test organisms. This may not present such a problem for toxicity testing as higher concentrations than would likely be encountered in the environment are commonly employed. The limited sensitivity may present some problems if applying this technique to biomonitoring of polluted sites, however, as compounds are likely to be present at relatively low concentrations. Environmental pollutants are also present in complex mixtures, thus to further validate this technique for biomonitoring applications it would be of interest to test mixtures of chemicals, both of known and unknown composition, especially if additive or synergistic relationships are anticipated.

In Chapter 3, we also investigated the effect of light sources on DNA strand breaks in *C. reinhardtii.* The results show that culturing algae under different wavelengths of light can have drastic effects on the levels of DNA strand breaks detected. One of the light sources employed, a marine blue actinic bulb, had a UVA component

(predominant output of 380-480nm) and UVA radiation is known to induce oxidative DNA damage via the generation of reactive oxygen species (see Chapter 3 for more detail). Under this bulb, significantly higher levels of DNA strand breaks were detected, and a decrease in population growth was also observed. For comparison, the algae were cultured under a Gro-Lux tube, which provided illumination outside of the UV spectrum (blue: 440-490nm and red: 625-750nm respectively), thus substantially reducing any potential for UV-induced DNA damage. These data raise concerns as to light-induced genotoxicity in the environment, particularly given the associated population-level effects. Algae are continuously exposed to UV light in the environment, and thus, based on the data presented in Chapter 3, would be expected to suffer sustained levels of DNA damage, potentially leading to population effects. The link between UV exposure and DNA damage is well documented, particularly in skin, where UVB light has been demonstrated to be the most effective light inducer of skin cancer in animals (Ichihashi *et al.,* 2003). Nucleic acids strongly absorb high-energy UVB quanta and this can result in the production of a number of structural modifications, the two most common of which are cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts (Petersen and Small, 2001). CPDs are cytotoxic as they block both DNA and RNA polymerases, thus inhibiting replication and expression of the genome (van de Poll *et al.,* 2002). Moreover, UV radiation (both UVA and UVB) can generate ROS, which has also been implicated in benign and malignant cutaneous tumours (Ichihashi *et al.,* 2003). It has been demonstrated that UV radiation can impact on a range of processes in phytoplankton, such as productivity, growth, photosynthesis and nutrient uptake (Buma *et al.,* 1997). These effects of UV exposure are of particular importance when considering the depletion of ozone levels in the stratosphere, primarily from anthropogenic compounds

such as CFCs (Allen *et al.,* 1998), as this has led to an increase in the levels of UV-B (280-315nm) radiation reaching the Earth's surface at mid and high latitudes in the Northern hemisphere (de Bakker *et al.,* 2005). Thus in the natural environment algae are likely to endure significant UV-induced DNA damage, which has the potential to impact on population levels, as indicated by decreased growth rates in our study (See Chapter 3, Section 3.4 for more detail). Since algae are the main primary producers, this has the potential to impact on other organisms within the ecosystem, as a decreased amount of food for those organisms that feed on algae may lead to population level effects on higher organisms. Moreover, current testing protocols do not specify wavelengths of light under which test cultures should be maintained. Thus, there is potential for UV-induced damage confounding test results.

Studies in algae, particularly *C. reinhardtii,* have provided evidence of direct DNA repair (of which photoreactivation of UV-induced damage in the best characterised), BER, and NER, as well as mismatch and recombinational repair (for more detail see Chapter 1, Section 1.8.1). Our results from the Comet assay showed higher levels of percentage tail DNA following exposure to UV light (marine blue actinic bulb) compared to chemical treatment, even though the concentrations used were close to lethal levels. Given the well documented ability of *C. reinhardtii* to repair UV-induced DNA damage via photoreactivation using photolyase, and CPDs via excision repair, it is possible that a proportion of the strand breaks detected following UV exposure were as a result of excision repair. It could therefore be inferred that algae have better protection against light-induced damage than chemical adduct damage.

The application of the Comet assay to *D. magna* did not provide a sensitive measurement for exposure to genotoxicants. A variable response was seen, resulting in no statistically significant differences in strand breaks between control and exposed organisms being detected despite using concentrations approaching lethality (Chapter 3). As discussed in Chapter 3, Section 4, limitations of the applied technique may have contributed to the variability, particularly the use of multiple cell types, and evidence that there is higher variability from *in vivo* studies. For example, Wilson *et al.* (1998) observed much less marked increases in DNA strand breaks from *in vivo* exposures compared to *in vitro* cells, and high variability between individual animals was observed by Nacci *et al.* (1996). Evidence of exposure and effects of the sodium dichromate-BaP mixture was provided by the application of *D. magna* microarrays to measure gene expression changes. Up-regulation of genes involved in the responses to DNA damage and oxidative stress was observed. This may be indicative of the induction of oxidative stress and DNA damage by these compounds, although alternatively it may reflect reactions of reactive metabolites and not necessarily DNA damage *per se*. Specifically, up-regulation was mostly observed after a 6h exposure, thus it could be postulated that although DNA damage may have been induced by these genotoxicants, repair processes had been activated to return the level of strand breaks to baseline levels by 24h. It is, therefore, possible that the Comet assay may be more sensitive at an earlier time point, although there are numerous studies with other organisms indicating that 24h is a sensitive time point for this assay (e.g. mussels, Labieniec *et al.,* 2007). Moreover, genes mainly involved in BER and NER, which repair oxidised bases and DNA-protein cross-links, were up-regulated in adults in our study, but not in neonates. If these types of DNA damage also represent the major types of lesions induced in neonates, a lack of

induction of the appropriate repair processes may provide an explanation for a lack of sensitivity of the Comet assay in *D. magna*. During the repair process a strand break is created when the damaged base or cross-link is excised, and this break in the DNA is detected by the Comet assay. Thus, the results of the Comet assay in neonates may reflect an underestimation of the level of DNA damage occurring. The FPG-modified version of the Comet assay was employed in an attempt to measure oxidative DNA damage, and this did appear to show a trend for greater percentage tail DNA following FPG excision of oxidised pyrimidines, although this trend was not statistically significant based on the number of replicates (n=3) and the degree of variability. Thus while the gene expression data provides some answers as to the potential reasons for a lack of response using the Comet assay, the variability may be genuine and simply reflective of the fact that we were working at the limit of sensitivity.

The novel strategy of using the array was to identify whether a generic response could be used in place of the rather insensitive measure of actual damage, and our data indicates some degree of success. This approach acts as a surrogate for genotoxicity, identifying gene expression changes indicative of exposure to genotoxicants, rather than detecting actual damage. Several studies have highlighted the utility of this technique to identify unique gene expression patterns indicative of exposure to specific classes of chemicals. For example, a study by Poynton *et al.* (2007) identified unique gene expression profiles following exposure to different metals and differential gene expression patterns have been obtained for genotoxic and non-genotoxic carcinogens in rat liver (Ellinger-Ziegelbauer *et al.,* 2004, 2005, 2008, 2009) and HepG2 cells (van Delft *et al.,* 2004).

Another important finding from the microarray was that expression of some DNA repair associated genes was higher in un-treated adults than neonates, and correspondingly, a greater response of these genes to the genotoxic insult was observed. Current testing requirements recommend the use of neonates (<24h), thus we used neonates to optimise the Comet assay with a view for potential application in regulatory testing. Reflecting on the gene expression data, neonates may not be the most appropriate age group to use for studies investigating the effects of genotoxicants, as they are potentially less able to respond to, and repair, DNA damage. It would be of interest and benefit to evaluate the levels of strand breaks in adult daphnids following exposure to genotoxicants, and also to conduct the experiment over a shorter time frame. Considering these data for their environmental implications, it is possible that neonates will be more susceptible to DNA damage induced by genotoxicants, which may ultimately lead to population level effects, as fewer daphnids may survive to reproductive maturity, or hereditable mutations may be produced that affect subsequent generations.

Another factor to consider is the apparent inefficiency of this organism to bioactivate pro-genotoxicants. The data presented in Chapter 4 show that EROD activity, which is representative of activity similar to CY1A in mammals, although detectable, was low in adult *D. magna.* Furthermore, the majority of the up-regulated genes identified from the microarray study indicated that activation of BaP was not extensive, since they are largely involved in the response to oxidative stress or repairing oxidative DNA damage, reflective of the mode of action of sodium dichromate. In light of this it might be concluded that *D. magna* have limited ability to activate pro-carcinogens that are metabolised by this route, of which BaP is an example. This conclusion is supported by a

reported lack of induction of CYP activity by βNF in *D. magna*, a known inducer of CYP1A (Baldwin and Le-Blanc, 1994), and Akkanen and Kukkonen (2003) showed that 80% of the parent compound BaP remained after 24h.

In contrast, two published studies have indicated that *D. magna* can metabolise the PAH pyrene (Akkannen and Kukkonen, 2003; Ikenaka *et al.,* 2006), and CYP3A was up-regulated in our microarray study, which has been shown to be involved in the activation of BaP in the channel catfish *Ictalurus punctatus* (James *et al.,* 2005; see Chapter 5, Section 5.3.4 for more detail). Thus in daphnids this gene may represent the primary route for activation of xenobiotics, particularly PAHs, rather than CYP1A. Nevertheless, the data presented in this thesis suggest that the capacity for *D. magna* to activate pro-genotoxicants is limited. This has potential implications for such compounds in the environment, as it seems unlikely that they will be metabolised to any great extent by daphnids. Moreover, drawing on the ability for *C. reinhardtii* to take up xenobiotics but the limited capacity of this alga to metabolise them, the potential for bioconcentration of these compounds is enhanced.

Data in Chapter 5 provide an indication that population level effects could result from exposure to the sodium dichromate-BaP mixture. Induction of ATP synthase was observed in both adults and neonates following exposure, indicative of a higher energy (ATP) requirement. An increase in stress has been shown to increase the energy consumption of an organism, which can result in a reduction of energy budgets (De Coen and Janssen, 2003). Moreover, the energy status is reported to affect the capacity of an organism to cope with stress: organisms with a low energy status are likely to be less able to deal with stress (Smolders *et al.,* 2005). Most of an organism's energy budget is

required for basal metabolism, growth and reproduction, thus an increase in basal metabolism to deal with toxic stress may result in decreased reproduction and growth (De Coen and Janssen, 2003). Mobilisation of the lipid fraction in response to genotoxicant exposure was indicated by the up-regulation of genes involved in lipid metabolism. In adults, alteration of the lipid status by toxicants may ultimately lead to population level effects, as there may be fewer lipids available for reproduction, in order to allow survival of the mother, while in neonates it may lead to long lasting effects on survival, growth and reproduction (De Coen and Janssen, 2003). It is clear then that acute exposure to sub-lethal concentrations of genotoxicants has the potential to induce molecular level effects that may potentially lead to population responses. Moreover, we have shown that microarray analysis of modulated gene expression can provide potential predictors of population level effects, which has great benefit as acute studies are shorter and require fewer animals.

It is important to consider the implications of these findings for environmental monitoring. It is unlikely that daphnids develop cancer as a result of exposure to genotoxicants since tumours are seldom seen in lower organisms (Kurelec, 1993). This may be as a result of the short life span of this organism; a daphnid reaches sexual maturity after about 7-8 days, and produces its third brood of neonates after approximately 14 days. Although a daphnid can live for 40-50 days under ideal conditions, they are less likely to live this long in the natural environment for reasons such as predation and variations in food availability. It is known that chemical carcinogenesis arises as a result of the accumulation of several mutations in a single cell, the so-called 'multi-hit' hypothesis (Owens *et al.,* 1999). Thus while daphnids may have multiple exposures to genotoxicants in the environment, their likely short life may

prevent the accumulation of sufficient 'hits' in order for carcinogenesis, and thus tumours, to develop. It is, therefore, likely that other effects of genotoxicants, such as hereditary mutations, may be of greater importance as these can impact on population dynamics. In contrast, it could be argued that genetic damage may be advantageous in the environment, as it may enhance biodiversity. It is known that harmful mutations are selected against and quickly eliminated from populations, thus it could be postulated that the impact of enhanced mutation frequency from mutagen exposure may not have immediate harmful effects. It may, in fact, serve to accelerate adaptation (Wurgler and Kramers, 1992).

In this study we have shown that for *D. magna,* microarrays offer a more sensitive tool for monitoring exposure to genotoxicants than the Comet assay, providing evidence of exposure and effect. In addition, this technique provides a greater amount of information regarding exposure to genotoxicants, or toxicants in general, in terms of identifying the induction of oxidative stress in this study. With respect to current testing strategies, genomics techniques offer a more "high information" approach. As this study has shown, a wealth of information was generated regarding being able to differentiate between life stages, identifying potential biomarkers of exposure and elucidating the mechanism of action of compounds. There is also the potential to convert this technique to a more high-throughput approach by using custom or, for example, real time PCR arrays. In particular, when presented with a mixture, this study has highlighted the potential for dominant stressors to be identified using gene expression analysis. Furthermore, we have demonstrated the potential value of microarray studies using acutely exposed daphnids to provide early warning signals for reproductive and population level effects, endpoints currently identified from chronic studies.

In all, the data presented in this thesis have gone some way to meeting the aims set out in Chapter 1. Both *C. reinhardtii* and *D. magna* respond to genotoxicants as measured by Comet assay and microarray analysis respectively. Indication of at least limited xenobiotic metabolic capabilities for both species was provided by measurable, but low, EROD activity as well as the concentration-dependent increase in strand breaks following exposure to chrysoidine. Evidence of DNA repair was provided by the gene expression data in *D. magna*, but this was not determined for *C. reinhardtii.* Both the Comet assay and microarray studies are high throughput, particularly as the 8-pack array format has been adopted for the commercial *D. magna* arrays. Moreover, the microarray data provided evidence that this technique can act as an early warning system of exposure and effect.

When considering the future directions for research in this area, an important direction would be a comparison of the gene expression data gained from the acute study with that from a chronic study, to confirm the potential predictors of chronic exposure. This could be taken further and the system could be validated using daphnids exposed to environmental samples or effluents of unknown complex mixtures. Currently under development is a more comprehensive and better annotated oligonucleotide microarray for *D. magna* (Vulpe, unpublished data), which will provide greater insight into the molecular level effects of genotoxicants, as well as other toxicants, on this organism.

Currently there is also interest in ultra-high-throughput methods of sequencing to measure gene expression, as these enable thousands of megabases of DNA to be sequenced in just a few days (Marioni *et al.,* 2008). Of the methods currently available,

two techniques, pyrosequencing, such as 454 sequencing, and the Illumina platform, have been shown to be a very useful tool for measuring gene expression levels (Torres *et al.,* 2008; Marioni *et al.,* 2008). Moreover, the study by Marioni *et al.,* (2008) identified a greater number of differentially expressed genes when compared to an array-based method. With regards to *D. magna*, as of July 2009, a Solexa sequencing run had been completed and 454 sequencing was planned (Vulpe, unpublished data). These have the potential to provide more comprehensive readouts of altered gene expression.

The gene expression data included some changes that may reflect a 'transcriptome fingerprint' indicative of exposure to sodium dichromate, and potentially of metals in general given the commonality between other genomics studies investigating metal toxicity (for more detail see Chapter 5). The identification of a 'fingerprint' of gene expression changes for individual compounds, or classes of chemicals may represent a useful strategy for identifying biomarkers of exposure.

It is clear from the data presented in this thesis for *D. magna* that the variability that arises from analysing multiple cell types simultaneously can mask the effects of genotoxicants, particularly in terms of strand breaks, but also in relation to gene expression changes, as fewer statistically significant differences were obtained than would commonly be detected in other organisms, such as fish. Techniques that enable individual tissue types to be analysed, such as laser-capture microdissection, may inform on specific targets of different compounds and would help to overcome the variability from using multiple tissues. In fact, recent work has shown this to be a real possibility: Hicks *et al.,* used haemolymph extracted from 6 adult *D. magna* for metabolomic analysis (Viant, 2008), showing the possibility of obtaining a specific tissue

or fluid from *D. magna,* and the head of *D. pulex*, which contains the brain/CNS, has been isolated by manual microdissection for use in neural peptide identification (Gard *et al.,* 2009).

As discussed earlier, the results from the Comet assay in *C. reinhardtii* indicated a much greater impact of UV light exposure compared to chemical exposure. This highlights the need to better understand the relative importance of chemical versus natural sources of genotoxicity in algae as they may be more susceptible to natural sources of genotoxicity. Algae are, however, well equipped to defend against UV-induced lesions, having well characterised photoreactivation systems that utilise photolyases to directly reverse the dimers (for more detail see Chapter 1, Section 1.8.1). Moreover, when coupled with exposure to anthropogenic genotoxicants, algae may have greater susceptibility as a result of high background levels of DNA damage induced by UV-radiation. There is also the potential for interactions between UV light and chemicals. For example, it has been found that PAHs have greater toxicity following exposure to simulated solar radiation, as upon absorption of UV light, PAHs become excited and can transfer their energy to molecular oxygen producing ROS, which can damage DNA (Dong *et al.,* 2000).

In conclusion, it has been shown that the invertebrates in this study are susceptible to DNA damage by a range of stressors. It is of fundamental importance to determine the extent to which such DNA damage is a problem in organisms in the environment, and to realise the importance of such damage from combined exposures to different sources. Furthermore, the viability and reproductive success of individuals may be compromised, leading to population, and potentially ecosystem effects.

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APPENDIX 1

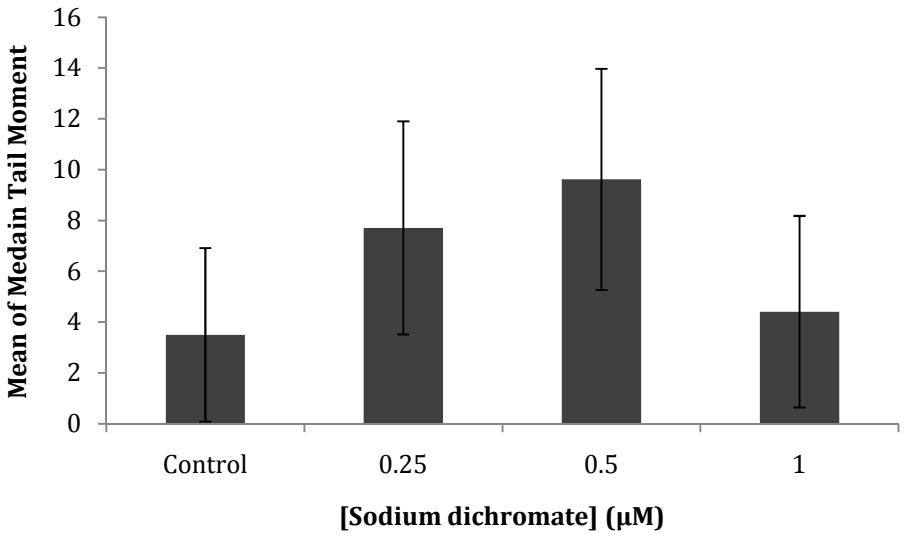


Figure 1 Tail Moment following exposure to increasing concentrations of sodium dichromate for 24h measured in neonate (<24h) Daphnia magna by Comet assay. Data are presented as means of median values ± standard error of the mean, n=4.

APPENDIX 2

Table 1 Apparently differentially expressed genes following a 24h exposure to a low or high dose of a mixture of sodium-dichromate and benzo[a]pyrene identified from cDNA microarray analysis. Differentially expressed genes are based on a 2-fold cut-off. Duplicate genes have been removed.

Gene most similar to	**Species**	**Accession Number**	**E value**
12S ribosomal RNA	*Daphnia magna*	DQ116603	4.00E-81
16S ribosomal RNA	*Daphnia magna*	DQ470575	1.00E-29
16S ribosomal RNA	*Daphnia magna*	AY921452	2.00E-52
16S rRNA	*Megacopta cribraria*	AB240165	0.26
18S ribosomal RNA	*Daphnia pulex*	AF014011	5.00E-43
60S ribosomal protein L13	*Ornithodoros parkeri*	EF633952	1.00E-38
60S ribosomal protein L38	*Artemia franciscana*	EF675775	8.00E-36
Actin	*Daphnia magna*	AJ292554	4.00E-129
Actin	*Ornithodoros moubata*	AY547732	2.00E-107
Actin (Act1)	*Rhipicephalus appendiculatus*	AY254899	3.00E-84
Actin (clone paract403)	*Artemia*	X52605	2.00E-90
Actin, isoform 4	*Daphnia pulex*	AJ245733	2.00E-70
Actin2	*Sitobion avenae*	AY581122	1.00E-34
Actin-87E, transcript variant 1	*Apis mellifera*	XM_623823	1.00E-84
Actin-depolymerizing factor	*Artemia franciscana*	EF547824	7.00E-55
ADP/ATP translocase	*Aedes aegypti*	DQ440004	1.00E-75
ADP-ATP translocator	*Ethmostigmus rubripes*	AF401758	2.00E-56
ADP-ribosylation factor	*Bombyx mori*	NM_001098285	1.00E-62
ADP-ribosylation factor 1	*Locusta migratoria*	U90609	4.00E-93
Aldehyde dehydrogenase 1 family, member L2	*Apis mellifera*	XM_623795	3.00E-78
Alpha-amylase 1	*Diatraea saccharalis*	AY330289	1.00E-41
Alpha-tubulin	*Cryptocercus punctulatus*	DQ925204	9.00E-47
Aminoacylase 1 (acy1),	*Xenopus laevis*	NM_001093437	9.00E-33
Arginine kinase	*Litopenaeus vannamei*	DQ975203	9.00E-69
Ariadne isoform A	*Apis mellifera*	XM_623341	6.00E-102
ATP synthase a chain	*Daphnia pulex*	Q95782	3,50E-26

ATP synthase a chain	*Daphnia pulex*	NP_008625	7.00E-10
B-3501A hypothetical protein	*Cryptococcus neoformans*	XM_770824	3.00E-15
Basic transcription factor 3-like 4, transcript variant 2	*Macaca mulatta*	XM_001111420	2.00E-49
Beta-1,4-mannanase	*Haliotis discus*	AB222081	1.00E-38
Beta-actin	*Litopenaeus vannamei*	AF300705	6.00E-73
Bnip3	*Apis mellifera*	XP_393165	6,00E-14
C1q-like adipose specific protein	*Danio rerio*	XM_001341847	0.003
C48A7.2 (Phosphate transporter family)	*Tribolium castaneum*	XM_965086	8.00E-41
Calcium-transporting ATPase	*Artemia franciscana*	X72713	<1.E-05
Calmodulin (LOC692927)	*Bombyx mori*	NM_001046769	6.00E-96
Camp-dependent protein kinase R1	*Apis mellifera*	XM_396167	4.00E-68
Carbonic anhydrase 1	*Drosophila melanogaster*	NM_078837	3.00E-08
Carboxypeptidase A2 (pancreatic)	*Rattus norvegicus*	NM_001013083	2.00E-08
Cathepsin L-like protease precursor	*Artemia franciscana*	AF147207	1,90E-45
Cell surface protein	*Lucili Lycopersicon*	DQ665309	2.00E-102
Cellulose-growth-specific protein (cel1)	*Agaricus bisporus*	M86356	1.3
Chymotrypsin B gene	*P.vannamei*	Y10665	1.00E-12
Cleavage stimulation factor-like gene	*Daphnia pulex*	EF077803	4.00E-21
Collagen type X alpha 1 (COL10A1)	*Canis familiaris*	AY903956	1.00E-07
Collagen, type I, alpha 1 (col1a1)	*Xenopus laevis*	NM_001087352	5.00E-13
Cuticle protein	*Artemia franciscana*	EF660903	4.00E-06
Cuticle protein	*Artemia franciscana*	EF660897	1.00E-28
Cuticle protein	*Lipaphis erysimi*	AY217538	1.00E-19
Cuticle protein	*Rhopalosiphum maidis*	AY217541	4.00E-04
Cuticle structural protein post-ecdysial PCP16.7	*Tenebrio molitor*	S78003	2,00E-15
Cuticular protein (CPR) 30, RR-1 family	*Anopheles gambiae*	XM_316043	0.26
Cyclic AMP-regulated protein	*Bombyx mori*	NM_001046966	1.00E-38
Cytochrome b	*Daphnia galeata*	S67569	8.00E-05
Cytochrome b	*Daphnia pulex*	ABD19355	8,50E-40
Cytochrome b	*Daphnia pulex*	DQ340836	<1.E-05
Cytochrome b	*Daphnia pulex*	NP_008632	<1.E-05
Cytochrome b (Cytb)	*Tockus alboterminatus*	AF346925	0.088

Cytochrome B561	*Anopheles gambiae*	Q7Q9L3	<1.E-05
Cytochrome c oxidase subunit	*Xiphopenaeus sp.*	DQ084376	6.00E-83
Cytochrome c oxidase subunit 1	*Carabus kyushuensis*	AB047574	<1.E-05
Cytochrome c oxidase subunit 1	*Daphnia pulex*	AAB53197	<1.E-05
Cytochrome c oxidase subunit 1	*Daphnia pulex*	ABD19215	<1.E-05
Cytochrome c oxidase subunit 1	*Daphnia pulex*	NP_008622	<1.E-05
Cytochrome c oxidase subunit 1	*Daphnia pulex*	Z15015	<1.E-05
Cytochrome c oxidase subunit 1	*Drosophila subquinaria*	AY154457	<1.E-05
Cytochrome oxidase	*Megacephala cuprascens*	DQ152151	2.00E-39
Cytochrome oxidase	*Megacephala pilosipennis*	DQ152177	9.00E-32
Cytochrome oxidase subunit 1 (COI)	*Daphnia magna*	DQ166849	7.00E-103
Cytochrome oxidase subunit I (COI)	*Daphnia magna*	AY803049	4.00E-89
Cytochrome oxidase subunit I (COI)	*Daphnia magna*	AY803065	1.00E-106
Cytochrome oxidase subunit I (COI)	*Daphnia magna*	AY803047	5.00E-96
Cytochrome oxidase subunit III	*Odontocheila confusa*	AF438928	4.00E-35
Cytochrome P450 (CYP45)	*Homarus americanus*	AF065892	5.00E-06
Cytochrome P450 monooxygenase	*Apis mellifera*	DQ244075	6.00E-90
Cytosolic juvenile hormone binding protein	*Bombyx mori*	NM_001044203	5.00E-64
Cytosolic small ribosomal subunit S21	*Aedes aegypti*	AY433229	2.00E-34
Cytotoxis NVH 391-98	*Bacillus cereus*	CP000764	0.19
Death associated protein 1a	*Danio rerio*	AF231127	<1.E-05
Dhb2 mRNAfor hemoglobin	*Daphnia magna*	AB021136	2.00E-134
Di-domain hemoglobin precursor	*Daphnia pulex*	AF074722	3.00E-30
Dmagvtg1 (vitellogenin fused with superoxide dismutase)	*Daphnia magna*	AB114859	3.00E-117
Dmagvtg2 (vitellogenin fused with superoxide)	*Daphnia magna*	AB252738	5.00E-112
Dmagvtg2, dmagvtg1 (vitellogenin fused with superoxide dismutase)	*Daphnia magna*	AB252737	2.00E-103
Duodenase	*Bos taurus*	XM_001252800	1.00E-06
Dynein, axonemal, light chain 4 (dnal4)	*Xenopus tropicalis*	NM_001006783	2.00E-31
Elongation factor 1-alpha	*Cyprinus carpio*	AF485331	1.00E-76
Elongation factor 2	*Oxyuranus scutellatus*	AY691668	9.00E-71
Elongation factor EF1-alpha.	*H.vulgaris*	Z68181	1.00E-11
Elongation factor-1 alpha gene, partial	*Diaphanosoma brachyurum*	AF526279	1

Elongation factor-2	*Hexagenia limbata*	AY305510	1.00E-81
Erythrocyte membrane	*Plasmodium falciparum*	AM116207	0.47
Erythrocyte membrane protein 1 (IT4_var18)	*Plasmodium falciparum*	EF158074	1.2
Esterases and lipases	*Anopheles gambiae*	XM_317954	4.00E-11
Eukaryotic translation elongation factor	*Oscheius tipulae*	AY928339	6.00E-59
Eukaryotic translation elongation factor 1 alpha 2	*Xenopus laevis*	NM_001087387	1.00E-19
Eukaryotic translation initiation factor 5	*Danio rerio*	AY577010	3.00E-53
Fast tropomyosin isoform (ftm)	*Homarus americanus*	AF034954	4.00E-45
Fatty acid binding protein 1 (FABP1)	*Mesocricetus auratus*	AY762568	2.00E-06
Fatty acid binding protein 3	*Anguilla japonica*	AB038695	2,00E-26
Fatty acid-binding protein	*Schistosoma japonicum*	AF331756	2.00E-10
Ferritin	*Daphnia pulex*	ABK91576	7.00E-01
Ferritin	*Daphnia pulex*	AJ245734	1,60E-40
Ferritin 3-like protein C	*Daphnia pulex*	DQ983433	4.00E-84
Ferritin 3-like protein	*Daphnia pulex*	DQ983429	4.00E-25
Fructose 1,6-bisphosphate aldolase	*Oncometopia nigricans*	AY725784	8.00E-73
Gaba(A) receptor associated protein	*Branchiostoma belcheri tsingtaunese*	AY616184	1.00E-71
General transcription factor II A, 1	*Mus musculus*	NM_031391	4.00E-07
Globin 1 (Glob1)	*Apis mellifera*	NM_001077823	4.00E-21
Glutamate permease	*Synthetic construct*	AJ005323	<1.E-05
Glutathione peroxidase 5 (GPX5)	*Equus caballus*	XM_001504880	2.00E-02
Glutathione S-transferase (GST)	*Haemaphysalis longicornis*	AY298731	1.00E-64
Glyceraldehyde-3-phosphate	*Daphnia magna*	AJ292555	7.00E-112
Glycine-rich protein	*Lycopersicon esculentum*	AY026037	4.00E-04
H+-transporting two-sector atpase protein 6	*Daphnia pulex*	T11354	5.00E-66
Haemoglobin 1	*Daphnia magna*	U67067	1.00E-89
Haemoglobin 2	*Daphnia magna*	AB021136	0,00E+00
Heat shock protein 70	*Locusta migratoria*	AY178988	9,50E-71
Hemoglobin (Dhb1)	*Daphnia magna*	U67067	7.00E-40
Hemoglobin 4	*Daphnia magna*	AY737794	9.00E-118
Histone H2B	*Gallus gallus*	P02279	<1.E-05

Hypothetical protein (An07g09140)	*Aspergillus niger*	XM_001391951	0.04
Inducible T-cell co-stimulator	*Rattus norvegicus*	NM_022610	0.19
Insect cuticle protein	*Tribolium castaneum*	XM_961240	2.00E-21
Insect cuticle protein (Chitin_bind_4)	*Aedes aegypti*	EAT45700	1,00E-18
Karyopherin alpha 1	*Drosophila melanogaster*	AE003515	<1.E-05
KH domain-containing transcription factor B3	*Xenopus laevis*	AF042353	6.3
L-3-hydroxyacyl-coa dehydrogenase	*Armigeres subalbatus*	AY441295	7.00E-60
Lacrimal lipase	*Oryctolagus cuniculus*	AF351188	<1.E-05
Large subunit ribosomal	*Daphnia magna*	AF346515	4.00E-157
Larval cuticle protein 12.3	*Apriona germari*	AF518323	4.00E-14
Lateral Signaling Target family member (lst-4)	*Caenorhabditis elegans*	NM_182290	4.00E-31
Lcp22 (loc692363)	*Bombyx mori*	NM_001043363	9.00E-23
Ldla domain containing chitin	*Apis mellifera*	XM_623720	9.00E-52
Leucine rich repeat (in FLII) interacting protein 2 isoform 2	*Apis mellifera*	XP_396457	1.00E-17
Lipase	*Antheraea yamamai*	AB180932	1.00E-14
Major ampullate gland peroxidase	*Nephila senegalensis*	AF516694	2.00E-06
Mitochondrial ATP synthase	*Homalodisca coagulata*	AY588070	3.00E-15
Mitochondrial genome	*Daphnia pulex*	AF117817	8.00E-29
Mitochondrial triosephosphate isomerase (tpia)	*Neotyphodium lolii*	EF370415	4.2
MPA13 allergen	*Periplaneta americana*	AY792955	3.00E-08
Mrna for chymotrypsin 1	*P.vanameii*	X66415	3.00E-29
Mtrr protein.	*Danio rerio*	XM_684065	1.00E-24
Multiple inositol polyphosphate phosphatase 2	*Apis mellifera*	XM_001121719	4.00E-37
Myosin	*Apis mellifera*	XM_393371	6.00E-51
Myosin heavy chain (MYO1)	*Chlamydomonas reinhardtii*	AF077352	0.86
Myosin light chain	*Daphnia obtusa*	EF077789	5.00E-60
Myosin regulatory light chain 2 (MLC-2)	*Tribolium castaneum*	XM_969595	3.00E-04
N(4)-(beta-N-acetylglucosaminyl)-L-asparaginase precursor (AGA), variant 1	*Apis mellifera*	XM_394866	0.007
Na,K-atpase alpha-1 subunit	*Artemia franciscana*	AJ389883	9.00E-24
NA,K-atpase beta subunit	*A. salina*	X55780	1.00E-40
Na+/K+-atpase alpha subunit	*Penaeus monodon*	DQ399797	0.013
NADH dehydrogenase subunit 1	*Daphnia pulex*	NP_008633	<1.E-05

NADH dehydrogenase subunit 2 (ND2)	*Daphnia magna*	DQ132627	2.00E-108
NADH dehydrogenase subunit 3	*Daphnia pulex*	DQ340837	<1.E-05
NADH dehydrogenase subunit 3	*Daphnia pulex*	NP_008627	<1.E-05
NADH dehydrogenase subunit 4	*Chrysomya chloropyga*	AF352790	<1.E-05
NADH dehydrogenase subunit 4	*Daphnia pulex*	NP_008629	<1.E-05
NADH:ubiquinone oxidoreductase	*Triatoma infestans*	EF639026	3.00E-27
NADH-ubiquinone oxidoreductase 42 kDa subunit	*Tribolium castaneum*	XM_966758	2.00E-60
Nam1	*Ajellomyces capsulatus*	XM_001544487	0.81
NEDD8-conjugating enzyme	*Apis mellifera*	XM_394551	4.00E-77
Neurospecific receptor kinase	*Daphnia magna*	AB193327	0.017
NRRL 1 dead box ATP-dependent RNA helicase	*Aspergillus clavatus*	XM_001272185	0.11
Olfactory receptor pseudogene	*Cercocebus agilis*	AY455044	0.14
Opsin bcrh1	*Hemigrapsus sanguineus*	D50583	1.00E-29
Ornithine decarboxylase antizyme	*Aedes aegypti*	AY064120	1.3
Oxidative stress protein	*Aurelia aurita*	AY836662	4.00E-13
PAN adenosine triphosphate	*Cherax destructor*	AY153870	7.00E-24
Pancreatic lipase precursor	*Tribolium castaneum*	XM_964126	6.00E-48
Peritrophic membrane chitin binding protein	*Trichoplusia ni*	AAY46199	7,00E-13
Peroxinectin	*Penaeus monodon*	AF188840	4.00E-29
Phenylalanyl-trna synthetase beta-subunit	*Homo sapiens*	BC006502	2,00E-43
Phospholipid hydroperoxide glutathione peroxidase	*Tribolium castaneum*	XM_964844	4.00E-60
Poly(A) binding protein, cytoplasmic 4 (inducible form), transcript variant 2	*Monodelphis domestica*	XM_001365301	9.00E-51
Protective antigen 4D8	*Dermacentor marginatus*	DQ159969	2.00E-22
Protein phosphatase 1, catalytic subunit, gamma isoform, transcript variant 1	*Apis mellifera*	XM_392943	2.00E-54
Putative defense protein	*Antheraea mylitta*	DQ666502	1.00E-09
Rab proteins geranylgeranyltransferase component A 1 (Rab escort protein 1) (Choroideraemia protein) (TCD protein)	*Apis mellifera*	XM_001121784	5.00E-09
Rab7	*Oncometopia nigricans*	AY725788	3.00E-80
Required for meiotic nuclear division 5 homolog A	*Strongylocentrotus purpuratus*	XM_001176353	2.00E-21
Ribosomal protein	*Culicoides sonorensis*	AY603568	6.00E-84
Ribosomal protein	*Tribolium castaneum*	XM_971032	1.00E-30
Ribosomal protein L10Ae	*Biphyllus lunatus*	AM049020	2.00E-49

Ribosomal protein L13	*Apis mellifera*	XM_624893	5.00E-70
Ribosomal protein L15 (rpl15)	*Xenopus tropicalis*	NM_001011478	4.00E-42
Ribosomal protein L18	*Lysiphlebus testaceipes*	AY961532	4.00E-07
Ribosomal protein L28e (rpl28e gene).	*Carabus granulatus*	AM049101	7.00E-17
Ribosomal protein L3	*Apis mellifera*	XM_624818	5.00E-79
Ribosomal protein L31	*Artemia franciscana*	EF062489	5.00E-43
Ribosomal protein L34	*Bombyx mori*	NM_001099606	9.00E-43
Ribosomal protein L38	*Plutella xylostella*	AB180440	3.00E-29
Ribosomal protein L4	*Apis mellifera*	XM_392071	5.00E-108
Ribosomal protein L7	*Spodoptera frugiperda*	AY072288	3.00E-38
Ribosomal protein l7e	*Agriotes lineatus*	AM048999	1.00E-38
Ribosomal protein L7e	*Biphyllus lunatus*	AM049000	2.00E-71
Ribosomal protein S13	*Danio rerio*	NM_001002079	3.00E-63
Ribosomal protein S16	*Gallus gallus*	XM_416113	2.00E-81
Ribosomal protein S16-like	*Taeniopygia guttata*	DQ213540	5.00E-68
Ribosomal protein S17	*Lysiphlebus testaceipes*	AY961580	6.00E-19
Ribosomal protein S2	*Urechis caupo*	U30454	7.00E-48
Ribosomal protein S27	*Xenopsylla cheopis*	EF179453	1.00E-45
Ribosomal protein S3	*Culicoides sonorensis*	AY752836	3.00E-81
Ribosomal protein S30	*Lysiphlebus testaceipes*	AY961508	4.00E-40
Ribosomal protein S4	*Apis mellifera*	XM_623047	2.00E-102
RNA binding protein	*Homo sapiens*	AF119121	<1.E-05
S5e ribosomal protein	*Dascillus cervinus*	AJ783868	4.00E-63
Serine collagenase 1 precursor	*Uca pugilator*	U49931	3.00E-27
Signal sequence receptor beta-like protein	*Crassostrea gigas*	AJ563481	1.00E-53
Similar to CG6647-PA, isoform A, transcript variant 1	*castaneum*	XM_962387	4.00E-57
Strain 3 mitochondrion	*Daphnia melanica*	DQ340845	1.00E-83
Strain A1 mitochondrion	*Daphnia pulex*	DQ340828	4.00E-15
Strain A12 mitochondrion	*Daphnia pulex*	DQ340830	1.00E-64
Strain A8 mitochondrion	*Daphnia pulex*	DQ340825	2.00E-65
Strain S1 mitochondrion	*Daphnia pulex*	DQ340836	2.00E-92
Strain S11 mitochondrion	*Daphnia pulex*	DQ340832	2.00E-57

Strain S6 mitochondrion	*Daphnia pulex*	DQ340839	3.00E-42
Strain S7 mitochondrion	*Daphnia pulex*	DQ340834	6.00E-23
Sulfotransferase	*Bombyx mori*	NM_001043537	5.00E-26
Syntaxin	*Apis mellifera*	XM_624477	4.00E-15
TATA-box-binding protein	*Apis mellifera*	XM_623085	1.00E-76
Telomere-associated protein RIF1	*Homo sapiens*	AK022932	<1.E-05
TEP15 (alpha-2-macroglobulin family)	*Anopheles gambiae*	XM_317088	2.00E-54
Tetratricopeptide repeat domain 11	*Apis mellifera*	XM_623646	8.00E-39
Thread matrix protein 2C	*Mytilus galloprovincialis*	EF535516	3.00E-04
Transaldolase 1	*Tribolium castaneum*	XM_961492	7.00E-85
Transcription factor BTF3 homolog 4	*Mus musculus*	Q9CQH7	6,80E-42
Translation initiation factor 5A	*Aedes aegypti*	DQ440058	2.00E-22
Translation initiation factor 5A (eif5a)	*Spodoptera exigua*	AF109730	2.00E-53
Translation initiation factor eif-4E, long splice form	*Xenopus laevis*	D31837	<1.E-05
Tropoelastin 1 (eln1)	*Xenopus tropicalis*	NM_001078709	0.13
Troponin I (Wupa)	*Bombyx mori*	NM_001043830	6.00E-11
Troponin T (tnt)	*Libellula pulchella*	AF133521	3.00E-13
Troponin T (tpnt)	*Apis mellifera*	NM_001040258	3.00E-13
Trypsin	*Strongylocentrotus purpuratus*	XM_001193194	2.00E-10
Trypsin	*Aplysina fistularis*	Q8I9P2	2,40E-16
Trypsin	*Drosophila melanogaster*	Q9VRS6	<1.E-05
Trypsin	*Aplysina fistularis*	AF486488	1.00E-13
Type 6 nucleoside diphosphate kinase	*Bos taurus*	XM_881360	2.00E-40
U1 small nuclear ribonucleoprotein A	*Tribolium castaneum*	XM_963178	2.00E-43
Ubiquinol-cytochrome c reductase	*Bombyx mori*	DQ521661	6.00E-33
Urate oxidase	*Aedes aegypti*	AY432512	1.00E-48
Vacuolar atpase G subunit-like protein	*Graphocephala atropunctata*	DQ445525	5.00E-16
Very low-density lipoprotein receptor precursor isoform	*Bombyx mori*	DQ443146	4.00E-25
Vinculin CG3299-PA	*Apis mellifera*	XR_014976	7.00E-62
Vitellogenin 1	*Daphnia magna*	AB114859	4,00E-108
Voltage-dependent anion-selective channel protein 2	*Meleagris gallopavo*	P82013	2,10E-74
XPA (xeroderma pigmentosum, complementation group A; XP1, XPAC)	*Homo sapiens*	AL445531	0.22

APPENDIX 3

Table 1 Genes that were statistically significantly more highly expressed in un-treated adult *Daphnia magna* compared to un-treated neonates. Differentially expressed genes are based on a 2-fold cut off, with duplicate genes removed. Significance was determined using a Welch T test, P<0.05 with a Benjamini and Hochberg False Discovery Rate (FDR) multiple testing correction applied (<0.05).

Fold Change	Common Name	Genbank
2.171	Acidic coiled-coil containing protein 3	AC015798
5.84	Apolipophorin precursor	AC154492
2.609	ATP dependent transmembrane transporter protein	BX664601
15.18	Aurora kinase A	AF267176
3.519	Aurora kinase A	XM_395732
2.331	Barrier-to-autointegration factor	BC084726
2.049	B-box type zinc-finger protein ncl-1	
4.351	Beta-tubulin	
3.139	Beta-tubulin	M20419
2.164	Beta-tubulin	BC043974
2.805	Bicaudal c homolog 1	AC092905
3.219	Bicaudal d	
2.225	Brca2 and cdkn1a-interacting protein	AC027184
4.309	Brix domain containing 2	CT005237
2.44	Calcyclin binding protein	CT025843
2.133	Carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase	BT025065
2.086	Cathepsin L precursor	AY795056
2.596	Caveolin 1	
2.571	Checkpoint kinase	AL844865
8.317	Chromobox homolog 1	AY813253
2.015	Coatomer	AC128649
9.859	Condensin subunit Smc	AC111145
7.31	Cub and sushi multiple domains 3	AC120004
3.827	Cuticular protein	AC004936
18.07	Cyclin a1	
9.86	Cyclin B	XM_473508
6.505	Cyclin b3	XM_966649

6.123	Cyclin b3	
6.746	Cyclin B4	AC103612
2.346	Cysteinyl-tRNA synthetase	XM_674094
2.261	Cytochrome P450 3A	AC026475
2.331	Damage-specific DNA binding protein 1, 127kda	AC119986
2.566	Dead box atp-dependent rna helicase	XM_461495
2.147	DEAD box ATP-dependent RNA helicase	AB258895
2.849	Dehydrogenase/reductase SDR family member 7B	XM_415576
10.15	Deoxyuridine triphosphatase isoform 1 precursor	XM_001084330
39.77	Diazepam binding inhibitor	AJ430511
3.899	Di-domain hemoglobin precursor	U67067
2.121	Dna topoisomerase i	XM_001088021
2.285	DNA-binding nuclear protein p8	AC147079
2.24	Dodo	NM_001008109
2.018	Elongation factor 1 gamma	AC163628
3.354	Elongation factor 2	CR860551
3.392	Epithelial cell transforming sequence 2 oncogene	
2.19	Eukaryotic translation initiation factor 2 subunit 2	XM_858344
2.033	Eukaryotic translation initiation factor 3, subunit 6 interacting protein	AJ851472
2.3	Exonuclease nef-sp	AC122007
2.205	Exosome component 2	AC099544
2.736	Fatty acid binding protein	AC182677
4.901	Fatty acid binding protein 3, muscle and heart	BT017516
3.624	Fed tick salivary protein 6	AC022536
5.049	Fibrillarin cg9888-pa	XM_624375
2.149	G protein pathway suppressor 1	
5.575	Glutathione peroxidase 3	AC117255
2.45	Glutathione S-transferase	AF133268
9.469	Growth and transformation-dependent protein	AC005138
2.017	Growth and transformation-dependent protein	AC078845
4.905	H2A histone family member V	BC074203
2.156	Heat shock 70kda protein 4-like	

4.94	Heat shock protein 90	AC018659
3.204	Heat shock protein 90	AF254880
2.203	Heat shock protein Hsp19.5	AC167928
7.397	Hemoglobin	U67067
2.971	Hemoglobin	AC164164
5.48	Heterochromatin protein 1-beta	BA000022
2.19	Heterogeneous nuclear ribonucleoprotein H3 (2H9)	AJ851718
2.809	Hir histone cell cycle regulation defective homolog a	AC102105
2.355	Histone H2A	X56335
2.047	HLA-B associated transcript 1	AF075691
2.267	Homologue of Sarcophaga 26,29kda proteinase	AC159976
84.08	Immune-related protein	AL023095
3.507	Inosine monophosphate dehydrogenase 1	XM_580404
5.408	Karyopherin alpha 2	AY034378
2.042	Kinectin 1	
4.046	Lactate dehydrogenase a	AC163672
3.059	Ly1 antibody reactive clone	AC104102
2.133	Lysyl-trna synthetase	NM_167196
2.159	Mannose-6-phosphate protein p76	AC150978
2.035	MCM2 mini-chromosome maintenance deficient 2, mitotin	AL161793
3.169	Melanoma antigen family B, 18	DP000011
2.722	Mortality factor 4 like 1	BC002936
2.101	M-phase phosphoprotein 10 (U3 small nucleolar ribonucleoprotein)	AL591202
2.407	Neuronal acetylcholine receptor subunit alpha-4	BC091817
4.847	Nhp2 non-histone chromosome protein 2-like 1	XM_396907
2.536	Nicotinic acetylcholine receptor subunit alpha9	AC116856
2.018	NTF2-like export factor 1	AC166572
2.179	Nucleolar protein family member 3	XM_633931
2.445	Nucleoporin 160kda	AC023508
2.082	Nucleoside diphosphate kinase	X97902
3.454	Nucleosome assembly protein 1, like 1	BC111130
2.93	Nucleosome assembly protein 1-like 1	AC154694

2.047	Nudc domain containing 1	BX649437
5.263	Origin recognition complex protein 1	AL603792
2.391	P23-like protein	AL109853
3.041	Perilipin	AP006430
39.38	Peroxidase CG3477-PA	AY013246
61.95	Peroxinectin	AE017355
6.085	Peroxinectin	AF022977
2.822	Peroxinectin	BX908798
3.819	Polyadenylate binding protein 2	
2.435	Polymerase (RNA) III (DNA directed) polypeptide B	BC046238
2.092	Prefoldin 5	XM_395405
50.19	Proline-rich extensin-like family protein	CR388124
2.611	Proteasome (prosome, macropain) subunit, alpha type 6	BC077442
2.243	Proteasome (prosome, macropain) subunit, beta type, 6	XM_642096
2.485	Proteasome beta-subunit	BC065608
3.411	Protective antigen 4d8	BC000764
2.871	Protein disulfide isomerase	AF008300
2.278	RAN binding protein 1	XM_395776
2.023	Receptor accessory protein 5	NM_001013536
2.149	Reticulon nogo	AC110177
5.401	Ribonucleoside-diphosphate reductase M1 chain	XM_633634
2.403	Ribophorin I	AC007694
2.685	Ribophorin ii	AB092486
2.352	Ribosomal protein l36	AB180419
2.263	Ribosomal protein l8e	AM049008
2.314	Ring finger protein 26	AC025257
2.066	SCP-Like extracellular protein family member (scl-11)	AF472440
22.21	Serine protease	AC113533
2.224	Serine proteinase inhibitor	AB210286
4.271	Serum amyloid A	AM158910
2.006	Signal peptidase complex subunit 3	XM_780814
2.611	Similar to Acylamino-acid-releasing enzyme (AARE) (Acyl-peptide hydrolase)	AC121264

2.589	Similar to High mobility group protein DSP1 (Protein dorsal switch 1)	BC046759
2.621	Similar to inositol hexaphosphate kinase 1	XM_867999
2.916	Similar to nuclear membrane protein XMAN1	AC079969
2.531	Similar to P-element somatic inhibitor	XM_967085
2.536	Similar to ubiquitin specific protease 14	XM_963963
3.274	Similar to zinc finger protein	AC013417
2.608	Sjogren syndrome antigen b (autoantigen la)	AC158148
4.582	Small nuclear ribonucleoprotein polypeptide D3	NM_001017093
2.02	Sorting nexin	
3.587	Speckle-type POZ protein	AC084675
2.508	Spermine oxidase	AC087644
2.018	S-phase kinase-associated protein	XM_392758
2.251	Stall cg3622-pc	AC113988
2.565	T-complex protein 1 subunit alpha	XM_001097535
2.935	T-complex protein 1 subunit delta (TCP-1-delta) (CCT-delta)	XM_779820
2.66	Tissue specific transplantation antigen p35b	CP000020
2.372	TPX2, microtubule-associated protein homolog	
2.376	Trypsin-like serine protease	AY372551
2.216	Tryptophanyl-trna synthetase	AL512665
3.186	Tubulin alpha chain	DQ096839
2.009	Two pore channel 1	BX000439
2.455	U1 small nuclear ribonucleoprotein A	XM_393440
2.231	Ubiquitin carboxyl-terminal esterase L4	NM_117857
3.284	Ubiquitin conjugating enzyme 7 interacting protein	AC134530
3.115	Ubiquitin specific peptidase 10	
2.335	Ubiquitin-conjugating enzyme E2	X92663
2.734	Ubiquitin-conjugating enzyme E2G 1	XM_965939
2.304	Ubiquitin-conjugating enzyme E2I	AJ720047
2.911	Uridine cytidine kinase I	
2.557	Vasa RNA helicase	AB193324
2.175	Vitamin k epoxide reductase subunit 1-like 1	
2.484	Vitelline membrane outer layer 1	AY862390

2.22	Vitellogenin	AL691432
216.4	Vitellogenin fused with superoxide dismutase	AC007030
129.1	Vitellogenin fused with superoxide dismutase	AL772226
103.9	Vitellogenin fused with superoxide dismutase	AB114859
7.04	Vitellogenin-like protein	AY421769
5.062	Vitellogenin-like protein	AC152063
2.217	Zinc finger protein	AC068324

Table 2 Genes identified with known proteins that had statistically significantly lower expression in un-treated adult *Daphnia magna* compared to un-treated neonates. Differentially expressed genes are based on a 2-fold cut off, with duplicate genes removed. Significance was determined using a Welch T test, P<0.05 with a Benjamini and Hochberg False Discovery Rate (FDR) multiple testing correction applied (<0.05).

Fold Change	Common Name	Genbank
0.241	ADP-ribosylation factor (ARF1 gene)	AJ421017
0.353	Alpha amylase	AF136603
0.407	Alpha-amylase	AF071045
0.338	Arrestin1 precursor	AJ303080
0.395	ATP synthase F0 subunit 6	AF408194
0.176	Calcium-binding protein	AC149291
0.477	Calcyphosphine	XM_792007
0.481	Carboxylesterase	XM_001089888
0.431	Carboxypeptidase a1	DP000009
0.45	Cathepsin d	AY878724
0.419	Cathepsin f-like cysteine protease	DQ372943
0.306	Cathepsin L-like	X74171
0.317	CCAAT/enhancer binding protein (C/EBP), beta	NM_001016719
0.494	Chemosensory protein 3	AJ851673
0.304	Chitin deacetylase 2 isoform a	XM_320594
0.291	Chitinase 10	XM_308858
0.486	Chitooligosaccharidolytic beta-N-acetylglucosaminidase	AY368703
0.206	Chondroitinlycan family member (cpg-2)	AL157774
0.232	Chymotrypsin	XM_778874
0.318	Compound eye opsin BCRH2	AF385330
0.43	Cuticle protein	Z54327
0.188	Cuticular protein 50Cb CG6305-PA	U82989
0.128	Cuticular protein 74, RR-1 family	AC078805
0.177	Cuticular protein cpr54	XM_396564
0.281	Cytochrome c oxidase subunit I	AY803045
0.451	Cytochrome oxidase subunit II	AY055541

0.414	Daphnia magna haplotype H39 cytochrome oxidase subunit I (COI)	AY803078
0.375	Daphnia melanica strain 3 mitochondrion, partial genome	DQ340845
0.306	Daphnia pulex mitochondrial genome, complete sequence	AF117817
0.438	Delta class glutathione S-transferase	AY174095
0.376	Endocuticle structural glycoprotein sgabd-1	AC112950
0.373	Endoglucanase 2	AF206716
0.414	Fatty acid binding protein	XM_503900
0.483	Fatty acid-binding protein, muscle (M-FABP)	XM_810384
0.439	Glutaryl-Coenzyme A dehydrogenase	BC083397
0.383	Glutathione S-Transferase family member (gst-11)	AC024213
0.17	Hemoglobin	U67067
0.231	Hemoglobin 4	AB021137
0.246	Hemoglobin 4	AB021134
0.229	Hypothetical protein	AF014011
0.367	Hypothetical protein Mhun_0530	AF242738
0.369	Larval cuticle protein 8	AC154428
0.295	Liver basic fatty acid binding protein	AL672066
0.232	Lysozyme	AE009951
0.393	Meprin a subunit beta	AY909437
0.489	Methylmalonate-semialdehyde dehydrogenase	XM_778510
0.331	Methyltransferase like 7A	AE008384
0.439	Microsomal dipeptidase	AC104553
0.383	Niemann-Pick Type C-2, putative	AL713853
0.35	Obstractor b	
0.4	Obstructor-A CG17052-PA	AC011705
0.487	Opsin	AC008628
0.382	Ovochymase 1	AC150401
0.188	Peptidoglycan binding domain-containing protein	AC159286
0.448	Phage-related lysozyme	AB015932
0.433	Pmp22 peroxisomal membrane	DP000010
0.433	Poly A binding protein	BC052100
0.34	Procollagen, type IV, alpha 1	

0.221	Putative chitin binding protein	XM_581280
0.234	Saposin isoform 1	AC096051
0.203	Serine collagenase 1 precursor	AF461035
0.269	Serine protease	XM_422464
0.407	Similar to CLCA family member 1, chloride channel regulator [	AC187767
0.233	Similar to dopamine-beta-hydroxylase	CR728870
0.496	Similar to pancreatic triglyceride lipase	U23521
0.355	Similar to vitelline membrane outer layer 1 homolog	AB015609
0.44	Sodium bicarbonate cotransporter	AB055467
0.202	Sptzle 2-like protein	AC136222
0.289	Sptzle 2-like protein	AL928628
0.365	Sptzle 2-like protein	AP002920
0.448	Sulfate transporter	
0.27	Sulfotransferase	XM_001074172
0.496	Sulfotransferase	BC061149
0.387	Tetraspanin 96f cg6120-pa	XM_640919
0.33	Transglutaminase-like protein	CP000127
0.199	Trypsin	AK061101
0.204	Trypsin	DQ149980
0.345	Trypsin	U58751
0.311	Vitellogenin fused with superoxide dismutase	AB114859

Table 3 Apparently differentially expressed genes identified with known proteins in adult and neonate *Daphnia magna* following a 6h or 24h exposure to the low or high dose of the sodium-dichromate-benzo[a]pyrene mixture derived from oligonucleotide Agilent microarray analysis. Differentially expressed genes are based on a 2-fold cut-off. Duplicate genes have been removed.

Name	**Neonates**				**Adults**				**Accession**
	6h Low	**6h High**	**24h Low**	**24h High**	**6h Low**	**6h High**	**24h Low**	**24h High**	
14-3-3 protein zeta			0.474			2.465			DQ311235
14-3-3epsilon CG31196-PA	0.388				2.136	3.415			AC167684
15.9 kda midgut protein						0.062	0.105		AL161627
16S ribosomal RNA gene	0.43	0.458							AF200971
18 bac ch230-129o15				0.264					AC107505
18S ribosomal RNA gene			0.381	0.187	0.462		2.26		AF144216
26S protease regulatory subunit	0.381	0.445			2.603	3.829		2.588	BC058462
28S ribosomal protein S16						0.44			CR361562
28S ribosomal RNA (partial)				3.01					Z49904
39S ribosomal protein L44	0.461					2.01			Z50755
3-hydroxyacyl-coa dehydrogenase					2.267	2.159			XM_420490
3-hydroxybutyrate dehydrogenase precursor				0.446		2.091			AC187028
3'-phosphoadenosine 5'-phosphosulfate synthase 2						0.269	0.272		AC154203
4-hydroxyphenylpyruvate dioxygenase				0.478	3.059	3.995			BC060451
5' nucleotidase, ecto				0.455		2.047			BX649534
50 kda midgut protein						0.091	0.304		XM_637858
5-aminolevulinic acid synthase	0.412	0.46			3.118	5.629		5.376	AY232150
60S acidic ribosomal protein P2		0.466	0.473	0.049		0.439	0.081	2.534	XM_861618
6-4 photolyase					2.21	2.112			NM_165334
7-dehydrocholesterol reductase				2.081			2.427		AC122526
Abc transporter		2.064		2.165					AY107792
Acidic coiled-coil containing protein 3	0.457								AC015798
Actin-depolymerizing factor		0.491							XM_963085
Activating transcription factor						2.438			
Acyl-coa synthetase short-chain family member 3					2.152	3.171			AK112532

Acyl-Coenzyme A binding domain containing 5 isoform a				2.333					AL591363
Acyl-coenzyme ac-4 to c-12 straight chain		2.013							BC095591
Adhesion regulating molecule 1		0.472			2.51	3.246			XM_396744
ADP ribosylation factor 79F CG8385-PB, isoform B	0.47					2.086			DQ311145
ADP-ribosylation factor (ARF1 gene)						0.057	0.22	5.529	AJ421017
ADP-ribosylation factor-like 5A				2.25					AK147525
Advillin						0.03	0.032	2.951	AE017198
Alanine-glyoxylate aminotransferase 2-like 1				2.226					XM_970732
Alanyl-trna synthetase (Alanine--trna ligase)		0.461				2.193			AJ720131
Alcohol dehydrogenase		2.007				0.426	0.401		CP000083
Alcohol dehydrogenase 5				0.364	2.39	2.956			AC093789
Aldehyde dehydrogenase 7 family, member A1	0.439	0.463				2.216			XM_538607
Aldehyde dehydrogenase type III	0.258				2.566	2.884			BX055538
Aldehyde reductase 1							0.498		AE008503
Aldose 1-epimerase, putative						2.186			XM_966959
Alkyldihydroxyacetone phosphate synthase				2.134					NM_172666
Alpha 1 type I collagen		2.334				2.481			AF336820
Alpha amylase	2.317	2.202		0.275		0.103	0.248		AF136603
Alpha isoform of regulatory subunit A, protein phosphatase 2						2.219		3.502	AJ876407
Alpha-actinin		2.16							NM_166920
Alpha-amylase						0.121	0.143	3.879	AC122289
Alpha-amylase	2.25					0.119	0.247		AF071045
Alpha--fucosyltransferase c						0.262	0.252		AY648302
AMP dependent coa ligase			0.479		0.429	0.356			AL833785
AMP-activated protein kinase					2.142	2.264			XM_969409
Amylase 2-pancreatic				0.439		0.463	0.407		AY867254
Amyloid precursor protein						0.436			
Angiotensin-converting enzyme						2.262			CT025525
Annexin x	0.415				3	3.1	2.158	3.039	CR937960
Annulin (Transglutaminase)			0.491	0.388		2.384			XM_967617

Antigen 5/SCP domain	2.255	2.374		0.217		0.215	0.237	3.221	AC159225
Apolipoprotein d	2.784	2.273				0.043	0.04	3.146	AC125538
Arginine kinase		0.494			3.692	4.962		4.439	AF233357
Ariadne ubiquitin-conjugating enzyme E2 binding protein					2.33	2.713			XM_396912
Ariadne-2 zinc finger protein	0.395								NM_001005678
ARP2 actin-related protein 2 homolog		0.479							XM_392831
Arrestin1 precursor					2.223	2.277			AJ303080
Aspartate aminotransferase					2.363	2.569			NM_059012
ATP synthase					2.35	3.376		5.84	DQ311340
ATP synthase F0 subunit 6		2.007			0.496				AF408194
ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit		2.53			3.179	5.394		5.926	DQ087452
ATP synthase-gamma chain CG7610-PA					2.761	3.275			DQ445536
Atpase inhibitor-like protein					0.461				AL592185
Atpase, H+ transporting, lysosomal accessory protein 1	0.412					0.396			AF266102
ATP-dependent protease La (LON) domain-containing protein				2.412					AC004764
Aurora kinase A						2.117			XM_395732
Basic leucine zipper and W2 domains 2		0.482							DQ440242
B-cell receptor-associated protein 31				2.938					AE017198
Beta-1,4-galactosyltransferase		0.454		0.369					AC100269
Beta-1,4-mannanase precursor				0.365		0.167	0.161	4.814	BX950851
Beta-hexosaminidase b						2.199			AF127936
Beta--mannanase precursor				0.463		0.096	2.035		AC022101
Beta-tubulin			0.488	0.406	2.115	2.863			BC043974
Beta-tubulin			0.45	0.344					M20419
Bridging integrator	2.598	2.302			0.361				AC093550
Bridging integrator					0.458	0.391			
Bridging integrator						2.1			XM_313953
Calcium-binding protein		2.394		2.503					AC149291
Calmodulin		2.055				0.475		2.746	

Calpain c (calcium-activated neutral proteinase homolog c)			2.987					XM_969000
Camp-dependent protein kinase R1 CG3263-PC	0.336			2.735	4.058		5.903	NM_001017732
Caprin 2 protein					0.08	0.469		AC087775
Carbonyl reductase 3			2.673	2.825	4.896	0.246		AP008934
Carboxylesterase		2.702			2.097			AL022720
Carboxylesterase			0.371		0.102	0.487		XM_001089888
Carboxypeptidase a1		2.219			0.06	0.408		DP000009
Carboxypeptidase A2 (pancreatic)			0.445		0.142	0.123		CP000383
Carnitine/acylcarnitine translocase					2.033			AY810580
Casein kinase 1, alpha 1	0.424	0.329			0.485	2.372		
Casein kinase II beta subunit					2.262			DQ413196
Cathepsin C						2.037		BC060335
Cathepsin d			0.188		0.22	0.343		AY878724
Cathepsin d			0.269		0.028	0.035	2.947	XM_392857
Cathepsin l	0.469			2.06	2.432			AC125409
Cathepsin L precursor			2.337					AY795056
Cathepsin L-like				2.308	0.488	0.104		X74171
Cathepsin L-like		2.181			0.488	2.245		AC125381
Cationic trypsinogen				2.115	2.809			CR954247
Caveolin 1				0.357	0.382	2.075	3.033	
Caveolin 1				0.444	0.456	2.958		
CCAAT/enhancer binding protein (C/EBP), beta		2.88					2.764	NM_001016719
CD9 antigen					0.302			AE010299
Cell Division Cycle related family member (cdc-48.2)			2.423					BC046949
Ceramidase					0.116	0.479		AC186358
Ceramidase					0.087	0.145		AY770505
Chaperonin				2.41	3.458			AY880346
Cheerio cg3937-isoform b		3.791			2.048		2.892	XM_624840
Chemosensory protein 2		3.794	2.008					AF068711
Chemosensory protein 3		2.205	5.004					AJ851673
Chitin deacetylase 2 isoform a		3.446		3.45	4.162		17.55	XM_320594

Chitinase						0.344	0.423		AC174609
Chitinase 10		2.093				0.077	0.201	17.23	XM_308858
Chitinase A1		2.152							AL138765
Chondroitinlycan family member (cpg-2)				0.495		0.061	0.188	2.725	AL157774
Chromatin modifying protein 2B	0.461				2.046				AK173513
Chromatin modifying protein 2b				2.782					AC011458
Chromobox homolog 1					2.438	2.001			AY813253
Chymotrypsin				0.288		0.063	0.084		XM_778874
Chymotrypsin BI						0.041	0.046	4.28	AP004943
Chymotrypsin-2 (chymotrypsin ii)		2.589					0.499	3.318	AL136120
Cklf-like marvel transmembrane domain containing 8						2.027			AY190685
Cleft lip and palate transmembrane protein 1						2.529			AC006615
CLIPD1 protein				0.417		2.135			AF486486
Clone AL_149 ribosomal protein L41		0.423							AY826139
Coatomer		0.47							AC128649
Collagen type I alpha 1		2.11							AC154127
Complement factor D						0.028	0.033	3.173	AC090980
Condensin subunit Smc				6.665			0.437		AC111145
Cop9 complex subunit 7a	0.466	0.358							XM_508964
Cral trio domain-containing protein						0.312			NM_068165
C-type lectin family member (clec-6)						0.057	0.304		AC102860
C-type lectin, mannose-binding						0.188	0.243		BA000041
C-type lectin, superfamily member 14 isoform 2						0.04	0.04	2.951	AP008230
Cub and sushi multiple domains 3					0.469				AC120004
Cullin	0.317				2.063	2.288			BC097675
Cuticle protein	2.22	4.608				0.364			AF329064
Cuticle protein		4.007		2.451			2.124	18.14	Z54327
Cuticular protein		0.394	0.337	0.062			0.467		AC004936
Cuticular protein				0.177			0.128	10.8	AC007655
Cuticular protein 49Aa CG30045-PB		4.668			3.827	0.401	0.46	11.02	AF026266
Cuticular protein 49Ae CG8505-PA	2.559	6.617		2.442		0.401	0.233		CR628337
Cuticular protein 50Cb CG6305-PA		6.6				0.142	0.091	12.36	U82989

Cuticular protein 65Az CG12330-PA		2.298			2.898		0.275		CR524203
Cuticular protein 74, RR-1 family	2.455	7.319		2.493		0.39	2.649		AC078805
Cuticular protein 97ea cg6131-pa				0.488			3.443	40.82	AC090231
Cuticular protein cpr54	2.524	3.078				0.495	2.304	17.22	XM_396564
Cuticular protein RR-3 family (agap006931-pa)		0.374	0.491	0.078		3.014	0.449	16.82	
Cyclin B					2.895	2.198			XM_473508
Cyclin b3					2.675	2.444			XM_966649
Cyclin B4					2.797	4.029			AC103612
Cyclin g				2.543					
Cystathionase (cystathionine gamma-lyase)					2.178	2.86			AC012160
Cystathionine beta-synthase						2.407			CP000024
Cysteine-rich protein		2.126	4.178	3.039					
Cytochrome c oxidase subunit I	0.338	0.426	0.432	0.443	2.806	5.402	2.416	4.055	AY803045
Cytochrome c oxidase subunit vic						0.494			AC167689
Cytochrome oxidase subunit 3				2.094					DQ152174
Cytochrome oxidase subunit II				0.338					AY055541
Cytochrome P450 314 family				2.81					AY156052
Cytochrome P450 3A							0.411		AC026475
Cytochrome P450 3A				2.531		0.102	0.091		XM_965322
Cytochrome P450 3A39					2.065	2.489	0.462		AL806527
Cytochrome P450 4 family							0.432		XM_779837
Cytochrome P450, family 3, subfamily a, polypeptide 11							0.412		AL806527
Cytoplasmic carbonic anhydrase					3.693	3.574			AC115361
Cytosolic phosphoenolpyruvate carboxykinase 1					2.054	2.666		4.33	CR956420
D-amino acid oxidase	0.499								AY147913
Daphnia longispina pola small subunit ribosomal RNA gene		0.208	3.602	2.114		0.369	2.481	2.553	AY730404
Daphnia magna haplotype H39 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	0.484	0.494		0.393	3.051	5.792		2.928	AY803078
Daphnia pulex mitochondrial genome, complete sequence	4.245	2.254			0.492				AF117817
Daphnia pulicaria clone wfms0000183 microsatellite							2.121		AY619192

marker Dp179 sequence								
DCP1 decapping enzyme homolog B			0.498		2.253			XM_001097703
Delta class glutathione S-transferase	3.102	6.101	2.571		4.755		2.515	AY174095
Delta-n p63 p73-like protein					2.31			AC114424
Deoxyribonuclease I					0.241	0.302		AJ133437
Diaphorase (NADH) (cytochrome b-5 reductase)					2.264			AF296833
Di-domain hemoglobin precursor	0.466	0.396		3.251	3.486		2.616	AF074722
Di-domain hemoglobin precursor	0.449	0.46			2.696		2.619	U67067
Diphosphoinositol polyphosphate phosphohydrolase, putative		0.498						AC025265
DNA directed RNA polymerase II polypeptide L				0.491				DQ214207
DNA-damage inducible protein					2.095			AC013449
Dnaj (Hsp40) homolog, subfamily A, member 2					2.337			AJ720637
Dnaj (Hsp40) homolog, subfamily B, member 12	0.347				2.073			NM_001031375
Dnaj homolog subfamily A member 1	0.442	0.487		2.256	2.18			DQ311436
Dnaj homolog subfamily A member 1			2.625					AC160962
Domon domain-containing protein			5.064		2.05			
Dorsal switch protein 1 CG12223-PD,						2.772		XM_794817
Dusky-like cg15013-pa		0.476	3.576	0.275	2.436	3.843	2.776	
Dusky-like cg15013-pa			6.799	0.454	2.587	9.415	2.835	
Ecdysteroid-regulated protein	2.667		0.404		0.471	0.045	3.23	BX470150
Elongation factor 1 alpha	0.139	0.122						
Elongation factor 1 gamma			0.477					AC163628
Elongation factor tu	0.308	0.383			2.557			XM_391880
Endocuticle structural glycoprotein sgabd-1		2.474		3.761	0.379	0.294		XM_788610
Endocuticle structural glycoprotein sgabd-1	2.273	7.156	2.368		0.379			AC112950
Endoglucanase 2			0.494		0.077	0.423		AF206716
Endou protein		0.336	0.348		0.022	0.023		AF475907
Enolase		0.49			0.486			
Epsilon-trimethyllysine 2-oxoglutarate dioxygenase					0.408	0.463		AL136967
Ets domain-containing protein				0.387				X51826
Eukaryotic translation elongation factor 2		2.15						AY439652

Eukaryotic translation initiation factor 1A					2.317	2.819			NM_001006082
Eukaryotic translation initiation factor 3, subunit E interacting protein		0.487		0.482		2.409			BC061351
Extracellular matrix protein papilin 2		2.454		2.155	2.46	2.44			CR956377
Fasciclin 2					0.484				
Fascin	0.413	0.388							XM_396175
Fatty acid binding protein						0.158	3.046	3.116	XM_503900
Fatty acid binding protein				0.311					AC182677
Fatty acid binding protein 3, muscle and heart	2.203					0.48			BT017516
Fatty acid-binding protein, muscle (M-FABP)						0.119	0.161	3.101	XM_810384
Fed tick salivary protein 6		0.445		0.479					AC022536
Ferritin	18.16	10.45	3.102		0.371		0.205		AJ292556
Ferritin 1-like protein A					0.469	0.373	0.272		XM_751417
Ferritin 2 isoform 2						0.32	0.314		AC093163
Ferritin 3-like protein			2.458	3.221	2.078	2.183	0.213		AJ245734
Ferritin 3-like protein						0.201			AJ292556
Ferrochelatase precursor				2.042					AC099975
Fg-gap repeat family protein						0.141			AF526219
Fibrillarin cg9888-pa								2.743	XM_624375
Fk506-binding protein		0.49							AB090307
Flavodoxin/nitric oxide synthase				2.287					CR354422
Formiminotransferase cyclodeaminase								3.3	AC012170
Fringe glycosyltransferase (O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase)				0.499					XM_623566
Fructose-1,6-bisphosphatase CG31692-PB, isoform B						2.128		4.029	AC116999
G protein pathway suppressor 1						0.5			
Galactose kinase			2.058						AC136003
Galactosidase, beta 1-like 2						0.097	0.089		AE011952
Galectin						0.119			BC031381
Gametocyte specific factor 1		2.031							CP000252
Gamma-butyrobetaine hydroxylase	0.374				2.244	2.533		3.054	AB230823
Gamma-subunit,methylmalonyl-coa decarboxylase,		2.019							AC005816

putative									
GAMMA-VPE (Vacuolar processing enzyme gamma); cysteine-type endopeptidase						2.274			NM_119448
Gastric caeca sugar transporter		2.211							CP000099
Gem (nuclear organelle) associated protein 8		0.489							AL928632
Globin 1				0.403					AL023805
Glucosamine-6-phosphate deaminase 1				2.632					BC022322
Glucosidase, beta, acid						0.184	0.24		AY630608
Glutamate-gated chloride channel								2.484	AF081674
Glutaminyl-peptide cyclotransferase-like					2.114	2.338			XM_395412
Glutaryl-Coenzyme A dehydrogenase			2.02	2.486	2.713	2.969		3.846	BC083397
Glutathione peroxidase		2.478				2.027	2.104		AC117255
Glutathione S-transferase		2.976		2.063					AF133268
Glutathione S-transferase 3						0.272	0.268		L23126
Glutathione S-Transferase family member (gst-11)					2.979	9.196			AC116337
Glutathione S-Transferase family member (gst-11)	2.483	5.329	8.065	13			2.528	2.525	AC024213
Glutathione S-transferase mu class			0.426	0.327	0.453				AL928729
Glyceraldehyde-3-phosphate dehydrogenase					3.457	3.458			AJ292555
Glycogen synthase kinase 3 beta							2.068		
Gram negative bacteria binding protein 2	2.345	2.146				0.169	0.301	3.224	DQ091256
Growth and transformation-dependent protein	2.893	4.706							AC078845
Growth hormone inducible transmembrane protein				2.284					XM_630501
GTP binding protein 4		2.254				2.304			BX070587
GTP-binding protein SAR2				2.011					AY440152
H+ transporting atpase V0 subunit D	0.468								AY750873
H3 histone, family 3A	2.034			2.032					XM_786159
Headcase protein			0.477	0.401					AC008365
Headcase protein				0.324					AC154484
Heat shock protein 70		0.437				2.884			DQ660140
Heat shock protein 90						2.247			AC018659
Heme binding protein 2			0.46		0.296	2.145	7.256		
Heme binding protein 2			2.617	2.812					BX649621

Heme-binding protein 2 (protein soul) (placental protein 23)				2.233		2.347		2.606	Z49703
Hemoglobin		0.332	0.407		2.58	4.593	3.58	2.679	AB021136
Hemoglobin						3.431	2.896		AC163676
Hemoglobin (Dhb1) mrna, complete cds		0.453		0.433	2.008	2.633	3.335	2.626	U67067
Hemoglobin 4		0.412			0.476	0.471	2.474	2.683	AB021134
Hemoglobin 4	4.255	6.514	4.368	11.3		0.424			AY929914
Hemoglobin dhb3		0.46			0.493	2.086	2.778	2.6	AB021137
Hepatocellular carcinoma-associated antigen 127	0.466					2.783			XM_395763
HIG1 domain family member 1A			3.031	3.629				2.764	AC122460
High-mobility group 20a							2.058		
Histidyl-trna synthetase	0.494					2.331			BC007680
Histone deacetylase						2.072			XM_394976
Histone H2A								6.722	X56335
HLA-B associated transcript 1					2.295	3.438			AF075691
Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1			2.225	4.734					
Homogentisate 1,2-dioxygenase				0.24					AK065189
Homogentisate-dioxygenase (homogentisate oxidase)				0.229		2.497			BA000012
Hsp70-interacting protein, putative						2.297			BC049337
Hydroxysteroid dehydrogenase like 2	0.431				2.272	2.685		3.851	AK174129
Hypoxia up-regulated 1		0.469							
Innexin 2		2.155				2.756			AC023698
Innexin-1		0.49							
Inter-alpha (globulin) inhibitor H5-like	0.494	0.493				2.071			AF458102
Interferon gamma-inducible protein 30						0.022	0.027		AC024606
IRE1 kinase related family member (ire-1)				5.366		0.394			AC110040
Juvenile hormone esterase						2.037			AL118556
Juvenile hormone-inducible protein					2.086		0.358		
Karyopherin alpha 2				0.483					AY034378
Kinectin 1				0.44					
Kiser			2.158	2.859					BX014764

Kynurenine 3-monooxygenase			2.162	3.058					XM_628470
Lactase						0.068	0.387		AY528410
Lactase						0.454	0.425		XM_781915
Lactase				0.395		0.08	0.097	3.436	XM_967089
Larval cuticle protein 8	2.035	4.917					0.35		AC154428
Larval visceral protein D CG8694-PA							0.362		XM_320159
Lectin 4 c-type lectin	2.068	6.783							DQ440163
Legumain	0.481					2.006			BC021064
Light chain 3				2.217					AY570553
Lipase						0.38	0.376	3.093	BC084493
Lipase				2.578					AY866426
Liver basic fatty acid binding protein						0.054	0.054	3.067	AL672066
Loc495958 protein	2.212	2.692	2.591	2.827					BX294189
Ly1 antibody reactive clone		0.433							AC104102
Lysozyme	3.017	2.14		0.418		0.122	0.329		AE009951
Lysyl-trna synthetase		0.464				2.533			AC144791
Maelstrom homolog						2.051			XM_645585
Malate dehydrogenase	0.432				2.071	2.332			AY441194
Malate dehydrogenase	0.347				2.566	2.719			XM_394487
Mannosidase, alpha, class 2B, member 1					0.244		0.35		AC099764
Mannosidase, beta A, lysosomal					0.173		0.21		CP000141
Map kinase-interacting serine/threonine kinase		2.185			2.294	3.725			XM_312850
Masquerade-like protein 2		2.314	2.212	0.39	2.797	3.988			AC138671
Membrane associated guanylate kinase, WW and PDZ domain containing 2					0.428	0.481			AY225148
Meprin a subunit beta					0.138		0.17	3.234	AY909437
Metalloproteinase, putative								3.363	AY030534
Metallothionein 3	3.742	6.608	2.356				0.432		AC158586
Methionine sulfoxide reductase		2.417			2.319				DP000010
Methylenetetrahydrofolate reductase	0.478	0.459							AL445532
Methylmalonate-semialdehyde dehydrogenase	0.33				3.243			3.378	XM_778510
Methylmalonyl coa epimerase			2.336	2.58	2.253	2.838			AL592494

Methyltransferase like 6	0.463				2.617				AC151337
Methyltransferase like 7A			2.72	7.571	0.278			6.891	AE008384
MFS family transporter: glycerol-3-phosphate					0.113		0.13		BC062990
Microsomal glutathione s-transferase 3				0.423	0.446				CR376724
Mitochondrial 28S ribosomal protein s18c					0.457				XM_745276
Mitochondrial aldehyde dehydrogenase	0.439				2.607	3.342			XM_781694
Mitochondrial ATP synthase F chain		2.167							DQ445507
Mitochondrial cytochrome c oxidase subunit via		2.048							AC161265
Mitochondrial import receptor subunit tom40	0.475				2.425				AC113084
Mitochondrial phosphate carrier protein CG4994-PA					4.244	4.663		2.48	AY105321
Mitochondrial processing peptidase beta subunit					2.084	2.616		3.609	AY880325
Mitochondrial ribosomal protein L52 CG1577-PA					0.491				XM_965776
Monooxygenase, DBH-like 1	2.955				0.113		0.088	4.908	AF125446
M-spondin cg10145-pa					3.501				BT009347
Multiple inositol polyphosphate phosphatase 1					2.291	2.6			Z22181
Muscle LIM protein		3.188							AY440627
NAD(P) transhydrogenase		2.678			2.277				BX927162
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 13					0.472				AL929204
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2, 8kda					0.473				CT025289
NADH dehydrogenase (ubiquinone) flavoprotein 1, 51kda				2.164					XM_965763
Nadh dehydrogenase 1 alpha subcomplex					0.496				BX571736
NADH dehydrogenase subunit 2				2.307					DQ132627
NADH dehydrogenase subunit 3		0.328			0.47		2.042		XM_424472
NADH dehydrogenase-ubiquinone Fe-S protein 2 precursor	0.358				2.83	3.219		2.862	BC109437
NADH:ubiquinone reductase 42kd subunit precursor CG6343-PA					2.046	0.495			AC091483
NADPH cytochrome P450 reductase				2.044	2.184				CP000095
NADPH-specific isocitrate dehydrogenase				2.005	2.266				CP000383

Neuralized-like protein 2	2.71	2.814	2.331	2.979					XM_705313
Neuroendocrine differentiation factor	0.406								XM_532972
Neuronal acetylcholine receptor subunit alpha-4					3.243	5.439			BC091817
Neutral endopeptidase 24.11					0.082		0.087	4.874	XM_969466
Nhp2 non-histone chromosome protein 2-like 1								6.484	XM_396907
Nicotinic acetylcholine receptor subunit alpha9					2.986	5.11			AC116856
Niemann-Pick Type C-2, putative		5.472		4.956	3.222				
N-myc downstream regulated					2.197	2.742			AC186294
N-terminal asparagine amidohydrolase	0.494				2.54				CP000057
Nuclear hormone receptor FTZ-F1 beta					0.378		2.28		Z92846
Nucleolar protein family member 3	2.04								XM_633931
Nucleolar protein nop56					2.14				DP000011
Nucleosome assembly protein 1-like 1		0.465			2.258	3.586			AC154694
Nucleotidase 4F8					2.041				BC077999
Obstractor b	2.649	6.151					2.008		
Obstractor D	2.502	3.979					2.176		AC167234
Obstructor-A CG17052-PA	3.199	6.348							AC011705
Opsin		2.873			2.139	2.158	0.395		AC008628
Ornithine aminotransferase	0.497				2.645	2.931			D50331
Ornithine decarboxylase		0.418			2.137	3.478		3.984	AC162792
Ornithine decarboxylase 1				2.08					AC148756
Ornithine decarboxylase antizyme				2.051					NM_179667
Outer mitochondrial translocase	2.053				0.451				AB084514
Oviductin		2.227							AC158170
Ovochymase 1				0.446	0.186		0.201	7.356	AC150401
Ovomucoid					0.301		0.208		AL731596
Oxidase peroxidase	0.47	2.136		0.303	3.666				DP000117
P23-like protein		0.482			2.146	2.282			AL109853
Papain family cysteine protease containing protein					2.048	2.138		2.866	NM_114696
Paramyosin		0.447			2.4	3.176	2.125	2.501	XM_393281
Paraplegin		2.958	3.421	5.319	2.061			2.811	XM_964017
Peptidase S1 and S6 chymotrypsin/Hap					0.158		0.175		AC130003

Peptidase S1 and S6 chymotrypsin/Hap					2.268			3.805	AL513354
Peptidoglycan binding domain-containing protein	2.401	2.578		0.362	0.141		0.217	3.212	AC159286
Perilipin					3.58				AP006430
Peritrophic membrane chitin binding protein					0.053		0.053	20.64	AL596168
Peritrophin a		2.228							AB229130
Peroxidase CG3477-PA							0.456	3.325	AY013246
Peroxinectin					2.264				BX908798
Peroxinectin		2.001		2.224	2.685	2.826			AF022977
Peroxinectin				0.47	3.225		2.4	2.878	AC150651
Peroxiredoxin			2.2						
Peroxiredoxin 6					2.59				BC054309
Peroxiredoxin V protein				2.992					XM_533241
Phage-related lysozyme	2.132			0.364			0.444		AB015932
Phosphoenolpyruvate carboxykinase								3.444	Z48544
Phosphoethanolamine N-methyltransferase					3.202	3.57			CR635070
Phospholipid scramblase					0.277		0.278		BC085715
Phytanoyl-coa 2-hydroxylase isoform a precursor					2.7		0.455		XM_535184
Pmp22 peroxisomal membrane					0.134		0.426		DP000010
Poly A binding protein					0.379		0.275	2.901	BC052100
Polyadenylate binding protein 2	0.495	0.356	2.8						
Polyadenylate-binding protein				2					
Polypyrimidine tract binding protein					2.04				XM_318405
Probable long chain fatty acid coa ligase		2.56							AC091073
Procollagen, type IV, alpha 1		5.671			0.361		5.01	2.501	
Programmed cell death 6-interacting protein					2.143	3.232			BC025689
Prohibitin				2.375					NM_166312
Prohibitin protein wph			2.132	3.197					CR388159
Prosaposin					2.527				X52944
Prostamide/PG F synthase					2.442	3.388		3.024	AL139420
Proteasome (prosome, macropain) 26S subunit, atpase 2					2.141				XM_623740
Proteasome 26S ATPase subunit 3	0.477				2.44	3.337		2.579	XM_855649

Proteasome beta-subunit					2.119				AY432919
Proteasome subunit alpha type 1					2.231				BC009576
Protein C kinase 98E		2.12							AF289084
Protein disulfide isomerase		0.397		0.226					AF008300
Protein disulfide-isomerase like protein erp57	0.341	0.307	0.273	0.236	2.156	3.476		2.767	AB210112
Protein phosphatase 1, catalytic subunit, alpha					2.414				NM_001004527
Protein phosphatase 1B, magnesium dependent, beta isoform	0.393				2.496			3.523	NM_001016158
Protein phosphatase 1k (pp2c domain containing)					2.011	2.314			AC126445
Protein phosphatase V CG12217-PA					2.434				XM_394400
Protein-glutamine gamma-glutamyltransferase				0.386					AY432897
Pupal cuticle protein				0.064			0.136	9.796	BC021036
Putative alpha-2-macroglobulin immunity protein							0.449		AY540092
Putative carboxypeptidase B					0.104		0.124		AF448416
Putative chitin binding protein	2.502	6.614							XM_581280
Putative defense protein		2.236							
Putative fructose-bisphosphate aldolase	0.469	0.5			2.498	4.312		4.24	AC008211
Putative gram negative binding protein							0.351		AY540101
Putative mitochondrial ATP synthase epsilon chain	2.103				0.465				AC159324
Pyruvate dehydrogenase	0.424				2.708	3.115		3.955	AY433089
Pyruvate kinase CG7070-PB, isoform B					2.567			3.511	AY608678
RAB family member (rab-39)				2.033					XM_640363
RAB11B, member RAS oncogene family					2.038	2.23			M38392
Rab5 member ras oncogene family					2.023				
RAD23 homolog B	0.465	0.494			2.345				NM_001037687
RAN binding protein 1					2.827	2.796			XM_395776
RAP1B, member of RAS oncogene family					2.14				AY423018
Repressor of RNA polymerase III transcription MAF1	0.418				2.244				AE017355
Reticulon nogo					2.704				AC110177
Retinol dehydrogenase 12					0.126		0.128		AY973038
Rho-like CG9366-PA					0.257		0.49		XM_384244
Ribonuclease X25 CG8194-PA					2.127	2.689			Y17445

Ribophorin I		0.439	0.492				AC007694
Ribophorin ii		0.473	0.465				AB092486
Ribosomal protein l18a		2.075					AY961511
Ribosomal protein L29	2.175			0.45			AF429975
Ribosomal protein L3	0.407			2.529	3.717		XM_001106805
Ribosomal protein L4				3.033		2.565	AY769271
Ribosomal protein L6				2.468			AC118630
Ribosomal protein l7e				2.241	3.072		X15109
Ribosomal protein l8e						2.537	AM049008
Ribosomal protein s13		2.585					X62673
Ribosomal protein S19		2.346					BC056505
Ribosomal protein s21		0.497					NM_201191
Ribosomal protein s29e				0.415			AM040022
Ribosomal protein s6				0.485			
Ring finger protein 10				2.04			BX324225
Rogdi cg7725-isoform a				2.878			CP000095
S-adenosyl methionine synthetase		0.493					BX935026
S-adenosyl-L-homocysteine hydrolase	0.449			3.399	5.3		XM_787396
Salivary secreted protein	2.325			0.03		0.031	2.998 AL021470
Saposin isoform 1	2.375	5.26	6.793				AC096051
Sarco /endoplasmic reticulum Ca-ATPase		3.054		2.833		5.606	X63009
Sarcosine dehydrogenase				2.351			XM_001100018
Scamp cg9195-isoform a				0.111			CR790378
Scarface cg11066-isoform b		3.212	0.279	2.214		0.388	2.717 AC155099
SCP-Like extracellular protein family member (scl-11)		2.234					AF472440
SEC13-like 1		0.436		2.164			XM_629792
SEC61, gamma subunit isoform 1				0.36	0.472		AY281321
Secreted protein acidic and rich in cysteine				2.537			AY751536
Secretory carrier membrane protein 2				0.272		0.336	AB014965
Selenophosphate synthetase 1		0.436					BT022124
Sequestosome 1, oxidative stress protein		2.967	3.948	2.349			AC116337
Serine (or cysteine) proteinase inhibitor, clade B			0.362				AP008208

(ovalbumin), member 10									
Serine collagenase 1 precursor	2.099			0.48	0.019		0.025		AY536261
Serine collagenase 1 precursor				0.448	0.042		0.269		AF461035
Serine collagenase 1 precursor				0.311	0.121		0.18	2.492	AP007167
Serine protease	2.236	3.642	2.207	5.012			2.014	3.928	XM_422464
Serine protease		2.084							DQ234084
Serine protease 7	2.35	5.025	2.185	4.795					AY814086
Serine proteinase inhibitor				0.222	0.408	0.467		2.737	AB210286
Serine/threonine kinase NLK				2.039					NM_168250
Sgt1 protein homolog ecdysoneless				0.48	2.142	2.564			AC111067
Short-chain dehydrogenease/reductase					2.078	2.564			AE014292
Sideroflexin					2.569				BC044027
Signal peptide peptidase-like 2B							2.033		BX957298
Signal sequence receptor beta subunit					0.45				AC146542
Similar to Aspartate aminotransferase, mitochondrial precursor		2.261							AK070560
Similar to brain chitinase and chia					0.057		0.439		BC056337
Similar to Cad87A CG6977-PA				0.46					AC135805
Similar to Calcium-independent phospholipase A2-gamma				2.025	2.347				AP008212
Similar to CG6202-PA					3.066	3.823			NM_076569
Similar to CLCA family member 1, chloride channel regulator [Ciona intestinalis]					0.051		0.322		AC187767
Similar to dopamine-beta-hydroxylase	2.303				0.09		6.392	5.332	CR728870
Similar to Eukaryotic initiation factor 4A (eif4a)	0.405				2.989	4.651		6.752	XM_511961
Similar to inositol hexaphosphate kinase 1				2.076					XM_867999
Similar to intestinal mucin					0.131		0.153		AC119410
Similar to miple CG1221-PA					0.481				CP000301
Similar to pancreatic triglyceride lipase				0.35			0.301		U23521
Similar to P-element somatic inhibitor					2.026	2.446			XM_967085
Similar to Placental protein 11 precursor (PP11)					0.23		0.157		AC092229
Similar to polyubiquitin				2.522					NM_171868

Similar to protease m1 zinc metalloprotease					0.108		0.378		CP000087
Similar to Protein Star				0.464	0.394				
Similar to ubiquitin specific protease 14	0.447	0.463							XM_963963
Similar to vitelline membrane outer layer 1 homolog				0.317	0.488		0.098	8.621	AB015609
Similar to zinc finger protein 425				2.144	0.457				AC092138
Small glutamine-rich tetratricopeptide repeat-alpha	0.354	0.45			2.202				BC074059
Sodium bicarbonate cotransporter		2.197							AB055467
Sodium chloride cotransporter 69 CG4357-PA			0.306	0.168				2.447	NM_172663
Sodium/potassium-transporting atpase subunit beta					2.343	3.208			CP000024
Solute carrier family 25, member 36a		2.104							AL592044
Solute carrier family 35 (UDP-N-acetylglucosamine (UDP-glcnac) transporter), member 3					0.388		0.491		AC169816
Solute carrier family 5 (sodium glucose cotransporter) member 1								2.46	
Sorting nexin 4					2.05	2.157			CP000393
Sparc	0.463				2.936				AC183301
Spermine oxidase		0.462	0.469	0.289					AC087644
Sphingosine-1-phosphate lyase 1		2.082		2.1	0.284		0.315		XM_600800
Splicing factor 3a, subunit 2					2.289				AY915588
Sprouty		2.485							CP000384
Sptzle 2-like protein		3.891			2.089	0.328	2.18		AL928628
Sptzle 2-like protein		3.494							AP002920
Sptzle 2-like protein		5.214							AC136222
Staphylococcal nuclease domain containing 1	0.479	0.495			2.073	2.988		2.88	CP000380
Stearoyl-coa desaturase (delta-9-desaturase)					2.589				BC096632
Steroid dehydrogenase					0.243		0.2		AE016853
Steroid dehydrogenase					2.217	3.262			AC131792
Stretch regulated skeletal muscle protein, putative	2.545				0.467				AC095247
Stubble CG4316-PA					0.041			2.766	AL732506
Succinate dehydrogenase			2.118	2.387	2.097			3.955	XM_459635
Sucrase-isomaltase					0.303		0.276		NM_167161
Sulfate transporter				0.472	2.064			2.476	AC163676

Sulfotransferase					0.078		0.399		XM_001074172
Sulfotransferase				2.803					BC061149
Surfeit 1	2.171	2.632	3.817	5.718					XM_635462
Syndecan binding protein (syntenin)					2.673	2.767			DP000011
Syntaxin 8	0.491								AC158200
Syntaxin-like protein					2.193	2.308			XM_880093
TAF10 RNA polymerase II, TATA box binding protein (TBP)-associated factor		2.23							CR450704
T-complex protein 1 subunit epsilon	0.39	0.355			2.544				XM_393315
Tetraspanin 74F CG5492-PB					2.244				CR731758
Tetraspanin 96f cg6120-pa		2.488						2.502	XM_640919
Tetraspanin D107					2.747	2.175			AY705947
Tetraspanin domain containing protein					0.382	0.343			AL732589
Thioester-containing protein (AGAP008364-PA)					2.284				AB231867
Thioredoxin domain containing 1	0.45								X62678
Thioredoxin peroxidase 1		2.07	2.374						CR937859
Threonine dehydrogenase		2.127							AC149040
Thymosin isoform 2				0.468					AJ012719
Thymus-specific serine protease					2.168	2.375			AK113308
Tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)					2.189				AB015609
Tissue specific transplantation antigen p35b	0.475				2.025	2.567			XM_625131
Titin a					2.309				AY559246
Toll-like receptor protein					0.053		0.041	3.229	BT016258
Toll-like receptor protein					0.186		0.222		AC124535
Transaldolase 1	0.357				3.001	2.792		3.954	BC084118
Transcription factor B1, mitochondrial				2.228	2.716				AC187997
Transgelin		2.148							XM_321834
Transglutaminase-like protein		2.051					0.367	3.86	CP000127
Translation elongation factor 2		2.078			3.925				AY305509
Translocase of inner mitochondrial membrane 8 homolog a	0.485				2.53				AF365697

Translocation associated membrane protein		0.434			2.486			2.491	NM_012288
Translocon-associated protein subunit alpha		0.421		0.482	2.882				AC124417
Transmembrane and ubiquitin-like domain containing 1		2.02							AE009925
Transmembrane protein 167a		0.485							AY605481
Transmembrane protein Tmp21 precursor (21 kda transmembrane-trafficking protein) (Integral membrane protein p23)		0.411							XM_786597
Trehalase (brush-border membrane glycoprotein)					0.083		0.324		AB059269
Triosephosphate isomerase 1	0.475				2.262	2.51			L38975
Tropomodulin cg1539-isoform c		2.001							XM_965199
Tropomyosin				2.038	2.144				
Troponin t, invertebrate	2.958		2.173		2.06	3	2.07	3.226	AY440402
Trypsin					0.03		0.034	4.076	XM_541470
Trypsin					0.03		3.29	5.281	DQ399327
Trypsin					0.034		0.041	5.009	AY231989
Trypsin					0.036		0.04	5.167	AK061101
Trypsin					0.041		0.039		U58751
Trypsin					0.043		0.039	4.965	XM_549363
Trypsin				0.41	0.051		0.353		BA000037
Trypsin					0.056		0.058	3.93	DQ149980
Trypsin				2.429	0.057		0.085		AC093476
Trypsin-like serine protease				0.193					NM_005577
Trypsin-like serine proteinase		0.369	0.387		0.268		0.405	3.321	XM_514836
Tubulin alpha chain		0.481		0.316					DQ096839
Tubulin alpha-1 chain		0.486		0.322	2.307	3.773			DQ440241
Tubulin alpha-1 chain				0.396	3.296			2.492	XM_790183
U1 small nuclear ribonucleoprotein A								6.624	XM_393440
Ubiquinol-cytochrome c reductase core protein II					2.133	2.359			AF526228
Ubiquitin carboxyl-terminal esterase L4					2.697	3.492			NM_117857
Ubiquitin-conjugating enzyme E2G 1					2.106	2.477			XM_965939
Ubiquitin-conjugating enzyme e2h					2.417				NM_003344

UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2			0.477		2.22				BX950854
Universal minicircle sequence binding protein					2.665				Z74033
Urate oxidase					2.343				X57113
Uroporphyrinogen decarboxylase	0.473	0.493			2.211				XM_967364
Vacuolar ATP synthase catalytic subunit A		0.4							AY813539
Vacuolar ATP synthase subunit G-like protein	0.485				0.476				XM_624343
Vacuolar H[+]-atpase 55kd B subunit CG17369-PB, isoform B	0.496				2.117			2.903	X64354
Vacuolar H+ ATP synthase 16 kDa proteolipid subunit					0.442				BT012440
Vacuolar protein sorting 36	0.499								DP000086
Valosin containing protein		0.485			2.259				CT032896
Vasa intronic gene cg4170-isoform a		0.467			3.074				AC121805
V-ATPase subunit a	0.486				2.159			5.739	AY864912
Vinculin				0.397	2.004				X96601
Vitelline membrane outer layer 1			0.43	0.228					AY862390
Vitelline membrane outer layer 1 homolog				0.42	0.03		0.466	8.354	X72378
Vitellogenin		2.08			2.678		0.449	7.615	AL691432
Vitellogenin fused with superoxide dismutase	3.871	4.928			0.253	0.244	2.429		AB114859
Vitellogenin fused with superoxide dismutase		0.482							AL772226
Vitellogenin-like protein		0.448		0.479	4.954	9.52			AC152063
Vitellogenin-like protein		0.309							AY421769
Von Hippel-Lindau tumor suppressor		0.449							AC152981
Wd repeat and socs box-containing 1	2.001	2.155							CP000390
WD repeat domain 18					2.034				AL136372
Wd repeat domain 75					2.007				AC108483
Y-box protein	0.379	0.397	0.441		2.242	3.605		6.179	XM_393344
YKT6 v-SNARE protein	0.476				2.454				AF309793
Zinc carboxypeptidase				2.179	0.308	0.464	3.483	4.211	NM_143745
Zinc carboxypeptidase A 1					0.154		0.12	3.414	AB180425
Zinc finger CCHC-type and RNA binding motif 1					2.785				AC135668
Zinc finger protein					2.128	2.41	2.178		

Zinc finger, AN1-type domain 2A			2.04				BC018415
Zinc metalloproteinase nas-12		2.424		0.059		0.174	AY909437
Zinc proteinase Mpc1			0.494	3.028	3.584		BX088708
Zygote-specific protein	4.218	3.569		0.024		0.197	AL358532

Table 4 Genes identified with known proteins that had statistically significant differential expression in adults and neonates following a 24h exposure to the high dose of the sodium dichromate – benzo[a]pyrene mixture compared to the time-matched control. (Parametric test, P<0.1, FDR, 2 fold cut-off.)

Fold Change	Gene Name	Accession Number
Adult		
40.82	Hemoglobin	U67067
Neonate		
2.333	Acyl-Coenzyme A binding domain containing 5 isoform a	AL591363
2.25	ADP-ribosylation factor-like 5A	AK147525
0.364	Alcohol dehydrogenase 5	AC093789
2.412	ATP-dependent protease La (LON) domain-containing protein	AC004764
0.365	Beta-1,4-mannanase precursor	BX950851
0.344	Beta-tubulin	M20419
0.279	Beta-tubulin	
0.269	Cathepsin d	XM_392857
0.188	Cathepsin d	AY878724
2.423	Cell Division Cycle related family member (cdc-48.2)	BC046949
2.285	Cg11035 cg11035-pa	AP008207
3.282	Cg11658-isoform a	AC151789
2.782	Chromatin modifying protein 2b	AC011458
0.288	Chymotrypsin	XM_778874
6.665	Condensin subunit Smc	AC111145
2.531	Cytochrome P450 3A	XM_965322
0.477	Elongation factor 1 gamma	AC163628
0.311	Fatty acid binding protein	AC182677
0.479	Fed tick salivary protein 6	AC022536
3.221	Ferritin 3-like protein	AJ245734
13	Glutathione S-Transferase family member (gst-11)	AC024213
0.324	Headcase protein	AC154484

2.812	Heme binding protein 2	BX649621
4.734	Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	
0.229	Homogentisate-dioxygenase (homogentisate oxidase)	BA000012
5.366	IRE1 kinase related family member (ire-1)	AC110040
2.859	Kiser	BX014764
2.217	Light chain 3	AY570553
0.418	Lysozyme	AE009951
0.423	Microsomal glutathione S-transferase 1	AP006662
4.956	Niemann-Pick Type C-2, putative	AL713853
5.319	Paraplegin	XM_964017
0.364	Phage-related lysozyme	AB015932
0.226	Protein disulfide isomerase	AF008300
0.236	Protein disulfide-isomerase like protein erp57	AB210112
2.033	RAB family member (rab-39)	XM_640363
0.465	Ribophorin ii	AB092486
3.948	Sequestosome 1, oxidative stress protein	AC116337
0.362	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 10	AP008208
0.424	Serine collagenase 1 precursor	AY536261
0.311	Serine collagenase 1 precursor	AP007167
0.433	Similar to C18B2.5a	AC183583
2.025	Similar to Calcium-independent phospholipase A2-gamma	AP008212
0.35	Similar to pancreatic triglyceride lipase	U23521
0.317	Similar to vitelline membrane outer layer 1 homolog	AB015609
0.289	Spermine oxidase	AC087644
2.1	Sphingosine-1-phosphate lyase 1	XM_600800
5.718	Surfeit 1	XM_635462
0.397	Vinculin	X96601

Table 5 Genes associated with DNA repair according to gene ontology terms present on the array.

Common name	Nr accession
Damage-specific DNA binding protein 1, 127kda	XP_396048
Similar to dna excision repair protein ercc-6	Cag08547
60S acidic ribosomal protein P0	CAH04310
Ga18248-pa	Eal28996
Inosine triphosphate pyrophosphatase	Xp_461884
RAD23 homolog B	XP_392856
Three prime repair exonuclease 1	Eat48911
6-4 photolyase	Baa97126
Similar to DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 11	XP_582719
Wd-repeat protein	Xp_397060
Bloom syndrome	EAL28826
Similar to CG8583-PA	XP_971689
Nei endonuclease VIII-like 1	NP_082623
Delta-n p63 p73-like protein	Aat72302
Replication-factor-C 40kd subunit CG14999-PA	AAM11182
Similar to CG8583-PA	XP_971689
DNA mismatch repair protein	AAM40505
Ubiquitin-conjugating enzyme e2a (rad6 homolog)	Eaa06004
Histone H2A	XP_876451
GADD45A gene for growth arrest and DNA-damage-inducible alpha	P00769
WD repeat domain 8	AAH50515

APPENDIX 4

Table 1 Gene Ontology (GO) terms that were statistically significantly over-represented in un-treated adult *Daphnia magna* (7 days) compared to un-treated neonates (<24h old). Differentially expressed genes used were based on a 2 fold cut-off. GO terms are ordered most to least significant. A cut off of a P value <0.05 and a minimum of 2 genes in the test group per category was used.

Category	**P-Value**
GO:43283: biopolymer metabolism	8.85E-09
GO:5634: nucleus	1.27E-08
GO:6139: nucleobase, nucleoside, nucleotide and nucleic acid metabolism	1.92E-06
GO:6396: RNA processing	4.44E-06
GO:6259: DNA metabolism	4.60E-06
GO:30532: small nuclear ribonucleoprotein complex	1.43E-05
GO:5488: binding	2.57E-05
GO:398: nuclear mRNA splicing, via spliceosome	3.90E-05
GO:375: RNA splicing, via transesterification reactions	3.90E-05
GO:377: RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	3.90E-05
GO:16070: RNA metabolism	4.85E-05
GO:5681: spliceosome complex	7.03E-05
GO:785: chromatin	9.37E-05
GO:51276: chromosome organization and biogenesis	0.000101
GO:6333: chromatin assembly or disassembly	0.00012
GO:8380: RNA splicing	0.00012
GO:7001: chromosome organization and biogenesis (sensu Eukaryota)	0.000177
GO:16684: oxidoreductase activity, acting on peroxide as acceptor	0.000194
GO:4601: peroxidase activity	0.000194
GO:3723: RNA binding	0.000197
GO:6364: rRNA processing	0.000202
GO:3676: nucleic acid binding	0.000268
GO:16209: antioxidant activity	0.000289
GO:7049: cell cycle	0.00032
GO:6397: mRNA processing	0.000393
GO:6325: establishment and/or maintenance of chromatin architecture	0.000414
GO:3724: RNA helicase activity	0.000422
GO:43176: amine binding	0.000427
GO:6323: DNA packaging	0.000543
GO:5732: small nucleolar ribonucleoprotein complex	0.000589
GO:16071: mRNA metabolism	0.000633
GO:43170: macromolecule metabolism	0.000743
GO:279: M phase	0.000792
GO:5694: chromosome	0.000874
GO:7304: eggshell formation (sensu Insecta)	0.00124
GO:7306: insect chorion formation	0.00124
GO:30703: eggshell formation	0.00124

GO:5684: major (U2-dependent) spliceosome	0.00139
GO:6996: organelle organization and biogenesis	0.00151
GO:6260: DNA replication	0.00162
GO:166: nucleotide binding	0.00305
GO:5524: ATP binding	0.00376
GO:4004: ATP-dependent RNA helicase activity	0.00381
GO:8186: RNA-dependent ATPase activity	0.00381
GO:6334: nucleosome assembly	0.00392
GO:16072: rRNA metabolism	0.00392
GO:6261: DNA-dependent DNA replication	0.00401
GO:15630: microtubule cytoskeleton	0.00467
GO:6800: oxygen and reactive oxygen species metabolism	0.00468
GO:7098: centrosome cycle	0.00476
GO:31023: microtubule organizing center organization and biogenesis	0.00476
GO:51297: centrosome organization and biogenesis	0.00476
GO:30554: adenyl nucleotide binding	0.00507
GO:228: nuclear chromosome	0.00518
GO:17076: purine nucleotide binding	0.00551
GO:15464: acetylcholine receptor activity	0.00571
GO:30594: neurotransmitter receptor activity	0.00571
GO:4889: nicotinic acetylcholine-activated cation-selective channel activity	0.00571
GO:42165: neurotransmitter binding	0.00571
GO:42166: acetylcholine binding	0.00571
GO:43234: protein complex	0.0066
GO:42254: ribosome biogenesis and assembly	0.00673
GO:790: nuclear chromatin	0.00708
GO:5720: nuclear heterochromatin	0.00708
GO:5689: minor (U12-dependent) spliceosome complex	0.00708
GO:792: heterochromatin	0.00708
GO:5892: nicotinic acetylcholine-gated receptor-channel complex	0.00708
GO:87: M phase of mitotic cell cycle	0.0087
GO:7067: mitosis	0.0087
GO:6979: response to oxidative stress	0.00902
GO:3682: chromatin binding	0.0097
GO:7017: microtubule-based process	0.00978
GO:16818: hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	0.0108
GO:16462: pyrophosphatase activity	0.0108
GO:278: mitotic cell cycle	0.0115
GO:16817: hydrolase activity, acting on acid anhydrides	0.0117
GO:3677: DNA binding	0.0117
GO:3729: mRNA binding	0.0117
GO:7051: spindle organization and biogenesis	0.0147
GO:5623: cell	0.0152

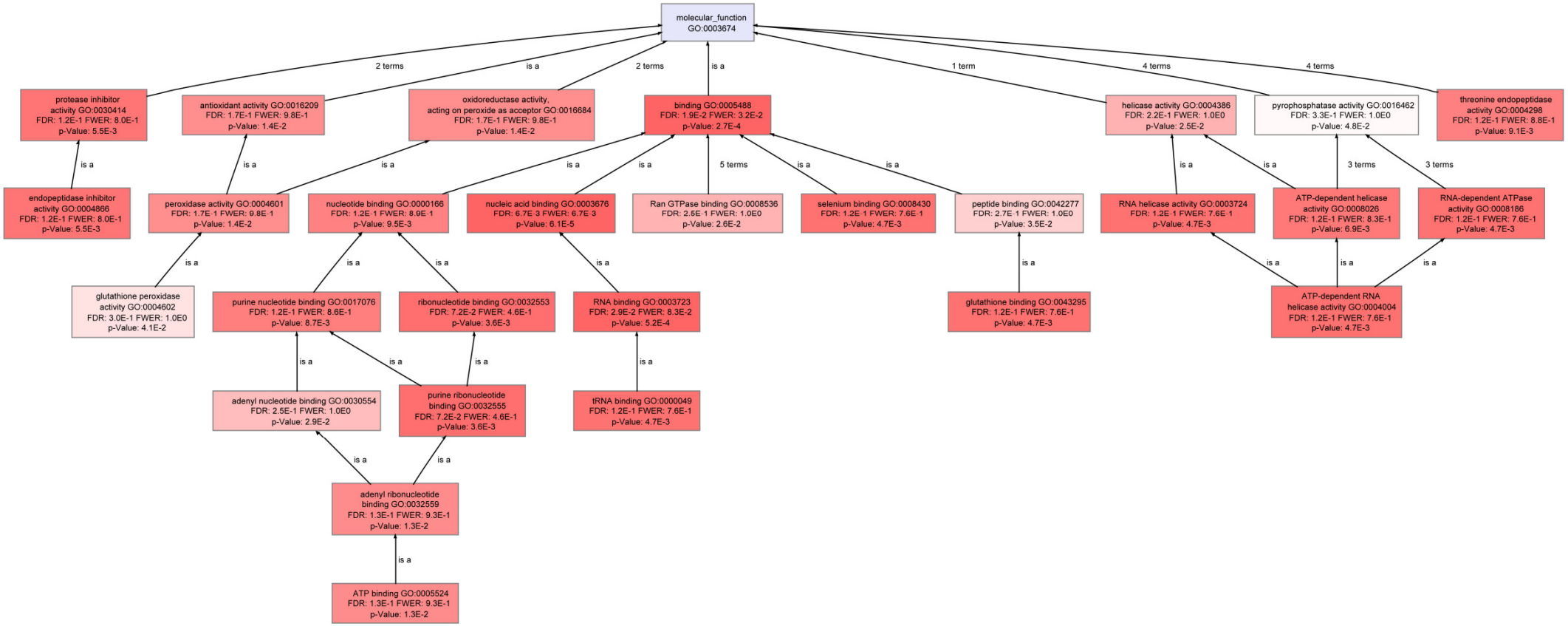
GO:40009: regulation of growth rate	0.0156
GO:40010: positive regulation of growth rate	0.0156
GO:19941: modification-dependent protein catabolism	0.0158
GO:6511: ubiquitin-dependent protein catabolism	0.0158
GO:19953: sexual reproduction	0.0162
GO:4576: oligosaccharyl transferase activity	0.0163
GO:4579: dolichyl-diphosphooligosaccharide-protein glycotransferase activity	0.0163
GO:5230: extracellular ligand-gated ion channel activity	0.0163
GO:5231: excitatory extracellular ligand-gated ion channel activity	0.0163
GO:31497: chromatin assembly	0.0172
GO:30705: cytoskeleton-dependent intracellular transport	0.0172
GO:7018: microtubule-based movement	0.0172
GO:30163: protein catabolism	0.0174
GO:74: regulation of progression through cell cycle	0.0178
GO:4298: threonine endopeptidase activity	0.018
GO:5261: cation channel activity	0.018
GO:12505: endomembrane system	0.0183
GO:51082: unfolded protein binding	0.0189
GO:6183: GTP biosynthesis	0.0189
GO:6268: DNA unwinding during replication	0.0189
GO:46039: GTP metabolism	0.0189
GO:31570: DNA integrity checkpoint	0.0189
GO:7028: cytoplasm organization and biogenesis	0.019
GO:7046: ribosome biogenesis	0.0194
GO:45211: postsynaptic membrane	0.0201
GO:5686: snRNP U2	0.0201
GO:775: chromosome, pericentric region	0.0201
GO:8250: oligosaccharyl transferase complex	0.0201
GO:42600: chorion	0.0201
GO:16043: cell organization and biogenesis	0.0203
GO:50875: cellular physiological process	0.021
GO:8026: ATP-dependent helicase activity	0.0224
GO:44257: cellular protein catabolism	0.0226
GO:51603: proteolysis during cellular protein catabolism	0.0226
GO:6512: ubiquitin cycle	0.0234
GO:43285: biopolymer catabolism	0.0236
GO:5839: proteasome core complex (sensu Eukaryota)	0.024
GO:5622: intracellular	0.0245
GO:5730: nucleolus	0.0249
GO:4386: helicase activity	0.0258
GO:45927: positive regulation of growth	0.0289
GO:5515: protein binding	0.0298
GO:17111: nucleoside-triphosphatase activity	0.0311
GO:5874: microtubule	0.0318

GO:5815: microtubule organizing center	0.0338
GO:5813: centrosome	0.0338
GO:5319: lipid transporter activity	0.0345
GO:9628: response to abiotic stimulus	0.0348
GO:7338: fertilization (sensu Metazoa)	0.0358
GO:6220: pyrimidine nucleotide metabolism	0.0358
GO:9147: pyrimidine nucleoside triphosphate metabolism	0.0358
GO:7346: regulation of progression through mitotic cell cycle	0.0358
GO:40008: regulation of growth	0.036
GO:5685: snRNP U1	0.0379
GO:30312: external encapsulating structure	0.0379
GO:5789: endoplasmic reticulum membrane	0.0386
GO:51258: protein polymerization	0.042
GO:51327: M phase of meiotic cell cycle	0.042
GO:7126: meiosis	0.042
GO:51321: meiotic cell cycle	0.042
GO:42175: nuclear envelope-endoplasmic reticulum network	0.0456
GO:6461: protein complex assembly	0.0473
GO:6950: response to stress	0.0486
GO:42623: ATPase activity, coupled	0.0488
GO:4602: glutathione peroxidase activity	0.049
GO:4532: exoribonuclease activity	0.049
GO:3697: single-stranded DNA binding	0.049

Table 2 Gene Ontology (GO) terms that were statistically significantly over-represented in un-treated neonate *Daphnia magna* (<24h) compared to un-treated adults (7 days). Differentially expressed genes used were based on a 2 fold cut-off. GO terms are ordered most to least significant. A cut off of a P-value of <0.05 and a minimum of 2 genes in the test group per category was used.

Category	**P-Value**
GO:30246: carbohydrate binding	1.71E-06
GO:42302: structural constituent of cuticle	1.91E-06
GO:1871: pattern binding	3.64E-06
GO:30247: polysaccharide binding	3.64E-06
GO:5214: structural constituent of cuticle (sensu Insecta)	5.96E-06
GO:6030: chitin metabolism	6.04E-06
GO:5976: polysaccharide metabolism	1.01E-05
GO:6040: amino sugar metabolism	1.08E-05
GO:6041: glucosamine metabolism	1.08E-05
GO:6044: N-acetylglucosamine metabolism	1.08E-05
GO:44264: cellular polysaccharide metabolism	1.83E-05
GO:8061: chitin binding	2.13E-05
GO:5576: extracellular region	2.94E-05
GO:9605: response to external stimulus	0.000969
GO:4263: chymotrypsin activity	0.00279
GO:8146: sulfotransferase activity	0.00289
GO:4252: serine-type endopeptidase activity	0.00338
GO:9077: histidine family amino acid catabolism	0.00338
GO:6548: histidine catabolism	0.00338
GO:9075: histidine family amino acid metabolism	0.00338
GO:6547: histidine metabolism	0.00338
GO:6807: nitrogen compound metabolism	0.00351
GO:9308: amine metabolism	0.00351
GO:6508: proteolysis	0.00369
GO:4295: trypsin activity	0.00418
GO:4175: endopeptidase activity	0.00504
GO:16782: transferase activity, transferring sulfur-containing groups	0.00567
GO:8233: peptidase activity	0.00658
GO:8236: serine-type peptidase activity	0.00671
GO:5975: carbohydrate metabolism	0.00769
GO:6584: catecholamine metabolism	0.00819
GO:18958: phenol metabolism	0.00819
GO:9583: detection of light stimulus	0.00819
GO:7602: phototransduction	0.00819
GO:9613: response to pest, pathogen or parasite	0.00854
GO:43207: response to external biotic stimulus	0.00996
GO:51606: detection of stimulus	0.0113
GO:9581: detection of external stimulus	0.0113

GO:9582: detection of abiotic stimulus	0.0113
GO:44262: cellular carbohydrate metabolism	0.0114
GO:16715: oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, reduced ascorbate as one donor, and incorporation of one atom of oxygen	0.0136
GO:4500: dopamine beta-monooxygenase activity	0.0136
GO:16490: structural constituent of peritrophic membrane (sensu Insecta)	0.0136
GO:5504: fatty acid binding	0.0136
GO:9416: response to light stimulus	0.0148
GO:9063: amino acid catabolism	0.017
GO:9310: amine catabolism	0.0191
GO:44270: nitrogen compound catabolism	0.0191
GO:6955: immune response	0.0191
GO:9314: response to radiation	0.0231
GO:8289: lipid binding	0.0302
GO:6046: N-acetylglucosamine catabolism	0.0482
GO:6032: chitin catabolism	0.0482
GO:6573: valine metabolism	0.0482
GO:44247: cellular polysaccharide catabolism	0.0482
GO:272: polysaccharide catabolism	0.0482
GO:42829: defense response to pathogen	0.0482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protease inhibitor activity GO:0030414 FDR: 1.2E-1 FWER: 8.0E-1 p-Value: 5.5E-3
antioxidant activity GO:0016209 FDR: 1.7E-1 FWER: 9.8E-1 p-Value: 1.4E-2
oxidoreductase activity, acting on peroxide as acceptor GO:0016684 FDR: 1.7E-1 FWER: 9.8E-1 p-Value: 1.4E-2
binding GO:0005488 FDR: 1.9E-2 FWER: 3.2E-2 p-Value: 2.7E-4
helicase activity GO:0004386 FDR: 2.2E-1 FWER: 1.0E0 p-Value: 2.5E-2
pyrophosphatase activity GO:0016462 FDR: 3.3E-1 FWER: 1.0E0 p-Value: 4.8E-2
threonine endopeptidase activity GO:0004298 FDR: 1.2E-1 FWER: 8.8E-1 p-Value: 9.1E-3
endopeptidase inhibitor activity GO:0004866 FDR: 1.2E-1 FWER: 8.0E-1 p-Value: 5.5E-3
peroxidase activity GO:0004601 FDR: 1.7E-1 FWER: 9.8E-1 p-Value: 1.4E-2
nucleotide binding GO:0000166 FDR: 1.2E-1 FWER: 8.9E-1 p-Value: 9.5E-3
nucleic acid binding GO:0003676 FDR: 6.7E-3 FWER: 6.7E-3 p-Value: 6.1E-5
Ran GTPase binding GO:0008536 FDR: 2.5E-1 FWER: 1.0E0 p-Value: 2.6E-2
selenium binding GO:0008430 FDR: 1.2E-1 FWER: 7.6E-1 p-Value: 4.7E-3
peptide binding GO:0042277 FDR: 2.7E-1 FWER: 1.0E0 p-Value: 3.5E-2
RNA helicase activity GO:0003724 FDR: 1.2E-1 FWER: 7.6E-1 p-Value: 4.7E-3
ATP-dependent helicase activity GO:0008026 FDR: 1.2E-1 FWER: 8.3E-1 p-Value: 6.9E-3
RNA-dependent ATPase activity GO:0008186 FDR: 1.2E-1 FWER: 7.6E-1 p-Value: 4.7E-3
glutathione peroxidase activity GO:0004602 FDR: 3.0E-1 FWER: 1.0E0 p-Value: 4.1E-2
purine nucleotide binding GO:0017076 FDR: 1.2E-1 FWER: 8.6E-1 p-Value: 8.7E-3
ribonucleotide binding GO:0032553 FDR: 7.2E-2 FWER: 4.6E-1 p-Value: 3.6E-3
RNA binding GO:0003723 FDR: 2.9E-2 FWER: 8.3E-2 p-Value: 5.2E-4
glutathione binding GO:0043295 FDR: 1.2E-1 FWER: 7.6E-1 p-Value: 4.7E-3
ATP-dependent RNA helicase activity GO:0004004 FDR: 1.2E-1 FWER: 7.6E-1 p-Value: 4.7E-3
adenyl nucleotide binding GO:0030554 FDR: 2.5E-1 FWER: 1.0E0 p-Value: 2.9E-2
purine ribonucleotide binding GO:0032555 FDR: 7.2E-2 FWER: 4.6E-1 p-Value: 3.6E-3
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2 terms
is a
1 term
4 terms
5 terms
3 terms
Enriched Graph

ABSTRACTS AND PUBLICATIONS

Abstracts

Xenobiotic Metabolism and ability of direct-acting and pro-genotoxic agents to produce DNA strand breaks in the unicellular alga *Chlamydomonas reinhardtii*.

Rhiannon M David[1], Matthew J. Winter[2], and J. Kevin Chipman[1]

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Poster presentation at SETAC Europe Annual Meeting, Warsaw, Poland, May 25-29th 2008

Towards the identification of a characteristic gene expression profile indicative of genotoxicant exposure in *Daphnia magna.*

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Platform presentation at SETAC UK Annual Meeting, Reading, UK, September 9-10th 2008

Identification of molecular level indicators of genotoxicant activation and exposure in *Daphnia magna.*

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Poster presentation at PRIMO 15, Bordeaux, France, May 17-20th 2009

Publications

Induction of DNA strand breaks by genotoxicants in the alga *Chlamydomonas reinhardtii*

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